Luca Giovanella *Editor*

Atlas of Thyroid and Neuroendocrine Tumor Markers

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To our patients and their families for giving meaning to our work.

Foreword

For me this has been an exciting and rewarding 50 years following and participating in the saga of the gastro-entero-pancreatic (GEP) system as an endocrine organ and contributing to the classification of neuroendocrine neoplasms (NENs). I also went after the evolution of the classification and histogenesis of both follicular cell-derived and parafollicular C cell-derived thyroid neoplasms (TNs), including recent findings that revise mammalian C cell ontogeny and suggest that medullary thyroid carcinomas should be reclassified to the family of NENs with endodermal ancestry (1). In my opinion Professor Luca Giovanella's project combining the study of biological markers of TNs and NENs has several reasons: (1) TNs and GEP-pulmonary NENs represent the most frequent endocrine neoplasms; (2) The incidence of both TNs and NENs has been significantly increased in the last 40 years, due in part to improved technology enabling early and even "over" diagnosis. Meanwhile, many cases of TNs and NENs have a good prognosis and the mortality rate has only minimally increased during the same period. (3) New advances in molecular imaging and molecular methods have been improved to better define TNs and NENs, and a number of studies demonstrating risk stratification systems that can be changed over time allow for personalization of diagnosis, initial treatment, and follow-up strategies. These systems are also helpful to avoid overdiagnosis or overtreatment for tumors that are indolent.

In the first chapter of this book Drs. Rossi and Fadda report the characteristic morphological features of thyrocyte derived carcinomas and critically examine the advantages and limits of immunohistochemical markers which help in the definition of malignancy and prognosis of well-differentiated, poorly differentiated, and undifferentiated carcinomas. Drs. La Rosa, Bongiovanni, and Uccella review the current classification of both GEP and pulmonary NENs; in addition they explore the entire spectrum of epithelial proliferation, site-specific, prognostic, and predictive markers of NENs. Successively, the definition and methodology for evaluation of blood or body fluid biomarkers, including monoanalyte and multianalyte approaches, are widely discussed by Dr. Holdenrieder. In the next chapter, Prof. Verburg examines what is known about the diagnosis, clinical aspects, and therapy of differentiated thyroid cancer (DTC).The following three contributions report recent advances in the study of thyroid biomarkers detected and measured in blood samples. The clinical utility of both thyroglobulin and thyroglobulin-antibody measurements for patients with DTC is sharply outlined by Professor

Giovanella and coauthors. Dr. Durante and coauthors look at the feasibility of liquid biopsy to guide thyroid cancer management. Drs. Winkens, Pachmann, and Freesmeyer describe preliminary, encouraging experiences on circulating tumor cells in patients with DTC, mainly oriented on the method for their isolation. Medullary thyroid carcinoma (MTC) is considered for diagnostic, therapeutic, and follow-up aspects by Prof. Alevizaki and coauthors; for the value of calcitonin and CEA as accurate markers of response to treatment by Prof. Costante and coauthors; and for the significance of procalcitonin as a complement or possible substitute of calcitonin in the diagnosis and follow-up of MTC by Dr. Trimboli and colleagues The clinical and pathological features of aggressive variants of TC, poorly differentiated TC, and anaplastic TC are reviewed by Dr. Salvadori and colleagues. The topic of de-differentiation of both DTC and MTC and of related de-differentiation markers is brightly addressed in the chapter by Prof. Giovanella and Drs. D'Aurizio and Tozzoli. The utility of measurements of thyroid/parathyroid markers on fine needle washouts is highlighted by Dr. Trimboli and Prof. Giovanella that also emphasize the necessity of standardization of these procedures. The three last chapters cover the subjects of diagnosis and clinical management of neuroendocrine tumors (Prof. Ferone and colleagues), of their circulating markers (Drs. Seregni and Lorenzoni), and related gene transcripts (Dr. Bodei and coauthors).

On the whole this book brings together chapters written by distinguished experts in their fields covering the wide range of biological markers relevant to thyroid and neuroendocrine neoplasms and will be helpful to those who want to have a comprehensive overview of a complex field.

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Reference

1. Nilsson M, Williams D. On the origin of cells and derivation of thyroid cancer: C cell story revisited. Eur Thyroid J. 2016; 5: 79-93

Preface

Thyroid and neuroendocrine tumors were traditionally considered rare but have been increasing in incidence in the last two decades, mainly due to the wide use of high-sensitive imaging procedures in daily life practice. Both tumor groups encompass a wide spectrum of different diseases from slow-growing tumors to highly aggressive and rapidly fatal cancers. A crucial step for the correct management of these diseases is the early diagnosis, the proper differential diagnosis, and an accurate disease staging and tumor characterization. Different circulating biomarkers are representing a key factor to evaluate thyroid and neuroendocrine tumors: Ideally diagnosis should be a staged process initiated by the identification of individuals at risk, followed by the performance of a targeted screen using an easily accessed sample (such as blood or urine), localization of the lesions with molecular imaging and tumor biopsy to confirm the diagnosis, assess activity, and select therapy. Our book aims to provide a general background on thyroid and neuroendocrine tumor biomarkers, to describe their current usage and limitations in current practice as well as contemporary strategies under evaluation, including the identification of novel analytes, as gene transcripts, microRNA, and circulating tumor cells, that identify disease. We strongly believe that a multidisciplinary approach is needed for the proper diagnosis and management of thyroid and neuroendocrine tumors in the clinical practice. Accordingly, several highly qualified international experts were involved as authors of different chapters, including experimental and clinical endocrinologists, laboratory medicine physicians, nuclear medicine and molecular imaging physicians, pathologists, and oncologists.

The first section discusses pathology and molecular pathology of thyroid and neuroendocrine tumors and tumor markers biology and clinical application. The remainder of the book focuses on applications of biochemical, genomic, molecular, and cellular biomarkers of differentiated, medullary and aggressive thyroid cancers and neuroendocrine tumors. To put biomarkers in an appropriate clinical context any section is introduced by an overview provided by expert clinicians. I strongly hope that our work will be useful to colleagues involved in the diagnosis and therapy of thyroid and neuroendocrine tumors at both the laboratory and clinical settings. Last but not least, I would like to sincerely thank each of the authors for their invaluable collaboration and the patients who have endured my requests.

Bellinzona, Switzerland — Luca Giovanella, M.D., Ph.D., TP-UZH

Contents

Part I

Pathology and Pathobiology

1 Pathology and Immunohistochemistry in Thyroid Tumors

Esther Diana Rossi and Guido Fadda

1.1 Introduction

Thyroid neoplasms represent the first endocrine tumors with an incidence of 8.7 cases/100,000 people per year in Europe although its overall mortality rate is less than 0.1% of all tumor cases [1, 2].

The classification of thyroid neoplasms [3] includes benign and malignant epithelial tumors, the latter being derived either from the follicular cells or from the parafollicular C cells. Papillary thyroid carcinoma (PTC) is the most frequent tumor type, accounting for almost 90% of all thyroid tumors, and usually it pursues a favorable course characterized by frequent nodal spreading though uncommon distant metastatic spread. PTC encompasses two main tumor variants: classic and follicular. The former exhibits the distinctive papillary structures from which the name derives, while the histological hallmark of the latter is the predominant microfollicular pattern. Regardless of the structure, the diagnosis of papillary carcinoma relies upon the distinctive nuclear features (clearing, elongation, and pseudoinclusions) which can be detected in all histotypes.

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Some cases of PTC show an obvious infiltration of either the capsule or the adjacent vessels which witnesses the malignant nature of the tumor. However, some cases do not show aggressive features so that the histological definition of carcinoma can be questionable.

The field of epithelial tumors encompasses also the follicular thyroid carcinoma (FTC), which accounts for about 5% of all thyroid malignant tumors. It is characterized by a follicular pattern made up of follicular cells (thyrocytes) showing variably pleomorphic and dark nuclei. The distinction between FTC and its benign counterpart (follicular adenoma, FA) relies upon the detection of histologic parameters of aggressiveness, notably capsular and vascular invasion. If only one of such features is observed in a follicular-patterned neoplasm, the diagnosis of follicular carcinoma is warranted. The same diagnostic parameters of malignant evolution apply to tumors composed exclusively by Hurthle (or oxyphilic or oncocytic) cell carcinomas (HCC) which represent hypoxic changes of the thyrocytes. FTC and HCC are less likely to metastasize to regional nodes rather than to distant sites such as lungs and bones.

Less frequent carcinomas arising in the thyroid gland are poorly differentiated (PDTC, also called insular thyroid carcinoma) and undifferentiated (anaplastic, ATC) carcinomas. They represent no more than 3% of all thyroid tumors and pursue a less favorable course than the pre-

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vious types. From the thyroid tissue, some other tumors may arise which are not of follicular derivation: the most important are medullary thyroid carcinoma (MTC) and primary malignant non-Hodgkin lymphoma (PML).

1.2 Immunochemistry in Follicular-Derived Tumors

Immunohistochemistry (IHC) has been introduced since the beginning of the 1970s in the routine pathology practice. It has been traditionally used in thyroid pathology for the identification of the cell origin in differentiated tumors arising in the gland or metastasizing outside it, such as thyroglobulin, calcitonin, or parathyroid hormone [4]. There are some tumor types which immunophenotyping deserves a more detailed discussion. Hyalinizing trabecular tumors (HTT) are uncommon neoplasms sharing with PTC some clinical and pathological features. In fact HTT exhibits a trabecular pattern with hyalinization of the stroma composed by thyrocytes showing nuclear pseudoinclusions resembling those of PTC, and in some cases, RET/PTC rearrangements have been identified [5, 6] . On the other hand, this tumor generally pursues a benign course with uncommon malignant forms [7]. Leonardo and coll. [8] have proposed an immunohistochemical method for differentiating HTT from PTC: the cytoplasmic expression of MIB-1 antibody, directed toward the cell-cycle protein Ki-67, instead of the usual nuclear expression of Ki-67 which is used as proliferative index in many tumors. The unusual experimental conditions (room temperature instead of 37 °C) and the strict histological criteria for diagnosing pure forms have somewhat hindered the diffusion of this diagnostic marker.

Hurthle cell tumors (or oxyphilic and oncocytic tumors, HCT) are usually included in the category of FTC although they do not share all its histological characteristics. Hurthle cells represent a different status of the follicular cells induced by either local hypoxia or hormone withdrawal: their morphology is quite distinctive as it shows large pleomorphic nuclei and abundant cytoplasms which, at the ultrastructural level, are engulfed with mitochondria. This unique cytoplasmic composition is responsible of the granular oxyphilic staining of the cytoplasms of HC and, at immunohistochemistry, of the mild non-specific positivity of these cells for the majority of the IHC reactions. Thus, the real positivity of Hurthle cells should be assessed only in the presence of a strong expression in the majority of the cellular component (like thyroglobulin usually does) or when the antibody expression is mostly at the nuclear level (like thyroid transcription factor-1, TTF-1). The immunohistochemical stainings which are helpful in the other differentiated tumors (such as galectin-3, HBME-1, and cytokeratin 19; see below) provide controversial results in HCT and are regarded as unreliable to discriminate benign from malignant neoplasms. Some studies involving HCT have reported that some proliferative markers such as Ki-67 and cyclin D1 may be of help in this differential diagnosis [9]. A different approach to oncocytic tumors has been studied by Gasparre and coll. [10]. They have observed that the oncocytic metaplasia, originated by a marked increase of the mitochondrial component in the cytoplasm of the follicular cells, is often associated to a nonsense mutation of the ND-5 subunit of the respiratory chain complex I of the mitochondria. The expression of the human mitochondrial antibody (HMA) against this subunit reveals the presence of oncocytic cells, regardless of their malignant nature, in every lesion in which they are present. In this case, the HMA does not represent a marker of malignancy, but, nonetheless, this is an important parameter to take into account for a diagnosis of follicular-patterned neoplasm since oncocytic cells may sometimes be misdiagnosed as PTC cells. From a clinical viewpoint, the diagnosis of PTC does not need the presence of capsular or vascular invasion unlike the oncocytic carcinoma, which is diagnosed only when histological features of invasion are detected. Thus, the expression of HMA in a wholly encapsulated follicular neoplasm favors the diagnosis of benign oncocytic adenoma, whereas its negativity suggests a papillary carcinoma.

Anaplastic thyroid carcinoma is made up of highly undifferentiated cells which lose the dis-

tinctive immunophenotype of thyroid carcinoma, such as the expression of thyroglobulin (TGB) and the thyroid transcription factor-1 (TTF-1) (Figs. 1.1 and 1.2).

Thus, the diagnosis of ATC relies mainly on the cellular morphology with the nonspecific cytoplasm expression of low- and high-molecular-weight cytokeratins [11]. Another important diagnostic marker is p53 which is often overexpressed in the majority of nuclei of ATC and represents an important marker for this tumor since it is not present in differentiated and PDTC [12] (Fig. 1.3).

Although the decreasing frequency of ATC would not justify the introduction of specific antibodies for the undifferentiated cells, a study [13]

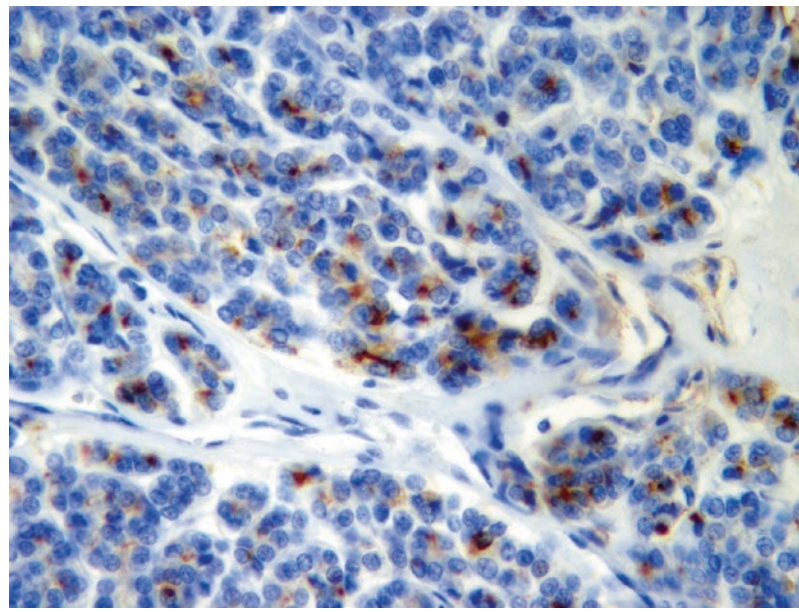

Fig. 1.1 The cytoplasmic expression of thyroglobulin in poorly differentiated thyroid carcinomas may result focally (250×, ABC)

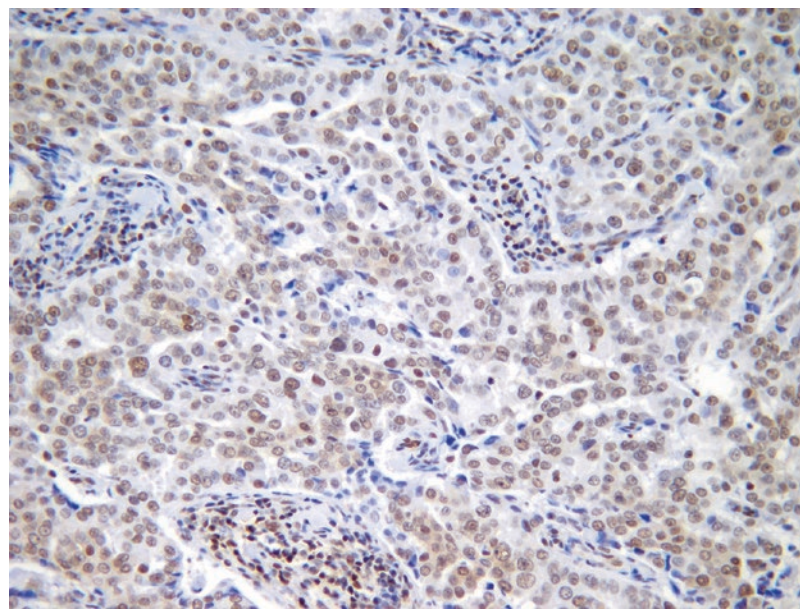

Fig. 1.2 A papillary thyroid carcinoma showing a clear-cut nuclear expression of TTF-1 (125×, ABC)

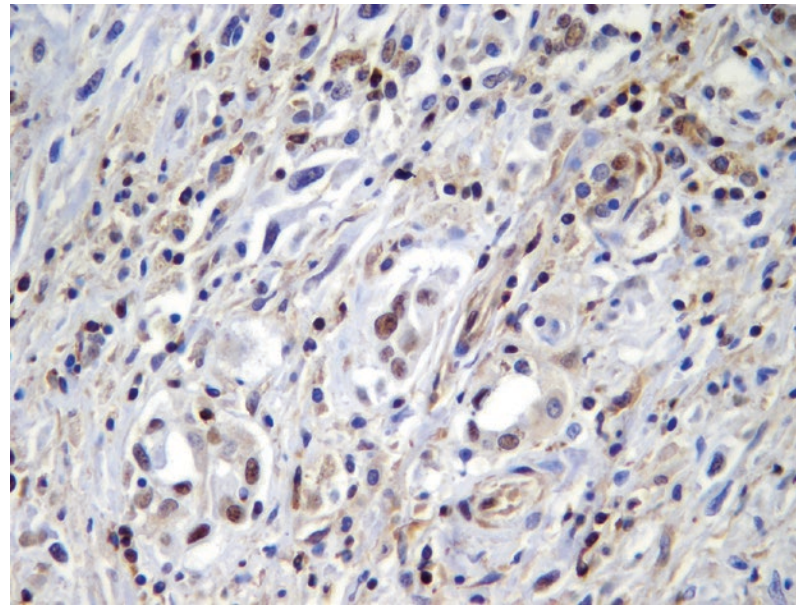

Fig. 1.3 Expression of the p53 antibody in a large proportion of neoplastic cells from an anaplastic thyroid carcinoma (250×, ABC)

has focused on the expression of some cancer stem cell markers (SOX-2, ABC, CXCR4, MRP-1, and LRP antibodies) in this tumor in respect to the differentiated carcinomas with the perspective of using these antibodies as prognostic or predictive markers (see also below).

1.3 Immunomarkers of Malignancy

One of the most puzzling problems in thyroid histological diagnosis is the differentiation between benign and malignant follicular-patterned neoplasms [14]. The traditional identification of features of aggressiveness of the tumor capsule and its surrounding structures (normal parenchyma, vessels, skeletal muscle) is the pivotal criterion on which this distinction is founded. Although some cases of papillary carcinoma do not exhibit the abovementioned features, nonetheless the nuclear features of PTC warrant a diagnosis of malignant tumor. However, a few cases of encapsulated lesions exhibiting a follicular structure either do not show the nuclear hallmark of PTC in a large amount of cells or display only focal nuclear clearing and irregularity which cannot allow a reliable diagnosis of malignancy.

The introduction of the markers of malignancy, which may distinguish malignant from benign lesions irrespective of the histological features of carcinomatous transformation (especially capsular or vascular invasion), has represented a pillar of the morphologic diagnostics of thyroid cancer [15]. HBME-1 (Hector Battifora mesothelial-1) antigen, originally produced for being applied in the discrimination between mesothelioma and adenocarcinoma of the lung, has been one of the first antibodies to be used for the diagnosis of thyroid carcinoma [16]. Since then more than 20 antibodies have been tested with the perspective of overlooking the problem of the nuclear features of the thyrocytes. A meta-analysis by Correia Rodrigues and coll. [17] has evaluated the clinical results of 25 antibodies which have been tested in about 100 different studies on thyroid FNA, and similar findings were registered by Griffith and coworkers [15]. The most studied markers of malignancy are HBME-1, galectin-3, and cytokeratin 19. Apart from HBME-1, whose epitope in the microvilli of the mesothelial is still unknown, the others are well-characterized molecules: for example, galectin-3 is a member of the lectin family molecules which recognize and binds beta-galactoside residues in glycoproteins and glycolipids which have extensively

been studied in histological and cytological samples [18–20]. Cytokeratin 19 is a low-molecular-weight intermediate filament of the cytoplasm which has also been extensively studied [21, 22].

Each of the abovementioned antibodies exhibits a characteristic expression pattern which may be helpful in some diagnostic settings. HBME-1 membranous staining is specific for the malignant thyrocytes and is often useful in identifying microcarcinomas as small as a few millimeters which are hardly detectable during t4e usual histological examination (Fig. 1.4).

Galectin-3 shows a strong cytoplasmic positivity which highlights the largest lesions as compared to the HBME-1 expression but may be detected in nonmalignant cells with clear cytoplasms: it also marks the wall of small vessels which can be used as internal positive controls. Cytokeratin 19 exhibits a strong cytoplasmic expression which emphasizes the presence of the neoplastic cells although the same staining may be detected also in benign thyrocytes [22]. Many studies have been undertaken concerning the reliability of each one of these antibodies in distinguishing benign from malignant neoplasms on both histological formalin-fixed paraffin-embedded (FFPE) sections and fine-needle aspiration biopsy material. The unanimous conclusion has been that no single antibody achieves such a high accuracy for being used in this crucial diagnostic task. Thus, the best combination of these antibodies, and others that will be mentioned below, has been investigated with the aim of harmonizing the high positive predictive value (PPV) of cytokeratin 19 and HBME-1 with the high negative predictive value (NPV) of galectin-3. According to the review of Griffith and coll. [15], the best results on FFPE sections were obtained with the panel made up of cytokeratin 19, galectin-3, and HBME-1 with different combinations of two of them. De Matos and coll. [23], in their large series of thyroid neoplasms, reported a good sensitivity (SE, 84–96.5%) and diagnostic accuracy (DA, 84.9%) for the combination of the three markers in the diagnosis of PTC, whereas the same parameters resulted significantly lower for FTC (63.1%). Similar results were obtained by Park and coll. [24], Scognamiglio and coll. [25], and Rossi and coll. [26] using different combinations of the abovementioned antibodies: they reported a diagnostic accuracy higher than 90%. Indeed, a recent paper by Nechifor-Boila and coll. [27] has investigated the accuracy of a panel made up of four antibodies (HBME-1, galectin-3, CK19, and CD56) in providing the correct discrimination between a

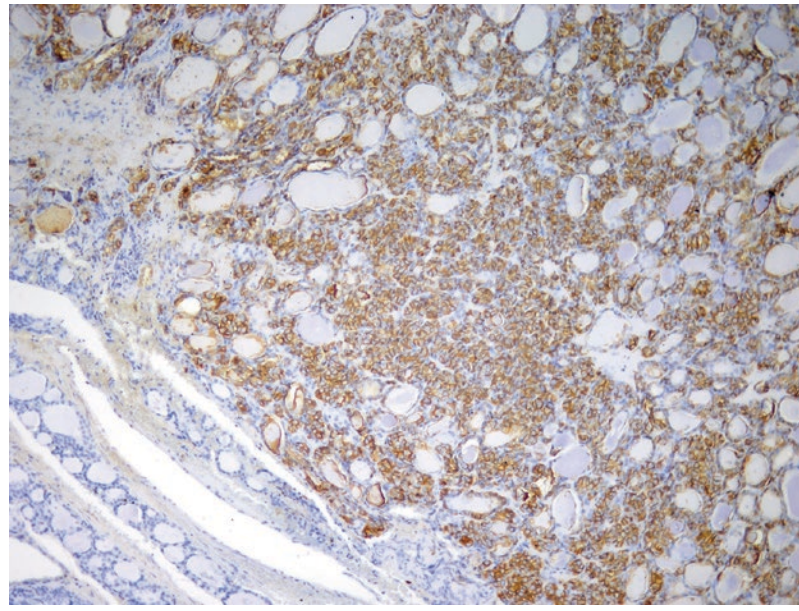

Fig. 1.4 The follicular variant of papillary thyroid carcinoma shows a marked membranous positivity for HBME-1 (125×, ABC)

malignant and a benign follicular neoplasm. Their results do not support the use of a combination of more than three antibodies especially when the diagnosis concerns the follicular variant of PTC. In the review by Correia Rodrigues and coll. [17], galectin-3 has proven to be the most reliable and effective malignancy marker in both FFPE and cytological materials followed by HBME-1, thyroid peroxidase (TPO), and cytokeratin 19. These authors, evaluating a great amount of studies, concluded that although galectin-3 shows on average a positive predictive value (PPV) and negative predictive value (NPV) of, respectively, 84.1% and 81% and a DA of about 83%, these results were not sufficient for recommending the use of this antibody alone for the differential diagnosis between malignant and benign differentiated neoplasms. Nonetheless, in the field of thyroid cytology, galectin-3, especially in combination with HBME-1, may be helpful in identifying neoplastic and malignant lesions in the indeterminate cytological categories which represent a clinical problem [28–30].

Among the antibodies which have been tested as malignancy markers in thyroid differentiated tumors, TPO, CD57, CD44v6, and Rb-1 [27, 31, 32] have also been proven to be effective in identifying malignant neoplasms irrespective of the presence of either capsular or vascular invasion, although the results are controversial because of the poor reproducibility of some experiments (TPO and Rb1).

Eventually, there are some recently investigated markers of malignancy which have revealed a good accuracy in identifying the malignant cells of a follicular-patterned neoplasm. FGFR-2 is an isoform of the fibroblast growth factor receptor family which is underexpressed in PTC and FA but exhibits a strong cytoplasmic positivity in normal and hyperplastic thyrocytes [33]. The retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), including isoforms A and B, have shown good sensitivity (RARs) and specificity (RXRs) in PTCs. It is remarkable that the expression of these antibodies is different in malignant and benign cells: the latter present a strong nuclear expression, whereas the former are identified by a marked cytoplasmic staining [34].

The malignancy markers have been also studied in thyroid tumors different than PTC and FTC [35]. In these instances, the IHC does not focus on the identification of malignant cells, since the histological hallmarks of malignancy are usually well detectable, but on the correct recognition of the poorly differentiated component which can be important for the prognosis and the treatment of the tumor.

1.4 Prognostic and Predictive Markers

The immunohistochemical expression of antibodies which may either anticipate the degree of aggressiveness or predict the clinical course of a malignant tumor has since long been investigated. Traditional proliferative markers such as Ki-67, p27/kip1, and cyclins D1 and E have been tested first in the differential diagnosis of malignant versus benign differentiated tumors and then as prognostic parameters in the same neoplastic category with controversial results [36–38].

In this setting, the paper by Wang and coll. [39] has underlined that a score based on the expression of the connective tissue growth factor (CTGF, also known as CCN2) may predict with high statistical significance the possibility of a high tumor stage at diagnosis and the likelihood of regional nodal metastases. Similar results were reported by Saffar and coll. [40] who tested MMP2 (matrix metalloprotease 2) and CCP3 (caspase-3) in PTC. MMP2 did not show a significant correlation with necrosis or extrathyroid invasion but was significantly associated with a higher likelihood of lymphatic spread, whereas CCP3 showed a specular correlation with the same prognostic parameters. The authors suggest the combined use of both antibodies for assessing the aggressiveness of PTC for therapeutic purposes. The expression of NCAM (CD56) and OCIAD-1 in thyroid differentiated tumors is associated with a lower aggressiveness, whereas a decreased or absent positivity in the neoplastic cells may be predictive of nodal or distant metastatic spread: it is noteworthy that in a few cases the metastatic thyrocytes retrieve the

expression of CD56 [41]. In our paper, CD56 was negative in 96% of the PM, while 68.5% of the BNs showed cytoplasm positivity for this marker, with an overall high sensitivity (96%) but lower specificity (69%). In specific, our 96% of the PMs did not show any follicular cell with CD56 expression [42].

More interesting are the recent investigations leading to the application of prognostic markers to less differentiated and undifferentiated tumors, especially ATC, for which new pharmacological treatment (sorafenib, axitinib, withaferin A) with monoclonal antibodies directed against the growth factor receptors of the neoplastic cells has been recently introduced [43, 44]. In this setting, the expression of cancer stem cell markers, which have been mentioned in the previous paragraph [13], has been reported as associated with a worst prognostic course of the tumor, and it might be evaluated in patients who are candidate for target therapies.

PDTC is an uncommon finding in routine practice, and it is usually observed as a pattern in the context of a differentiated carcinoma. Nevertheless, many authors have reported a decrease of the disease-free interval and of the overall survival in tumors showing a predominant insular pattern (usually higher the 40% of the tumor area). Recently, a paper by Rossi and coll. [35] has found, in a small series of pure PD insular carcinomas, a statistically significant correlation with the expression of beta-catenin compared to more traditional malignancy markers (HBME-1 and galectin-3) in PTC.

1.5 Antibodies Directed Against Mutated Genes

The recent discoveries of the involvement of the most important signaling pathways of the follicular cells in the thyroid carcinogenesis have led the investigation to the role of each single-gene mutation in the cell transformation. As a consequence, the studies involving the mutations of the genes of the MAP kinase cascade have provided brilliant data supporting the pivotal importance of RAF and RAS mutations in the origin of thyroid carcinoma, and the point mutations of the RET gene have been regarded as a key mechanism for MTC [45–47]. Many of these investigations have been carried out at the molecular level, but, as a natural consequence, some antibodies directed toward the mutated proteins have been produced and released for different clinical purposes.

RET proto-oncogene, an antibody with cytoplasmic expression, has been used at the beginning of this century as a malignancy marker for follicular differentiated tumors [15, 26]. Unfortunately, because of technical problems or of a significant lack of specificity, its application for this important differential diagnosis has been discontinued in many institutions.

For similar reasons, the use of PAX8/PPARgamma antibody, which was regarded as a robust diagnostic marker of FTC [47–49], showed a significant rate of positive expression in in FA (31%, [50]) which blurred its role in this diagnostic field.

More recently, an antibody directed toward the *B-RAF* mutated protein kinase (VE-1) has been commercialized as diagnostic and prognostic marker [51]. Its expression, according to the authors who have published on this subject, would reveal the presence of the V600E mutation of the *B-RAF* gene, which is the most common gene mutation in PTC, and it is primarily involved in the occurrence of this tumor [46, 47, 52]. The identification of a B-RAF mutation can be helpful in two different clinical settings: (a) on cytological material, when an indeterminate diagnosis is made on a fine-needle aspiration biopsy, and (b) on both cytological and FFPE materials, when the diagnosis of PTC is already evident. In the former case, the *B-RAF* mutation, as is regarded as 99% specific for PTC, identifies those patients who should be addressed to the surgical thyroidectomy, sparing many unnecessary thyroid removals. In the latter case, the identification of *B-RAF V600E* mutation would provide the surgeon with an additional tool for a more aggressive approach to either the tumor or the central neck compartment nodes [53].

Rossi and coll. [54] suggest that also in case with a diagnosis of suspicious for carcinoma,

which is associated with a high likelihood of papillary carcinoma (60–80%), the identification of a *B-RAF* mutation might induce the surgeon to a more aggressive approach to suspicious nodules at cytology [54, 55]. The most important condition for the use of the antibody VE-1 as a morphologic substitute of the molecular change is the nearly complete overlapping between molecular and IHC findings [56].

Conclusions

The recent insights in the molecular mechanisms of thyroid carcinogenesis, also prompted by the need of understanding the processes of occurrence and progression of the infantile tumors induced by the exposure to ionizing radiation after the Chernobyl accident, have led to a flourishment of studies involving new antibodies. With the introduction of new molecular targeted therapies, these new antibodies may represent useful predictors of therapeutic response in tumors which either do not respond any more to the radioiodine treatment (dedifferentiated and oncocytic carcinomas) or are not sensitive to any conventional antitumor treatment. At the same time, some new antibodies are being tested for the identification of tumors which, in some instances, may be misdiagnosed as malignant (HTT and oncocytic tumors), with negative consequences for the patients. Some markers of malignancies have been introduced although their efficacy has to be tested on both large series by evaluating different prognostic parameters than before. Finally, the use of antibodies directed to proteins generated by mutated genes may represent a cost-effective method for diagnosing and managing patients affected by thyroid tumors.

A nosological entity recently described by Nikiforov YE and coll. [57] has been defined NIFTP (noninvasive follicular thyroid neoplasm with papillary-like nuclear features). It represents the less aggressive counterpart of the follicular variant of papillary carcinoma. Although their morphologic and molecular features have been somewhat described in the abovementioned study, the immunohistochemical profile of NIFTP needs a more detailed investigation, especially in order to identify the most distinctive features helpful in distinguishing this prognostically favorable tumor from its aggressive counterpart.

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2 Pathology of Neuroendocrine Neoplasms: Morphological, Immunophenotypical, and Circulating Molecular Markers

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2.1 Introduction

Neuroendocrine neoplasms (NENs) are a heterogeneous group of epithelial neoplastic proliferations arising in a large number of body organs. Irrespective of their primary site and of their grade of differentiation, neoplastic cells share features of neural and endocrine differentiation including the presence of secretory granules, synaptic-like vesicles and the ability to produce amine and/or peptide hormonal products.

The high heterogeneity of biological and clinical features of NENs represents a challenge for oncologists and pathologists, and a correct diagnostic approach is crucial for the management of patients. Indeed, NENs encompass a wide spectrum of neoplasms ranging from "benign" or very indolent tumors to highly aggressive neuroendocrine carcinomas. Accordingly, the morphological features of these neoplastic proliferations are variable and must be carefully identified by pathologists in order to produce a correct and complete histopathological report, which is the starting point for the correct treatment and follow-up of each patient [1]. The clinicopathological features are related to the morphology and immunohistochemical profile, which also depend on the site of origin. Most cases arise in the bronchopulmonary and gastroenteropancreatic (GEP) systems, while neoplasms arising in other sites, including pituitary gland, parathyroid, thyroid, adrenal glands, and paraganglia are rarer. In addition to these sites, NENs may occasionally arise in other organs, including nasal and paranasal cavities, salivary glands, skin, and urogenital and gynecological organs, showing peculiar clinicopathological features. The systematic description of all of these is beyond the scope of the present chapter, which will discuss the most frequent neoplasms arising in the digestive and bronchopulmonary systems.

Both GEP and lung NENs are relatively rare tumors, although their incidence is steadily increasing. Poorly differentiated neuroendocrine carcinomas (NECs), mainly of the small cell type, are more frequent in the lung than well-differentiated tumors (carcinoids), whereas in the GEP system well-differentiated neuroendocrine tumors (NETs) occur more commonly than NECs [2]. Although the diagnosis of GEP and lung NENs is generally simple in routine practice, there are critical aspects that need to be taken into account during the diagnostic workup.

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The most challenging tasks include the difficulty in achieving the correct prognostic evaluation of NETs by recognizing their metastatic/aggressive potential and the identification of NECs. To this aim, a comprehensive morphological, immunohistochemical, and molecular investigation represents the most reliable tool for pathologists.

2.2 Nomenclature and Classification

2.2.1 Nomenclature

Although the terms used to define NENs change in relation to the site of insurgence (lung versus GEP system), the WHO classifications of lung and digestive tumors separate both lung and GEP NENs into two main groups with different morphological, clinical, and molecular features: well-differentiated and poorly differentiated neoplasms [3, 4]. This distinction is crucial for the different prognoses and therapeutic approaches of the two categories, which are distinct entities despite a similar neuroendocrine phenotype [5, 6].

The terminology used to define NENs has been a matter of debate in the last 100 years creating some confusion among clinicians and pathologists. At the beginning of the twentieth century, Siegfried Oberdorfer described a series of six tumors of the small intestine, which he called *"carcinoid"* (i.e., carcinoma-like) on the assumption that they were similar to intestinal adenocarcinomas but showed a more bland morphology and benign/indolent behavior [7]. This term, which became very popular among clinicians and pathologists, underlined that these neoplasms were different from the more frequent exocrine carcinomas of the same site, although they showed an epithelial morphology. The neuroendocrine nature of these lesions was unknown at that time and remained obscure for several years, until it was demonstrated by Claude L. Pierre Masson, who developed the argentaffin reaction and called this tumor type *"argentaffinoma"* [8]. Forty-five years later, the term *"APUDoma"* was introduced by Antony G. E. Pearse, who proposed the so-called "APUD concept": all neuroendocrine cells of the body are capable of amine precursor uptake and decarboxylation (APUD cells) and represent the cells from which neuroendocrine tumors derive, including those of nonintestinal sites [9]. Nevertheless, the term carcinoid has remained very popular among pathologists and clinicians and it has been widely used to define the wide spectrum of NENs arising in digestive and nondigestive sites. However, since digestive NENs originate from several different neuroendocrine cell types showing different clinical, pathological, and molecular features, the term "carcinoid" has failed to adequately convey the variety of such tumors and, in 1995, it was replaced by the term "neuroendocrine tumor" by Capella and coworkers who proposed a prognostic classification of GEP NENs [10]. Since then the use of the term "carcinoid" has been discouraged in diagnostic practice in favor of "neuroendocrine tumor (NET)", maintaining the term carcinoid solely in the context of the "carcinoid syndrome" [4, 11, 12]. Conversely, in the lung the term carcinoid has been retained over the years because the heterogeneous spectrum of NENs, including different immunophenotypes and clinical syndromes, is extremely reduced if compared with those of GEP NENs. Based on morphological features, lung carcinoids are divided into typical and atypical (see below).

As a general principle, lung carcinoids correspond to NETs as classified in the GEP system because of their well-differentiated morphology. It is worth noting that all these tumors are now considered to be malignant and potentially metastatic with different biological aggressiveness which mainly depends on proliferative indices, presence of specific endocrine syndromes and metastases at the time of diagnosis. Conversely, NECs of both small and large cell types are high grade, very aggressive cancers with an ominous prognosis without significant difference in outcome between lung and GEP cases. Moreover, the therapeutic strategy for NEC seems to be the same, independently of their origin [13].

2.2.2 Classification of Neuroendocrine Neoplasms

2.2.2.1 GEP System

Due to the need to develop a classification scheme able to separate aggressive NECs from more indolent NETs and neoplasms with different behavior among the latter, several changes in the classification of GEP NENs have been necessary in the past few years, with the aim of providing tools to better stratify patients in different prognostic groups [10, 11, 14]. The current WHO classifications identify two main categories [4, 15] (Table 2.1): *neuroendocrine tumors* (NETs), broadly corresponding to "carcinoid tumors" or "well-differentiated neuroendocrine tumors/carcinomas" of previous classifications [11, 14] and *neuroendocrine carcinomas* (NECs), corresponding to poorly differentiated neuroendocrine carcinomas of the previous classification [11]. In the 2010 WHO classification NETs are further divided in two groups (G1 and G2) based on the mitotic count (<2 per 10HPF or 2–20 per 10HPF, respectively) and/or Ki67 index (<2% or 3–20%, respectively). NECs, including small cell and large cell subtypes, present high mitotic count (>20 per 10 HPF) and Ki67 index (>20%) and, by definition, are graded G3. Although this classification is easy to use, it has highlighted a controversial issue represented by a subset of well-differentiated neoplasms with an elevated Ki67 index, generally ranging from 20 to 55%. Indeed, the application of the 2010 WHO classification has emphasized the existence of these cases which show well-differentiated morphology and a high proliferation rate [16–22]. Several recent studies have shown that these cases have an overall survival rate that is worse than NET G2, but significantly better than poorly differentiated NECs. Additionally, they do not seem to benefit from platinum-based therapy [18–20, 23]. Furthermore, they show a molecular profile more similar to that of well-differentiated NETs rather than that of NECs [24]. These findings have confirmed the existence of a new tumor category characterized by a well-differentiated morphology associated with a high proliferation rate. Some authors have proposed defining such a group of tumors as NET G3, and this terminology has been introduced in the 2017 WHO classification of pancreatic NENs (Table 2.1) [15]. It may also be discussed in the future WHO classification of tubular NENs. Moreover, in the 2017 WHO classification of pancreatic NENs, the Ki67 cut-off to separate G1 from G2 NETs has been moved from 2 to 3%, to avoid the gap existing in the previous 2010 WHO classification. This choice has been made by considering that there are papers in the literature demonstrating that

Table 2.1 Comparison of 2010 WHO classification of digestive neuroendocrine neoplasms and 2017 WHO classification of pancreatic neuroendocrine neoplasms

	WHO 2010		WHO 2017*	
	Mitotic index	Ki67 index	Mitotic index	Ki67 index
#Well-differentiated NEN				
NET G1	<2/10HPF	<2%	<2/10HPF	<3%
NET G2	2–20/10HPF	3–20%	2–20/10HPF	3–20%
NET G3	–	–	>20/10HPF	>20%
#Poorly differentiated NENs				
NEC	>20/10HPF	>20%	>20/10HPF	>20%
Mixed neoplasms				
	MANEC		MiNENs	

NEN neuroendocrine neoplasm, *NET* neuroendocrine tumor, *NEC* neuroendocrine carcinoma, *MANEC* mixed adeno-neuroendocrine carcinoma, *MiNEN* mixed neuroendocrine/nonneuroendocrine neoplasm, * only for pancreatic NENs, # morphologically well differentiated or poorly differentiated, *HPF* high power field

the cut-off of 3% is better in differentiating G1 from G2 tumors [25, 26]. It is worth noting, that a Ki67 cut-off of 5% has been proposed by some authors as the best one to differentiate G1 from G2 pancreatic NETs [27–29]. However, studies on large series have not demonstrated an improved performance of this threshold, compared to the old cut-off, sufficient to justify such a great change (from 2 to 5%) [30].

In addition to pure NENs, there are cases composed of both neuroendocrine and nonneuroendocrine components. Such mixed neoplasms have been defined in the 2010 WHO classification with the term "mixed adenoneuroendocrine carcinomas (MANECs)" [4]. By definition, they are composed of both exocrine and neuroendocrine components and each must represent at least 30% of the lesion. However, this term does not adequately convey the different types of mixed neoplasms. In fact, both exocrine and neuroendocrine components can have variable morphological features, ranging from adenomas to adenocarcinomas or squamous cell carcinomas in nonneuroendocrine components and from well to poorly differentiated NENs in neuroendocrine components [31]. The different combinations of these tumor types gives rise to different prognostic categories ranging from indolent neoplasms composed of adenoma and NET G1 or G2, to very aggressive neoplasms characterized by NECs associated with a nonneuroendocrine component (adenoma, adenocarcinoma or squamous cell carcinoma). For this reason, MANEC should not be considered as a single entity but as a spectrum of different neoplastic diseases. We have recently suggested modifying the term MANEC with "mixed neuroendocrine/nonneuroendocrine neoplasms (MiNENs)" [31], which has been included in the 2017 WHO classification of pancreatic NENs [15].

2.2.2.2 Pulmonary System

The classification of pulmonary NENs has maintained the same general approach and terminology over the past 20 years, and in the last WHO classification published in 2015 lung NENs have been grouped in the same chapter (Table 2.2). In the previous WHO classification, small cell carcinomas were considered separately from other lung NENs, while large cell neuroendocrine carcinomas were included in the chapter on large cell carcinomas [32]. Lung NENs are divided into four categories: typical carcinoids, atypical carcinoids, large cell neuroendocrine carcinomas, and small cell carcinomas [3]. The distinction among the different categories is purely morphological and the histological criteria will be described subsequently. In the WHO classification of GEP NENs, the Ki67 index represents a crucial tool in distinguishing between the different categories, whereas in the WHO classification of lung NENs it is considered as an ancillary tool which is helpful in the diagnosis of small biopsies with crush artifacts. However, the prognostic value of the Ki67 proliferative index in lung carcinoids has been recently proved [33]. Cases composed of both neuroendocrine and nonneuroendocrine components are also present in the lung where they are defined as combined carcinomas. They are characterized by the combination of small or large cell NECs with a nonneuroendocrine component (adenocarcinomas or squamous). This term is also used to define neuroendocrine carcinomas composed of an admixture of small and large cells [3].

Table 2.2 WHO classification of lung neuroendocrine neoplasms

Histological feature	Typical carcinoid	Atypical carcinoid	LCNEC	SCNEC
Neuroendocrine morphology	Yes	Yes	Yes	Yes
Degree of cell differentiation	WD	WD	PD	PD
Mitoses × 2 mm2	<2	2–10	>10	>10
Necrosis	No	Focal	Yes	Yes

LCNEC large cell neuroendocrine carcinoma, *SCNEC* small cell neuroendocrine carcinoma, *WD* well differentiated, *PD* poorly differentiated

2.3 Morphological Aspects

2.3.1 Cytological Features

The cytological diagnosis of NENs is based on well-defined cytomorphological features [34–36] although, whenever possible, it should be confirmed using immunocytochemistry. Furthermore, cytological diagnosis of NENs has added value, as several patients, mainly those with poorly differentiated neoplasms, do not undergo surgical procedures due to the extension of the disease at the time of diagnosis. Thus, cytological preparations remain the only available material for diagnosis and molecular analyses which are useful for choosing the most appropriate therapy. In general, NETs are easily diagnosed by means of the most common cytological procedures: expectorations, aspiration, brushing, lavage, and fine-needle aspiration (FNA), performed either with the assistance of computed tomography (CT) (transcutaneous) or with ultrasound (US) guidance (transcutaneous and/or endoluminal). Rapid on-site evaluation (ROSE) is becoming more commonly requested by clinicians and provided by cytopathologists. This procedure allows the increase in yield of collected material and consequently the best triage of the specimen can be achieved. Subtyping NENs in cytological specimens is strongly encouraged because of the clinical implications. In most cases, specific cytomorphological features permit the distinction between low grade (well differentiated) and high grade (poorly differentiated) neoplasms (Fig. 2.1).

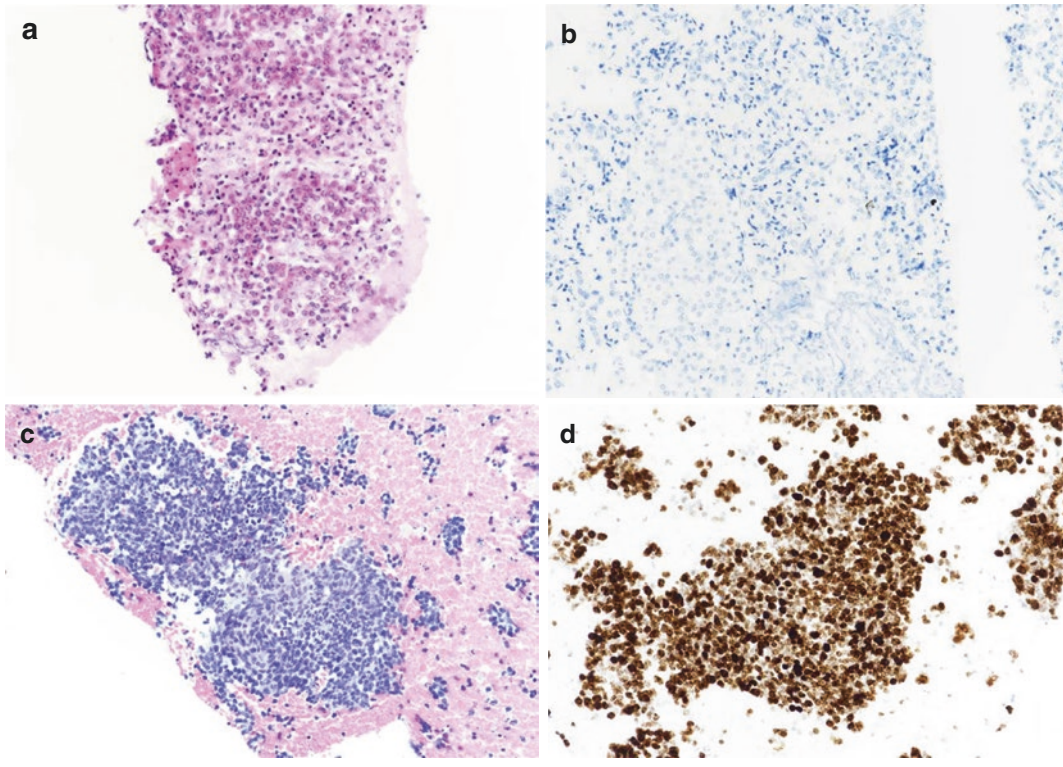

Fig. 2.1 Cytological features of neuroendocrine neoplasms compared with Ki67 labeling index. (**a**, **b**): Well-differentiated neuroendocrine tumor (NET) with clear background, showing cohesive groups of medium-sized cells with moderately abundant eosinophilic cytoplasm and round nuclei with mild pleomorphism; the Ki67 labeling index in this field is less than 1%. (**c**, **d**): Poorly differentiated neuroendocrine carcinoma (NEC), small cell type, with necrotic background, composed of small to medium sized cells with a high nucleocytoplasmic ratio, nuclear molding, inconspicuous nucleoli and numerous apoptotic bodies; the Ki67 labeling index is very high, approaching 90%

2.3.1.1 Well-Differentiated Neuroendocrine Neoplasms (Typical Carcinoids, Atypical Carcinoids, Well-Differentiated Neuroendocrine Tumors)

These usually show a clean or hemorrhagic background and tumor necrosis is absent (necrosis is usually associated with aggressive behavior also on cytology). FNA material is highly cellular and contains predominantly monotonous and cohesive groups of medium-sized cells. Cells can also be isolated, but there are usually fewer than in poorly differentiated cases. Architecturally, trabeculae, nests, ribbons, acini, and rosettes are the most frequently encountered structures. Cells are round, columnar, or plasmacytoid in shape and contain eosinophilic cytoplasm with granular nuclei, stripped chromatin, and moderate pleomorphism. Nucleoli are small, and molding is usually absent. Mitoses are absent or very rare. Vascularity can be present on the FNA material as branching capillaries surrounded by neoplastic cells, especially in pancreatic NETs. In cases of cystic NETs, which are more frequently observed in the pancreas representing up to 15% of cases, the background can be proteinaceous and viscous, with abundant macrophages [37]. CEA and amylase levels are usually low in these cystic lesions [38]. In cases of mixed forms, nonneuroendocrine malignant cells (squamous, adenocarcinomatous or undifferentiated) may also be seen.

The differential diagnosis between typical (TC) and atypical carcinoids (AC) of the lung is not easy on cytological grounds since the diagnostic criteria including the presence of necrosis (often punctate and focal) and mitotic count are difficult to evaluate in cytological specimens [3]. However, nuclear pleomorphism with occasional molding and a more disorganized architecture can be observed in ACs. Cells can be fusiform or spindle in peripherally located carcinoids. In cases of hypocellular smears and abundant crushing artifacts, the differential diagnosis with poorly differentiated neuroendocrine carcinomas, especially of the small cell type, is a difficult task. A mitotic count of less than 10/mm^2 does not allow the diagnosis of AC rather than SCLC and reaspiration or a biopsy should be requested.

2.3.1.2 Poorly Differentiated Neuroendocrine Carcinomas (NEC) (Large Cell and Small Cell Types)

Morphologically, the diagnosis of poorly differentiated NECs does not cause any problems for the differential diagnosis with well-differentiated forms, except in the case of paucity of material. The main challenge for the cytopathologist is to think about the possibility of a NEC rather than a lymphoma, small blue round cell tumor or poorly differentiated nonneuroendocrine carcinoma, and the use of immunocytochemical markers is mandatory. Morphologically, the slide background is generally dirty and occupied by necrotic debris and cellular ghosts. Necrosis can be very abundant. Cells are variable in size: small, twice the size of a resting lymphocyte in the case of small cell NECs and large, plasmacytoid-appearing in the case of a large cell NECs. Cells are usually isolated and discohesive. They have a high nucleocytoplasmic ratio in small cell NECs and their most distinctive features are nuclear molding, marked nuclear pleomorphism and nuclear crushing artifacts. Small cell NECs have typical salt-and-pepper chromatin with inconspicuous nucleoli and scant cytoplasm, while large cell NECs have visible nucleoli, coarsely granular chromatin and abundant cytoplasm. Nuclear molding is not so frequently observed as in small cell NECs. Mitotic count is high in both lesions (more than 10 mitoses/mm^2 is necessary in case of lung small cell NECs and large cell NECs). Diffuse nuclear molding is frequently observed in cases of small cell NECs while a vaguely basaloid/rosettiform appearance is most evident in cases of large cell NECs (especially on cytoblock material) [39, 40]. Other features associated with malignancy include spindling or multinucleation of tumor cells. Combined forms of well-differentiated neoplasms exist, and should also be reported on cytological specimens.

2.3.2 Histological Features

GEP and lung NENs are morphologically heterogeneous (Fig. 2.2). A useful and effective framework for the diagnosis is based on the degree of

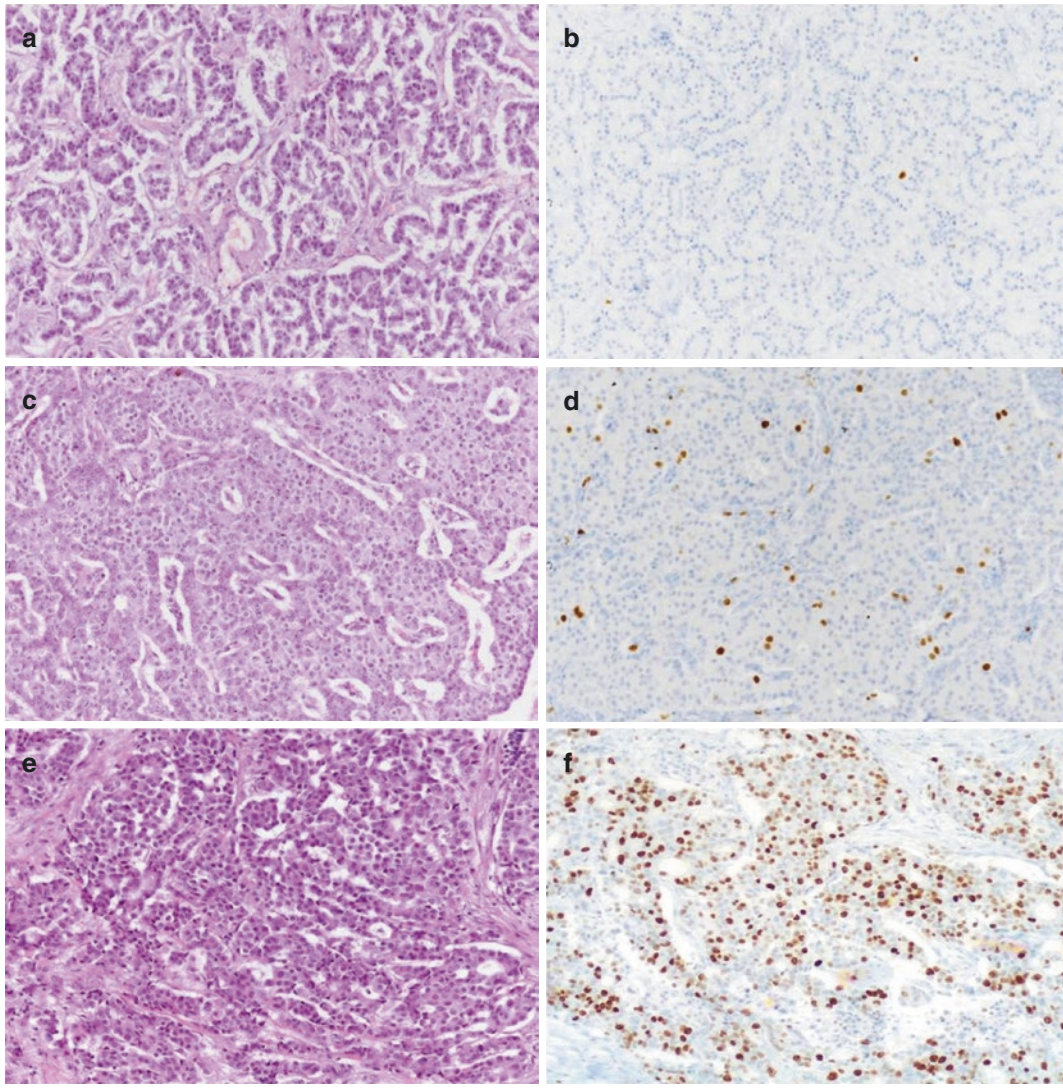

Fig. 2.2 Histological features of neuroendocrine neoplasms compared with Ki67 labeling index. (**a**, **b**): Well-differentiated neuroendocrine tumor (NET) with trabecular pattern of growth, composed of uniform medium sized cells with moderately abundant eosinophilic cytoplasm and round nuclei with only bland atypia; the Ki67 labeling index in this field is less than 1%. (**c**, **d**): NET G2 growing in solid nests. The cytological features are similar to those seen in NET G1. The Ki67 labeling index in this field is 5%. (**e**, **f**): NET G3 of the pancreas with solid and pseudoglandular pattern of growth, composed of cells with moderate pleomorphism, but with still differentiated nuclear and cytoplasmic features; the Ki67 labeling index in this field is higher than 30%. (**g**, **h**): Large cell neuroendocrine carcinoma (NEC) growing in solid sheets of large pleomorphic cells with vesicular nuclei showing prominent nucleoli and abundant eosinophilic cytoplasm: mitotic figures are evident; the Ki67 index is higher than 40%. (**i**, **j**): Small cell neuroendocrine carcinoma (NEC) growing in ribbons and solid sheets, composed of small sized cells with high nucleocytoplasmic ratio, dark nuclei with inconspicuous nucleoli and high mitotic count; the Ki67 labeling index is very high, approaching 80%

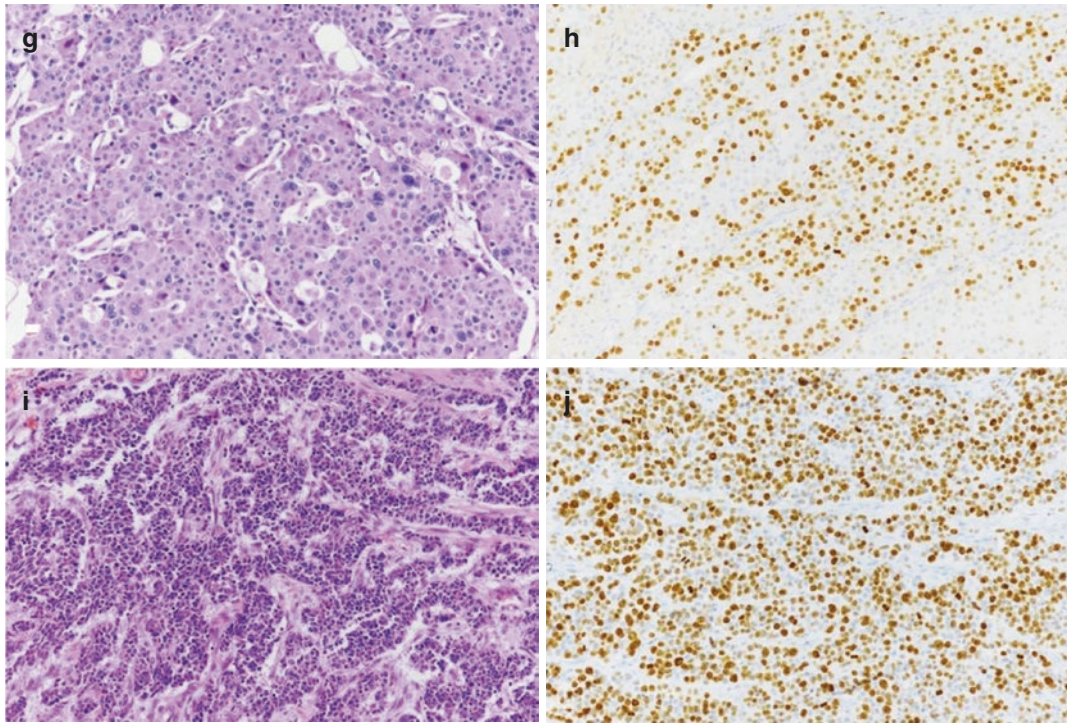

Fig. 2.2 (continued)

differentiation of these neoplasms, which are subdivided into two broad categories: *well-differentiated neuroendocrine tumors* and *poorly differentiated neuroendocrine carcinomas*. It is worth noting that a third category composed of a neuroendocrine and a nonneuroendocrine component (usually adenoma, adenocarcinoma, or squamous cell carcinoma) represents a distinct group with peculiar morphological and clinical features [4, 31].

Well-differentiated neuroendocrine tumors (NETs) are characterized by an organoid proliferation of uniform cells, with moderately abundant granular and eosinophilic cytoplasm containing numerous secretory granules. Nuclei are generally round, with clumped or finely granular ("salt and pepper") chromatin and small nucleoli. Nuclear atypia may be moderate in some cases, and pleomorphic cells with large atypical nuclei may be present. It is worth noting that their presence is not related to an increased biological aggressiveness [41]. NETs can show different architectural features, including small to medium size solid nests, trabecular, pseudo-glandular, or diffuse patterns of growth. These different architectural aspects may be related to the site of origin: ileal and appendiceal NETs are mostly characterized by nests, rectal NETs are frequently trabecular, while ampullary NETs show a characteristic pseudoglandular architecture. As NETs may behave in a malignant fashion, it is important to look for morphological clues that can be associated with tumor aggressiveness, such as the invasion of blood and lymphatic vessels and of perineural spaces, which represent histological signs of malignancy. These morphological features need to be carefully searched and indicated in the pathology report. Other important histological features to be included in the pathology report are the presence of necrosis and the mitotic count, which have prognostic value for GEP-NETs and are the crucial histological features in the differential diagnosis between typical and atypical carcinoids of the lung.

Poorly differentiated neuroendocrine carcinomas (NECs) are highly aggressive neoplasms. Macroscopically, they are poorly circumscribed, may show large areas of necrosis and hemorrhage, and are frequently metastatic when diagnosed. Microscopically, NECs are characterized by a solid proliferation of cells, in large nests or in sheets with large areas of "geographic chart"

necrosis. They are divided into small and large cell subtypes, based on the morphological features of the neoplastic cells, but this distinction does not have a relevant prognostic impact. Small cell carcinomas are composed of small to medium-sized (2–4 times the size of a small lymphocyte), round to oval cells with scant cytoplasm, indistinct cell borders and hyperchromatic nuclei with inconspicuous nucleoli. Large cell subtypes are composed of large cells with vesicular nuclei showing prominent nucleoli and abundant eosinophilic cytoplasm. Although tumor cells grow forming sheets or large nests, in the large cell subtype more structured organoid architecture can be observed. Mitotic figures are extremely frequent and the mitotic count >10 mitoses/10HPF is the cut-off for the distinction between atypical carcinoids and large cell neuroendocrine carcinoma of the lung (Table 2.2). The neuroendocrine nature of the neoplastic proliferation has to be confirmed by immunohistochemical analyses, as the differential diagnosis may be a challenging task and includes a number of poorly differentiated nonendocrine epithelial neoplasms, as well as nonepithelial tumors such as PNET, Ewing sarcoma, desmoplastic small round cell tumors, and myeloid and lymphoid leukemia.

Mixed neuroendocrine/nonneuroendocrine neoplasms (MiNENs) are neoplasms with both a neuroendocrine and a nonneuroendocrine component, each representing at least 30% of the tumor mass (Fig. 2.3). The spectrum of mixed

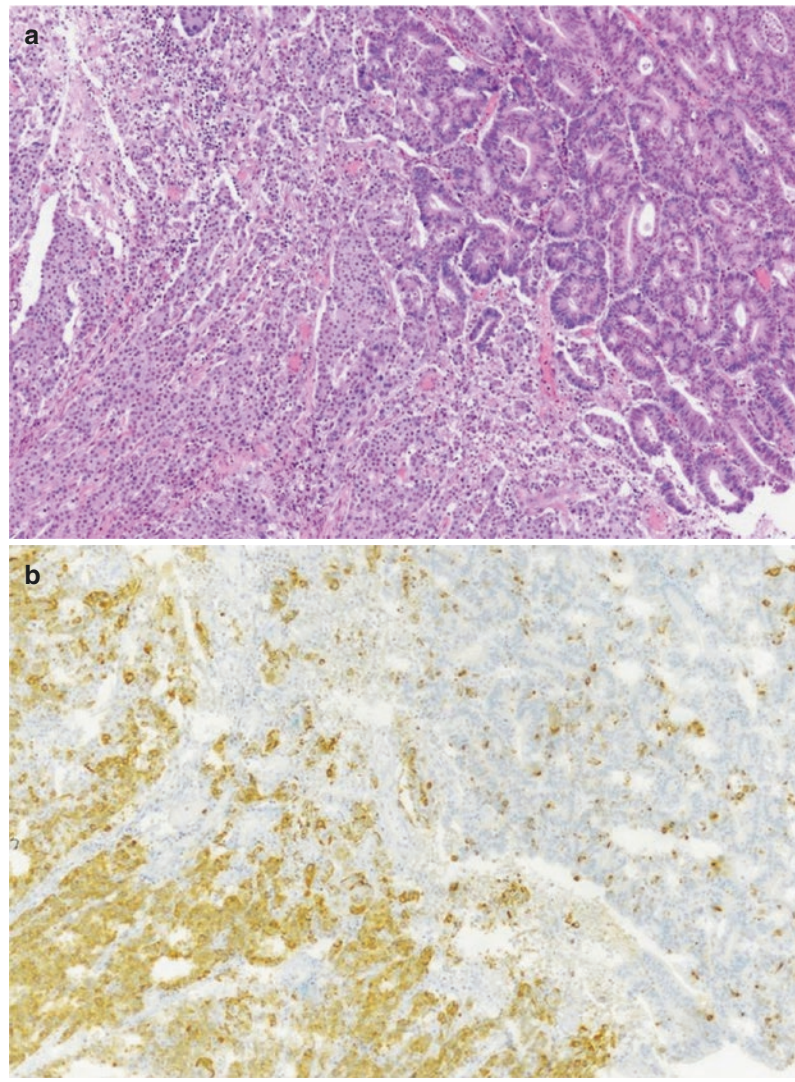

Fig. 2.3 Mixed neuroendocrine/nonneuroendocrine neoplasm (MiNEN) of the colon, composed of a moderately differentiated adenocarcinoma and a large cell neuroendocrine carcinoma (**a**). The immunostaining with anti-chromogranin A antibody highlights the neuroendocrine component and a number of neuroendocrine cells interspersed in the adenocarcinomatous component (**b**)

neoplasms is wide and encompasses all the possible combinations between neuroendocrine neoplasms (NETs and NECs) and other epithelial tumors (adenomas, adenocarcinomas, and squamous cell carcinomas). Consequently, their biological and clinical behavior is variable and depends on the grade of malignancy of each component [31].

2.4 Immunohistochemical Markers

Immunohistochemistry is a cornerstone for both the diagnosis and the prognostic classification of neuroendocrine neoplasms (NENs). In addition, immunohistochemical markers provide important clues to the possible primary site of origin of NENs presenting as metastatic lesions.

The diagnosis of NENs relies on the confirmation of their neuroendocrine nature, by means of *general neuroendocrine markers*. In addition, the demonstration of the epithelial nature of the neoplastic proliferation, using *cytokeratins*, is important to rule out the possibility of neural-derived and other neoplasms that can express general neuroendocrine markers. The histopathological classification of NENs is the result of a careful cytomorphological analysis, including mitotic count. However, the use of an immunohistochemical *marker of cell proliferation* is highly advisable to improve the prognostic stratification. In fact, the Ki67-related proliferative index is incorporated in the WHO classification of GEP NENs and has a pivotal role in distinguishing different prognostic categories among well-differentiated NETs [4]. In addition to morphological parameters and the proliferative index, a number of potential *prognostic markers* have been proposed, mostly related to specific sites. Finally, in NENs clinically presenting as metastatic lesions, the use of *site-specific markers* may help in identifying the primary site of origin, with important prognostic and therapeutic implications.

2.4.1 General Neuroendocrine Markers

2.4.1.1 Chromogranin A

Chromogranin A, chromogranin B and secretogranin II (chromogranin C) are the best characterized members of the granin family; a group of glycoproteins which represent the major constituents of neuroendocrine secretory granules [42, 43]. While anti-chromogranin A commercial antibodies are widely used in routine diagnostics (Fig. 2.4a, b), antibodies against chromogranin B and secretogranin II are available but are not generally used. Chromogranin A is strongly expressed in normal neuroendocrine cells and, together with synaptophysin (see later in the text) it is the first-choice marker to confirm the neuroendocrine nature of a neoplasm. It is a very specific neuroendocrine marker, however the immunostaining, which is strong and diffuse in NETs, may be weak, focal or even absent in NECs, whose cells contain few secretory granules. Furthermore, a subset of NETs, mainly including L-cell NETs of the hindgut, may be negative for chromogranin A [44]. For these reasons, using a panel of neuroendocrine markers, including at least synaptophysin, is always advisable.

2.4.1.2 Synaptophysin

Synaptophysin is an integral membrane calcium-binding glycoprotein (38,000 kDa), which is the main constituent of synaptic vesicles of neurons [45]. In normal and neoplastic neuroendocrine cells, synaptophysin is present in cytoplasmic

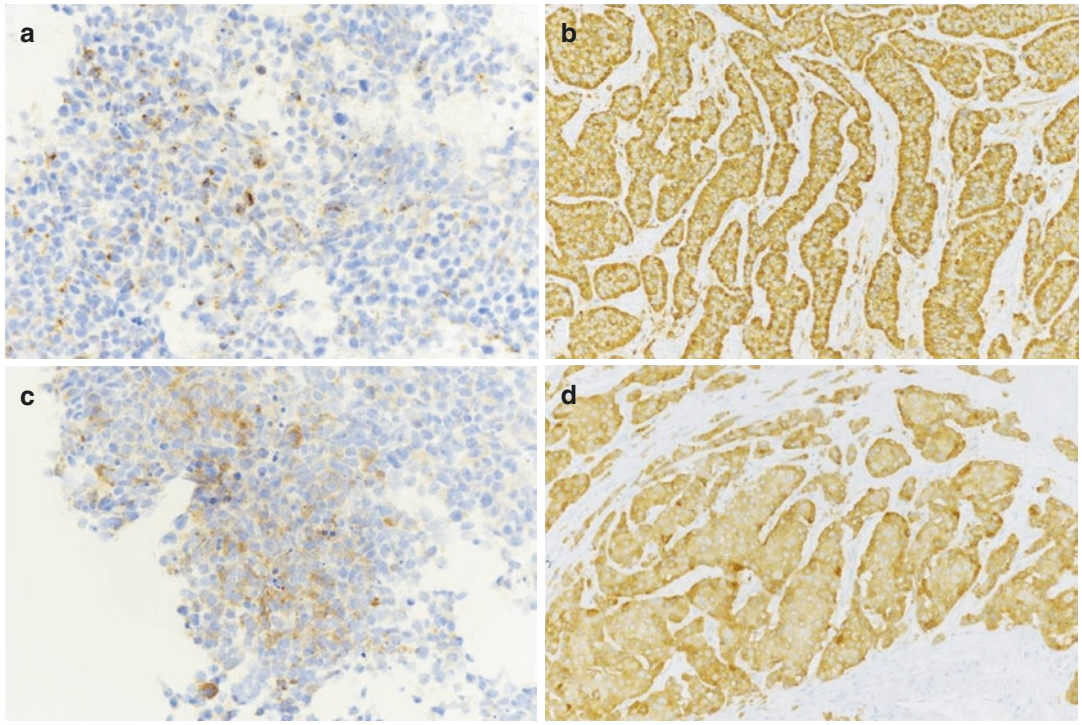

Fig. 2.4 General neuroendocrine markers expression in neuroendocrine neoplasms. Dot-like paranuclear immunostaining for chromogranin A (**a**) in a cytological preparation of a poorly differentiated carcinoma, which is also positive for synaptophysin (**c**). Intense and diffuse immunostainings for chromogranin A (**b**) and synaptophysin (**d**) in histological slides of a well-differentiated neuroendocrine tumor

microvesicles, and not in secretory granules [45], and it represents the most sensitive general neuroendocrine marker, being expressed both in well differentiated and in poorly differentiated NENs (Fig. 2.4c, d). However, it is not a specific neuroendocrine marker, as it is also expressed in non-neuroendocrine tissues and neoplasms, such as adrenal cortical carcinomas, neuroblastomas, olfactory neuroblastomas, and Ewing sarcomas/PNETs [45, 46]. Again, it is evident that a panel of antibodies is mandatory for a correct diagnosis of the NEN.

Other proteins associated with synaptic vesicles are present in normal and neoplastic neuroendocrine cells and may be used as neuroendocrine markers. Among these, *synaptic vesicle protein 2 (SVP-2)* [47], *vesicular monoamine transporters 1 and 2 (VMAT1 and VMAT2)* (Fig. 2.5a, b) [48, 49], and *L-type amino acid transporter (LAT) 1 and 2* [50] have been investigated the most.

2.4.1.3 Other Markers

A number of antigens have been proposed and used in immunohistochemistry as general neuroendocrine markers. As a whole, most of them are not as sensitive and specific as synaptophysin and chromogranin A. However, they may be of use when the immunostaining for one of these mark-

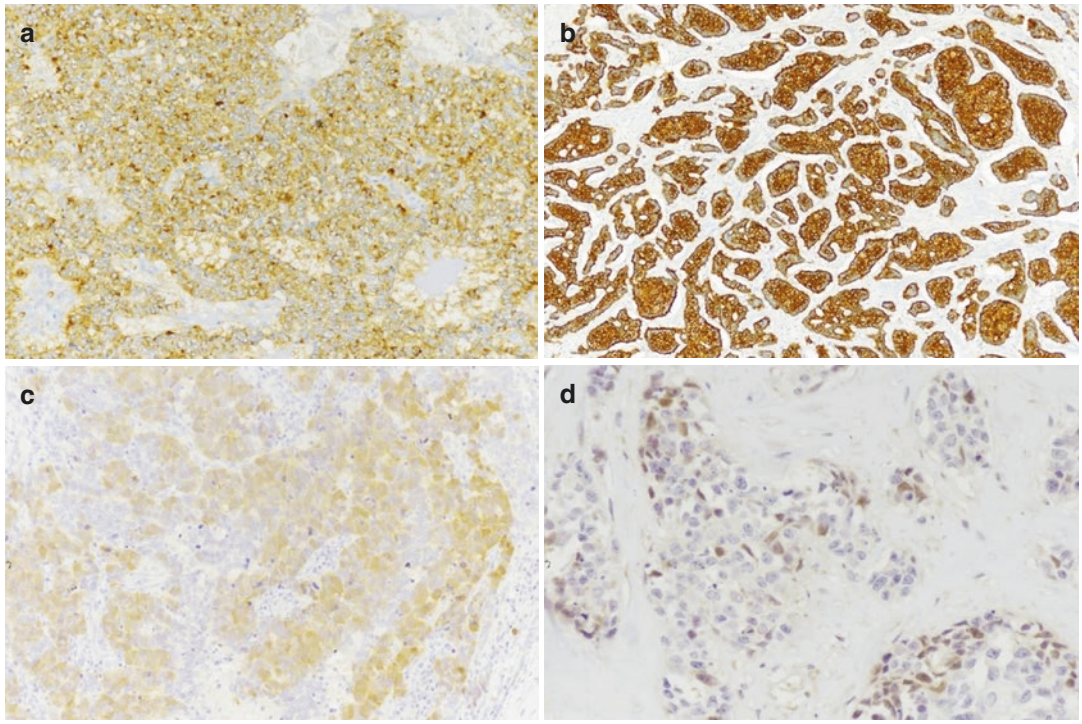

Fig. 2.5 Additional general neuroendocrine markers in neuroendocrine neoplasms. v-MAT2 is intensely expressed in a gastric ECL-cell NET (**a**). v-MAT1 immunostaining is strong and diffuse in an ileal EC-cell NET (**b**). Immunoreactivities for histidine decarboxylase (**c**) and ASH1 (**d**) are present in poorly differentiated neuroendocrine carcinomas

ers, like chromogranin A, is weak or absent which may happen in NECs or in specific types of NETs (i.e., hindgut NETs).

Neuron-Specific Enolase and Other Enzymes Involved in Hormone Synthesis and Metabolism

Historically, *neuron-specific enolase (NSE)*, the gamma-gamma isoform of enolase, has been widely used as a general neuroendocrine marker. However its specificity has been debated and it has been demonstrated that anti-NSE antibodies cross-react with other dimeric isoforms of enolase, expressed in nonneuronal and nonneuroendocrine cells [51]. This marker has currently been removed from the diagnostic immunohistochemical panels for NENs. Other enzymes, such as the *protein gene product 9.5 (PGP9.5)/ubiquitin-C-terminal hydrolase 1 (UCHL-1)* [52], *L-DOPA decarboxylase* (*L-aromatic amino acid decarboxylase*) [49, 53], *tyrosine-hydroxylase*, *dopamine β-hydroxylase*, *phenylethanolamine N-methyltransferase*, and *histidine decarboxylase* (Fig. 2.5c) [49, 54] have been variably used as general neuroendocrine markers, although their use is not currently recommended in daily diagnostic practice.

Surface Antigens

Two cell surface proteins, classified as a cluster of differentiation (CD), have also been suggested as general neuroendocrine markers. *CD56*, which is the *neural cell adhesion molecule (N-CAM),* has high sensitivity in identifying the neuroendocrine phenotype of a neoplasm. However, it lacks specificity, as anti-CD56 antibodies also stain other neoplasms, such as well-differentiated thyroid carcinomas, hepatocellular carcinoma, cholangiocarcinoma, renal cell carcinoma, ovarian carcinoma, endometrial carcinoma, Wilms' tumor, neuroblastoma, and plasma cell myeloma [55–60].

The second cell surface protein employed as a general neuroendocrine marker is *CD57*, which, again, is not specific for neuroendocrine differentiation, as it is expressed in a variety of other normal and neoplastic cell types, including oligodendroglial cells, Schwann cells and epithelial cells [61, 62].

Achaete-Scute Homologue 1

Achaete-scute complex-like 1 (ASCL1), or Achaete-scute homologue1, termed *mASH1* in rodents and *hASH1* in humans, is a member of the basic helix-loop-helix family. It is a crucial transcription factor for neuroendocrine cell differentiation and it has been shown to be involved in the development of neuroendocrine cells of the thyroid, adrenal medulla, and foregut [63, 64]. The high value of ASH1 in the immunohistochemical workup of NENs relies on the fact that it seems to be expressed exclusively in poorly differentiated NECs (Fig. 2.5d), whereas in NETs its expression is lacking, except for a subset of lung carcinoids [65, 66]. In small biopsies, ASH1 may thus be helpful, together with Ki67, in the differential diagnosis between high grade and low grade NENs [26, 27]. In addition, as ASH1 expression seems to be restricted to NECs, it can also be used as a general neuroendocrine marker in the differential diagnosis of high grade neoplasms.

2.4.2 Epithelial Markers

The demonstration of the epithelial nature of neoplastic cells is important in the diagnosis of NEN, as both NETs and NECs have nonepithelial mimickers, and the differential diagnosis is important for the correct management of patients. Although a fraction of NENs may not express epithelial markers, cytokeratin-negative NETs should be differentiated from paragangliomas [67], as well as cytokeratin-negative NECs, particularly in small biopsies, which may be confused with other neuroectodermal neoplasms and with neuroendocrine markers-expressing sarcomas [68, 69].

The most useful epithelial markers are cytokeratins, which are intermediate filaments, present in the cytoskeleton of epithelial cells. Antibodies directed against low- and high-weight cytokeratins (CK AE1/AE3) and anti-cytokeratin 8 (CAM 5.2, which cross-reacts with CK18) are most commonly used in routine immunohistochemical practice to detect the epithelial nature of a neoplasm. Antibodies directed against specific cytokeratins may be used in the differential diagnosis with nonneuroendocrine neoplasms (e.g., high weight cytokeratins are absent in lung neoplasms, while they are expressed in squamous cell lung cancer), in the search for an unknown primary origin (e.g., CK20 is expressed in Merkel cell carcinoma and not in lung NENs, whereas CK7 may give a clue to pulmonary or pancreatic origin), or in assessing prognosis (e.g., CK19 expression in pancreatic NENs) [70–72].

2.4.3 Markers for Proliferation: Ki67

Alterations in cell growth and proliferation are key events in neoplastic transformation and in cancer progression. The proliferative fraction of a neoplastic population is correlated with tumor grade and to its biological aggressiveness and has important clinical implications in terms of patients' outcome and management [73]. As mitotic count represents only one aspect of the proliferating cell, in order to better assess the proportion of neoplastic cells in all of the phases of the cell cycle, immunohistochemical markers of proliferation have been optimized.

Ki67 antigen is a cell proliferation marker expressed in the nuclei of normal and neoplastic proliferating cells, along all cell cycle phases (G1, S, G2, and M), while it is absent from resting cells in the G0 phase. These properties make the Ki67 labeling index (i.e., the percentage of immunoreactive neoplastic nuclei of the total neoplastic nuclei) a good proliferation marker, with a close correlation to the real growth fraction of the neoplastic population [74]. The important prognostic value of the Ki67 labeling index in NENs has gained almost universal consensus, and the neuroendocrine proliferations of the GEP tract are currently classified by the World Health Organization (WHO) on the bases of morpho-

logical differentiation, mitotic count, and Ki67 index [4, 15]. In the recent new edition of the WHO classification of pulmonary NENs, Ki67 index is not integrated in the definition of the different entities. However, the diagnostic and prognostic value of the Ki67 index is recognized, and the range of values are inserted among the diagnostic criteria for the first time: 50–100% for small cell carcinomas, 40–80% for large cell carcinomas, up to 20% for atypical carcinoids and up to 5% for typical carcinoids [3].

2.4.4 Site-Specific Markers

NENs are frequently metastatic at clinical presentation and in up to one third of the cases the site of origin of the tumor is unknown [75]. The identification of the site of the primary neoplasm is important in making the correct treatment decision, especially in the case of well-differentiated NETs, in which therapeutic protocols may vary also according to the site of origin. In poorly differentiated NECs, the major clinical problem is represented by cutaneous neoplasms, in which the distinction of Merkel cell carcinomas from visceral NEC is crucial for correct management. Imaging techniques, including positron emission tomography, are able to identify the primary NEN in a consistent proportion of cases, but in more than 15% of patients it remains occult [72]. The pathologist is therefore asked to give clues to the possible primary site, and the use of a correct panel of immunohistochemical markers is a powerful tool to answer this question.

2.4.4.1 Well-Differentiated NETs

Transcription Factors

Caudal Type Homeobox 2 (CDX2)

CDX2 is a homeobox domain-containing transcription factor, which is involved in gut development and the maintenance of the intestinal phenotype in epithelial cells. It is expressed in the epithelium of the small and large intestine [76]. CDX2-expressing cells are present not only in pancreatic centroacinar, intercalated and intralobular duct cells, but also in scattered ductal cells [77]. CDX2 immunostaining is widely used in diagnostic pathology to assess the intestinal differentiation of adenocarcinomas. It is expressed in the vast majority of intestinal and appendiceal adenocarcinomas, but also in intestinal type adenocarcinomas of the stomach, esophagus, pancreas, gallbladder and extrahepatic biliary tract, ovary, uterine cervix, urinary bladder, and nasal cavity [78–80].

CDX2 immunostaining (Fig. 2.6a, b) in well-differentiated NETs is highly sensitive and fairly specific for a midgut origin [81–83]. A meta-analysis of 14 papers assessing CDX2 expression in NETs of different sites (lung, stomach, duodenum, pancreas, jejunum/ileum, cecum, colon, and rectum) has revealed that a strong and diffuse CDX2 immunostaining is present in more than 90% jejunoileal and appendicular NETs. By contrast, CDX2 immunoreactivity was detected only in about 30% of duodenal and rectal primaries, and in about 15% of gastric and pancreatic tumors, with a faint and patchy staining. As little as 3% of lung carcinoids have been found to show CDX2 expression [71].

Thyroid Transcription Factor-1 (TTF-1)

TTF-1 is another homeodomain-containing transcription factor, and it is involved in the development of the thyroid, of the lung and of the diencephalon. This marker is widely used in the diagnostic pathology of lung and thyroid neoplasms. TTF-1 expression in well-differentiated NET is a very specific marker of pulmonary origin (Fig. 2.6c, d), but its sensitivity is not high and it has been reported as very variable in different papers. Interestingly, peripheral spindle cell carcinoids seem to express TTF-1 more frequently than central carcinoids [84]. In the NENs context, one should also bear in mind that TTF-1 expression is nearly always present in medullary carcinoma of the thyroid and immunostaining for calcitonin and CEA may be of help in defining the diagnosis [67].

Paired Box Gene 8 (PAX8)

PAX8, a member of the paired box transcription factors family, is involved in thyroid and kidney

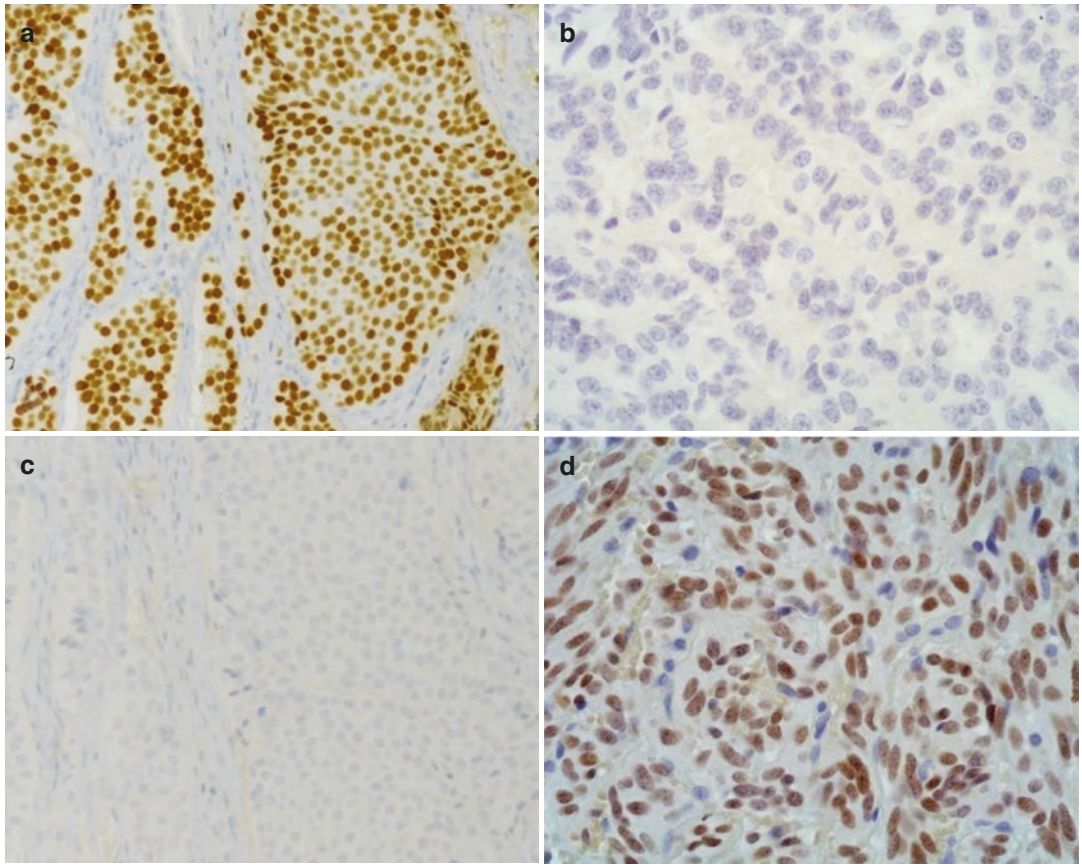

Fig. 2.6 Transcription factors are useful in the identification of the site of origin of well-differentiated neuroendocrine tumors (NETs). CDX2 is intensely expressed in an ileal NET (**a**), which is negative for TTF-1 (**c**). By contrast, pulmonary carcinoids are CDX2-negative (**b**), and TTF-1-positive (**d**)

development and is expressed in carcinomas arising in these organs. It has also been described as a good marker for Müllerian duct-derived neoplasms [72]. PAX8 immunostaining has also been detected in pancreatic islet cells and it has been demonstrated that it can be used as a marker of a pancreatic origin in well-differentiated NETs [67, 72]. Of note, it has been reported that duodenal and rectal NETs express PAX8, whereas ileal NETs are not immunoreactive [72].

Insulin Gene Enhancer Binding Protein Isl-1 (Islet 1)

Islet 1 is another homeodomain-containing transcription factor, which is important in the embryonal development of neuroendocrine and neural cells and is highly expressed in Langerhans' islet cells [85]. It is a good marker of pancreatic origin in well-differentiated NETs and its sensitivity is superior to PAX8, with which it shares the immunostaining of ileal and rectal NETs [72].

Pancreatic and Duodenal Homeobox 1 (PDX1)

This transcription factor is crucial in the development of the pancreas and of the duodenum. In the adult, its expression is restricted to pancreatic islet cells, whereas it is absent in acinar and ductal structures [86]. Among well-differentiated NETs, PDX1 expression is neither a specific nor a sensitive marker for primary pancreatic neoplasms. However, as it has been detected in a subset of pancreatic, duodenal and gastric NETs, whereas it is absent in ileal and pulmonary carcinoids, the

main utility of positive immunostaining for PDX-1 seems to be the exclusion of an ileal or pulmonary neoplasm [87].

Amine and Peptide Hormones

Commercial antibodies to a wide variety of peptide hormones are available, including serotonin, substance P, calcitonin, gastrin, pancreatic hormones (insulin, glucagon, somatostatin, and pancreatic polypeptide) and intestinal hormone peptides (gastric inhibitory peptide, motilin, secretin, cholecystokinin, vasoactive intestinal polypeptide, glicentin, and peptide YY). Apart from calcitonin, which is, along with CEA, a very sensitive and specific marker for medullary carcinoma of the thyroid, the use of these antibodies is of limited clinical utility.

2.4.4.2 Poorly Differentiated NECs

As previously mentioned, the differential diagnosis between a Merkel cell carcinoma (MCC) and a cutaneous metastasis of a poorly differentiated visceral NEC represents the single most relevant situation in the management of metastatic NECs. It has been demonstrated that the use of an immunohistochemical panel including TTF-1, cytokeratin 20 and, more recently, MCC polyomavirus (MCPyV), represents an effective approach to this problem. The immunoreactivity for cytokeratin 20 and MCPyV, in the absence of TTF-1 immunostaining is diagnostic for MCC [88, 89]. As for TTF-1 and other transcription factors, the pathologist should be well aware that their expression may be not site-specific (Fig. 2.7) and should not be used in to search for an occult primary [90].

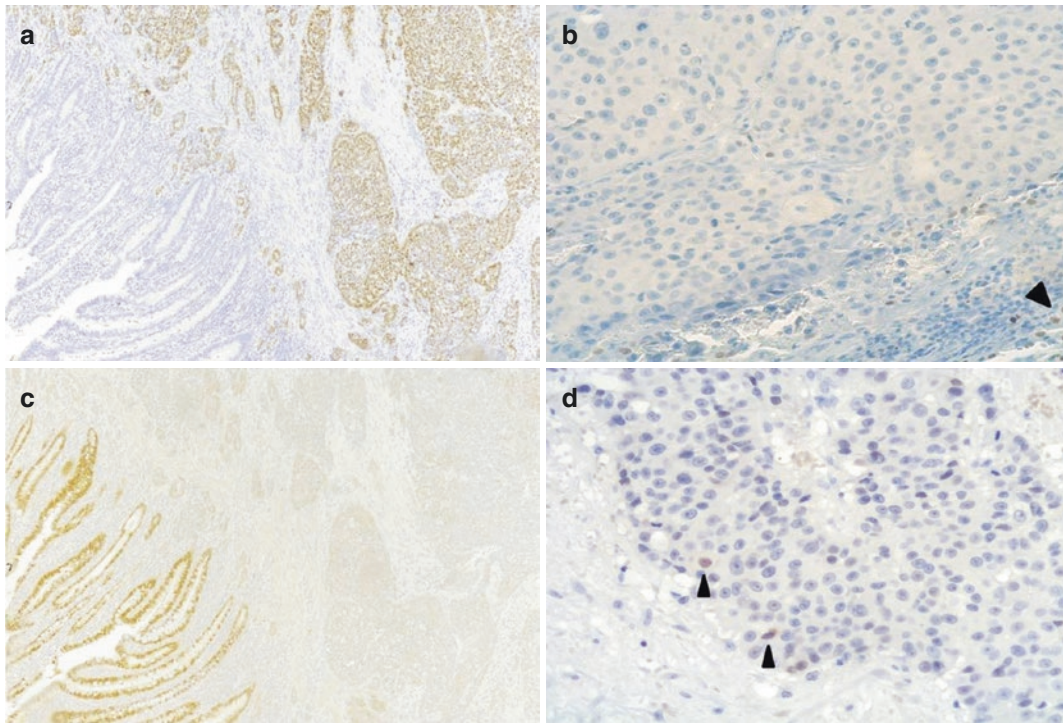

Fig. 2.7 Transcription factors are not useful in the identification of the site of origin of poorly differentiated neuroendocrine carcinomas (NECs). TTF-1 may be expressed in primary colonic NECs (**a**) and not in NECs of the lung (**b**) (*arrowhead* indicates positive internal control, represented by normal pneumocytes). On the other hand, CDX2 immunoreactivity may be absent in colonic NECs (**c**) (positive control in the overlying colonic mucosa, in the left side of the picture), whereas it may be expressed by scattered cells in a pulmonary NEC (**d**) (*arrowheads* indicate two positive nuclei. With permission from La Rosa et al. Virchows Arch 2004;445:248–54, reference #82)

2.4.5 Prognostic and Predictive (Theranostic) Markers

2.4.5.1 Somatostatin Receptors

Somatostatin is a peptide hormone produced in regions of the central nervous system and by D cells in the GEP tract, where it suppresses the release of several hormones. The cell sensitivity to somatostatin is mediated through members of the somatostatin receptors family (SSTRs), composed of at least five subtypes (SSTR1, 2, 3, 4, and 5). SSTRs are frequently expressed by NENs, both in NETs and in NECs, and this is the rationale of the OctreoScan, in which the somatostatin analog octreotide is coupled to ^{111}In to allow the identification of NENs with nuclear medicine imaging. In addition, somatostatin analogs are used as antisecretory drugs in functioning tumors (including patients with carcinoid syndrome) and seem to have a tumoristatic activity in NETs [91]. Somatostatin analogs used in diagnostic and therapeutic settings have the highest affinity for the type 2A receptor (SSTR2A), for this reason its detection in tumor tissues with immunohistochemistry has been implemented (Fig. 2.8). The availability of a monoclonal anti-SSTR2A antibody has improved the specificity and sensitivity of the immunostaining on formalin-fixed and paraffin-embedded samples [92]. Volante and coworkers have proposed a three-tiered scoring system for the evaluation of SSTR2A immunoreactivity in neuroendocrine tumors, taking into consideration both the subcellular localization and the extent of the staining. Pure cytoplasmic immunoreactivity without membranous staining corresponded to score 1, whereas score 2 and 3 were attributed to cases with membranous immunoreactivity in less or more than 50% of cells, respectively. Importantly, only membranous immunoreactivity had a good correlation with positivity to somatostatin receptor scintigraphy and a good response to cytostatic therapy with somatostatin analogues [93]. Interestingly, unrelated groups have recently demonstrated an independent prognostic role of SSTR2A immunohistochemistry in GEP and pulmonary NETs. In fact, SSTR2A membranous immunoreactivity has been reported to be associated to a longer overall and progression-free survival, both in NETs and in NECs [94–96].

2.4.5.2 Cytokeratin 19

Cytokeratin 19 (CK19) is an acidic cytokeratin highly expressed in the exocrine component of the human adult pancreas, including duct and centroacinar cells, whereas it is absent in normal islets of Langerhans [97, 98]. Aberrant CK19 expression was found in a subset of pancreatic

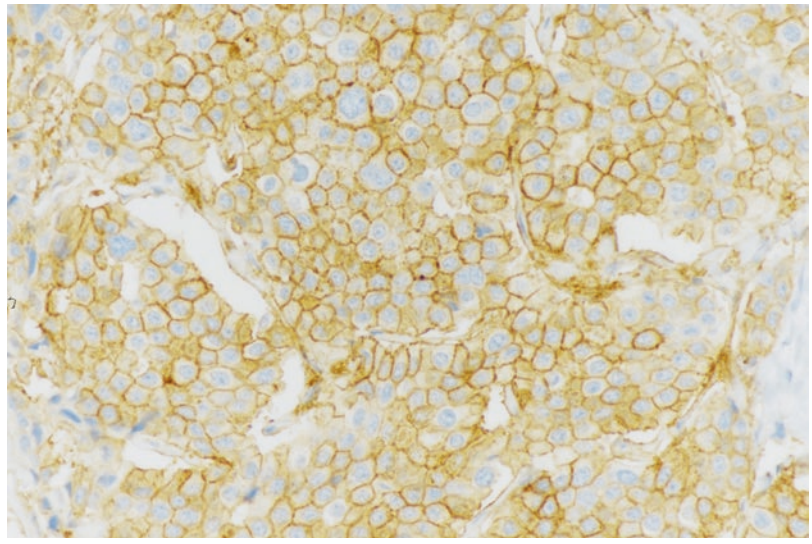

Fig. 2.8 Intense and complete membranous immunoreactivity for SSTR2A in a duodenal gastrinoma

well-differentiated NETs and it has been reported to be an independent prognostic marker in these tumors [99, 100]. However, a subsequent study from our group did not confirm the independent adverse prognostic role of CK19 immunoreactivity, which, although more frequently observed in aggressive NETs, failed to reach statistical significance in multivariate analysis. In addition, we reported that the sensitivity and specificity of the anti-CK19 antibodies in detecting aggressive pancreatic well-differentiated NETs, depend on the clone employed [101].

2.4.5.3 CD117

CD117, also named c-kit, is a type 3 tyrosine kinase receptor of the platelet-derived growth factor subfamily. It is expressed in multiple cell types and it has been shown to be a marker of progenitor cells in the human pancreas. In pancreatic NETs, CD117-immunoreactivity was found to be an independent prognostic marker, being preferentially expressed in aggressive tumors [102, 103]. A number of studies have demonstrated significant overexpression of CD117 in high-grade NECs, including pulmonary, GEP and cutaneous (Merkel cell carcinoma) carcinomas [104–106], and an adverse prognostic significance of this marker's expression has been reported [90, 102, 107]. However, CD117 immunoreactivity in NENs does not seem to be related to an underlying activating mutation of the c-Kit gene, and this could be an explanation of the poor effectiveness of imatinib mesylate (Gleevec) therapy in these patients [108, 109].

2.4.5.4 Mismatch Repair Proteins

The coexistence of microsatellite instability (MSI) and widespread gene methylation is a predictor of a better outcome in patients with gastrointestinal NECs [107, 110]. Since there is a good correlation between MSI and the immunohistochemical loss of mismatch repairs proteins, immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 may be included in the diagnostic panel to identify a lower risk class among these very aggressive neoplasms.

2.5 Practical Applications of Immunohistochemistry in Routine Diagnosis

2.5.1 Cytology

Ancillary techniques should be employed all the time to confirm the morphological diagnosis [67]. In general, the same immunohistochemical markers used in histology can be applied on the cytoblock preparation or on smears (Fig. 2.4). The most frequently used markers include, in order of importance and in case of limited material, CD45 and pan cytokeratin, and then the panel of neuroendocrine markers such chromogranin A, synaptophysin, CD56 and NSE. Ki-67 can be used to gain an understanding of the proliferative rate in the case of particularly crashed material, but it is not mandatory in cases of pulmonary NETs and NECs. On the contrary, for pancreatic neuroendocrine lesions or metastatic pancreatic NECs, Ki67 is used for grading purposes and has also been validated for its use on cytological material (Fig. 2.1) [111, 112]. Hormones and predictive markers can also be applied in cytological specimens, if needed.

2.5.2 Histology

The minimal immunohistochemical tests recommended by different guidelines are: chromogranin A, synaptophysin, and Ki67 [4, 113, 114]. While chromogranin A and synaptophysin can be regarded as diagnostic tests (Fig. 2.4), Ki67 has to be considered as both a diagnostic and prognostic marker. The usefulness of chromogranin A and synaptophysin for defining the neuroendocrine nature of a tumor has been discussed above in the specific paragraph. The possible pitfalls of the use of chromogranin A in the diagnostic workup of NECs, where it may be focal or absent reflecting the reduction or absence of secretory granules, which depends on the deficient differentiation of neoplastic cells, have also been underlined. In these cases the use of other general

neuroendocrine markers such as the cytosolic components NSE and PGP 9.5, or the membrane molecule CD56 may be necessary, though this depends on the experience of the pathologist. It is worth noting that chromogranin A immunoreaction can also be negative in some NETs, in particular in those of the rectum, even in the presence of abundant intracellular neuroendocrine secretory granules and heavy immunoreactivity for the hormones glicentin, PP, and PYY. As a general rule, at least two positive general neuroendocrine markers are needed to substantiate the neuroendocrine differentiation of a tumor.

The minimal number of hormonal markers necessary for the routine diagnostic workup includes antibodies against insulin, gastrin, and serotonin, either by a clinician's request and/or to provide information for a better evaluation of the clinical profile and for the patient's follow-up. Similarly, on specific clinical request, the assessment of SSTR2A in tumor tissue may be necessary. To this end, the use of the score recently proposed by Volante et al. [93] is recommended.

The use of a minimal immunohistochemical panel including transcription factors CDX2, TTF1 and the hormones serotonin, gastrin and insulin is also recommended for the workup of liver or lymph node metastases from occult NETs [75].

2.6 Circulating Molecular Markers

In the last 10 years, several attempts have been made to elucidate the molecular mechanisms associated with the development and progression of NENs and a large amount of information is available in the literature to date. It is now clear that poorly differentiated NECs show distinct molecular alterations compared to well-differentiated NETs. NECs are mainly characterized by p53 and Rb1 alterations, independently of their site of origin [5]. Conversely, gene alterations found in NETs are more heterogeneous and can be associated with the site of origin; *ATRX*, and *DAXX* gene mutations are more frequently observed in pancreatic NETs [115], while MEN1 mutations and/or losses are found in variable percentages of lung and GEP NETs [116]. It is worth noting that, in addition to gene mutations, epigenetic mechanisms are involved in NET development and progression and gene methylation has been recently demonstrated to play a developmental and prognostic role in pancreatic NET [117]. In general, the analysis of molecular alterations is not needed for tumor diagnosis and classification, which is generally easily achieved using morphology and immunohistochemistry. However, the detection of specific molecular features may be useful for the prognostic evaluation and prediction of therapy effectiveness. The systematic review of all the molecular alterations involved in GEP and lung NENs is beyond the scope of the present chapter, where only new information regarding a practical or potential role of new molecular plasma markers in clinical workup is discussed.

Traditional biomarkers that can be identified in the blood stream, including chromogranins and various hormones, have been proved to present several limitations in terms of assay reproducibility, sensitivity, and specificity with the consequent need to find more efficient biomarkers [118, 119]. Detection of circulating transcripts, microRNA, and circulating tumor cells is a new intriguing and promising approach to the diagnosis and management of patients with NENs. Currently, the most widely investigated biomarker tool is the blood-based multianalyte transcript analysis [120, 121]. The multianalyte-derived NET gene signature encompasses the expression of 51 genes which are assessed by four different prediction algorithms and seems to give information on tumor state and evolution, from stability to progression [120]. This approach defines the circulating fingerprint of the tumor showing a higher sensitivity and specificity than traditional secretory markers. The gene expression profile is mathematically analyzed using specific algorithms, which define tumor activity. Interestingly, recent data have suggested that Circulating Transcript Analysis (NETest) may

identify tumor categories with a different prognosis and response to somatostatin analogues and peptide receptor radionuclide (PRRT) therapy [122–124]. However, this new approach shows some problematic issues including technical complexity, which restricts the analysis to specific laboratories. However, a Delphic consensus assessment has considered that circulating RNA detection seems better than traditionally employed general NEN biomarkers. It has been decided that circulating multianalyte mRNA (NETest) may have clinical utility in both the diagnosis and monitoring of therapeutic efficacy. Overall, it has been concluded that a combination of tumor spatial and functional imaging with circulating transcripts (mRNA) would represent the future strategy for real-time monitoring of disease progress and therapeutic efficacy [125].

In addition to RNA multigene analysis, miRNAs have been considered as potentially useful circulating biomarkers; miRNA are a class of small noncoding RNAs functioning as post-transcriptional regulators. They can be deregulated in neoplasia and may have a potential role as biomarkers. Global miRNA profiles have been evaluated in GEP and lung NETs and have shown nonoverlapping expression among different NET types [126, 127]. Upregulation of miR-103 and miR-107 and downregulation of miR-584, miR-1285, miR-550-002410, and miR-1825 were found in pancreatic NETs [128, 129] and, interestingly, downregulation of serum miR-1290 was able to differentiate pancreatic NETs from adenocarcinomas [129]. In small intestine NETs, other miRNAs seem to be involved and some of them were found to be upregulated (miR-96, miR-182, miR-183, miR-196, and miR-200) or downregulated (miR-31, miR-129-5p, miR-133a, and miR-215) [130]. In the lung, NENs have different miRNA expression profiles that correlate with different tumor categories [127]. However, weak correlations between miRNA expression levels in both tumor tissue and serum have been reported and the fact that some miRNAs are upregulated while others are downregulated suggests that the use of this marker is a complex task which needs to be considered with caution. The American College for Clinical Chemistry underlined several problems related to use of miRNAs in NETs including the low reproducibly and accuracy of the tests used, so additional clinical information is needed before using miRNAs in clinical practice.

Detection of circulating tumor cells, already approved for monitoring breast, prostate, and colon cancers is a novel and interesting approach to the study of NENs. In a recently published study, the prognostic value of circulating tumor cells was demonstrated [131], but further investigations are needed to corroborate the prognostic role of this marker. Recently reported guidelines established that circulating tumor cell analysis is not a sensitive and specific diagnostic tool for NETs [119].

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3 Circulating Biomarkers: Biological Basis, Methods, and Interpretation Criteria

Stefan Holdenrieder

3.1 Biological Basis and Use of Biomarkers

Biological markers are alterations on the cellular, biochemical, or molecular level that can be objectively measured in the tissue, blood, or other bodily fluids and that indicate a physiological or pathophysiological condition or a response to a therapeutic intervention [1, 2]. Biomarkers are frequently used for multiple indications such as risk assessment or prediction of a disease, diagnosis, estimating prognosis, or monitoring the disease course during or after therapy. Thereby they complement other diagnostic approaches such as imaging or clinical exams. Blood or body fluid biomarkers comprise cells, cellular particles, and diverse molecules such as proteins, peptides, amino acids, carbohydrates, lipids, nucleic acids, drugs, and others. In cancer disease, cell surface and secreted proteins and peptides are most frequently used. Newer approaches include circulating nucleic acids that are released from cancer cells into plasma and serum (CNAPS) such as cell-free tumor DNA (ctDNA) with its genetic or epigenetic characteristics, gene expression fingerprints, as well as patterns of regulative noncoding RNAs (miRNAs and lncRNAs). These markers can also be extracted from circulating cancer cells and exosomes that constitute an enrichment compartment for cancer-specific markers [3, 4]. To detect and quantify biomarkers reliably, highly sensitive and specific techniques are needed, and rigorous quality controls have to be performed in laboratories dedicated to patient diagnostics.

This chapter focusses on blood-based biomarkers that are in use for the diagnosis and management of patients with neuroendocrine tumors. While definitive diagnosis still requires imaging and tissue exams, this approach has several advantages as blood drawing is only minimal invasive and can be done serially in individuals. Furthermore, analyses are objective, quantitative, highly sensitive, robust, cost-effective, and highly quality controlled. During course of cancer disease, biomarkers that circulate in the blood can be employed to answer many questions that are highly relevant for the management of health and disease in a specific person. In detail they are applied for the following indications (Fig. 3.1):

- The screening of presumably healthy persons (without any symptoms)
- The monitoring of persons at risk for cancer disease (but without symptoms)
- The risk estimation of a person with suspicious symptoms or signs

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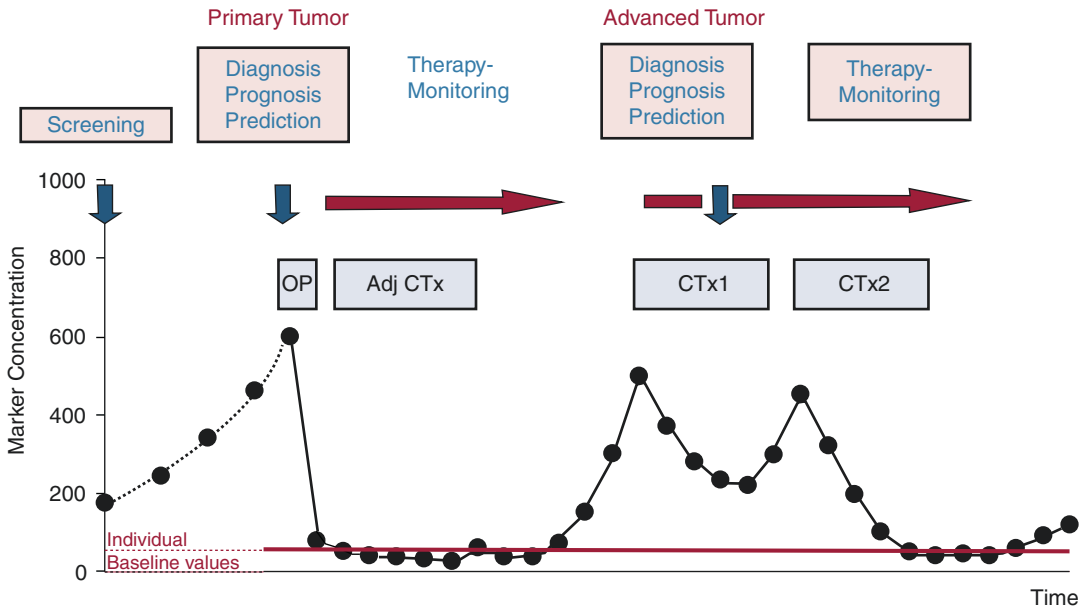

Fig. 3.1 Blood-based biomarkers can be used for many indications during the course of cancer disease, for (1) cancer detection and differential diagnosis, (2) estimation of prognosis, (3) prediction and monitoring of therapy response, (4) early detection of therapy resistance and of recurrent disease. Biomarker changes in relation to individual baseline values often sensitively mirror the course of disease. Cancer screening is the most challenging indication for circulating biomarkers (Adapted from [5]; with permission from Springer)

- The definitive estimation of differential diagnosis in persons with specific symptoms
- The estimation of severeness (and staging) of a cancer disease
- The estimation of prognosis in patients with a defined cancer diagnosis
- The stratification of cancer patients for a specific therapy
- The monitoring of the response to anticancer therapy
- The early estimation of therapy response as a special application
- The monitoring of a patient after the primary therapy
- The early detection of recurrent disease

In cancer patients, one-time biomarker determinations (often a combination of several markers) are performed for (1) *screening* purposes, (2) supporting *differential diagnosis*, and (3) estimating *prognosis*. In contrast, the monitoring of serial biomarker testings is mostly applied for (1) the *screening* of patients who are at risk for cancer disease, (2) the *monitoring response* to local or systemic therapies, and (3) the *early detection of disease recurrence* after the primary therapy has been finished. Newer biomarkers such as CNAPS markers are highly meaningful as *companion diagnostics* to stratify patients for a newly developed targeted therapy and to monitor the responsiveness of this therapy as well as for the *detection of drug resistance and biochemical recurrence* in order to enable an early and specific therapy adaptation on an individual basis [5].

3.2 Methods and Quality Requirements for Biomarkers

In order to give reliable and meaningful results that can be used for patient guidance, circulating biomarkers and the methods that are applied for

their determination have to fulfill the highest methodical, preanalytical, and clinical quality criteria if they are to be implemented into patient care. There are several methodical preconditions biomarker assays have to meet [6, 7], among others:

- A high analytical sensitivity (the analyte is detected at very low concentrations)
- A high analytical specificity (only the analyte is measured)
- A high accuracy including a high intra- and between-run imprecision
- A high recovery and dilution linearity in the given matrix
- A high robustness against potentially disturbing factors

Analytical performance of the assays has to be regularly controlled by internal and external quality controls.

Preanalytical aspects may greatly influence the results of biomarker measurements. Therefore, preanalytics should be standardized for routine diagnostics as well as for study settings. The following aspects have to be considered:

- The conditions of the patient and the blood drawing
  (time, fasting, position of the patient, tourniquet time, type of needle, etc.)
- The conditions of the material
  (type of blood matrix, i.e., serum or plasma, additives, tubes, volumes, etc.)
- The conditions of the transport to the lab (time, temperature, pneumatic delivery, etc.)
- The conditions of the centrifugation (time, temperature, speed, braking, etc.)
- The conditions of the sample handling
  (storage time, temperature, extraction, deep freezing, thawing frequency, etc.)

Potentially influencing preanalytical factors have to be considered prior to marker analysis as well as for the interpretation of marker results [5, 6].

3.3 Clinical Performance of Biomarkers

If biomarkers are applied to diverse clinical indications, some measures are informative about their clinical performance. For differential diagnosis of cancer disease, the *clinical sensitivity and specificity* of cancer biomarkers are greatly meaningful. The sensitivity indicates the percentage of positive results in the cancer patient group while the specificity is the percentage of negative results in the control group. Because for many cancer biomarkers the value ranges of cases and controls often overlap, it is hardly possible to define optimal cutoffs that enable cancer detection with 100% sensitivity and specificity. This is even more difficult if cancer patients are to be distinguished from the differential diagnostically relevant group of patients with organ-related nonmalignant diseases [8].

The diagnostic performance of a biomarker can best be demonstrated by *receiver-operating characteristic (ROC) curves* showing the complete profile of sensitivity and specificity. This graph gives the sensitivity and specificity at all possible cutoff points and is highly informative when the performances of different biomarkers are compared with each other. Meaningful measures are (1) the area under the curve (AUC), (2) the sensitivity at a defined specificity (e.g., 95%), or (3) an optimized sensitivity-specificity combination illustrated by the point closest to the left upper corner (Fig. 3.2). Most important is the choice of the groups that are compared by ROC curves. Best results are obtained if patients with advanced cancer disease are compared with young healthy individuals. However, in the clinical situation, it is more meaningful to distinguish coeval persons with suspicious symptoms who may suffer from an early cancer or a nonmalignant pathology. In these cases, the curves often will be less optimistic [8, 9].

Beyond diagnostic applications, ROC curves are also used to illustrate the performance of a biomarker for the staging of disease (e.g., early stage cancer vs. metastatic cancer) or for the staging of therapy response (e.g., remission vs.

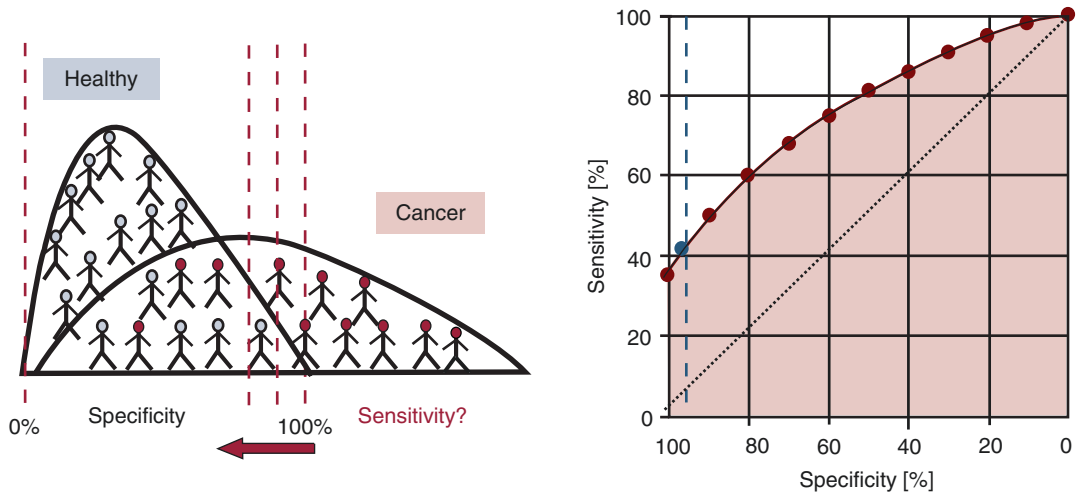

Fig. 3.2 Levels of many cancer biomarkers overlap with those from healthy individuals. Receiver-operating characteristic (ROC) curves are an elegant tool to illustrate the diagnostic performance of a biomarker over the whole value range. To establish the ROC curve, the percentages of correctly negative controls (specificity) and correctly positive cancer patients (sensitivity) are delineated for all possible cutoff points (decreasing stepwise from 100% specificity) and transferred to the scheme. The area under the curve (AUC) and the sensitivity at a fixed specificity (e.g. 95%) are most informative measures for the comparison of diverse diagnostic biomarkers. As control groups, healthy individuals and patients with benign organ-related diseases that are relevant for differential diagnosis are considered (Adapted from [5]; with permission from Springer)

non-remission). In the monitoring of disease, also kinetic information (increases or decreases of marker values) are used as marker variables.

It has to be pointed out that for screening purposes the *positive and negative predictive values (PPV and NPV)* are more important than the sensitivity and specificity. While PPV indicates the probability of disease if the value is positive, NPV gives the probability of being disease-free if the value is negative. This measure also takes the prevalence of a disease into account. Because the prevalence for cancer diseases in the normal population is quite low, PPV may be low even if the sensitivity and specificity are higher than 90%. Further, predictive values are informative if patients are stratified for specific therapies and responses are anticipated [5].

While *prediction* always relies to the response of a specific therapy, *prognosis* is related to the time of disease-free (DFS), progression-free (PFS), or overall survival (OS). Clinical and biomarker values can be obtained before or during a therapy. When monitoring therapy response, biomarker information that is available at the same time as the radiological staging can support the accurate estimation of the individual therapy response. If the information is available prior to the radiological staging, i.e., after one application of chemotherapy, the biomarker determination leads to a time advantage in terms of early estimation of therapy response that would enable an early and individual adaptation of the therapy strategy.

When a new cancer biomarker is evaluated on its clinical performance, a relevant number of patients with the target cancer disease have to be compared with healthy controls and patients with the organ-related benign diseases that are relevant for differential diagnosis [8, 10]. To get a whole picture of the usefulness of a biomarker, further cancer diseases and benign diseases that are involved in the marker catabolism such as renal and hepatic disorders have to be included as well. For therapy monitoring studies, a meaningful number of patients with a certain cancer that undergo a homogeneous type of therapy with favorable and non-favorable outcome have to be

considered. Recently published guidelines support the professional validation of biomarkers for diagnostic and monitoring purposes [11, 12] as well as for the development and incorporation of biomarker studies in early clinical trials [7].

A new biomarker will only be implemented into patient care if it is superior to existing biomarkers or offers additive diagnostic, predictive, or monitoring information. Therefore, new biomarkers should always be compared with those that currently are used in clinical routine [8, 10]. Although only few single markers demonstrate a clear and reproducible advantage in these tough comparisons, the combination of multiple biomarkers could lead to a significant improvement of sensitivity and specificity. These combinations may result from a bottom-up approach that assembles biologically complementary markers or from a top-down approach that extracts meaningful markers out of a plentitude of markers. While the first approach is supported by logistic regression, supporter vector machine, or neuronal network models, the latter one often comprises cluster analysis or even more complex algorithms. In all cases a validation in an independent patient set is paramount to confirm the findings [5].

3.4 Monitoring Cancer Disease by Biomarkers

In order to monitor the state of cancer disease or response to anticancer therapy, biomarkers are frequently determined when clear clinical correlates are present, e.g., after tumor resection, at time of recurrent disease, before start of systemic therapies, and at time of radiological staging. Then biomarker levels are ideally assumed to be only influenced by disease activity or therapy response. However, it is necessary to develop rules which changes of biomarker levels are relevant for clinical decision making for the markers are implemented into clinical routine. For the individual interpretation of marker changes over time, several aspects have to be considered:

- The biological variation of a biomarker in individual patients
- The role of influencing factors
- The disease state when the therapy is applied
- The type of therapeutic interventions
- The monitoring schedule for a biomarker and the data interpretation
- The accuracy of biomarker monitoring and its consequences for patient management

For some biochemical markers, it is well known that their blood concentrations depend on age, gender, and ethnicity and can vary due to diurnal, mensal, annual, or other cycles. Further influencing factors are fasting; hydration; medication; the position at blood drawing; marker-specific factors such as stress, sports, etc.; and comorbidities or drug-related immune reactions. Although influencing factors cannot be ruled out completely, standardized procedures for blood collection are recommended [5, 7]. As heterogeneity among individuals is considerable for many markers, relative marker changes on an individual basis are preferred to absolute cutoff rules orientated at diseased patient groups.

Disease states of cancer patients may be very different including (1) local manifestations, (2) dissemination to distant lymph nodes or other sites in the body, (3) recurrences, or (4) continuous progressions. All these states have in common the presence of malignant masses that should be reduced by the therapy.

Treatment options comprise the local tumor eradication such as by surgery, external or internal radiotherapy, or local application of cytotoxic drugs and further systemic approaches if the cancer disease is already in an advanced stage, such as endocrine therapies, cytotoxic chemo- or radiotherapies, biological (targeted) therapies, and immune, gene, vaccine, or other therapies. All these therapies are assumed to reduce the tumor mass with different velocities suggesting a differentiated monitoring plan for each situation. This applies also to the different types of treatment strategies like neoadjuvant therapy before surgery, as well as primary, recurrent, or palliative therapy without surgery.

Sometimes no direct evidence of cancer disease is present, e.g., when monitoring is applied in (1) individuals at risk of developing cancer disease and (2) in patients after successful tumor eradication. Although biomarker monitoring has not been widely established in routine patient management, a sensitive detection of micrometastases could trigger early intervention trials that lead to improved tumor control and better outcomes in recurrent or advanced tumor stages [8].

To guide the individual patient management by biomarkers, a prospective scenario of *appropriate determination intervals* has to be defined that allows the sensitive and accurate estimation of therapy response or tumor (re)occurrence. These intervals depend on the one hand side on the efficiency of the therapy and on the other hand side on the expected half-life of the biomarker response.

It is recommended that biomarker assessments are not only done at the regular stagings with imaging exams but do also cover the initial phase of the therapy, e.g., the first hours or days after the initial treatment application but at least prior to every new therapy cycle, to enable a very early estimation of the biochemical response. Then they offer a real-time advantage over conventional strategies and may trigger an early adaptation of the therapeutic plan. This may be beneficial for the patient in terms of more efficient therapies, less toxic side effects and comorbidities, and considerable cost reduction [5].

Generally, there are three major indications for the *early estimation of therapy response*:

- Monitoring the completeness of surgical tumor eradication and potentially suggesting adjuvant therapies
- Monitoring response to systemic therapies (neoadjuvant, primary, palliative) and potentially suggesting alternative or additional therapies
- Monitoring resistance to a part of the (targeted) therapies and potentially suggesting an alternative approach

For patients presenting with no evidence of disease (NED) who are monitored to early detect micrometastases or recurrence of cancer disease, the intervals will depend on the reoccurrence probability of the tumor and the regular follow-up program [13]. Nevertheless, the intervals should be close enough not to miss incidental recurrences and to offer a real-time advantage to regular radiological exams. However, biomarker monitoring will only be implemented into standardized patient guidance programs if it leads to earlier therapeutic interventions and to a clear benefit in terms of better overall survival and life quality [5].

3.5 Biomarkers in Neuroendocrine Tumors (NETs)

Neuroendocrine tumors (NET) display a very heterogeneous group of neoplastic diseases with respect to their localization, morphology, histology, and biochemical and clinical characteristics. They are quite rare with an incidence of 2–5 cases per 100,000 population. They can be subdivided into well-differentiated grade 1 and 2 NETs and poorly differentiated grade 3 neuroendocrine cancers. Often clinical symptoms of NETs are non-specific or appear only late leading to their diagnosis in an advanced stage of disease [14]. Around two thirds of NETs are localized in the gastroenteropancreatic tract (GEP) such as carcinoids, gastrinoma, insulinoma, vipoma, or glucagenoma. Other types of NETs develop in the lung such as small cell lung cancer (SCLC) and some large cell lung cancer types and in other organs like the medullary C-cell cancer in the thyroid or neuroendocrine subtypes of prostate cancer. Some of them grow locally, while others show a disseminating growth pattern with multiple manifestations. One feature they have in common is the production of peptide hormones, prohormones, or neuropeptides with paracrine or endocrine effects. These can be measured as cancer-associated biomarkers in the tissue, blood, urine, or other bodily fluids and support the diagnosis and monitoring of neuroendocrine cancer disease [2, 14]. Among the monoanalytes that are used in NET diagnostics, there are more general

neuroendocrine biomarkers such as chromogranin A (CgA), neuron-specific enolase (NSE), progastrin-releasing peptide (ProGRP), NT-pro-brain natriuretic peptide (NT-proBNP), and cytokine markers released from diverse NETs. In addition, there are markers with higher specificity for one NET subtype such as serotonin and urine 5-hydroxyindoleacetic acid (5-HIAA) for carcinoids (APUDoma), gastrin for gastrinoma, glucagon for glucagenoma, insulin and C-peptide for insulinoma, vasoactive intestinal peptide (VIP) for vipoma, pancreatic polypeptide (PP) for pancreatic NETs, and calcitonin for medullary C-cell carcinoma of the thyroid, as well as diverse markers in neuroendocrine tumors of the pituitary gland or ectopic manifestations thereof (Table 3.1). These markers can also be elevated in combination, particularly in the case of multiple endocrine neoplasias (MEN) [2, 14].

Most analytical and clinical evidence is available for the biomarkers CgA, NSE, and ProGRP. Chromogranin A is a 68 kDa acidic glycoprotein that is most frequently used for the diagnosis of GEP-NETs. It is expressed in secretory dense core granules of neuroendocrine cells and is released upon stimulation along with other peptide hormones and neuropeptides. As there are various forms of CgA, specificity and affinity of antibodies used in the immunoassays are essential for detection of CgA subtypes [14]. Sensitivity for NET detection ranges between 60 and 80% depending on primary site, grade, and status of the disease. It is mainly elevated in carcinoids and other ileal or pancreatic NETs and correlates with tumor burden, presence of metastases, recurrence, and prognosis. For interpretation of CgA results, it has to be considered that non-specific elevations are seen in patients with renal failure, cardiac diseases, inflammatory disorders, and other types of cancer as well as in patients treated with proton pump inhibitors [2, 14].

Neuron-specific enolase (NSE) is a 100 kDa glycolytic enzyme that is present in neurons and neuroendocrine cells. It is a sensitive biomarker for the diagnosis and therapy monitoring of small cell lung cancer. Moreover, it is used for diagnosis of other NETs as well as in neuroblastoma and Wilms tumors of pediatric patients [15]. Thereby NSE correlates with tumor burden, poor histological differentiation, and high cellular turnover. NSE is a cytoplasmic enzyme

Table 3.1 Biomarkers that are used in the diagnosis or monitoring of neuroendocrine tumors (NETs)

Biomarker	MW	Diseases
Chromogranin A	68 kDa	Diverse, particularly gastroenteropancreatic (GEP)-NETs
Neuron-specific enolase (NSE)	100 kDa	Diverse, particularly bronchopulmonic NETs
Progastrin-releasing peptide (ProGRP)	125 AA, 16.2 kDa	Diverse, particularly bronchopulmonic and medullary C-cell NETs
NT-pro-brain natriuretic peptide (NT-ProBNP)	76 AA, 8.5 kDa	Carcinoid heart disease
Cytokeratin fragments (e.g., CYFRA 21-1)	36 kDa	Diverse NETs, also epithelial cancers
Serotonin	1 AA, 0.2 kDa	Carcinoid
5-hydroxyindoleacetic acid (5-HIAA)	1 AA, 0.2 kDa	Carcinoid
Gastrin	17 AA, 2.1 kDa	Gastrinoma, Zollinger-Ellison-Syndrome
Glucagon	29 AA, 3.5 kDa	Glucagenoma
Insulin (and Proinsulin)	51 AA, 5.7 kDa	Insulinoma
C-peptide	31 AA, 3.0 kDa	Insulinoma
Vasoactive intestinal peptide (VIP)	28 AA	VIPoma
Pancreatic polypeptide (PP)	36 AA, 4.2 kDa	Pancreatic NETs
Calcitonin	32 AA	Medullary C-cell carcinoma of the thyroid

that is not actively secreted and has a lower sensitivity for the diagnosis of GEP-NETs (30–50%) as compared with CgA. Beyond these indications, NSE can also be elevated in various solid tumors particularly in metastatic stages. In addition, it is non-specifically increased in benign lung diseases, uremia, and neurodestructive diseases such as stroke, trauma, etc. Accurate preanalytic sample handling is essential for NSE interpretation as erythrocytes contain high concentrations of NSE and hemolysis may cause false-positive results [15].

ProGRP is a 16 kDa precursor protein of gastrin-releasing peptide (GRP). In contrast to 27 amino acidic GRP with a half time of 2 min in serum, recombinant ProGRP (31–98) with 125 amino acids is much more stable in the blood. ProGRP is the most specific and sensitive marker for the differential diagnosis of neuroendocrine lung cancers, particularly SCLC. It is one of the few tumor markers that is almost exclusively released from one tumor type in high concentrations and is considered as diagnostic marker of SCLC if values are >300 pg/mL [8, 16]. Solely patients with medullary C-cell cancer of the thyroid and other neuroendocrine cancers may achieve similar high value levels at times [17]. In other cancer types or in nonmalignant conditions, only occasionally slight elevations up to 100 pg/mL are observed. However, renal failure is a well-recognized source of false-positive results which has to be taken into consideration for interpretation of ProGRP values [8, 16]. Further, differences with regard to the preanalytic stability of serum samples are observed for some immunoassays [17].

3.6 Diagnostic Performance of Monoanalytes in NETs

In a comprehensive study on patients with GEP-NETs, CgA has shown superior diagnostic performance in grade 1 and 2 NET and large cell neuroendocrine cancer (LCNEC) with AUCs of 0.86, 0.91, and 0.90 when compared with healthy controls followed by cytokeratin fragments (AUC 0.76, 0.86, 0.88) and NSE (AUC 0.54, 0.80, 0.83). CgA was strongly elevated in all three NET stages, while cytokeratins and NSE mainly increased in G2 NET and LCNEC; in consequence cytokeratins were prognostic in all stages, NSE only in LCNECs in multivariate analyses. ProGRP had no diagnostic relevance in GEP-NETs. However, in patients with small cell neuroendocrine cancer mainly in the lung, ProGRP was the most sensitive marker (AUC 0.86) particularly at high specificities (73% sensitivity at 95% specificity) followed by cytokeratins (AUC 0.87), NSE (AUC 0.79) and CgA (0.77). Once again cytokeratins and NSE were prognostically relevant [18]. Best differentiation of lung NETs from non-lung NETs as well as between grade 1 and 2 NETs was found for ProGRP, too. Regarding survival, additive prognostic value of ProGRP and CgA was reported [19].

Recently, a large multicentric trial across Europe and China with more than 2500 patients confirmed earlier results regarding the excellent methodical, preanalytical, and diagnostic performance of ProGRP for small cell lung cancer (SCLC) [17]. Thereby strong elevations were only observed in SCLC patients while healthy individuals, patients with NSCLC, benign lung diseases, other benign or cancer disease had no or only slightly increased, and patients with renal failure moderately elevated values. Most remarkably, ProGRP discriminated with high sensitivity and specificity not only between SCLC and benign lung diseases but also between SCLC and NSCLC (AUC 0.89 in Europe and 0.94 in China, respectively) underlining its high clinical utility for histological subtyping in case of unclear lung masses [17]. Earlier, a multiparametric score involving ProGRP, NSE, and CYFRA 21-1 achieved higher AUC for differentiation of SCLC and NSCLC than single markers did [20]. Molina et al. included ProGRP, NSE, CEA, CYFRA 21-1, SCC, and CA 15-3 in an algorithm that supported the diagnosis and histological subtyping of lung cancer [21]. In addition, ProGRP, NSE, and cytokeratin fragments have shown to be valuable markers for the monitoring and early prediction of response to systemic therapy in SCLC patients [22].

A consensus paper on the use of biomarkers for NET disease outlined the need for circulating biomarkers for diagnosis, prognosis, monitoring therapy response, identifying minimal residual disease, and detection of recurrent disease. While the limitation of monoanalytes in sensitivity and specificity for GEP and lung NETs was recognized, more accurate diagnostic tools were looked for. Current research approaches address circulating DNA, mRNA, microRNA, and metabolomic biomarkers as well as circulating tumor cells; however their clinical utility still has to be proven [23].

3.7 Perspective: Multianalyte Approaches

Great potential is seen in multianalyte approaches such as a multi-transcript molecular signature for PCR-based blood analysis with algorithmic evaluation that was specifically developed for GEP-NETs. The so-called NETest includes 51 genes involved in transcription, DNA repair, antigen processing and presentation, apoptosis, cell adhesion, cell division, immune response, and several metabolic processes. When investigating the assay in three independent blood sets, the gene-based classifier reached high sensitivities (85–98%) and specificities (93–97%), as well as positive (95–96%) and negative (87–98%) predictive values for NET diagnosis clearly outperforming CgA. In particular, the classifier indicated NET in more than 90% of patients with low CgA levels [24]. While superior performance of NETest over CgA was confirmed in a subsequent study, it showed to be elevated in all grades of NET, in both local and disseminated disease, and was not normalized by somatostatin analog therapy [25]. Importantly, it was unaffected by proton pump inhibitors (PPI), while CgA levels were increased in 83% of PPI-treated patients as well as in 26% of controls revealing a high rate of false-positive results [25]. These findings demonstrate that NETest meets the qualitative expectations in a sensitive and accurate diagnostic biomarker [23]. Unmet questions, however, are preanalytical and analytical quality control issues including standardization and harmonization, the high workload and hands-on time, as well as cost-efficiency and reimbursement issues. In relation with benefits from early NET diagnosis and improved quality of life for the patients, considerable cost savings of the society are expected [25]. However, these aspects have to be acknowledged from health insurances if such high-performance biomarker assay is to be implemented in future NET patient's guidance.

Conflict of Interest Statement The author declares to have no conflict of interest.

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Part II

Differentiated Thyroid Cancer

Differentiated Thyroid Cancer: Diagnosis, Therapy, and Follow-Up

4

Frederik A. Verburg

4.1 Introduction

Although it concerns fewer than 1% of all cancer cases, and its incidence varies throughout the world [1], thyroid carcinoma is the most common endocrine malignancy [2]. Thyroid carcinoma spans a considerable oncological width with respect to natural history and prognosis. About 10% of cases concern an anaplastic thyroid carcinoma (ATC), which originates from thyroid cells and is one of the most lethal human cancers. It has a cause-specific mortality of nearly 100% medullary thyroid carcinoma (MTC). The 5-year cause-specific mortality of this entity is about 50%. The remaining 80% of thyroid carcinoma cases comprise the so-called differentiated thyroid carcinoma (DTC): papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC). These tumors derive from the follicular thyrocytes and are referred to as "differentiated" thyroid cancer because the tumor cells retain some of normal thyrocytes' properties. Most importantly the ability to take up and store iodine and to respond to thyrotropin (thyroid-stimulating hormone, TSH) stimulation is retained, which allows for treatment and imaging using radioactive iodine analogues. The relative frequency of FTC and PTC in part depends on the iodine sufficiency and therefore varies by geographic area, but generally, PTC is more common [3]. DTC cases typically have a good prognosis, with long-term survival ranging from about 70% to more than 95%, depending on the extent of disease at the time of diagnosis [4]. Consequently, in >85% of DTC patients, life expectancy is unimpaired [5, 6].

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4.2 Epidemiology and Clinical Behavior

4.2.1 Incidence

The incidence of DTC has steadily increased over the past few years [7], although this rise now seems to be abating [8]. The rise in incidence for the largest part appears to be due to an increasing use and quality of ultrasound diagnostics. DTC is more frequent in females than in males, with reported incidences of 2.0–3.8 per 100,000 in females vs. 1.2–2.6 per 100,000 in males [1, 8–11]. DTC-specific mortality varies from 0.4 to 2.8 per 100,000 in females and from 0.2 to 1.2 per 100,000 in males [12]. The gender differences are most prominent in the reproductive period. The mean age at diagnosis is about 45 years, with younger patients almost exclusively having papillary thyroid cancers and older patients showing an increased frequency of follicular carcinomas [4].

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4.2.2 Histology and Clinical Behavior

4.2.2.1 Papillary Thyroid Carcinoma

The classical form of PTC is an unencapsulated tumor with papillary and follicular structures. It is characterized by overlapping cell nuclei that have a ground-glass appearance and longitudinal grooves, with invaginations of cytoplasm into the nuclei [13, 14]. PTC histologic variants among others include the encapsulated, follicular, tall-cell, columnar cell, clear-cell, diffuse sclerosing, solid or trabecular, and oxyphilic forms [2, 15]. PTCs are often multifocal, with many of the lesions of different clonal origin, i.e., arising independently [16]. PTC metastasis tends to be lymphogenic, before spreading to the lungs and bones.

4.2.2.2 Follicular Thyroid Carcinoma

FTC is characterized by follicular differentiation, without the nuclear changes seen in PTC [13, 14]. FTCs are encapsulated tumors, distinguishable from follicular adenomas by the presence of invasion of the capsule and/or vessels. According to the pattern of invasion, FTCs can be divided into two categories: minimally invasive and widely invasive. FTCs are less often multifocal than are PTCs. FTC tends to metastasize to the lungs, bone, and liver; regional lymph node metastases are much less common than in PTC. Hürthle cell carcinoma is a variety of FTC that consists of at least 75% oxyphilic cells [15]. An important characteristic of Hürthle cell carcinomas is their reputedly poor or even absent iodine uptake, which renders this entity more difficult to treat.

4.3 Diagnosis

4.3.1 Presentation

The clinical presentation of a differentiated thyroid carcinoma in many cases is a solitary thyroid nodule. Occasionally metastases to lymph nodes, lungs, or bones are the first sign of disease. Patients are usually euthyroid at the time of presentation. Especially in iodine-deficient countries where endemic goiter is still a clinical problem, thyroid carcinoma is often encountered upon pathological examination of a thyroidectomy specimen after surgery for benign indications (e.g., obstructive goiter or symptomatic cold nodules).

4.3.2 Ultrasound

Thyroid ultrasound is the most important diagnostic modality for the identification of nodular thyroid disease. It should be performed with a good-quality ultrasound machine with an ultrasound frequency of at least 7.5–10 MHz. Most newer machines are capable of at least 12–18 MHz. Such modern machines allow the diagnosis of nodules as small as 2–3 mm diameter [17]. The typical sign of malignancy—in over 90% of cases—is a solid hypoechogenic nodule. Isoechogenic or hyperechogenic nodules are rarely malignant [18]. Several criteria for malignancy such as the absence of a halo sign, unsharp delineation of nodules, or the presence of microcalcifications have been examined for their diagnostic accuracy regarding the malignancy of a thyroid nodule. Each of these criteria separately does not have sufficient sensitivity or specificity to be useful for diagnosis or exclusion of malignancy of a thyroid nodule [17, 19]. As an illustrative example, an increased blood flow was found in 67% of malignant and 50% of benign nodules [19]. The only truly reliable indicators for the presence of malignancy were a sonographically visible locally invasive extrathyroidal growth or the unequivocal presence of lymph node metastases [17]. The American Society of Radiologists has tried to summarize the available literature regarding the usefulness of sonographic criteria for the diagnosis of thyroid malignancy; this summary has been adapted in Table 4.1.

Other attempts to combine the different ultrasound characteristics are the TI-RADS system [21] and the risk classification currently recommended by the American Thyroid Association guidelines on diagnosis and treatment of patients with thyroid nodules and dif-

Table 4.1 Sonographic criteria for the diagnosis of thyroid malignancy (adapted from Society of Radiologists in Ultrasound Consensus Statement [20])

Sonographic feature	Sensitivity (%)	Specificity (%)
Microcalcifications	26–59	86–95
Hypoechogenicity	27–87	43–94
Irregular delineation or failing halo sign	17–78	39–85
Solid	69–75	53–56
Intranodular vascularisation	54–74	79–81

ferentiated thyroid cancer [22]. The use and usefulness of these systems are however not universally accepted.

4.3.3 Scintigraphy

Thyroid scintigraphy is one of the oldest forms of targeted imaging. It provides information on the functional status of both the entire thyroid and individual nodules, provided they are >1 cm in size (in smaller nodules scintigraphy lacks the necessary resolution to achieve a reliable results, especially where "cold" nodules are concerned). It is performed using the iodide analogue Tc-99m-pertechnetate. Contrary to iodide, Tc-99m-pertechnetate is not organified in the form of thyroid hormones. Therefore, the maximum tracer accumulation is relatively low. In contrast, autonomous, also called "hot," nodules show a much higher uptake than the surrounding tissue. Proper thyroid scintigraphy requires the use of a high-resolution collimator [23]. The scintigraphic hallmark of malignancy is the so-called cold nodule. This indicates a nodule that shows a lower degree of radionuclide uptake than the surrounding healthy tissue. This has been known for many decades; in studies dating back as far as 1965, it was already described that 11% of cold nodules showed histological evidence of malignancy in patients between 45 and 65 years of age; in patients over 65 years of age, this rate was as high as 25% [24]. Although the precise frequency of malignancy in cold nodules may have changed over the years, the use of thyroid scintigraphy as a selection method for further analysis by increasing the *"a priori"* likelihood of a positive test remains valid as ever [25]. It is important to realize that in contrast the risk of malignancy in autonomous "hot" nodules is negligible. In the context of iodine deficiency, this is especially relevant, as a large proportion of nodules will be "hot" rather than "cold." However, in many international guidelines, such as those from the American Association for Clinical Endocrinology and the Associazione Medici Endocrinologi [26], the American Thyroid Association [22, 27], or the European Thyroid Association [28], it is recommended to perform fine needle aspiration biopsy (FNAB) in nodules >1 cm in diameter suspicious in US examination. Considering that 10–20% of all FNABs results in a need for histological confirmation, this would result in a large number of unnecessary thyroid surgeries in areas of iodine deficiency. Schicha et al. were able to clearly show that purely mathematically speaking, the addition of thyroid scintigraphy in areas of iodine deficiency can help in selecting those nodules with an a priori higher risk of malignancy and thereby greatly reduce the rate of unclear findings resulting in "diagnostic" thyroid surgeries [25].

A further technique which has proven useful in clinical practice is thyroid scintigraphy using Tc-99m-MIBI. This tracer, which was developed for myocardial scintigraphy, was shown to be taken up in increased relative quantities compared to pertechnetate by malignant thyroid nodules. Indeed, it was also shown that an uptake which relative to pertechnetate scintigraphy is normal or reduced is associated with a negligible chance of malignancy. Hence Tc-99m-MIBI scintigraphy has become a valuable tool in the evaluation of thyroid nodules, especially in patients in whom fine needle biopsy results are equivocal or nonassessable. It's high negative predictive value ensures that patients with a negative scintigraphy need not needlessly undergo a diagnostic (hemi)thyroidectomy [29–33]. Its availability and comparatively low price make this method of additional investigation of thyroid nodules of an unclear nature more cost-effective than those based on genetic analysis of fine needle biopsy [32].

4.3.4 Fine Needle Aspiration Biopsy

Especially in areas with endemic goiter, a good selection of patients for fine needle aspiration biopsy (FNAB) is a must. Ultrasound and scintigraphy combined form an effective way of selecting which nodules to examine cytologically: as discussed above thyroid scintigraphy is a good way to select those nodules with a strongly elevated risk of malignancy, provided the nodules are >1 cm in diameter. Independent from ultrasound and scintigraphy, FNAB is mandatory in fast-growing nodules, especially in younger patients. The use of ultrasound for guidance of FNAB is always preferable. It will help identify the nodules for FNAB and assist in guiding the needle through the nodule; then it is obligatory in non-palpable nodules or multinodular goiter. For instance, in a study from Carmeci et al., it was shown that ultrasound-guided FNAB has considerable advantages over palpation-guided FNAB: the rate of malignancies found rose from 40 to 59% [34]. The rate of false-negative FNABs is not well known as patients with negative FNAB are only rarely operated on; a false-negative rate of about 0.7% is estimated [35]. Next to inadequate sampling, there are several major problems with FNAB. The first one is that the thyroid is a well-perfused organ, causing FNAB samples to contain a lot of blood which may conceal the thyrocytes. The second problem concerns follicular lesions, which remain of an unclear nature by FNAB alone as for the diagnosis or exclusion of malignancy in such nodules, the entire capsule of the nodules and the vascular and lymphatic structures in it need to be assessed by microscope. This means that any such lesion needs to be removed surgically. In recent years a method has been developed which may reduce the need for surgery in such lesions based on genetic expression analysis [36]. This so-called gene expression classifier (GEC) is characterized by a high negative predictive value, thus potentially greatly reducing the number of unnecessary surgery by complementing the high sensitivity seen in FNAB cytological analysis. However, clinical effectiveness in daily clinical practice is still debated, and the use of this gene expression classifier is hampered by the high associated costs in many countries.

4.4 Treatment

In the treatment of DTC, multiple modalities are involved, each of which will be discussed separately.

4.4.1 Surgery

Surgery is the first and most important component of the primary treatment of DTC. In Europe, the Americas and much of Australasia, (near) total thyroidectomy is usually performed in almost all patients. Only for papillary microcarcinoma hemithyroidectomy is deemed to suffice in most patients [16, 37–44]. More recently, a discussion has erupted on the need for total thyroidectomy in nonmetastatic, not locally invasive DTC with a diameter up to 4 cm [22]; this strategy however has yet to prove itself. For DTC in general, the vast majority of authors have found the therapeutic effect to be related to the extent of surgery [37]. Relapse-free survival and thyroid cancer-specific survival are better after (near-) total thyroidectomy than after unilateral thyroid lobectomy. Moreover, in >50% of patients undergoing a completion thyroidectomy after initial unilateral lobectomy, malignant tissue is found in the thyroid remnant [44]. This observation is unsurprising in light of the frequently multifocal, multiclonal nature of PTC [16]. Arguments against total thyroidectomy in early-stage PTC are also available. PTC often is found in postmortem studies without a single clinical symptom during life [45, 46], raising questions regarding the clinical relevance of papillary microcarcinomas.

The prognosis of DTC and the effectiveness of adjuvant treatment with I-131 (also called ablation; see below) are so good [47–49] that without at least cytologic proof of the necessity of a radical neck dissection, this procedure, which is associated with significant morbidity, appears to be unjustifiable in any case. A modified radical

lateral neck dissection should be performed only after a diagnosis of lymph node metastases [2]. The less invasive central or pre-tracheal compartment lymph node dissection is however advocated as a standard procedure in thyroidectomy for any DTC by many authors, as it both facilitates the accurate staging with regard to lymph node status and may prevent locoregional DTC recurrence [50]. Current guidelines, however, suggest to refrain from this procedure in T1 to T2 DTC [22] as potentially associated morbidity is not justified by a significant clinical benefit in such cases.

The most serious potential complications of thyroid surgery are hypoparathyroidism and recurrent laryngeal nerve damage [51, 52]. The incidence and impact of complications can be reduced by performing the procedure in expert centers [52]. Serum calcium levels should be monitored frequently in the immediate postoperative phase. Identification and electronic monitoring of the recurrent laryngeal nerve can significantly reduce the rate of nerve damage [53].

4.4.2 Thyroid Hormone Replacement Therapy

As by definition the production of endogenous thyroxine is discontinued by thyroidectomy procedure, DTC patients require thyroid hormone (levothyroxine, LT4) replacement therapy [22]. Differentiated thyroid cancer cells still react to TSH stimulation; for this reason LT4 is usually administered in such doses that TSH levels fall to very low levels of <0.1 mU/l [54]. Especially for low-risk patients, TSH suppression is not generally advocated [22]. The exact dose of LT4 required to achieve TSH suppression varies from patient to patient and depends in part on body weight.

4.4.3 Radioiodine (I-131) Treatment

The majority of current guidelines recommend postsurgical application of an "ablative" I-131 activity as a second component of the primary treatment of DTC in some or most (near) totally thyroidectomized patients [27, 55–57]. The radioiodine is administered as sodium iodide (NaI), either orally or by intravenous injection. I-131-NaI closely approaches the ideal oncologic drug: due to thyroid cells' role as the body's main iodine reservoir and primary locus of NIS expression, I-131 is largely specific for the target cancer cell and generally has relatively limited side effects. Additionally, this isotope emits therapeutically useful beta radiation as well as gamma rays suitable for imaging the drug distribution.

In clinical practice, I-131 ablation has three goals [2]:

- To destroy occult microscopic DTC foci, thereby decreasing the long-term risk of recurrent disease [37, 40, 48, 58, 59].
- To eliminate any remaining healthy thyroid tissue, thereby increasing the specificity of detectable serum thyroglobulin (Tg) and positive WBS as markers for persistent or recurrent DTC cells [2, 58, 60]. Additionally, by destroying healthy thyroid cells, ablation may remove a locus for new neoplastic transformation [61], given the multiclonal nature of many DTC cases [16].
- Through the use of a large I-131 activity, to permit sensitive post-ablation WBS to detect previously unknown persistent locoregional disease or, because the abundance of tracer overwhelms any "monopolization" of uptake by the thyroid bed, to detect previously unknown metastases [62, 63]. Post-ablation scintigraphy also allows precise probe-guided removal of the newly detected disease foci in selected cases [64].

Although the medical use of the second goal can be debated in the current age of highly sensitive and robust Tg assays, the first (and in a sense also the third) objective solidly positions I-131 treatment as an adjuvant oncological therapy. Achieving these goals should lead to decreased rates and to more timely detection of persistent or recurrent disease and, more importantly, to improved tumor-specific survival in (near) totally thyroidectomized patients with DTC [37, 40, 65].

The preferred I-131 activity for initial postoperative I-131 treatment has been a matter of debate [66–72]. Although good results may be obtained with 1.1 GBq [67, 68], some literature has suggested that remnant eradication rates seem to improve with increasing activities [72]. A plateauing of the I-131 activity-response curve has been noted for ablation activities over 1.85 GBq [66]. On the other hand, in low- as well as in high-risk DTC patients, higher activities in long-term follow-up were associated with lower DTC-related death and recurrence rates. Nonetheless, published papers have contained no clear answer from randomized controlled trials regarding the precise activity that optimizes the chance for successful ablation. Those trials that have been published [73–75] unfortunately only targeted successful ablation, and no long-term follow-up in patients is as yet available from these trials. Although as noted above, I-131 is highly specific for thyroid cells, physiological uptake occurs in other tissues expressing NIS, including the salivary glands and the breasts. Relatively high radiation exposure also occurs in the gastrointestinal and urinary tracts, since the body excretes from iodine via the feces and, especially, the urine. Measures to minimize extrathyroidal radiation exposure include abundant hydration to encourage frequent micturition, lemon juice or lemon candy ingestion to stimulate salivary secretion (but started only with at least 24 h distance to I-131 administration as otherwise the radiation dose to the salivary glands will paradoxically be increased), and laxative administration to decrease fecal transit time.

Iodine also is secreted in milk [76], so breast-feeding must be discontinued at least 6–8 weeks before I-131 administration. This discontinuation serves two objectives: (I) preventing I-131 ingestion by the infant and (II) avoiding unduly high radiation doses to the mother's breasts. Discontinuation of breast-feeding stops milk production and consequently decreases the mammary gland's uptake of circulating iodine. However, as the mammary glands require some time to reduce their mass and activity once lactation stops, a waiting period between discontinuation of lactation and I-131 is in order.

Relative contraindications for radioiodine therapy in patients with thyroid cancer include:

- High-grade bone marrow depression in cases of treatment with high activities of radioiodine
- Considerable reduction of pulmonary function in patients with lung metastases and high radioiodine uptake
- Considerable xerostomia due to proven impairment of salivary gland function

Side effects and complications from I-131 therapy potentially include acute or chronic salivary gland problems ranging from mild sialadenitis to complete xerostomia, the latter occurring only in a small minority of patients. Other potential complications include temporary loss of taste or smell and transient hematologic abnormalities. The incidence of such complications depends on the administered activities as well as on the cumulative lifetime activity [77, 78]. Evidence of earlier onset of menopause was found in women treated with I-131 for DTC [79]. Additionally, an excess of second primary malignancies can be observed after I-131 treatment of DTC [80–82]. The risk of second primary malignancies also may relate to administered radioiodine activities; that risk presumably is smaller when only a single activity, especially a lower one, is given for ablation versus when multiple radioiodine therapy courses are applied for metastatic disease, as was the case in many patients in epidemiological studies examining second primary malignancy incidence in the radioiodine-treated DTC setting. Also, whether the I-131 therapy is the sole cause of excess second primary malignancies, or whether a genetic predisposition to cancers among DTC patients also is involved, is open to debate [83].

4.4.3.1 A Short "How-to"

Before I-131 treatment, the patient should be prepared adequately. A low-iodine diet for 2–3 weeks before iodine administration is recommended, even more important is the avoidance of iodine-containing drugs (e.g., X-ray contrast media, reagents for disinfection, ophthalmologic agents,

amiodarone, iodide medication) or food and food additives with high iodine content (e.g., seaweed, kelp, dietary supplements) [84–88].

The application of a diagnostic activity of I-131 immediately before radioiodine treatment may induce "stunning," i.e., reduced I-131 uptake or changed I-131-kinetics during consecutive radioiodine therapy that may be the cause for significant impairment of the efficacy of radioiodine therapy [89]. Therefore, it is recommended not to use diagnostic activities of I-131 necessary for scintigraphy, i.e., more than 10–20 MBq [90]. With smaller activities of I-131 (< 10 MBq), 24 h uptake measurements may be performed to estimate roughly the mass of thyroid remnant after surgery [91]. As an alternative for I-131, short-lived I-123 (half-life 13.2 h, gamma emitter) may be used. The advantage of I-123 is that no stunning is involved. I-124 is an interesting positron and gamma-emitting radionuclide suitable for high sensitive and specific PET-CT imaging with half-life 4.2 days, but so far high costs and limited availability have limited its clinical use.

Ablative radioiodine treatment demands sufficient TSH stimulation of thyrocytes to induce sufficient radioiodine uptake in thyroid remnants and/or tumor tissue. This may be either achieved through endogenous stimulation after levothyroxine (LT4) withdrawal or exogenous stimulation by injection of recombinant human thyrotropin (rhTSH). Although for many years it was thought TSH must exceed 30 mU/l, newer data have shown that a strict adherence to this level is not necessary in patients who have been stimulated for at least 4 weeks.

Usually standard I-131 activities of between 1 and 3.7 GBq are recommended for treatment; a standard activity of 3.7 GBq is prescribed in the registration studies of rhTSH. Between 1 and 3.7 GBq I-131, higher activities seem to coincide with higher success rates [92] and better recurrence-free and overall survival most likely due to a higher bioavailability of I-131 [93].

In the case of microscopically non-radical resections, aggressive subtypes of differentiated thyroid cancer, or other high-risk constellations, higher standard activities of up to 7.4 GBq I-131 can be administered without special precautions. If higher activities of radioiodine are considered for ablative treatment, dosimetric approaches should be used to estimate the radiation dose to the bone marrow [61, 94].

4.4.3.2 I-131 Therapy of Advanced DTC and Dosimetry

For I-131 therapy, patients usually are given a standard fixed activity. This activity reflects the physician's or institution's estimation of the amount of radioiodine needed to deliver the highest safe radiation dose to neoplastic foci, given the patient's tumor burden or histology, age group, etc. However, because there is great inter- and even intra-patient (i.e., between different lesions in the same individual) variation in radioiodine kinetics, such fixed activities frequently pose a risk of either underdosing the patient or—much more worrying—of exceeding commonly accepted safety limits [95, 96]. To avoid both risks, it is possible to perform a pre-therapeutic dosimetry using a small test activity of I-131. The rationale for basing treatment activities on such dosimetry is to allow the administered activity and, with it, the absorbed dose to iodine-avid thyroid tissue, to be maximized for each individual while avoiding bone marrow toxicity, pulmonary fibrosis, or (presumably) other severe or serious side effects. One well-accepted concept for treating advanced DTC uses the activity that is as high as safely administrable (AHASA) and based on calculation of the individualized activity to be administered to deliver a 2 Gy absorbed dose to the blood. Blood, as a surrogate for the red bone marrow, was considered the critical organ at risk in the approach originally reported by Benua et al. [97] and Benua and Leeper [98]. This "safety dosimetry" concept also specifies that the whole-body retention should be <4.4 GBq at 48 h after radioiodine administration. Additionally, in the presence of diffuse pulmonary metastases, lung uptake should be <3 GBq at 24 h post-therapy to avoid pulmonary fibrosis [98]. Recently, the EANM published a standard operational procedure (SOP) for determining the AHASA I-131 activity for DTC therapy using the 2 Gy absorbed blood radiation dose threshold [94]. In brief, patients should be given 5–10 MBq

I-131, followed by whole-body scanning together with a standard 1 h, 4 h, 24 h, 48 h, 72 h, 96 h, and 168 h after I-131 administration; at the same time, blood samples should be drawn. Based on the results of these measurements, the AHASA activity can then be calculated. Initial clinical experience with this methodology indicates that it is safe and effective [99]. Iodine-124 PET-CT has been advocated because it can also be used for tumor dosimetry [100–103]. Using three-dimensional voxel-based dosimetry, highly variable dose estimates were found for individual metastases in individual patients. Although large-scale data correlating these estimates with response are lacking, the prospect of calculating the minimally effective activity/dose while at the same time being able to calculate the activity that is as high as safely administrable (AHASA) using whole-body retention, organ and blood/bone marrow dose to individualize treatment seems very appealing and of high oncologic relevance.

4.4.3.3 Treatment of I-131 Negative Disease

A minority of DTC cases will either before diagnosis or in the course of treatment lose the ability to concentrate iodine in sufficient quantities to allow for therapeutically effective radiation doses to the DTC lesions. For these patients traditionally the only registered treatment used to be intravenous chemotherapy with doxorubicin, even though this only provided a response in a small minority of patients. However, in recent years new options have become available in the form of multi kinase inhibitors. Currently two drugs from this substance class have been registered for advanced, iodine refractory DTC: sorafenib and lenvatinib. These drugs were both shown to induce a marked period of progression-free survival (or even remission) in patients with progressive, I-131 refractory DTC. However, thus far it has not yet been shown beyond a doubt that these drugs are able to increase cancer-specific survival, and they are associated with significant, sometimes potentially lethal side effects. It is therefore as yet unclear which patients will actually benefit from DTC in terms of an increase in quality adjusted life years, and many clinicians are hesitant to employ drugs from this substance class unless patients suffer from significant DTC-related symptoms, have lesions which in the short term may present a threat to patients' lives, or show extremely rapid progression.

4.5 Follow-Up

Contrary to most other cancer patients, thyroid carcinoma patients traditionally were never considered "cured," as recurrences could occur more than 30 years after initial treatment. In more recent years, the availability of more sensitive follow-up tools has in clinical practice nearly eliminated the phenomenon of late recurrences [49], as (nearly) all such patients now show at least minor biochemical abnormalities. The question is therefore whether the follow-up of patients with differentiated thyroid cancer still should be lifelong.

4.5.1 Thyroglobulin Measurements

As thyroglobulin (Tg), a 664 kilodalton glycoprotein, is produced only by (normal or neoplastic) thyroid follicular cells, detectable serum levels signal the presence of recurrent or metastatic disease. Tg is the best available tumor marker for PTC and FTC after a (near) total thyroidectomy and subsequent radioiodine ablation of remaining thyroid tissue. As Tg measurement will be dealt with extensively elsewhere in this volume, it will only summarily be discussed here. Most currently available methods in use for measuring Tg are immunometric assays. While these are highly sensitive, these also lead to problems with the measurement of Tg:

– The presence of circulating autoantibodies against Tg (TgAb) is a problem for the detection and the interpretation of serum thyroglobulin levels. TgAb can cause either over- or underestimation of Tg levels. Tg tests should therefore always be combined with TgAb tests; if TgAb test positive, Tg values are unre-

liable. Tg autoantibodies themselves have been proposed as a surrogate tumor marker as TgAb react to the presence or absence of thyroid cells and of Tg [104–107].
- Although this problem appears to be rare, heterophilic antibodies can interfere with Tg measurements [108–111].
- There is a significant inter-assay variation [112]. Despite CRM-457 standardization, this variation between assays supersedes within-person variability.
- The most sensitive Tg measurements are obtained during TSH stimulation [113]. On the other hand, high TSH levels also induce thyroid (cancer) cell proliferation. This problem appears to be less acute now that highly sensitive Tg assays have a high enough clinical accuracy even without TSH stimulation [114].

Tg measurement is a key element at every stage of treatment once the diagnosis of DTC has been made and thyroidectomy has taken place. Its measurement will provide crucial information on the patients' response to treatment as well as on the patients' status with regard to possible recurrent disease after successful treatment.

4.5.2 I-131 Whole-Body Scintigraphy

In many countries, 6–12 months after I-131 ablation, diagnostic whole-body scintigraphy (dxWBS) is performed during TSH stimulation to evaluate whether the ablation was successful. A minimal uptake in the former thyroid bed is generally no longer considered pathological. If pathologic I-131 uptake is still observed, especially in the presence of elevated Tg levels, a second dose of I-131 is administered to achieve complete ablation.

The I-131 activity used for follow-up dxWBS ranges from 74 to 370 MBq. Higher dosages increase the sensitivity of the test but may also induce stunning of thyroid remnants and consequently lessen the efficacy of a therapeutic I-131 dosage.

With the advent of highly sensitive Tg tests, diagnostic I-131 WBS has become controversial; a negative I-131 dxWBS may be observed in the presence of detectable serum Tg levels, and, in most of these cases, foci of iodine uptake can be observed after administration of a therapeutic I-131 dosage. Conversely, positive I-131 dxWBS at undetectable serum Tg levels has become a rare observation. Furthermore, ultrasound of the neck is more sensitive than I-131 dxWBS for detecting lymph node metastases. Therefore, the medical sense of I-131 dxWBS in some countries is being questioned; further studies will be required to show its continuing utility.

I-131 dxWBS during LT4 suppression medication is quite insensitive. To achieve adequate sensitivity, TSH stimulation is required. This can be realized by prolonged discontinuation of LT4 medication, but the issuing hypothyroid state is poorly tolerated by many patients. The more tolerable alternative is the use of recombinant human TSH (rhTSH), both in diagnostic and in therapeutic settings. Similar sensitivity, specificity, positive, and negative predictive figures are observed after LT4 withdrawal or administration of rhTSH.

4.5.3 Ultrasound

The use of ultrasound (US) for the evaluation of thyroid nodules was first described in the early 1970s. US was primarily used to distinguish between cystic and solid thyroid lesions. Over the years the spatial resolution of ultrasound imaging has progressively improved, and hence its clinical usefulness has expanded. As discussed previously, US-guided fine needle aspiration of thyroid nodules has a distinct role in the primary diagnostic process of thyroid carcinoma; this technique can however also be applied to suspicious lymph nodes during follow-up. Also during follow-up, ultrasound has a clear added value: ultrasound imaging is presently the most sensitive imaging modality for the early detection of locoregional recurrence and/or metastases, especially cervical lymph node metastases. Size and location of cervical lymph nodes are the most

important predictors of metastatic disease. Clinically, US of the neck has for a long time been recommended as a standard procedure during follow-up of thyroid carcinoma [115]. Recently, however, some studies have revealed that this might produce more false than true positive results. If confirmed, this may in the future reduce the importance of cervical US during follow-up.

Conclusion

The diagnosis, treatment, and follow-up of DTC are a complex matter with which entire books can be filled. Although some principles such as I-131 therapy have been employed with little change for over 70 years, many aspects of DTC care are rapidly evolving and will continue to change in the future. The most urgent need for new solutions exists in patients with I-131 negative or refractory advanced disease, where life expectancy is still impaired.

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Thyroglobulin and Thyroglobulin Antibodies

5

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5.1 Introduction

Thyroglobulin (Tg) is a large glycoprotein that in healthy thyroid tissue is stored in the follicular colloid of the thyroid gland where it acts as a substrate for the synthesis of thyroid hormones. As it is produced by normal or well-differentiated malignant thyrocytes only, its tissue-specific origin makes it highly useful as a tumour marker [1]. Tg is released into the bloodstream together with thyroid hormones both upon physiological and pathophysiological stimulation but also upon destruction of the thyroid gland [2] (Table 5.1).

The advent of Tg measurement in the early 1980s greatly improved the follow-up of DTC, and due to the gradual improvements in the sensitivity and precision of Tg assays, the measurement of serum Tg has thus become the cornerstone in the follow-up algorithms for management of thyroid carcinomas after successful treatment [3, 4]. However, until recently, optimal sensitivity of Tg assays for the detection of smaller disease foci required stimulation of endogenous Tg production by high serum TSH concentrations, obtained after expensive exogenous injections with recombinant human TSH or after withdrawal of the

Table 5.1 Causes of increased Tg levels into the bloodstream

TSH receptor stimulation by TSH, TSH receptor antibodies and human chorionic gonadotropic hormone (hCG)
Increased proliferation (e.g. *benign and malignant nodules*, *goitre*)
Iodine deficiency
Destructive thyroiditis (e.g. *subacute*, *postpartum and silent thyroiditis*, *hashitoxicosis*)
Thyroid surgery
Radioactive iodine therapy
Fine needle aspiration cytology and core needle biopsy
Thermal ablation of thyroid nodules
Serious manipulation of the thyroid gland (e.g. *anterior neck trauma*, *strangulation*)

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patient's levothyroxine (LT4) replacement therapy, resulting in profound hypothyroidism [5–8].

Over the years, the sensitivity and precision of Tg assays have improved by multiple orders of magnitude, and nowadays new highly sensitive Tg assays are available. In fact, such assays are sufficiently sensitive to obviate the need for TSH stimulation in most patients with DTC [9–12].

Therefore, the increasing adoption of these assays in clinical practice has considerable implications, such as a reduction of costs of DTC follow-up and avoidance of hypothyroidism [13].

However, measuring Tg is technically challenging, and in addition, criteria adopted to define assay sensitivity by different manufacturers, laboratories and clinicians may diverge considerably. In the present chapter biological basis, advances and challenges in Tg measurement techniques and their impact on clinical management of DTC patients are reviewed.

5.2 Thyroglobulin: Biochemistry and Physiopathology

The thyroid gland is responsible for the production of thyroid hormones, mainly the prohormone thyroxine (T4), which contains four iodide molecules [14]. The gland consists of thyroid follicles; i.e. epithelial cells that border a lumen with their apical membranes and are in contact with the blood circulation through their basal membranes, respectively. Thyroidal proteins involved in thyroid hormone synthesis are the thyroid-stimulating hormone receptor (TSH-R); i.e. a seven-transmembrane receptor located in the basal membrane. Upon stimulation of the TSH-R, several processes, including Tg synthesis, are upregulated in the thyroid cells to favour thyroid hormonogenesis [15]. The most abundant protein in the thyroid gland is Tg which functions as a scaffold protein for thyroid hormonogenesis and as a storage protein for thyroid hormones and iodide. Initial transcription of the Tg gene (>300 kb) is regulated by thyroid-specific transcription factors (TTF-1 and TTF-2) and Pax8 [16]. After translation of the mRNA, the post-translational route of Tg starts with the signal peptide directing the uptake in the endoplasmic reticulum (ER), where the first mannose and glucose residues are added and the Tg protein is folded. Therefore Tg is directed to the Golgi apparatus, where glycosylation proceeds. At this point, Tg molecules have a molecular weight of 300,000, contain 10% carbohydrate structures and are routed through to the follicular lumen of the thyroid cells where homodimers with a molecular weight of 660,000 are formed [17]. The sodium iodide symporter (NIS), located in the basal membrane, and pendrin, located in the apical membrane, are responsible for the iodide supply and transport within the gland. Iodination of specific tyrosine residues in Tg and coupling of these iodinated residues to form thyroid hormones are done by the thyroid peroxidase (TPO) anchored in the apical membrane and on one or more thyroid oxidases (Tox) that provide the H_2O_2 [18]. Before the secretion of thyroid hormones, Tg is taken up by the follicular cells through a process involving endocytosis and phagocytosis, and thyroid hormones (mainly T4) are released by proteolytic enzymes into the bloodstream [19]. For a long time, it was assumed that no Tg secretion or leakage from the healthy thyroid could occur. However, when more sensitive methods to measure Tg became available, low concentrations of circulating Tg were demonstrated in virtually all healthy subjects [20–22].

As previously mentioned different benign and malignant thyroid diseases release significant amounts of Tg into the blood (i.e. Graves' disease, goitre, destructive thyroiditis, differentiated thyroid carcinoma) with a wide overlap between them. As a consequence, serum Tg measurement cannot be used to diagnose and differentiate different thyroid diseases, and Tg is mainly useful as a tumour marker only after thyroidectomy (ideally followed by thyroid remnant radioiodine ablation).

However, in rare cases of patients with proven distant metastases, high Tg levels may serve as a useful tool to identify an unknown primary thyroid cancer. Finally, serum Tg may be measured to aid in the differential diagnosis of congenital hypothyroidism and *thyrotoxicosis factitia*.

Interestingly, serum Tg levels recently proved to be a useful complementary tool in the challenging management of thyroid nodules with indeterminate cytology reading [23].

5.3 Thyroglobulin Measurement: Methods and Analytical Performance

Immunoassay has been the main analytical technique used for the measurement of serum Tg, first by competitive immunoassay and later by immunometric (reagent excess) assays. More recently, also mass spectrometric methods have been developed. Each new assay format has been developed to attempt to overcome the major analytical challenges in measuring Tg, a heterogeneous analyte of large molecular weight, in the presence of interfering antibodies. It is notable, however, that there is no reference method system including a reference method procedure available for Tg, and use of the BCR® 457 certified reference material (formerly CRM 457) has not completely eliminated the notable differences in results obtained by different methods [24]. Issues of commutability of the BCR® 457 material and the need for harmonization have yet to be addressed [25].

5.3.1 Standardization and Harmonization

Tg is a large (660 kDa), highly glycosylated dimeric molecule that is heterogeneous in serum due to differential splicing of Tg mRNA as well as carbohydrate and iodide heterogeneity. In addition, biosynthesis of the mature Tg molecule may become deregulated in thyroid tumour cells resulting in differences in the structure of circulating Tg protein. These changes can lead to exposure or masking of epitopes and hence differences in Tg immunoreactivity. Different Tg assays employ a number of antibodies against Tg, with varying specificity for different epitopes. Potentially this introduces variability in the measurement of different Tg isoforms and ultimately to differences in Tg concentration reported by the assays [26, 27]. Early international collaborative studies showed that serum Tg concentrations varied by as much as 40–60% between methods [28, 29]. The introduction and use of the BCR® 457 have significantly reduced inter-method variability to about 30% but have not completely eliminated it [30]. Consequently, any change in Tg assay has the potential to disrupt serial monitoring and prompt inappropriate clinical decisions. For longitudinal consistency of clinical care, consecutive measurements of Tg concentrations should be performed in the same laboratory using the same assay each time. If an assay change is unavoidable, a new baseline of the individual patient's serum Tg concentrations should be established through parallel Tg measurements using both the old and the new assay [26, 27, 30]. Furthermore, internal and external quality control programmes, including samples at low and very low Tg concentrations, are of pivotal importance for checking the precision, reproducibility (internal quality control) and accuracy (e.g. lack of bias of analytical results) of assays to ensure optimal patient care. Laboratories providing Tg measurement are required to participate in a certified national or international programme of quality assurance [31].

5.3.2 Analytical Performance

Laboratory specialists are familiar with the necessary experiments that must be performed in order to verify assay performance, namely, assessment of linearity, measuring range, trueness (measurement bias), comparability through patient comparison studies and limit of detection/limit of quantitation/functional sensitivity. It is worth considering some particular points with regard to serum Tg [32]. Commercial assays may be provided with an assay diluent and specify a dilution value (e.g. 1 in 10 v/v). Laboratories may wish to consider whether this covers the range of concentrations that is required from a clinical perspective (i.e. monitoring of metastatic disease). The concentration at which the high-dose hook effect has been excluded should also be

determined. The feasibility of using an in-house human serum pool as diluent (with undetectable TgAb as measured by a suitable assay and Tg concentration less than 0.1 μg/L) may need to be investigated and the linearity over a wider concentration range determined. Estimation of recovery of added Tg has been proposed as a method of assessing whether there is interference by endogenous antibodies (i.e. Tg antibodies, TgAb and heterophilic antibodies, HAb), although this is not advocated by current guidelines (see section on Interferences). Nevertheless, determination of quantitative recovery should be performed as part of the method validation of the assay [32]. Studies have shown that the measured recovery is dependent on the protocol used—in particular the Tg concentration, source of Tg (degree of iodination) and incubation time. Assessment of recovery using a source of Tg independent of the kit calibrators is suggested, though manufacturers may recommend a protocol if recovery is being determined in the context of assessing assay interference [26].

5.3.2.1 Analytical Sensitivity

The growing recognition of the clinical need for improved precision of assays at low Tg concentrations has been paralleled by improvements in assay sensitivity with the original radioimmunoassays (RIAs) reporting down to 2–5 μg/L, the first immunometric assays (IMA) down to 1 μg/L and, more recently, IMAs with limits of about 0.1 μg/L. However, these broad comparisons are limited because of differences in bias between different assays and because different experimental and statistical methods were used to determine the sensitivity of the assays. In the first instance, analytical sensitivity has often been determined by repeat analysis of the zero calibrator and determination of the apparent concentration equal to the zero plus 2 or 3 standard deviations of the signal for immunometric assays (minus for competitive assays), which is known as the limit of the blank (LOB). In the majority of cases, the measured sensitivity will be below the concentration of the lowest concentration calibrator. Although of limited use in understanding the precision of low concentration samples, the LOB can be useful when optimizing conditions during assay development. The limit of detection (LOD) is defined as the lowest analyte concentration that can be distinguished from the LOB using replicate analysis of a sample of known low concentration. Lastly, the limit of quantitation (LOQ) is similar to the functional sensitivity but does have an additional requirement for predefined goals for bias and imprecision and is increasingly used as a measure of sensitivity for both immunometric and mass spectrometric assays. The relationship between these estimates of sensitivity is LOB < LOD ≤ LOQ. Manufacturers should quote LOB, LOD and LOQ as determined by regulatory authorities and national guidelines (e.g. those of the Clinical and Laboratory Standards Institute EP17-A2) [33, 34]. Functional sensitivity (FS) was introduced as a measure of analytical sensitivity and was originally described for assessing the sensitivity of TSH assays. The NACB protocol [4] indicates that FS may be determined from between batch precision of Tg measurement:

- In patients serum pools
- In the same test mode (singleton or duplicate) as the patient samples
- Over the clinically relevant concentration range
- Over two different lots of reagents and calibrators
- Over a period of >6 months

The patient pools should be TgAb negative. The protocol specifies three different concentration ranges for the patient pools. From the calculated precision profile, a cut-off value corresponding to a CV of 20% (somewhat arbitrarily) is taken as the FS (Table 5.2).

The difference in FS between Tg assays has created a "generational" nomenclature system with each subsequent generation exhibiting a substantial improvement (i.e. tenfold). It should be recognized, however, that there are limitations to this approach when determining the sensitivity of an assay (i.e. potential matrix effects) and differences in the statistical approach to the calculation of the precision profile, principally to

Table 5.2 Definitions of different parameters describing analytical sensitivity

Parameter	Definition	Protocol
Limit of blank	Highest measurement that is likely to be observed for a blank sample [$mean_{blank}$ + 1.645(SD_{blank})]	IFCC
Limit of detection	Lowest amount of that can be detected, but not quantified as an exact value LOB + 1.645 ($SD_{low\ concentration\ sample}$)	IFCC
Limit of quantitation	Lowest amount of analyte in a sample that can be quantitatively determined with stated total error, understated experimental condition	IFCC
Functional sensitivity	Lowest amount of analyte that can be quantitatively determined with an inter-assay coefficient of variation <20%	NACB

do with the identification of outliers and the confidence intervals of the profile. In facts confidence intervals of the precision profile may vary significantly over the concentration range indicating the confidence with which the calculated FS can be viewed. In addition, the published literature is often not helpful since details of the procedure used to generate the precision profile are rarely provided (e.g. whether within or between batch precision was used, how many samples were analysed and whether samples were analysed in singleton or duplicate). Therefore, with such sparse data, the relationship between imprecision and concentration can only be poorly estimated [35]. Finally, given that assay performance can vary with time, operator, reagent lot, calibration, equipment maintenance and other factors monitoring of sensitivity whether as FS or LOQ should be ongoing, and laboratories should determine their own FS/LOQ rather than just quoting manufacturers' data [36]. Consequent to above arguments, it becomes clear that in comparing the performance of different Tg assays and in order to provide clinicians with realistic interpretations of Tg results, it is necessary to know exactly how "sensitivity" has been determined such that like can be compared with like. Some examples of these approaches are illustrated in Table 5.3.

5.3.2.2 Reference Range

Valid estimations of a reference range require sizable groups of subjects; all of whom must be correctly identified according to the absence of disease by methods other than the diagnostic tests being evaluated [37]. Selection criteria of reference population are critical as, for example, Tg reference values are geographically sensitive, since serum Tg is influenced by iodide availability and intake [38]. According to the National Academy of Clinical Biochemistry, the reference range could be evaluated in healthy non-smokers with thyroid-stimulating hormone (TSH) within the normal reference range for the population, with no personal or familial history of thyroid disease, no palpable or visible thyroid gland nor positive antibodies against Tg (TgAb) or thyroperoxidase (TPOAb). The Tg reference range should be expressed as median ± 2 standard deviations obtained after log-transformation of data [39, 40]. Reference ranges of Tg in widely employed Tg assays are summarized in Table 5.4.

The tissue-specific origin of Tg biosynthesis dictates that Tg in serum will be absent in DTC patients treated by thyroid ablation (i.e. expected Tg values theoretically correspond to zero). As a consequence, the clinical impact of Tg reference range in thyroid healthy subjects is limited. However, a reliable reference range is useful to properly evaluate the assay performance, especially at low Tg concentrations. In fact, assays which provide the greatest distinction between the lower limit of the euthyroid reference range and the analytical limit of the assay offer the most clinical sensitivity for detecting small amounts of thyroid tissue even in the TSH-suppressed state [4]. Subnormal to undetectable serum Tg levels (despite a negative TgAb test results) are occasionally encountered in clinical practice in DTC patients with clear disease foci or significant thyroid remnants [41–43]. Such false-negative results may erroneously suggest complete biochemical response and may occur when the spatial conformation of Tg is changed, leading to decreased immunoreactivity or when the ability

Table 5.3 Analytical sensitivity of some commercially available Tg assays as reported in the literature or quoted by manufacturers

Assay	Manufacturer	Methodology	Parameters (ug/L)
sTg KRYPTOR	BRAHMS	TRACE	LOB = 0.02 (M), LOD = 0.04 (M) LOQ = 0.1 (M), total allowable error of ≤40%
Tg II COBAS	ROCHE	ECLIA	LOB 0.02 (M), LOD 0.04 (M) LOQ 0.1 (M), total allowable error ≤30% FS 0.1 inter-assay CV = 20%
Tg Access	Beckmann	CLIA	LOD 0.1 (M) FS 0.1 precision profile from ten pools at a CV = 20% FS 0.05 according to NACB
Dynotest Tg Plus	BRAHMS	IRMA	LOD 0.16 (M) FS 0.4 (M) Values corrected for difference in BCR® 457 standardization (i.e. correction factor: x 2)
eIASON	Iason GmbH	ELISA	FS 0.2 (M)

Legend: *LOD* limit of detection, *LOQ* limit of quantification, *FS* functional sensitivity, *M* manufacturers, *TRACE* time-resolved amplified cryptate emission, *ECLIA* electrochemiluminescent immunoassay, *CLIA* chemiluminescent immunoassay, *IRMA* immunoradiometric assay

Table 5.4 Tg reference ranges in different assays

Assay	Manufacturer	Methodology	Reference range (μg/L)
RIA Tg-plus	BRAHMS	IRMA	2.00–51
sTg KRYPTOR	BRAHMS	TRACE	2.40–48
Tg II COBAS	ROCHE	ECLIA	3.50–77
Tg IMMULITE 2000	DPC	CLIA	1.60–60
Tg Access	Beckmann	CLIA	1.59–50

Legend: *IRMA* immunoradiometric assay, *TRACE* time-resolved amplified cryptate emission, *ECLIA* electrochemiluminescent immunoassay, *CLIA* chemiluminescent immunoassay

to secrete Tg is lost by cancer cells. In other cases false-negative Tg results may occur in the presence of an undetected TgAb interference [44].

As preoperative Tg below the lower reference limit may be detected in these cases, a measurement of Tg and TgAb before thyroidectomy was proposed in all DTC patients. This strategy provides "baseline" Tg and TgAb concentrations which serves as benchmark for the subsequent follow-up and could theoretically allow assessment of the reliability of post-surgery Tg and TgAb measurements [4, 44]. However, this simple concept is not widely accepted in clinical practice [despite pre-therapy measurements are recommended for other tumour marker such as calcitonin in patients with medullary thyroid carcinoma.

5.4 The Relationship Between Thyrotropin and Thyroglobulin

Like the physiological thyroid secretion, tumoural secretion of Tg mostly displays a TSH dependency as follicular-derived tumour

cells mostly preserve TSH receptors. As a consequence, Tg concentrations measured under maximum TSH stimulation (i.e. stim-Tg) exceed Tg values under TSH suppression (i.e. on Tg) by one order of magnitude. In clinical practice TSH stimulation is obtained by withdrawing levothyroxine (~4 weeks) or by administering recombinant human TSH (rhTSH). The protocol for Tg stimulation approved by regulators in every country where rhTSH is marketed consists of an intramuscular injection of 0.9 mg of rhTSH in the buttock, followed by a second injection of rhTSH 0.9 mg 24 h later. A serum stim-Tg is obtained 72 h after the second rhTSH injection [45]. A significant correlation was found between peak of Tg after hormone withdrawal and administration of rhTSH; however, rhTSH-stimulated Tg levels are usually (significantly) lower than off-Tg ones [46]. Several explanations may be offered for the Tg increments after thyroid hormone withdrawal being higher than those after rhTSH. Tg synthesis and secretion are more continuous and prolonged during endogenous TSH stimulation, and Tg clearance rate may be lower, compared with exogenous stimulation. Whatever the cause, this finding poses relevant problems in the interpretation of the serum Tg results obtained by different stimulation protocols. The TSH level is necessary to achieve adequate Tg stimulation after thyroid hormone withdrawal has not been determined, and the commonly used cut-off is derived from the level thought necessary for radioactive iodine imaging. Valle and colleagues [47] reported that TSH and Tg levels continuously rise throughout 4 weeks of thyroid hormone withdrawal, and the minimal TSH cut-off of >30 μUI/mL may be inadequate to detect many patients that eventually demonstrated a stimulated Tg ≥2 μg/L. A TSH cut-off of >80–100 μUI/mL was more reliable to detect these patients, suggesting that consistent methods and intensity of stimulation are necessary for adequate comparisons when monitoring patients with DTC. Interestingly, when the Tg was undetectable (<0.2 μg/L) at a TSH level > 20 μUI/mL, their final Tg did not stimulate to ≥1–2 μg/L during 4 weeks of thyroid hormone withdrawal with 91% and 100% certainty, respectively. Similar problems when using rhTSH stimulation were also reported regarding body surface area, lean body mass and age, as these factors may affect the TSH concentration that is reached after rhTSH administration [48–50]. All in all, consistency in the method and manner in which stimulated Tg is performed is needed, and differences should be acknowledged when consistency is not possible. Additionally, in contrast with clinical guidelines and current practice, the same cut-off thresholds should not be used for different stimulation methods. In fact, when Tg measurements were obtained using both stimulation methods in the same patients, it was observed that Tg levels after rhTSH stimulation were fourfold lower than after withdrawal of thyroxine replacement, respectively [51]. In practice, clinicians should be prompted to recognize the dynamic variables of TSH, weeks of thyroid hormone withdrawal and method of Tg stimulation when they compare different stimulated Tg results in a DTC patient. Overall, by considering the principle factors influencing serum Tg concentrations (i.e. thyroid tissue mass, injury and TSH), it is evident that the trend in basal Tg, measured when TSH is suppressed, should reflect changes in thyroid tissue mass and thus provide more accurate clinical information than stimulated Tg testing [4]. A follow-up strategy centred on unstimulated Tg is now possible by employing highly sensitive Tg assays. Using an assay with a functional sensitivity of 0.4 μg/L, Giovanella et al. measured unstimulated serum Tg in 117 low-risk DTC patients: the negative predictive value of a Tg level < 0.4 μg/L was 96% and increased to 99% when combined with neck US. In this study, rhTSH-stimulated Tg measurement only detected one additional recurrence in 104 patients with an undetectable unstimulated Tg [10, 13]. After that, a number of studies were performed to investigate the performance of basal highly sensitive Tg measurement in the follow-up of DTC patients [12, 44, 52–57]. Recently, Giovanella et al. reviewed and meta-analysed data from nine studies including 3178 DTC patients and confirmed the very high negative predictive value (98–100%) of an undetectable basal highly sensitive Tg (e.g. <0.1 μg/L). Importantly, these assays also have an adequate

sensitivity for detection of recurrent disease (88–98%) [58]. All in all, the negative predictive value of a rhTSH-stimulated Tg value below 1–2 μg/L is comparable to a basal high-sensitive Tg value below 0.10–0.20 μg/L. Therefore highly sensitive assays obviate the need of TSH stimulation in DTC patients with undetectable basal Tg levels. Additionally, although the low frequency of DTC recurrences impacts the ability to study positive predictive values (PPVs), the PPV of an rhTSH-stimulated thyroglobulin above 1–2 μg/L appears comparable to a basal highly sensitive Tg above 0.10–0.20μg/L [59, 60]. In addition, the trend in basal hsTg, measured when TSH is lowered/suppressed at constant level, should reflect changes in thyroid tissue mass and thus provide a sensitive parameter for disease [4, 44]. This is also supported by a growing number of studies showing the prognostic utility of monitoring the basal hsTg trend and thyroglobulin doubling time [61–66].

5.5 Thyroglobulin Antibodies and Other Interferences on Thyroglobulin Measurement

Together with thyroperoxidase antibodies, TgAb are important pathogenic markers of thyroid autoimmune disease, present in approximately 10% of most female populations, depending on, e.g. the iodine intake [2, 67]. In DTC, on the other hand, TgAb are detected in 15–40% of patients, i.e. roughly twice or more as often as in the general population. It has also been noted that the frequency of a previous or current history of thyroid autoimmunity is higher than expected in DTC patients [68]. Epitope recognition patterns of TgAb were recently shown to be restricted to immunodominant clusters in 58% of patients with different thyroid cancer, whereas the rest were either broadly heterogeneous (16%) or nonreactive (26%). However, median Tg recovery did not differ between sera with restricted and unrestricted specificities (69% vs 80%; $p > 0.05$). Tg recovery in these sera was inversely correlated with the total number of epitopes recognized by sera ($r = -0.66$; $p < 0.001$). TgAb with both restricted and broad specificities were present in patients with differentiated thyroid cancer. TgAb interference was related to the number of epitopes recognized by sera rather than the pattern of epitope recognition [69]. In an earlier study, Ruf et al. showed that Tg epitope specificity of thyroid cancer TgAb was similar to that of thyroid healthy subjects with low TgAb concentrations but different in patients with overt thyroid autoimmune thyroid diseases such as Graves' disease and Hashimoto's thyroiditis [70]. Anyway, regardless of whether the presence of TgAb is due to true autoimmune disease or not, the possibility of compromising serum Tg measurements as tumour marker in DTC is not negligible. The initially established radioimmunoassays for measurement of serum Tg used double antibody techniques, which could result in either falsely high or falsely low serum Tg quantification, depending on the nature of the second antibody in the assay. The influence of the presence of TgAb in serum, however, will always be unidirectional resulting in a false lowering of the Tg concentration when using current immunometric assays. In these cases, Tg contained in a patient's serum is "sandwiched" between capture and detection antibody. TgAb can prevent binding of capture or detection antibody (or both) by blocking access to their respective epitopes on Tg, thereby resulting in false low Tg measurements [71] (Fig. 5.1).

5.5.1 Screening for TgAb Interferences in Tg Measurement

5.5.1.1 Semiquantitative and Indirect Methods

Semiquantitative assays make use of percentage of spiked labelled Tg that can be precipitated as immunocomplexes, gelatin agglutination and immunofluorescent methodologies with serial sample dilution. These methods are not used in current practice due to their low sensitivity and specificity, manual format and technical com-

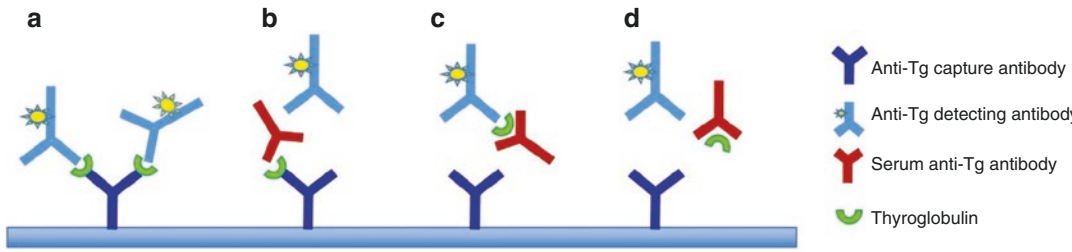

Fig. 5.1 Schematic representation of TgAb interference in Tg immunoassay. Legend (**a**) Normal interaction between anti-Tg capture antibody, Tg and anti-Tg labelled antibody; (**b**) TgAb binds Tg preventing interactions between anti-Tg labelled antibody and the complex Tg-anti-Tg capture antibody; (**c**) TgAb binds Tg preventing interactions between anti-Tg capture antibody and the complex Tg-anti-Tg labelled antibody; (**d**) TgAb sequesters Tg, preventing the binding with the capture and the labelled antibody

plexity. Indirect assays are based on comparing observed recoveries with expected recoveries of defined amounts of exogenous Tg spiked into patient samples (i.e. recovery test) and were widely adopted in clinical practice. However, recoveries may be influenced by exogenous Tg as multiple Tg isoforms have been found in both serum and the tissue-derived Tg preparations typically used for recovery testing. Conventional recovery testing with serum buffers containing 40–50 μg/L of Tg is considered undisturbed by most manufacturers if recovery rates are >70–80%; that is, a Tg concentration of 10–15 μg/L can be missed without finding a pathologic recovery. Given such a wide reference range, only strong interferences will be shown when using a conventional recovery buffer containing 40–50 μg/L Tg. In earlier days, when the lower detection limit of Tg assays was approximately 5 μg/L, this low sensitivity of recovery measurement might have been adequate. In modern clinical practice, a level of less than 1 μg/L is considered relevant, and as a consequence, conventional recovery testing is no longer adequate, and its use in clinical practice is discouraged [72–74]. A new development in recovery testing is the introduction of the so-called mini-recovery, in which recovery measurement is performed by adding serum with a low (i.e. 1–5 μg/L) Tg concentration. Theoretically, this mini-recovery test should be able to identify a detection loss of about 1 μg/L Tg, which, as already described, represents a clinically relevant limit. Preliminary clinical data are promising, but the performance of this mini-recovery has not yet been investigated extensively in DTC patients [75–77].

5.5.1.2 Thyroglobulin Antibodies Immunoassay

The determination of TgAb started 50 years ago with little sensitive qualitative techniques such as complement fixation, indirect immunofluorescence tests, passive hemagglutination, particle agglutination and immunodiffusion [78]. Later, in the 1980s, more sensitive competitive RIAs became available; they detected TgAb as a function of ^{125}I-Tg binding and reported results in kIU/L relative to the International Reference Preparation (IRP) and Medical Research Council (MRC) 65/93 [72]. Over the past decade, most of the laboratories have preferred non-isotopic, competitive or noncompetitive, automated IMA methods. They are currently considered the "gold standard", and their use is recommended by both clinical and laboratory guidelines [4, 8, 44, 79]. The main analytical performances of the current TgAb automated IMA methods are described in Table 5.3. They employ defined Tg-derived antigens in controlled concentrations, and most of them claim to be standardized against the IRP MRC 65/93 reporting results in kIU/L (= IU/mL). However, despite IRP standardization, several studies demonstrated the persistence of marked differences between TgAb IMAs: high variability of LOD, LOQ, FS and TgAb results for the same specimen measured by different methods (variation up to 100-fold) [78, 80–90]. The sources of TgAb inter-method variability are

manifold. First, the IRP MRC 65/93 dates back about 60 years, and it is made up of a pool of human plasma samples containing TgAb with Tg epitopic specificities which are more representative of thyroid autoimmunity than DTC [69, 91, 92]. In addition, some methods use their own internal standards being not standardized directly with IRP MRC 65/93 (Table 5.1), and so the difference between the epitope specificity of the reference preparation and of the secondary standards could result in widely discrepant numeric TgAb values for the same serum specimen (Table 5.3) [84, 86]. Another source of variability is the heterogeneous Tg immunoreactivity: differential splicing of Tg mRNA, various post-translational modifications (glycosylation, sialic acid content, iodination and sulfation) and alterations of biosynthesis regulation in thyroid tumour cells lead to exposure or masking of epitopes with resulting differences in Tg immunologic structure [93]. If the variations of Tg during the follow-up of DTC patients affect TgAb, characteristics are a matter which merits to be studied [94]. The assay discordance could also be assigned to the distinct specificity of patient's circulating TgAb for Tg. It seems that each patient has a typical IgG subclass and specificity for recognizing the Tg assay reagents [95]. Latrofa et al. [92], using recombinant human monoclonal TgAb, demonstrated the existence of two different models of TgAb epitopes: autoimmune thyroid disease (AITD) model and non-AITD model; the first displayed a more restricted epitope pattern with higher inter-method concordance; the non-AITD model had heterogeneous epitope pattern with consequent discrepant results in different assays. Interestingly, papillary thyroid carcinoma with lymphocytic thyroiditis resembled a model similar to that AITD with less epitope heterogeneity [94]. TgAb heterogeneity has two main implications: first, the ratios between TgAb measurements made with different assays remain constant during the serial monitoring of individual patients [73]. Secondly, even when different assays correspond well with each other in most patients, they might give discrepant results in certain individuals, probably due to dissimilarities in the Tg-derived antigens used in the assays, which might be recognized differently by distinct patient's TgAb [71, 96]. The determination and the interpretation of the cut-off of positivity are another important point to be addressed; in fact, the analysis of several studies showed a relevant discrepancy between the upper reference limits (URLs), according to the method employed [84, 85, 88]. A comparative study with most of the currently marketed immunometric automated methods in order to determine the experimental URL (e-URL) at the 97.5th percentile for each TgAb method was conducted according to the CLSI standard C28-A3c (submitted data) [97–99]. A panel of 120 sera obtained from healthy subjects were tested for TgAb. A wide variability of the e-URLs was found between methods but also, within the same method, between the manufacturer's URL (m-URL) and the e-URL (Table 5.5).

Particularly, with the exception of the Architect i1000 and the Maglumi 2000 Plus, e-URLs were lower than those claimed in the package inserts. These discrepancies could be related to the lack of strict criteria in the selection of the subjects for the reference group; in fact, there could be racial differences as most of the studies, sponsored by manufacturers, were performed in the geographical area of the production line and consequently difficult to reproduce in other settings. Moreover, the use of non-stringent criteria in the choice of subjects could have led to the enrolment of individuals with subclinical AITD and so high levels of TgAb, causing the raise of the URL. All in all, the differences between m-URL and e-URL highlight the need for individual laboratories to confirm the appropriateness of the reference intervals according to the method they use and the patient population they serve [85, 99–102]. Theoretically, a simple relationship exists between Tg and TgAb and the higher the TgAb concentration, the higher the Tg concentration that can be concealed by TgAb. Effectively, a logarithmic relationship was reported between TgAb concentrations (measured by IMAs) and surrogate measures of TgAb interference in Tg assays, such as the prevalence of undetectable Tg concentrations in patient populations or abnormal Tg spike recovery. Interference rates in two studies, for example,

Table 5.5 Analytical performance characteristics of the main current TgAb automated immunoassays

Manufacturer	Platform	Method	tracer/enzyme	Assay	Imprecision (%): *intra-; inter-; total*	LOD[d] (kIU/L)	LOQ[d] (kIU/L)	Assay range (kIU/L)
Abbott Diagnostics	ARCHITECT	CLIA	Acridinium esters	NC	1.7–6.6[b]; nd; 2.7–8.2[b]	0.07	0.31	0.07–100,000
Beckman Coulter	Access	CLIA	Lumi-phos 530/alkaline phosphatase	NC	3.6–5.7; 1.7–5.2; 4.8–7.7	0.9	nd	0.9–25,000
bioMerieux	VIDAS 3	ELFA	4MUP/Alkaline phosphatase	NC	2.6–9.5; 5.5–16.0; nd	6.2	6.4	6.4–8000
Thermo Fisher Scientific BRAHMS	Kryptor[a]	TRACE	Europium cryptate/XL 665	C	1.5–3.5; 6.8–20.0; nd	10	33	10–850
DiaSorin	Liaison	CLIA	Isoluminol derivatives	NC	2.3–3.2; 4.4–8.9; nd	5	10	5–5000
Fujirebio	Lumipulse G	CLEIA	AMPPD/ Alkaline phosphatase	NC	1.8–4.6; nd; 2.5–5.3[c]	5.152	5.152	5.152–3,000,000
Phadia AB, Thermo Fisher Scientific	Phadia 250	FIA	4-methyl-umbellipheryl-β-D-galactoside/ β-galactosidase	NC	3.3–5.6; 2.6–6.5; nd	12	nd	12–4794
Roche Diagnostics	Cobas/Elecsys	ECLIA	Ruthenium derivatives	C	1.3–5.6[c]; 2.1–8.7[c]; nd	10	nd	10–4000
Siemens Healthineers	ADVIA Centaur[a]	CLIA	Acridinium esters	C	2.9–5.5; 1.8–2.0; 3.5–5.8	10	30[e]	10–500
Siemens Healthineers	Immulite XPi	CLIA	Adamantyl dioxetane phosphate/ Alkaline phosphatase	NC	3.2–4.9; 4.6–5.8; nd	2.2	nd	20.0–30,000

(continued)

Table 5.5 (continued)

Manufacturer	Platform	Method	tracer/enzyme	Assay	Imprecision (%): *intra-; inter-; total*	LOD[d] (kIU/L)	LOQ[d] (kIU/L)	Assay range (kIU/L)
Snibe	MAGLUMI	CLIA	ABEI	NC	2.8–9.1; 5.2–9.8; nd	10	nd	10–2800
Tosoh Bioscience	AIA	FEIA	4MUP/Alkaline phosphatase	NC	4.3–5.1; nd; 5.5–6.0	0.12	nd	0.12–200,000
Tosoh Bioscience	AIA CL2400	CLEIA	DIFURAT®/Alkaline phosphatase	NC	5.1–5.5; 5.8–6.6; nd	0.005	nd	0.005–2,500,000

Legend: *4MUP* 4-methyl-umbelliferyl phosphate, *ABEI* N-(aminobutil)-N-(ethyl)-isoluminol, *AMPPD* alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, *C* competitive immunoassay, *CLIA* chemiluminescence immunoassay, *CLEIA* chemiluminescence enzyme immunoassay, *DIFURAT®* 3-(5-tert-Butyl-4,4-dimethyl-2,6,7-trioxabicyclo[3.2.0]hept-1-yl) phenylphosphate disodium salt, *ECLIA* electrochemiluminescence immunoassay, *ELFA* enzyme-linked fluorescence assay, *FEIA* fluorescence enzyme immunoassay, *FIA* fluoroimmunoassay, *NC* noncompetitive immunoassay, *nd* not declared, *TRACE* time-resolved amplified cryptate emission

[a]All methods are standardized with the reference preparation MRC 65/93 and use international units (kIU/L) except for Centaur and Kryptor which refer to a secondary standard and use arbitrary units (kAU/L)

[b]Precision defined by the NCCLS Protocol EP5-A

[c]Precision defined by the modified NCCLS Protocol EP5-A2

[d]LOD and LOQ defined by the CLSI protocol EP17-A

[e]Functional sensitivity defined as TgAb concentration with total CV ≤20%, determined for a period of 2 days using one lot of reagents and testing, by four instruments, multiple samples from normal patients

were about 5–8% percentages at TgAb concentrations of less than 4–6 kIU/L but rose quickly to approximately 30% just above this level and continued to rise asymptotically to 70–85% at 50–100 kIU/L with minimal further increment in prevalence thereafter [69, 103]. However, low concentrations of TgAb may be sometimes associated with strong interference, and, conversely, patients with high concentrations of TgAb show no evidence of interference with the Tg measurement. On the basis of these considerations, the term positivity (that means TgAb concentration higher than the URL) is used inappropriately, and many specimens with interfering TgAb were proved to be misclassified as TgAb negative using m-URL. In fact, since the relationship between TgAb concentration and their interference in Tg IMAs is not clear, with even low concentrations of TgAb being able to cause false-negative results, TgAb URL must be interpreted with caution, never forgetting that it is usually calculated to diagnose AITD and not to exclude the presence of potentially interfering TgAb. Most likely, false-negative misclassifications could be reduced or even eliminated by using the LOD/FS of the method employed. Thus, it is advisable to use high-sensitivity quantitative TgAb assays, in order to allow detection of the majority of possible TgAb interferences [44]. Therefore, according to these findings, two different cut-offs for TgAb could be defined, one for the diagnosis of AITD and one for the effects of TgAb on Tg measurement [104]. However, it is noteworthy that the LOD/FS cut-off is associate with about 20% false-positive cases. In addition, LOD/FS cut-off also has an inherent 15–20% between-run imprecision, leading to false fluctuations in TgAb status [4, 105]. Finally, the relevant difference between URLs supported concerns regarding inter-method bias; in fact, despite the attempt of harmonization, TgAb methods were too qualitatively and quantitatively variable to come up with any definitive and common cut-off or to establish conversion factors that would allow a change in method without disrupting serial TgAb monitoring [89]. The lack of satisfactory agreement between methods has an important practical implication: on the one hand, the clinicians/patients had to use always the same method to measure TgAb in the follow-up of DTC, and on the other hand, laboratories had to notify in time any change in TgAb methods to facilitate re-baselining. Moreover, as the qualitative characteristics of the TgAb secreted by individual patients remain constant over time during long-term monitoring, independent of changes in TgAb concentration, it is advisable to store a patient's sample in order to establish, if possible, the ratio between the old and the new method, allowing re-baselining and so the monitoring of TgAb trend [44, 91, 104, 106].

5.5.1.3 Comparison of Tg Measurement by Radioimmunoassay Versus Immunometric Assay

Competitive Tg assays (usually radioimmunoassay, RIA), using polyclonal antibodies, were reported to be less susceptible to TgAb interference than Tg IMA methods, and discordance between Tg measured by RIA versus IMA was adopted as an additional methodological benchmark for detecting TgAb interference [4, 107, 108]. It is unlikely, though, that this observation can be generalized to all Tg RIAs, as some authors have found the opposite [109]. Although TgAb interference with IMA methodology is always unidirectional (i.e. underestimation), the influence of TgAb on RIA measurements is variable and assay dependent. Early studies reported that TgAb caused overestimation of Tg measured by RIA, presumably when endogenous TgAb sequestered [^{125}I-Tg] tracer. In contrast, more recent studies have suggested that TgAb causes underestimation of Tg measured by RIA, presumably when the second antibody reagent precipitates endogenous TgAb-[125I-Tg] complexes [1]. Moreover, most RIA methods are less sensitive in detecting Tg than IMA ones. Hence, any benefits gained in robustness against TgAb interference might be negated by the inability to detect the low Tg levels that characterize cure [110, 111]. In summary, any sample with a positive TgAb result, measured by a sensitive test, should be considered unreliable for measuring serum Tg concentrations in patients with DTC. Several analytes have been investigated as alternatives to

Tg measurement to assess relapse/metastases in TgAb-positive DTC patients such as oncofetal fibronectin, Tg messenger RNA (mRNA), thyrotropin receptor mRNA, thyroid peroxidase mRNA and circulating mutated BRAF. However, in most studies it proved impossible to achieve clinically useful sensitivity and specificity levels for these analytes [8, 112].

5.5.1.4 Tg measurement by Tandem Mass Spectrometry: Liquid Chromatography (MS/MS-LC)

Tandem mass spectrometry-liquid chromatography (MS/MS-LC) recently emerged as a promising method to overcome interferences in Tg measurement [113, 114]. The MS/MS-LC workflow for protein measurements involves a digestion of the sample with trypsin. Trypsin cleaves protein in a predictable fashion into peptides, which are measured and identified by protein database matching. In fact trypsin digests all proteins in a sample, including Tg and any TgAb or other antibodies, by cleaving them at predictable sites. One can then specifically look for tryptic peptides that are proteotypic for Tg (based on predicted cleavage), without any interference by TgAb [115] (Fig. 5.2).

Current MS/MS-LC Tg assays have shown comparability to immunoassays in samples that are TgAb negative. In TgAb-positive samples, which have detectable Tg by Tg immunoassay, all published MS/MS-LC Tg assays still correlated with the immunoassay but demonstrated a slope of ~1.5, consistent with systematic under recovery of Tg (~50–60%) in the immunoassays. Finally, in samples that are TgAb positive but have an undetectable Tg by sensitive immunoassay, the Tg MS assays can detect Tg in 20–25%

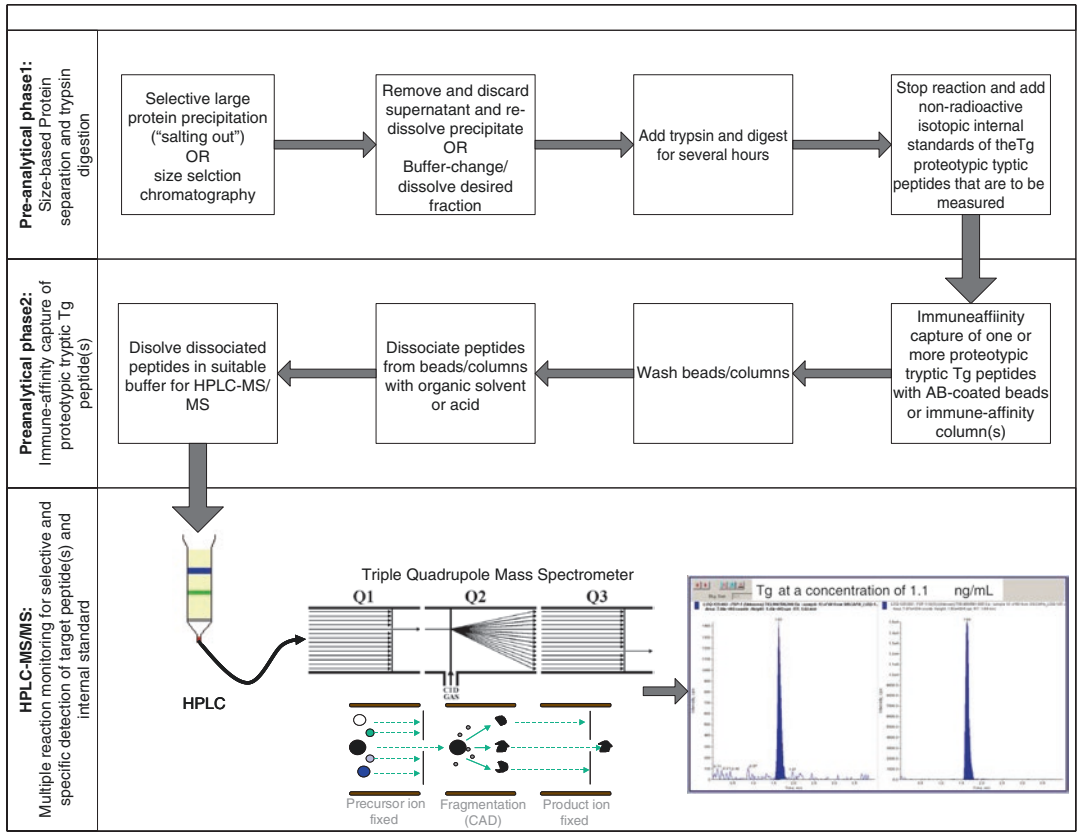

Fig. 5.2 Schematic depiction of the workflow for current (high-pressure) liquid chromatography tandem mass spectrometry (LC-MS/MS; HPLC-MS/MS) measurement of Tg (from [32], permission obtained)

of cases [116]. In practice, however, many problems affect Tg measurement by MS/MS-LC. First, one faces formidable "signal to noise" problems in identifying the quite low concentration of Tg peptides in a trillion-fold higher background abundance of all the other peptides from all the other proteins, and sample enrichment is required (i.e. immune affinity purification of the desired Tg target peptides from all the other peptides). Second, MS/MS-LC-based Tg assays are manual, complex, with a the long turnaround time (largely due to the several hours of tryptic digest). Third, the LOQ of current MS/MS-LC Tg assays is 0.5–1 μg/L, higher than that of high-sensitive Tg immunoassay (FS/LOQ ~0.1 μg/L) resulting in a suboptimal clinical sensitivity. Azmat and colleagues [117] recently evaluated the frequency of detectable Tg-MS/MS-LC with the functional sensitivity (FS) of 0.5 μg/L in patients with structural disease and compared performance of Tg MS/MS-LC versus Tg IMA, using either Immulite® assay with a FS of 0.9 μg/L or Beckman® assay with a FS of 0.1 μg/L in detecting structural disease in patients with positive TgAb. In patients with structural disease and positive TgAb, Tg MS/MS-LC was undetectable in 43.7% of patients. In the 26 patients with positive TgAb where Immulite assay was used, the sensitivity and specificity for detecting structural disease were at 44.4% and 94.1% for Tg MS/MS-LC assay and at 33.3% and 88.2% for Immulite assay. In the 74 patients with positive TgAb where Beckman assay was used, the sensitivity and specificity for detection of structural disease were 62.6% and 93.7% for the Tg MS/MS-LC and 72.7% and 71.4% for the Beckman assay, respectively. Overall, Tg MS/MS-LC was frequently undetectable and was less sensitive for detecting disease than a Tg immunoassay with functional sensitivity at 0.1 μg/L questioning the clinical usefulness of reflexing Tg measurement to MS/MS-LC in TgAb-positive patients [118]. Furthermore, in TgAb-positive patients with negative recovery measurement, RIA/IMA comparison and MS/MS-LC, falsely low Tg levels may still occur due to a faster biological clearance of TgAb-bound Tg [119].

5.5.2 Interfering Heterophilic Antibodies

Heterophilic antibodies (HAb) can bind animal antigens and form a bridge between capture and detection antibody leading to a falsely elevated (or, rarely, falsely decreased) Tg measurement in immunometric assays. These interferences are usually eliminated by the manufacturers by adding blocking agents to the assay, but a small percentage of patients (~1–3%) still show HAb interference on Tg measurement [120]. HAb interference may be detected either by recovery measurement or measurement of Tg in serially diluted sera (providing Tg concentrations are sufficiently high). An additional method, which is more specifically geared towards HAb interference, is to pretreat a serum aliquot with proprietary blocking agents and then compare the Tg result with an aliquot that was not pretreated [121]. In addition, as HAb interference is generally assay specific, the use of an alternate assay may both identify a false-positive sample and provide the correct test value. From the practical point of view, routine screening for the presence of HAb is not recommended; however, one or more of these tests should be performed in patients with discordant clinical findings, such as high serum Tg but negative imaging workup, positive imaging but undetectable Tg in the absence of TgAb and/or an unusual clinical course of Tg concentrations [122, 123].

5.5.3 Hook Effect

Thyroglobulin, as other tumour marker tests employing two-site noncompetitive IMAs, is prone to the so-called high-dose hook effect: this phenomenon is reported for the first time by Miles [124]. The excessively high concentrations of the analyte simultaneously saturate both capture and detecting antibodies. This prevents the formation of detectable antibody leading to the formation of stable capture antibody/analyte/detecting antibody complexes with a plot resembling a "fish hook" (Figs. 5.3 and 5.4) [3]. It affects mainly solid-phase assays where the capture antibody concentrations may be limiting.

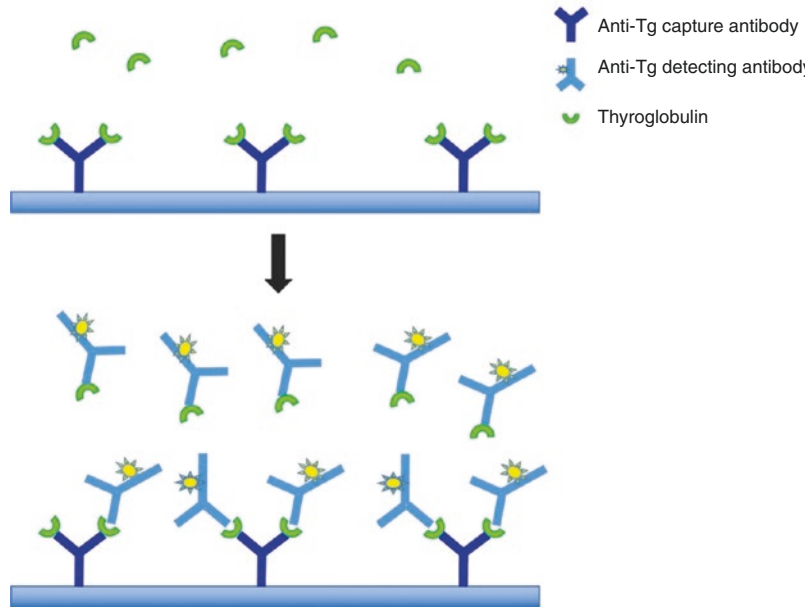

Fig. 5.3 High-dose hook effect: scheme of the principle

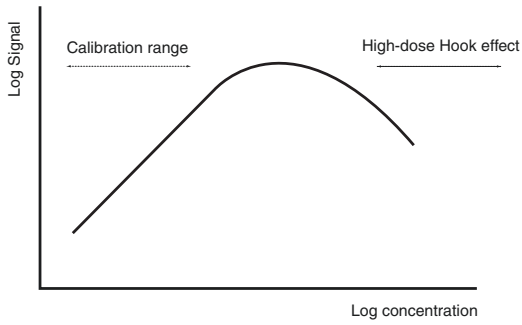

Fig. 5.4 Graphic representation of the high-dose hook effect

In this case a falsely low serum Tg measurement may have important clinical consequences. This phenomenon is demonstrated for alphafetoprotein, prolactin, chorionic gonadotropin, PSA, NSE, calcitonin and other tumour markers [125, 126]. In the case of Tg, published data show that the recent two-step sandwich IMAs are very resistant to high-dose hook effect, probably occurring only at very high concentrations of Tg (up to 200,000 mg/L) that is an unusual situation (about 0.1% of routine samples) [3, 127]. However, all laboratories providing tumour marker assays (i.e. Tg) should be alert to the possibility of hook effect ensuring a constant vigilance for this phenomenon: to avoid reporting falsely low results, some laboratories perform the Tg measurement on both neat and diluted serum (for cost reasons possible in few laboratories) or make a pool of patient specimens comparing it to the expected average of the batch of samples [128].

5.6 Role of Thyroglobulin and Thyroglobulin Autoantibodies in Managing Patients with Differentiated Thyroid Cancer

As previously mentioned preoperative Tg measurement is considered to have limited diagnostic value, although a number of studies report that an elevated preoperative serum thyroglobulin is a risk factor for nodular malignancy [23, 129, 130] and serum Tg measurement is sometime employed to strengthen or exclude a suspicion of DTC in patients with widespread metastases of unknown origin. Also, the relationship between the serum Tg levels and tumour burden may give indication of the efficiency of the tumour cells to secrete Tg and thereby determine the significance of postoperative serum Tg changes [3, 4, 44, 131].

5.6.1 Patients Treated by Total Thyroidectomy and Radioiodine Ablation

Current international clinical guidelines agree that Tg is a pivotal sensitive method for monitoring patients with DTC for the presence of residual or recurrent disease during follow-up after total thyroidectomy and, ideally, adjuvant ^{131}I remnant ablation. For a long time DTC follow-up was based on stimulated serum Tg in all patients who had had remnant ablation and negative cervical US and undetectable TSH-suppressed Tg within the first year after ablation. A "negative" TSH-stimulated Tg measurement and no other evidence of recurrent disease (i.e. negative clinical examination, neck US or additional imaging procedures, when indicated) predicted a very low risk of recurrence. The prognosis is excellent and life expectancy normal if response to the treatment is achieved within 6–12 months after treatment, both for low- and high-risk patients [132]. This approach (i.e. *dynamic risk stratification system*) allows tailoring follow-up intensity on an individual basis according to the response achieved after primary treatment [133] (Table 5.6).

Traditionally, "biochemical cure" was defined by a Tg levels <1–2 μg/L following TSH stimulation. More recently, however, novel highly sensitive Tg assays (with a functional sensitivity ≤0.1–0.2 μg/L) have been developed and became commercially available [9]. As rhTSH typically stimulates basal Tg approximately tenfold, the negative predictive value of a rhTSH-stimulated Tg value below the fixed cut-off of 1–2 μg/L is comparable to a basal high-sensitive Tg value below 0.10–0.20 μg/L, as consistently confirmed by literature [44]. Such concept was introduced in recent ATA 2015 guidelines [8] where interpretation criteria for basal and stimulated Tg were provided, as summarized in Table 5.7.

These criteria are simple and practical and allow clinician to modulate the intensity of further follow-up and to avoid inappropriate diagnostic procedures with relevant impacts on patients' comfort and overall costs. However, a relevant point of discussion is that most guidelines do not sufficiently address differences between different Tg assays in terms of the lower reporting limit (i.e. functional sensitivity or LOQ), analytical and clinical performance and appropriate cut-off limits. In fact, despite calibration against an international reference standard

Table 5.6 Response assessment after total thyroidectomy and radioiodine ablation according to ATA 2015 criteria [8]

Response	Definition
Excellent response	No clinical, biochemical or structural evidence of disease
Incomplete biochemical response	Abnormal Tg or rising anti-Tg antibody levels in the absence of localizable disease
Incomplete structural response	Persistent or newly identified loco-regional or distant metastases
Indeterminate response	Nonspecific biochemical or structural findings that cannot be confidently classified as either benign or malignant

Table 5.7 Response assessment after total thyroidectomy and radioiodine ablation: imaging and biochemical criteria according to ATA 2015 criteria [8]

Response	Imaging	Thyroglobulin [μg/L]
Excellent response	Negative	Basal Tg <0.2 μg/L *OR* Stimulated <1 μg/L
Incomplete biochemical response	Negative	Basal >1 μg/L *OR* Stimulated >10 μg/L
Incomplete structural response	Positive	Any value
Indeterminate response	Indeterminate findings	Basal 0.2–1 μg/L Stimulated 1–10 μg/L

(BCR®457), multiple assays analysing the same samples report different values due to heterogeneity in both Tg structure and assay reactivity. Additionally, different protocols are used by manufacturers to define analytical characteristics of different assays, and an assay with a declared higher functional sensitivity value may have a clinical performance equal to or better than one with a lower declared functional sensitivity. Accordingly, clinical thyroidologists and laboratory specialists are strongly advised to carefully evaluate the analytical and clinical performance of any newly introduced (highly sensitive) Tg assay, including a comparison between basal and stimulated values in the same assay, and to confirm cut-off and decision limits in their own DTC patient populations [44]. As a practical recommendation, it should be noted that Tg results cannot be reliably interpreted from samples collected immediately after surgery (i.e. post-surgical Tg half-life: 2–4 days) or up to 3 months after radioiodine therapy [62, 134]. Therefore, waiting 6–8 weeks after surgery and 3 months after radioiodine therapy is recommended [4, 44]. Finally, it is important to note that most patients who were enrolled in available studies on high-sensitive Tg were affected by low-risk and, even if less frequently, intermediate-risk DTC. Data on patients with high-risk tumours, however, are sparse and less robust. As a consequence, further studies in a broader spectrum of high-risk DTC patients are needed before applying the same approach in these cases, and for the moment, using a combination of ultrasound, stimulated Tg and diagnostic whole body scan is suggested [135].

5.6.2 Is There a Role for Tg Measurement in Patients Treated with Surgery Alone?

5.6.2.1 Lobectomy

According to current clinical guidelines, it is sufficient to treat low-risk patients with a thyroid microcarcinoma by resection of the affected thyroid lobe only, without complete thyroidectomy or ^{131}I ablation. In this situation, measuring Tg using either highly sensitive or conventional assays is essentially useless as Tg levels will not depend on the presence or absence of tumour foci but rather on the remaining thyroid lobe volume, current iodine status and TSH concentration. In such patients, the options for DTC follow-up are to perform cervical US and, if recurrence or metastasis are suspected, to secure the diagnosis through a fine needle biopsy [44]. More sophisticated Tg reference intervals, mathematically normalized to TSH level and residual thyroid tissue, tailored to individual patients should be useful in these cases [1]; for the moment, however, no reliable interpretation criteria are available.

5.6.2.2 Thyroidectomy

In patients with tumours <10–20 mm, no lymph node and/or distant metastases, a (near-)total thyroidectomy without radioiodine ablation is now considered a reasonable treatment [8]. These non-ablated patients may have a considerable thyroid remnant, and a TSH stimulation during follow-up is not useful as Tg will be detectable due to remaining healthy thyroid tissue and will obscure any possible tumour-related Tg level rise. In addition, the absolute Tg concentration will be significantly less useful in this scenario. However, a decrease of Tg concentrations over time was reported, and the number of patients with undetectable Tg significantly increased after 5 years of follow-up [136]. A retrospective evaluation of 86 patients with low-risk DTC treated by total thyroidectomy only (i.e. without radioiodine ablation) using a highly sensitive Tg assay (i.e. functional sensitivity, 0.1 μg/L) was reported [137]. Of the 76 patients without TgAb, the first Tg measurement (on T4), obtained at a mean time of 9 months after surgery, was ≤0.1 μg/L in 62% of cases, ≤0.3 μg/L in 82%, ≤1 μg/L in 91% and ≤2 μg/L in 96% of cases. After a median follow-up of 2.5 years, one patient had persistent disease, an unstimulated Tg concentration of 11 μg/L and an abnormal neck US, while two patients had Tg levels >2 μg/L with normal neck US. Within the first 2 years after total thyroidectomy, the unstimulated Tg level was <0.3 μg/L in 86% and ≤2 μg/L in 96% of the cases, respectively. However, the authors emphasize that the

results were strictly dependent on the completeness of surgery by a dedicated surgeon in a referral centre. More recently, Tg cut-off levels were proposed by Momesso and colleagues for DTC patients treated without radioiodine ablation [138]. However, relevant methodological problems hampered such study and precluded any reliable conclusion for the clinical practice. In facts, they employed five different Tg immunoassays over time with different functional sensitivities and arbitrarily selected Tg cut-off [139]. Then, although a stable low Tg concentration, combined with normal neck US, may be helpful in assessing whether there is concern for progressive disease in these patients, reliable interpretation criteria of Tg testing are still lacking, as underlined in the last version of ATA guidelines [8]. It is true that the trend in basal Tg should reflect changes in thyroid tissue mass and low Tg concentrations arising from small post-surgical thyroid remnants are expected after thyroidectomy, typically in the 0.1–0.5 μg/L range when TSH is suppressed [59]. However, (1) the volume of thyroid remnants is "surgeon dependent" with a high variability [140], and (2) TSH-suppression is no longer recommended in most low-risk and intermediate-risk DTC patients and TSH levels ranged between 0.1–0.5 and 2 mUI/L in such cases. Of course, Tg reference values may be significantly different in patients with small remnants and suppressed TSH after surgery and those with large remnants and non-suppressed TSH levels, respectively. Again, Tg values should be normalized to the volume of remnant tissue and TSH levels to obtain a reliable clinical information. Additionally, TSH levels may also impact the longitudinal evaluation of Tg [i.e. trend and doubling time] that recently showed prognostic utility [141]; in facts, aspecific TSH fluctuations can induce relevant Tg changes that, in turn, may falsely alert or reassure both patients and physicians. All considered, it seems very difficult, if not impossible, to provide general interpretation criteria for serum Tg in non-ablated DTC patients. Then, for the moment, Tg interpretation in these patients requires stable TSH levels, consistency of Tg assay employed across the follow-up and cautious clinical interpretation.

5.6.3 Managing Patients with Positive Thyroglobulin Antibodies

Serum levels of TgAb are not correlated with the tumour load of the patient but rather indicate the activity of the immune system. Furthermore, the mere presence of TgAb in serum has thus far not conclusively been shown to correlate with a worse or better overall prognosis. Chiovato et al. [142] demonstrated that the concentration of TgAb after thyroid ablation for thyroid carcinoma of 182 patients with thyroid autoimmune disease before treatment had a mean disappearance time of 3 years, indicating that the actual TgAb concentration is not very useful during that period for outcome prediction. However, TgAb can be used as "surrogate tumour marker" as disease-free patients with high TgAb concentrations typically display a progressive TgAb decline over time, even if some patients may not achieve full TgAb negativity, possibly because of the long-lived memory of plasma cells. In this context the trend is more important than the absolute level: a consistent reduction in the serum TgAb concentration thus seems to indicate that the patient is likely to be free of disease, while a consistent rise or *de novo* appearance of serum TgAb raises suspicion of recurrence, and an unchanged serum TgAb concentration must be regarded as indeterminate [109, 143, 144]. Unfortunately, robust quantitative criteria for the interpretation of a specific "rising" or "falling" of serum TgAb concentration are still lacking [74], and few caveats should be also mentioned (1) a very abrupt and extreme rise in serum Tg as in rapid development of metastatic thyroid carcinoma may compromise correct quantification of TgAb measurements by immune complex formation with falsely low TgAb concentrations; (2) a rapid, but transient, increase in measured TgAb concentration may occur shortly after thyroidectomy in patients with prior positive TgAb; and (3) a similar but slower reaction is seen after radioactive iodine treatment [145–147]. As a guide for TgAb trend interpretation, different authors have suggested that disease-free patients typically display a > 50% drop in TgAb in the

first post-operative year [148–150]. However, this figure is very likely to depend on the initial pretreatment concentration, and more studies and evidence are thus required to be able to use this as early risk assessment in clinical practice, in order to avoid uncertainty and additional expensive imaging procedures.

Finally, it should be emphasized that the recommendation to use serial TgAb concentrations as a surrogate tumour marker necessitates continuity of the method in the laboratory as changing methods disrupts TgAb monitoring. In this context it is worth noting that despite numeric differences between methods, the ratio between any two different TgAb methods appears constant for a given patient but different for different patients, reflecting TgAb heterogeneity. Establishing the ratio between an old and proposed new method on a specimen from the patient can be used to *re-baseline* the new method, which is an important approach in order to avoid misinterpretation of the long-term outcomes. However, this is rarely done in clinical practice [91]. Currently the data on TgAb use for thyroid cancer management is based on an initial treatment strategy of total ablation, since insufficient ablation will hamper both Tg and TgAb as tumour markers. For TgAb the important issue is continued presence of autoantigens as long as remnant thyroid cells are still present. In practice, while TgAb together with the other thyroid autoantibodies generally decrease after removal of the thyroid tissue by total ablation [142], their disappearance is not expected in other cases since autoimmunity will be continuously stimulated by the presence of thyroid autoantigens as well as by intrathyroidal lymphocytes. Importantly, no clinical data are currently available on the management of TgAb-positive patients treated by either lobectomy alone or thyroidectomy without radioiodine ablation.

Conclusions

The post-surgical follow-up of DTC is aimed at early identification of the small proportion of patients who have residual disease or will develop recurrence. In the absence of TgAb and heterophilic antibodies, Tg measurements are nowadays the reference standard for clinical management of patients previously treated for DTC. Even though the introduction of high-sensitive Tg assays is not without challenges, there is an increasing body of evidence that an undetectable highly sensitive Tg during LT4 treatment is sufficient with a high negative predictive value to forgo TSH stimulation. In the presence of TgAb, it is possible to follow the dynamic trend of TgAb themselves as surrogate markers. Robust data are urgently needed to define the clinical role, the interpretation criteria and the limitations of these markers in the increasing number of patients treated with lobectomy alone or total thyroidectomy not followed by radioiodine ablation. For the time being, the issue remains largely unaddressed, and no clear-cut recommendations for the clinical practice can be delivered before well-designed, large and multicentric studies addressing these issues have been published.

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Circulating Molecular Biomarkers in Thyroid Cancer

6

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6.1 Introduction

Histological examination of tumor tissues is still regarded as the gold standard method for assessing cancer biomarkers. However, it entails invasive collection procedures, which are not only costly and time-consuming but also associated with a variety of potentially serious adverse effects [1]. It is therefore poorly suited for serial use in evaluating the evolution of tumors and their response to treatment. Biomarkers that can be detected and measured in blood samples offer a number of potential practical advantages in this setting, as well as potential shortcomings involving their diagnostic/prognostic performance.

The principal serum biomarkers used to detect and monitor thyroid cancer are thyroglobulin and calcitonin [2, 3]. Thyroglobulin, the protein precursor of thyroid hormone, is produced exclusively by follicular thyrocytes (normal or neoplastic). Assays of its levels in the serum are of little use in the preoperative work-up of patients with thyroid nodules since elevations can be associated with most types of thyroid disorders [4]. However, in patients with differentiated thyroid cancer (DTC) who have undergone total thyroidectomy and radioiodine remnant ablation, detectable thyroglobulin levels in the serum have long been considered a sensitive and specific indicator of persistent or recurrent cancer [5]. In terms of sensitivity, the major shortcoming of this approach is the possible interference by circulating autoantibodies directed against thyroglobulin itself, which are present in approximately 20–25% of patients with DTC [6]. Specificity issues are becoming more and more relevant as the management of DTC evolves to meet the challenges of the rising incidence and earlier diagnosis of these tumors. The specificity of serum thyroglobulin assays declines substantially in the presence of residual normal thyroid tissue [7–10], and this situation is being encountered with increasing frequency as treatment choices shift toward the use of less extensive surgery, more selective use of radioactive iodine remnant ablation, and in some cases even active surveillance alone [5, 11–15]. The use of serum calcitonin levels as a marker of MTC recurrence is also limited by problems of specificity. This polypeptide hormone is produced mainly (but not exclusively) by the C cells of the thyroid gland, and immunoassays of calcitonin levels in the serum thus provide information on the activity of the cells that give rise to MTC and to the related but benign condition known as C-cell hyperplasia [16]. However, hypercalcitoninemia can also be caused by a number of other diseases, including chronic autoimmune thyroiditis and several non-thyroid-related conditions (e.g., neuroendo-

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crine tumors, hypergastrinemia, hypercalcemia, chronic kidney disease) [16]. To further complicate matters, serum calcitonin levels are influenced by a number of analytical factors, drug use, and physiological variables, such as age, sex, diet, and lifestyle. As a result, reliable, standardized cutoffs for interpreting serum calcitonin assays are lacking [16].

6.2 Liquid Biopsy: Concept and Potentialities

One of the solutions being developed to meet clinicians' increasing need for cancer biomarker assays that combine ease of use and low cost with high-level diagnostic accuracy is the concept known as "liquid biopsy." The term refers to all diagnostic procedures performed on cancer-derived materials that can be isolated from a peripheral blood sample rather than from a tissue biopsy or resected tumor [17]. This new and powerful approach for the study of cancer has been made possible by recent advances in technologies, such as next-generation sequencing (NGS) and digital PCR [18]. They allow detection and quantitative analysis of materials in the bloodstream (including those present at very low levels), which originate specifically from solid tumors [18].

Aside from its practical benefits, the liquid biopsy approach offers several other potential advantages over tissue-based analyses. For example, compared with tissue biopsy or aspirates for cytological analysis, which depict very restricted areas of the tumor, examination of various tumor derivatives that find their way into the bloodstream are likely to furnish a more comprehensive picture of intra-tumoral heterogeneity. Indeed, used serially, liquid biopsies can potentially provide ongoing documentation of the evolving genetic panorama of the entire tumoral landscape, including metastatic lesions [19]. This knowledge may be prognostically informative, as shown in studies of breast cancer and other types of cancer, where certain genetic alterations display correlation with disease stage, vascular invasion, lymph node metastasis, as well as overall and disease-free survival [1]. It might also improve decisions regarding appropriate targeted therapy and other aspects of clinical management [20].

A growing body of evidence suggests that liquid biopsies are potentially valuable tools for identifying early-stage malignancy and for monitoring tumor burden, including minimal residual disease [18]. Use of this approach could conceivably eliminate some of the frequent drawbacks of imaging studies, which are currently used for this purpose, including high cost, exposure to ionizing radiation, and limited sensitivity for the detection of micrometastases and/or for monitoring minimal residual disease [1]. Uninformative imaging findings are often bolstered with supplementary information on circulating levels of protein biomarkers, such as prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and cancer antigens (CA) 19-9 and 125. Here, too, liquid biopsy offers potential advantages, particularly when reliable, disease-specific protein markers for the patient's cancer are simply not available. Moreover, protein biomarkers persist in the circulation for weeks, whereas the turnover of circulating cancer cells or circulating free DNA is much more rapid. As a result, liquid biopsy should be able to detect tumor changes long before they are revealed by imaging findings or protein biomarker studies [21].

The most important areas in which liquid biopsies are expected to make major contributions are the prediction and monitoring of responses to treatment. The presence of a single genetic alteration in the tumor can decisively influence the selection of targeted drug therapies, particularly for advanced-stage cancers. Today, these decisions are made on the basis of genetic analysis of archived tumor tissues collected weeks or months earlier, which are probably poorly representative of the current genetic status of the disease. In contrast, a liquid biopsy can provide a "real-time" picture of the current molecular status of the tumor [1, 22]. Moreover, recent studies have shown that serial biopsies collected during the course of treatment can facilitate earlier detection of multiple drug-resistant clones [21], allowing prompt discontinuation of expensive, potentially toxic drug therapy that is unlikely

to be effective and rapid initiation of more suitable treatment [1].

The expected benefits of liquid biopsy appear to be supported by growing bodies of evidence in several types of cancers. However, before this approach can be used in the clinic, numerous hurdles must be overcome, including the lack of standardized techniques, and the preliminary findings must be substantially strengthened by the addition of data from larger studies and prospective trials [1, 18, 22].

6.3 The Cancer-Derived Materials Found in the Bloodstream

As noted above, whole tumor cells as well as cell-free nucleic acids (cfDNA, cfRNA, and circulating miRNAs) can all be found within the peripheral blood [18]. Whole tumor cells can enter the bloodstream after breaking free from primary or metastatic tumors. It is unclear whether this represents an active invasion of the vascular tree or is simply the result of passive shedding of tumor cells [23]. One of the most plausible hypotheses is that individual CTCs or clumps of CTCs detach from the tumor and penetrate the bloodstream via an active process that probably involves the epithelial-to-mesenchymal transition (EMT) [23]. Cell-free nucleic acids (DNA and RNA) can be found in the bloodstream as freely circulating (cfNAs) species or encapsulated in extracellular vesicles (EVs). EVs are membranous lipid structures produced by healthy and non-healthy cells for specific purposes, such as intercellular communication and immunoregulation [24]. The mechanisms by which cfNAs are introduced into the blood vessels are unclear. They probably include passive release during apoptotic or necrotic events occurring in the tumor microenvironment, as well as the active secretion of cfNA fragments, alone or incorporated into protein or lipid complexes [1, 24–26]. It is also conceivable that cfNAs can be released by CTCs after they enter the bloodstream, although conclusive evidence of this mechanism has yet to be presented [1].

The following section provides a closer look at circulating RNAs as a potential class of biomarkers for thyroid cancer.

6.4 Circulating RNAs

Messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) can all be found in the human bloodstream. Most of the research conducted thus far on circulating RNAs has focused on the analysis of mRNAs and more recently miRNAs. The presence and stability of RNAs in body fluids was surprising, given the high levels of RNase in the extracellular environment. The circulating RNAs were found to be protected from these enzymes by their incorporation in lipoprotein complexes or sequestration within lipid vesicles [27]. Notably, miRNAs were found to be the most abundant RNA species in lipid vesicles [28]. As compared to miRNAs, mRNAs were reported to be more susceptible to degradation [29]. For this reason, the past decade has witnessed a shift in the focus of research toward the study of circulating miRNAs, which are tissue-specific and highly stable, even after exposure to high temperatures, low or high pH, prolonged storage at room temperature, and multiple freeze-thaw cycles [26, 30].

6.4.1 Isolation and Detection

Research on circulating mRNAs and miRNAs as potential biomarkers has produced highly variable results, clearly reflecting the absence of methodological standardization in this field [31]. This variability can be caused by multiple factors, including sample-related factors (i.e., patient's sex, sample collection time, diet, exercise), pre-analytical factors (i.e., sample type, processing, and/or storage conditions), and experiment-related factors (i.e., RNA isolation protocol, quantification methods) (Table 6.1).

Table 6.1 Summary of factors that affect reproducibility of circulating RNA analysis

Factors	Note
Sample-related factors	
Gender	Some circulating miRNAs show gender-specific disease association [40]. Additionally, events like menstrual cycles [41–44] and pregnancy [45, 46] also altered the overall circulating miRNA profile
Sample collection time	Some circulating miRNAs are affected by the circadian cycle [47, 48]
Diet	Nutrition modulates circulating miRNA expression [49]
Exercise	Aerobic exercise influences circulating miRNAs [50]
Platelet contamination	Platelet-derived miRNAs have significant influence on circulating miRNA profile [51]
Hemolysis	Plasma levels of miR-16 were higher in hemolyzed samples [52]
Sample type	Use of whole peripheral blood or its mononuclear cell layer is not advised since both contain normal blood cells
	Plasma and sera generally have similar miRNA expression patterns [30], but in specific instances, significant differences between these biological fluids are apparent [32]
Experimental-related factors	
Sample processing	Heparin inhibits the activities of reverse transcriptase and DNA/RNA polymerases commonly used in various methodologies (e.g., qPCR, NGS, and microarray) [53]
Sample storage	Circulating levels of several miRNAs including miR-16 increased from 24 to 72 h in either room temperature, 4 or −20 °C [54]
RNA isolation method (phenol/chloroform-based technique or silica-based methods)	Silica-based methods are faster, can be automated, and allow to isolate RNA samples with higher purity. Among these, miRNeasy Serum/Plasma Kit (Qiagen) showed a better overall performance [34, 35]
RNA measurement	
Microarray	Pros: High throughput
	Cons: Low sensitivity, low specificity, no absolute quantification [34, 35]
qPCR (relative quantification)	Pros: Scalability, high sensitivity, high specificity
	Cons: Absence of universally invariant endogenous control in body fluids [34, 35]
NGS	Pros: High throughput, identification of new miRNAs, sequence information (isoforms, RNA editing)
	Cons: Expensive, data analysis requires bioinformatics support, does not provide absolute copy numbers per milliliter [34, 35]
qPCR (absolute quantification)	Pros: Scalability, high sensitivity, high specificity, provides absolute copy number per mL (fundamental for setting diagnostic and prognostic tests)
	Cons: Require the use of standard curves [34, 35]
dPCR	Pros: High sensitivity, high specificity, absolute number of copies/ml (fundamental for setting diagnostic and prognostic tests), no need of standard curves, less susceptible to PCR inhibitors
	Cons: Expensive, low throughput [38, 39]

Different biological fluids can be expected to have different circulating RNA profiles, and yet assays continue to be performed on whole peripheral blood samples, as well as their serum, plasma, and mononuclear cell fractions. In more recent studies, serum and plasma samples tend to be preferred since they eliminate the interference caused by the presence of normal blood cells. Cell-free mRNA in plasma or serum undergoes rapid degradation and is thus difficult to identify, even with highly sensitive techniques [29]. For this reason, circulating mRNA is almost always isolated from the mononuclear cell layer of peripheral blood, and transcripts for thyroid-specific genes are assumed to be derived from circulating tumor cells. Rare attempts have also made to extract mRNA from whole-blood samples for this purpose.

Plasma and serum generally have similar miRNA expression patterns [30], but in certain instances, significant differences between these biological fluids are evident [32]. Indiscriminate use of plasma and serum specimens within a given study is not recommended, and comparing results from studies in which different sample collection protocols were used is also unadvisable. If plasma is used, care should be given to the choice of anticoagulants and EDTA, since citrate and heparin can inhibit the activities of reverse transcriptase and DNA/RNA polymerases [33], which are commonly used in circulating RNA detection methods (qPCR, NGS, microarray).

Other factors affecting circulating RNA analysis are experiment-related: these include the method used for total RNA isolation as well as the choice of a measurement platform. Older RNA extraction methods use a phenol/chloroform-based technique that is often facilitated by the addition of guanidinium thiocyanate. The newer methods, which are faster and in some cases automated, use phenol/chloroform extraction with mini-columns, which allows the inclusion of multiple wash steps and produces RNA samples with higher purity [34]. Farina and coworkers tested two of the most widely used extraction kits, mirVana PARIS and miRNeasy Serum/Plasma Kits, and found the overall performance of the latter to be superior [35].

Several technologies are currently available for quantifying circulating RNAs, including microarrays, quantitative PCR (qPCR), NGS, and digital PCR (dPCR), and each has advantages and limitations (Table 6.1). Microarrays are the least sensitive and specific, whereas quantitative real-time PCR is probably the most popular. It can be used to quantify single RNAs or hundreds of RNAs; it is also relatively cheap, easy to carry out, and sensitive. As for miRNAs, several companies offer quantitative PCR-based assays for the detection of specific miRNAs, including some based on the stem-loop real-time PCR technique used for the relative quantification of low-abundance circulating miRNAs [36]. However, characterizing circulating miRNA expression using relative quantification is limited by the absence of a universally invariant endogenous control in body fluids [37]. This limitation can be overcome with innovative methodologies like NGS and digital PCR, which allow quantitative analysis of circulating miRNA expression without internal controls. NGS is ideal for profiling circulating RNAs, because it provides comprehensive, definitive information on low-abundance species, including sequence data, which can be used to distinguish different isoforms of mRNA and miRNA and to identify new miRNAs and changes related to RNA editing. It cannot, however, be used for absolute quantification of circulating RNAs. Digital PCR is currently the only technique that can directly quantify the absolute number of circulating RNA copies. For this reason, dPCR should be the method of choice for validation studies and for setting diagnostic and prognostic tests. It also eliminates the need for the standard curves and endogenous controls required for qPCR, and it is also superior to the latter in terms of sensitivity, precision, and susceptibility to PCR inhibitors [38, 39].

6.4.2 Progress Toward the Analysis of Circulating mRNAs in Thyroid Cancer

Circulating thyroid-specific mRNAs have been evaluated as potential diagnostic and prognostic biomarkers in thyroid cancer (see Table 6.2). Several studies have shown that assays of circulating *TSHR* and *TG* mRNA levels can improve the diagnosis of most (78–85%) thyroid nodules with indeterminate FNA [55, 56, 59, 60]. The findings of one study suggested that these markers could be particularly useful for distinguishing follicular adenomas from carcinomas, which is difficult with FNA cytology, although this conclusion needs to be confirmed in an extended cohort of patients [55]. Combined assessment of circulating *TSHR* mRNA levels and neck ultrasound findings in patients with cytologically indeterminate thyroid nodules resulted in more sensitive detection of all types of DTCs, but it also reduced specificity [56]. Rates of assay positivity for circulating *TSHR* mRNAs reportedly vary with the thyroid cancer histotype. Detectable

Table 6.2 Circulating messenger RNA as biomarkers of thyroid cancer

	mRNA	Collection time[a]	Sample type	mRNA detection	Nodules with indeterminate cytology (no.)	Diagnosed nodules with mRNA analysis (rate)	Diagnosed nodules with mRNA analysis + US (rate)	Ref
Diagnosis	*TSHR*, *TG*	Pre	PBMC	RT-PCR	18	12/18	na	[55]
	TSHR	Pre	PBMC	Q-PCR	63 (29 with US data)	45/63	21/29	[56]
	TSHR	Pre	PBMC	Q-PCR	57	29/57	na	[57]
	TSHR	Pre	PBMC	Q-PCR	182	123/182	159/182	[58]
	TSHR	Pre	PBMC	Q-PCR	54	46/54	49/54	[59]
					Patients (no.)	*Prognostic value*		
Prognosis	*TSHR*	Pre	PBMC	Q-PCR	61 dDTC, 27 rDTC	dDTC < rDTC	na	[56]
		Pre			6 distant metastasis, 43 node negative	Distant metastasis > node-negative patients		
		Post-24 h			1 residual disease, 2 metastatic disease	High in post-24 h residual/metastatic disease		
	TSHR	Pre	PBMC	Q-PCR	6 DTC with LNM ≥45 years, 13 DTC with LNM <45 years	Correlated with lymph node metastasis in patients ≥45 years	na	[57]
	TSHR, *TG*	Post	PBMC	RT-PCR	19 DTC with metastases, 37 DTC without metastases	Detected in all patients with local or distant metastasis	na	[60]
	TSHR, *TG*, *NIS*, *TPO* and *PDS*	Post	WB	Q-PCR	29 DTC with residual/metastatic disease, 26 DTC with no evidence of disease	High in residual/metastatic disease	na	[61]
	TPO	Post	PBMC	RT-PCR	23 PTC stage I, 8 PTC stage II, 3 PTC stage III	Stage I > stage II > stage III	na	[62]
	TG	Post	WB	Q-PCR	15 DTC with NED, 15 DTC BED, 10 DTC with SED (7 distant, 3 proximal metastases)	High in SED	na	[63]
	TSHR	Pre- and post-24 h	PBMC	Q-PCR	7 TC with persistence of disease, 39 TC NED	Associated with persistence	na	[59]
		Post (long-term follow-up)			31 TC with recurrence of disease; 222 TC NED	Associated with recurrence		

dDTC DTC at diagnosis, *rDTC* recurrent DTC, > higher mRNA levels, < lower mRNA levels, *PBMC* peripheral blood mononuclear cell, *WB* whole blood, *NED* no evidence of disease, *BED* biochemical evidence of disease, *SED* structural evidence of disease, *DTC* differentiated thyroid cancer, *TC* thyroid cancer

[a]Pre- and/or post-surgery; *na* not available

levels are found mainly in PTC patients (principally in tall cell variants). Assay negativity is more common in patients with micro-PTCs, follicular variant of PTCs, FTC, and dedifferentiated or anaplastic thyroid cancers [57, 59].

The possible utility of circulating thyroid-specific mRNA assays to detect thyroid cancer recurrence after total thyroidectomy has been explored as an alternative to serum thyroglobulin assays, which are unreliable in several situations,

such as the presence of Tg autoantibodies. Historically, *TG* mRNA is the marker candidate that has been most widely studied for this purpose. Studies conducted to evaluate the value of circulating *TG* mRNA assays for detecting local or distant metastasis in DTC patients showed that the most reliable results were obtained only with primers carefully designed to detect all *TG* splice variants [60, 64]. The specificity of *TG* mRNA in this setting is more controversial, since ectopic *TG* expression was also detected in lymphocytes [64]. In one study, *TG* mRNA assay identified distant or local DTC recurrence with high sensitivity and specificity (100% and 94%, respectively). The results obtained with *TG* mRNA assay showed a high concordance with *TSHR* mRNA data [60]. Higher *TG* mRNA levels have also been found in metastatic vs. nonmetastatic DTC, with more pronounced increases in those with distal vs. proximal metastasis [63]. Other investigators found that circulating *TG* mRNA levels were of no value in predicting post-thyroidectomy recurrence of disease [65]. Moreover, it is not yet clear if it could represent a more sensitive marker than serum thyroglobulin assays [61, 63, 66, 67].

Higher presurgery *TSHR* mRNA levels appear to predict a higher risk for thyroid cancer recurrence [56, 59, 60], but they displayed no association with lymph node metastasis, multifocality, or extrathyroidal extension [56]. Assays of *TSHR* mRNA levels (beginning on the day after surgery) were a powerful posttreatment surveillance tool for identifying patients with residual/metastatic disease [56, 59]. Notably, *TSHR* mRNA assay positivity is reportedly associated with a higher responsiveness to radioactive iodine treatment [57].

Comparative analysis of the performance of circulating *TG*, *NIS*, *TSHR*, *TPO*, and *PDS* (Pendred syndrome) mRNA assays in the detection of residual or recurrent thyroid cancer found that, under thyroid hormone suppressive therapy, *TPO* and *TSHR* mRNA offered good specificity but low sensitivity. In contrast, circulating *TG*, *NIS*, and *PDS* mRNA assays were unaffected by TSH status (suppressed or stimulated) and displayed good sensitivity but limited specificity [61]. The high specificity in this setting of *TPO* mRNA levels was also confirmed in a more recent study, where assay positivity displayed correlation with early stage PTC [62].

6.4.3 Progress Toward the Analysis of Circulating miRNAs in Thyroid Cancer

A growing body of evidence points to circulating miRNAs as an ideal class of biomarkers for many cancer types, owing mainly to their tissue-specific expression patterns and impressive stability in biological fluids [30, 68–70]. However, their widespread use in clinical practice has been delayed for several reasons. First of all, it is becoming increasingly clear that circulating miRNA levels can be affected by a number of physiological factors and pathological conditions. Second, studies conducted on circulating miRNAs thus far are characterized by marked methodological diversity, and consequently the results vary widely and are often inconclusive.

As far as thyroid cancer is concerned, little or no attention has been given to the expression of circulating miRNAs and their clinical significance in less common forms, such as MTC, PDTC, and ATC. The majority of studies published thus far have been conducted on patients with PTC, and in this setting, circulating miRNAs show undeniable promise as novel diagnostic and prognostic biomarkers. However, as shown in Table 6.3, the protocols used and the results obtained from these studies are highly variable. Importantly, only few groups have validated their findings in a larger cohort of patients [71, 73, 74, 77, 80] or specified the isoforms of the miRNAs identified [74, 75, 77, 80, 81].

Circulating levels of miR-146b-5p, miR-221-3p, and miR-222-3p in PTC patients have been found to be higher than that in healthy controls [71, 72, 80], while miR-222 and miR-146b levels also reportedly discriminate between PTCs and benign nodules [71, 76, 79]. Plasma levels of miR-21 in FTC patients are reportedly higher than those found in patients with benign nodules or PTC, whereas miR-181a is more highly expressed in PTC patients than in those with FTC [78].

Table 6.3 Circulating microRNAs as biomarkers of thyroid cancer

	MicroRNA	Expression	Sample type	miRNA detection	Ref
Diagnosis	let-7e, miR-151-5p, miR-222	High in PTC vs. HC, PTC vs. BN	Serum	NGS + qPCR	[71]
	miR-221, miR-222, miR-146b	High in PTC vs. HC, in MNG vs. HC	Plasma	qPCR	[72]
	miR-190	High in PTC vs. HC, PTC vs. NG	Serum	qPCR	[73]
	miR-95	Low in PTC vs. HC, PTC vs. NG			
	miR-25-3p, miR-451a, miR-140-3p, let-7i	High in PTC vs. HC, PTC vs. BN	Plasma	Microarray + RT-PCR	[74]
	hsa-let7b-5p and hsa-miR-191-5p	High in BN vs HC	Plasma	qPCR	[75]
	hsa-miR-150-5p and has-miR-342-3p	Low in BN vs. HC			
	hsa-let7b-5p, hsa-miR-191-5p, hsa-miR-93-5p	High in PTC vs. HC			
	hsa-miR-150-5p, has-miR-342-3p, hsa-miR-146a-5p	Low in PTC vs. HC			
	hsa-let7b-5p and hsa-miR-10a-5p	High in PTC vs. BN			
	hsa-miR-146a-5p and hsa-miR-199b-3p	Low in PTC vs. BN			
	miR-146b and miR-155	High in PTC vs. BN	Plasma	qPCR	[76]
	miR-124-3p and miR-9-3p	High in PTC vs. BN	Plasma	qPCR	[77]
	miR-126-3p, miR-145-5p, miR-31-5p	High in PTC vs. BN	Plasma-derived exosomes	qPCR	[78]
	miR-21	High in FTC vs. BN			
	miR-21	High in FTC vs. PTC			
	miR-181a	High in PTC vs. FTC			
	miR-222	High in PTC vs. MNG	Serum	qPCR	[79]
	miR-21	Low in PTC and MNG vs. HC			
	miR-146a-5p, miR-28-3p, miR-103a-3p, miR-222-3p, miR-191-5p, miR-24-3p, miR-146b-5p, miR-221-3p	High in PTC vs. HC	Serum	qPCR	[80]

(continued)

Table 6.2 (continued)

	MicroRNA	Expression	Sample type	miRNA detection	Ref
Follow-up	miR-151-5p and miR-222	Decreased after tumor excision	Serum	qPCR	[71]
	miR-221, miR-222, miR-146b	Decreased after tumor excision in PTC and MNG	Plasma	qPCR	[72]
	miR-25-3p and miR-451a	Decreased after tumor excision	Plasma	Microarray + RT-PCR	[74]
	miR-126-3p, miR-145-5p, miR-146a-5p, miR-181a-5p, miR-206, miR-21-5p, miR-221-3p and miR-223-3p, miR-31-5p	Decreased after tumor excision	Plasma-derived exosomes	qPCR	[78]
	miR-221, miR-222, miR-151-5p and miR-31	Decreased after tumor excision	Serum	qPCR	[79]
	miR-146a-5p, miR-221-3p, miR-222-3p, miR-146b-5p, miR-28-3p, miR-103a-3p, miR-191-5p, miR-24-3p	Decreased after tumor excision	Serum	qPCR	[80]
	miR-146a-5p and miR-221-3p	Postoperative serum levels were consistent with ATA responses			

HC healthy controls, *BN* benign nodule, *PTC* papillary thyroid cancer, *FTC* follicular thyroid cancer, *MNG* multinodular goiter, *MTC* medullary thyroid cancer, *ATA* American Thyroid Association

In PTC patients, circulating levels of miR-146b-5p, miR-221-3p, miR-222-3p, and miR-146a-5p have been shown to decline after tumor excision [71, 72, 78–80]. Notably, miR-221-3p and miR-146a-5p levels in PTC patients have been shown to predict clinical responses, with significantly increased levels observed at the 2-year follow-up in patients with structural evidence of the disease, including some in which serum thyroglobulin assays remained persistently negative [80].

The association of circulating miR-146b-5p, miR-221-3p, and miR-222-3p with the presence of thyroid cancer is strengthened by the evidence of their upregulated expression in PTC [82, 83], FTC [84, 85], and ATC [84] tissue. Upregulated tumor tissue expression of miR-21 has been found in these tumors as well as in MTC [86].

Conclusions

The liquid biopsy technique can potentially change clinical practice by providing us with more specific and reliable biomarkers for the early detection of cancer. The rapid, low-cost, noninvasive nature of sample collection makes this new technique ideal for ongoing real-time assessment of neoplastic disease, including

changes in tumor burden, intra- and interlesional genetic heterogeneity, and response to therapy. Recent studies have shown that assays of circulating RNAs can be used for the early diagnosis of thyroid cancer and for monitoring treatment responses. Compared with circulating mRNAs, circulating miRNAs are emerging as the more promising candidates, due to their higher stability and tissue-specific origin. Realization of this enormous potential will depend largely on our ability to successfully address the outstanding issue of low reproducibility for the biomarkers that have been discovered. A major goal of future research in this field should therefore be the development of standardized methods for evaluating circulating miRNAs and validation of their performance in clinical settings.

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Circulating Epithelial Tumor Cells in Thyroid Carcinoma

7

Thomas Winkens, Katharina Pachmann, and Martin Freesmeyer

7.1 Introduction

Patients with differentiated thyroid carcinoma (DTC) show a good overall survival rate of >90% [1]. A major reason for this favorable outcome is radioiodine therapy (RIT) performed according to current guidelines, which irradiates all postoperatively remaining thyroid carcinoma cells. This goal is reached due to the fact that thyroid-derived cells accumulate and store iodine [2–4]. Nevertheless, distant metastases occur in 15–27%, worsening patient prognosis [5, 6]. The links between the primary tumor and distant metastases are tumor cells which stem from the primary tumor and are shed into circulation. Subsequently, these cells leave the blood vessels at the site of metastasis and form tumor cell clusters which proliferate, destroy surrounding tissue, and release more tumor cells into circulation [7–9]. The tumor cells located in blood vessels are called circulating tumor cells (CTC). This book chapter gives an overview of the role of CTC in patients with DTC.

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There are a lot of different methods to detect and isolate CTC from venous blood samples [9]. Reviewing the current literature about CTC, breast cancer, colon cancer, and prostate cancer are the most frequently investigated carcinoma entities [9]. All different methods are based on one key step in CTC isolation: differentiation of CTC and physiologically present blood cells, i.e., leukocytes and erythrocytes. One concept of distinguishing between these cells is called positive selection, which is mainly performed using the epithelial cell adhesion molecule (EpCAM) (CD 326). EpCAM is overexpressed on most carcinoma tissues; however, the tissue carcinoma arises from, i.e. the epithelium, also expresses EpCAM albeit to a much lower intensity [10, 11]. This EpCAM overexpression is used for a majority of CTC isolation methods. In addition to positive selection of CTC, negative selection of regular blood cells is also necessary. This consists of erythrocyte lysis (eliminating erythrocytes) and size differences, density gradient, electrical properties, and/or surface markers (eliminating leukocytes) [9].

The most frequently used method of CTC detection is the FDA-approved CELLSEARCH® (Janssen Diagnostics, Raritan, USA). After a fixation step, anti-EpCAM antibodies coupled to magnetic beads are added to the blood sample, binding to all EpCAM-positive cells. Subsequently, a magnet is used to accumulate all cells which have been recognized by this

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antibody. A microscopy approach is used for negative selection of leukocytes, and further selection steps are performed [12].

A less frequently used method of CTC isolation represents MAINTRAC® (SIMFO, Bayreuth, Germany), which detects more CTC than the CELLSEARCH® method. This difference is caused by less elaborate selection steps; thus, MAINTRAC® is regarded more sensitive, finding more EpCAM-positive cells per milliliter venous blood [12]. The experiences with CTC in patients with DTC are based on the MAINTRAC® method; therefore, this CTC test is described more detailed in this book chapter.

Both aforementioned CTC isolation methods, MAINTRAC® and CELLSEARCH®, initially aimed at determining the number of CTC per milliliter blood in patients with newly diagnosed cancer, drawing a conclusion about the potential to develop a recurrence and/or metastases over time [13–18]. Additionally, the change of CTC numbers during antineoplastic therapies (e.g., chemotherapy, radiation) has been investigated, representing a surrogate parameter of therapy response. Recently, much effort has been made to investigate additional properties of CTC besides the plain number of CTC. This allows to investigate (1) differences between the primary tumor and the CTC responsible for metastases (e.g., Her2neu expression), (2) in vitro sensitivity of CTC regarding different chemotherapeutic substances (chemosensitivity), and (3) the location of the primary tumor in case of cancer of unknown primacy (CUP syndrome) [9, 19, 20]. Therefore, detection and characterization of CTC are also referred to as "liquid biopsy" [9, 15, 21]. In addition, it is desirable and necessary to exactly determine the origin of the CTC, especially in view of the much higher sensitivity of MAINTRAC® compared to CELLSEARCH®, detecting a thousand times more CTC, however, presumably associated with a reduced specificity. Determining the origin of CTC enables conclusions about the similarities with the primary tumor as well as malignant properties of the CTC themselves.

There are only few studies investigating the role of directly isolated CTC in patients with DTC. Besides our experiences with more than 350 blood samples, only two recently published articles can be found in a current literature research [22, 23].

However, there are studies focusing on *indirect* CTC detection in patients with DTC. As these examinations focus on the quantification of messenger RNA (mRNA) typical for thyroid tissue taken from venous blood samples, the methodological approach is very different from the one that we used, because the whole blood sample is used for mRNA detection without isolation of *single* tumor cells. As early as 1996, authors concluded that CTC are responsible for thyroid mRNA detected in venous blood samples and, therefore, CTC were indirectly determined [24–28].

The question is whether CTC represent a valuable marker of tumor load and prognosis in patients with DTC, applicable in clinical routine. Human thyroglobulin (hTg) is an excellent marker which is organ specific and thus can serve as a reliable tumor marker in patients with DTC, once the thyroid remnants have been removed by ablative RIT [26, 29]. If hTg shows rising values after postablative negativity, this is a strong indicator of tumor recurrence [2–4]. hTg is a robust tumor marker which is frequently expressed even by dedifferentiated thyroid carcinomas [30]; however, there are patients in which anti-hTg antibody interferes with correct detection of hTg, thus disabling reliable follow-up [2, 26]. This constellation might represent an application field for CTC in the follow-up of DTC patients. Furthermore, it is desirable to know properties of CTC in case of metastases and to compare those to the properties of the primary tumor (if histological examination of a metastasis is not possible), thus identifying therapeutic strategies directly aiming at metastases. Hence, as "liquid biopsy," determination of CTC and their properties might represent a supplement or an alternative to invasive conventional biopsy of a metastatic lesion. Another advantage of CTC determination might be the independence from expensive recombinant human thyroid-stimulating hormone (rhTSH) which increases sensitivity of hTg and is frequently used in clinical routine [26].

The term CTC—circulating *tumor* cells—is frequently used in literature; however, in patients with thyroid diseases including DTC, it can only

be used with restrictions according to our experiences and findings. We detected EpCAM-positive cells in patients with benign thyroid diseases and healthy volunteers (without any history or signs of cancer) as well [31]. The presence of circulating *tumor* cells in healthy people would reduce the applicability of this method in cancer patients due to the questionable specificity. Therefore, we did not use the term CTC in previous publications, but used circulating *epithelial* cells (CEC) instead [31–33]. It is important to emphasize that CEC in this meaning is different from the term circulating *endothelial* cells, which has also been reported in the literature, however, in a very different context not related to cancer [32].

The key issues we investigated included the detection of CEC in patients with different thyroid diseases comprising patients with benign thyroid diseases before RIT, patients with newly diagnosed DTC, patients with DTC and metastases, and patients with a history of DTC without any signs of recurrence. Additionally, we investigated the influence of RIT on the number of CEC in different groups of patients with DTC, and finally we determined the origin of CEC in a subpopulation of these patients.

7.2 Methods

The isolation method described in this book chapter is the one introduced by Pachmann et al., which differs from the CELLSEARCH® approach in the way of positive and negative cell selection and enrichment steps [12, 14, 16, 31]. One milliliter of peripheral venous blood in EDTA is needed for CEC isolation. The first step consists of an erythrocyte lysis and the remaining cell suspension is separated from the plasma by centrifugation. The resulting pellet consisting of leukocytes and CEC is resuspended and incubated with a mix of fluorescence-bound markers. Anti-EpCAM-fluorescein isothiocyanate (FITC) identifying EpCAM and 7-aminoactinomycin D (7-AAD) indicating cell viability are used. After 15 min of incubation, a predefined volume of the cell suspension is scanned with a semi-automated fluorescence microscope (Scan_R, Olympus, Hamburg, Germany), acquiring transmission and fluorescence images which are manually evaluated afterwards. The cells' morphologic appearance, the distribution of the FITC-signal and viability as shown by absence of 7-AAD accumulation within the cell nucleus are assessed (Fig. 7.1).

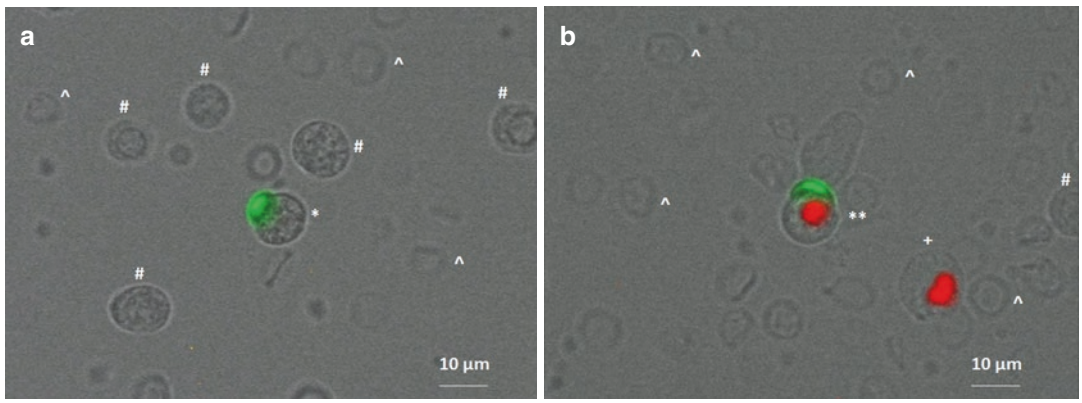

Fig. 7.1 Fluorescence image of a vital circulating epithelial cell (CEC) in (**a**) (*asterisk*). The clear signal (*green*) generated by the epithelial cell adhesion molecule (EpCAM) coupled to fluorescein isothiocyanate (FITC) indicates that the cell is of epithelial origin. The cell nucleus in (**a**), in comparison to (**b**), does not contain any red fluorescence generated by 7-aminoactinomycin D (7-AAD), indicating the vital status of the CEC. For comparison, see a dead CEC in (**b**) (*double asterisk*) with a *green signal* but also a *strong red* 7-AAD signal in the nucleus. Cell fragments, debris of lysed erythrocytes (*caret*), and non-stained leukocytes (*hash*) are also visible in (**a** and **b**). A dead leukocyte (*plus*) is additionally visible in (**b**) (image and figure caption from Winkens T, Pachmann K, Freesmeyer M. Circulating epithelial cells in patients with thyroid carcinoma. Nuklearmedizin 2013; 52: 7–13)

Only viable cells are counted for further analysis. As predefined volumes are used throughout the whole isolation procedure, it is possible to calculate the number of CEC per milliliter blood and, thus, the CEC load in the patient, based on the number of positive events on the Scan_R system.

Determination of the number of CEC at a certain time point only represents a snapshot. Recently, CEC determination at different time points has been found useful in monitoring the response to antineoplastic therapy. This course of CEC was able to predict tumor recurrence in certain situations [13, 14]. We performed a study on 28 patients with DTC, investigating the course of CEC numbers in a group with their first ablative RIT after surgery for DTC as well as a group with patients who were subjected to a repeated RIT during their DTC therapy. The number of CEC was determined before RIT as well as after RIT at different time points (i.e., after 2, 14, and 90 days). The results were compared with hTg and clinical therapy response after 3 months. Special attention was paid to the individual course of CEC in relation to the initial CEC count before RIT.

One focus of our research is to determine the exact origin of CEC as this kind of cells has been found in individuals with different benign diseases [31, 34]. DTC is suitable to investigate this topic because thyroid cells produce proteins that can be exclusively or almost exclusively found in the thyroid gland and nowhere else in the human body; thus, differentiation between CEC deriving from the thyroid gland and CEC deriving from other tissues is possible. The most important protein is thyroglobulin (Tg), i.e., the substrate for thyroid hormone biosynthesis. Thyroid-stimulating hormone receptor (TSH-R) is a membrane-bound molecule which is not only expressed on the surface of thyroid cells but is also detectable on adipocytes, fibrocytes, and leukocytes, however, to a lesser degree [35, 36]. TSH-R is responsible for activation and proliferation of thyroid cells. Thyroid peroxidase (TPO) is regarded thyroid specific and has therefore been included in the analyses, too [29, 37]. Another protein typical for thyroid tissue is the natrium-iodide symporter (NIS), which is known to be also found in the salivary gland, stomach, and choroid plexus [38, 39].

It seems promising to investigate if the CEC contain mRNA for the abovementioned proteins typical for thyroid cells. In contrast, direct visualization via fluorescence-coupled antibodies targeting these structures is not preferable because only viable CEC with an intact cell membrane are analyzed; therefore, intracellular proteins (e.g., Tg) are not easily accessible using these antibodies. Extracellular proteins (TSH-R, NIS, TPO) can be detected using antibodies; however, their specificity is less compared to Tg [40].

Analysis for thyroid mRNA at a single cell level is performed by selecting individual CEC which are separately screened for the presence of mRNA. Isolation and visualization of CEC are identical to the CEC identification for CEC number evaluation. Once an EpCAM-positive CEC has been identified on the fluorescence microscope, it is aspirated into a glass capillary and stored in a cap. The next step consists of a quantitative real-time polymerase chain reaction (qRT-PCR) separately comparing the amount of thyroid mRNA (Tg, TSH-R, TPO, NIS) to the amount of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Looking at the expression level of mRNA in normal thyroid tissue and leukocytes, it can be concluded that a CEC contains thyroid mRNA in a relevant quantity, if the amount of thyroid mRNA copies is the same as the amount of GAPDH copies [32, 41–43]. A CEC is regarded to originate from the thyroid gland if it contains at least three different thyroid mRNAs [32].

7.3 Results and Discussion

Circulating epithelial cells can be detected in patients with differentiated thyroid carcinoma. This finding is in analogy to other epithelium-derived tumors (i.e., breast cancer, prostate cancer), for which determination of circulating tumor cells is a widely used method.

However, according to currently available literature, it is important to emphasize that determining the number of CEC is not able to correctly identify patients with a high tumor load (metastases) or without residual thyroid cancer tissue (after initial

surgery, R0 resection). We found CEC in many patients with different DTC disease presentations (i.e., residual tumor tissue, tumor recurrence or metastases, patients without residual tumor after initial surgery, and patients in complete remission). Although there was a correlation between hTg levels and the number of CEC in patients with DTC, this method is far away from being capable of distinguishing between a patient in complete remission and a patient with residual tumor as this is commonly practiced using the established tumor marker hTg. In our pilot study, we found high cell numbers (14,128 ± 14,630; range 240–56,490) for patients with "clinically active" DTC (i.e., local recurrence and/or metastases). In patients who had been subjected to thyroidectomy due to DTC, even higher CEC numbers were found before starting the first ablative RIT (20,324 ± 21,502; range 1460–84,560); however, this difference was not significant. We concluded that the recently performed surgery (about 4 weeks prior to the CEC determination) was the explanation for the elevated CEC numbers in the latter group as this finding has been reported for breast cancer and lung cancer and in patients with surgery for benign reasons (e.g., cholecystectomy) [17, 44, 45]. However, it is noteworthy that the patients after thyroidectomy usually do not contain any residual tumor tissue as most of the DTC are completely resected (R0). In these patients, RIT is mainly performed to destroy any residual *benign* thyroid tissue, thus enabling the reliable use of hTg as a tumor marker. Nevertheless, in the postoperative group, highest CEC numbers were present. Besides these two groups (in which presence of CEC is plausible), CEC were also detectable in the following control groups: patients with DTC in complete remission (9769 ± 11,406; range 0–42,610); patients with benign, RIT-requiring thyroid diseases (10,483 ± 10,488; range 0–53,040); and a group of healthy volunteers (4857 ± 5779; range 240–19,850). These results are not fully understood, and a valid explanation for these findings cannot be provided; however, we presume that there might be CEC of unclear significance that stay in the circulation over years in patients with DTC in complete remission. This theory had been proposed based on animal testing [46]. Additionally, there are patients that exhibit a low amount of hTg over years without clinical signs of tumor presence or progress [47]. In patients with benign thyroid diseases, there was a positive correlation between the number of CEC and the thyroid volume which can be regarded as indication of the CEC origin. However, in these patients, CEC do not represent carcinoma/tumor cells. Proliferation and inflammation are known to cause increased expression of EpCAM at the cell membrane, and the shedding of these benign cells into circulation has been reported as well, e.g., for colitis and hepatitis [34, 48, 49]. Benign thyroid diseases comprise goiter (i.e., proliferation) and autoimmune thyroiditis (i.e., inflammation), so the CEC detected in this group might represent benign epithelial cells.

In contrast to our results, Xu et al. found CTC in only 1 of 14 patients with DTC using the CELLSEARCH® method [23]. On one hand, this supports the fact that CELLSEARCH® generally detects lower CTC numbers than MAINTRAC®. On the other hand, this might represent an indication for the lower specificity of the method developed by Pachmann et al. This needs to be investigated in future studies.

RIT has an effect on the number of CEC in a part of patients with DTC, and an association with hTg can be found. Some patients showed an early decrease of CEC numbers (compared to pre-therapeutic numbers), coinciding with an increase of hTg. This was interpreted as a therapeutic effect, i.e., the RIT destroys thyroid-derived CEC, on the one hand, leading to a decreased detectability of CEC and shedding of Tg stored within these CEC into circulation, thus increasing hTg levels, on the other hand (Fig. 7.2).

Shedding of tumor cell contents into circulation following antineoplastic therapy is well described for different tumor entities [50]. However, the pattern of simultaneous decrease of CEC numbers and increasing hTg levels was only observed in part of the patients; therefore, it can be concluded that there are other yet unknown factors that influence the number of CEC after RIT. Additionally, it was shown that a reduction of CEC numbers 2 days after RIT was able to predict therapy response after 3 months. It is important to know, however, that only 28 patients were included in this study.

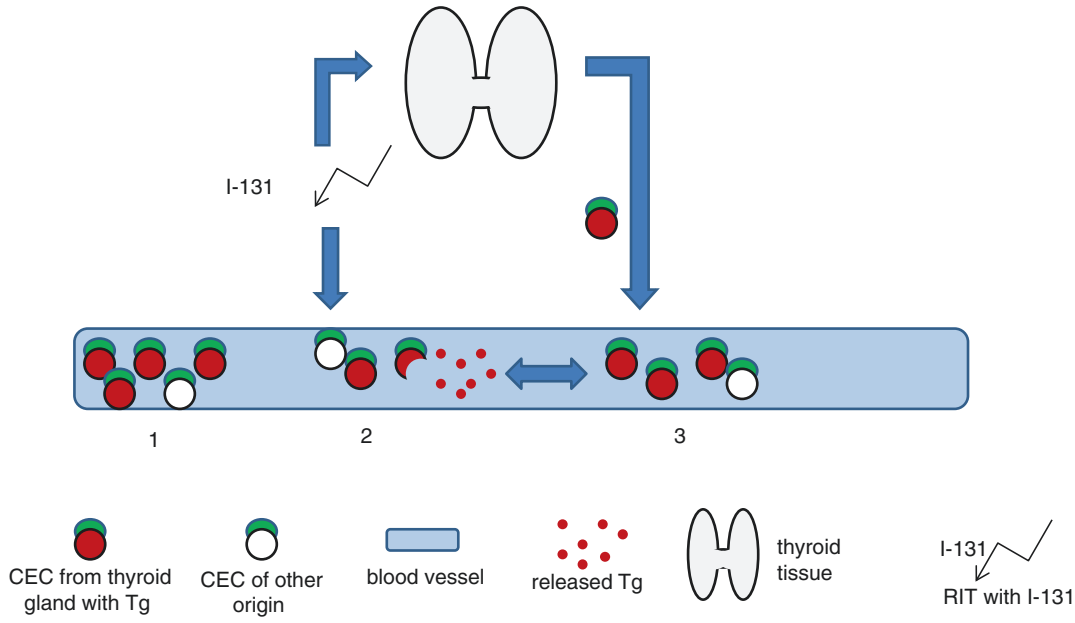

Fig. 7.2 Schematic representation of possible effects of RIT on blood CEC and on thyroid tissue, the latter either as post-thyroidectomy remnant or as local persistence/distant recurrence. (*1*) Baseline conditions: thyroid-derived CEC containing thyroglobulin (Tg) (*in red*) and CEC of different tissue sources. (*2*) In response to RIT, the Tg-containing CEC undergo cell damage or death and release Tg into circulation at measurable levels (hTg). (*3*) The RIT targets also normal thyroid tissue, where inflammatory changes caused by treatment lead to overexpression of EpCAM and mobilization of cells into the circulation. The CEC in blood, therefore, do not distinguish between EpCAM-positive carcinomatous cells and inflammation-activated but benign cells. The balance of these two sources may change inasmuch as a predominance of cell destruction causes a CEC decrease and predominance of mobilization causes a CEC increase (image and figure caption from Winkens T, Pachmann K, Freesmeyer M. The influence of radioiodine therapy on the number of circulating epithelial cells (CEC) in patients with differentiated thyroid carcinoma—a pilot study. Exp Clin Endocrinol Diabetes. 2014;122(4):246-53.© Georg Thieme Verlag KG)

The method of single cell origin analysis of CEC was proven technically successful and feasible. 9/16 cells were found positive for at least three different kinds of thyroid mRNA in a patient with DTC metastases; therefore, these cells were regarded as truly originating from the thyroid carcinoma metastases. Furthermore, a patient with elevated hTg (3.4 ng/mL) showed an even higher percentage of thyroid mRNA-positive CEC: 7/8 cells derived from the thyroid according to our definition. However, this patient did not show clinical signs of tumor recurrence on neck sonography, I-131 whole-body scan, and F-18-FDG PET/CT. Of the 3 patients in complete remission, only 3/24 CEC were identified as thyroid derived. Thus, it was concluded that it is very probable that CEC originate from thyroid carcinoma tissue and that in DTC patients with metastases and elevated hTg, more thyroid-derived CEC can be found compared to patients in complete remission. These results were obtained using three different kinds of thyroid mRNA as cutoff for a CEC to stem from the thyroid. However, this cutoff was chosen arbitrarily; therefore, further studies should investigate if a cutoff of only two thyroid mRNAs produces differing results. It is noteworthy that 92% of the CEC that were investigated showed an expression of TSH-R-mRNA. Furthermore, expanding the panel of mRNA using a marker for thyroid carcinoma seems promising. Thyroid transcription factor-1 (TTF-1) is commonly found on thyroid carcinoma cells; therefore, future studies should investigate this marker, too.

Considering the abovementioned methods, there is a tool available that is able to verify the thyroid

origin of CEC. Reviewing the literature, there are other methods aiming at identifying CTC and obtaining information about their origin. Dent et al. report on a fluorescence and transmission microscope method to directly screen for the presence of EpCAM, Tg, and NIS [22]. In analogy to the experiences we have with single cell analysis in patients with DTC, their results are based only on a few patients. Interestingly, they report on a patient with DTC metastases, exhibiting CTC with a sparse expression of EpCAM, only identifying the CTC by their strong Tg and NIS expression [22]. This can be seen as an indication that there are thyroid-derived CTC that do not express EpCAM; thus, this kind of cells cannot be detected by the MAINTRAC® or CELLSEARCH® approach. These results and the RIT-induced change of CEC with regard to their origin should be investigated in future studies.

Conclusion

The role of CEC/CTC remains unclear in patients with differentiated thyroid carcinoma, and their use in clinical routine cannot be recommended based on the few studies available. CEC can be detected in patients with DTC and metastases as well as patients with benign thyroid diseases, raising the question of CEC origin. Single cell analyses of CEC show that mRNA typical for thyroid tissue can be found in part of the CEC; therefore, thyroid origin is highly probable. Considering the influence of RIT on the number of CEC, there are patients in which patterns can be identified, presuming a destruction of CEC following RIT. The experiences presented in this book chapter are based on a limited number of patients and served as pilot studies to establish the method of CEC detection in DTC patients. Further studies are required to investigate the clinical performance of this method.

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Part III

Medullary Thyroid Carcinoma

8 Medullary Thyroid Carcinoma (MTC): Diagnosis, Treatment and Follow-Up

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8.1 Introduction

Medullary thyroid carcinoma (MTC) is a rare thyroid tumour accounting for approximately 2–5% of all thyroid malignancies. It derives from the thyroid parafollicular C-cells which do not play a role in thyroid function; they secrete amines, polypeptides and prostaglandins. Calcitonin (CT) is the main secretory product and serves as a marker for the diagnosis of this disease. It is also used in the follow-up of MTC patients after thyroidectomy for the identification of relapse or progression of disease [1, 2]. Recently procalcitonin has been identified as a potential marker for disease progression in specific cases [3]. CEA is another non-specific marker for MTC.

MTC is more aggressive than follicular cell-derived carcinoma. At presentation the patients frequently have lymph node involvement, and about 10% already have distant metastases [4, 5]. However the epidemiology of this disease has recently changed. In recent years routine CT measurement has been introduced as a screening in patients with nodular goitre, and the disease may thus be diagnosed at an earlier stage [1, 6].

MTC is inherited in 25% of cases, frequently in the context of multiple endocrine neoplasia syndromes. Mutations in the "rearranged during transfection" (*RET*) proto-oncogene are responsible for the transmission of the familial cases. Inherited disease comprises familial MTC (fMTC), MEN2A and MEN2B variants. Although fMTC and MEN2A are distinct entities, some overlapping may occur. Genetic screening in all patients diagnosed with MTC helps identify familial disease within the group of apparently sporadic cases; early intervention may be performed in gene carriers. The time of prophylactic thyroidectomy is planned according to the risk level for aggressive disease [2]. Investigation for the presence of other tumours such as pheochromocytoma (PHEO) and primary hyperparathyroidism (PHP) in MEN2 syndromes is also mandatory.

The standard treatment for MTC is thyroidectomy and lymph node dissection in the majority of cases. The initial successful surgery is of great importance for cure of the disease. Stage at diagnosis, tumour size, lymph node metastases and postoperative CT levels are important predictors for disease progression and disease-free survival [4, 7–9]. The overall 10-year survival rate is

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65% but depends on the stage at diagnosis. In stage I it is 95%, stage II 93%, stage III 71% and 20–40% in stage IV patients.

The majority of MTC cases with undetectable postoperative CT are cured. On the other hand elevated postoperative CT is the main marker of disease persistence; the clinical course of these cases varies. Estimation of CT and CEA doubling time helps predict progression and outcome. Local therapies for metastatic lesions are important as palliative or adjuvant therapy depending on the site. Two tyrosine kinase inhibitors (TKIs), vandetanib and cabozantinib, have been approved for the treatment of metastatic MTC; these agents can induce clinical response and stabilization of the disease [10, 11]. The recent developments in the presentation, diagnosis, clinical course and management of both sporadic and inherited MTC are presented in this chapter.

8.2 MTC Presentation and Diagnosis

Sporadic MTC may present at any age but is most frequently encountered in the fourth to sixth decades of life. According to recent studies, 0.5–1.3% of multinodular goitres may harbour unsuspected small MTCs; thus its incidence may be higher than previously believed. The introduction of CT as a screening test in multinodular goitre as well as the use of high resolution ultrasound has resulted in an increase in diagnosis of small MTCs: the clinical significance of these may be different from the classical MTC. It should be noted that "incidental" MTCs may be found in 0.2–0.8% of autopsies; the majority of these are microcarcinomas. Interestingly, in 10–15% of MTCs, the diagnosis is available only after thyroidectomy [12, 13].

"Classical" MTC appears as a firm hard nodule in the upper middle region of thyroid lobes or less frequently with palpable lymph nodes. Ultrasound shows features of malignancy such as microcalcifications and hypoechogenicity with irregular margins and occasionally small infiltrated lymph nodes. Fine needle aspiration (FNA) cytology is diagnostic in only 50% of the cases [14]. It may be false negative or indicate papillary thyroid cancer (PTC) or unspecified malignancy. In such cases CT measurement in the FNA washout fluid (FNA-calcitonin) and the immunohistochemical staining for markers such as CT, CEA and chromogranin A (CgA) may increase the FNA accuracy [15]. Routine CT measurement along with FNA-calcitonin may improve the diagnostic accuracy in patients at risk for MTC and avoid false-negative or inconclusive results from cytology [2, 16]. Hereditary MTC is frequently bilateral, multicentric and associated with C-cell hyperplasia.

At diagnosis, lymph node involvement is frequent and may be detected in 20–30% of MTC patients with microcarcinoma (≤1 cm). The prevalence of lymph node involvement increases with increasing primary tumour size and may affect the central and lateral but also contralateral compartments [17]. The clinical picture of MTC may be variable, and patients are usually asymptomatic. Unexplained diarrhoea may be the presenting symptom, probably due to prostaglandin release by the tumour. Flushing due to the release of biogenic amides and Cushing's syndrome due to ectopic ACTH secretion by the MTC cells may rarely occur. Distant metastases may be present already at diagnosis in approximately 10% of patients; the most frequent sites are the lung, liver, bones and less frequently the brain and skin [9]. In MEN2 symptoms of PHEO may appear first. PHEO is bilateral in 60–80% of the cases. Hyperparathyroidism may appear during the third decade of life in 10–25% of MEN2A patients and is usually mild and slow progressing [18]. Yearly screening for PHEO (serum-free metanephrines or urine metanephrines and catecholamines) and for PHP (albumin-corrected or ionized calcium and PTH) is recommended in familial cases.

The preoperative diagnosis is made through serum CT measurements with sensitive immunochemiluminometric two-site assays (ICMAs) [19]. Usually CT levels are associated with tumour size [20]. Rarely a "hook effect" (falsely low CT in the laboratory result due to very high serum CT levels

interfering with the immunoassay) may occur. This should be examined when inappropriately low serum CT levels are found in a patient with large tumour burden. Rarely calcitonin-negative tumours or tumours with low calcitonin secretion have been reported; these MTCs are poorly differentiated in histology and usually have rather aggressive biological behaviour [21]. In these cases other markers such as procalcitonin (ProCT) and CEA may be useful [3, 22, 23].

CT measurement in the evaluation of multinodular goitre may lead to an earlier diagnosis of MTC and hence to earlier intervention and higher cure rates of the disease [1, 6]. The national organizations do not all recommend routine screening [24, 25]. CT may be slightly elevated in the absence of MTC. C-cell hyperplasia, a frequent cause of marginally elevated CT, precedes the tumour development in familial MTC but is also innocently present in Hashimoto's thyroiditis, in PTC, in association with proton pump inhibitors intake, in chronic renal failure and in smoking; CT may also be increased in patients with neuroendocrine tumours. One cause of falsely elevated CT may be the presence of heterophilic antibodies [26]. It has been reported that up to 50% of patients who have been operated for MNG on the basis of elevated CT levels did not have MTC in the final histology. In a recent study, a cut-off value of 65 pg/mL for basal CT levels has been proposed as a threshold for detecting MTCs larger than 1 cm. However, microMTCs or even C-cell hyperplasia cannot be always discriminated [27].

For the investigation of marginally elevated CT levels stimulation, tests have been used either with i.v. calcium or with i.v. pentagastrin [28–30]. In both familial and sporadic MTCs, a threefold increase of CT levels or an absolute level of >100 pg/mL after stimulation may be considered abnormal. Gender-specific CT thresholds may better discriminate between C-cell hyperplasia and MTC cases [28, 30, 29]. It is advised that laboratories should determine their own reference levels for stimulated CT. CT measurement appears to be the most sensitive marker for the evaluation of disease persistence and progression during follow-up [5, 31, 32]. CEA can also serve as a marker during follow-up; in cases of declining CT levels postoperatively, increasing CEA levels may be a marker of dedifferentiation [33].

One of the limitations of CT assays has to do with its instability in vitro. Procalcitonin (ProCT), the prohormone of calcitonin, is not subject to this limitation and could be another promising marker both for diagnosis as well as for the follow-up of MTC patients. Indeed, ProCT has been found to have a strong negative predictive value and be at least as accurate as CT for MTC diagnosis [21, 34]. It could also serve as a marker of clinical course and disease progression, mainly when it is correlated with CT levels. Moreover ProCT may be a useful biomarker particularly in the small proportion of MTCs that are CT negative or secrete low levels of CT. A value of 0.1 ng/mL has been proposed as threshold for normal ProCT in most studies [22]. A high ProCT/CT ratio has been associated with worse disease prognosis [35].

Other peptides of the CT gene family, such as calcitonin gene-related peptide (CGRP) and amylin/islet amyloid polypeptide (IAPP), have been studied and occasionally used for the detection and follow-up of MTC [36, 37]. Katacalcin (PDN-21) a 21-amino-acid peptide adjacent to the carboxyl terminus of CT has shown a high correlation with CT levels, and it was suggested that it could be of some use especially in patients with borderline CT [38, 39]. However none of these markers have proved to be superior to CT, and therefore they have not been established for routine use.

CEA is produced and secreted by the cancerous cells and thus serves as another marker in MTCs although it is not specific for this type of malignancy [40, 41]. Its levels have been associated with the extent and aggressiveness of MTC, roughly reflecting the tumour burden. When CEA levels are >100 ng/mL at diagnosis, lymph node involvement and probably also distant metastases may be present [42]. The gastrointestinal tumour marker carbohydrate antigen 19.9 (Ca 19.9), although not routinely used, could potentially serve as a marker of metastatic potential as the negative tissue staining has been

associated with better prognosis [43]; accordingly, elevated serum levels of Ca 19.9 have been associated with poor prognosis [44]. Serum chromogranin A (CgA) may be elevated only in case of bulky disease but cannot be used for the initial diagnosis [38]. Elevated CgA levels in a patient with moderately elevated CT levels may indicate the presence of PHEO or other neuroendocrine tumour [45]. MTC may also ectopically produce other hormones, bioactive amines and neuropeptides, such as ACTH, histaminase, NSE (neuron-specific enolase), prostaglandins, serotonin, somatostatin, tryptase, GIP, VIP, etc. [46, 47]. However these peptides are of limited or no use for MTC diagnosis and follow-up. One characteristic of MTC histology is the amyloid deposition in the stroma found in >50% of MTCs, usually composed of full-length calcitonin. Immunohistochemistry is positive for CT and CEA staining and frequently for various cytokeratins and CgA. A summary of the serum and immunohistochemistry markers that have occasionally been used as well as the secretory products of MTC is shown in Table 8.1.

Table 8.1 Serum and immunohistochemical markers that have been studied for MTC diagnosis and follow-up

	Serum	Immunohistochemical[a]	Diagnosis	Follow-up	Comments
CT	+	+	+	+	Routine use—specific [1, 40]
CEA	+	+	+ reflecting tumour burden	+ disease progression	Routine use—not specific [40, 41]
ProCT	+	+	+ ProCT/CT ratio	+	Not routinely used "ideal" for CT-negative MTC [3, 34]
CgA	+	+	±	±	Not routinely used "ideal" for CT-negative MTC [38]
CGRP	+	+	± (additional to CT)	−	Not routinely used [37]
Katacalcin (PDN-21)	+	−	±	±	Not routinely used [38]
Ca 19-9	+	+	−	+ predictive of poor prognosis	Not routinely used marker of metastatic potential [43, 44]
IAPP	+	+	−	−	Assessed in one clinical study [36]
ACTH	Secretory products with limited or no use for diagnosis and follow-up [46, 47]				
Histaminase					
NSE					
Prostaglandins					
Serotonin					
Somatostatin					
Tryptase					
GIP					
VIP					

CT calcitonin, *CEA* carcinoembryonic antigen, *ProCT* procalcitonin, *CgA* chromogranin A, *CGRP* calcitonin gene-related peptide, *Ca 19-9* cancer antigen 19-9, *IAPP* amylin/islet amyloid polypeptide, *ACTH* adrenocorticotropic hormone, *NSE* neuron-specific enolase, *GIP* gastric inhibitory peptide, *VIP* vasoactive intestinal peptide

[a]FNA fluid and/or tissue

8.3 Familial Disease

About 25–30% of MTC cases are familial. These represent variants of the multiple endocrine neoplasia (MEN2) syndromes: MEN2A, MEN2B syndrome or familial MTC (fMTC). In MEN2A, practically all patients develop MTC, 30–50% also have PHEOs, and 25–30% of the cases also have PHP. Rarely, cutaneous lichen amyloidosis and Hirschsprung disease may also be present in MEN2A patients. fMTC is a clinical variant of MEN2A characterized by the isolated existence of MTC not accompanied by any other endocrine neoplasia. It should be noted that cases initially considered as fMTC only have been subsequently reclassified as MEN2 as PHEOs may develop at later generations [48]. In MEN2B, which is more rare, MTC is associated with PHEO (45%), mucosal neuromas, ganglioneuromatosis of the intestinal tract, medullated corneal nerves, musculoskeletal abnormalities and a characteristic phenotype with Marfanoid habitus.

The gene responsible for the transmission of the predisposition for hereditary MTC is the *RET* (rearranged during transfection) proto-oncogene. The *RET* gene is located in chromosome 10q11.2, spans 21 exons and encodes a tyrosine kinase transmembrane receptor. It has three distinct domains: an extracellular ligand-binding segment (ECD) with a cadherin-like region, a calcium-binding site and a juxta-membrane cysteine-rich region critical for receptor dimerization, a hydrophobic transmembrane domain (TD) and an intracellular part (ICD) with two tyrosine kinase (TK) subdomains that mediate the downstream signalling pathways (Fig. 8.1). In the physiologic state, *ret* dimerization is required for autophosphorylation of the intracellular tyrosine residues and receptor activation. The physiologic role of *RET* during development is to transmit signals in cells of neural origin. In hereditary MTC, germline gain-of-function mutations are present in 95–98% of cases resulting in ligand-independent dimerization of mutant *ret* proteins and constitutive activation of the TK domain and its downstream transduction pathways [49]. These molecular abnormalities result in cell proliferation and differentiation of tissues derived from neural crest cells, including C-cells and adrenal medulla cells.

Novel *RET* mutations are continuously recognized. To date more than 150 germline *RET*

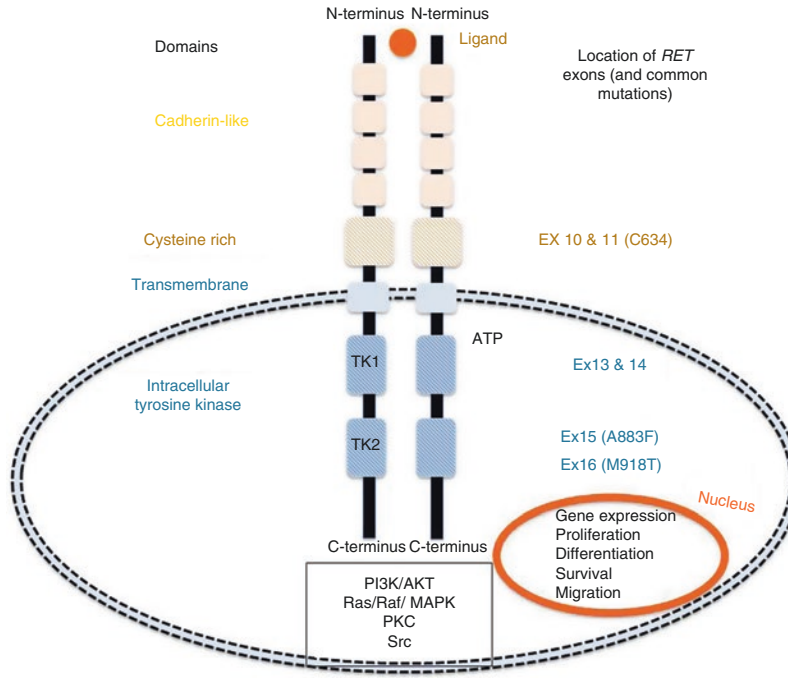

Fig. 8.1 Schematic representation of the RET tyrosine kinase receptor. Abbreviations: *TD* transmembrane domain, *TK* tyrosine kinase domain, *P* phosphorylation site, *ATP* adenosine triphosphate, *PI3K* phosphatidylinositol 3-kinase, *AKT* protein kinase B, *ERK* extracellular signal-regulated kinase, *PKC* protein kinase C, *Src* proto-oncogene tyrosine-protein kinase Src

mutations have been identified. These are accessible in electronic databases (e.g. www.arup.utah.edu/database/MEN2). A list of pathogenic *RET* mutations associated with MEN2 syndromes is shown in Table 8.2. The majority of recognized mutations are located in exons 5, 8, 10, 11 and 13–16; this panel is screened first [24]. According to the ETA (European Thyroid Association) guidelines for the management of hereditary MTC, the *RET* gene should be screened in all patients presenting with MTC [24]. Most typical MEN2A cases (i.e. with coexisting endocrine tumours) harbour mutations in the cysteine-rich region of the ECD, specifically in exon 10 (codons 609, 611, 618, 620) and exon 11 (630 and 634) [50]. Interestingly, the same gene is mutated in 50% of sporadic MTC tumour cells [51].

A genotype-phenotype correlation has been identified [24, 52]. In the presence of codon 634 mutations, the prevalence of PHEO and PHP is significantly higher [53]. A few European large-scale studies have investigated the distribution of *RET* mutations [50, 54, 55] and have shown slightly different mutation spectra. The prevalence of the detected mutations has changed over the last decades. In earlier studies, mutations involving codon 634 in exon 11 were the most frequent, while recently mutations in exons 10, 13, 14 and 15 are increasingly recognized [56]. The exon 11 mutation is frequently associated with the full-blown MEN2A including PHEOs, which may have a more prominent clinical picture; these were the cases originally identified as familial. One further reason may be the more extensive testing that now includes more than just the original "hot spot" regions. Concerning MEN2B in >95% of cases, the mutation is localized in codon 918 of exon 16 (ATG to ACG) and rarely in codon 883 of exon 15 [2].

The widespread application of genetic screening has revolutionized the management of MTC patients and the counselling of family members carrying the mutated *RET* gene. Early identification of gene carriers allows timely prophylactic thyroidectomy at an early stage without lymph node involvement [57]. This approach significantly decreases the incidence of persistent or recurrent disease [58, 59]. Several studies have evaluated the beneficial role of prophylactic thy-

Table 8.2 Overview of the ATA recommendations regarding prophylactic thyroidectomy for *RET* mutation carriers

ATA risk category (mutation)	Perform thyroidectomy	Consider central neck dissection	Annual cervical US and serum basal CT levels
HIGHEST M918T	• Within first year of life	• When suspicious LN present • Aim to preserve parathyroid glands	
HIGH C634 A883F	• By age 5 years**or** • Sooner if elevated CT levels	• When CT levels over 40 pg/ml • With positive imaging • When suspicious LN identified during surgery	Start at age 3 years
MODERATE G533C C609,611,618, 620,630 D631Y K666E E768D L790F V804 S891A R912P	• When CT levels elevated **or** • ≈ age 5 years if long-term FU difficult	• When CT levels elevated	Start at age 5 years

ATA American Thyroid Association, *CT* calcitonin, *LN* lymph nodes, *FU* follow up, *US* ultrasound

roidectomy in asymptomatic carriers; the site of the mutation, patient's age and basal CT levels are important parameters with direct impact on the long-term outcome [54].

In the recent ATA guidelines *RET* mutations have been classified according to risk level and aggressiveness of MTC in three groups: moderate (ATA-MOD), high (ATA-H) and highest (ATA-HST) risk level of aggressiveness [2] (Table 8.2). The ATA-HST category includes patients with MEN2B (mutation in *RET* codon M918T), the ATA-H category includes MEN2A patients with a C634 *RET* mutation, and the category ATA-MOD includes the rest of fMTC patients [25]. ATA-HST mutation harbours the highest risk for MTC developing very early in life with increased metastatic potential and prompts for total thyroidectomy within the first year of life. For ATA-H mutation, it is advised that gene carriers undergo surgery within the first 5 years of life. Concerning the third class, ATA-MOD, the risk of MTC developing at a young age is lower, and although thyroidectomy before age 5 is advocated, it is suggested that it could be postponed if CT values (baseline and stimulated) and neck ultrasound are still normal (Table 8.2). Age-appropriate prophylactic thyroidectomy according to the risk classification of the mutations may improve disease-free survival [60]. Recent studies demonstrated that stimulated CT values could represent a safe and reliable tool to personalize timing of thyroidectomy independent of the *RET* mutation and gene carrier age [61].

The wide application of genetic testing has also resulted in the recognition of previously undiagnosed hereditary disease in cases among those considered as sporadic. In a study with 729 MTC patients by Romei et al., direct sequencing of eight exons of the *RET* gene (5, 8, 10, 11, 13–16) led to the reclassification of 6.5% of MTC from sporadic to hereditary. 41.1% gene carriers within the MTC kindreds were identified; half of them underwent total thyroidectomy, and 90% remained disease-free after a 6-year follow-up [62]. Accordingly, one study from Greece recently reported an appreciable 7.7% incidence of the G533C exon 8 mutation in apparently sporadic MTCs [63, 64]. Polymorphisms in *RET* genetic analysis may be found; however their significance in MTC pathogenesis is undetermined. The European Thyroid Association suggests that it is important to validate the oncogenicity of novel *RET* mutations with in silico and in vitro testing [24, 65].

8.4 Treatment Strategies

The initial treatment of MTC is surgical. Preoperative neck ultrasound is necessary to determine the extent of local disease. A systemic staging with appropriate imaging should be performed in patients with evidence of metastatic disease and CT levels >500 pg/mL. Contrast-enhanced CT scan can visualize neck, chest and abdomen metastases; MRI for the liver and brain and bone scintigraphy or MRI are more sensitive methods in detecting metastases. In patients with familial MTC, pheochromocytoma should be excluded before thyroidectomy is performed. A management algorithm for MTC patients is shown on Fig. 8.2.

Total thyroidectomy is recommended. CT and CEA preoperative levels are important for determining the extent of the initial surgery [2, 19, 42]. Patients with no lymph node metastases in preoperative US and no evidence of distant metastases should undergo central compartment dissection at the initial surgery as lymph node invasion is frequently already present at diagnosis [2]. In patients with preoperatively confirmed cervical lymph node involvement, lateral and central compartment dissection should be performed [17]. In those with basal preoperative CT >200 pg/mL and positive ipsilateral lymph nodes, a contralateral neck dissection should be considered [2, 66]. Thoracic surgery may be needed for infiltrated upper mediastinum lymph nodes [2]. Of patients with lymph node metastases at diagnosis, only 20–30% will have remission [5, 17]. In familial disease, the prognosis is better because they are diagnosed and treated at an earlier age.

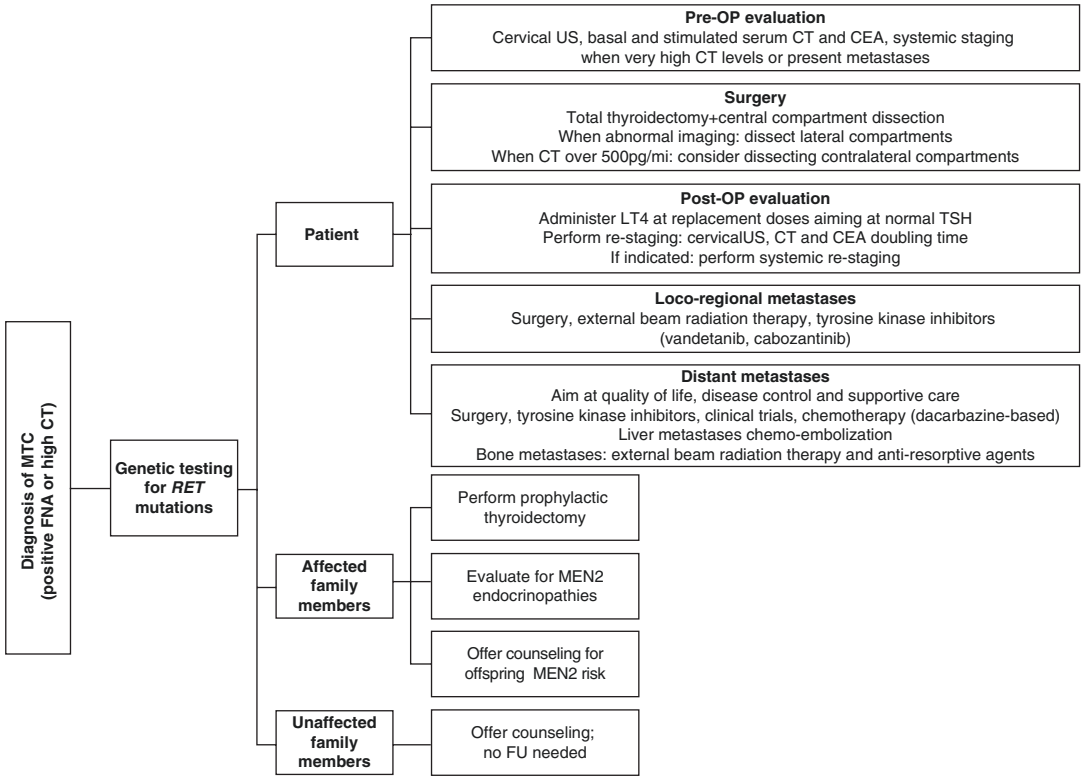

Fig. 8.2 Evaluation and management algorithm for MTC patients

Undetectable postoperative CT decreases the probability of relapse to 0–5% [5, 67]. Many patients with lymph node metastasis and/or elevated postoperative CT will have a long survival [32]. When postoperative CT exceeds 100–200 pg/mL, restaging with localization of distant metastases is very important for the appropriate therapeutic decisions [68]. Patients with postoperative CT levels >400–500 pg/mL are likely to have distant metastases. When present, the 10-year survival is 20–40%.

Short doubling time of CT and CEA has been proposed as the best indicator of disease progression according to the RECIST (Response Evaluation Criteria in Solid Tumors) [69, 70]. Specifically, CT doubling time shorter than 2 years correlates with structural disease progression [40, 70]. Fluctuations in successive measurements of CT levels (20–30%) do not indicate disease progression. Tumour progression must be determined by imaging techniques, similar to those used preoperatively. FDG-PET/CT and F-DOPA-PET/CT have poor sensitivity unless the tumour progresses rapidly [2, 71, 72]. The RECIST evaluates the structural disease progression before and during systemic treatment.

In cases of locally invasive tumours endangering aerodigestive structures, external radiation is proposed. In distant metastases palliative locoregional therapies may provide local control. Chemoembolism may decrease tumour mass in hepatic metastases. External radiation in bone metastases may offer pain relief, protect from fractures and preserve motility. External mediastinum radiotherapy may be used to avoid pressure symptoms on the trachea. Surgery and/or stereotactic irradiation for brain metastases and radiofrequency ablation in lung, bone and liver metastases have been used [2, 73]. Local interventions should preferably be performed before the initiation of systemic therapy.

The recent progress in the understanding of the oncogenic pathways in MTC and the identification of specific molecular alterations has

played a crucial role in the development of molecular-targeted therapies, mainly tyrosine kinase inhibitors (TKIs) (Table 8.3). Their main targets are the RET kinase, the vascular endothelial growth factor (VEGF) and other factors participating in signalling downstream pathways involved in tumorigenesis and angiogenesis [74, 75]. Two multikinase inhibitors, vandetanib and cabozantinib, have recently been approved for the progressive metastatic MTC. In two randomized phase 3 trials, they showed, compared to control group, increased progression-free survival (PFS) and objective response rate [10, 11] (Table 8.3). Both drugs have been associated with disease stabilization in 30% and partial regression in 35% of cases [11, 76]. However, the estimation of benefit of these agents on overall survival is difficult because the majority of patients have slow disease progression and long-life expectancy.

The rare cases with symptomatic or rapidly progressive metastatic disease are candidates for receiving molecular-targeted therapy [2, 77, 78] which aims to stabilize disease and to prolong the overall survival. Such therapy should not be used in patients with only biochemical disease progression, no structural progression or in asymptomatic patients with small metastatic lesions and no evidence of progression [2, 78]. The presence of either germline or somatic *RET* mutation may predict response to TKI treatment [79, 80]. In sporadic tumours the most common is *RET* M918T (85%), and its presence is associated with higher proliferation rate and more aggressive disease. RAS mutations, usually without coexisting *RET* mutations, are also present in 0–43% of intermediate risk MTC tumours [81]. Therapy failure due to insufficient drug dose or to resistance may occur. The TKIs treatment should be discontinued in cases with disease progression. A switch to another TKI may be helpful in maintaining disease control. TKIs have substantial adverse effects in 30–60% of patients (Table 8.3). Serious adverse events (SAE) occur in 2% of patients. Other TKIs (sorafenib, sunitinib) have also been used. New multikinase

Table 8.3 Tyrosine kinase inhibitors that have been used in patients with metastatic MTC

Tyrosine kinase inhibitors	Molecular targets	PFS (*vs.* placebo) in months	ORR (%)	Major adverse events
Vandetanib [10]	RET, VEGFR2, VEGFR3, EGFR, PDGFR	30.5 (19.3)	45	QT prolongation, fatigue, rash, dry skin, photosensitization, folliculitis, diarrhoea, decreased appetite and weight, hypertension
Cabozantinib [11]	VEGFR2, RET, c-MET, KIT, AXL, FLT3, Tie2	11.2 (4)	28	GI perforation, haemorrhage, fistula formation, diarrhoea, abdominal discomfort, fatigue, hypertension, mucositis, hand-foot syndrome
Sorafenib	RET, VEGFR, PDGFR, RAF, c-KIT, FLT3	17.9	21	Hand-foot syndrome, hypertension, diarrhoea, infection, leukopenia, musculoskeletal pain
Sunitinib	RET, VEGFR1-3, PDGFR, c-KIT, FLT3, CSF1R		32	Leukopenia, fatigue, diarrhoea, hand-foot syndrome, musculoskeletal pain
Pazopanib [82]	VEGFR1-3, c-KIT, FGFR, PDGFR	9.4	14	Fatigue, anorexia, diarrhoea, abnormal liver tests, hypertension
Lenvatinib [83]		12.6	50	Weight loss, hypertension, fatigue, diarrhoea, dehydration, proteinuria

PFS progression-free survival, *ORR* objective response rate, *AE* adverse events, *GI* gastrointestinal

inhibitors (pazopanib, lenvatinib) are studied showing promising responses [82, 83] (Table 8.3). Novel drug molecules as well as combined targeted therapies may prove efficient. Finally, other treatments (labelled antiCEA antibodies and DTPA, octreotide analogues labelled with yttrium-90) have shown some response; however they present significant toxicity. Classical cytotoxic chemotherapy does not prolong survival. Radioiodine has no place in the management of MTC [2].

Conclusions

MTC diagnosis and prognosis have substantially improved in recent years because of the routine CT screening in nodular disease, the availability of better quality ultrasound, the wide application of genetic screening and perhaps the improved surgical procedures. Perhaps the most important development in the field has been the recognition of the genetic defect which allows screening and early intervention in familial disease. This should be performed in all MTC patients, those with positive family history as well as those with the apparently sporadic form. The most sensitive marker for diagnosis and follow-up is serum calcitonin, although procalcitonin also appears promising in some cases. The course of disease may vary but is generally of slow progression. Tyrosine kinase inhibitors constitute an important advancement in the management of patients with distant metastases and progressive disease.

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9 Calcitonin and Carcinoembryonic Antigen for the Diagnosis and Management of Medullary Thyroid Carcinoma

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9.1 Introduction

The parafollicular cells of the thyroid gland are also recognized as C cells because of their ability to secrete the calcitonin (CT) hormone [1]. Their neoplastic proliferation is generically defined C cell disease and may occur as either medullary thyroid carcinoma (MTC) or C cell hyperplasia (CCH) [2, 3].

MTC is a rare cancer, representing 4–10% of all thyroid malignancies [4]. Approximately 75% of MTCs are sporadic tumors [4], and 25% of them are hereditary forms [4], associated to germline mutations of the *rearranged during transfection* (RET) proto-oncogene [4–6]. Familial MTC may be isolated or develop in the context of multiple endocrine neoplasia (MEN) type 2A and type 2B [3, 4].

CCH with nuclear and/or cytoplasmic aspects of atypia was initially described in association with hereditary MTC. In this context, CCH represents a C cell carcinoma in situ [2]. C cells with morphometric characteristics similar to those present in familial CCH may occasionally be observed in individual cases of severe chronic lymphocytic thyroiditis [2], but also in thyroid glands from normal subjects [5]. The significance of this sporadic CCH is not exactly clear, and its progression to MTC has never been demonstrated.

Preoperative diagnosis of C cell disease is difficult. In familial forms, the introduction of genetic testing has greatly contributed to the early identification of the subjects at risk, allowing prompt radical surgery and great improvement of the outcome [3, 4, 6]. In the sporadic form, CCH can only be recognized at surgical pathology, and MTC typically presents as a thyroid nodule. Although some clinical findings (pain on palpation, location in the upper third of a lobe, presence of enlarged lymph nodes) and suspicious for malignancy US features (i.e., hypoechogenic nodule, microcalcifications, lymph node abnormalities) could suggest the possibility of MTC, its diagnosis is often a challenge. At variance with differentiated thyroid cancer of follicular derivation, the identification of C cell lineage malignancies has taken limited advantage from cytology by fine needle aspiration biopsy (FNAC). This technique, in fact, displays a relatively poor MTC detection rate [3, 7, 8], thus exposing to the risk of inadequate preoperative evaluation and late diagnosis.

Neoplastic C cells fully maintain CT expression and secretion [2–4]. Therefore, the hormone

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constitutes a very sensitive pre- and postoperative marker of C cell disease, with high prognostic value [3, 4].

Carcinoembryonic antigen (CEA) [9, 10] is one of the oldest and most widely used tumor markers. In addition to colorectal cancer (CRC) and other adenocarcinomas, MTC patients also present increased serum CEA levels [4]. At variance with circulating CT, however, circulating CEA displays a poor preoperative sensitivity for MTC diagnosis and a more limited efficacy as a prognostic factor [4].

9.2 Circulating Calcitonin in Healthy Subjects and in C Cell Disease

9.2.1 CT Assay

CT is the product of cleavage and posttranslational processing of procalcitonin, a precursor peptide derived from pre-procalcitonin. The term "mature" CT represents the bioactive hormone and defines a small monocatenar peptide (32 amino acids) with a disulfide bridge at its amino terminal end. Other "immature" CT forms may also be found in MTC tissue and in the serum [11, 12]. Additionally, procalcitonin may be released in the circulation during sepsis or other general inflammatory conditions by tissues that do not normally express the CT gene [12, 13].

Because of its molecular heterogeneity, serum CT levels measured by assays utilizing antisera directed against different epitopes of the hormone can yield discrepant results [3]. Two-site immunoassays combining monoclonal antibodies recognizing distinct portions of the unique bioactive monomer avoid the interference of procalcitonin or other calcitonin-related peptides/precursors and allow accurate determination of mature CT concentrations [3]. Such immunoassays, based on radioisotopic, enzymatic, or luminescent labeling, represent the most sensitive tool for measuring serum CT levels [11]. Importantly, CT measurements obtained with different commercial assays may widely vary [3, 11]. Therefore, the reference ranges of basal and stimulated serum CT measurements should be defined in each laboratory, making it imperative that comparison of CT values in individual patients should be performed using the same method [11].

Heterophilic antibodies (human antibodies induced against external antigens and displaying reactivity with antibodies of other animal species) can interfere with two-site immunoassays, causing either spuriously elevated or, with a lesser frequency, artificially lower serum CT levels [14]. A "hook effect" (i.e., falsely low results due to interference caused by extremely high analyte concentrations) may seldom occur also with the most recent immunochemiluminescent assays (ICMAs) [15]. This kind of interference should always be suspected when low CT levels are observed in MTC patients with a large tumor burden [3, 11].

9.2.2 Circulating Calcitonin Levels in Healthy Subjects

CT levels are slightly higher in men than in women, probably because men have a larger C cell mass than women [16, 17]. They are also weakly influenced by age, body mass index (particularly in males), and smoking [18]. Depending on the employed assay method, 90–97% adults have CT levels <10 pg/ml, and more than 50% of normal subjects have serum CT concentrations below the functional sensitivity of the assay [18]. Notably, the most recent MTC guidelines released by the American Thyroid Association [19] do not recommend definite reference ranges of basal or stimulated serum CT levels. Data from young children are limited. It is suggested that CT levels <40 pg/ml should be considered normal during the first 6 months of life, with a progressive decline thereafter [16]. After the third year of life, CT values reach levels indistinguishable from those observed in adults [16].

9.2.3 Calcitonin Levels in Diseases

In MTC patients, serum CT levels rise early and parallel tumor progression [3, 4], thus representing a sensitive disease marker [3, 4]. A more

limited CT elevation is usually observed in CCH [3], even though the original report by Guyetant et al. [5] showed that the sporadic form could also occur in the absence of CT increase. In addition to C cell disease, a rise in serum CT levels has also been described in patients with other pathological conditions (Table 9.1) such as chronic renal failure, autoimmune thyroiditis, hypergastrinemia, sepsis, type 1A pseudohypoparathyroidism, and mastocytosis [3]. Concerning the influence of thyroid autoimmunity, the association with increased serum CT is still controversial [3]. Some studies, in fact, have reported decreased CT levels in smaller groups of Hashimoto's patients, possibly due to atrophy and/or fibrosis associated to destruction of both follicular and C cells [3]. Moreover, a recent study [20] has shown that circulating CT levels in patients with positive anti-TPO antibodies were not higher than in controls (4.71 ± 6.46 vs. 4.84 ± 13.11pg/ml; $P > 0.05$) and the frequency of "suspicious" (>10 pg/ml) CT values was not significantly different between the two groups of patients (3.9 vs. 3.0%) [20].

Table 9.1 Increased levels of serum calcitonin not related to C cell disease

Laboratory artifacts
Heterophilic antibodies
Pharmacological treatments
Proton pump inhibitors
Pathological conditions
Nonneoplastic
Chronic renal failure
Autoimmune thyroiditis
Sepsis
Pernicious anemia
Pancreatitis
Hyperparathyroidism
Nonneoplastic hypergastrinemia
Mastocytosis
Type 1A pseudohypoparathyroidism
Neoplastic
Small cell lung carcinoma
Breast cancer
Neuroendocrine tumors of the lung or gastrointestinal tract
Zollinger's syndrome
Follicular thyroid tumors
Papillary thyroid micro-carcinoma

Increased circulating CT levels, up to values comparable to MTC, are often observed in patients with non-C cell-derived malignancies (Table 9.1), such as small cell and large cell lung cancers and in neuroendocrine tumors of the gastrointestinal tract [3]. In such instances, the correlation between CT increase and tumor burden is not so tight as it is in MTC patients [3].

9.2.4 Calcitonin Provocative Testing

C cells from MTC patients are able to release CT in response to several pharmacological agents, the most effective resulting Ca^{2+} and pentagastrin (PG) [21]. The same response is also observed in CCH, but to a lesser extent than in MTC [3]. Since patients with limited C cell disease may have normal serum CT levels, these provocative tests have been widely used to disclose C cell abnormalities [11, 22]. In addition, these tests constitute important tools for differentiating increased serum CT associated to C cell disease from those occurring in non-thyroid malignancies [3]. In fact, CT response to Ca^{2+} and PG stimulation is virtually absent in lung and gastrointestinal neuroendocrine tumors [4].

9.2.4.1 The Pentagastrin Stimulation Test

The most widely used method for stimulating CT secretion consists in the slow intravenous administration of a PG bolus (0.5 μg/kg body weight) and measurement of serum hormone levels before and 3 and 5 min after starting the infusion [11]. Because some discomfort or potentially dangerous side effects (e.g., tachycardia, bradycardia, nausea, vomiting, dizziness, flushing, substernal tightness) may occur, this test is contraindicated in patients with coronary artery disease and/or hypertension and is not recommended in subjects >60 years of age [11].

Similarly to basal CT, stimulated hormone levels are on average higher in men than in women [3]. In 80% normal subjects, the maximum CT

increase remains <10 pg/ml, and the peak levels do not exceed 30 pg/ml in 95% of cases [3]. A stimulated CT response >100 pg/ml is usually considered suggestive of C cell disease [3, 4]. Milder CT elevations may also be found in adults with other thyroid abnormalities [11]. In patients with MTC and elevated basal serum CT levels, a fivefold to tenfold increase occurs after PG stimulation [11]. A very limited increase (0–2 times) is observed in the case of other neuroendocrine tumors [23].

9.2.4.2 The Calcium Stimulation Testing

CT secretion may also be stimulated by a short intravenous Ca^{2+} infusion [11, 24, 25]. This approach represents an alternative to PG in countries (e.g., the USA) where PG is not available. Ca^{2+} infusion may also be combined with PG stimulation to increase sensitivity [22, 26]. Serum CT levels measured after a 30-s infusion of calcium gluconate (2.5 mg/kg) are comparable to those stimulated by PG administration in both normal subjects and patients with C cell disease [11].

A recent report has accurately defined the procedure, the cutoffs of CT response, and the safety of the calcium gluconate testing [27]. A 25 mg calcium gluconate dose (i.e., 2.3 mg or 0.12 mEq of elemental calcium)/kg body weight resulted safe and well tolerated. To avoid an overdose in obese patients, an adjusted body weight calculation has been recommended (www.manuelsweb.com/IBW.htm, for ideal body weight and adjusted body weight calculator). The procedure should start with a basal CT determination. Calcium gluconate should be administered i.v., during a minimum 3 min infusion time (5 ml/min). Additional CT determinations at 2, 5, and 10 min after stopping the infusion should be performed [27]. The optimal CT threshold peaks for MTC diagnosis were reported >79 pg/ml for female and >544 pg/ml for male patients [27].

9.3 The Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) was one of the earliest tumor markers to be identified and characterized [9, 10]. It was first described in 1965 by Gold and Freeman who identified an antigen present in both fetal colon and colon adenocarcinoma, but absent in normal human colonic tissue; for this reason it was defined carcinoembryonic antigen [9].

9.3.1 Molecular Characteristics and Physiological Role

CEA and other CEA-related antigens are encoded by the CEA family genes, belonging to the immunoglobulin superfamily. The human CEA family has been fully characterized and is composed by 29 genes, 18 of which are expressed. Seven of them belong to the CEA subgroup and 11 to the pregnancy specific glycoprotein subgroup [28].

CEA molecule is a cell surface glycoprotein normally produced in gastrointestinal tissue during fetal development, its production ending before birth [29]. For this reason, it is present only in very small amounts in the blood of healthy adults and in different normal tissues. Its molecular weight ranges from 150 to 300 (average 185) kDa. The protein component consists of a 30-amino acid single polypeptide chain, with a lysine residue at the N-terminus. The carbohydrate moiety contains fucose, mannose, and galactose residues [29].

Functionally, CEA appears to play a role in cell adhesion, acting as a glycosyl phosphatidyl inositol (GPI) cell surface-anchored glycoprotein [29]. The sialofucosylated glycoforms serve as functional colon carcinoma L-selectin and E-selectin ligands, which may be critical for the metastatic dissemination of colon carcinoma cells [30].

9.3.2 Measurement

CEA can be utilized as tumor marker by immunohistochemical staining of tumor tissue and by immunoassay in serum.

Several techniques have been developed and compared over time for quantitative evaluations of circulating CEA, namely, radioimmunoassay,

immunoradiometric assay, and luminescent oxygen channeling immunoassay [31, 32].

9.3.2.1 Reference Range of Circulating CEA Levels

Small amounts of CEA are normally present in the circulation of the great majority of normal subjects [33]. Its turnaround time is generally 1 day and the half-life in plasma approximates 3 days, varying from 1 to 5 days [29].

The upper limit of CEA in the healthy population is 2.5–3 μg/l for non-smokers and 5 μg/l for smokers, being slightly higher in men than in women [33].

Several nonneoplastic disorders (Table 9.2) such as liver diseases (cirrhosis, viral hepatitis, chronic active hepatitis, obstructive jaundice), digestive diseases (diverticulitis, inflammatory bowel disease, peptic ulcers, polyps, pancreatitis), chronic lung diseases, and renal failure may determine increased serum CEA concentrations. CEA levels are not elevated in maternal serum during pregnancy, since this glycoprotein does not cross the placental barrier [29].

9.3.2.2 CEA as Tumor Marker

Circulating CEA is one of the most widely used tumor markers worldwide, mainly in CRC. Disappointingly, it displays a poor sensitivity for early diagnosis of disease [29].

Since nonneoplastic disorders may determine increased serum CEA concentrations, its specificity is also poor [29]. In addition, increased CEA levels were reported in up to 19% of active smokers and in 3% of a healthy control population [33]. For all these reasons, circulating CEA measurement is not appropriated as a screening procedure.

Among malignancies (Table 9.2), most types of adenocarcinomas (breast, gastric, lung, esophageal, ovarian, and pancreatic tumors) may present increased serum CEA levels (35). Supranormal CEA concentrations may also occur in MTC, mesothelioma, melanoma, and lymphoma.

Irrespective of cancer type, CEA is rarely elevated in patients with localized disease [34, 35]. In addition, both CEA levels and the proportion of patients with elevated CEA values tend to increase with progressive disease stage [34]. In CRC patients, it has been suggested that CEA levels could have prognostic value [34], being positively correlated with stage and negatively correlated with disease-free survival [34]. Importantly, several studies have shown that CEA concentration per gram of total protein is remarkably higher in well-differentiated CRCs tissue samples as compared to poorly differentiated specimens [35].

In clinical practice, circulating CEA is mainly employed to document progressive disease, to monitor response to therapy, and to detect recurrence of gastrointestinal malignancies. In the case of CRC, several studies have shown that an intensive surveillance regimen which included irregular CEA measurements following curative surgery resulted in a significantly better patient outcome than a follow-up lacking CEA testing [34]. Most expert panels in Europe [34] and the USA [34], therefore, recommend serial measurements of CEA after curative surgery for CRC. Indeed, there is no agreement concerning the amplitude of serum CEA variation that should be considered of clinical relevance in terms of disease progression. In addition to surveillance following curative resection of CRC, the second main clinical application of circulating CEA is the monitoring of treatment in advanced CRC, particularly when the disease progression cannot be evaluated by standard criteria.

Table 9.2 Increased levels of serum carcinoembryonic antigen not related to medullary thyroid cancer

Nonneoplastic	Neoplastic
Liver disease	*Adenocarcinoma*
Cirrhosis	Breast
Viral hepatitis	Gastric
Chronic active hepatitis	Lung
Obstructive jaundice	Esophagus
Digestive disease	Pancreas
Peptic ulcer	Ovary
Inflammatory bowel diseases	*Mesothelioma*
Diverticulitis	*Lymphoma*
Polyps	*Melanoma*
Pancreatitis	
Chronic lung diseases	
Renal failure	

9.4 CT and CEA Measurement for the Preoperative Diagnosis of C Cell Disease

Preoperative identification of C cell disease may be arduous, and the diagnosis is often performed at an advanced stage [3, 4]. This section will mainly focus on the preoperative diagnostic procedures currently used for MTC diagnosis, with particular attention to the contribution of CT and CEA determination.

9.4.1 The Contribution of FNAC

All historical MTC series showed that the great majority of patients identified during the clinical work-up of thyroid nodules already presented lymph node involvement and/or distant metastases, with an unfavorable prognosis in most cases [4]. FNAC, in fact, displays a remarkably low sensitivity for MTC diagnosis [3, 8]. Overall, approximately 50% of MTC cases included in most studies would have been missed based on FNAC alone [3, 8], with only two studies reporting a reasonably good sensitivity of cytology for MTC [36, 37]. Also in recent years, a multicenter study conducted among 12 centers in the USA and Europe [7] and a meta-analysis [8] have confirmed that preoperative investigation for MTC cannot rely on FNAC only, because of an unacceptably high false negative rate.

9.4.2 Circulating CT for the Preoperative Diagnosis of MTC

Since early diagnosis and radical surgical treatment are necessary requirements to improve MTC morbidity and mortality [4], several efforts have been made over the last two decades to identify the most convenient approach for an early diagnosis of this malignancy.

The available evidence shows that the sensitivity of serum CT measurement for preoperative MTC identification approximates 100% [3]. However, prospective, randomized, large-scale, long-term studies are lacking, and the reported results were obtained using assays with great differences in both analytical sensitivity and normal reference values (Table 9.3) [3]. A 100% positive predictive value (PPV) for MTC, comprising also some incidentally discovered microscopic tumors (whose progression to clinically manifest neoplasms had never been proven), has been demonstrated only for basal CT elevation >100 pg/ml [38]. Moreover, increased basal CT levels in most series of unselected thyroid nodular disease patients (Table 9.3) included also a relevant proportion of false positives [3]. By consequence, the PPVs resulted rather low (ranging from 10% to 40%), except for two studies from the same group reporting a PPV >90% [39, 40]. To increase specificity, a two-step approach using also a pro-

Table 9.3 Positive predictive values of basal calcitonin levels in different studies

Author	Year	Subject number	Assay method	Sensitivity (pg/ml)	Cutoff (pg/ml)	Increased basal CT	
						Frequency (%)	PPV (%)
Pacini F	1994	1385	IRMA	2	20	0.58	100
Niccoli P	1997	1167	IRMA	2	10	3	26.5
Vierhapper H	1997	1062	IRMA	1	5	6.7	8.4
Hahm JR	2001	1448	IRMA	0.8	10	3.8	17.8
Iacobone M	2002	7276	IRMA	2	10	0.9	68.2
Elisei R	2004	10,864	IRMA	2	20	0.43	93.6
Karanikas G	2004	414	ILMA	1	10	6.8	12.5
Gibelin H	2005	5018	IRMA	2	10	1.3	43.3
Vierhapper	2005	10,157	IRMA	2	10	4.9	6.3
Papi G	2006	1425	ILMA	1	10	1.61	39.1
Costante G	2007	5817	ILMA	1	20	1.15	23.1

PPVs were calculated by considering only positive patients who underwent surgery

vocative test was necessary in most studies [3]. Disappointingly, even after a positive CT response to PG or Ca^{2+} stimulation, CCH could not easily be preoperatively distinguished from MTC [3]. In fact, employing this two-step procedure, a rather elevated frequency (30–75%) of CCH in the absence of MTC was reported by most groups, particularly in patients with basal CT levels between 20 pg/ml and 100 pg/ml and a positive response to PG stimulation [3].

To avoid unnecessary surgery for sporadic CCH, it would be important to distinguish this condition from MTC before surgery. To this purpose, the amplitude of the stimulated CT peak might be of help (Fig. 9.1), though the proposed thresholds varied among different studies [3]. Particularly, one study reported that a CT response to PG stimulation >1000 pg/ml had a 100% PPV for MTC, while a CT peak >100 < 1000 pg/ml exhibited an 80% PPV for CCH [38]. In another report, a CT increase to 275 pg/ml after PG displayed a 100% PPV for MTC diagnosis, while a positive response below this threshold had a PPV of 89% for CCH [41]. These results seem encouraging, but further studies are required for a more precise definition of the appropriate threshold windows of CT response capable of discriminating CCH from MTC.

For all the reasons illustrated above, the usefulness of universal CT screening in thyroid nodular disease patients remains controversial, and no straightforward recommendations have eventually been disclosed from the different scientific societies [42]. In fact, the latest guidelines released by the American Association of Clinical Endocrinologists (AACE) in conjunction with the European Thyroid Association (ETA) and the Italian *Associazione Medici Endocrinologi* (AME) [43] advocated CT screening only in the presence of clinical risk factors for MTC, while those from the American Thyroid Association (ATA) declined to recommend for or against such a procedure, while recognizing that a basal CT level > 100 pg/ml is highly suspicious for MTC [44, 45].

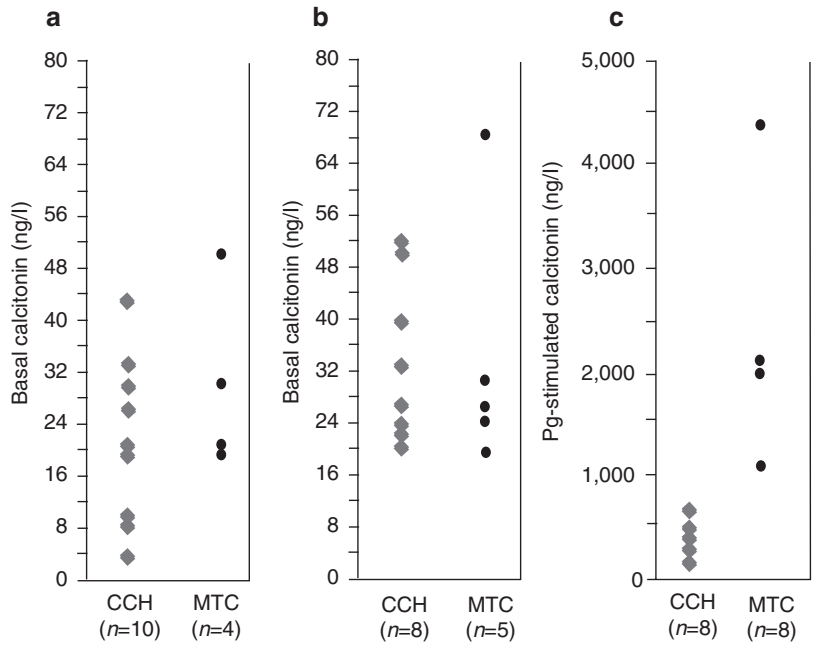

Fig. 9.1 Preoperative calcitonin levels in patients with either medullary thyroid carcinoma or C cell hyperplasia. (**a**) Basal calcitonin values in patients submitted to prophylactic thyroidectomy for hereditary MTC (familial MTC or MEN type 2). (**b**) Basal calcitonin levels in patients with surgical histology indicative of sporadic C cell disease (CCH and MTC). (**c**) Stimulated calcitonin levels in patients with surgical histology indicative of sporadic C cell disease (CCH and MTC). From Costante et al., Nat Clin Pract Endocrinol Metab. 2009;5:35–44

9.4.3 CT Measurement in the Washout Fluid of FNAC

During the last decade, the determination of CT in the washout fluid of FNAC samples (FNAC-CT) has been proposed as an ancillary method for the confirmation of preoperative MTC diagnosis in both primary lesions presenting as thyroid nodules and suspicious lymph nodes [8]. In all cases, the reported sensitivities of FNAC-CT were remarkably improved as compared to cytology alone, varying from 80% to 100% [8]. Importantly, a relevant proportion of these MTCs were identified in subjects with basal circulating CT <100 pg/ml. On the other hand, none of the published studies found undetectable CT levels in the washout fluids from patients eventually confirmed negative for MTC at surgical pathology, possibly due to the presence of nonneoplastic C cells in the FNAC aspirates [46–48]. Consequently, "arbitrarily" defined cutoff values of FNAC-CT have been proposed, not validated for the most frequent non-C cell-derived thyroid disorders such as autoimmune thyroiditis and colloid goiters [49] and with a wide variation (from 7.4 pg/ml pg to 67 pg/ml) among studies [8]. In addition, the lack of established results demonstrating the suitability of commercial assays for CT measurements on samples different from serum or plasma and the heterogeneity of the sample preparation procedures represent important limiting factors, making it difficult to make comparisons and to draw univocal conclusions. Finally, the cost to benefit effectiveness of CT-FNAC has never been appropriately addressed.

The latest ATA guidelines [44] recommend FNAC-CT in case of cytology suggestive for MTC or inconclusive FNAC results.

9.4.4 CEA for MTC Diagnosis

Analogously to CT [2, 3], CEA is also expressed in hyperplastic and malignant C cells [29]. In fact, both CT and CEA positive immunostaining in the absence of thyroglobulin represent a standard procedure for MTC diagnosis [2, 19]. Notably, CT immunostaining may vary in intensity and extent and is often reduced in undifferentiated tumors, whereas staining for CEA is almost always strongly positive [19, 50].

9.4.4.1 CEA Serum Levels for MTC Diagnosis

Although generally expressed by neuroendocrine tissues [29], CEA is not a specific biomarker for MTC diagnosis, also because its levels do not increase following calcium or PG stimulation [4, 19]. For these reasons, CEA measurement is considered to have lower diagnostic accuracy than CT [19]. Therefore, its determination is not suitable for MTC screening and of little use for the preoperative tumor diagnosis. Indeed, CEA is frequently increased at diagnosis, and, as in the case of circulating CT, the serum CEA levels might be of help for the risk stratification of MTC patients [51–53].

Although very limited data are available concerning the role of serum CEA as an alternative marker for the diagnosis of CT-negative MTCs [54], it can be used as a marker for the follow-up of MTCs that do not secrete CT [42, 50, 55]. In this respect, one study found CEA and CT almost uniformly expressed in CCH, micro-MTCs, and tumors with intra-thyroid extension [50]. Conversely, the patients with more aggressive MTCs presented an immunohistochemistry pattern characterized by an indistinct or absent CT and an intense CEA staining. Based on these data, it was suggested that CEA expression would be retained as a marker of early epithelial differentiation, while CT expression would more rapidly decline, representing a late differentiation phenomenon in C cell differentiation [50].

Rarely, patients with advanced MTC present normal or low serum levels of both CT and CEA [4, 19]. In such instances, either a misdiagnosis or an advanced MTC dedifferentiation, associated to a poor prognosis [56, 57], should always be taken into account. In this respect, a study [55] conducted on a large series showed that <1% of advanced sporadic MTC patients did not present increased circulating levels of both CT and CEA. In such instance, the occurrence of features such as poorly differentiated histology, high Ki-67

proliferation index, and high proportion of RET codon M918T mutations was consistent with a more aggressive behavior of these MTCs [55].

9.4.5 Screening for Familial C Cell Disease

Approximately 25% of MTCs are observed as autosomal dominant syndromes in the context of MEN type 2A or 2B familial cancer syndromes, which include MTCs and other neuroendocrine tumors [4, 6]. Prior to the advent of the molecular biology approach, all members of the affected families were periodically screened for MTC by both basal and PG-stimulated serum CT measurement [3], with all difficulties arising from the elevated false positive rate, borderline results, and inconveniences due to repeated PG testing. The discovery that germline mutations of the RET proto-oncogene are responsible for these syndromes allowed a much simpler and more effective management of these families [3, 6, 19]. Genetic screening can, in fact, identify subjects at risk before cancer development, with excellent sensitivity and specificity (virtually 100%). In this context, preoperative circulating CT measurement is recommended in subjects at risk, to help planning in terms of both optimal timing and extent of thyroidectomy [19]. In the absence of RET mutations, the risk for MEN-related cancers for nonaffected members is not greater than that of the general population, and they can be excluded from further surveillance with CT screening [19].

Because approximately 7% of apparently sporadic MTCs harbor germline RET mutations (generally involving exon 13, 14, or 15), genetic testing should be performed in all such patients [3, 6, 19].

9.5 Role of CT and CEA Measurement for Postoperative Management of MTC

At the time of diagnosis, locoregional metastases can be observed in up to 70% of MTCs presenting as palpable thyroid nodules, and up to 7–23% of such patients display already distant metastases [3, 4]. After initial treatment, repeated surgery, external beam radiation therapy, or other local treatment modalities may effectively be employed for cervical/mediastinal recurrence and for limited distant metastatic disease [4]. Systemic treatment should be deserved to patients with significant tumor burden or rapidly progressive MTC, defined according to the Response Evaluation Criteria in Solid Tumor (RECIST) [58]. Unfortunately, the survival rate is approximately 25% at 5 years and 10% at 10 years after the discovery of MTC distant metastases [45]. For all these reasons, accurate postsurgical follow-up for early detection of persistent/recurrent disease is necessary, for improving the outcome of MTC patients.

9.5.1 Postoperative Detection of MTC Persistence/Relapse

MTC persistence/recurrence can be suspected based on biochemical evidence and should subsequently be identified with imaging procedures [19, 59, 60]. In this context, serum CT and CEA determinations play a pivotal role [19, 59, 60].

9.5.1.1 The Role of Postoperative CT

After initial surgery, the normalization of serum CT may require up to 4 weeks, depending on preoperative hormone levels [61]. Therefore, postoperative CT assessments should be postponed after the second month following thyroidectomy. In patients with postoperative basal CT <10 pg/ml, a provocative PG or calcium stimulation testing may be performed, to confirm the absence of small residual tumor tissue foci. Patients with normal basal and stimulated CT levels on two consecutive follow-up evaluations are probably disease-free [4]. Since a tiny proportion of these patients (generally <5%) can experience MTC recurrence, a long-term follow-up should be recommended, with neck ultrasound examination and periodic CT determination on a regular basis [19, 60]. The same protocol may apply to patients with normal basal CT levels and mild to moderate elevations after provocative stimulation.

9.5.1.2 The Role of Postoperative CEA

Similarly to CT, serum CEA levels also decline after surgery. Nevertheless, the kinetics of CEA decline are not so well defined as in the case of CT. Usually also serum CEA levels increase at the time of relapse in patients with initially undetectable marker and parallel disease progression [3, 19]. From a clinical standpoint, it is currently accepted that either a marked elevation in the serum CEA level out of proportion to a lower serum CT level or normal/low levels of both serum CT and CEA are indicative of poorly differentiated MTC [3, 19].

Notably, postoperative CEA levels were found normal also in some patients with occult metastatic disease [62–66].

9.6 Prognostic Value of CT and CEA in MTC Patients

The prognosis of any type of cancer is related to both the tumor burden as determined by imaging procedures and the cancer progression rate, estimated according the RECIST criteria [58]. These estimates can be complicated in MTC patients, where metastatic disease often involves multiple lesions in different organs, some of which (e.g., liver metastases) may be difficult to visualize [4]. For these reasons, several attempts have been made to identify simple surrogate markers. Indeed, none of the candidates (mitotic rate, the Ki67 labeling index, and 18F-fluorodeoxyglucose uptake on PET scan) appeared reliable enough for use in clinical practice [19, 60].

At present, CT and CEA represent the only available markers that have been proven of some prognostic utility [19, 60].

9.6.1 Prognostic Value of Preoperative CT

In a large series, preoperative CT levels exhibited a significant correlation with the maximum MTC diameter and with the postoperative hormone levels [67]. Only 2% of MTC patients with preoperative levels <50 pg/ml presented postoperative CT elevations [67], while increased hormone levels after surgery were reported in 17% of patients presenting preoperative levels <100 pg/ml [38] and in 37% of those with CT levels >500 pg/ml [68].

The magnitude of the CT increase after PG stimulation may also reflect the extent of disease. A strong relationship has, in fact, been demonstrated between a less than tenfold CT response to PG stimulation and lymph node involvement, distant metastases, extrathyroidal extension, and normalization of postoperative CT levels [69].

9.6.2 Prognostic Value of Preoperative CEA

The contribution of abnormal preoperative CEA levels on the recognition of more invasive MTCs, requiring aggressive surgery, remains presently unclear. Indeed, limited data seem to indicate that serum CEA concentrations are increased at diagnosis in up to 50% of the patients and its preoperative levels can be correlated to disease progression and prognosis [53]. Based on the hypothesis that increased CEA levels might herald advanced disease, a retrospective analysis conducted on a large MTC series addressed the question of the relationship between preoperative CEA levels and tumor progression [53]. On multivariate analysis, abnormal preoperative CEA concentrations were significantly associated with the initial surgery rather than re-intervention, larger primary tumors, lymph node, and distant metastases. Data analysis limited to the patients with increased CEA levels before primary surgery showed a significant association between progressively increasing CEA levels, lymph node, and distant metastases [53].

As reported earlier, patients with poorly differentiated and more aggressive MTCs frequently show a disproportionately high CEA/CT ratio [3, 19].

9.7 CT and CEA for the Postoperative Management of MTC Patients

As mentioned above, the delay necessary for reaching nadir levels of serum CT levels after MTC surgical treatment is still controversial [3]. Some studies have suggested that 3 months after surgery is the optimal time to reach nadir levels of serum CT in MTC patients [70, 71]. Due to a more protracted half-life [29], CEA serum levels may require a longer interval to reach the postoperative nadir. Therefore, both the ETA and the ATA guidelines have recommended that serum levels of CT and CEA should be measured 3 months after surgery [19, 60]. In patients with undetectable or normal postoperative levels, both markers should be measured every 6 months for the first year and then yearly [19, 60].

Even though the determination of CEA and CT serum levels is important for postoperative surveillance, there is no definitive evidence showing that they are clearly related to progressive or stable status of the disease. In current practice, persistently elevated postsurgical CT levels >10 pg/ml indicate residual MTC tissue [11]. In a relevant proportion of such patients, no other evidence of disease may be demonstrated. Indeed, this finding is compatible with long-term survival [72, 73], with one to two thirds of such patients not developing symptomatic disease for several years after surgery and presenting a 10-year recurrence rate approximating 40% [73]. Importantly, the extent of circulating CT elevation allows a reliable prediction of disease extension (Fig. 9.2, Table 9.4),

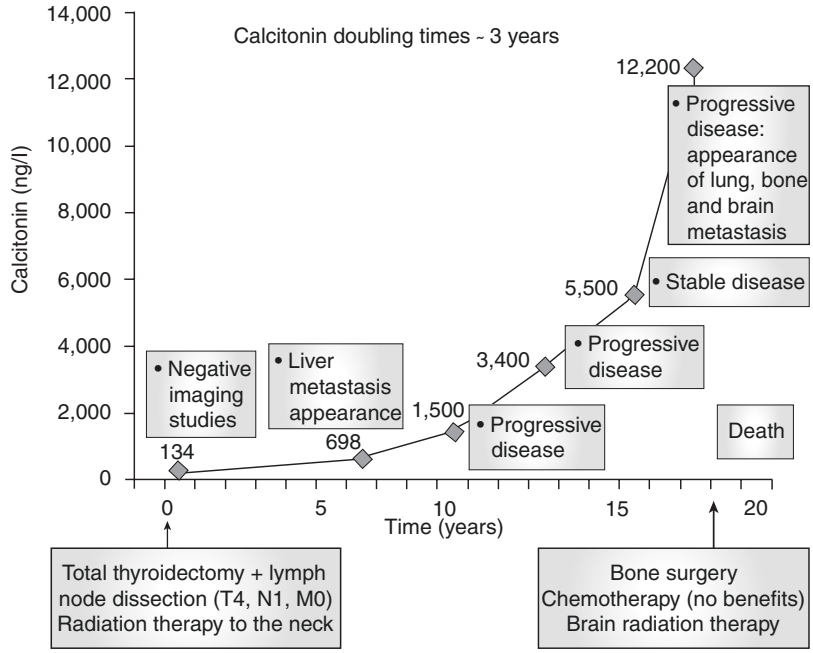

Fig. 9.2 Correlation between serum calcitonin levels and disease progression in MTC patients. Basal plasma calcitonin levels are shown for a patient with advanced MTC followed for 20 years. The *boxes* highlight disease evolution during the follow-up period. After the primary treatment (total thyroidectomy, lymph node dissection, and radiation therapy), the patient displayed only biochemical evidence of persistent disease (calcitonin levels 134 ng/l). The calcitonin doubling time was approximately 3 years. Distant metastases in the liver appeared 7 years later, with disease progression despite chemotherapy. Death occurred after a 20-year period of follow-up. Abbreviations: *M0* no distant metastases, *MTC* medullary thyroid carcinoma, *N1* regional lymph node metastases, *T4* primary tumor extension beyond the thyroid capsule and invading the subcutaneous soft tissues, larynx, trachea, esophagus, or recurrent laryngeal nerve. From Costante et al., Nat Clin Pract Endocrinol Metab. 2009;5:35–44

Table 9.4 Clinical interpretation of pentagastrin-stimulated calcitonin values

Stimulated CT peak	Clinical interpretation
<10 pg/ml	Absence of C cell disease
>10 < 100 pg/ml	Indeterminate (likely false positive)
>100 < 500 pg/ml	Probable C cell hyperplasia
>500 < 1000 pg/ml	Probable medullary thyroid carcinoma
>1000 pg/ml	Medullary thyroid carcinoma

being the probability of local or distant metastases strongly related to its levels [3, 4]. In particular, lower concentrations (e.g., <150 pg/ml) are more likely associated to disease confined to the neck, whereas higher levels are more suggestive of distant metastases [3, 4]. The likelihood of structural disease (e.g., tumor tissue identified by imaging studies) is also related to circulating CT increase [3, 4]. In this respect, it is recommended that patients with serum CT >150 pg/ml should undergo appropriate imaging procedures for the localization of distant metastatic lesions [19].

The relationship between tumor burden, levels of CT and CEA, and progression status according to RECIST criteria was analyzed in MTC patients presenting elevated CT levels and submitted to sequential imaging procedures [74]. That study indicated that CT and CEA levels were actually correlated with tumor burden. Nevertheless, no correlation could be demonstrated between CT and CEA levels and survival [74].

9.7.1 CT and CEA Doubling Times as Prognostic Factors

In the case of sporadic MTC patients presenting persistently elevated CT and CEA levels, the assessment of the progression status according to RECIST may often be complicated, even using standardized imaging procedures, particularly in case of slowly progressive tumors [58]. Moreover, CT levels can vary substantially (30% or more) within short periods of time [7, 13]. In these MTC patients, the dynamics of elevated CT and CEA levels can be more precisely quantified by calculating their doubling times (DT) [75] and compared to tumor growth rate in relation to RECIST evaluation [76, 77]. Notably, evaluation of DTs can be a long procedure [75], requiring measurements at multiple time points (at least four consecutive determinations over a minimum of 2 years). In case of DTs <6 months, the calculations should be performed based on CT and CEA levels over 1 year postoperatively [19].

A study of 45 MTC patients showed strong correlation between CT-DT and CEA-DT and RECIST findings in over 80% of patients. Additionally, 94% of those with CT-DT and CEA-DT <24 months had RECIST findings indicative of progressive disease, while 86% of the patients with longer DTs (>24 months) had disease classified as stable based on RECIST criteria [75]. Based on these results, the 2012 ETA guidelines recommended to determine CT-DT for the assessment of MTC progression rate and to eventually confirm it by imaging procedures according to RECIST criteria [58]. Analogously, the most recent ATA guidelines for MTC management [19] recommended the determination of CT-DT and CEA-DT by measuring their serum levels at least every 6 months.

In this respect, a retrospective study evaluated 65 patients treated by total thyroidectomy and bilateral lymph node dissection, from 2.9 to 29.5 years after surgery [75]. In patients with serum CT-DT <6 months, the survival rate was 25% at 5 and 8% at 10 years, while it increased to 92% and 37%, respectively, for DTs between 6 and 24 months. Additionally, it has been reported that a DT of circulating CT <6 months carried a 75% risk of cancer-related death within the following 5 years, while DTs of >2 years were associated with no tumor-related death [74].

In rare cases, an MTC patient with a large tumor burden may present with low CT levels. If the possibility of a "hook effect" can be excluded [16], this finding may reflect a poorly differentiated tumor [3]. In cases of this type, CEA levels are usually increased, and the CEA-DT is likely to be reduced [4]. Such a

discrepancy could herald aggressive disease, particularly if the CEA-DT is shorter as compared to the CT-DT [74].

In contrast to CT, only few studies have investigated the relationship between CEA-DT and survival outcomes. The available evidence suggests that CT-DT could be a more effective predictor of survival than the CEA-DT (7, 78). Nevertheless, a study [74] reported an 80% frequency of concordant CT and CEA DTs, with an average DTs of both CEA and CT of 12 months in patients with progressive disease, while stable disease was observed for DTs of 48 months for CEA-DT and 58 months for CT-DT. Importantly, progressive disease was evident in 94% of patients with DTs of both markers <24 months, while no evidence of progression could be demonstrated in 86% of patients with CT-DT and CEA-DT >24 months. For discordant DTs, progressive disease was observed in more than 50% of patients presenting a DT <25 months for either CT or CEA.

In a meta-analysis from Meijer et al., CEA-DT displayed a more pronounced impact on the prognosis [78]. This study showed that both CT-DT and CEA-DT were significant risk factors for recurrence and death caused by MTC for the cutoff value of 1 year. In the subgroup of patients for whom both CT and CEA were available, the model with CEA-DT <1 year had a higher predictive value for survival as compared to CT-DT. To explain the higher predictive value of CEA-DT, the authors speculated that CT and CEA production per individual MTC cell may not be constant during the course of the disease, particularly during the process of dedifferentiation, when CT production can be relatively normal [57, 79] or even decreased, while an increase in CEA production may occur [80].

In clinical practice, therefore, the DTs of both CT and CEA could be important for an adequate risk stratification of MTC patients. In this respect, both the ATA and ETA guidelines [19, 60] agreed that the CT-DT and CEA-DT of less than 2 years should be considered negative prognostic factors for MTC in patients with persistent or recurrent disease.

9.8 CT and CEA Measurement for the Assessment of Treatment Response

Because circulating CT and CEA levels are directly correlated to MTC tumor burden, their variations not only represent a useful parameter for estimating tumor progression but can also provide important information on the response to treatment. Indeed, their clinical utility for the decision-making process and for the evaluation of response to local (i.e., surgery, radiotherapy, interventional procedures) treatment in early and advanced/recurrent MTC is well known since many years [4].

Recently, tyrosine kinase inhibitors (TKIs) vandetanib and cabozantinib demonstrated improved objective response rates as compared to cytotoxic chemotherapy, with a significant increase in progression-free survival (PFS) [81–83], and have eventually been approved by both FDA and EMA for use in rapidly progressive recurrent/metastatic MTCs. Sequential determinations of serum CT levels have been used as potential marker for assessing MTC response to these drugs, based on the assumption that decreased CT concentrations during these treatments could effectively reflect targeting of tumor cells [59] and consequent tumor shrinkage or at least inhibition of either or both CT synthesis/secretion. Unfortunately, all trials evaluating RET inhibitors were unable to confirm the reliability of such a paradigm. No clear correlation could, in fact, be demonstrated between the variation in either CT or CEA levels and the response to TKI in terms of degree or duration of the observed objective responses [81, 84, 85]. Moreover, fluctuations in CEA and CT serum levels were observed in MTC patients irrespective of either response to treatment or tumor progression on TKI treatment [59]. Additionally, paradoxical increases in both biomarkers occurred in some responders [82, 86]. Eventually, most MTC patients presenting tremendously decreased CT and CEA levels after receiving TKIs frequently exhibited rebounds and oscillations up to 30% magnitude, without any correlation in terms of RECIST criteria [81–84, 87]. Dissociated effect of RET inhibitors on the proliferative and the secretory pathways of thyroid

C cells has been suggested to explain such results [88], at least for CT. No direct effect of RET inhibition on CEA expression has been observed [89].

Recently, a retrospective study has addressed the issue of CEA and CT variations as predictive factors for tumor progression in MTC patients treated with vandetanib [89], showing that objective progressive disease (PD) could be predicted with 82% accuracy in case of 40% increase in serum CT levels. In contrast, no value for prediction of structural progression during TKI treatment could be demonstrated for variations of CEA levels.

A retrospective review [90] aimed at evaluating CT or CEA levels as surrogate markers of cell toxicity induced by traditional cytotoxic agents in MTC patients reported no correlation between early circulating CT or CEA variation and overall survival (OS) during the first 3 months of chemotherapy. No significant relationship between changes in CT levels and either tumor response or progression-free survival (PFS) could be demonstrated, though an increase in CEA levels was associated to a significantly shorter PFS. Based on this observation, the authors suggested that discontinuation of cytotoxic chemotherapy should be considered in case of CEA progression after 3 months of treatment. A prospective study is necessary for definitive validation of these results.

9.9 Conclusions and Key Points

CT and CEA are useful tools for the diagnosis and the management of MTCs.

1. In the preoperative settings, circulating CT is the most specific and sensitive disease marker for MTC, while CEA displays a lower diagnostic accuracy, and both are of prognostic utility, allowing accurate estimation of tumor burden.
2. During the postsurgical follow-up of MTC patients, the evolution of both CT and CEA levels reflects MTC progression, and their increase reliably heralds persistence/relapse.
3. Discrepant results with disproportionately low circulating CT as compared to CEA levels should alert the clinician suggesting dedifferentiated, more aggressive MTCs.
4. CT and CEA doubling times (DT) can be used as prognostic factors, representing CT-DT a more precise indicator of MTC progression than CEA-DT.
5. Circulating CT and CEA levels are accurate markers of response to local treatment in early and advanced/recurrent MTC, but their suitability for the evaluation of response to systemic therapy needs confirmation.

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Procalcitonin: Are We Ready for Clinical Use?

10

Pierpaolo Trimboli, Alicia Algeciras-Schimnich, and Luca Giovanella

10.1 Introduction

Medullary thyroid carcinoma (MTC) is a malignant tumor originating from the thyroid parafollicular C cells and accounting for up to 5–10% of all thyroid cancers [1]. The diagnosis of MTC, since its first description [2], is still a challenge in clinical thyroidology [3–5]. Challenges in the diagnosis of MTC include the following: (1) cytologic examination by fine needle aspiration cytology (FNAC) has been shown to detect approximately only one-half of all MTC lesions [3, 4], (2) traditional ultrasound risk factors for thyroid malignancies have mostly been developed based on papillary thyroid cancer (PTC) and are not always present in MTC [6, 7], and (3) routine measurement of serum calcitonin (CT) in patients presenting with thyroid nodules is not universally accepted in clinical practice [5]. Regarding the latter issue, one of the main limitations is that no specific CT thresholds have been identified to diagnose MTC, and various potential cutoff values have been reported in literature. Hence, interpretation of CT might be confusing for clinicians. Furthermore, rare cases of serum CT-negative MTC have been described in literature [8]. For all the above reasons, a not negligible rate of MTC might be incidentally found after a thyroidectomy performed for nodular goiter [1]. Because the prognosis of MTC patients is strongly influenced by the initial treatment, additional tools to preoperatively detect MTC are desired.

In the last years, the use of procalcitonin (ProCT) has been investigated for the preoperative diagnosis and postoperative follow-up MTC [9]. ProCT is the precursor of CT and is expressed by the C cells of the thyroid gland and other neuroendocrine cells in the lungs and bowel. In comparison to CT, ProCT has a longer in vivo half-life (20–24 h) and is more stable after sample collection [8]. These features have generated growing interest in the use of ProCT as a diagnostic and prognostic marker of MTC alone or in combination with CT.

10.2 Rationale for the Use of ProCT to Detect MTC

CT, a 32-amino acid monomeric peptide, is considered the most reliable tool in the identification of MTC both before and after surgery. CT belongs

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to the calcitonin gene-related peptide (CGRP) superfamily, is generated by alternative splicing of the calcitonin gene (CALCI), and is the main product of thyroid C cells, while alternative expression of the gene results in the formation of CGRP in neural tissue. The biosynthetic secretory pathway for CT involves a complex series of progressive modifications [10]. After the biosynthesis and folding of precursors, subsequent proteolytic processing occurs both within the Golgi apparatus and later within the secretory granules due to the action of prohormone convertase (PC) enzymes. The precursor of CT is pre-procalcitonin (Pre-ProCT) containing 141 amino acids with a signal peptide of 25 residues [11]. ProCT (26–141) is processed to mature calcitonin, amino acids 85–116, and the carboxyl-terminus peptide-1 (CCP-1, also known as katacalcin), amino acids 121–141 (Fig. 10.1).

Under normal conditions, CT is involved in calcium homeostasis and its release is induced by increases in calcium concentrations. In disease, besides MTC, CT is elevated in bacterial infections, hypercalcemia (hyperparathyroidism), renal failure, autoimmune thyroiditis, leukemia, mastocytosis, small cell lung carcinoma, and breast or pancreatic cancer [5]. Hypergastrinemia, treatment with proton-pump inhibitors, and less frequently heterophilic antibody interference may be also associated with hypercalcitoninemia [5]. Serum CT concentrations in healthy subjects are low but detectable. When measured by manual immunoradiometric assays (IRMA), serum CT concentrations are <10 pg/mL in healthy subjects, while a CT concentration >100 pg/mL is universally recognized as a reliable marker of the presence of MTC. The major challenge for CT interpretation is in those subjects with CT concentrations between 10 and 100 pg/mL. A stimulation test with an infusion of pentagastrin or calcium gluconate might be required in these cases, and stimulated CT values >100 pg/mL should prompt additional investigation and possibly surgery due to high suspicion for MTC [5]. It is important to emphasize that this diagnostic recommendation, adopted by several clinical guidelines and widely used in the clinical practice, was established by specific CT IRMA methods [12, 13]. In recent years, most laboratories have moved CT measurement from IRMA methods to automated chemiluminescent or fluorescent ones, and significant inter-assay differences as well as method-specific reference intervals have been reported making the use of the above cutoffs questionable. There are pre-analytical and analytical challenges with the measurement of CT. In the pre-analytical phase, there is marked variation of CT during the day due to its pulsative secretion which may also be influenced by food intake. In addition, once the sample is collected, CT is rapidly degraded at room temperature and decays by 23% after 12 h, by 35% after 24 h, and by 65% after 7 days [12, 13]. Therefore, the blood sample should be drawn in the morning after an overnight fasting. It is necessary to centrifuge the sample immediately after blood coagulation and transport frozen to the laboratory. The analytical methods of CT assay have been

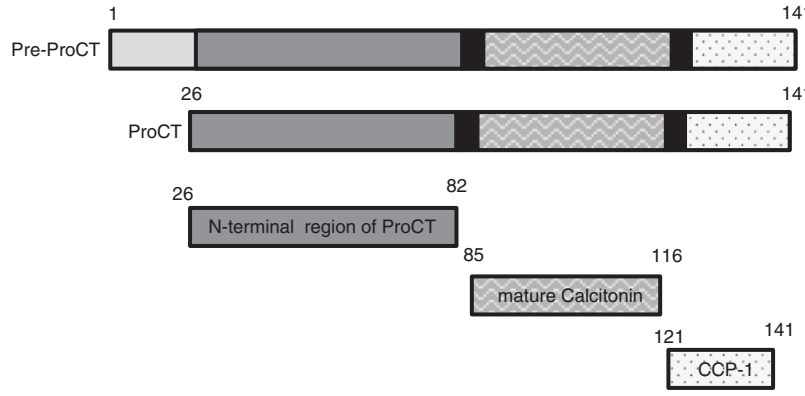

Fig. 10.1 Schematic of posttranslational processing of procalcitonin in medullary thyroid carcinoma. CCP-1 stands for carboxyl-terminus peptide-1 (also known as katacalcin)

evolving over time, and several laboratories have moved routine CT measurement from manual IRMA methods [12, 13] to automated chemiluminescent immunoassay (ICLA) platforms with comparable analytical performance [14]. However, the variable level of the different products of the CT gene results in several circulating immunoreactive isoforms and fragments [12, 13], and, consequently, poor inter-assay and interlaboratory agreement was demonstrated [15]. In the post-analytical phase, establishment of CT reference intervals in healthy subjects is still an unsolved challenge, and lack of standardization between assays has resulted in different reference intervals on the commercially available assays [16]. In addition, the need for gender-specific reference intervals has to be considered due to the differences in the number of C cells, which is approximately two times higher in men than women [16]. To overcome these problems, other potential MTC serum markers, such as CEA, chromogranin A, and ProCT, have been investigated in the last two decades. Of these molecules, ProCT, as the most recently reported, has appeared also the most promising. In fact, ProCT has been reported as a new accurate serum marker for the preoperative diagnosis of MTC and long-term follow-up of MTC patients.

10.3 ProCT Expression

Under normal condition, ProCT is only produced by the C cells of the thyroid where it is rapidly cleaved as described above. Only a small amount of PCT is released into circulation with concentrations of <0.10 microgram/L in healthy individuals [17]. In the presence of bacterial infection and inflammatory conditions, PCT production is activated in non-thyroidal tissues and concentrations rapidly increase [18]. PCT assays are currently approved by the Food and Drug Administration (FDA) to aid in the risk assessment of critically ill patients for progression to severe sepsis and septic shock. The role of PCT in patient with MTC is described below. Nevertheless, relevant limits of CT measurement in clinical practice have affected its accuracy [19, 20].

10.4 ProCT Analytical Considerations

The stability of PCT after sample collection has been reported to be superior to CT. PTC has been shown to be stable when stored at 4 °C or room temperature for 24 h. Decreases of 10–12% from the original ProCT concentration have been reported under these conditions [21, 22]. ProCT concentrations significantly decrease after 48 h at room temperature with a median decrease of 30% from the original concentration [23]. When stored frozen, ProCT up to three cycles of freezing and thawing does not significantly affect ProCT concentrations [24].

The original ProCT assay was developed as a luminometric immunoassay (LIA) using a coated tube system with two monoclonal antibodies and a luminometric tracer. Based on this, a number of automated assays have been developed using TRACE (time resolved amplified cryptate emission), ELFA (enzyme-linked fluorescent assay), CLIA (chemiluminescent immunoassay), and ECLIA (electrochemiluminescence immunoassay) technologies for use on the automated platforms BRAHMS Kryptor®, BioMérieux VIDAS®, Siemens ADVIA Centaur®, and Roche Elecsys®, respectively. There is currently no reference method for ProCT; however, all automated and ProCT assays have been developed using the same set of antibodies and the results compared with the LIA method resulting in improve between-assay comparability. Figure 10.2a shows patient samples' result agreement within 10% for the BRAHMS Kryptor® assay and the recently introduced in the United States Roche Elecsys® assay with a R2 of 0.98. Comparability between assays could also be assessed by the analyzing proficiency testing results. The College of American Pathologists proficiency testing surveys (2014, 2015, 2016) show larger than expected differences between platforms (Fig. 10.2b). The differences between the assays seem to be more pronounced at ProCT concentrations > or = 1.8 ng/mL where a > 20% deviation from all-methods mean is observed. It is possible that these differences are due to a matrix effect of the proficiency testing material in the different assays

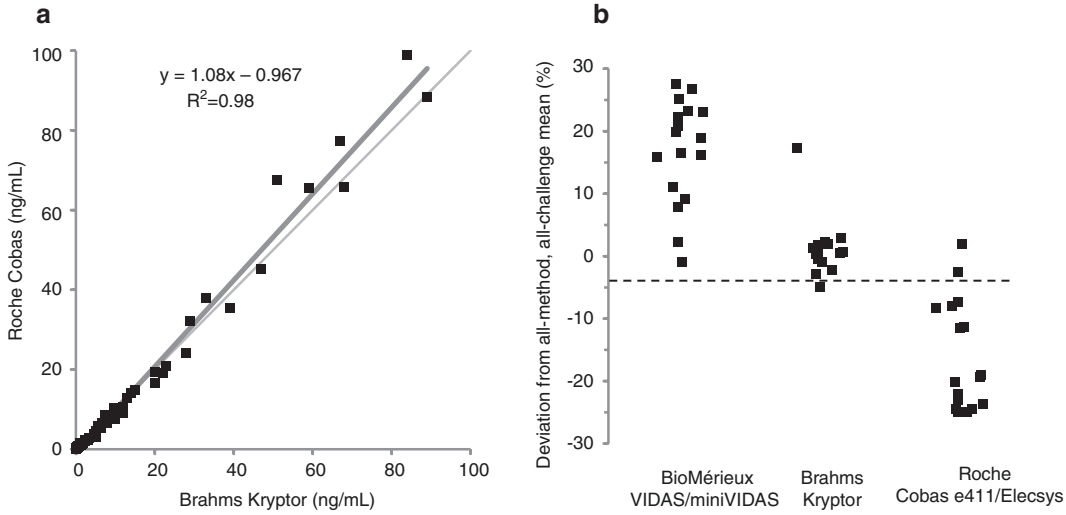

Fig. 10.2 (**a**) Ordinary least-squares fit regression analysis for the BRAHMS Kryptor (x) and Roche Elecsys (B) assays. Analysis includes 65 samples with concentration ranging from 0.1 to 90 ng/mL. (**b**) Result agreement of three ProCT immunoassays. The results of the College of American Pathologists proficiency testing survey (12 challenges) for ProCT for the years 2014, 2015, and 2016 are plotted. Individual challenges consist of three samples containing low, medium, and high concentrations. Each method is used by at least 20 different laboratories. The results are plotted as percentage deviation of their respective all-methods, all-challenges mean. If assays were in perfect agreement, then all data points would lie on the dotted line that runs through 0% deviation

or the introduction of a calibration bias over time. Although these differences might not be as relevant in the context of sepsis when measurement will occur within a short timeframe (weeks), these potential differences need to be considered in the context of MTC patient monitoring which will occur over the course of years.

The effect of degradation products interference in the ProCT assay has not extensively been studied. However, it has been suggested that one of the potential advantages of ProCT is the lack of susceptibility to interfering isoforms or fragments, which could cause falsely low CT concentrations. In a report of two patients with falsely low CT concentrations due to interfering fragments, the concentrations of ProCT were consistently elevated [21].

10.5 ProCT Clinical Studies

To date, a number of about 50 articles reporting the use of ProCT for the diagnosis of MTC have been published. A part of these studies used ProCT in the preoperative identification of MTC, while the remaining ones measured ProCT during a follow-up of patients previously treated for MTC.

10.6 Use of ProCT for Preoperative Detection of MTC in Thyroid Nodule Patients

To date, two studies [25, 26] investigated the potential role of ProCT as a marker for the initial diagnosis of MTC in patients with thyroid nodule(s). All subjects included in these two studies underwent surgery with a final histologic diagnosis of MTC. One prospective study [25] measured both CT and ProCT in a consecutive series of 1236 patients with thyroid nodules, and 14 of these reported increased (i.e., above 10 pg/mL) CT levels. These cases were selected for pentagastrin stimulation. At final histology, two MTCs were recorded and they had basal CT >100 pg/ml and ProCT >0.1 ng/ml. The main findings of this study were that basal and pentagastrin-stimulated CT had some false positive results, while all patients without MTC had both basal and stimulated undetectable

ProCT (100% PPV and 100% NPV). These data prompted the authors to recommend measuring a basal ProCT instead of performing a pentagastrin-stimulated CT in those patients with moderately elevated basal CT. Very relevant data about the prognosis of patients was described in another paper [26]. Machens and colleagues evaluated a number of 457 consecutive patients with untreated MTC, 112 of whom had ProCT determined before the initial surgery and 107/112 had ProCT >0.1 ng/ml (95.5% sensitivity). The ROC analysis revealed equal diagnostic accuracy for ProCT and CT, yielding comparable areas under the curve for primary tumors, extrathyroidal extension, lymph node, and distant metastasis, respectively. However, as an interesting result, ProCT levels were significantly correlated with the number of lymph nodes involved and distant extent of disease. Also, when ProCT levels increased, the biochemical cure rates declined, being 71% for patients with ProCT 1 ng/ml, 36% for those with 5 ng/ml, 23% for those with 10 ng/ml, and only 10% for the remaining with ProCT of 50 ng/ml. Lastly, a recent case report described the determination of ProCT in the washout fluids from FNAC of a MTC with ultrasound presentation of purely cystic. This study demonstrated the technical feasibility of the measurement of ProCT in biological fluids other than serum/plasma [27]; however, its clinical role should be evaluated in larger studies and compared to the well-established FNA-CT measurement.

ProCT appears to be useful in those rare MTC cases where CT is undetectable both pre- and postoperatively and therefore lacks this marker of disease activity [8]. In one MTC patient with negative CT, Brutsaert and colleagues [28] tested ProCT before and after surgery and its value converted from detectable (0.21 ng/ml) to undetectable (<0.1 ng/ml), respectively.

10.7 ProCT for the Assessment of MTC Patients During Postoperative Follow-Up

Several papers [21, 29–34] used ProCT determination in MTC patients during their follow-up after surgery. The most relevant article was published by Algeciras-Schimnich et al. [21] reporting on a cohort of 133 MTC patients (91 active and 42 cured disease), 83/91 (91.2%) of the recurrent MTC had ProCT >0.15 ng/mL, while no cured MTC with positive ProCT were recorded (100% specificity). Out of the active MTC group, those with stable disease had the lowest ProCT levels (mean 3.6 ng/ml), patients with recurrent/metastatic disease had highest value (mean 241.7 ng/ml), and patients with newly diagnosed cancer had intermediate concentrations (mean 13.8 ng/ml). The ProCT concentration giving the best diagnostic accuracy was 0.16 ng/ml, corresponding to upper reference limit for normal subjects (≤0.15 ng/ml). These findings prompted the authors to quote that ProCT is a promising complementary MTC tumor marker due to its greater analytical stability. A relevant contribution was reported by Kratzsch and colleagues [34]. They compared ProCT and several CT assays (Immulite, Liaison, and IRMA Medipan) in different groups of patients with high CT levels, such as subjects affected by recurrent or persistent MTC, chronic kidney disease (CKD), or Hashimoto's thyroiditis. The MTC patients had CT ranging from 18 to 1511 pg/ml and ProCT between 0.226 and 11.6 ng/ml. Assuming the highest CT and ProCT value in CKD patients, the authors found a diagnostic sensitivity for MTC of 100% for ProCT and IRMA-CT, but lower for Immulite-CT (90%) and Liaison-CT (80%). According to the authors' conclusion, a ProCT <0.25 ng/ml can exclude MTC. Of importance for clinical practice, ProCT concentrations appeared to have better stability than CT. Kaczka and colleagues reported two interesting series [31, 32]; one study recorded undetectable (i.e., <0.1 ng/ml) ProCT in 20/23 cured MTC (87% specificity) and detectable ProCT (ranging from 0.63 to 5.52 ng/ml) in active MTC (100% sensitivity) [31]. Later, these authors evaluated ProCT in four persistent MTC, in two MTC patients before and after surgery, and in other 23 MTC in remission [32]; the most relevant finding was that 20/23 (87%) disease-free patients had undetectable (<0.1 ng/ml) ProCT levels while CT was undetectable (<5 pg/ml) in only 15 cases, with mean ProCT of 3.5 ng/ml in active MTC and

0.06 ng/ml in MTC in remission. The study by Walter et al. [33] evaluated a group of 69 MTC during follow-up, and ProCT was detectable (i.e., >0.06 ng/ml) in 67 cases; the best cutoff for discriminating MTC from controls was 0.5 ng/ml (84% sensitivity and 84% specificity), and the threshold to distinguish MTC from C cell hyperplasia was 0.16 ng/ml (59% sensitivity and 100% specificity). Also, ProCT:CT ratio was as an accurate predictor of progression-free survival and correlated with the clinical outcome. Finally, two older papers have to be cited. High performance of ProCT in detecting recurrent MTC was also recorded by Bolko et al. [29]; however, the assay used in this study was a sandwich assay with anti-katacalcin as the detection antibody and anti-CT as the capture antibody; therefore, the results were not perfectly comparable with more recent studies. The study by Bihan et al. [30] investigated a new chemiluminescent assay which detects both intact ProCT molecule and CT-joined C-terminal cleavage carboxyl-terminus peptide-1 (CCP-1) but does not distinguish between the two; therefore, unfortunately, the results of PCT were not specified.

The majority of the published reports described ProCT measurement during follow-up of MTC patients after initial surgical treatment. Importantly, cured patients had very rarely detectable ProCT, while positive ProCT levels were present in those cases with persistent disease, with both sensitivity and specificity nearly to 100%. A few studies reported the evaluation of ProCT as a marker for the initial preoperative detection of MTC. In this scenario, ProCT values correlated with the extent of disease, and the biochemical cure rates were inversely correlated with ProCT levels [26]. In another study [25], pentagastrin-stimulated CT and ProCT were tested in patients with moderately elevated CT, and both basal and stimulated undetectable ProCT had 100% PPV and NPV.

Conclusions

In view on the data published to date, serum ProCT has great potential to be combined with or replace serum CT as a new standard of care in the management of MTC. An advantage of ProCT is its improved stability when compared to CT. On the other hand, a limitation might be that measurement of ProCT is still not so widely diffused. However, due to the increasing use of ProCT in internal medicine and antibiotic therapy, the availability of ProCT is expected to be wider during the next years [35]. As the most critical challenge, further studies are needed to identify the optimal cutoff level of ProCT to distinguish active (i.e., recurrent/persistent) MTC patients from cured ones.

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Part IV

Aggressive and Rare Thyroid Carcinoma

Aggressive Variants of Thyroid Carcinoma

11

Massimo Salvatori, Claudio Altini, Luca Zagaria, Alfonso Verrillo, and Germano Perotti

11.1 Introduction

Thyroid carcinoma of follicular origin is the most common endocrine neoplasia and the cancer with the greatest increasing rate of incidence. In 2016, the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute estimated 64,300 new cases corresponding to 3.8% of all new cancer cases in the USA [1]. (https://seer.cancer.gov/statfacts/html/thyro.html). Thyroid carcinoma is currently the fifth most common cancer diagnosis in women. By the year 2030, it is estimated that it will be the second leading cancer diagnosis in women and the ninth leading cancer diagnosis in men.

Most thyroid carcinomas of follicular origin (papillary thyroid carcinoma, PTC, and follicular thyroid carcinoma, FTC) are well-differentiated tumors (WDTC) characterized by a papillary and/or follicular architecture on histologic examination, show an indolent clinical course, and have an excellent prognosis with a 98% of survival rate at 5 years. In 2016 has been estimated 1980 deaths, corresponding to 0.3% of all cancer deaths (https://seer.cancer.gov/statfacts/html/thyro.html).

However, certain histological variants of primary thyroid carcinomas have an aggressive course characterized by extensive vascular invasion, extensive tumor necrosis and/or mitoses, and frequent extrathyroidal extension (ETE) [2–4]. Recommendation 46 of the 2015 American Thyroid Association (ATA) "*Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer*" recommends that pathology reports should contain data on the histopathologic variants of thyroid carcinoma associated with more unfavorable outcomes (Table 11.1) [5].

Although underreporting and misclassifying due to non-standardized criteria for defining variants, some studies seem to indicate that the incidence of the aggressive variants, particularly the tall-cell variant (TCV), is now outpacing the incidence of classic PTC [3]. These tumors have a higher risk of distant metastases and tumor-related mortality compared to WDTC, observed mostly in patients with an advanced disease stage at presentation. Furthermore, there exists the possibility that initial WDTC may have undergone considerable genomic and histologic changes during the course of time progressing to the histologically aggressive subtype in the metastasis/recurrence [2–4].

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Table 11.1 Important features in a surgical histopathology report

• Histopathologic tumor type
• Histopathologic variants with favorable or unfavorable outcomes
• Tumor size
• Multifocality
• Extrathyroidal invasion
• Status of resection margins
• Number of lymph nodes examined
• Number of lymph nodes involved with tumor
• Size of the largest tumor-involved lymph node
• Extranodal invasion
• Presence of vascular invasion and number of invaded vessels

Table 11.2 Histological aggressive variants of primary thyroid carcinomas

Papillary thyroid carcinoma
• Tall-cell variant
• Columnar-cell variant
• Hobnail-cell variant
• Solid variant
• Diffuse sclerosant variant
Poorly differentiated thyroid carcinoma (PDTC)
• Insular thyroid carcinoma (ITC)
• Hürthle cell carcinoma (HCC)
Anaplastic thyroid cancer (ATC)

The present chapter will focus on the clinical and pathologic characteristics of thyroid cancers more aggressive than the classic WDTC, following the classification reported in Table 11.2. In addition to the aggressive variants of the PTC (tall cell, columnar cell, hobnail, solid, diffuse sclerosing), this classification considers poorly differentiated thyroid carcinoma (PDTC) (insular thyroid carcinoma, Hürthle cell carcinoma) and anaplastic thyroid carcinoma (ATC), too.

11.2 Clinicopathologic Characteristics

Except for PDTC and ATC for which sufficient careful data are available, most of the aggressive variants of the PTC present similar uncertainties and lack of data affecting management and indication to treatment. This is can be due to several reasons.

First, due to their rarity, most studies of aggressive variants of PTC in the literature consist of single-institution series that often enrolled few patients [3]. The lacking size of the records determine difficulties to define precise prognostic and therapeutics indications.

Second, the discrepancies in diagnostic definitions contribute to reduce the magnitude of the clinical records and can to explain the various reported incidence. Different diagnostic criteria are applied in various geographic areas, resulting in wide discrepancies among pathologists and clinicians worldwide. For example, there is controversy in the literature about the percent of the tumor that must have tall-cell features to classify the tumor as a TCV. The two most common recommendations in the literature are a 10% and 30% minimum tall-cell component [6, 7].

Third, in some cases, it is uncertain if the presence of the histologic cell type in itself is an important prognostic factor or whether the presence of the unusual cell type simply correlates with a higher incidence of the other factors known to influence prognosis unfavorably [2]. Some studies have indicated that higher clinical stage and grade, and not variant histology, explain differences in outcome [2, 8, 9]. Others, by using multivariate analyses to determine the independent effect of aggressive variants on survival, concluded that histology alone leads to a worse prognosis [10, 11, 3].

However, the 2015 ATA Initial Risk Stratification System recommended to classify patients with aggressive variants of PTC (e.g., tall-cell, hobnail variant, columnar-cell carcinoma) at ATA intermediate risk of disease recurrence and/or persistence.

11.2.1 Aggressive Variants of Papillary Thyroid Carcinoma

11.2.1.1 Tall-Cell Variant

More than ten microscopic variants of PTC have been documented in the literature [12].

TCV represents one of the most frequent aggressive variants, characterized by high predominance of tall columnar cells showing the same characteristic nuclear features of the PTC but in addition abundant eosinophilic cytoplasm to the point that tumor cell height is at least three times their width.

According to current literature, thresholds for the requisite percentage of tall cells range from 10% [7] to 30% [6]. However, this threshold has not been strictly defined, and there is a wide variability in the definitions of TCV used by different pathologists. This can explain the various reported incidence (ranging between 3 and 19% of cases) and the possibility that the majority of TCV cases are missed by pathologists [11].

Compared with classical PTC, TCV present with an older age at diagnosis (it is rare among pediatric and young patients), a higher rate of ETE, increased incidence of regional metastases, and decreased disease-specific survival. Even when to the initial diagnosis the tumor doesn't show ETE, some studies found a higher rate of disease recurrence and poorer survival when compared with classic PTC without ETE, and this was independent of patient age and tumor size and stage [5].

The largest series of TCV from Memorial Sloan Kettering Cancer Center (MSKCC) reported 278 cases identified between 1999 and 2006 by using the SEER registry [11]. A matched-pair analysis between TCV and classical PTC performed by these authors showed a mortality rate nearly doubled, from 10 to 19 cases out of 278 TCV (81.9% versus 91.3%, $p = 0.049$). The absolute difference in disease-specific survival reported by Morris et al. [11], although modest in absolute terms (9.4%), represents a clinically significant distinction for WDTC.

Kazaure et al. reported for TCV a significant increase in mortality risk for each centimeter increase, suggesting accelerated aggressiveness with small incremental increases in size [3]. The authors found a 29% increase in mortality risk for each centimeter increase in TCV tumor size in multivariate analyses of survival [3]. Machens et al. [13] found that after controlling for other major prognostic variables, the risk of distant metastasis was four greater in patients with TCV that in patients with WDTC.

Some characteristics at a molecular level could explain the oncologic aggressiveness of TCV, such as Muc1 protein and Type IV collagenase overexpression, RET/PTC3 rearrangements, and BRAF point mutations (up to 80%) [11].

11.2.1.2 Columnar-Cell Variant

An extreme variant of PTC is the columnar-cell variant (CCV), characterized by predominance of elongated tumor cells with scant cytoplasm and pronounced elongated hyperchromatic pseudostratified nuclei. The morphology of CCV is often reminiscent of endometrial or colonic carcinoma, with some of the nuclear features of PTC [14]. As with TCV, CCV may by occur in tumors with other patterns of differentiation and there are multiple reports of tumors with mixed tall- and columnar-cell features.

The notion that CCV are clinically aggressive was later challenged by observations that tumor circumscription and/or encapsulation may confer a more favorable outcome. The CCV can be separated into circumscribed and widely invasive subtypes corresponding to the clinicopathological indolent and aggressive carcinomas, respectively. Chen et al. [14] reviewed the English literature to better define the clinicopathological characteristics of these rare neoplasms, founding 20 clinically indolent and 23 aggressive neoplasms. Of cases with aggressively behaving and clinical follow-up, 13 died of disease ranging from 7 to 126 months after the diagnosis, and 5 patients were alive but with disease ranging from 16 to 108 months follow-up. The BRAF V600E mutation is found in one-third of these tumors [14].

11.2.1.3 Hobnail Variant

Hobnail variant (HV) is a rare, recently described variant of the PTC characterized by the predominance of small papillary clusters surrounded by lacunar spaces, hobnail features and high nuclear/cytoplasmic ratio [15]. This subtype of PTC has been considered aggressive and rare, with a prevalence accounting for less than 1% of the whole PTC histotype based on the few series

reported in the literature [16]. Although HV of the PTC has been only recently identified, its clinicopathologic features suggested an aggressive behavior, as documented by larger volume, common ETE, increased rate of spread to lymph nodes and distant organs [15, 16]. The more aggressive behavior of this tumor could be referred to the p53 overexpression (> 25% of the tumor cells in the series). Besides, the BRAF V600E mutation is found in about 80% of HV and in 20% of patients a RET/PTC1 variation is present [15].

11.2.1.4 Solid Variant

The solid variant (SV) of PTC is characterized by a nested pattern of growth with the nests separated by a delicate capillary network and fibrous stroma, vague papillary formations, and the follicular pattern partly maintained [17]. This variant, which has been best defined in studies of the thyroid cancers occurring in children living in the countries of the former Soviet Union affected by the Chernobyl nuclear accident, has a propensity to occur in childhood, and it may be related to radiation exposure and/or dietary iodine deficiency [12, 17, 18]. Although this variant is associated with aggressive behavior (about 15% of cases with distant metastases and 42% with vascular invasion) and slightly higher mortality rate (10–12%), in the experience of the post-Chernobyl group of SV, very few children and adolescents have died of thyroid cancer (<1%) during the first 10 years of follow-up [19].

11.2.1.5 Diffuse Sclerosant Variant

Diffuse sclerosant variant (DSV) of PTC is a rare neoplasm characterized by diffuse and extensive involvement of one or both lobes usually without forming a dominant mass [3, 10, 20]. The prevalence of DSV varies from 0.7 to 6.6% of all PTC and is especially high in regions affected by increased radiation exposure, such as in Belarus (the area affected by the Chernobyl accident) [20]. Also, aside from the association with radiation, the prevalence of this entity is high in the pediatric population and tends to occur more frequently in women (male-to-female ratio 1/5) and more often in patients in their third decade [20].

On microscopic examination, DSV have characteristic nuclear features of PTC. In addition, the carcinoma shows marked squamous metaplasia, numerous psammoma bodies, extensive interstitial fibrosis, and heavy lymphocytic infiltration with formation of germinal centers. The presence of numerous psammoma bodies implicates a high sensitivity of diagnosis by fine needle aspiration (FNA).

DSV has a high incidence of lymph node (80%) and distant metastases (5%), predominantly affecting the lung. In the data analysis reported by Pillai et al., approximately 14% (89/641) of the patients with DSV showed recurrence of cancer, and disease-related mortality was noted in 3% (19/641) of the patients [20]. DSV shows different expression patterns of epithelial membrane antigen when compared to conventional PTC (galectin 3, cell adhesion molecules, p53 and p63) and common (activation of RET/PTC rearrangements) and uncommon events (BRAF and RAS mutations) at genetic analysis [20].

11.2.2 Poorly Differentiated Thyroid Carcinoma

Defining criteria for the diagnosis of PDTC is a source of controversy. For many years, the recognition and definition of this group of neoplasm was not clear. Some authors have advocated using architectural features such as a solid/trabecular growth pattern, whereas others propose using a proliferate grading system to establish the diagnosis of PDTC.

A new consensus diagnostic criterion for PDTC was proposed in 2007 by an international group of pathologists convened in Turin, Italy [18]. These authors require the presence of (1) solid/trabecular/insular microscopic growth pattern, (2) lack of well-developed nuclear features of papillary carcinoma, and (3) convoluted nuclei (evidence for partial loss of differentiation in papillary cancer), tumor necrosis, or three or more mitoses per ten high-power fields [18].

The most common variants of PDTC encountered in the clinical practice are the insular thyroid carcinoma (ITC) and the Hürthle cell carcinoma (HCC).

11.2.2.1 Insular Thyroid Carcinoma

ITC is a rare tumor classified since 2004 by the World Health Organization (WHO) as a component of the larger group of PDTC. ITC is characterized by nests or "insulae" of small, uniform, neoplastic cells, surrounded by a hyaline stroma, and associated with areas of tumor necrosis as well as microfollicles of thyroglobulin [18, 21].

Recently, Pezzi et al. evaluated characteristics and outcomes of 508 patients with ITC reported from 1998 to 2012 by the National Cancer Data Base (NCDB), a joint program of the Commission on Cancer of the American College of Surgeons and the American Cancer Society [21]. It accounts for only 0.14% of all thyroid cancers in the NCDB, and its incidence has remained relatively stable throughout the study period, with respect to the increased incidence of PTC and FTC.

Compared to PTC and FTC, patients with ITC are older (mean age of 61.4 years), have larger tumors (mean tumor size 6.1 cm vs. 1.8 cm and 3.6 cm, respectively), and present more frequently distant metastases (20.8% vs. < 1% and 4.2%, respectively) and a lower overall 5-year survival (57%). Despite the fact that ITC is believed to arise from FTC, the rate of lymph node metastasis in ITC (26.3%) is closer to that seen in PTC than in FTC [18, 21].

11.2.2.2 Hürthle Cell Carcinoma

HCC is regarded to be an oxyphilic variant of follicular FTC according to the WHO classification. It comprises about 3% of all thyroid malignancies and is characterized by the presence of acidophilic, granular cytoplasm and hyperchromatic or vesicular nuclei with large nucleolus [22]. Hürthle cells show the follicular growth pattern and have abundant granular eosinophilic cytoplasm, due to the accumulation of abundant mitochondria [22]. Tumors classified as HCC are required to contain more than 75% Hürthle cells and are divided in minimally and widely invasive HCC on the basis of the gross invasion and the vascular invasion. Minimally invasive carcinomas are fully encapsulated tumors with less than four foci of capsular or vascular invasion, in contradistinction to widely invasive tumors, which have extrathyroidal invasion and more than four foci of capsular or vascular invasion [22].

According to the ATA and National Comprehensive Cancer Network (NCCN) treatment guidelines, HCC follows the same risk stratification as that of FTC and can be considered at low, intermediate, and high risk of residual or recurrent disease on the basis of grade of capsular or vascular invasion, metastatic lymph nodes, incomplete tumor resection, and distant metastases. Clinically, the widely invasive form of HCC is the most important because it can be locally invasive, can metastasize into neck lymph nodes (5.3–13%), and develops a high incidence of distant metastases, often refractory to radioactive iodine [23]. Patients with widely invasive HCC have a poor prognosis with respect to non-aggressive variants of WDTC, with a recurrence rate of 31% and disease-specific mortality of 25%. If distant metastases are present, the mortality rate rises to 80% [24].

The largest group of patients with distant metastases of HCC and long follow-up in all the literature has been reported by Besic et al. [25]. The authors analyzed 32 patients with proven metastases from HCC followed for 1–226 (median 77) months. The most common sites of metastases were the lungs, bone, mediastinum, kidney, and liver. After thyroid surgery radioiodine (RAI) ablation of thyroid remnant was performed in 30 patients, while 20 of them had RAI therapy (median 4 times). Chemotherapy was used in 13 patients and external beam radiation therapy (EBRT) in 19 patients. Estimated 10-year disease-specific survival for all patients was 60%, while the estimated median disease-specific survival after the diagnosis of metastatic disease for all patients was 77 months [25].

11.2.3 Anaplastic Thyroid Carcinoma

ATC is one of the most aggressive solid tumors to affect humans, with a median survival on the

order of 3–5 months following diagnosis. In an analysis of survival of ATC patients from the SEER database from 1983 to 2002, which included patients who survived for more than a month, the median survival was 4 months [26]. It contributes up to 14–50% of the annual mortality associated with thyroid cancer, and 1-year and 10-year survival rates are estimated at 10–20% and less than 5%, respectively [26].

The incidence of ATC is estimated at one to two per million population per year, corresponding to less than 1–3% of all thyroid cancers. The trend has been decreasing even though the incidence of WDTC has been increasing; studies published from Italy showed a reduction of ATC from 4% to 1% between 1969 and 1973, while another study in India showed a decline from 8% to 4% between 1989 and 1993 [26–28].

ATC is primarily a disease of the elderly, more than 60% of patients are ≥70 years old, and females constitute 70% of ATC patients. Some studies have reported an association between goiter and ATC; in 25% of ATC cases, patients had a prior history of thyroid goiter and another 10% a family history of goiter [27]. It is also known that ATC is more common in places with endemic goiter, and thus with improvements in iodine supplementation, the incidence of this tumor would be expected to decline [27, 28].

All ATCs are considered stage IV by the International Union Against Cancer (UICC)-TNM staging and American Joint Commission on Cancer (AJCC) system, and even in the absence of metastatic disease, they are considered to have systemic disease at the time of diagnosis [29]. Frequently, ATC presents local symptoms such as a rapidly evolving central neck mass, noticeable dysphagia, voice change or hoarseness, and regional symptoms including a noticeable lymph node mass and neck pain [27]. A study analyzing the SEER Program from 1973 to 2000 reported that only 7.5% of patients with ATC had an intrathyroidal carcinoma, whereas 37.6% and 43.0% had extrathyroidal invasion with regional lymph node metastasis and distant metastasis, respectively. Distant metastases [more frequently in the lungs (~80%), bone (~ 12%), and brain (~ 8%)] are found in 50% of patients at presentation, while the 25% of patients develop metastasis during the course of the disease [26].

11.3 Diagnostic and Therapeutic Procedures for Clinical Management

11.3.1 Radioiodine Whole-Body Scan and Therapy

Since most thyroid cancers retain a degree of iodide-concentrating capacity, RAI represents a major diagnostic and therapeutic tool in the management of WDTC patients. RAI enter both the normal and neoplastic thyroid cells against its electrochemical gradient by an active transport mechanism at the basolateral membrane, which is mediated by the sodium iodide symporter (NIS). NIS is an intrinsic membrane glycoprotein of the thyroid follicular cells characterized by 13 putative transmembrane domains, an extracellular aminoterminus and an intracellular carboxyl-terminus [30].

Radioiodine uptake in primary WDTC can be reduced in comparison with normal thyroid tissue. Due to a process of dedifferentiation, metastases from WDTC may display a different pattern of expression of thyroid-specific proteins, including NIS and thyroid peroxidase (TPO) gene expression, with the consequence that one-third of thyroid cancer metastases do not concentrate radioiodine [31].

Decreased iodide uptake is even more pronounced in aggressive variants of PTC and in PDTC. The diminished radioiodine uptake in such tumors can be due to decreased levels of NIS expression or other possible mechanisms, including alterations in NIS gene structure, NIS gene and protein regulation, post-translational modification, NIS protein synthesis, and cellular localization of NIS [30].

Structurally evident locoregional and distant metastases from aggressive variants of thyroid carcinoma are often RAI-refractory (RAI-R). To define a DTC as a RAI-R tumor, it should fulfill at least one of the following criteria: (1) the

malignant/metastatic tissue is unable to take up iodine and the post-RAI therapy whole-body scan (TxWBS) is negative; (2) the malignant/metastatic tissue, previously able to take up RAI, loses this ability over time; (3) RAI uptake is present only in some lesions but not in others; and (4) the disease progresses despite its ability to take up RAI [5]. When a patient is classified as RAI-R and is in progressive structural disease, there is no indication for further RAI therapy and should be considered for other treatment options [5].

Very few studies examined the levels of NIS expression and the diagnostic/therapeutic role of RAI in aggressive variants of PTC. Wei et al. [32] found positive NIS staining in 73% of patients with conventional PTC, 32% of patients with TCV, and 40.0% of patients with DSV. In TCV and DSV with positive NIS staining, the NIS protein was located in the nuclear membrane and nucleus, when is well known that the only location where NIS is functional is the cellular membrane [32]. Kazaure et al., by using the SEER database, evaluated the largest cohort of DSV and TCV patients present in the literature and reported improved survival among DSV patients who received RAI and to a lesser extent in TCV patients [3].

Most studies on the value of RAI therapy in aggressive variants of thyroid carcinomas regard patients with HCC, and there is controversy about the efficacy of therapy on such patients. However, the majority of studies evaluating the effect of RAI therapy for HCC are small, retrospective, single-institutional series, likely due to the rarity of HCC [22].

Lopez-Penabad et al. [24], evaluating 89 patients with HCC, reported that 38% of the patients with known metastases showed RAI uptake and received RAI therapy. The treatment had no overall effect on disease-specific mortality and disease progression (40% of patients died of thyroid carcinoma), but a subgroup of patients receiving RAI adjuvant therapy had lower disease-specific mortality rates and a longer time to disease progression compared with patients who either did not receive treatment or had only RAI remnant ablation. Similarly, Besic et al. [25] reported RAI uptake in 16 of 30 patients (53%) who had distant metastases, and Pryma et al. [33] evaluating 33 patients with HCC reported a sensitivity of 50% for the diagnostic RAI whole-body scan (DxWBS), increasing to 72% after evaluation of TxWBS.

At a nationwide level, Jillard et al. [34], evaluating 1162 (60.9%) out of 1909 patients with HCC who received RAI, demonstrated an improvement of 5- and 10-year survival rates and a 30% reduction in mortality compared with untreated patients. On the contrary, among 22 patients with elevated thyroglobulin (Tg) levels after total thyroidectomy for HCC, Yen et al. [35] identified only 4 (18%) patients with positive DxWBS, confirming the poor RAI uptake and retention in HCC. At Mayo, DxWBS (either TSH-stimulated or in hypothyroid state) was performed in 40 patients with metastases, and RAI uptake was seen in only 9 cases (22.5%) [36].

In conclusion, our view is that the RAI therapy by using high RAI activities (5.5–7.4 GBq) could be an effective treatment option in patients with aggressive variants of thyroid carcinoma and should be considered at least once in all patients, especially in light of the low efficacy dearth of other effective systemic therapies.

11.3.2 FDG PET/CT

In the last years, FDG PET/CT has emerged as important tools for the management of patients affected by thyroid carcinoma, widely used in selected clinical situations, such as in patients with increasing Tg levels and negative DxWBS/TxWBS; in the follow-up of high-risk patients with aggressive histological subtypes; in the identification of patients who are at the highest risk of disease-specific mortality; in the management of patients with RAI-R disease; and in clinical trials of novel targeted therapies [37].

The fundamentals for the use of FDG PET/CT in oncology lies in the accelerated rate of glucose metabolism mediated by the overexpression of key regulatory glycolytic enzymes and transporters observed in malignant transformed cells (Fig.11.1). However, in thyroid tumors, an

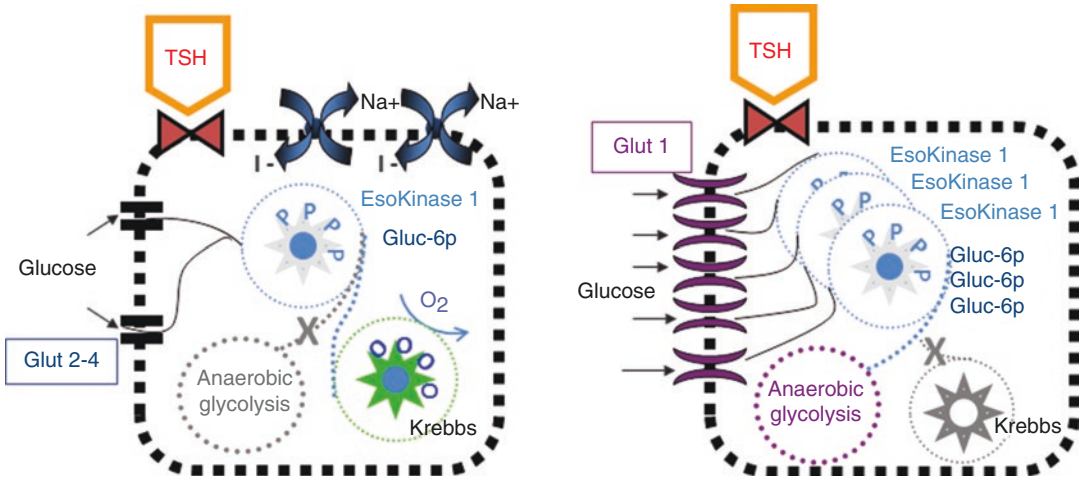

Fig. 11.1 Molecular basis of glucose uptake in normal thyroid cells (*left*) and in thyroid tumor cells (*right*) (Modified from [38])

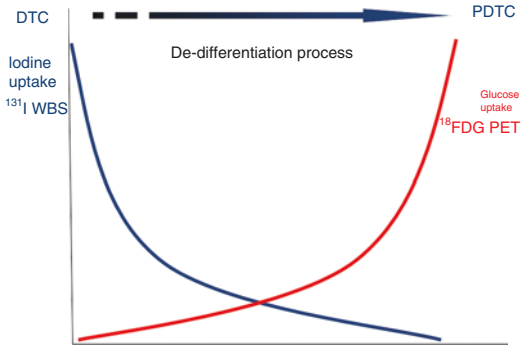

Fig. 11.2 Molecular basis of the flip-flop phenomenon in dedifferentiated thyroid carcinoma. Progressive reduction of iodine uptake at WBS with simultaneous increasing of glucose uptake at FDG PET/CT (Modified from Bongiovanni M et al., Clin Transl Imaging (2013) 1:149-161)

increased uptake of FDG is restricted to more aggressive and high-grade tumors, and no or low uptake can occur in WDTC [37]. In 1996, Feine et al. [38] reported an inverse relationship between RAI and FDG uptake in thyroid carcinoma (the so-called flip-flop phenomenon), which was thought to be the result of a loss in RAI concentration capacity during dedifferentiation combined with an increased demand of tumor cells for glucose (Figs.11.2 and 11.3).

Few reports evaluated the role of FDG PET/CT in specific subgroups of aggressive variants of thyroid carcinomas [39] and, as for the use of RAI therapy, most studies regard patients with HCC [33,40,41].

The largest study to date was the one conducted by Pryma et al. [33]. They enrolled 44 patients with HTC who underwent FDG PET/CT at the initial postoperative staging, and they found that this technique had an excellent diagnostic accuracy with sensitivity and specificity of 95.8% and 95%, respectively. The authors reported that FDG PET/CT improved the diagnostic information obtained with CT alone and RAI DxWBS and provided important prognostic information. The authors reported a 5-year overall survival in patients with SUVmax <10 and SUVmax >10 of 92% and 64%, respectively ($p < 0.01$).

Plotkin et al. [40] evaluated 17 HCC patients submitted to FDG PET/CT and concluded that the method is highly sensitive in the diagnosis of recurrent HCC. In the same study, the authors performed a meta-analysis including 35 patients of two previous studies reporting a sensitivity of 92%, a specificity of 80%, a positive predictive value of 92%, a negative predictive value of 80%, and an accuracy of 89%.

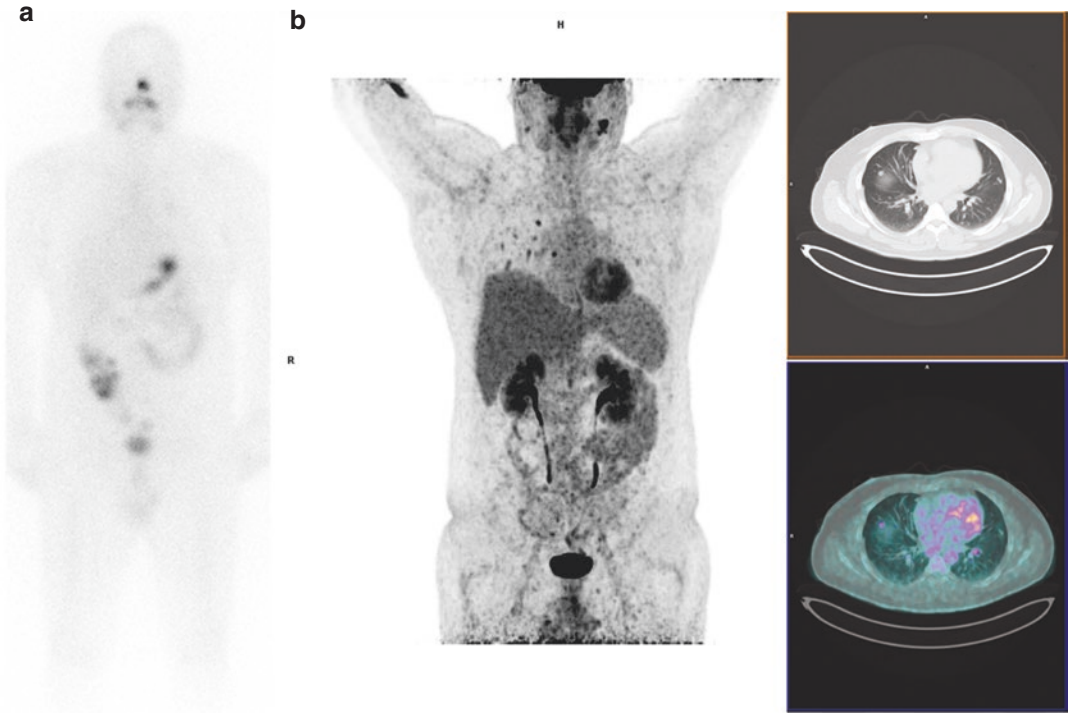

Fig. 11.3 Radioiodine anterior whole-body scan (A) and FDG PET/CT scan (B) in a 45-year-old male patient submitted to total thyroidectomy and radioiodine therapy for lung metastases from columnar variant of papillary thyroid carcinoma. Note the absence of radioiodine pulmonary uptake and lung bilateral foci of FDG uptake

Lowe et al. [41] reported an FDG PET/CT sensitivity of 92% in 14 patients with HCC; PET findings were positive in all but one patient with known disease, and in seven patients, FDG PET/CT scans alone diagnosed disease that was not identified by other techniques. FDG PET/CT provided additional information on disease extent that led to a change in patient care for 50% (7/14) of the scans obtained. The change in care was due to better characterization of indeterminate abnormalities on CT or sonography for three scans and to detection of unsuspected disease for four scans.

ATC usually do not concentrate significant amounts of RAI, so several studies have evaluated the role of FDG PET/CT in this histologic subtype of thyroid carcinoma to provide information at the initial diagnosis about the extent of the disease and prognostic information. Several studies observed that FDG PET/CT was the reference imaging modality for ATC both in the initial staging and in evaluation of the response to treatment, because it was more effective than RAI and other imaging technics, such as CT alone, in detecting cervical and mediastinal lymph nodes, in the early assessment of tumor volume, and in influencing the clinical management. These studies conclude that FDG PET/CT could improve disease staging and thereby potentially change the clinical management of patients with ATC [42–44].

Poisson et al. [42] evaluated the value of FDG PET/CT for initial staging, prognostic assessment, therapeutic monitoring, and follow-up in 20 consecutive ATC patients. They reported that FDG PET/CT was more effective than CT and bone scintigraphy in detecting cervical and mediastinal lymph nodes and bone metastases, respectively. Besides, the volume and the intensity of FDG uptake were significant prognostic factors for survival and the FDG PET/CT was also more efficient in the early assessment of

tumor volume and in the response to treatment than CT was. The authors concluded that FDG PET/CT appears to be the reference imaging modality for ATC at initial staging and seems promising in the early evaluation of treatment response and follow-up. These results can be explained by the high percentage and intensity of GLUT1 expression found in ATC by Grabellus et al. [43]. Bogsrud et al. [44] investigated the role of FDG PET/CT in the management of 16 patients with ATC by comparing PET data with other diagnostic tools and with clinical follow-up. In all of the patients, PET records were true positive for primary tumors, and in 50% of patients PET data influenced the clinical management. These authors concluded that FDG PET/CT could improve disease staging and thereby potentially change the clinical management of patients with ATC.

11.3.3 Surgery

Concomitant surgical and radiation-based therapy should be considered as the standard intervention for aggressive variants of thyroid carcinoma, regardless of tumor size. Although the current ATA guidelines do not specifically address the extent of thyroidectomy for patients with aggressive variants, when the diagnosis is advanced by preoperative FNA, the initial surgical procedure should include a near-total or total thyroidectomy and gross removal of all primary tumors [5].

In case of diagnosis of aggressive variant obtained by preoperative cytology, prophylactic central-compartment neck dissection (ipsilateral or bilateral) should be considered in patients with clinically uninvolved central neck lymph nodes (cN0) or clinically involved lateral neck nodes (cN1b). Therapeutic lateral neck compartmental lymph node dissection should be performed for patients with biopsy-proven metastatic lateral cervical lymphadenopathy [5].

The high propensity for lymph node involvement in DSV suggests that adjuvant radioiodine therapy may be considered in all cases. Initial radical surgery followed by radioiodine treatment and a long-term follow-up are the common management strategies for DSV [10, 20].

The typical therapeutic approach for ITC consists of total thyroidectomy and lymph node dissection whenever feasible, followed by RAI ablation and potentially additional therapy for residual or recurrent disease even though the role of adjuvant therapies such as EBRT and chemotherapy remain poorly established [18, 21].

In rare cases of patients with ATC, multimodality treatment consisting of surgery when feasible combined with EBRT and chemotherapy is generally recommended. The surgery can permit to achieve a good local disease control and to confer short-term palliative and survival benefit, but it must not compromise the functional anatomy of the cervical structures [26, 27].

11.3.4 Directed Therapies

In patients with RAI-R disease in whom the treatments imposed by the specific situation are more localized, directed approaches may have greater potential to control localized disease and symptoms compared to systemic therapies [5].

Several local treatment modalities other than surgery may be used to treat brain, lung, liver, and bone lesions from thyroid carcinoma. Interventional radiology (thermal ablation and cement injections) and intensity-modulated radiation therapy (IMRT) are the most frequently used techniques. In selected patients, these techniques may be an alternative to surgery as first-line treatment, and they may induce local tumor control with a similar efficacy to surgical resection [5].

Percutaneous thermal ablation is aimed at destroying tumor foci by increasing (radiofrequency ablation) or decreasing (cryoablation) temperatures sufficiently to induce irreversible cellular damages. Cryoablation is a safe technique to treat or to stabilize bone lesions; it is frequently associated with cementoplasty to consolidate purely lytic bone metastases from thyroid cancer and rapidly to control long-lasting pain [45, 46].

It remains unknown whether IMRT might reduce the risk for recurrence in the neck following adequate primary surgery and/or RAI treatment in patients with aggressive histologic subtypes. Although there are reports of responses among patients with locally advanced disease and improved relapse-free and cause-specific survival in patients over age 60 with ETE but no gross residual disease, the 2015 ATA management guidelines for thyroid cancer suggest there is no role for routine adjuvant IMRT to the neck in patients with DTC after initial complete surgical removal of the tumor [5].

However, adjuvant IMRT may be considered to obtain a good local control or to confer short-term palliative and survival benefit in the context of certain individual patients, such as patients with positive surgical margins, gross incomplete surgical resection, or disease that invade the vital local structures in the neck [5].

11.3.5 Systemic Therapies

Systemic therapies in selected clinical contexts appear to provide clinical benefit in treating metastatic RAI-R patients, including improved progression-free survival or induced durable tumor regression [5, 26, 47]. However, randomized clinical trial data are not yet available to address the effects of systemic therapies of various types on survival and quality of life or to address critical issues of optimal patient selection/inclusion/exclusion criteria for therapy and duration of treatment. Besides to date, no clinical trial has demonstrated an overall survival advantage or improved quality of life from the use of any therapy in RAI-refractory DTC [5].

Tyrosine kinase inhibitors (TKIs), many of which share the common target of the VEGF receptor (VEGFR), have recently emerged as highly promising therapies for metastatic RAI-refractory thyroid carcinomas [5]. The antitumoral activity of TKIs is related to their ability to block tyrosine kinase receptors (TKRs) by competing with ATP at its binding site, thus limiting angiogenesis and lymphangiogenesis [47]. At present, two different TKIs, lenvatinib (Lenvima®; Eisai Co Ltd., Tokyo, Japan) and sorafenib (Nexavar®; Bayer Health Pharmaceutical, Leverkusen, Germany), recently approved by both the US Food and Drug Administration and European Medicines Agency, can be used for the treatment of RAI-R DTCs [5].

However, TKIs have high probability of negatively impacting quality of life and/or necessitating dosage reductions in many patients and treatment discontinuation in up to 20% of patients. Some patients treated with TKIs can develop the so-called escape phenomenon, likely due to the development of a drug resistance determined by both the activation or upregulation of alternative pro-angiogenic signaling pathways and the selective pressures of the microenvironment during malignant progression [5, 47]. Patients who progress through first-line TKI therapy commonly respond to a second similarly targeted agent, and thus they should be considered candidates for second-line TKI therapy. Besides, TKIs are associated with numerous adverse effects, including fatal risks such as therapy-related death (~1.5–2%) [5].

Unfortunately, TKIs appear to be less effective in controlling bone metastases in comparison to pulmonary and lymph nodes metastases. Hence, TKIs cannot be relied upon to control diffuse bone metastases in many patients with RAI-refractory DTC because progression of bone metastases commonly occurs despite maintained benefit with respect to disease at other metastatic sites. Bisphosphonate therapy should be considered in such patients, either alone or concomitantly with other systemic therapies [48, 49]. Adequate renal function and calcium level should be documented prior to each therapeutic dose, and dental evaluation should take place before initial use [48, 49].

Although no randomized trial data conclusively demonstrated that survival is prolonged or quality of life improved in ATC patients in response to treatment with cytotoxic chemotherapy, anecdotal and nonrandomized studies support its use in selected ATC patients and perhaps also in some patients with progressive RAI-R metastatic disease from PDTC unresponsive to TKIs. Several drugs and treatments have been tried in such patients, but to date, doxorubicin

remains the single most effective and approved cytotoxic chemotherapy for the treatment of these patients, and all the others have poor response rates with no long-term survival [27].

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12 Circulating Mucins and Cytokeratins in Aggressive Thyroid Cancers

Luca Giovanella, Federica D'Aurizio, and Renato Tozzoli

12.1 Introduction

Follicular cell-derived thyroid carcinomas constitute a biological *continuum* progressing from the highly curable DTC to the much more aggressive, and almost always fatal, undifferentiated, or anaplastic thyroid carcinoma (ATC) [1]. Poorly differentiated thyroid carcinoma (PDTC) and aggressive variants of DTC, such as tall cell and columnar cell, frequently serve as intermediates in this progression model of dedifferentiation [2]. In fact, the gradual loss of typical papillary and follicular growth patterns and the simultaneous appearance of a solid growth pattern, with increased mitoses, necrosis, and nuclear pleomorphism, are frequently observed in aggressive thyroid carcinomas. Otherwise, residual foci of differentiated carcinoma may be frequently detected in aggressive tumor forms [3]. While radioactive iodine (I-131) is an effective treatment in thyroid cancers exhibiting a differentiated phenotype, there is a large body of information demonstrating that patients whose metastases concentrate ^{131}I have a higher survival rate and thus a better prognosis than patients with ^{131}I-refractory metastases [4]. Dedifferentiation of thyroid cancer may consist of loss of expression of the TSH receptor, natrium-iodide symporter (NIS), and loss of thyroglobulin (Tg) production and radioiodine cannot be longer employed for monitoring and treatment. In turn, this subset of tumors frequently shows avid ^{18}F-fluorodeoxyglucose (FDG) uptake in positron-emission tomography/computed tomography scans (FDG-PET/CT) [5]. These iodine-negative/FDG-positive tumors have a poor survival, and, consequently, alternative treatment options are required which may include observation, additional surgery, external beam radiation, interventional radiology (i.e., radio-frequency ablation), or systemic treatments [6].

Chemotherapy has shown limited success at best, while tyrosine kinase inhibitors (TKIs) have been introduced and tested in recent clinical trials. The DECISION trial using sorafenib showed a significant improvement in progression-free survival (PFS) of 10.8 months (vs. 5.8 months in the placebo group) [7]. In the SELECT trial, lenvatinib could demonstrate significantly increased PFS in patients with progressive radioiodine-refractory DTC. In comparison to sorafenib,

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lenvatinib even represented the most active agent with a better tumor response rate and an improved PFS of 18.3 months [8].

Based on these results, both drugs have been approved by the FDA for the treatment of locally recurrent or metastatic, progressive DTC that no longer responds to radioactive iodine treatment.

In order to assess effectiveness of TKI treatment, morphologic tumor measurement based on computed tomography is routinely used to monitor patients. Both Tg biosynthesis and secretion are partially retained in dedifferentiating DTC cells. However, the synthesis and secretion rate is reduced (i.e., poor Tg secretors) in comparison to normal thyrocytes and well-differentiated cancer cells. Consequently, a large dedifferentiated tumor mass could be associated to low levels of circulating Tg, and, consequently, the role of serum Tg is rather limited in this scenario.

In a recent study on a small cohort of iodine-refractory patients with progressive disease undergoing treatment with lenvatinib, serum Tg fluctuations were frequently detected but do not necessarily reflect morphologic tumor alterations, especially shortly after lenvatinib dose reductions. However, whereas patients with controlled disease presented with oscillating tumor markers after an initial nadir without morphologic tumor progression, patients with true progression demonstrated a continuous rise in serum Tg [9]. Other authors also reported a decrease of Tg levels in most patients receiving TKIs, but neither baseline Tg nor Tg changes consistently correlated with the degree or duration of objective response [10].

A progressive dedifferentiation may be also observed in medullary thyroid carcinoma (MTC) cells, translating in a more aggressive tumor behavior especially in some of the patients with locally advanced or metastatic disease. As discussed in other sections of the present book, serum calcitonin (CT) and carcinoembryonic antigen (CEA) levels are related to tumor burden, though production of these markers may differ between tumors (i.e., tumors with low expression of calcitonin and higher production of CEA may be more aggressive) [11]. Nowadays, tyrosine kinase inhibitors (TKIs), especially vandetanib, have been recommended as first-line therapy in the case of aggressive metastatic MTC patients based on phase II and phase III trials in MTC patients that reported higher objective response rates compared with cytotoxic chemotherapy [12].

Indeed, no strict correlation has yet been reported between early changes in CT and CEA levels, and response to TKI and even paradoxical increase in biomarkers was observed in responders [13].

Overall, currently available results suggest that the mechanisms leading to tumor control and tumor marker Tg, CT, and CEA secretion are likely dissociated in the setting of TKI administration in patients with advanced radioiodine-refractory DTC and advanced MTC as well. As previously remarked, changes in serum Tg, CT, and CEA should always be confirmed by imaging in the setting of TKI treatment of advanced DTC and MTC, respectively. However, conventional imaging criteria (i.e., RECIST) may also have their own limitations when determining the effects of TKIs on tumor volume [14]. Therefore, new circulating biomarkers are warranted to help identify patients most likely to benefit from these therapies. Recently, among a series of candidate tumor markers, carbohydrate antigen 19-9 (CA 19-9) and cytokeratin fragments 19 (Cyfra 21.1) emerged as potentially useful prognostic predictors in both advanced DTC (CA 19-9 and Cyfra 21.1) and MTC (CA 19-9), respectively. Biology and physiopathology, assay methods and laboratory pitfalls, current clinical data, and potential applications of such tumor markers will be addressed in following sections.

12.2 Carbohydrate Antigen 19-9 (CA 19-9)

Mucins (MUCs) are heavily glycosylated, high molecular weight glycoproteins with an aberrant expression profile in various malignancies. So far 19 mucin genes have been described; eight of them are now well-characterized (i.e., *MUC1-4*, *MUC5B*, *MUC5AC*, *MUC6*, and *MUC7. MUC8, MUC9, MUC11,* and *MUC12*

have also been partially sequenced, but their characterization is yet to be completed, *MUC13* was identified as a cell surface mucin expressed by epithelial cells as well as hematopoietic cells, and *MUC16* was characterized from a partial cDNA sequence encoding a mucin that has long been known as the ovarian cancer marker CA125) [15]. Mucins are synthesized either as membrane-bound or as secreted glycoproteins by epithelial cells in the lungs, stomach, intestines, eyes, and several other organs. Under normal circumstances, they line the apical surface of epithelial cells and protect the body from infection by pathogen binding to oligosaccharides in the extracellular domain, preventing the pathogen from reaching the cell surface [16]. In addition, their involvement in the renewal and differentiation of the epithelium, modulation of cell adhesion, as well as cell signaling has also been proposed. Of relevance, alterations in the expression and in the structure of mucins have been reported in both preneoplastic and neoplastic lesions [17]. Mucin 1, cell surface-associated (MUC1) or polymorphic epithelial mucin (PEM), is a mucin encoded by the *MUC1* gene in humans. MUC1 is extensively O-linked glycosylated in its extracellular domain. MUC1 is overexpressed in colon, breast, ovarian, lung, and pancreatic cancer cells, and its associated glycans are shorter than those of nontumor-associated MUC1 [18]. Indeed, mucins were proved to be involved in dedifferentiation of tumor tissues and to promote resistance to treatments (Fig. 12.1).

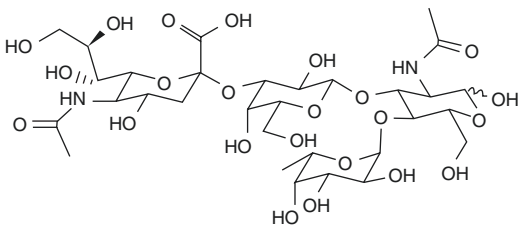

Fig. 12.1 Expression and release of CA 19-9 in cancer progression. Galli C et al. CA 19-9: handle with care Clin Chem Lab Med 2013;51(7):1369–1383 (permission obtained)

In fact, the heavy glycosylation in the extracellular domain of MUC1 creates a highly hydrophilic region which prevents the drugs from reaching their targets and allows cancer cells which produce a large amount of MUC1 to concentrate growth factors near their receptors, increasing receptor activity and the growth of cancer cells. MUC1 cytoplasmic tail has been also shown to bind p53 and to increase expression of Bcl-xL preventing both p53- and mitochondrial cytochrome c-mediated apoptosis. Additionally, MUC1 may prevent the interaction of immune cells with receptors on the cancer cell surface through steric hindrance. Finally, increased expression of MUC1 promotes cancer cell invasion through beta-catenin, resulting in the initiation of epithelial-mesenchymal transition which promotes the formation of metastases. The most commonly used clinical tests for mucins as tumor markers are serum-based immunoassays for blood group-related antigens and glycoproteins like CA 125, CA 15-3, CA 242, and CA 19-9. The CA 19-9 or carbohydrate antigen sialyl-Lewis is a frequently used tumor marker for cancers of the digestive tract. Although the maximum tissue expression of CA 19-9 occurs in pancreatic cancer, this antigen is not tissue specific, because it has been demonstrated in cancers involving other organs, such as the stomach, lung, colon, breast, ovary, and uterus [19]. Increase of this antigen expression in tissues and blood depends on the tumor-related hypoxia that, in turn, induces the transcription of several glycogenes involved in sialyl-Lewis a synthesis and is associated with a greater probability of the patient developing hematogenous metastasis. It has recently been reported that there is another form of the molecule, named *disialyl-Lewis a*, that is predominantly expressed in nonmalignant epithelial cells, connected to two sialic acid molecules. This molecule normally helps to maintain immune homeostasis of the gastrointestinal mucosa. In the early stages of carcinogenesis, inhibition of the sialyl-transferase gene causes a partial synthesis because of the incomplete connection of the second residue of sialic acid and the resulting accumulation of monosialyl Lewis

in cancer cells [20]. Although it was characterized almost three decades ago, according to the current international guidelines, based on available evidence, CA 19-9 is still the most commonly used serum tumor marker for the monitoring of pancreatic cancer: the levels should fall when the tumor is treated, and they may rise again if the disease recurs [21]. Thus far, the lack of specificity does not support its use for the diagnosis of the early forms of pancreatic cancer [20, 22], and its potential applications for the differential diagnosis are still debated. In fact, its sensitivity (70–80%) and specificity (68–91%) are not considered sufficient [21, 23]. Positive findings may occur in several situations other than malignant neoplasia such as patients with inflammatory processes (chronic and acute pancreatitis, cholangitis, and liver cirrhosis), characterized by high concentrations of CA 19-9 [24], which decrease to normal values after appropriate treatment. In these cases, the positivity is common for almost all methods. The increases of CA 19-9 in patients with benign disease are quite unavoidable, and the only current analytical approach to better define the nature of such elevations could be the assessment of the relationship between *sialyl-Lewis a* and *disialyl-Lewis* a, which is not elevated in malignant disease [25]. In addition, the finding of elevated CA 19-9 levels in healthy subjects for whom this test should not be ordered may have important implications from a psychological point of view with the requirement of many diagnostic tests to be performed [26, 27].

The dependence of tissue expression and circulating levels of *sialyl-Lewis a* on the Lewis blood group influences the sensitivity of testing for CA 19-9. In fact, false-negative results will always be found in subjects with *Lewis a*-negative genotype, representing 5–10% of the Caucasian population, whereas no data on other races are available [21]. Recently, it has been reported that low or medium (higher than 100 kU/L) levels of CA 19-9 may be found in some patients with *Lewis a*-negative genotype and suffering from advanced pancreatic cancer [28]. It was probably due to the situation of homozygosity for the secretory gene and overproduction of glycan precursors [29].

12.3 Measurement of Circulating CA 19-9

A commercial assay for CA 19-9 was developed in 1983 [30]. Radioimmunoassays were first used for the determination of CA 19-9 in the blood and other biological fluids, but they were quickly replaced by immunoassays (IMAs) [20] (Table 12.1).

Almost all of the IMAs for the quantitative detection of CA 19-9 utilize a sandwich assay format and depend on the use of the monoclonal antibody 1116-NS-19-9, named Centocor, that recognizes a sialylated lacto-N-fucopentaose II epitope occurring on the mucin, and it is related to the Lewis a blood group [20, 31] (Fig. 12.2).

The original hybridoma secreting the monoclonal antibody 1116-NS-19-9 was developed by immunizing mice with the SW1116 human cancer cells. The minimal structure recognized by this antibody is the terminal tetrasaccharide of the CA 19-9 antigen. Removal of the fucose residue or the sialic acid moiety cancels or decreases the antigen-antibody interaction [32, 33].

The interpretation of CA 19-9 results is often altered by nonspecific elevations both in diseased and healthy subjects, either because of associate morbidity (see above) or IMA interference, leading to misdiagnosis and further unnecessary and expensive examinations [33, 34].

In particular, similarly to other IMAs, also for the CA 19-9, different studies in the literature have reported cases of interference due to rheumatoid factor and heterophilic antibodies. The latter were responsible for 44.4% of the discrepancies observed between two automated IMAs for CA 19-9 as reported by Passerini et al. in a recent paper [33, 35, 36].

Rheumatoid factor and heterophilic antibodies are endogenous autoantibodies found in serum/plasma, mainly of IgM class, that can bind to immunoglobulins (preferably IgG) of other species. Thus, they usually affect the "sandwich" assay by bridging the capture and detection antibodies causing an increased signal and consequently a falsely elevated measured concentration [37]. A nonlinear response to dilution is suggestive of antibody interference. However, it must be

Table 12.1 Analytical performance characteristics of the main current CA-19.9 automated immunoassays (Manufacturers' data)

Manufacturer	Analyzer	Methodology	Assay principle/tracer	Monoclonal antibodies	Imprecision (CV): intra-; inter-; total (%)	LoD (kU/L)	Assay range (kU/L)	Cutoff (kU/L)	High-dose Hook effect (kU/L)
Abbott Diagnostics	ARCHITECT	CMIA	Noncompetitive; heterogeneous/ acridinium esters	1116-NS-19-9	2.3–8.0; nd; 3.4–8.5	2.0	0–1200	37	> 1,750,000
Beckman Coulter	Access	CLIA	Noncompetitive; heterogeneous/ Lumi-Phos 530	Anti-CA 19-9 antibodies	1.7–6.4; 2.4–5.7; 3.0–8.9	0.8	0.8–2000	35	> 800,000
bioMerieux	VIDAS 3	ELFA	Noncompetitive; heterogeneous/4MUP	1116-NS-19-9	2.2–2.9; 2.5–4.2; nd	3	3–500	37	> 1000,000
Thermo Fisher Scientific BRAHMS	Kryptor	FEIA (TRACE)	Noncompetitive; homogeneous/ europium cryptate-XL665	1116-NS-19-9	1.4–3.0; 4.8–5.9; nd	1.2	1.2–700	37	> 600,000
Diasorin	Liaison	CLIA	Noncompetitive; heterogeneous/ isoluminol derivatives	1116-NS-19-9	1.9–10.0; 4.9–7.5; nd	0.3	0.3–1000	37	> 1,550,000
Fujirebio	Lumipulse G	CLEIA	Noncompetitive; heterogeneous/ AMPPD	1116-NS-19-9	0.8–1.2; nd; 1.5–3.4	0.894	2–500	37	nd
Ortho Clinical Diagnostics	Vitros	CLIA	Noncompetitive; heterogeneous/ luminol derivatives	1116-NS-19-9	0.8–1.4; nd; 2.6–5.7	1.4	1.4–1000	37	> 965,000
Roche Diagnostics	Cobas/Elecsys	ECLIA	Noncompetitive; heterogeneous/ ruthenium derivatives	1116-NS-19-9	1.2–4.4; 1.9–8.0; nd	0.6	0.6–1000	34	> 500,000
Siemens Healthineers	Advia Centaur	CLIA	Noncompetitive; heterogeneous/ acridinium esters	1116-NS-19-9	3.4–10.4; 2.4–5.3; 4.3–11.7	1.2	1.2–700	37	> 5,800,000
Siemens Healthineers	Advia Dimension Vista	CLIA (LOCI)	Noncompetitive; homogeneous/ Phthalocyanine-olefin	1116-NS-19-9	1.1–5.8; 2.7–8.9; nd	2.0	2–1000	37	> 1000,000
Siemens Healthineers	Immulite XPi	CLIA	Noncompetitive; heterogeneous/ adamantyl dioxetane phosphate	Anti-CA 19-9 antibodies	4.2–7.5; 5.5–6.9; nd	1.0	1–1000	18.4	> 50,000
Snibe	Maglumi	CLIA	Noncompetitive; heterogeneous/ ABEI	Anti-CA 19-9 antibodies	4.76–6.04; 8.52–9.64; nd	1.0	1–1000	37	> 10,000

(continued)

Table 12.1 (continued)

Manufacturer	Analyzer	Methodology	Assay principle/tracer	Monoclonal antibodies	Imprecision (CV): intra-; inter-; total (%)	LoD (kU/L)	Assay range (kU/L)	Cutoff (kU/L)	High-dose Hook effect (kU/L)
Tosoh Bioscience	AIA	FEIA	Noncompetitive; heterogeneous/4MUP	Anti-CA 19-9 antibodies	2.3–2.8; nd; 3.9–4.4	1.0	1–400	37	> 200,000
Tosoh Bioscience	AIA CL2400	CLEIA	Noncompetitive; heterogeneous/ DIFURAT®	Anti-CA 19-9 antibodies	3.6–4.5; 3.9–5.1; nd	0.17	0.5–1500	34.4[a] 37.7[b]	nd

4MUP 4-methyl-umbelliferyl phosphate, *ABEI* N-(aminobutil)-N-(ethyl)-isoluminol, *CLIA* chemiluminescence immunoassay, *AMPPD* alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, *CLEIA* chemiluminescence enzyme immunoassay, *CMIA* chemiluminescent microparticle immunoassay, *DIFURAT®* 3-(5-tert-Butyl-4,4-dimethyl-2,6,7-trioxabicyclo[3.2.0]hept-1-yl) phenylphosphate disodium salt, *ECLIA* electrochemiluminescence immunoassay, *ELFA* enzyme-linked fluorescence assay, *FEIA* fluorescence enzyme immunoassay, *LOCI* luminescent oxygen channeling immunoassay technology, *LoD* limit of detection, *nd* not declared

[a]Defined in Asian population

[b]Defined in Caucasian population

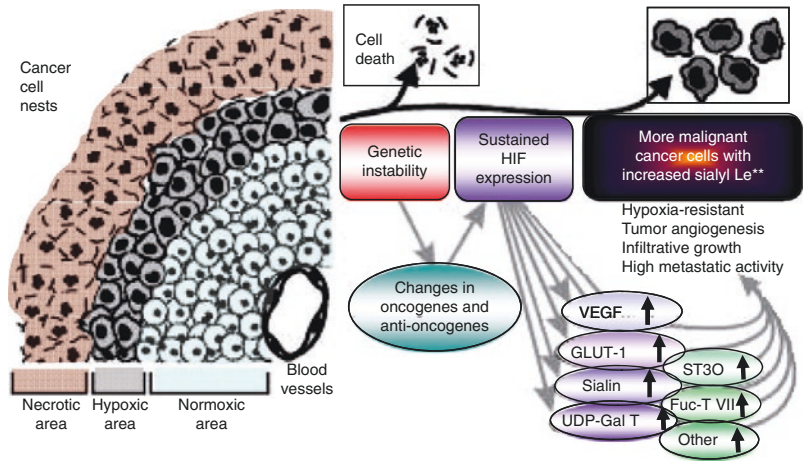

Fig. 12.2 Chemical structure of the sialyl-Lewis *a* determinant. Galli C et al. CA 19-9: handle with care Clin Chem Lab Med 2013; 51(7): 1369–1383 (permission obtained). *FUC-T-VII* fucosyltransferase VII, *GLUT-1* glucose transporter, *HIF* hypoxia-inducible factor, *Sialin sialic* acid transporter, *ST3O* sialyl-transferase, *UDP-GALT* UDP galactose transporter, *VEFG* vascular endothelial growth factor

noted that a nonlinear response can also result from the hook effect or cross-reactivity. Moreover, nonlinear dilution, with increased recovery of the antigen, is common in IMAs for mucins. This is probably due to a variety of factors (i.e., the inherent property of mucins to aggregate and disaggregate into a range of molecules species, the presence of anti-carbohydrates, and of other not well-known matrix-related effects, etc.) [32]. Therefore, it has been suggested that nonlinear response to dilution may not be an appropriate method to detect interference [32, 38].

All in all, the laboratory should be aware of IMA interference and should apply a systematic approach in the investigation of such phenomenon: first, the repetition of the analysis to confirm the result and then, if possible, the use of an alternative IMA to inquire about the discrepancy. Of course, the close interaction between the laboratory and the physicians is essential [36, 39].

An additional important limitation of CA 19-9 determination is the particular sensitivity of the assay to viral and bacterial neuraminidases resulting in false negatives. Thus, samples should be carefully prepared to avoid bacterial contamination [32].

The interpretation of CA 19-9 results is also made difficult by the inter-method discrepancy showing significant CA 19-9 disparities in the same individual samples. In fact, the studies performed in the last decade focusing on this issue reported similar data and reached analogous conclusions: the recent automation of IMAs has certainly improved the assay imprecision but has also helped to impair the concordance between the results obtained with different methods [40–46].

Another relevant aspect related to the CA 19-9 analytical specificity and the recurrent inter-method discrepancy is the concept of threshold/cutoff. Undoubtedly, the cutoff for CA 19-9 depends on the background: no values can be suggested to strictly distinguish between benign and malignant disease. Moreover, the upper reference limit is usually established at the 95th or 97.5th percentile in a healthy reference sample, not representing the "real-life" sample that will be tested for this biomarker. Finally, it would be important considering ethnic diversities in the reference population [41, 42]. All in all, the authors concluded their papers on this issue suggesting the use of the same method for the monitoring of patients with cancer and inviting laboratories to indicate in the report the name of the method employed [20]. Taken into consideration the aforementioned aspects, the scientific societies and the manufacturers should work together in order to improve CA 19-9 harmonization, making available an international reference material and following existing programs for method assessment and correction of bias, as was the case with other IMAs. In fact, the differences between methods are attributable to numerous variables involved in IMAs such as assay technology, reaction kinetics, incubation times,

dilution, and, overall, the use of different antibodies [20, 44]. With regard to this last point, Partyka et al. [47] have recently demonstrated the improvement of cancer detection by using the antibody with broader specificity beyond the sialyl-Lewis a antigen, suggesting that the additional glycans were also elevated in a cancer-specific manner. All in all, Partyka's analysis was useful for understanding the factors that may further improve upon the CA 19-9 assay, suggesting that the use of different antibodies can lead to a better sensitivity in patients with malignant neoplasia without an elevation of reactivity in non-malignant disease.

12.4 CA 19-9 and Thyroid Cancer

The natural history of medullary thyroid cancer (MTC) varies from rapid progression and survival over a few years to a very slow progression, or stable disease, that extends for decades. Overall, the prognosis of MTC patients is related to the extent of the disease at the time of diagnosis (i.e., median 5-year survival rate is 50% in the presence of distant metastases). As discussed in other section of the present book, calcitonin and procalcitonin are very sensitive and specific serum marker for the diagnosis of MTC, and carcinoembryonic antigen (CEA) is also employed while monitoring MTC patients. In 2011, Milman and colleagues [48] described the case of a 56-year-old woman with multiple endocrine neoplasia 2B syndrome presented with extensive metastatic spread of MTC to the lungs and liver, 47 years after the original diagnosis. The patient's calcitonin level decreased from 2950 to 261 pg/mL over a 20-year period. The serum CEA level was elevated at 6800 ng/mL; serum CA 19-9 and CA 125 tumor markers were also measured and found to be significantly elevated, at 39,334 U/mL and 96.2 U/mL, respectively. Immunostaining of the metastatic MTC tissue showed patchy staining for calcitonin, strongly positive staining for CEA and CA 19-9, and weakly positive staining for CA 125. Basing on this picture, they postulated that high serum levels of CA 19-9 could be considered a marker of MTC dedifferentiation and disease aggressiveness. Two years later, Elisei and colleagues [49] reported the peculiar case of a young patient with MEN 2A who rapidly died from aggressive MTC 10 months after initial diagnosis. Her CA 19-9 increased up to >10,000 IU/mL, and immunohistochemistry of the thyroid nodule was performed at the autopsy and demonstrated positive staining for CT and CA 19-9 in the primary tumor. Then, the same group measured CA 19-9 in 100 advanced structural recurrent/persistent MTC patients and in 100 MTC patients cured or with biochemical but structural disease [50]. Sixteen percent of patients with advanced diseases had high CA 19-9 and concomitantly higher levels of CEA and CT compared with the group with normal CA 19-9 levels. None of patients with controlled disease had high CA 19-9 levels; moreover, among patients with advanced disease those with high CA 19-9 levels showed a higher mortality rate than patients with normal CA 19-9 serum levels. Overall, these results demonstrated that increased CA 19-9 levels in serum is an adverse prognostic factor in patients with advanced MTC and identifies those cases with a higher risk of short-term mortality. Recently, Milman and colleagues [51] evaluated whether positive CA 19-9 staining of primary MTC tissue predicts metastatic potential. Among specimens from 16 patients, 63% stained positive for CA 19-9; indeed, all specimens from patients with advanced (i.e., stage IV) MTC stained positive for CA 19-9, compared to only 40% of cases with stages I to III. Importantly, 100% of the primary specimen with associated metastatic spread over time stained positive for CA 19-9. As a consequence, a negative CA 19-9 staining excludes a stage IV MTC with a 100% negative predictive value.

Similarly, serum CA 19-9 levels were reported to be elevated in some cases of anaplastic thyroid carcinoma and in papillary thyroid carcinomas with poor differentiated features, aggressive tumor behavior, and a worse prognosis [52].

12.5 Cytokeratin Fragment 19 (Cyfra 21.1)

The cytoskeleton of eukaryotic cells is responsible for the mechanical integrity of the cell and is a critical participant in several cellular

processes, such as cell division, motility, and cell/cell contact. It is composed of three different types of distinct filamentous structures: microfilaments, intermediate filaments (IF), and microtubules [53].

The IF protein family includes several hundred of different members. In turn, these are classified basing on structural similarities. Intermediate filament types I and II constitute the cytokeratins (acidic and basic proteins, respectively). The type III group includes desmin, vimentin, and glial fibrillary acidic proteins; type IV includes the neurofilament proteins (NF-L, NF-M, and NF-H) and internexin, while type V proteins are known as nuclear lamins, exclusive to the cell nuclei. The remaining IF proteins, sometimes called type VI, include filensin and phakinin [54].

The expression of cytokeratins varies with epithelial cell type, extent of differentiation, and development of the tissue. During the transformation of normal cells into malignant cells, the cytokeratin patterns are usually maintained, and this property has enabled cytokeratins to be applied as tumor markers [55, 56]. In the cytoskeleton, cytokeratins demonstrate poor solubility, but when present in the circulation, cytokeratins are detected either as partially degraded single protein fragments, as small complexes, or as large polymeric protein complexes, while intact, nondegraded, cytokeratins have not yet been detected in the bloodstream. The release of soluble cytokeratin fragments into the circulation involves multiple pathways including proteolytic degradation of cytokeratins in dying cells, abnormal mitosis, spillover of monomeric cytokeratin polypeptides from proliferating cells, apoptosis, and neoangiogenesis. Upon release from the tumor cells, cytokeratins can be detected in blood as well as in other body fluids. In normal, apparently healthy individuals, the level of cytokeratins in the circulation is low. However, levels rise significantly in patients with epithelial cell-associated carcinomas. Stratified squamous epithelia express mostly cytokeratins 1–6 and 9–17, while cytokeratins 7, 8, and 18–20 are identified in simple epithelia. Of the latter, cytokeratins 8, 18, and 19 are the most abundant ones in malignancy [57]. The most widely applied cytokeratin tests use the monoclonal-based assay tissue polypeptide antigen (TPA), cytokeratin fragment 19 (Cyfra 21.1), and tissue polypeptide-specific antigen (TPS). TPA (tissue polypeptide antigen) measures cytokeratins 8, 18, and 19 in serum samples [58] and is an example of a broad-spectrum cytokeratin assay demonstrating high sensitivity in cancer patients with various epithelial cell-associated carcinomas such as breast cancer, colorectal cancer, lung cancer, head and neck cancer, and bladder cancer [59–63].

The TPS is a specific cytokeratin-based assay, which detects a defined epitope structure on human cytokeratin 18 using the M3 monoclonal antibody. It was evaluated and proposed in various epithelial cell-associated carcinomas such as breast cancer, ovarian cancer, prostate cancer, and gastrointestinal cancer [64–66]. Finally, an assay measuring soluble cytokeratin 19 fragments in the circulation, Cyfra 21.1, exemplifies a monospecific cytokeratin assay [67]. Unlike the majority of epitopes, detectable by useful tumor markers such as CEA, CA 15-3, and CA 19-9, which are glycoproteins, Cyfra 21.1 is unique in the fact that its epitope is a polypeptide, probably released as a result of cell death [68].

Most reports in the literature have focused on the clinical use of Cyfra 21.1 in lung cancer and in head and neck cancer [69–71]. Although based on detection of the same type of proteins in serum, the individual cytokeratin immunoassays may give different profiles of reactivity likely due to the different detector antibodies employed and the different release of cytokeratin fragments into the circulation from one cytokeratin to another. All in all, as with most tumor markers, the cytokeratin assays are not interchangeable.

12.6 Measurement of Circulating Cyfra 21.1

Five decades ago, for the first time, two IMAs for the measurement of Cyfra 21.1 were introduced: a two-site sandwich immunoenzymometric assay (IEMA) and a two-site sandwich immunoradiometric assay (IRMA), respectively [68, 72].

These methods used two mouse monoclonal antibodies (KS 19-1 and BM 19-21) directed against two different epitopes of a fragment of cytokeratin 19, which is referred to as serum Cyfra 21.1. The target sites of the two monoclonal antibodies lie within amino acids 346–367 for BM 19.21 and 311–335 for KS 19.1: both epitopes are located in C-terminal helical domain of the molecule. These monoclonal antibodies were obtained by immunization against the MCF-7, a breast cancer cell line. Afterward, a large number of other automated methods have been developed, including monoplex immunoassays, electrochemiluminescence immunoassay (ECLIA) [73–78], chemiluminescent microparticle immunoassay (CMIA) [79, 80], heterogeneous chemiluminescent immunoassay (CLIA) [81], chemiluminescent enzyme immunoassay (CLEIA) [45, 82, 83], and luminescent proximity oxygen channeling immunoassay (LOCI) [84], and multiplex immunoassays, addressed laser bead immunoassay (ALBIA) [85] and lateral flow immunoassay (LFIA) [86]. All these recent automated methods are based on the same principle of the first IMAs; in particular, they are heterogeneous and noncompetitive, "two-step" sandwich, automated or automatable, and characterized by the use of two monoclonal antibodies: the first with acceptor function (KS 19.1), prevalently coated of a solid phase (paramagnetic microparticles, iron beads, streptavidin-coated microparticles, magnetic microbeads coated with anti-FITC, beads coated with fluorophores, nitrocellulose membranes, etc.) and the second (BM 19.21), with tracer function, coated to fluorophores (europium cryptate, phycoerythrin) or luminescent molecules (acridinium esters, alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, ruthenium derivatives, N-(aminobutil)-N-(ethyl)-isoluminol, phthalocyanine-olefin, etc.) or enzymes (alkaline phosphatase, etc.). In addition, these methods show good analytical performances in terms of sensitivity (the LoD is in general very low, ranging from 0.01 to 0.20 μg/L), specificity (no critical pre-analytical phases, no interferences with other analytes, Hook effect at very high concentrations, etc.), precision (intra-assay <3.0% and inter-assay <6.0%), and accuracy (good correlation between different methods). For the aforementioned reasons, the upper reference limits are quite similar between methods, ranging from 1.5 to 5.4 μg/L (Table 12.2).

12.7 Cyfra 21.1 and Thyroid Cancer

The cytokeratin 19 (CK19) is an acidic protein that is part of the cytoskeleton of epithelial cells. Tissue CK19 is highly expressed in DTC, mainly those with papillary histotype (PTC) [87]. Increased preoperative Cyfra 21.1 levels were found in patients with localized aggressive histotypes of primary epithelial thyroid cancers, while they are usually normal in patients with primary and metastatic classical DTC histotypes [88]. More recently, it was demonstrated that patients with ^{131}I-refractory DTC metastases had significantly higher serum Cyfra 21.1 levels than patients with ^{131}I-avid ones. Such differences argue that ^{131}I-refractory thyroid cancer cells (i.e., dedifferentiated cells) are likely the source of the increased serum Cyfra 21.1 [89]. No data are currently available on the relationship between serum and tissue Cyfra 21.1 expression in DTC; however, increased serum Cyfra 21.1 levels were previously reported in patients with primary aggressive thyroid carcinomas despite low or absent CK19 immunostaining in corresponding tumor tissues [90]. Previous studies in human lung and liver cancer cell lines showed that among CK19-producing cells, only those with caspase-3 (an enzyme involved in apoptosis phenomena) expression induced high Cyfra 21.1 levels in culture supernatants [91–93]. Indeed, serum caspase-3 enzyme activity is detectable in patients with metastatic^{131}I-refractory thyroid cancer [94].

Globally, thyroid tumors with high proliferation rate, diffuse apoptosis, and necrosis are likely to release Cyfra 21.1 via caspase-3 action. The fast processing of CK19 molecules may explain the coexistence of a negative tissue CK19 staining with high levels of CK19-soluble fragments in serum of patients with such

Table 12.2 Analytical performance characteristics of the current Cyfra 21.1 automated immunoassays

• Manufacturer	Analyzer	Methodology	Assay principle/tracer	Monoclonal antibodies	Imprecision (CV): intra-; inter- (%)	LoD (μg/L)	Assay range (μg/L)	Cutoff (μg/L)	High-dose Hook effect (μg/L	Ref
Abbott Diagnostics	**ARCHITECT**	CMIA	Noncompetitive; heterogeneous/acridinium esters	BM 19.21 – KS 19.1	1.0–5.0; nd; 2.0–6.0[a]	0.09	0.5–100	2.1	750	Manufacturer data
Thermo Fisher Scientific BRAHMS	**Kryptor**	FEIA (TRACE)	Noncompetitive; homogeneous/europium cryptate and XL 665	BM 19.21 – KS 19.1	1.7–3.6; nd	0.16	0.16–350	3.3	> 4000	Manufacturer data
Fujirebio	**Lumipulse**	CLEIA	Noncompetitive; heterogeneous AMPPD	BM 19.21 – KS 19.1	0.6–2.1; 2.7–3.5	0.01	0.5–100	1.5	nd	Patel, 2010
Roche Diagnostics	**Cobas/Elecsys**	ECLIA	Noncompetitive; heterogeneous ruthenium derivatives	BM 19.21 – KS 19.1	1.1–2.1; 2.8–3.3	0.20	0.1–500	5.4	> 2000	Sanchez-Carbayo, 1999
Snibe	**Maglumi**	CLIA	Noncompetitive; heterogeneous ABEI	Anti-Cyfra 21.1 antibodies	5.4–6.1; 8.3–9.8	0.20	0–600	2.7	> 12,000	Lumachi, 2014
Perkin-Elmer	**Alphalisa**	CLIA (LOCI)	Noncompetitive; homogeneous Phthalocyanine-olefin	Anti-Cyfra 21.1 antibodies	3.4–9.0; 4.0–10.0	0.08	0–500	nd	nd	He, 2013
Luminex	**MAGPIX**	FEIA	Multiplex immunoassay Phycoerythrin	BM 19.21 – KS 19.1	3.1–8.1; 2.6–14.1	nd	nd	nd	nd	Doseeva, 2015
nd	**aQcare TRF**	LFIA	Multiplex immunoassay Quantum-dot	Anti-Cyfra 21.1 antibodies	5.0–9.0; 6.9–9.6	0.16	0–480	nd	> 480	Chen, 2017

4MUP 4-methyl-umbelliferyl phosphate, *ABEI* N-(aminobutil)-N-(ethyl)-isoluminol, *CLIA* chemiluminescence immunoassay, *AMPPD* alkaline phosphatase-spiroadamantyl-methoxy-phosphoryloxy-phenyl-dioxetane, *CLEIA* chemiluminescence enzyme immunoassay, *ECLIA* electrochemiluminescence immunoassay, *FEIA* fluorescence enzyme immunoassay, *LOCI* luminescent oxygen channeling immunoassay technology, *LFIA* later flow immunoassay, *LoD* limit of detection, *nd* not declared

[a]Total imprecision

aggressive thyroid tumors [89, 90, 95]. Vice versa, low proliferation rate and absent of apoptosis phenomena explain low serum levels of Cyfra 21.1 in patients with classical DTC [88, 90]. Interestingly, high Cyfra 21.1 levels were found in ^{131}I-refractory patients even after exclusion of those patients with primary aggressive thyroid carcinomas. This is in line with previous reported differences between primary differentiated thyroid carcinomas and their metastases at the genetic level, as the number of chromosomal abnormalities increases as thyroid carcinomas progress [96]. Then, although the majority of primary thyroid carcinomas leading to ^{131}I-refractory disease were aggressive follicular and papillary histotypes, primarily well-differentiated tumors may be also responsible for ^{131}I-resistance and increased Cyfra 21.1 levels. As previously remarked, serum Tg measurement and RECIST assessment have their own limitations when determining the effects of TKIs. Therefore, new circulating biomarkers are warranted to help identify patients most likely to benefit from these therapies. Even if prospective randomized studies will be designed to independently validate its predictive and/or prognostic, serum Cyfra 21.1 may serve as a marker for recurrent ^{131}I-refractory thyroid cancer and is an important potential monitoring tool for alternative treatment approaches.

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Part V

Thyroid Biomarkers on Fine-Needle Washouts

Measurement of Thyroid Tumor Markers on Fine-Needle Washouts

13

Pierpaolo Trimboli and Luca Giovanella

13.1 Introduction

Differentiated thyroid carcinomas (DTC), such as papillary (PTC) and follicular (FTC), produce thyroglobulin (Tg). Then, DTC patients can be followed up by periodic evaluation of serum Tg levels and examination by local and whole-body imaging [1]. A rate up to 50% of these patients has metastatic neck lymph nodes at their initial presentation and/or during postoperative follow-up [1]. While ultrasonography (US) can detect neck lesions suspicious for DTC metastases, fine-needle aspiration (FNA) under US guide is generally performed to prove the metastatic involvement of these lesions. This approach is essential to allow a tailored surgical excision [1]. Because FNA samples from neck lymph node may be not adequate, the measurement of Tg in the fluids from FNA (FNA-Tg) is essential in combination with cytology [2, 3]. Specifically, FNA-Tg achieves high relevance in those cases involving small and/or partially cystic lymph nodes.

Based on the experience on FNA-Tg, the determination of calcitonin (CT) in washout fluids (FNA-CT) from thyroid nodules suspected for medullary thyroid carcinoma (MTC) has been recently described [4]. This approach was based on the limits of conventional cytology in detecting MTC [5]. In fact, poor sensitivity of cytology (i.e., 55–65%) was recorded in single- and multicenter series [6–8], and FNA-CT can reduce false-negative and inconclusive cytologic results with a sensitivity near to 100% [4]. These data prompted the board of ATA guidelines to recommend the use of FNA-CT in patients suspected for MTC [9].

The treatment of choice of hyperfunctioning parathyroid (HP), such as adenomas, hyperplasia, or more rarely carcinomas, is represented by surgical removal. Then, their identification and localization are pivotal to better address the therapy. In this context, various potential limits of different imaging techniques (i.e., ultrasonography, scintigraphy, magnetic resonance) have been reported [10]. The determination of parathyroid hormone in FNA washout fluids (FNA-PTH) was proposed as an improving tool in localizing HP, with controversial results [11]. Generally, Tg, CT, and PTH are measured in blood, and their determination in fluids other than serum/plasma has been developed in the last years. Although studies have reported overall satisfactory results, a good standardization of procedures has not yet been reached, and further efforts should be made in order to better define pre-analytical, analytical, and post-analytical aspects.

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13.2 FNA-Tg in Lymph Nodes Suspicious for Metastases from Differentiated Thyroid Carcinoma

The measurement of FNA-Tg in cervical lymph nodes/masses suspected to be metastases from DTC was firstly described in 1992 by Pacini et al. who demonstrated that the sensitivity of FNA-Tg was significantly higher than that of cytology (85%) [2]. Also, no FNA-Tg false-positive results were recorded. Later, several studies confirmed similar results, and a meta-analysis of 24 studies including 2865 lymph nodes reported overall sensitivity of 95% and specificity of 94% for FNA-Tg, with significant heterogeneity [12].

Even if many articles with concordant findings have published, a better standardization of analytical methods and cutoff levels is required. The cutoff of FNA-Tg to discriminate metastatic lymph nodes from negative ones has been largely debated; in the first studies, the threshold values ranged from 0.9 to 50 ng/mL [12]. The metastases diagnosed in the presence of thyroid gland and those detected in athyreotic patients were analyzed, and different FNA-Tg cutoffs were proposed in the presence (36 ng/mL) or absence (1.7 ng/mL) of thyroid [13, 14]. However, two different papers found a unique accurate threshold of 0.93 ng/punction [15] and 28.5 ng/mL [16, 17]. In general, both sensitivity and specificity of FNA-Tg were higher in surgically treated DTC than in those waiting for surgery [18, 19]. In this context, the use of high-sensitive Tg assays should provide more accurate data. Snozek and colleagues [20] measured FNA-Tg by an immunochemiluminometric assay (ICMA) with functional sensitivity of 0.1 ng/mL; 96% of non-malignant samples had values ≤1 ng/mL, and 100% of metastatic lesions had levels >1 ng/mL. Furthermore, using a high-sensitive immunoradiometric assay (IRMA) with functional sensitivity 0.2 ng/mL, a cutoff of 1.1 ng/mL provided 100% sensitivity, specificity, and accuracy [20]. These results were confirmed by others [14, 21–23]. As an ancillary information, a potential improvement of previous rhTSH was reported in patients with positive AbTg [24, 25]. Table 13.1 details the findings of main studies.

Based on the above demonstrations, FNA-Tg is included in all international guidelines. The ATA guidelines [1] quoted at Recommendation 32 that "US-guided FNA of sonographically suspicious lymph nodes > 8-10 mm in the smallest diameter should be performed to confirm malignancy if this would change management (strong recommendation, moderate-quality evidence); the addition of FNA-Tg washout in the evaluation of suspicious cervical lymph nodes is appropriate in select patients, but interpretation may be difficult in patients with an intact thyroid gland (weak recommendation, low-quality evidence)." The ATA board suggests FNA-Tg in lymph nodes with cystic changes, inadequate cytology, or cytologic/echographic divergences. Also, ATA underlines that false-positive FNA-Tg may occur in evaluating lymph nodes of central compartment of patients with thyroid. Finally, ATA guidelines highlight the lack of standardization of FNA-Tg procedures or assays with consequent potential difficult in interpreting data [1]. The guidelines by AACE/AME/ETA stated that "In the presence of

Table 13.1 Accuracy of FNA-Tg reported in the literature

First author (year)	Lymph nodes	Method	Cutoff (ng/mL)	Sensitivity	Specificity
Pacini (1992)	23	IRMA	21.7	100	100
Cunha (2007)	18	CLIA	0.9	100	100
Giovanella (2009)	126	IRMA	1.1	100	100
Kim (2009)	91	IRMA	50	100	80
Bournaud (2010)	98	IRMA	0.93	92.3	97.8
Salmashoglu (2011)	255	IRMA	28.5	100	96.4
Snozek (2007)	122	CLIA	1.0	100	96.2
Moon (2013)	528	RIA	1.0	93.2	95.9

IRMA immunoradiometric assay, *CLIA* chemiluminometric immunoassay, *RIA* radioimmunoassay

suspicious cervical lymphadenopathy, FNA biopsy of both lymph node and suspicious nodule(s) is essential (Grade B)" [26]. These guidelines suggest to wash the needle in 1 ml of saline solution, do not indicate a specific cutoff, and underline that in athyreotic DTC patients "the detection of even low thyroglobulin levels by UGFNA should be considered suspicious for malignancy." Guidelines for neck US and US-guided techniques for the management of DTC patient after treatment were published by ETA [27]; there, ETA suggests to report the results of FNA-Tg as "ng/FNA" ("a more suitable result which reflects the quantity of Tg in the needle") and propose to adopt value <1 ng/FNA as normal, values between 1 and 10 ng/FNA to be compared with the results from cytology, and levels >10 ng/FNA as positive for the presence of tumor tissue. Also, at Recommendation 12, they quoted that FNA cytology and FNA-Tg should take into account the stage and histology of cancer, size and location of lymph node, and serum Tg level. The most recent guidelines by AACE/ACE/AME recommend FNA-Tg according to clinical indication [28]. All in all, based on the evidence of literature and according to current guidelines [1, 26–28], FNA-Tg has to be measured in cervical lymph nodes suspicious for metastases from DTC.

13.3 FNA-CT in Thyroid Nodules and Lymph Nodes of Patients with Suspicious Medullary Thyroid Carcinoma

The first studies on this topic were published in 2007 [29, 30]. There, 100% MTC lesions (nodules and lymph nodes) were correctly identified by FNA-CT, and only a minor rate had positive cytology. Initially, Boi et al. [29] proposed an "arbitrary" FNA-CT cutoff of 36 pg/mL (i.e., corresponding to three times the highest value found in non-medullary lesions). Later, a multicenter experience showed that among 34 patients with a primary MTC (i.e., thyroid nodule), 21 (62%) and 34 (100%) were detected at conventional cytology and FNA-CT, respectively [31]. In this paper a cutoff of 39.6 pg/mL was calculated for practice use. Another interesting prospective study [32] compared FNA-CT to basal and pentagastrin-stimulated calcitonin and cytology; the recorded sensitivities were 100% for FNA-CT (using a cutoff of 17 pg/mL), 93.7% for basal calcitonin, 87.5% for stimulated calcitonin, and 12.5% for cytology. Other studies confirmed these results [33–35]. Finally, only one paper searched a reference range for FNA-CT [36]; there, in a series of 78 non-medullary thyroid nodules, the 97.5th upper FNA-CT value was 8.50 pg/mL for saline and 7.43 pg/mL for buffer solution. Table 13.2 details the findings from the major studies.

The promising results obtained in these studies prompted ATA expert board to include that in the MTC guidelines [9]. In these guidelines, FNA-CT is suggested in both lymph nodes and thyroid nodules. Specifically, at Recommendation 19 (grade B), it is reported that "FNA findings that are inconclusive or suggestive of MTC should have calcitonin measured in the FNA washout fluid and immunohistochemical staining of the FNA sample to detect the presence of markers." However, what cutoff level for FNA-CT has to be adopted has not been reported. In addition, AACE/ACE/AME indicates that FNA-CT can be used in enlarged

Table 13.2 Accuracy of FNA-CT reported in the literature

First author (year)	Lesions[a]	Analytic Method	Cutoff (pg/mL)	Sensitivity	Specificity
Boi (2007)	36	CLIA	36	100	100
Kudo (2007)	14	NR	67	100	ND
Diazzi (2015)	60	CLIA	17	100	88.8
Trimboli (2014)	90	CLIA	39.6	100	100
De Crea (2014)	62	CLIA	10.4	89	100

CLIA chemiluminometric immunoassay, *NR* not reported
[a]Thyroid nodules/lymph nodes

lymph nodes of patients with MTC or in suspicious thyroid nodules of patients at risk for MTC or MEN2 syndrome [28].

A consideration for clinical practice should be addressed. The experience of the authors of this chapter suggests to measure serum CT in patients undergoing thyroid FNA and to use FNA-CT in those subjects with elevated serum CT levels. This selection of patients at risk for MTC allows the use of FNA-CT in the same FNA sample and, of high relevance in clinical practice, provides useful information to the cytopathologist [4]. As a potential limitation of this approach, those MTC with no secretion of serum CT have to be taken into account [37].

13.4 FNA-PTH in Lesions Suspected for Hyperplastic Parathyroids

As the first, Doppman et al. reported FNA-PTH in seven enlarged parathyroids [38]. Later, other papers showed the relevance of FNA-PTH to localize parathyroid adenomas with specificity from 75 to 100% and sensitivity from 70 to 100% [11, 38–43]. The accuracy of FNA-PTH was higher than that of cytology [15, 38, 43] and MIBI scintiscan [42–44]. No fixed cutoff has been reported, and no consensus on reference range and upper reference limit exists. In clinical practice, a FNA-PTH/serum PTH ratio ≥ 2 should be considered as positive for parathyroid adenoma (Table 13.3).

13.5 Considerations on FNA-Tg, FNA-CT, and FNA-PTH Testing

The measurement of FNA-Tg, FNA-CT, and FNA-PTH has been recently developed and largely worldwide diffused in the last years. The technique is easy to perform, without a dedicated needle: samples can be collected from FNA for cytology by washing out the needle, after dispensation of the specimen onto the appropriate slides. Despite the achievement of satisfactory results, the determination of thyroid and parathyroid markers in fluids other than blood poses today one of the major challenges to laboratory medicine due to the lack of international standards for the performance and interpretation of the technique. The main technical features and relevant problems are summarized in Table 13.4.

13.5.1 Pre-analytical Factors

The first issue to be addressed is the appropriate sampling: samples should be representative of the lesion in the lymph node or in the thyroid bed [45, 46]. However, unlike the FNA cytology, it is possible to make a diagnosis by using FNA markers even though no epithelial cells were aspirated, since Tg, CT, and PTH present high levels both inside and in the neighboring area of the lesion [47].

Second, when determining the concentration of a marker in fluids other than serum/

Table 13.3 Accuracy of FNA-PTH reported in the literature

First author (year)	Lesions	Method	Cutoff (pg/mL)	Sensitivity	Specificity
Sacks (1994)	45	IRMA	20	82	100
Kiblut (2004)	170	CLIA	1000	87	75
Conrad (2006)	66	ECLIA	1000	80	100
Kwak (2009)	18	IRMA	PTH-FNA > PTH-serum	92.9	100
Boi (2012)	43	CLIA	103	100	100
Kuzu (2016)	57	NR	PTH-FNA > PTH-serum	89	100

IRMA immunoradiometric assay, *CLIA* chemiluminometric immunoassay, *ECLIA* electrochemiluminometric immunoassay, *NR* not reported

Table 13.4 Measurements of FNA-Tg, FNA-CT, and FNA-PTH: technical features

	FNA-Tg	FNA-CT	FNA-PTH
Overall reliability	High	High	High
Solution to be used	Saline, 1 mL	Saline, 1 mL	Saline, PTH-free serum
Cutoff to be adopted	< 1 μg/L negative > 10 μg/L positive	< 10 ng/L negative > 36 ng/L positive	FNA/serum PTH ratio > 2
Potential false positives	Ectopic normal thyroid tissue	Unknown assay interferences	PTH truncated fragments
Potential false negatives	TgAb, hook effect	Hook effect	Hook effect
Reliability in the presence of inadequate FNA cytology	Unchanged	Unchanged	Unchanged
Pre-analytic factors	Laboratory specialists must be informed of the suspicious DTC Collection, preparation, and management of the sample	Laboratory specialists must be informed of the suspicious MTC Collection, preparation, and management of the sample	Laboratory specialists must be informed of the suspicious IPTH Collection, preparation, and management of the sample
Post-analytic factors	FNA-Tg concentration expressed as ng/FNA units or ng/mL, cutoff	FNA-CT concentration expressed as pg/FNA units or ng/mL, cutoff	FNA-PTH concentration expressed as pg/FNA units or ng/mL, cutoff
Time of work	1 day	1 day	1 day
Costs	Up to 15 €	Up to 20 €	Up to 15 €

plasma, we have to consider the so-called matrix effects that are changes of the medium in which the marker is measured and could represent confounding factors [43, 44]. However, despite the demonstration of the matrix effect in some studies, the most advanced IMA for serum markers (i.e., Tg, CT, and PTH) do not seem to be affected by this type of interference, obtaining comparable results with the use of saline, marker free-serum, and kit buffer [43, 45, 46]. Consequently, saline solution is widely employed in current practice.

Third, plain tubes should be employed preferentially as lithium-heparin tubes slightly reduced the FNA-Tg concentration when compared to plain tubes in one study [3]. Also, the volume of fluid used to wash the FNA needle ranges from 0.5 to 3.0 mL with 1.0 mL as most widely utilized one (Table 13.4).

Fourth, CT and PTH are poorly stable peptides requiring precautions for preservation (i.e., need to be kept on ice through the entire process) [42, 48]. Finally, the sample could require a pretreatment such as mixing and centrifugation in order to discard cellular debris coming from blood and tissue contamination [49].

All in all, variability in marker measurement in FNA washouts should be reduced by using saline solution in fixed volume (i.e., 1 mL) and a plain tube and using special pre-analytical precautions when measurement of unstable molecules (i.e., CT, PTH) is required. Figure 13.1 illustrates the initial preparation of the sample to be used for measurement of Tg, calcitonin, or PTH in washout from FNA.

13.5.2 Analytical Factors

Many analytical problems (i.e., "hook effect," immunoassay interference, and analytical variability) are similar when Tg/CT/PTH are measured in serum/plasma or different fluids, respectively. However, measuring thyroid/parathyroid markers in fluids other than blood is more problematic due to the lack of experimental data to support the validity of results and absence of formal support for this application by commercial manufacturers. Then, full analytical validation to regulatory standards by laboratories is required [4, 49]. Additionally, the possible influence of the Tg autoantibodies in the determina-

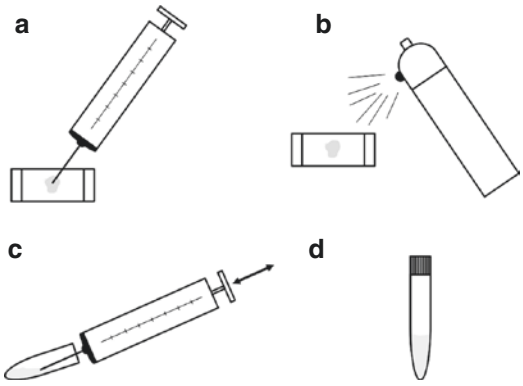

Fig. 13.1 Preparation of sample for the measurement of thyroglobulin, calcitonin, and PTH in fluids from FNA of neck lymph nodes, thyroid nodules, or suspicious parathyroids

tion of FNA-Tg was evaluated with inconclusive results; anyway the influence of TgAb on the clinical performance of FNA-Tg is limited, and Tg levels remained detectable in washouts from patients with malignant lesions [13, 14].

13.5.3 Post-analytical Factors

The marker measured in the FNA fluid is not the true concentration, but it reflects the dilution of the marker left in the needle in the arbitrary selected volume of the washout fluid. So, some authors and also the ETA guidelines suggest expressing Tg, CT, and PTH in ng/FNA units [24]. Nevertheless, several studies reported FNA marker in ng/mL, allowing for the comparison of the FNAB-marker and serum marker levels.

Moreover, in the interpretation of FNA marker levels, it is important to consider the clinical context of the patient such as pre-/post-thyroidectomy, histologic diagnosis, and serum TSH concentration [49].

Conclusion

Measuring endocrine biochemical markers in FNA washout fluids rapidly emerged as a powerful and relatively cheap tool to refine challenging clinical diagnosis in patients with thyroid/parathyroid tumors. In particular, both FNA-Tg and FNA-CT measurements are now included in current clinical guidelines. Nevertheless, we underline that results should be used in conjunction with information from the clinical evaluation of the patient and other diagnostic tools. A close cooperation between laboratory specialists and clinicians involved in thyroid/parathyroid diseases' care is mandatory to define the most appropriate pre-analytical procedures, to select accurate interpretation criteria, and to properly address cases with conflicting results. In our personal experience, the presence of laboratory specialist during FNA procedures was relevant, especially during the introduction of these techniques in daily clinical practice, to define an accurate work flow.

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Part VI

Neuroendocrine Tumors

Neuroendocrine Tumours: Diagnosis, Therapy and Follow-up

14

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14.1 Introduction

Neuroendocrine neoplasms (NENs) represent a clinically and pathologically heterogeneous group of tumours showing peculiar phenotypic characteristics and a common origin from cells of the diffuse neuroendocrine system [1–4]. NENs are commonly considered to be rare if compared to corresponding non-neuroendocrine neoplasms. Their frequency, however, has increased considerably in recent decades, as reported by epidemiological studies, such as the Surveillance, Epidemiology and End Results (SEER) registers in the USA, showing an increase of 1–5 new cases per 100,000/year [5]. In view of the more favourable prognosis of gastroenteropancreatic (GEP)-NEN than non-neuroendocrine neoplasms, the prevalence of GEP-NEN is 35 cases/100,000, shortly after colon adenocarcinoma and before all other gastroenteric adenocarcinomas [1].

Among thoracic NENs, pulmonary lesions represent 1–2% of all malignancies and 10–30% of all NENs [4]. The most common subtype is small cell lung cancer (SCLC), representing 20%, whereas large cell neuroendocrine carcinoma (LCNEC) represents 3%, atypical carcinoid (AC) 2% and typical carcinoid (TC) 1% [4].

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Major negative prognostic factors of NENs are site of the primary tumour (e.g. pancreatic have, generally, a worse prognosis than intestinal NENs), TNM stage and WHO histopathological classification with grading that is, to date, considered the strongest prognostic factor for NENs in general [6, 7]. Grading not only correlates significantly with overall survival (OS) but also with progression disease (PD) in patients with advanced pancreatic NEN (pNEN) and with tumour recurrence in patients undergoing pancreatic curative surgery [8–10]. Other prognostic factors are somatostatin receptors (sstrs) expression (which predicts a more favourable clinical behaviour than in case of negative lesions), tumour evolution and age of the patient.

Clinically, NENs are commonly divided into functional and non-functional tumours, according to the presence or not of a clinical syndrome related to the production and secretion of one or more hormones, as well as other bioactive molecules. Frequently, when the disease is asymptomatic, NENs are incidentally diagnosed, while when symptomatic, the most common symptoms are those related to the mass effect. The most frequent initial clinical symptom from single- or multicentric series, as well as from population-based data sources, is nonspecific abdominal pain (often assimilating irritable bowel syndrome) [11–15] which may be due to different reasons: dysmotility of the small bowel wall, small bowel obstruction and intermittent mesenteric ischaemia, caused by mesenteric root fibrosis, but also functional causes, such as secretory diarrhoea and bacterial overgrowth. Other symptoms are bleeding and jaundice. The carcinoid syndrome is seen in approximately 20–30% of patients with metastases, and this percentage is higher than previously indicated. Moreover, carcinoid syndrome is usually seen in 95% of all patients with liver metastases. Airways obstruction, cough, dyspnoea, haemoptysis are observed more frequently in thoracic primary, while a carcinoid syndrome can be observed in 1–5% of thoracic NENs, and this is related to the release of active peptides, such as serotonin [16].

Thymic NENs are very rare, with an incidence of <1/100,000/year [17], representing 2–5% of thymic and 2% of all mediastinal neoplasms [18]. They show an association rate with multiple endocrine neoplasia type 1 (MEN1) that is higher than pulmonary NENs. Males are more commonly affected by this tumour than females, with a 3:1 ratio [19, 20]. Thymic NENs show a more aggressive biological behaviour compared to abdominal neuroendocrine lesions. Therefore, these cases are more frequently diagnosed at a more advanced stage than the other NENs [21]. As many other NENs, thymic ones are often associated with endocrine disorders, such as Cushing's syndrome and MEN1- and MEN2-related syndromes [22]. The association between thymic NENs and myasthenia gravis is rare, and, differently from GEP-NENs, carcinoid syndrome is exceptional [23, 24].

14.2 Diagnosis

For the biochemical diagnostic markers, see the specific chapter. The functioning pNENs occur typically as nodular, hypervascular lesions, frequently smaller than 2 cm, while those non-functioning are often larger than 2 cm, capsulated and with intense and heterogeneous signals after contrast-enhanced imaging; sometimes they may show cystic-like features [11].

The ileal NENs are frequently multiple nodules, presenting as enhancing submucosal mass in distal ileum. The mesenteric extension of the tumour appears with discrete soft tissue mass, with calcification (up to 70% of cases) and demoplastic reaction due to mesenteric fibrosis. Tumour may show spiculation with a stellate pattern, with "fingerlike" projections of mass into adjacent mesentery [11].

At computed tomography (CT) scans, the pulmonary carcinoid tumours appear as nodular masses less than 5 cm, well circumscribed, usually associated to a perihilar tumour. In most cases the carcinoid has central location while rarely arise in peripheral pulmonary sites [25]. The LCNEC presents radiological features very similar to those of non-small cell lung cancer (NSCLC), and it is, therefore, difficult to distinguish them only on the basis of

morphological imaging. The LCNEC often is peripheral, while, in a minority of cases, has a central location, with pulmonary atelectasis [26]. Its margins are usually well defined, often displaying lobulation. However, there are also nodules with irregular margins (spiculated), cavitation, air bronchogram, as well as central necrosis [26]. The contrast enhancement is not characteristic of this type of tumour. The SCLC has central location, and the diagnosis is made almost always when the disease is at an advanced stage.

Thymic NENs are well-circumscribed mass, localized in the anterior mediastin, and it is very difficult to distinguish them from thymomas. The diagnosis of a thymic NEN occurs frequently at an advanced stage, and a magnetic resonance imaging (MRI) is critical to adequately define the staging, as well as to allow a possible surgical approach with curative intent [25].

14.2.1 Conventional Imaging

Morphological imaging plays an important role in diagnosis and staging of NENs, both thoracic (T-NENs) and GEPs [27]. The type of imaging and the acquisition technique should be individualized, based on the specific diagnostic question and the clinical presentation [27]. Transabdominal ultrasonography is a non-invasive method, spread and operator dependent, that may be used in patients with low body mass index (BMI) for the assessment of parenchymal organs. Its sensitivity can be reduced (13–27%) in the definition of pancreatic lesions due to the presence of intestinal meteorism [28]. This method, associated with the use of intravenous contrast, is called contrast-enhanced ultrasound (CEUS) and significantly increases diagnostic accuracy. A recent study on the identification of solid pancreatic lesions has highlighted a similar sensitivity between CEUS and CT (83% and 95%, respectively), while transabdominal ultrasound was confirmed of lower sensitivity (approximately 44%) [29]. CEUS can also be considered in patients with allergy to iodine agents or in those with impairment of renal function.

NENs generally appear as well-defined nodular hypervascular lesions, resembling encapsulated neoplasms with heterogeneous enhancement after administration of contrast agents [30], although sometimes they may look like cystic lesions [31]. Multislice CT (MSCT), as well as MRI of chest and abdomen, is the most useful exam to diagnose primary tumour and staging NEN disease [32, 33]. Both require the intravenous injection of a contrast agent and a three-phase approach (early arterial, late arterial and portal phases), critical for proper identification and characterization of the lesions. Recent studies have shown similar sensitivity between CT and MRI (69–94% for CT vs. 74–94% for MRI). MRI is better than the MSCT in detecting and studying small lesions and in the definition of primary pancreatic NENs [34, 35]. Sequences by diffusion-weighted imaging (DWI) increase diagnostic accuracy, especially in the identification of non-hypervascular lesions [36], while contrast-enhanced MRI is considered the first choice to study bone metastases and central nervous system when the MSCT is not diagnostic or contraindicated. CT and MRI enterography allow studying the small intestine after the distension of the intestinal loops. At this purpose, non-absorbable, iso-osmolar polyethylene glycol (PEG) solutions and hyperosmolar solutions (mannitol, sorbitol) are commonly used, because these agents prevent water absorption during transit through the small intestine. The enteric contrast agent can be orally administered or injected through a nasal jejunal feeding tube (enteroclysis). Enterography is faster, simpler and well tolerated than enteroclysis, although this latter one allows a greater relaxation of the jejunum and of the proximal ileum. Although some data indicate a similar diagnostic accuracy, there are no studies comparing the two different methods, supporting the superiority of one of these two approaches [37]. Several papers on the role of enteroclysis CT and MRI in evaluating small intestinal diseases, including NENs, demonstrate a high sensitivity (100% and 85%, respectively) and specificity (96% and 98%, respectively) in the detection of these neoplasms [38, 39]. Compared to CT, MRI presents an intrinsic

contrast within soft tissues, and, due to the absence of ionizing radiation, it can be used to study young patients at risk of NENs, such as those bearing MEN mutations. However, MRI is less widespread than CT, takes longer time and is more susceptible of artefacts from patients' movement [40, 41]. In case of suspected small bowel NEN, CT or MRI enterography are the gold standard for the detection and characterization of the lesions.

To study colorectal NENs, virtual colonoscopy (VC) is a non-invasive accurate imaging technique, well tolerated, probably better than barium enema [42], displaying a sensitivity comparable to conventional colonoscopy (CC) in the detection of colorectal cancer, as well as polyps [43, 44]. VC can be considered in case of incomplete CC or as an alternative to colonoscopy in the study of elderly patients and in general for those refusing the traditional exam [44].

In case of thymic NENs, combined neck ultrasound and CT is useful to assess the vascular extension of the disease. As an alternative, mediastinal MRI enables the assessment of the local extension of the disease, and, due to its high contrast resolution at the level of soft tissues, it may depict the infiltration of the adjacent structures [26].

14.2.2 Study of Liver Metastases

It is well known that the liver is frequently involved by metastatic NENs. Indeed, metastases are present at diagnosis in about half of patients. Liver metastases may have widespread pattern, and sometimes the diagnosis can be difficult with any method due to the small size of the lesions [44]. The role of transabdominal ultrasound to diagnose liver metastasis is controversial. Currently, there are only few studies that have evaluated the role of transabdominal ultrasound in liver patients with metastatic NENs, showing a variable sensitivity (14–88%) and specificity (92–100%) [27]. Conversely, MSCT and MRI have higher diagnostic accuracy. A multiphase contrast study is required due to the high vascularity of hepatic metastases from NENs. The high flow of contrast agent, as well as the high vascularization of the primary and secondary lesions, highlights the neoplastic areas in comparison with the normal tissue. The MSCT showed a sensitivity of 82–100% and a specificity of 83–100% [28]. The reported MRI diagnostic accuracy value is 80–85% [45], with a sensitivity of 55–79% and a specificity of 88–100% [46].

14.2.3 Endoscopy

In gastrointestinal (GI) NENs, endoscopy of the upper, as well as lower, digestive tract has a main role in detecting the primary tumour. For this purpose, to better display the region of the papilla of Vater, duodenoscopy should be performed in case of negative gastroscopy, whereas endoscopic evaluation of the terminal ileum should always be part of the pancolonoscopy [30].

Type 1 and 2 NENs develop in the gastric fundus, as well as body, and are usually small (<1 cm) and multiple lesions. Conversely, type 3 gastric NENs typically appear as isolated and polypoid lesions [47].

The duodenal NENs are generally located in the submucosal layer and appear rounded. In up to 70% of cases, the first and second duodenal portions represent the primitive site of gastrinomas. In these cases, endoscopic diagnosis may be difficult for the small size of the lesion (<1 cm), and echoendoscopy (EUS) can be of considerable aid [47].

Until a few years ago, the small bowel was considered difficult to explore by endoscopic techniques. However, the recent introduction of double-balloon (DBE), and even single-balloon (SBE) techniques, as well as the video-endoscopic capsule (VCE) allowed to virtually exploring the whole small bowel. DBE, SBE and VCE appear to be of particular clinical utility in the search for the primary lesion in cases of metastatic NENs with unknown origin. In particular, VCE showed a high diagnostic power (about 45%) in identifying and locating small intestine tumours [48]. DBE, in association with the histological sampling, has a good diagnostic accuracy in this subset of patients as well [49].

The EUS, using high ultrasound frequencies, can accurately visualize the layers of the GI wall, detecting 2–3 mm lesions, and precisely defining the invasion of NENs (T stage) and locoregional lymph nodes involvement (N stage). Finally, fine needle biopsy under EUS guidance can be performed for suspected adenopathies. For these reasons, EUS is recommended to evaluate the feasibility and safety of endoscopic resection of well-differentiated GI NEN, with low proliferative index, less than 1 cm diameter, confined to mucous and submucous layers in all the endoscopically accessible sites [50].

Flexible bronchoscopy is a fundamental method in the cytological characterization of lung NENs and is indicated in each patient with suspected central airway cancer [51]. Additional endoscopic diagnostic methods, such as endobronchial ultrasonography (EBUS) and transbronchial biopsy (TBNA), allow to obtain in a single procedure the histological and/or cytological definition and the staging of pulmonary neoplasms [51]. The most frequent endoscopic feature of TCs is a well-defined endoluminal lesion, with regular surface, sometimes polypoid and easily bleeding. ACs have intermediate endoscopic features between the typical one and the LCNEC. These lesions are frequently wider, with variable invasion of the bronchial wall and pulmonary parenchyma. LCNECs often appear as invasive airway necrotic lesions, with infiltration of the bronchial wall and, sometimes, of the adjacent mediastinal structures.

Tumours involving the central airway, such as endobronchial lesions detectable with standard bronchoscopy, can be histologically diagnosed, with high diagnostic accuracy, by biopsies [51]. The peribronchial central lesions, without the endoluminal component, can be diagnosed by the use of transbronchial needle aspiration, preferably under ultrasound guide (EBUS-TBNA). This latter approach has a high sensitivity and specificity, especially when used with sampling optimization methods, such as rapid on-site evaluation (ROSE), with the analysis of the adequacy of the sample, taken in the endoscopic chamber, by the cytopathologist, as well as with the use of a cell blocker for subsequent treatment as a histological withdrawal [52, 53]. The material obtained with EBUS-TBNA allows the immunocytochemical analysis with the scope of differentiate between the different types of lung NENs.

Peripheral lung lesions can be diagnosed endoscopically by transbronchial lung biopsies or, rarely, by TBNA. The diagnostic sensitivity of bronchoscopy in peripheral lesions varies depending on the morphology and size of the lesion.

14.3 Nuclear Medicine

Functional nuclear medicine techniques exploit the sstr expression (particularly subtype 2) of neuroendocrine tumour cells or the ability of these cells to metabolize ammine precursors. These techniques are essentially represented by the sstr scintigraphy with ^{111}In-pentetreotide (Octreoscan®) and by the more innovative positron emission tomography (PET) with ^{68}Ga-labelled analogues, or ^{18}F-DOPA, as well as PET using ^{11}C-5-hydroxytryptophan.

14.3.1 Functional Imaging

The specific goals of functional imaging in NENs are the localization of the primary tumour, the staging and characterization of the lesions in terms of sstr content, or the neuroamine metabolism and glucose consumption, as well as the restaging of the disease during and after treatments. An additional specific role of functional imaging is to select patients for, or even predict the response to, peptide radionuclide receptor therapy (PRRT) with radiolabelled somatostatin analogues. Scintigraphy or PET is currently considered standard of care in the management of patients with NEN [54, 55]. To date, scientific evidence indicates ^{68}Ga-PET the first choice, given to its superior diagnostic accuracy with respect to scintigraphy and single-photon emission computerized tomography (SPECT), even when combined with CT scan.

14.3.1.1 Octreoscan®

The rational for the use of sstr scintigraphy is the internalization of the labelled analogue/receptor complex and its retention in the cytoplasm. ^{111}In-pentetreotide has been the first approved radiopharmaceutical for imaging of NENs. It is important to remember that commonly used protocols (images taken 4 and 24 h and, if necessary, 48 h after injection) should include SPECT, preferably after 24 h, in order to have an adequate diagnostic sensitivity. In the whole-body scan, the physiological distribution of the radiopharmaceuticals in the spleen, liver and kidney, along with a variable visualization of pituitary, thyroid, bladder and intestine, is noted [56, 57]. Therefore, images should be interpreted in the light of clinical information, although in general, areas that show a higher than normal distribution in the healthy liver are classified as positive. However, in evaluating a scan, it is important to consider the false positive, which are mainly due to areas of flogosis (such as radiotherapy, a recent surgery or the presence of inflammatory bowel disease), physiological accumulation of radiopharmaceuticals in gallbladder, or skin contamination, frequently by the urine of the patient [56, 57]. Additionally, the possibility of a false negative may be related to an inappropriate application of the protocol (e.g. lower dose of the radiopharmaceutical or lack of SPECT analysis). However, more often it reflects the intrinsic resolution limit of the method, particularly if the lesions are 1 cm or less in size. Finally, among the causes of false negative, we should consider the lack of receptor overexpression, such occurs in poorly differentiated tumours or low-grade insulinomas [56, 57].

The sensitivity of Octreoscan® in GEP-NENs varies from 75% to 100% [58, 59]. Given their heterogeneity, NENs in general can be classified into tumours with high sensitivity (sensitivity of receptor scintigraphy >75%), such as pituitary adenomas, GEP-NENs, paragangliomas, SCLCs and tumours with intermediate sensitivity (between 40% and 75%), such as insulinoma, medullary thyroid carcinoma and pheochromocytoma [60].

14.3.1.2 PET with ^{68}Ga-peptides

To overcome the limits of spatial resolution of scintigraphic techniques, from the early 2000s, PET with somatostatin analogues labelled with ^{68}Ga (a positron emitter) was introduced. The three most commonly used analogues are DOTA-Tyr^3-octreotide (DOTA-TOC), DOTA-Tyr^3-octreotate (DOTA-TATE) and DOTA-Nal^3-octreotide (DOTA-NOC). These analogues retain an octreotide-like affinity profile and, in particular, a high affinity for sstr2. DOTA-NOC seems to display also a certain affinity for sstr3. Despite these differences in receptor affinity, a clear superiority of a compound compared to others has never been demonstrated in the clinical practice. The PET/CT with ^{68}Ga-DOTA-peptides offers several advantages compared to conventional scintigraphic techniques. Among these, especially the greater spatial resolution, which allows excellent picture quality, as well as the detection of lesions less than 1 cm, the rapidity of the examination, which takes place on a single day, and a more favourable bio-distribution of these PET tracers were compared with the scintigraphic ones (less liver and intestinal fixation). In addition, the possibility of a standardized semi-quantitative analysis of the capture areas using the standardized uptake value (SUV) parameter, which, for the same radiotracer used, allows an estimate of the change in capture areas over time and offers further advantages for better management of patients [61, 62]. These features have made PET/TC with ^{68}Ga-DOTA-peptides an exam that is increasingly used in experienced referral centres and certainly the first-choice method for studying well-differentiated NENs. As in the case of conventional scintigraphy, a normal examination shows the physiological visualization of liver, spleen, pituitary, kidney and urinary tract, as well as of adrenal glands. Moreover, minor uptake may be found in the thyroid and bowel [63]. Generally, clinical image interpretation is easier than the receptor scintigraphy, thanks to the better spatial resolution and the co-recording of CT images. Even in this case, areas showing a radiopeptide accumulation higher than that of normal liver are considered as positive and thus indicating the presence of a tumour lesion [63].

In the case of PET, it is necessary to mention that the interpretation of pancreatic findings requires caution, as this organ may exhibit a variable degree of physiological or para-physiological accumulation of ^{68}Ga-DOTA-peptides, both diffuse and focal, which can be found in 30–70% of cases, depending on the radiopharmaceutical used [64–66]. These findings must be correctly interpreted in the light of morphological investigations, since the pancreas and the duodenum are frequent sites of NENs. Other possible false positives are related to inflammatory phenomena with lymphoid infiltrate (such as actinic results), to the presence of small accessory spleens or urinary contamination.

In a group of 84 patients, 62 of whom with GEP-NENs, it has been demonstrated that PET with ^{68}Ga-DOTA-TOC has a greater sensitivity (97%) than CT (61%) and conventional scintigraphy with ^{111}In-pentetreotide (52%) for the detection of small lymph node or skeletal lesions or lesions in unusual locations, such as the breast, uterus and prostate [67]. Subsequent studies confirmed the high diagnostic accuracy of PET/CT with other DOTA-peptides and its impact on patient clinical management [61, 68].

14.3.1.3 Metabolic PET

There are alternative imaging modalities for NENs, such as PET with ^{18}F-DOPA, as well as with ^{18}FDG. PET with ^{18}F-DOPA has high sensitivity and accuracy for intestinal NENs (93% and 89%, respectively). In a cohort of 53 patients with carcinoid tumours, PET with ^{18}F-DOPA showed 100% sensitivity, detecting more lesions than the conventional CT and scintigraphy [69]. However, its physiological pancreatic distribution prevents the lesions from being studied at this site. Furthermore, given the inferiority of ^{18}F-DOPA compared to PET with ^{68}Ga-peptides in the study of NENs expressing sstrs, together with the technical difficulties related to its synthesis, its use is limited to those cases with poor/variable receptor expression (pheochromocytomas, medullary thyroid carcinoma) [69]. PET with ^{68}Ga-DOTA-peptides in some studies showed greater sensitivity than PET with "alternative" tracers [70, 71]. Moreover, compared to PET methods that trace the metabolism of the neuroendocrine cell, receptor systems also have the advantage to predict response to PRRT. PET with metabolic tracers is an unconventional test and should be considered only in selected cases when the receptor methods are negative.

14.3.1.4 PET with ^{18}FDG

Sensitivity of ^{18}FDG PET for NENs is generally low (58% in a recent prospective study including 96 patients) since well-differentiated neuroendocrine cells are usually characterized by a low glucose metabolism [72]. In contrast, G3 NENs exhibit high metabolic rate. Moreover, as with other solid tumours, even in NENs, FDG avidity is a prognostic factor: a study of 98 patients with NEN, enrolled after surgery and programmed for various therapies, showed that between ^{18}FDG, Ki67, chromogranin A (CgA) and the presence of liver metastases, the only parameter correlating with the prognosis was ^{18}FDG positive PET. In particular, it was shown that a PET SUV max > 9 and a high Ki67 index were predictors of OS, while a SUV max > 3 was the only predictor of progression-free survival [73].

The use of ^{18}FDG PET in well-differentiated NENs has a prognostic value and can identify less differentiated and, therefore, potentially less responsive lesions and more aggressive clinical behaviour of the tumours. It should be adopted, however, in particular cases, such as early progression in differentiated malignancies, with relatively high Ki67 index and with absent or low degree uptake on other functional imaging. To avoid unnecessary exams, this modality of PET should always be performed on the indication from a multidisciplinary team, including a nuclear medicine physician.

14.4 Therapy

In general, curative surgery should be always considered whenever possible, even in the presence of metastatic disease, including localized metastatic disease to the liver, if considered potentially resectable, and the patient does not

exhibit features that contraindicate surgery. In this chapter, however, we discuss only about medical treatments.

14.4.1 Somatostatin Receptor Ligands

Somatostatin receptor ligands (SRLs) are synthetic analogues of native somatostatin, because this neurohormone has a half-life of about 2–3 min and, therefore, cannot be used in a clinical setting.

Over 80% of NENs do express sstrs on the surface of the cell membrane, especially low-grade tumours [74]. SRLs represent the elective treatment of carcinoid syndrome, however are also indicated in functioning GEP-NENs associated with other paraneoplastic syndromes and in evolutive non-functional tumours [75–77]. In fact, preclinical data early indicated that SRLs also might have antiproliferative effect through direct activation of the surface-specific receptors and indirect antitumour effect, independent from the receptor, which can occur through the inhibition of growth factors, such as IGF-1 and EGF, or via anti-angiogenic effects, as well as modulation of the immune system [78–81].

First-generation SRLs are approved and in clinical use are octreotide and lanreotide. They have high affinity for two of the five known sstrs, particularly for type 2 and less for type 5, and are available in the rapid release formulations, as well as in the slow release, such as long-acting repeatable (LAR) octreotide or lanreotide autogel (ATG). Standard doses of short-acting octreotide range from 0.1 to 0.5 mg administered subcutaneously one to three times per day. These latter schedules are used mainly in case of refractory praneoplastic syndromes, in patients tacking slow release formulations, sometimes together with slow release in initial phase (induction/sensitization) and rarely as chronic therapy (e.g. insulinoma) [76]. About 40% of carcinoid syndromes are not fully controlled with the maximum dose of slow release SRLs. In these cases an increase in dose (high dose), a reduction in the intervals of administration (high frequency), or the addition of subcutaneous octreotide (rescue) can be considered [76, 77]. Side effects of SRLs are rare and include diarrhoea, bradycardia, hyperglycaemia and cholelithiasis.

14.4.1.1 Somatostatin Receptors Ligands in GEP-NENs

In general, SRLs improve clinical symptoms in over 60% of cases, stabilize tumour growth (SD) in about 60% and show partial tumour regression (PR) in rare cases (3–8%) [77]. There are extensive retrospective evidences, especially in GEP-NENs [82] and less frequently in lung lesions [83], about the stabilization of progressive tumours at baseline. Other data suggesting an impact on survival derive from single-centre retrospective studies on small intestinal NENs [84] or large epidemiological studies on NENs of various origins [5]. Only two randomized prospective studies evaluating the antiproliferative activity of the SRLs have been published so far, both versus placebo. The prospective, randomized, double-blind, phase III PROMID study compared treatment with octreotide LAR (30 mg every 4 weeks) vs. placebo in a population of naïve patients with neuroendocrine tumours (NETs) of the midgut (small intestine + proximal colon) [85]. Of the 90 patients included, 85 were randomized, 42 in the octreotide LAR arm and 43 in the placebo one. Both non-functioning and functioning tumours have been enrolled, with flushing or diarrhoea manageable without SRLs. Overall, 95% of the neoplasms had a Ki67 < 2%, and 74% was positive for scintigraphy with labelled octreotide. Baseline disease status (progression or stability) was unknown, and this represents one of the major limitations of the PROMID study. Octreotide LAR has more than doubled the time to progression (TTP), from 6.0 to 14.3 months, compared with placebo. At univariate and multivariate analysis of prognostic factors, the "liver tumour load" (<10 vs. >10%) was statistically significant [85]. This is the first randomized, prospective study that showed a statistically significant superiority of SRLs compared to the standard of care (placebo) in a GEP-NEN category. More recently, the randomized phase III CLARINET study confirmed and

extended the results of the PROMID trial. The CLARINET evaluated the antitumour effect of lanreotide ATG (120 mg/monthly vs. placebo) in patients with advanced, well- or moderately differentiated G1 and G2 (Ki67 < 10%) non-functioning NETs, not only from midgut but also from other gastrointestinal sites (pancreas and hindgut), as well as in NETs with unknown primary origin, with or without disease progression at the study entry [86]. Patients were randomized to receive lanreotide (101 patients) or placebo (103 patients). At randomization, 96% of patients had a stable disease (no tumour progression according to RECIST criteria in the 3–6 months before randomization), and 84% were untreated. Additionally, 32% of patients had a G2 tumour (Ki67 range 3–10%), and 33% had greater than 25% metastatic liver involvement. All patients had a positive octreotide scintigraphy. The study showed that, compared to placebo, lanreotide ATG was associated with prolonged progression-free survival (PFS). Indeed, the median PFS was not reached in the treatment arm vs. a median of 18 months in the placebo one ($p < 0.001$). Estimated PFS at 24 months in the lanreotide group was 61% (95% CI 54.0–74.1) vs. 33% (95% CI 23.0–43.3) in the placebo. Considering all patients, the hazard ratio (HR) for death and PD favoured lanreotide over placebo (HR 0.47, 95% CI 0.30–0.73), with a 53% risk reduction of PD or death. This advantage was maintained by dividing patients into subgroups, according to the primary tumour site, except for the smaller subgroup originating from the hindgut ($n = 14$), for which the confidence interval was too wide to draw definitive conclusions (HR = 1.47, 95% CI 0.16–13.34) [86]. Differently from PROMID, PFS according to hepatic tumour volume was consistent with overall population: HR 0.34, 95% CI 0.18–0.62 for patients with hepatic tumour volume < 25% ($n = 137$) and HR 0.45, 95% CI 0.23–0.88 for hepatic tumour volume > 25% ($n = 67$) [86]. Probably, the low number of patients with high liver burden enrolled in the PROMID can explain the apparent discrepancy. Similar to PROMID, CLARINET confirmed the efficacy of SRLs in midgut tumours and in NETs of unknown origin (often from the midgut). For pancreatic NETs the result was borderline significant (HR 0.58, 95% CI 0.32–1.04), maybe because of the early study termination [86]. However, we must consider that the subgroup population analysis was not designed for powered primary endpoint and was only a post hoc analysis. The primary endpoint was PFS in all NETs' population and not in a single subgroup. As stated before, the vast majority of patients enrolled in the CLARINET study had a stable disease, whereas in the PROMID trial there was no information on tumour progression before randomization. This could explain the significant difference in PD observed in the placebo group, almost twofold longer in the CLARINET compared to the PROMID study. As previously reported in PROMID, also in CLARINET OS did not differ significantly between the study groups [86]. This latter finding is consistent with the long-life expectancy of patients with G1 and low G2 NETs, although the crossover from the placebo to the lanreotide group, when PD occurred, complicates the analysis. Moreover, recent further information, published on the follow-up of patients from the PROMID trial, showed that octreotide had no impact on OS [87]. As far as quality of life and safety, in the CLARINET study, no treatment-related death was reported, and there were only few withdrawals due to adverse events (AEs). Moreover, AEs occurred in a similar proportion among patients of the two groups (88% lanreotide vs. 90% placebo) and were in line with the known safety profile of SRLs. Indeed, the most common one was diarrhoea (26% lanreotide vs. 9% placebo) [86]. At the end of the CLARINET core phase, eligible patients could enter a single-arm open-label extension (OLE) study, whose primary objective was to investigate the long-term safety of lanreotide ATG in this setting [88]. The secondary objective was to further investigate the efficacy of lanreotide, particularly to estimate the median PFS in patients originally randomized to receive lanreotide in the core study ($n = 101$) and the time to subsequent PD in patients switching to lanreotide after progressing on placebo in the core phase ($n = 32$). A total of 101 patients from the core were eligible to enter the OLE study.

However, among these, 88 were enrolled into the OLE: 41 had been receiving lanreotide and 47 placebo in the core phase. In most patients primary tumour originated from pancreas (38%) or midgut (39%) and 24% of patients had a hepatic tumour load >25%. The long-term safety profile and the tolerability of lanreotide ATG was favourable during median treatment duration of 40 months (continued lanreotide group; range: 26–74 months). Incidences of severe and serious AEs were similar between groups and across the two studies [86–88]. Most of the reported AEs in patients who received lanreotide in the core study have gradually improved with the increasing of treatment duration, particularly diarrhoea. Conversely, as expected, in patients switched from the placebo arm of the CLARINET, diarrhoea was higher in the OLE. The median PFS for patients randomized to lanreotide in the core, and that continued treatment in the OLE, was 32.8 months (95% CI 30.9–68.0). For patients from the placebo group, who progressed during the core study and then switched to OLE, median time from first to subsequent PD or death was 14 months (95% CI: 10.1, not calculable) [88]. The importance of the CLARINET results is to have strengthened and confirmed the rational use of long-acting SRLs as antitumour agents in patients with well-differentiated metastatic GEP-NETs. Indeed, compared to PROMID trial, CLARINET displayed the efficacy of SRLs not only in G1 midgut NETs with low hepatic tumour burden but also in low G2 NETs with a high hepatic tumour involvement. Moreover, although CLARINET failed to demonstrate a statistical significant benefit in particular subsets of patients according to the primary tumour site, for pNETs there was a clear significant trend of superiority in the treatment arm [86].

The use of SRLs as adjuvant therapy after radical resection of a localized or locally advanced GEP-NENs, to date, is not indicated because not sufficiently explored [75, 76, 89–91]. Also the use of SRLs after surgical (or radiological) "debulking" for metastatic GEP-NENs, again as adjuvant therapy, in patients with no evidence of disease and in asymptomatic patients is controversial. Unfortunately, no studies have been designed or conducted to specifically answer this question, and there is no data supporting this use so far [75, 76, 89–91].

14.4.1.2 Somatostatin Receptors Ligands in Thoracic NENs

Evidence of an antiproliferative effect of SRLs in thoracic NENs is mainly retrieved from retrospective studies on enteropancreatic NENs [82] or pulmonary carcinoids [83], showing significant percentages of SD as best response. There are basically no prospective studies with octreotide or lanreotide conducted exclusively in thoracic NENs. The only one is the multicentre three-arm, phase II, randomized LUNA study that compared pasireotide (a novel SRL binding 4 of the 5 sstrs) monotherapy with everolimus (a mTOR inhibitor) alone and in combination with pasireotide in patients with well-differentiated neuroendocrine carcinoma of the lung and thymus. The LUNA trial has been recently completed, whereas another multicentre, randomized, double-blind, PBO-controlled phase III study (SPINET, NCT02683941, EudraCT: 2015-004992-62) exploring safety and antitumour efficacy of lanreotide ATG 120 mg in well-differentiated typical or atypical, metastatic and/or unresectable lung NETs is currently recruiting.

14.5 Peptide Receptor Radionuclide Therapy

The expression of sstrs (mainly type 2) on the membrane of NET cells [92, 93] permits the use of PRRT, a therapy based on the use of a carrier molecule (octreotide derivatives) to which are attached a variety of different radionuclides, including indium-111 (^{111}In), yttrium-90 (^{90}Y) and lutetium-177 (^{177}Lu) [94]. The SRL is linked to the radionuclide via a specific chelator, most commonly DOTA or DTPA. Both the choice of the carrier molecule and of the specific radionuclide confer different benefits in targeting and delivering the radiation [95]. This complex radiopharmaceutical binds to the membrane sstrs and is internalized. Thus, radioactivity is transported into the intracellular receptor recycling

compartment of the tumour cell, where it exerts its action in proximity to the nucleus. The efficacy of PRRT is defined by different parameters: the expression of sstrs on the tumor cell membrane, a preserved receptor recycling's dynamic, that ensures properly internalization of the radioactive isotope and subsequent release of the radioactivity [96]. To estimate the radioactivity concentration, all patients are evaluated by ^{68}Ga-SRL-PET-CT to assess the sstr density in vivo. This imaging technique seems capable to estimate the clinical efficacy of treatment as well. In fact, the evidence of tumour lesion uptake greater than kidneys and/or spleen is correlated with objective response in 60% of patients [60]. The most extensively studied radioisotopes for PRRT, ^{90}Y and ^{177}Lu are characterized by different radiation energies. The ^{90}Y emits high-energy β-particles (Emax 2.27 MeV) and, consequently, thanks to its long pathway of 11 mm in soft tissue, might be useful to irradiate large lesions. On the other hand, ^{177}Lu β-particles (Emax 0.497 MeV) have a range of penetration of 2 mm in tissues, and it permits keeping energy inside lesions, especially in the small ones. The use of both radioisotopes, ^{90}Y and ^{177}Lu, could be useful particularly in patients with lesions of different sizes, including small metastases. Since ^{177}Lu also emits gamma particles (6.5% 133 KeV, 11% 208 KeV), it can be used also for post-treatment imaging, dosimetry and monitoring of the tumour response [97]. PRRT has been used for many years in uncontrolled trials, including different types of NETs and showing mainly a stabilization of disease and 15–35% rate of remissions [98]. Recently, the results of an international prospective, randomized, phase III study (NETTER 1 trial), that evaluated safety and tolerability of ^{177}Lu-DOTA-TATE plus octreotide LAR (30 mg/month) compared to high-dose octreotide LAR (60 mg/month) in progressive metastatic or advanced midgut NETs, have been reported. The objective response rate was 19% with PRRT and 3% with high-dose octreotide. Median PFS with PRRT was not reached (>27 months) while it was 8.4 months with high dose of octreotide [99]. A prerequisite for the use of PRRT was the expression of sstrs evaluated by ^{68}Ga-SRL-PET-CT. It remains unclear which patients have the highest benefit in terms of objective and durable response; however, a strong expression of $sstr_2$ seems to be remarkable. Tumour load, especially in the liver, and grading and performance status were relevant parameters in predicting PRRT outcome. Moreover pNETs were more responsive to PRRT compared with other types of NETs, although they frequently relapsed earlier [100]. Previously, Campana et al., in a multicentre retrospective analysis of 69 patients with G1-G2 GEP-NETs, also found that PRRT was more effective in patients with low tumour burden and low proliferation index [101]. Moreover, these authors showed that a previous treatment with hepatic transarterial chemoembolization (TACE) had a negative role in terms of objective response and PFS [101].

As far as the safety profile, PRRT is a relatively safe therapeutic procedure, well tolerated in the majority of patients, with a low occurrence of severe toxicity. The target organs of long-term toxicity are the kidneys and bone marrow, with loss of renal function (grade 3/4 toxicity in 3–9% of patients treated with ^{90}Y peptide, grade 4 toxicity in 0.4% of subjects treated with ^{177}Lu peptide), reduced bone marrow reserve and, more infrequently, myelodysplastic syndrome and leukaemia [102]. Haematological toxicity is the most common subacute side effect of bone marrow irradiation. More severe WHO grade 3 or 4 haematological toxicities occur in about <10% of patients, irrespective of the radiopeptide [102]. Bodei et al., in a large retrospective analysis on 807 patients, confirmed that PRRT with ^{177}Lu-octreotate was safer than with ^{90}Y-octreotide, alone or in combination, both in terms of haematological/renal toxicity and outcomes [102]. Because of its higher energy and longer penetration range, ^{90}Y irradiates the renal interstitium glomeruli more extensively than ^{177}Lu. The main risk factor for renal function impairment after PRRT is hypertension, along with poorly controlled diabetes and previous platinum-based chemotherapy. Risk factors associated with bone marrow toxicity are previous chemotherapy and other bone marrow risk factors, such as previous myelotoxic therapies and

anaemia. The authors concluded that their results suggest the existence of unidentified individual susceptibilities to radiation-associated disease beside known clinical risk factors [102].

14.5.1 Target Therapy

Among novel targeted therapies, different agents have been explored. Everolimus, an oral inhibitor of mTOR, as well as sunitinib, a tyrosine kinase inhibitor, has been the most extensively studied in NENs.

RADIANT-2, a randomized, double-blind, controlled phase III study comparing everolimus with placebo, both in combination with octreotide LAR (30 mg every 28 days), reported a greater median PFS in the everolimus group vs. placebo (16.4 months vs. 11.3 months). Treatment benefit with everolimus (plus octreotide LAR) was recorded irrespective of the previous therapies, including chemotherapy [103]. Moreover, the results from the randomized, double-blind, controlled phase III RADIANT-4 trial, evaluating efficacy and safety of everolimus compared with placebo in advanced, progressive, well-differentiated, non-functional NETs of lung or GI origin, showed that treatment with everolimus was associated with significant improvement in PFS (11.0 months in the everolimus group vs. 3.9 months in the placebo one) [104]. A retrospective post hoc analysis evidenced a consistent beneficial effect on PFS across predefined subgroups, based on the primary tumour origin. Specifically, the HR registered for the GI (that comprises stomach, colon, rectum, appendix, caecum, duodenum over ileum and jejunum tumours) was 0.56 (95% CI 0.37–0.84) while for lung was 0.50 (95% CI 0.28–0.88) [104]. On the basis of this evidence, everolimus was approved in non-functional progressive GI and lung NETs. Patients enrolled in the RADIANT-4 trial could have been previously treated with SRLs, interferon, one line of chemotherapy, PRRT or a combination of these treatments [104]. However, from data analysis we cannot determine whether patients previously treated with one therapeutic line have gain greater benefit or have experienced more severe AEs compared to patients treated with other approaches. The RADIANT-3, another large randomized, phase III, placebo-controlled trial, compared everolimus with placebo in pNET [105]. This study led to everolimus approval by FDA and EMA in patients with well- or moderately differentiated, advanced, progressive pNENs. The RADIANT-3 involved 410 patients with well- or moderately differentiated, advanced, progressive pNETs. Patients received everolimus 10 mg/day (207 patients) or placebo (203 patients), randomized 1:1. Patients who were in PD in the placebo arm were allowed to crossover towards the treatment arm. With a median follow-up of 17 months, PFS, that was the primary endpoint of the study, was higher in the everolimus arm (11.4 months, 95% CI, 8.4–13.9) compared to the placebo one (4.6 months, 95% CI, 3.1–5.4), with a HR of 0.35 (95% CI, 0.27–0.45, $p < 0.001$). Therefore, there was a probability of prolonging PFS in 65% of everolimus treated patients. The everolimus benefit was demonstrated for all subgroups defined at baseline, such as performance status, previous chemotherapy and previous treatment with SRLs [105].

In general, the main grade 3–4 toxicities observed during clinical trials with everolimus was represented by stomatitis (7–9%), anaemia (4–6%), hyperglycaemia (3–5%) and infections (5%). For grades 1–2, the most frequent toxicities have been aphthous stomatitis (64%), diarrhoea (around 30%), fatigue (around 30%) and infections (23–29%), mainly of the respiratory tract. Other AEs were non-infectious pneumonia, neutropenia, thrombocytopenia, hypercholesterolemia and hypertriglyceridemia [103–105].

The data regarding sunitinib treatment in NENs derives primarily from a prospective randomized, phase III, placebo-controlled study with sunitinib 37.5 mg/day in patients with advanced, well-differentiated pNEN, in radiologic PD [106]. Differently from the clinical trials with everolimus, no crossover was allowed for patients who progressed in the placebo arm. These patients in PD received sunitinib in a separate open-label study, similar to a protocol extension phase. The study was stopped earlier than planned, because a non-pre-planned analysis of the independent data

and the safety monitoring board found a statistically significant difference in terms of PFS. At that point 86 patients received sunitinib and 87 placebo. The PFS was 11.4 months in the sunitinib arm and 5.5 months in the placebo arm, with a HR 0.42 (95% CI, 0.26–0.66; $p < 0.001$). The most common grade 3–4 AEs were neutropenia (12%), hypertension (10%), palmo-plantar erythrodysesthesia (6%), diarrhoea (5%), asthenia (5%), abdominal pain (5%), stomatitis (4%) and thrombocytopenia (4%) [106].

A direct comparison study between sunitinib and everolimus in pNET, or in NENs in general, does not exist. Both studies were 1:1 randomized to placebo. About 50% of patients in RADIANT-3 [105] and 36% in the sunitinib trial [106] received SRLs prior to the study, and 40% and 28%, respectively, received SRLs during the study. Inclusion criteria were almost overlapping, with a few differences: radiological progression within the last year in RADIANT-3 and PD according to RECIST in sunitinib study, patients with well- and moderately differentiated tumours could enter the study with everolimus and those with only well-differentiated NENs entered the study with sunitinib [105, 106]. Results in terms of PFS between the two studies are superimposable. In terms of survival, in RADIANT-3, in relation to the study design that allowed the crossover from the placebo arm to the treatment one, in case of progression, the OS endpoint was not evaluable [105]. Concerning the sunitinib study, the data reported in the trial for survival benefit in treated patients compared to those receiving placebo were not confirmed by the subsequent analysis obtained with the extension of the follow-up [107]. Therefore, to date, the real impact of the two drugs on survival is not assessable.

14.6 Chemotherapy

Chemotherapy represents the most common therapeutic approach in advanced NENs defined as "poorly differentiated carcinoma" or "high-grade" lesions (NEC). Although these neoplasms appear relatively chemosensitive, their prognosis is poor.

14.6.1 Schedules in GEP-NENs

Based on the assumption that the clinical behaviour of GEP-NECs is similar to that of small cell cancers, the most commonly proposed chemotherapy regimen is cisplatin (CDDP)/etoposide (VP-16). However, the evidence is still scarce, and no controlled trials have been recently performed. In 1991, Moertel et al., among 45 patients with metastatic NENs, treated 14 GEPs with a VP-16 and CDDP-based regimen. However, overall in this study, only 18 patients had a NEC (not specified as many of the GEP tract). The objective rate of tumour response was clearly different between NEC (67%) and NET (7%). In NEC, TTP was 11 months and the OS 19 months, reflecting a poor prognosis [108]. According to this evidence, the CDDP/VP-16 regimen has been considered the reference therapy in NECs. In 1999, in a French retrospective analysis, 53 patients with advanced NENs received CDDP plus VP-16 every 3 weeks [109]. Forty-one patients had a NEC, and 20 of these were of GEP origin (13 pancreatic NECs). This chemotherapy was the first-line in 70% of cases, and the response rate was again clearly different between NECs (42%) and NETs (9%). The median PFS was 9 months in patients with NECs and 2 months in those with NETs. In contrast, OS was 15 months in NECs and 18 months in NETs [109]. In a recent retrospective analysis of 21 patients with NECs of the pancreatic/hepatopancreatic tract (10 pancreatic NECs) treated with CDDP/VP-16, a lower response rate (14%) with poor PFS (1.8 months) and OS (5.8 months) and high toxicity was recorded [110].

These studies show that the published results of chemotherapy with CDDP and VP-16, in GEP-NECs, are based on very low numbers, on different doses and schedules. In a recent Scandinavian retrospective analysis of over 200 patients with advanced GEP-NECs treated with chemotherapy (NORDIC Study), the use of cisplatin versus carboplatin did not affect the response and survival with statistically significance [111]. In this study, patients suffering from NECs with Ki67 < 55% responded less (15 vs. 42%; $p = 0.001$) but lived longer (14 vs. 10 months; $p < 0.001$) compared

to those with Ki67 > 55% [111]. On this basis alternative chemotherapeutic regimens to those containing platinum in NECs with Ki67 < 55% can be considered.

While second-line regimens have not been evaluated rigorously, options include temozolomide-, irinotecan- or oxaliplatin-based schedules, as main alternatives. Only recently, in the latest ENETS 2016 guidelines, attempts were made to answer this specific question by suggesting some therapeutic algorithms for GEP-NENs metastatic disease and for NECs and NETs G3 [90, 112]. A series of 19 patients with GEP-NECs, that underwent to a platinum-based chemotherapy as first-line, received FOLFIRI as second-line. The objective response was 31% and control of tumour growth was 62% [113]. In another published experience, a second-line temozolomide was used, alone or in combination with capecitabine (+/− bevacizumab). The response rate was 33%, with a median duration of 19 months. The overall PFS was 6 months and the OS was 22 months [114].

Despite the low numbers and heterogeneity of treated tumours, further studies have shown a clinical benefit of temozolomide alone or in combination of capecitabine in pNET or in advanced NENs of different grading and origins, as first- or second-line treatment [115–118]. A significant differences in PFS and OS after first alkylant use according to MGMT status was recently reported in advanced well-differentiated NETs (58% pNETs and 31% GI NETs) [119].

14.6.2 Schedules in High-Grade Thoracic NENs

Based on the meta-analysis published in the early 1990s that demonstrated how the use of radiotherapy in the limited disease results in a significant benefit, not only in terms of local disease progression but also of OS, the standard treatment of patients with SCLC with localized disease is chemotherapy and radiation therapy [120, 121]. The drugs currently considered most active in the treatment of SCLC are CDDP and VP-16, administered in combination, based on a randomized phase III study that compared the combination of CDDP/VP-16 with that of 3 drugs (cyclophosphamide, epirubicin and vincristine) [122].

To date, few data in the literature can provide solid recommendations on the treatment of LCNEC limited disease, most derived from retrospective analysis on minimal case series of patients. A Japanese study compared, prospectively, survival data of 15 patients with LCNEC, operated and treated with adjuvant chemotherapy with CDDP/VP-16 for two cycles, with those of a previously collected case series of 32 patients with LCNECs treated with surgery alone. The group treated with adjuvant chemotherapy had a survival at 2 and 5 years of 88%, while the group treated with surgery alone showed a survival at 2 and 5 years of 65% and 47% [123]. The LCNECs have an aggressive behaviour, which makes these cancers similar to SCLCs, therefore, the first-line treatment does not differ from the standard already validated treatment for the SCLC (CDD/VP-16), in the absence of alternative treatments. Therefore, in the clinical practice, regimens containing CDDP/VP-16 are the most commonly used, even in case of metastatic SCLCs and LCNECs [122, 124–128].

14.6.3 Schedules in Low-Grade Thoracic NENs

Systemic medical treatment with chemotherapy is reserved for locally advanced and metastatic cases [17, 129]. Low-grade thoracic NENs, due to their rarity, have often been included in chemotherapy studies designed for NENs of other anatomic districts. Therefore, there is currently no standard chemotherapy approach, and the therapeutic results do not appear uniform. Moreover, given their low proliferative activity, carcinoids are generally considered to be chemoresistant neoplasms [17, 129, 130].

Temozolomide, due to its oral administration, lower toxicity, the ability to cross the blood-brain barrier, the possibility of being associated with other cytostatic and to be used for long periods of time, it is among the most used and promising

drugs in this group of neoplasms. Some evidence from the literature shows that schemes containing temozolomide may be of benefit in the treatment of advanced lung carcinoids. In a retrospective study of 36 patients with NENs, including 7 thymic carcinoids and 13 bronchial carcinoids, temozolomide alone resulted in radiological responses in 14% of patients, with disease stabilization in another 53% [118]. The median time to progression was 7 months, and the most significant toxicity was the haematological (thrombocytopenia of grades 3 and 4 in 14% of cases) [118]. Another recent retrospective study evaluated temozolomide in 31 patients with metastatic bronchial carcinoid [131]. No CR was found; however, PR in 14% and disease stabilization in 52% of cases were achieved. The most commonly reported grade 3–4 toxicity was, as expected, thrombocytopenia [131].

14.7 Follow-up Programmes

In general, follow-up investigations should include clinical evaluation, biochemical parameters measurement, as well as conventional imaging. In patients with R0/R1 resected G1/G2 NENs, it is recommended that imaging (CT or MRI) is performed every 3–6 months (in G3 NEC every 2–3 months) for the first 2 years and then every 12 months. Functional imaging, using either Octreoscan® or PET/CT with ^{68}Ga-DOTA-TOC/-NOC/-TATE, or ^{18}F-DOPA, or ^{18}FDG, should be included in the follow-up on the basis of a suspicion suggested by conventional imaging [89, 129]. The type of surgery, the R status, the size of the primary and the presence of metastatic disease will also be taken into account to define the most appropriate interval of follow-up. According to the primary site of origin, different endoscopic techniques could be used in the follow-up of patients with NENs. Timing and duration of follow-up is, however, still largely debated. In view of their slow growth and the possibility of recurrences also many years after diagnosis, it is advisable to perform a long-term follow-up (at least 15 years or even lifelong) [89, 129].

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Circulating Markers in Neuroendocrine Tumors

15

Ettore Seregni and Alice Lorenzoni

15.1 Introduction

Neuroendocrine tumors (NETs) are a group of rare and heterogeneous neoplasm that are derived from cells throughout the nervous and endocrine systems [1]. These tumors are widely distributed throughout the human body including the lung, stomach, intestine, pancreas, adrenals, thyroid, and pituitary gland. The particular characteristic that distinguishes NETs from other solid malignancies is that NETs are composed of specialized cells that have the ability to produce, store, and secrete bioactive amines and peptide hormones [2]. NETs are broadly classified into two categories termed functional NETs or nonfunctional NETs according to whether these tumors give rise to a clinical syndrome. Functional NETs may be discovered when they are in the distinctive early stage, but they are often misdiagnosed on account of nonspecific and unpredictable symptoms. By contrast NETs with no symptoms or just local symptoms are not frequently identified until they have progressed to an advanced state, by which time metastasis has already occurred [3, 4]. Circulating tumor markers can offer relevant clinical information either in the diagnosis of this neoplasm or in the follow-up of the affected patients, i.e., clinical surveillance and therapy monitoring. At regard, different markers have been proposed in the last years as useful tools in clinical management of patients affect by NETs. From a practical point of view, these markers can be classified as "specific markers," i.e., the product of the individual endocrine cell or "pan-endocrine markers," i.e., a product that is common to all endocrine cells. Chromogranin A (CgA) is the most relevant pan-endocrine marker. In fact, it is present in the secretory granules in all endocrine and neuroendocrine cells. During the past several decades, a growing body of evidence has demonstrated that CgA is released in abnormal amounts by many neoplastic neuroendocrine cells and elevated circulating CgA levels have been confirmed to be a helpful biochemical marker for the diagnosis of various types of NETs [5]. At present, CgA is considered the most useful biomarker of both nonfunctioning and functioning NETs. In this review, we firstly consider CgA and then the specific markers of the major neuroendocrine neoplasm.

15.2 Chromogranin A (CgA): The Pan-endocrine Tumor Marker of NETs

Chromogranin A (CgA), a member of the granin family of acid proteins, is present in the secretory granule of a wide variety of endocrine and

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neuroendocrine cells. Granins have been proposed to play important roles in secretory granulogenesis, secretory protein sorting, and secretory granule maturation and condensation. Several granins interact with other components of the matrix of the secretory granule, such as catecholamines, serotonin, and histamine, suggesting that granins contribute to the formation of secretory granules [6]. Granins consist of single-polypeptide chains of approximately 180–700 amino acid residues, carrying an amino-terminal signal peptide that directs the movement of the preproteins from ribosomes to the endoplasmic reticular lumen and, hence, the Golgi complex, where further post-translational modifications occur [7]]. Nowadays, at least ten members of the granin family represented from chromogranins (chromogranin A and B) and secretogranin (SGII → Sg VIII) have been identified. The first member of the chromogranin/secretogranin family to be identified was CgA which was first isolated from chromaffin cells of the bovine adrenal medulla. The human CgA is an acidic protein with a length of 439 amino acids and with a molecular weight of 48–60 kDa, depending on glycosylation and phosphorylation status. The N-terminal sequence contains a disulfide bridge between the cysteinyl residues in position 17 and 38, which seems important for the intracellular sorting [8]. The intact CgA protein also contains nine dibasic and other basic cleavage sites, which are processed to a variable extent [9]. CgA biosynthesis is controlled both transcriptionally and posttranscriptionally. CgA, as well as the other granins, is characterized by (1) an acidic pH due to high percentage of acidic amino acids (glutamic acid and aspartic acid), (2) heat stability due to its high hydrophilic nature, (3) the presence of multiple dibasic cleavage sites, and (4) the capacity to form aggregates and to bind calcium. CgA is expressed by several normal or neoplastic cells of the diffuse endocrine and neuroendocrine systems or by some cancer cells that can undergo neuroendocrine differentiation and generally correlates with dense-core secretory vesicle number. Chromogranin A appears to be crucial for the formation of secretory granules and sequestration of hormones in neuroendocrine cells. The adrenal medulla is the main source of circulating CgA, while adrenergic nerve endings and neuroendocrine cells secrete CgA in peripheral tissues [10].

15.2.1 Biochemical and Biological Properties of Chromogranin A and Derived Peptides

The human CgA gene is located on chromosome 14q32.12, spans 12192bp, and is organized in eight exons and seven introns. The derived transcript of 2 kb is translated into the 457 residues CgA protein of about 48–52 kDa molecular weight that undergoes posttranslational processes and proteolytic cleavages by different enzymes such as prohormone convertase 1-3 (PC1-3) and cathepsin L [5–8]. The CgA maturation produces several biologically active peptides which include vasostatins 1 and 2, chromofungin, chromacin, pancreastatin, catestatin, WE14, chromostatin, GE25, parastatin, and serpinin (Fig. 15.1).

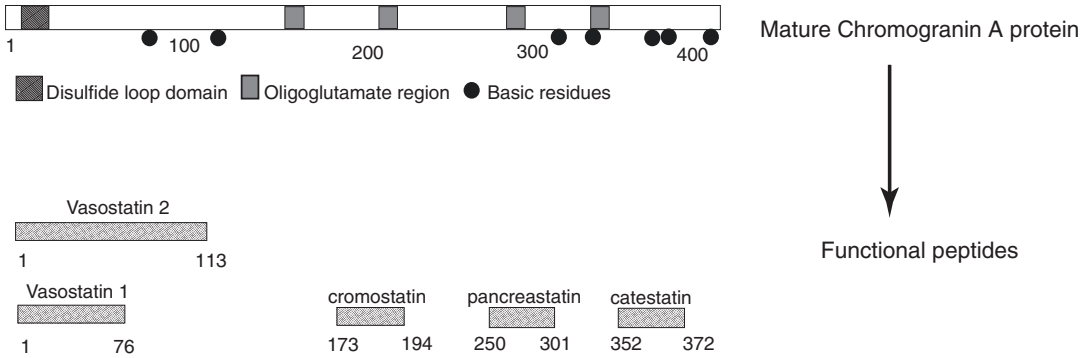

Fig. 15.1 Chromogranin A cleavage and derived functional peptides

These peptides have autocrine, paracrine, and endocrine activities. The CgA processing varies in a tissue-specific manner: in adrenal medulla and anterior pituitary gland, rate and processing are low, while in the endocrine pancreas and in gastrointestinal, the proteolytic processing is faster and more extensive [11]. The proteolytic processing of CgA may also occur after its release from neuroendocrine cells. CgA and its fragments constitute in plasma a highly heterogeneous mixture of proteins and peptides, whose complexity is further emphasized by the tissue-tumor and patient-specific processing. The impairment of chromogranin A expression by antisense RNA depletes secretory granules, inhibits regulated secretion of a prohormone, and reduces secretory granule protein in cells [12]. CgA contributes to the formation of the secretory vesicle when the immature vesicle buds from the trans-Golgi network. Furthermore CgA plays a fundamental role in intracellular calcium homeostasis, due to its high binding capacity but low affinity for Ca^{2+} which allows calcium intracellular deposit. Meanwhile, CgA facilitates the mobilization of ionized calcium into the cytoplasm with the activation of IP3R/Ca^{2+} channels [13, 14]. The complete extracellular functions of CgA remain to be elucidated. CgA seems to be implicated as a regulator in vascular homeostasis, angiogenesis, cardio-regulation, and tissue repair. At the central nervous system level, CgA may play an autocrine role as a glucocorticoid-responsive inhibitor regulating the secretion of peptides derived from proopiomelanocortin in the pituitary gland [15]. Moreover, CgA indirectly causes neuronal apoptosis by inducing microglial cells to produce both heat-stable diffusible neurotoxic agents and TNFα [16]. The cleavage peptides of CgA have several regulatory functions influencing the cardiovascular, the endocrine, and the immune system. Furthermore they control the glucose and calcium homeostasis (Table 15.1). Pancreastatin (CgA 250–301) was the first identified CgA-derived peptide in porcine pancreas in [17]. Released with catecholamines in stress situations, it appears to be involved in the modulation of energy metabolism with a general counter-regulatory effect to that of insulin. Pancreastatin activates a receptor signaling system that belongs to the seven-spanning transmembrane receptor coupled to a Gq-PLCbeta-calcium-PKC signaling pathway. Increased pancreastatin plasma levels, correlating with catecholamines levels, have been found in insulin resistance states, such as gestational diabetes or essential hypertension. It exerts multiple, potentially dysglycemic actions on isolated cells or organs in vitro, including inhibition of glucose-stimulated insulin release and inhibition of glucose uptake in adipocytes and hepatocytes [18]. Vasostatins 1 and 2 represent the N-terminal fragments of CgA and exert a large spectrum of homeostatic actions, including vasodilation, antifungal and antimicrobial effects, modulation of cell adhesion, and inhibition of parathyroid hormone secretion. Vasostatin inhibits VEGF-induced endothelial cell proliferation and migration and the formation of capillary-like

Table 15.1 Proposed actions of chromogranin A-derived peptides

Fragment	Biological action
Vasostatin 1 (CgA 1–76)	Inhibits vasoconstriction, promotes fibroblast adhesion, inhibits parathyroid hormone secretion from parathyroid chief cells, triggers microglial cell-mediated neuronal apoptosis, and exerts bacteriolytic and antifungal effects
Vasostatin II (CgA 1–113)	Inhibits vasoconstriction and parathyroid hormone secretion
Pancreastatin (CgA 250–301)	Inhibits insulin release from pancreatic islet beta cells; promotes hepatic glycogenolysis; decreases insulin-induced glycogen synthesis in skeletal myocytes, hepatocytes, and adipocytes; stimulates amylase release from pancreatic acini; decreases gastric acid release from parietal cells; diminishes glucose uptake by skeletal muscles in humans
Parastatin (CgA 357–428)	Inhibits parathyroid hormone secretion, inhibits insulin release
Catestatin (CgA 352–372)	Inhibits catecholamine release from the adrenal medulla
Chromostatin (CgA 124–143)	Inhibits catecholamine release

structures [19]. Catestatin acts at nicotinic cholinergic receptors as a potent autocrine inhibitor of catecholamine secretion [20].

15.2.2 Chromogranin A Measurement

CgA is usually measured in serum or plasma using immunometric techniques. It has been reported that plasma chromogranin tends to be markedly higher than that determined in the serum. The first competitive CgA assay was described by O'Connor and Bernstein in [21]. Three diagnostic techniques are available: enzyme-linked immunosorbent assay (ELISA), immunoradiometric assay (IRMA), and radioimmunoassay (RIA). In clinical practice, several kits are currently commercialized. Clinical interpretation of CgA results may be limited by the considerable heterogeneity between commonly available CgA assays. The diagnostic accuracy of an assay depends upon antibody specificity and the molecular forms it recognizes. An assay that recognizes more forms is likely to have better diagnostic accuracy. Although several commercial CgA assays are currently available, the forms of CgA detected by these assays vary owing to differences in antibody specificities and assay design. This makes direct comparison between assays problematic. Currently there is no universal, worldwide accepted diagnostic technique; thus caution is recommended when trying to compare the results from different centers. There are many studies comparing the sensitivity and specificity of available diagnostic methods. Stridsberg et al. compared the three commercially available kits in a group of NET patients and found sensitivities to vary between 67 and 93%, while specificities were 85% for all three [22]. A multicenter prospective study comparison between two methods, immunoradiometric and ELISA, found a 36% clinical discordance rate [23]. The results of CgA blood concentration may be influenced by various factors or coexisting pathological conditions. Among the factors causing a substantial increase of the blood CgA concentration are treatment with proton-pump inhibitors or H_2-receptor blockers, chronic atrophic gastritis (type A), impaired renal function, primary parathyroid hyperplasia, and thyroid C-cell hyperplasia. There are also many conditions which may have a moderate or little influence on the concentration of CgA such as inflammatory bowel disease (ulcerative colitis and Crohn's disease), deteriorating liver function, untreated essential hypertension, heart failure, hypercortisolism, and pregnancy. CgA increases under the influence of food intake and exercise. Maximum CgA concentrations are observed 30–90 min after a meal and reach two to three times the upper reference range. Therefore, it is recommended to measure CgA after rest and in fasting conditions. Furthermore the CgA concentration may vary up to 25% in the single subject. In conclusion proper assessment of the CgA results requires detailed knowledge about various factors, drugs, and pathological conditions influencing its concentration in blood. Factors affecting the concentration of chromogranin A are summarized in Table 15.2.

Table 15.2 Factors affecting the concentration of chromogranin A

Diseases of gastrointestinal tract	Chronic atrophic gastritis, inflammatory bowel diseases, irritable bowel syndrome, pancreatitis, chronic hepatitis, liver cirrhosis
Diseases of the cardiovascular system	Hypertension, heart failure, acute coronary syndrome
Renal diseases	Renal insufficiency
Inflammatory disorders	Systemic rheumatoid arthritis, systemic lupus erythematosus, sepsis, chronic obstructive pulmonary disease (COPD)
Endocrine disorders	Hyperparathyroidism, hyperthyroidism, hypercortisolemia
Non-neuroendocrine tumor	Prostate cancer, ovarian cancer, breast cancer, colorectal cancer, pancreatic cancer, hepatocellular carcinoma, hematological malignancies
Medications	Proton-pump inhibitors, histamine type-2 receptor antagonists, antihypertensive drug
Other	Food intake or exercise

15.2.3 Chromogranin A and Neuroendocrine Tumors

Plasma CgA and derived peptides are now commonly used as diagnostic and prognostic markers or to monitor several diseases, such as endocrine tumors, heart failure, hypertension, and neurodegenerative and neuropsychiatric diseases. Nowadays CgA is the most valuable marker of neuroendocrine tumors. In fact CgA is secreted by a number of neuroendocrine tumors, which include pheochromocytoma, medullary thyroid carcinoma, and pulmonary neuroendocrine tumors including small-cell lung cancer.

In general, the highest values of CgA and accuracy in the determination of CgA are observed the most frequently in tumors showing intense secretory activity, mainly NET of the small intestine, particularly causing carcinoid syndrome and in GEP-NETs occurring in MEN-1 syndrome [24, 25]. As regards the pancreatic neuroendocrine tumors, both functioning and nonfunctioning tumors reveal intermediate levels of CgA. Satisfactory accuracy of CgA is also observed in nonfunctioning neuroendocrine tumors. Chromogranin A concentration may be normal in the case of neuroendocrine tumors with mild proliferative potential such as appendicular NET. Furthermore in about 75% of insulinoma, CgA is not increased. CgA increase may be not observed in poorly differentiated NET which may lose neuroendocrine features [26–28]. Chromogranin A is widely expressed in adrenal medulla, and serum CgA assay showed to be very sensitive in pheochromocytoma diagnosis and follow-up. Chromogranin A concentrations and sensitivity depend mainly on the spread of cancer. In general, CgA is significantly higher in the case of disseminated rather than limited neoplastic disease. The presence of liver metastases may significantly increase the concentration of CgA, especially in case of multiple metastatic lesion [25, 29, 30]. An exception may be a gastrinoma, as here CgA is high even in the absence of metastases in the liver or in case of small volume tumor. The sensitivity and specificity of CgA for different types of neuroendocrine tumors are in the range 60–100% and 70–100%, respectively, with the highest values observed in the case of serotonin-secreting neuroendocrine tumors. In the case of serotonin-secreting neuroendocrine tumors originating from the midgut, CgA concentration is an independent prognostic factor, because its concentration is correlated not only with the size of the tumor but also with the biological activity. Plasma chromogranin A > 5000 μg/l were found to be an independent predictors of overall survival in midgut cancer [31]. A chromogranin A elevation three times the upper normal limit or more was found to be a negative prognostic factor in pancreatic NET [32]. CgA is the most reliable biomarker reflecting the clinical evolution of NETs. Bajetta and colleagues demonstrated that the increased level of CgA during follow-up is associated with progressive disease in 83% of patient, whereas a stable CgA is associated with stable disease in 54% of patients with NETs [33]. In patients with liver involvement, CgA increase is associated with local progression. The evaluation CgA concentration during follow-up may be also helpful in detecting potential recurrence of the disease. In a study performed by Welin et al. evaluating the usefulness of CgA in monitoring radically treated NET, the increase in CgA is the first marker to indicate tumor recurrence in the majority of radically operated midgut carcinoid patients [34]. Assuming that the concentration of CgA correlates with the volume of malignancies, the concentration of CgA is theoretically expected to decrease if treatment is effective. Several studies have shown that changes in CgA levels are associated with disease status and treatment responses with acceptable sensitivity (54–86%) and specificity (60–86%) [33, 35–38]. The concordance between biochemical response based on CgA measurement and tumor response based on Response Evaluation Criteria in Solid Tumors (RECIST) was found to be 74% in patient with nonfunctioning GEP-NETs in a recent study by Kim and colleagues [39].

Pancreastatin is present in NETs and is present in pM concentrations in normal serum primarily as the CgA (250–301) form and in higher molecular weight forms. Pancreastatin has been

proposed as an alternative biomarker, as its levels are less susceptible to nonspecific effects, the assay is more standardized, and early experience indicated a correlation with clinical outcomes. Higher pancreastatin levels are found to be significantly associated with worse progression-free and overall survival in small bowel and pancreatic NETs independently to primary tumor site and the presence of nodal or metastatic disease [40]. Furthermore pancreastatin may identify liver metastasis in patients with primary tumors of the small bowel with sensitivity and specificity of 85.7% and 66.7%, respectively, compared with sensitivity and specificity of 61.5% and 43.8% for chromogranin A [41].

Further investigation of pancreastatin's diagnostic and predictive value is warranted.

15.3 Specific Biomarkers of NETs

15.3.1 Gastroenteropancreatic (GEP) Tract

15.3.1.1 Serotonin and 5-Hydroxyindoleacetic Acid (5-HIAA)

Patients affected by NETs originating from the midgut may suffer of functional symptoms due to the secretion of vasoactive products and, particularly, of serotonin [5-hydroxytryptamine (5-HT)]. This syndrome (i.e., "carcinoid" syndrome), occurring in approximately 5% of patients with midgut NETs and characterized by flushing and diarrhea, is in part sustained by the stimulating action of 5-HT on smooth muscle cells [4]. 5-HT is synthesized and stored in enterochromaffin cells of the gastrointestinal tract (80% of total body serotonin), in dense granules of platelets, and in the serotoninergic neurons of the central nervous system. The majority of serotonin is metabolized by monoamine oxidase producing 5-hydroxyindoleacetaldehyde, which is further oxidized to 5-hydroxyindoleacetic acid (5-HIAA).

Determination of plasma or serum 5-HIAA is used in the diagnosis and monitoring of patients with midgut NETs and, particularly, in the clinical setting of the "carcinoid" syndrome. In fact, the overall sensitivity and specificity of urinary 5- HIAA in the presence of the "carcinoid" syndrome is about 70% and 90%, respectively [42]. The sensitivity is lower in patients with midgut carcinoid tumors without the "carcinoid" syndrome and in patients with fore-and hindgut NETs. Furthermore, sensitivity also depends on tumor volume and may very low in patients with nonmetastatic tumors. Correlation between 5-HIAA levels and the clinical severity of the "carcinoid" syndrome is not always apparent in all patients, and this can be related to a fluctuating release of serotonin from tumors. Conflicting data are available about the prognostic role of 5-HIAA in NETs patients. In fact, in two studies including 76 and 119 patients, respectively, high 5-HIAA levels were an independent survival factor [43, 44], while in other two studies performed in 256 and 139 patients with midgut NETs 5-HIAA levels were predictive of poor outcome only at univariate analysis [45, 46]].

5-HIAA determination is usually performed in 24-h urine samples, and high-performance liquid chromatography (HPLC) is the most frequently employed method to measure this analyte. Procedures and consideration for a correct analysis of 5-HIAA in urine are reported in Table 15.3.

Collection of 24-h urine samples is time-consuming and prone to errors during urine collection either in term of over- or under-collection. There is also inconvenience with respect to collection and impact on daily life during the period of collection. In addition, there are health and safety issues with 24-h urine collections for 5-HIAA related to the use of acid-containing bottles. For these reasons 5-HIAA determination in serum or in plasma should be preferred. Recent data show that plasma and urine 5-HIAA have very similar diagnostic sensitivities and specificities, with a good correlation between the two methods. In addition, it has been demonstrated, using plasma samples for 5-HIAA quantification, that patients only need to avoid serotonin-containing foods for 24-h prior to blood sampling, rather than the three-day dietary restriction that is currently recommended for 24-h urine collections [47].

Table 15.3 Urine 5-HIAA measurement: pre-analytical procedures and interferences

• Collect and measure urine in plastic containers with acid addition to ensure sterility and stability
• Store sample in a refrigerator until analysis
• When the test is required for diagnosis, perform two consecutive 24-h collections (in order to minimize intraindividual variation). A single specimen may be sufficient for follow-up
• Causes of false-negative results:
– Pathological status: renal impairment, hemodialysis
– Medications: chlorpromazine, heparin, isoniazid, levodopa, monoamine oxidase inhibitors, phenothiazines, promethazine, and tricyclic antidepressants, octreotide
• Causes of false-positive results:
– Pathological status: malabsorption, e.g., celiac disease, tropical sprue, Whipple's disease, cystic fibrosis
– Food rich in dietary tryptophan: plums, pineapples, bananas, eggplants tomatoes, avocados, and walnuts
– Medications: phenacetin, reserpine, cisplatin, fluorouracil, melphalan

15.3.1.2 N-Terminal Pro-brain Natriuretic Peptide (NT-proBNP)

Metastatic midgut NETs secrete serotonin and other vasoactive substances that are responsible not only for the "carcinoid syndrome," as previous described, but also for the long-term complication of "carcinoid heart disease" (CHD). CHD is characterized by thickening of the tricuspid and pulmonary valves, resulting in regurgitation and/or stenosis of the affected valve. Any or all of the cardiac valves can be affected, with tricuspid regurgitation being the most frequently observed pathology. Detecting the presence of CHD is important in determining the most appropriate management strategy and also has prognostic significance for long-term survival [48]. The identification of a sensitive and specific biochemical marker that can predict the presence and severity of CHD may be of clinical value. Among the proposed marker, the most useful to date is N-terminal pro-brain natriuretic peptide (NT-proBNP). NT-proBNP is released from cardiac myocytes with myocyte stretch being the main stimulus for its synthesis and secretion. Elevated levels reflect increased wall tension and pressure, making its measurement of value in CHD. Some studies have demonstrated that this marker can have both diagnostic and prognostic significance for cardiac involvement. For predicting CHD in NETs, it has a high sensitivity and specificity (87% and 80%, respectively) [49]. Expression is also strongly associated with survival; specifically, high levels (>90 ng/l) were negatively correlated with outcome (hazard ratio, 3.43) [50].

15.3.1.3 Insulin and Glucose

NETs secreting insulin are termed insulinomas and are almost exclusively intrapancreatic in nature. Excessive insulin secretion leading to hypoglycemia usually results in a combination of neurologic (diplopia, blurred vision, confusion, abnormal behavior and amnesia, seizures, coma, etc.) and autonomic (sweating, weakness, hunger, tremor, nausea, feelings of warmth, anxiety, palpitations) symptoms. Symptoms are usually related to the degree of insulin-induced hypoglycemia but may be nonspecific. Hypoglycemia-induced clinical signs are classically present in the early morning preprandial phase or may be exercise induced. The diagnosis is suggested in the presence of (1) symptoms of hypoglycemia, (2) glucose <40 mg/dl, and (3) relief of symptoms with administration of glucose [51]. This is known as Whipple's triad. The 72-h fast is the gold standard for diagnosing insulinoma and relates to the integrity of patients' endogenous suppression of insulin in the face of hypoglycemia. The fast attests to autonomous insulin secretion and the failure of appropriate insulin suppression in the presence of hypoglycemia. Procedure, methods, and consideration for a correct 72-h fast test are summarized in Table 15.4.

15.3.1.4 Gastrin

Gastrinoma is a gastrin-secreting tumor that is associated with Zollinger-Ellison syndrome (ZES). The majority of cases occur in the pancreas, followed by the duodenum. ZES is characterized by gastric hypersecretion, hyperacidity, and atypical peptic ulceration. Typical symptoms include abdominal pain, secretory diarrhea,

Table 15.4 Procedure, methods, and consideration for a correct 72-h fast test

• Patients should be hospitalized in a specialist unit experienced in performing the test
• Patients should stay off all foods except for plain water, black tea or coffee, and essential medications
• Absolute blood (venous) determinations should be performed at least two to four times per day and when the patient describes symptoms
• Blood should be drawn for insulin measurement concurrently with glucose estimations and assay for insulin and C-peptide when the hypoglycemia is confirmed
• Symptoms appear within 12 h for one third of patients, 80% within 24 h, 90% with 48 h, and approaching 100% within 72 h
• The endpoint of the test is documented hypoglycemia
• Absolute values of glucose and insulin are the most important variables, and any measurable insulin is abnormal when blood glucose drops to 45 mg/dl

Table 15.5 Procedure, methods, and consideration for a correct gastrin provocative secretin test

• If fasting serum gastrin (FSG) is >1000 pg/ ml, a secretin test is not necessary. When FSG lies between 200 and 1000 pg/ml, a secretin test should be performed
• Conditions leading to high FSG should be considered such as fundic atrophic gastritis, *Helicobacter pylori* infection, renal failure
– Secretin test
Preparation
– PPIs should be interrupted 10 days to 2 weeks prior to the test (PPIs for 2 weeks can be replaced by H_2 blockers), interruption of H_2 blockers for approximately 48 h prior to test
– Patient fasting overnight, 12–14 h
Execution
– Secretin (2 U/kg body weight) is given by intravenous bolus
– Serum gastrin baseline measured at –15 and –1 min before test and 2, 5, 10, 15, 20, and 30 min after secretin
– Samples stored on ice (immediate transfer to laboratory)
• Possible side effects of the secretin test include flush, allergic reaction
• Interpretation of results: delta gastrin at least 200 pg/ml any time during the test is considered as positive

esophagitis, and hypercalcemia. The diagnosis of ZES can be established by the demonstration of elevated fasting serum gastrin (FSG) in the presence of low gastric pH. FSG alone is not adequate to make the diagnosis of ZES because hypergastrinemia can be seen in patients with achlorhydria associated with chronic atrophic fundus gastritis (e.g., pernicious anemia) and in other conditions with hyperchlorhydria (e.g., *Helicobacter pylori* infection, gastric outlet obstruction, renal failure, antral G-cell syndromes, short bowel syndrome, retained antrum). In addition, the use of chronic proton-pump inhibitors (PPIs) leads to high FSG levels, and therefore gastrin provocative tests are needed to establish the diagnosis of ZES. Indeed, in a recent prospective analysis, up to two thirds of gastrinoma patients were found to have FSG values <tenfold normal [52]. The gold standard is the secretin test (Table 15.5). This hormone, when given intravenously, provokes an increase in serum gastrin and secondarily in gastric acid secretion. It has been demonstrated that patients with fasting gastrin <1000 pg/ml, the sensitivity of the secretin test, using the criterion delta (increase from prestimulation level) gastrin of >200 pg/ml, is of 85% [53]. The same group recently reported their prospective experience on gastrin provocative tests in patients with ZES and with < tenfold increase in respect to normal value. They found that a delta gastrin of >120 pg/ml has the highest sensitivity and specificity (94% and 100%, respectively) [54].

15.3.1.5 Neurokinin A (NKA)

Neurokinin A (NKA) is a member of the tachykinin family, and plasma concentrations are elevated in patients with midgut carcinoid tumors. Tachykinins are neuropeptides involved in nociception and smooth muscle contraction, and they are known to have effects on gastrointestinal motility, vasodilatation, and flushing. In one study elevated concentrations of NKA were found in the plasma of 46% of patients with midgut carcinoid tumors [46]. Furthermore, circulating NKA and age were shown to be the only independent indicators of poor prognosis in these patients. It was demonstrated that patients with circulating levels of NKA > 550 ng/L have a median survival of less than 2 years [55].

15.3.1.6 Circulating VIP, Glucagon, and Somatostatin

VIPomas, glucagonomas, and somatostatinomas are very rare NETs producing VIP, glucagon, and somatostatin, respectively. Patients affected by these NETs are characterized by specific symptoms and signs sustained by the overproduction of the individual hormone. Watery diarrhea, hypokalemia, and achlorhydria are associated with VIPomas; necrolytic migratory erythema, weight loss, and diabetes are often present in patients affected by glucagonomas; and gallstones and steatorrhea are frequent in somatostatinomas. Evaluation of circulating levels of VIP, glucagon, and somatostatin offers diagnostic information, and serial determinations of the individual gut hormone during the follow-up are useful tools to monitor the evolution of the disease and the efficacy of the adopted therapies.

15.3.2 Bronchial Carcinoid

15.3.2.1 Neuron-Specific Enolase (NSE)

Neuron-specific enolase (NSE) is present in neurons and neuroendocrine cells and can be raised in tumors originating from them, especially with a high tumor burden, poor histological differentiation, or a high rate of cell death. NSE is located in the cytoplasm and, unlike CgA, is not secreted. Its diagnostic sensitivity in GEP-NETs is low (32–47%) and is mainly the marker for poorly differentiated NEC or bronchial carcinoid [56–58].

15.3.2.2 Pro-gastrin-Releasing Peptide (proGRP)

Pro-gastrin-releasing peptide (proGRP) is a precursor of a neuropeptide hormone called "gastrin-releasing peptide (GRP)," and it is frequently elevated in patients affected with small-cell lung cancer (SCLC). ProGRP is a biologically active protein that stimulates tumor cell proliferation, and it can function as an autocrine growth factor. The growth-stimulating properties of proGRP may be responsible for more aggressive tumor behavior and can explain its prognostic significance [59, 60]. In patients with SCLC, circulating proGRP levels serve as a reliable marker for disease monitoring and for evaluating clinical response to therapies. Furthermore, proGRP is the most sensitive marker for discriminating SCLC from benign diseases of the lung. This marker, in fact, is rarely elevated in patients with benign conditions or other malignancies with the relevant exception of patients affected by medullary thyroid carcinoma and NETs. As regards a first cross-sectional marker study in which the possible role of proGRP was evaluated in addition to the established makers CgA and NSE in the diagnosis and prognosis of 573 patients with NET and NEC as well as 282 healthy controls, proGRP appeared to be the most sensitive marker for small-cell NEC, especially when located in the lung [61].

15.3.3 Pheochromocytoma (PHEO) or Paraganglioma (PGL)

15.3.3.1 Catecholamines and Their Metabolites

Pheochromocytoma (PHEO) or paraganglioma (PGL) are rare tumors derived by neuroendocrine chromaffin cells usually found in the adrenal medulla and other ganglia of the nervous system. In particular, PHEOs arise from adrenomedullary chromaffin cells producing catecholamines, while PGLs derive from extra-adrenal chromaffin cells of the sympathetic paravertebral ganglia (thorax, abdomen, pelvis) producing catecholamines or parasympathetic ganglia located in the neck and at the base of the skull, which do not produce catecholamines [62].

Catecholamines (e.g., adrenaline, noradrenaline, and dopamine) are amines synthesized from the amino acid tyrosine (Tyr) in sympathetic nerve terminals and in the adrenal gland or in sympathetic paravertebral ganglia. The catecholamine catabolism is different in sympathetic nerve and in extra-neuronal tissues. In fact, the sympathoneuronal pathway involves intraneuronal deamination of norepinephrine by monoamine oxidase (MAO). The extra-neuronal pathway, by contrast, involves *O*-methylation catalyzed by the membrane-bound enzyme

catechol-*O*-methyltransferase (COMT) with the intracellular production of the *O*-methylated metabolites, normetanephrine, and metanephrine. Sympathetic nerves lack of COMT with the relevant biochemical consequence that O-methylated metabolites are relatively specific markers of chromaffin tumors. Furthermore, in PGLs derived from the sympathetic paravertebral ganglia, the biosynthesis of the enzyme phenylethanolamine-N-methyltransferase converting norepinephrine to epinephrine is downregulated at both the mRNA and protein level. In these tumors, the increased production of norepinephrine results in an elevated concentration of normetanephrine in circulation. By contrast, in PHEO the increased production of epinephrine leads to elevated levels of circulating metanephrine [63]. So markers of pheochromocytoma and paraganglioma (PPGL) are plasma free metanephrines or urinary fractionated metanephrines, and, particularly, normetanephrine is associated with PPGL phenotype and metanephrine with PHEO phenotype. Different studies demonstrated the superior sensitivity of urine metanephrines over catecholamines and vanillylmandelic acid (VMA) for diagnosis of PPGLs [64].

In patients affected by symptomatic catecholamine-producing disease the diagnostic sensitivity of plasma free or urine fractionated metanephrines is very high. However, it is important to stress that all positive results indicate the presence of a tumor. Considering the low pretest prevalence of PPGLs (usually less than 1%), false-positive rate is much higher than true-positive rate (low positive predictive value). However, elevations of both normetanephrine and metanephrine and solitary increases in either normetanephrine or metanephrine elevated threefold or more above upper cutoffs are rare as false positives.

Liquid chromatography with mass spectrometric or electrochemical detection represents the methods of choice for the determination of metanephrines. Immunoassay methods suffer from imprecision, and they may underestimate the concentrations of metanephrine and normetanephrine. For correct measurements of plasma metanephrines, blood must be drawn with the patient in the supine position and after 30 min of supine rest. Different medications may cause falsely elevated test results for plasma and urinary metanephrines. These medications can directly interfere with measurement methods (e.g., acetaminophen, mesalamine, sulfasalazine in LC-ECD methods) or interfere with the disposition of catecholamines (e.g., tricyclic antidepressants).

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16 Neuroendocrine Gene Transcripts: The Role of Molecular Biomarkers in Diagnosis and Management

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16.1 Overview

16.2 Brief History of Biomarkers

Biomarkers as an entity broadly describe tools and technologies that can facilitate the prediction, cause, diagnosis, progression, regression, or outcome of treatment of disease. Their identification and measurement are used to evaluate and examine a number of processes including normal biological functions, pathological events, or pharmacologic responses to a therapeutic intervention. In general, biological markers (biomarkers) are considered "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids" [1]. Although the majority of previous biomarkers have assessed cell surface or secreted proteins, the current focus has been on the identification of "candidate" biomarkers expressed in the nucleus or cytoplasm. At present, there is limited data on this group, and not all of the putative markers are clinical accessible.

Although the term "biological marker" was introduced in the 1950s [2], widespread use of the term "biomarker" appeared much later in about 1980 [3]. In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [4]."

Biomarkers can be correlated with the presence or level of a disease state or assessment of the efficacy of diverse therapies (surgery, pharmacological agents, radiation, or tissue ablation). Objective assessment of a marker involves the application of a variety of diverse techniques including biopsy, imaging, cell collection, or complex genomic measurements. Many recent biomarkers are focused on the issue of treatment selection, and currently most require tumor biopsy samples and invasive protocols. Overall, the "biomarker space" is diverse and broad. It includes delineation of novel chemicals, the mechanistic development of more accurate/effective tests, clinical positioning, informatics, and subsequent regulation of tests. In most clinical

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circumstances, irrespective of the efficacy of a biomarker, adjunctive usage of an imaging modality is usually a requisite for precise identification of tumor location (Fig. 16.1).

16.2.1 Types

It has been proposed that a categorization of biomarkers would better capture the different areas that each might address. To this end, the NIH has offered a biomarker classification system based on validation and clinical usage which comprises three categories (Fig. 16.1) [5]. *Type 0* markers, "indicators of the natural history of disease," correlate with diagnosis, prognosis, and outcome, but their relationship with the disease may be limited. *Type I* "captures the effects of an intervention in accordance with the mechanism of action of the drug." Such a biomarker reflects general efficacy of treatment, without necessarily being linked to the specific mechanism being measured. *Type II* markers are surrogates for clinical endpoints which may variously reflect patient health, functionality, or survival. The application of this system to the development of NET biomarkers is particularly difficult since NET disease is not a single entity and comprises a heterogeneous group of neoplasia. Since these exhibit a broad spectrum of biological, pathological, and clinical manifestations, a more complex array of biomarkers would likely be needed to capture the spectrum of pathobiology.

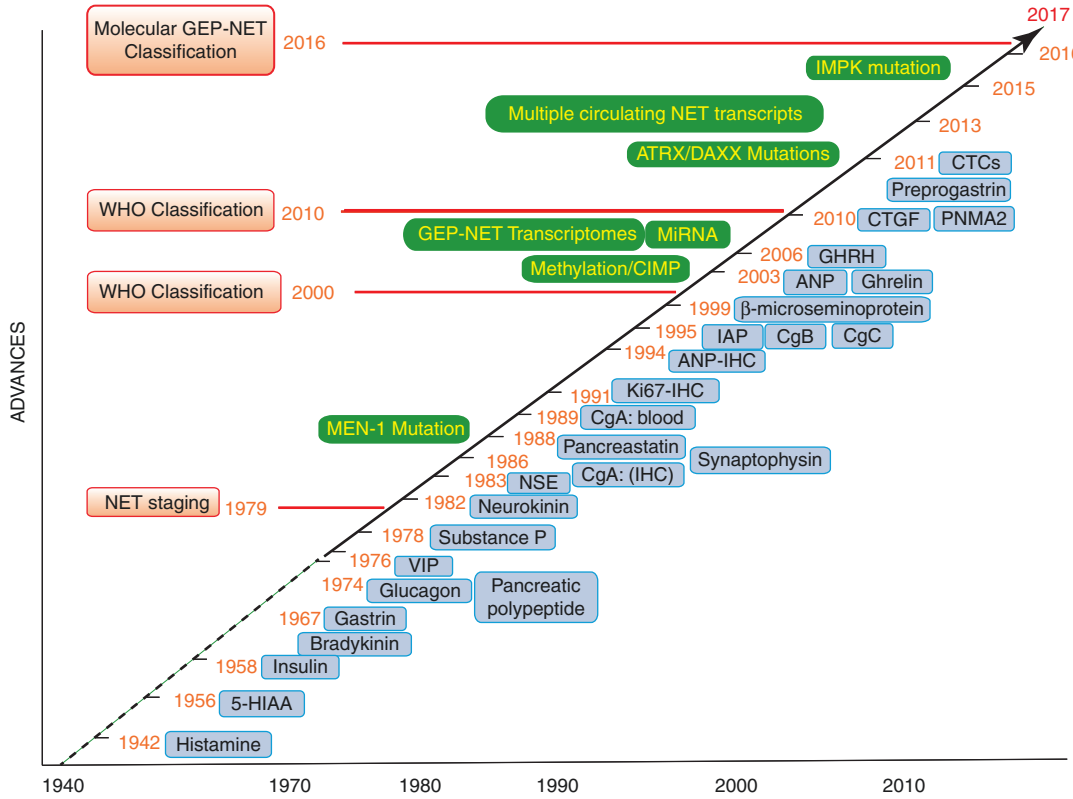


Fig. 16.1 Timeline of discoveries in monoanalyte and multianalyte NET biomarkers (1942–2016). WHO staging is provided for context (1979, 2000, and 2010) as are molecular classification advances including somatostatin receptor imaging and mutational status [31]. Previous monoanalyte biomarkers (*right side of the diagonal in blue*). Molecular-based strategies (*left side of the diagonal -green*) include mutation analysis, tumor transcriptomic classification, and miRNAs and multiple circulating NET transcripts (NETest)

16.2.2 Compartments

To date most biomarkers (tissue or blood/urine) have been single analytes with varying degrees of sensitivity and specificity. More recently, saliva and even lachrymal secretion have provided useful biomarker information [6].

16.2.3 Development

A variety of steps are required to develop and utilize a biomarker in the clinical setting. These include:

(a) *Proof of concept* (used to identify the specific characteristics of the biomarker).
(b) *Experimental validation* (required for the development of the most adaptable protocol for routine use. Simultaneously, it is possible to confirm the relevance of the protocol with various methods (histology, PCR, ELISA, etc.) and to define strata based on the results.
(c) One of the critical steps is *analytical performance validation* which serves to identify specific characteristics of the candidate biomarker before developing a routine test. Several parameters are considered including sensitivity, specificity, robustness, accuracy,and reproducibility.
(d) *Protocol standardization* is required to optimize the validated protocol for routine use, including analysis of the critical points by scanning the entire procedure to identify and control the potential risks. Ultimately, a biomarker, once defined and clinically validated, requires to be tested in a CLIA-certified laboratory.

16.2.4 Metrics

The scientific "power" of a biomarker is based upon its sensitivity and specificity. This is often colloquially referred to as "accuracy." While the individual significance of a test may be decided by clinical purpose, biomarkers as a group are valuable as objective quantifiers. Sensitivity is the rate of true positives, or the percentage of diseased individuals detected by a test. Specificity represents the percentage of individuals without the disease—hence those who are truly negative. Low sensitivity results in a high false-negative rate, while low specificity results in frequent false positives. The use of the term accuracy is best avoided since it does not adequately capture the effective power of a test if either parameter is too low. The ideal representation of the sensitivity and specificity balance is provided by the receiver-operating curve (ROC), in which *each point represents a conditional probability of a test result from a random diseased subject exceeding that from a random non-diseased subject* [7]. This creates a graphical representation where the area under the curve (AUC) represents a convenient ideogram of the diagnostic power of the test. The comparative magnitude of an AUC thus enables biomarkers to be effectively compared in the same graph. In general, the higher the AUC, the better the performance characteristics of a biomarker. An AUC greater than 0.8 (moderately accurate) is considered as reasonable and appropriate for clinical use [8]. In order to further amplify the information provided by a biomarker and increase the clinical utility, it is important to objectively define if an individual test result is reliable, whether positive or negative. This can be assessed by the development of positive and negative predictive values (PPV and NPV, respectively).

16.3 Current Circulating Biomarkers in NETs

16.3.1 Utility

Neuroendocrine tumors (NETs) are relatively unique in that they secrete bioactive products, either amines or peptides into the circulation. Such products are detectable and quantifiable. Circulating products include monoanalytes specific to individual cell types, e.g., gastrin and gastrinomas; co-secreted products that are common to all NETs, e.g., chromogranin A; or other individual secreted components, e.g., NSE. Other proposed biomarkers include pancreastatin [9, 10], a derivative of CgA, and neurokinin A

(Substance K), a ten-amino acid peptide translated from the pre-protachykinin gene [11]. Specific tumor secretory biomarkers (insulin/glucagon/VIP/gastrin) are usually effective serum indicators of specific tumor activity e.g., gastrin and gastrinoma, but since this group of lesions, e.g., gastrinoma, represents a minority of NENs (<3–5%), their broad utility is limited. Moreover, while useful in diagnosis and the identification of disease recurrence, such markers are disappointing in the assessment of disease progression, since they predominantly reflect secretory activity and evolving lesions may exhibit alterations in their secretory pattern during progression [12].

CgA is a constitutive product of the neuroendocrine cell secretory granule and is measurable in serum or plasma. It has been variously reported to correlate with tumor biology and mass and prognosticate survival [13, 14]. Despite initial promising results, the diverse limitations of CgA (poor laboratory metrics, nonspecificity, diagnostic inaccuracy) have become increasingly evident and resulted in a significant decrease in enthusiasm for its clinical utility [15].

16.3.2 Limitations

The most significant limitation of monoanalytes is that a high proportion of NETs (~30%) are "nonfunctional" and do not secrete detectable products. Furthermore, small tumors may be hypersecretory, while large tumors may exhibit low secretion. Specific receptor targeting agents, e.g., somatostatin analogs, also decrease secretion through inhibition of synthesis and secretory machinery. Abnormalities in the secretory pathway itself, e.g., differential expression of prohormone convertase enzymes, may also exist. The latter impacts CgA processing resulting in differential cleavage products and heterogeneous secretion into the circulation [16]. Irrespective of the output, measurement of products only reflects the secretory function and capacity of tumor cells, not their proliferative, metabolic, or metastatic potential.

Various assays have been developed to measure these agents, but no universally accepted monoanalyte assay or positive control/gold standard exists. For example, CgA levels from the same sample fluctuate extensively between test platforms, all of which have varying sensitivities and specificities [17], and widely differing coefficients of variations [18]. Despite these limitations, circulating secretory products have been evaluated as biomarkers to assess disease recurrence and progression in NETs. Although there has been some improvement regarding the use of comparable units of measurement, there is no reference CgA standard, and wide variations exist in the assay measurements in different laboratories [13]. Furthermore, the sensitivity of CgA ranges from 60 to 90% with a specificity <50% (depending on the population studied) [19]. This reflects the spurious CgA elevations associated with numerous non NEN-related conditions including renal failure, cardiac disease, and other neoplasia and drug administration (proton pump inhibitors) [13].

16.3.3 Mono vs. Multianalyte Measurement

The complexity and diversity of the biological behavior of a cancer or its response to therapy have been effectively addressed by Hanahan and Weinberg [20, 21]. As such, the limitations of secretory products of a cell alone to define the permutations of oncogenic genomic regulators are apparent and have led to the development of molecular technologies to better delineate cancer biology [22, 23]. This biological research has identified extensive interfacing mechanisms that delineate GEP-NEN neoplastic development [24]. Nevertheless, numerous questions regarding the regulation of tumor growth, metastasis, and immune interactions remain unresolved. Measurements of exocytotic and secreted proteins do not reflect the biological activities including cell proliferation, growth factor signaling, etc. that constitute the "hallmarks of cancer" [21]. Diverse oncological disciplines have therefore concluded that a dynamic and panoramic delineation of the biological topography of an evolving neoplasm can be optimally captured by a multidimensional assessment of the molecular genomic machinery of the tumor cell (Table 16.1).

Table 16.1 Biological and clinical topography of monoanalytes vs. multianalytes

Detection indices	Monoanalyte	Multianalyte
Pathobiology		
Mutations	No	Yes
Proliferation	No	Yes
Secretion	Yes	Yes
Metabolism	No	Yes
Epigenetic remodeling	No	Yes
Apoptosis	No	Yes
Signaling pathway activity	No	Yes
Cell of origin	Yes	Yes
Clinical		
Syndrome identification	Yes	No
SSR expression quantification	No	Yes
Prediction of therapy efficacy	No	Yes
Measurement of treatment response	No[a]	Yes
Identification of Residual disease	No[b]	Yes

SSR Somatostatin receptor
aOnly symptomatic therapy
bOnly in specific cases, e.g., gastrinoma/insulinoma

16.4 Contemporary and Novel Biomarkers in NETs

16.4.1 Tissue

Mutations: Molecular strategies have to date mostly focused on DNA alterations and have for the most part been clinically non-informative. Unlike most other cancers, activating mutations are infrequent, or largely unknown, in GEP-NENs [25]. Indeed, most GEP-NENs exhibit mutations in tumor-suppressor genes, either germline or sporadic. Inherited genetic alterations typically underlie ~5% of pancreatic NENs and are also occasionally (rarely) evident in small intestinal tumor types. Pancreatic NENs are associated with germline mutations in *MEN1* (menin) [26, 27], *VHL* (von Hippel–Lindau tumor suppressor) [28], *NF1* (nuclear factor 1) [29], and *TSC* (tuberous sclerosis complex) family [30]. Mutations in *MEN-1*, the predominant NEN mutation (pancreatic NENs), are not currently considered useful in clinical management, i.e., as a prognostic index or as a predictor of drug therapy efficacy [31]. Moreover, the clinical utility of alterations in ATRX, DAXX, mTOR signaling [32] and YY1 [33] (all principally identified as sporadic mutations in pancreatic NENs) remains to be proven.

Small bowel NENs represent one of the most genetically stable cancers and are characterized by low mutation (average 1 nucleotide variation per 10^7 base pairs) rates, [34] similar to myeloid leukemia or rhabdoid tumors [35]. Mutations in *MEN1*, *DAXX*, or *ATRX* have not been identified. However, 8% of SINENs exhibit small insertions or deletions in *CDKN1B,* which inactivate the cell cycle inhibitor that this gene encodes: cyclin-dependent kinase inhibitor 1B (also known as $p27^{KIP1}$) [36]. Genotype–phenotype correlations that are likely to be clinically relevant to management or outcomes of the disease are rare for mutations associated with GEP-NENs.

Methylation: Alterations in CpG island methylation are well recognized in GEP-NENs, and methylation-based classifications are currently being considered [31]. Gene-specific hypermethylation or hypomethylation is, however, less commonly observed in pancreatic than in gastrointestinal NENs [37]. In pancreatic NENs, aberrant methylation of tumor-suppressor genes is typically associated with advanced tumor stages and identifies molecularly distinct tumors (despite identical histological classifications) [37, 38].

The clinical utility of methylation patterns in GEP-NEN is unknown, but such methylation patterns could potentially be of use as prognostic markers [31].

Transcriptome: Gene-expression data has been developed for pancreatic and small bowel NEN. For pancreatic NENs, a broad overlap between the histology and gene-expression profiles suggests that molecular alterations largely recapitulate histological delineation and can be used to better define current categorizations. Two subtypes of small intestinal NEN have been identified through gene-expression profiling: the first synthesizes and secretes serotonin only and the

second subtype produces serotonin as well as substance P and other tachykinins [39].

Gene-expression data can also be used to confirm different organs of origin of NENs and thus have been used to develop clinically relevant tests for detecting tumor origin in patients with CUPs [40]. The most useful data from gene-expression profiling is the identification of circulating tumor RNA that can provide the basis for development of blood-based biomarker signatures (Table 16.2). The latter (discussed in detail below) have been shown to be clinically useful in the diagnosis of GEP-NENs [41] and directly recapitulate tumor-based gene-expression data that predict disease progression [24].

16.4.2 Circulation

mRNA: Recently, transcriptional profiling of tumor tissue has identified a series of neuroendocrine transcripts that are detectable in the circulation [42] and can be used clinically to evaluate GEP-NEN [41, 43–48] and paraganglioma–pheochromocytomas [49]. This blood-based multianalyte transcript analysis [41–47, 50–52] is also the most extensively investigated circulating molecular biomarker tool.

The NETest assesses biological activity using gene inference technology and cancer hallmark prediction [24]. Details of the PCR methodology, mathematical analysis, and validation have been published [24, 42, 46, 50]. Individual genes were selected by analyzing microarray data and sets of cellular profiles from fresh frozen tumors as well as from whole blood of NET patients to identify similarities in expression patterns [42]. Once identified, undertaking co-expression network inference with normal tissue eliminated genes that were considered unlikely to be neoplasia relevant [42]. A similar strategy was used to minimize tumor-associated genes from other tumor types, e.g., the breast. Using this analytic and computational strategy, candidate marker genes detectable in peripheral circulation were identified as representative of NET. Redundancy of expressed genes and systematically selected analytes enables this type of test to be significantly more robust than monoanalyte tests such as CgA or pancreastatin [48].

The multianalyte algorhythmic analysis (MAAA) procedure is well validated [50] and is undertaken in a CLIA-certified clinical laboratory environment. The test has been demonstrated to exhibit a reliably high level of sensitivity and specificity (both >95%) [42]. The PCR test is standardized and reproducible (inter- and intra-assay CV <2%) [50] and is not affected by age, gender, ethnicity, fasting, or PPI medication [43, 50]. The test utilizes a two-step protocol (mRNA isolation, cDNA production and PCR) [42, 50] from EDTA-collected whole blood (Fig. 16.2) [42, 50].

The expression of 51 NET marker genes includes analysis of clusters of biologically rele-

Table 16.2 Molecular abnormalities in GEP-NET

Organ	Familial mutation(s)	Somatic	Methylation CIMP	Transcriptome	miRNA
Stomach	MEN1	No data	No data	Yes	No data
Duodenum	MEN1	No data	No data	No data	No data
Pancreas	MEN1, VHL, NF-1, TSC	MEN1, ATRX, DAXX, mTOR pathway, YY1	Yes	Yes	Yes
Small bowel	IPMK	CDKN1B	Yes	Yes	Yes
Appendix	No data	No data	No data	No data	No data
Colon	No data	No data	No	No data	No data
Rectum	No data	No data	No	No data	No data

ATRX Alpha thalassemia/mental retardation syndrome X-Linked, *CIMP* CpG island methylator phenotype, *DAXX* death domain-associated protein, *CDKN1B* cyclin-dependent kinase inhibitor 1B (or $P27^{KIP1}$), *IPMK* inositol polyphosphate multikinase, *MEN1* multiple neoplasia type I, *NF-1* neurofibromatosis, *TSC* tuberous sclerosis, *VHL* von Hippel–Lindau, *YY1* yin-yang 1

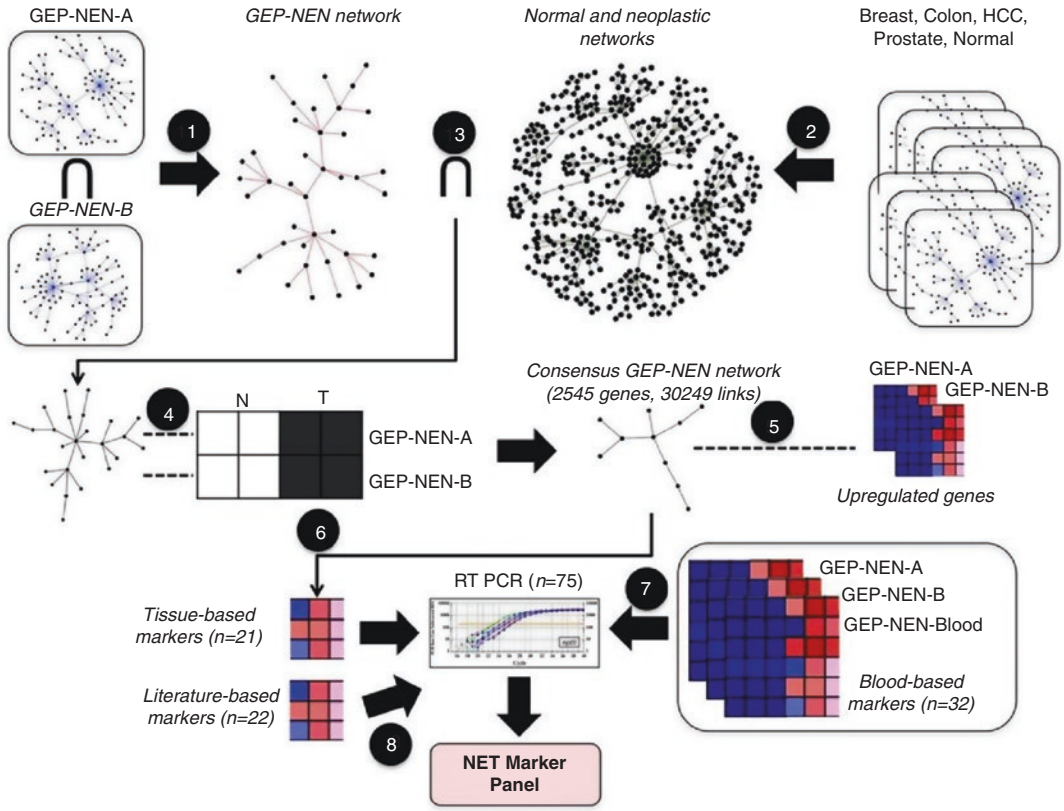


Fig. 16.2 Computational pipeline utilized to derive a set of marker genes, the "NET Marker Panel" that identifies GEP-NEN/NET disease in the blood. *Step 1*: Gene co-expression networks inferred from two independent datasets (GEP-NEN-A and GEP-NEN-B) are intersected to produce the GEP-NEN network. *Step 2*: Co-expression networks from neoplastic and normal tissue microarray datasets are combined to produce the normal and neoplastic networks. *Step 3*: Links present in normal and neoplastic networks are subtracted from the GEP-NEN network. *Step 4*: Concordantly regulated genes in GEP-NEN-A and GEP-NEN-B networks are retained; other genes are eliminated from the GEP-NEN network, producing the Consensus GEP-NEN network. *Step 5*: Upregulated genes in both the GEP-NEN-A and GEP-NEN-B dataset are mapped to the Consensus GEP-NEN network. *Step 6*: Topological filtering, expression profiling, and literature curation of putative tissue-based markers reveal 21 putative genes further examined by RT-PCR. *Step 7*: Identification of mutually upregulated genes in GEP-NEN blood transcriptome and GEP-NEN-A and GEP-NEN-B datasets, yielding 32 putative genes further examined by RT-PCR. *Step 8*: Literature curation and cancer mutation database search, yielding a panel of 22 putative marker genes for further RT-PCR analysis. A total of 75 marker genes was analyzed to identify and define the final NET Marker Panel

vant genes that constitute the different "omes" (SSTrome, proliferome, metabolome, secretome, epigenome, and pluromes) [24] which define the NET "fingerprint." Blood gene expression of tumor biomarkers closely correlates with tumor tissue expression levels. Cluster gene analysis captures the biology of neuroendocrine neoplasia thereby facilitating accurate molecular definition of clinical status [24]. Blood gene expression is normalized to housekeepers and quantified versus a population control [42]. Multianalyte algorithm analysis (MAAA) was undertaken (SVM, LDA, *KNN*, and *Bayes*) for categorization into different groups using "majority vote" [42]. This results in a 0–8 score [42, 50] which is mathematically converted to an activity ranging from 0 (low activity) to 100% (high activity) based on expression of "omic" genes [24]. Elevated expression of these genes is used to weight the score such that a high score, e.g., "8," when com-

bined with elevated "omes" (identified to differentiate progressive from stable disease [24]) is scaled to 100% (high activity). A score of "8" with a low "ome" is weighted to 53%. Activity score ranges to classify disease activity were then developed by assessing 3000 individual patient blood samples. The ranges that conform to clinical disease assessment in NETs are low activity, 0–40%; moderate activity, 40–79%; and high activity, 80–100% [24].

The signature can identify all types of GEP-NEN, bronchopulmonary NETs, and small nonmetastatic tumors. Comparison assessment with other NET biomarkers significantly outperforms monoanalyte-based assays for detection [42, 48]. In addition, levels correlate with clinical status, e.g., stable or progressive disease [53]. Mathematical analyses of multianalyte methodology determined this technique was superior to single-analyte assays in the detection of NETs [54].

CTC: Circulating tumor cells (CTCs) are currently detected through expression of EpCAM—an epithelial cell marker. Data CTC usage as prognostic markers remains scarce and is described from a single center with a correlation with tumor burden in metastatic NET disease [55, 56]. The low number of patients with detectable CTCs, coupled to heterogeneity in EpCAM expression, as well as the absence of significant relationships with therapeutic response has proved disappointing [15, 57]). The more recent identification that the majority of CTCs are somatostatin receptor negative is inconsistent with other scientific information regarding NETs, and a number of recent international consensus meetings have expressed concern regarding the scientific basis and clinical utility of CTC measurement in NET disease [15, 57]. Currently, it is concluded that CTC measurements in NENs are unproven as being of clinical utility as a biomarker of progression.

cfDNA: Circulating free tumor DNA is considered potentially useful in other cancers, but this has not been effectively studied in NETs. Measurements of specific tumor mutations may be relevant to identifying tumor progression in other neoplasia, e.g., prostate cancer [58]. The absence of clinically informative mutations in NETs, current known mutations in MEN-1 [59, 60], ATRX, DAXX, and mTOR signaling [32] and YY1 [33] or the recently identified *IMPK* mutation in a single small bowel carcinoid family [61] remain to be defined. It is likely that cfDNA measurement may be difficult to implement in NENs.

Methylation: The clinical usefulness of chemical-based DNA modifications, e.g., methylation, requires substantial elucidation. This largely negates the utility of cfDNA as markers of progression until significant data is available.

miRNA: MiRNAs are a class of small (19–25 nucleotides) noncoding RNAs that function as posttranscriptional regulators in diverse disease processes. Alterations in miRNA have been identified in the circulation, and levels may be decreased by SSA usage [62]. However, as for other monoanalyte assays, detection and quantification of miRNAs remain challenging for a number of reasons. Metrics are currently platform specific and dependent on vendor reagents, and data normalization remains problematic. The evaluation of miRNA as biomarkers in disease progression is challenging since currently no standardization and normalization methodology has not been adequately characterized.

Metabolomics: Metabolomic approaches have been suggested as an alternative for identifying potential biomarkers. While effective for differentiating NETs into different metabolic subgroups, e.g., functional versus nonfunctional [63], its use to identify markers of progression remains unsubstantiated (Fig. 16.3).

16.5 NETest

16.5.1 Diagnostic Utility (Fig. 16.4, *Left*)

Bronchopulmonary NETs: The NETest has been evaluated in over 150 bronchopulmonary neuroendocrine tumors including typical and atypical carcinoids as well as in large cell neuroendocrine carcinoma and small cell neuroendocrine carcinomas [64]. Detectable mRNA in blood was identified in all tumor types (100%) with a neuroendocrine phenotype. NETest levels,

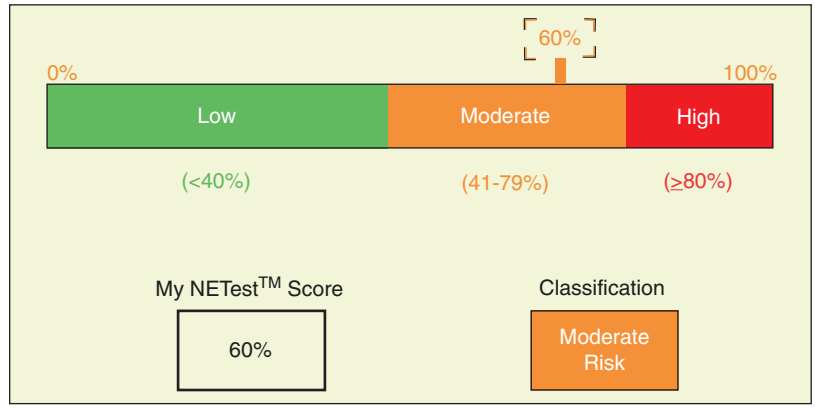


Fig. 16.3 The circulating NET transcript gene-expression data is expressed as a single score scaled 0–100%. This scaled GEP score—the NETest—delineates individual categories of low (<40%), moderate (40–79%), and high (≥80%) risk for disease activity

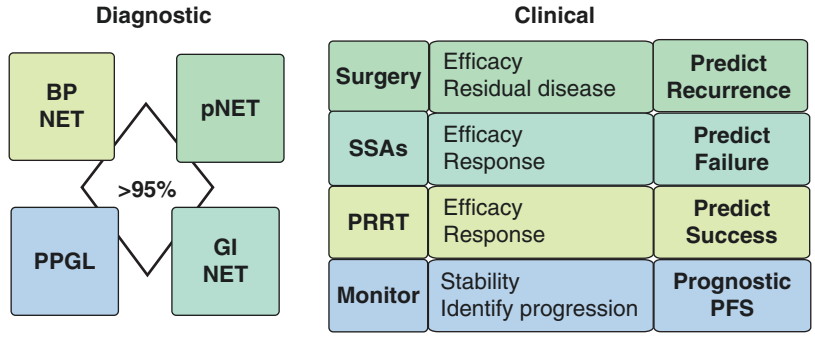


Fig. 16.4 Clinical utility of a multianalyte assay for neuroendocrine tumor diagnosis and management. *Diagnosis*: The MAAA can detect bronchopulmonary (BP), pancreatic (pNET), and gastrointestinal tract (GI) NET with ≥95% accuracy. In addition, the NETest is able to diagnose paragangliomas and pheochromocytomas (PPGL). *Management*: MAAA has clinical utility in four areas: (**a**) evaluate the efficacy of a surgical procedure and (**b**) identify residual disease. This allows for a prediction of disease "recurrence." (**c**) Evaluate treatment response to somatostatin analog (SSA) use, and (**d**) predict treatment failure (disease progress). Radioreceptor therapy (PRRT) response to therapy can be predicted (with tumor grade), and transcript levels monitor efficacy and response. Overall MAAA can identify disease stability and detect disease progression. It is prognostic and can predict PFS in GEP-NET

in particular, were significantly increased in those with RECIST-defined progressive disease than clinically stable disease or those considered surgical cures. Levels were greater in disseminated disease than local. As a comparator, chromogranin A was elevated in <50% and was not clinically useful as a biomarker.

Pancreas: In pancreatic disease, the NETest is useful for accurately confirming neuroendocrine disease (compared to other cancers and nonneoplastic diseases, e.g., chronic pancreatitis) [41]. In one study, the accuracy was 94% (96% NETs positive; two (6%) of intraductal papillary mucinous neoplasms (IPMNs) were positive) [41]. In comparison, the accuracy of CgA was 56% (only 29% of pancreatic NETs were CgA positive). Overall, the NETest was significantly more sensitive than CgA for the detection of pancreatic NETs.

Gut: In one study, the accuracy of the NETest for detecting small intestinal NETs was 93% (all NETs positive and three (12%) colorectal tumors were positive) [41]. CgA was positive in 80%, but 29% ($n = 7$) of colorectal cancers were CgA positive. Overall, the NETest was significantly more sensitive than CgA for the detection of small intestinal tumor (area under the curve 0.98 vs. 0.75 $p < 0.0001$). In this study, NETest scores

were elevated ($p < 0.05$) in extensive disease and were more accurate (76–80%) than CgA levels (20–32%) [41]. The metrics of the multianalyte NETest met the performance criteria (i.e., >80% accuracy) proposed by the NIH for biomarkers, whereas CgA measurement did not.

PPGL: Paragangliomas and pheochromocytomas (PPLs) are NETest positive (100%) [49]. A ROC analysis area under curve was 0.98 for differentiating PPGLs vs. controls. Although the mutation status was not directly linked to NETest levels, genetic and molecular clustering was significantly associated ($p < 0.04$) with NETest scores. Metastatic (80 ± 9%) and multicentric (64 ± 9%) disease had significantly ($p < 0.04$) higher scores than localized disease (43 ± 7%). Progressive disease had the highest scores (86 ± 2% vs. stable 41 ± 2%, $p < 0.0001$). Prolific, epigenetic, and somatostatin receptor gene expression was significantly elevated in progressive disease, while metabolic gene expression was decreased in those with SDHx mutations. In this study, successive NETest measurements accurately defined the clinical status, i.e., identified whether a patient was stable or exhibited progressive disease. Elevated NETest was noted to be prognostic in all cases when the NETest was elevated [49].

16.5.2 Management Utility (Fig. 16.4, *Right*)

Surgery: NETest has been evaluated in GEP-NEN undergoing surgery. In a prospective study [44], the score was elevated in all 35 patients (100%) preoperatively. In comparison, only 14 (40%) had elevated CgA. Resection reduced NETest from 80 ± 5% to 29% ± 5 ($p < 0.0001$). NETest decreases correlated with diminished tumor volume (R [2]=0.29, $p = 0.03$). CgA decrease was insignificant (14.3 ± 1.6 U/L to 12.2 ± 1.7 U/L) and did not correlate with tumor reduction. Interestingly, 4 (36%) of 11 R0s with elevated NETest at 1 month subsequently developed positive imaging (sensitivity 100%, specificity 20%) within 6 months of surgery. These results identify that blood NET transcripts delineate surgical resection/cytoreduction and facilitate early identification of residual disease.

The majority of monoanalyte studies are retrospective in nature. In one large, multinational retrospective study (339 patients who underwent surgical management for hepatic metastases), relapse was not anticipated by changes (increases) in CgA levels [65]. The absence or significant decreases in CgA levels in those who have detectable levels may be informative. A retrospective Danish study identified that normal postoperative CgA levels were associated with a 100% 5-year survival rate [66]. However, no data was provided as to the percentage of patients that initially expressed normal CgA levels. In a smaller study ($n = 22$), a ≥ 80% reduction in CgA levels following cytoreductive surgery for carcinoid tumors predicted subsequent symptom relief and disease control, despite incomplete cytoreduction [67].

Drug Efficacy: In a prospective, blinded study, the utility of the NETest was evaluated compared to CgA for the ability to determine somatostatin analog (SSA) efficacy [46]. In this study, a cutoff ≥ 80% was used as this had been shown to accurately differentiate stable from progressive disease. In the 28 patients on SSAs (14 on sandostatin, 14 on lanreotide), only NETest ($p = 0.002$) and tumor grade ($p = 0.054$) were associated with therapy response. Multiple regression analysis, however, identified that only NETest could predict PD ($p = 0.0002$). NETest changes occurred significantly earlier than image changes (146 days prior to image-defined progression) and occurred in 100% of patients who progressed. CgA was not identified as predictive of SSA therapy. This study [46] identified that NETest exhibited utility in predicting SSA treatment response.

Regarding monoanalytes, a 30% decrease in CgA (from pretreatment levels) may be predictive of a response to SSA confirming the secretory dimension measured [68]. This was not observed in the Cwikla et al. study [46].

PRRT Efficacy: Peptide radioreceptor therapy (PRRT) is a well-established NET therapy. Typically, patients are chosen based on degree of somatostatin receptor uptake, but not all respond

to therapy [69]. This identifies that the biological and genetic nature of the tumor may be important in defining those that will respond than arbitrary assessment of SSTR expression. Based on this premise, a molecular evaluation of prediction was undertaken using the NETest and relevant "omic" components (the growth factor signalome and metabolome) as predictors of PRRT efficacy. In this large (n = 54) prospective study, 94% of patients (GEP-NEN and BPNEN) were NETest positive prior to PRRT [47]. CgA was elevated in only 59%. Changes in the NETest accurately (89%, $p < 10^{-6}$) correlated with treatment response, while changes in CgA were only 24% accurate. Combination of tumor grade and gene cluster expression (growth factor signalome and metabolome) had an AUC of 0.90 for predicting tumor response to PRRT. Circulating transcripts correlated accurately (94%) with PRRT responders (97%) vs. nonresponders (91%). This study identified that PRRT efficacy could be accurately predicted using circulating NET transcripts and tumor grade.

Prognosis: A recent study has evaluated the utility of the NETest as prognostic [45]. This long-term (5-year) study in 34 patients identified that the NETest has predictive and prognostic utility for GEP-NETs, identifying clinically actionable alterations approximately 1 year before image-based evidence of progression. Cox modeling identified that the only factor associated with PFS was the NETest. Moreover, a baseline NETest >80% was significantly associated with disease progression (median PFS 0.68 vs. 2.78 years with <40% levels). In contrast baseline NETest levels >40% in those defined as clinically stable were 100% prognostic of disease progression. Baseline NETest values <40% accurately (100%) predicted stability over 5 years.

Changes in the NETest was more informative (96%) than CgA changes (>25%) in consistently predicting disease alterations. In addition, significant alterations in the NETest occurred at an earlier time-point change than imaging (~1 year). These data identify a role for the NETest as a prognostic factor.

Increases ≥25% in CgA have been proposed to have high sensitivities (78–86%) and specificities (86–91%) for the prediction of disease events [70]. Others have suggested that levels twice the upper limit of normal (~300 μg/l or 300 ng/ml) or higher (≥600 ng/ml) [71] may be effective predictors of shorter PFS. None of these parameters were identified to be effective in the study by Pavel et al. [45]. This is consistent with the observation that retrospective observations do not effectively translate into clinical practice (Fig. 16.5) [72].

Elevated pancreastatin has been suggested to be prognostic for disease recurrence, while

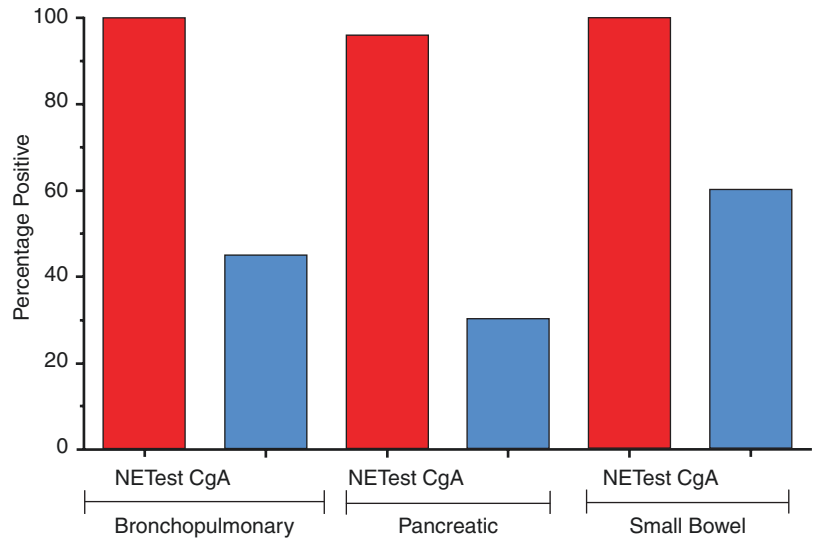
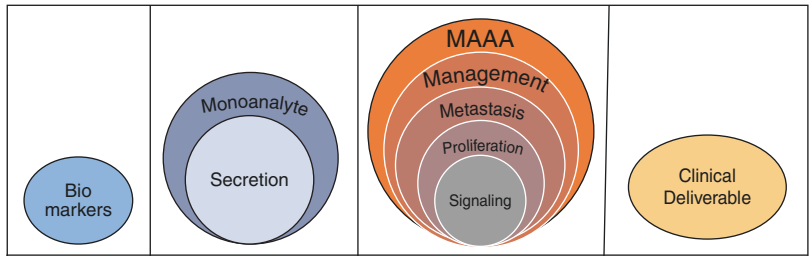


Fig. 16.5 Accuracy of the circulating multiple NET transcripts—NETest—compared to chromogranin A. The MAAA is positive in 96–100% of bronchopulmonary, pancreatic, and small bowel NET. Chromogranin A (CgA) in contrast is only positive, i.e., detectable as elevated in 30–60%. Normal CgA levels are normal in 40–70% of NETs, significantly limiting its clinical utility as a biomarker



Type 0	20-80%	>90%	Diagnostic
Type I	10-50%	>90%	Therapeutic
Type II	No prospective studies	>90%	Prognostic

Fig. 16.6 Comparison between monoanalytes and multianalyte markers as type 0–II biomarkers. Monoanalytes are markers of secretion, and their metrics exhibit a wide range between 10 and 80% as type 0/I markers. No prospective studies have validated monoanalytes as type II biomarkers. MAAAs capture a diversity of tumor-specific biological information that are clinically relevant and have diagnostic, therapeutic, and prognostic deliverables. Multianalyte assays have >90% metrics for type 0, type I, and type II markers. *MAAA* Multianalyte assays with algorithm analysis

elevated NKA levels are considered an independent indicator of NET prognosis. Both observations are based on retrospective studies [73, 74]. Enthusiasm for clinical utility is diminished by a database of retrospective studies coupled to variability in measurement platforms, differences in predetermined cutoffs, and the absence of gold standard assays. Significant development and clinical testing are required before any role can be ascribed to these secretory products for predicting progression.

16.6 Future Directions

A critical requirement is the development of molecular tools that can better identify an appropriate drug target in a particular tumor and, thereafter, define treatment response. The demonstrable utility of circulating RNA as a biomarker supersedes that of standard monoanalyte biomarkers and has diverse clinical applicability (Fig. 16.6).

Current data suggests added value for the transcript analysis in the monitoring of a variety of therapeutic modalities (surgery, drug therapy, and PRRT), particularly in conjunction with other clinical and imaging parameters to monitor disease progression. The most promising future strategy for refining and improving the evaluation of therapy will be provided by a combination of imaging modalities and the blood-based molecular information provided by transcriptome analysis. Optimization of such tools can be enhanced by the development of nomogram-based mathematical indices for objective analysis of multiple molecular and clinical variables.

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