Diagnostic correlation between RET proto-oncogene mutation, imaging techniques, biochemical markers and morphological examination in MEN2A syndrome: case report and literature review

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Abstract
Multiple endocrine neoplasia type 2 (MEN2) is a rare autosomal dominant monogenic disorder caused mostly by missense mutations in the RET (REarranged during Transfection) proto-oncogene on chromosome 10q11.2. MEN2A represents more than 50% of all MEN2 cases, having a regular pattern with medullary thyroid carcinoma (MTC) incidence of 90–100%, bilateral pheochromocytoma (PCC) incidence of 40–50% and primary hyperparathyroidism (HPT) incidence of 10–25%. Until recently, the diagnosis of MTC was most frequently based on fine-needle aspiration of thyroid nodules, after an ultrasound examination and endocrine evaluation of serum calcitonin levels. Nowadays, RET gene screening (starting with exons 10 and 11) is a mandatory test used for identification of both symptomatic and asymptomatic MTC carriers or for exclusion of healthy individuals from subsequent periodical clinical/biochemical screening. In this context, and in the idea of PCC preceding MTC, the early detection of germline RET mutations is highly suggestive for hereditary disease. PCC diagnosis is established in classical manner by abdominal ultrasound imaging or computed tomography confirming the presence of adrenal gland masses, elevated plasma metanephrines and normetanephrines values and histopathological examination. Additional HPT diagnosis is acknowledged by serum ionized calcium and parathormone levels. Here we report a hereditary case of MEN2A in a two-generation Romanian family, along with data presenting the importance of correlative plurifactorial diagnostic scheme in this syndrome and a short literature review.

Keywords: MEN2A syndrome, RET gene, medullary thyroid carcinoma, pheochromocytoma, fine needle aspiration, serum calcitonin level.

Introduction
Multiple endocrine neoplasia type 2 (MEN2) is a rare autosomal dominant monogenic disorder caused by missense mutations in the RET (REarranged during Transfection) proto-oncogene, which activate an encoded transmembrane tyrosine kinase receptor [1]. It is a complex neoplastic neurocristopathy [2] with three distinct subtypes (A, B and F), according to the variable penetrance of medullary thyroid carcinoma (MTC), pheochromocytoma (PCC) and primary hyperparathyroidism (HPT).

MEN2A (Sipple syndrome, OMIM 171400) represents 50–75% of all MEN2 cases [1, 3]. It has the most regular pattern of all subtypes, with MTC incidence of 90–100%, generally bilateral PCC incidence of 40–50% and variable HPT incidence of up to 35% [1, 2, 4, 5]. MTC is generally the first clinical manifestation in young adults but tends to be present in the fifth or sixth decade of life with a slight female preponderance. The diagnosis of MTC is most frequently based on fine-needle aspiration (FNA) of thyroid nodules, after an ultrasound examination and endocrine evaluation of serum calcitonin (CT) levels. PCC can precede (13–27% of cases), be concomitant or appear after MTC. Diagnosed at an early age, it usually is bilateral and has a 4% risk of malignancy [4]. The presence of adrenal gland masses is established by ultrasound imaging of the abdomen or computed tomography followed by detection of elevated plasma metanephrines and normetanephrines values; still, the final confirmation of PCC is based on histopathological examination. Due to the inconstant presence of HPT, biochemical assays (e.g., serum ionized calcium [iCa] and parathyroid hormone [PTH] levels) must be done in order to verify the diagnostic.

MEN2B (less than 10% of MEN2 cases) has the most aggressive type of MTC, which is due to de novo germline mutations in more than half of patients [1]. Besides MTC (100% of cases), MEN2B is also characterized by PCC (50% of patients), but no HPT; patients have a characteristic appearance (i.e., marfanoid habitus with skeletal abnormalities and joint laxity, everted eyelids,
Familial MTC (FMTC) accounts for more than 30% of all MEN2 cases [1], with very low penetrance of PCC and HPT [1]. Usually, it is diagnosed when at least four members of the same family are affected by MTC after the age of 50, with no evidence of either PCC or HPT in more than 10 carriers [7]. Due to significant overlapping of gene mutations in both FMTC and MEN2A, differential diagnosis between the two syndromes can be challenging.

Since MTC is usually the main cause of death due to low response to chemo- and radiotherapy, the only therapeutic option is early detection or prophylactic surgery [5, 8]. Biochemical markers such as CT can be used for MTC diagnosis but only after the disease has developed. In view of this, genetic testing was introduced in clinical practice for early detection of mutations causing the disease [8].

RET gene on chromosome 10q11.2 is the mutated gene identified by high-resolution melting (HRM) analysis and sequencing, in nearly all MEN2 patients: 98% of MEN2A cases, more than 98% of MEN2B cases and about 95% of FMTC cases [9]; more than 50 different mutations and even more variants are so far described [6]. Very few cases, sporadic or familial, are caused by NTRK1 gene mutations [7]. Molecular analysis shows that in MEN2A patients, the frequently found RET gene missense mutations affect the cysteine (Cys)-rich extracellular domain encoded by exons 10 and 11 (most of them affecting the Cys residue from codon 634); other different RET mutations are associated with MEN2B or FMTC [1, 6, 7, 10]. Patients identified to be gene mutation carriers, have a stratified risk for malignancy [5, 11]. Germline RET mutations are present in hereditary cases but somatic mutations, which are restricted only to the tumor cells, can only be found in sporadic MTC.

Nowadays, RET molecular analysis starting with codons 10 and 11 [2] is extensively used for identification of both symptomatic and non-symptomatic carriers or for exclusion of healthy individuals [12] in families with MEN2 syndrome. Surgical procedures are recommended based on the genetic testing, according to the type of RET mutation [1, 11]. In case of any genetic testing, patients should receive appropriate genetic counseling and be presented the individual and familial risks and benefits. Family members of these patients have a 50% risk of inheriting the same RET gene mutation, due to the fact that the mutations are transmitted in an autosomal dominant manner, so they should be tested as well and offered prophylactic thyroidectomy if necessary [5].

Here we report a hereditary case of MEN2A in a two-generation Romanian family (a female patient and her children), along with data presenting the importance of correlutive pluriformal diagnostic scheme in this syndrome and a short literature review.

The study was developed in accordance with the WMA Declaration of Helsinki and was approved by the University Ethics Committee. Informed consent was obtained from all individuals included in the study prior to any clinical, biological and genetic testing.

**Patients, Methods and Results**

In June 2012, a 55-year-old, non-obese female patient with a medical history of left adrenalectomy for PCC 18 years ago, was admitted to the Endocrinology Clinic with permanent hypertension. Once every few days, symptoms worsened with blood pressure reaching 210/110 mmHg, concomitant headache, chest pain, tachycardia, sweating and intense panic. Ischemic stroke was documented one year prior to hospital admission.

Laboratory evaluation revealed normal serum fasting glucose (108 mg/dL), increased serum cholesterol (224 mg/dL) and normal blood cells count and serum electrolytes, including ionized calcium (iCa) levels.

Thyroid status evaluation showed serum thyrotropin (TSH) level of 0.76 mU/L (normal range 0.5–4 mU/L) and serum free thyroxin (FT4) level of 0.81 ng/dL (normal range 0.8–1.4 ng/dL). However, the anti-thyroid peroxidase antibodies (anti-TPO) titer was elevated by more than 1400% (700.3 U/mL vs. normal 50 U/mL), confirming a chronic autoimmune thyroiditis, which was previously suggested by the inhomogeneous, hypo-echoic and multinodular thyroid on neck ultrasound examination. Some enlarged cervical lymph nodes were also found (Figure 1).

![Figure 1 – Inhomogeneous, hypo-echoic and multinodular thyroid on ultrasound examination.](image)

Endocrine evaluation of the adrenal medulla function revealed elevated plasma metanephrines levels of 275.8 pg/mL (normal <90 pg/mL) and normetanephrines levels of 220.3 pg/mL (normal <180 pg/mL).

Ultrasound imaging of the abdomen followed by computed tomography confirmed the presence of a right, well-delineated adrenal gland mass of 44/34 mm in diameter.

In this context, the diagnosis of right PCC was established and the patient was put on combined alpha and beta-adrenergic blocker therapy with Doxazosin (Kamiren®) 3 mg/day and Metoprolol 50 mg/day, which normalized the blood pressure. After two weeks, a right laparoscopic adrenalectomy was performed, followed by lifelong substitutive therapy with gluco- and mineralocorticoids.

After the ablation of the tumor, microscopic examinations of the sections have reconfirmed the diagnosis
of PCC (Figure 2, a–d). Hematoxylin–Eosin (HE) revealed a tumor proliferation of medium-size cells grouped into alveolar structures and solid nests; tumor cells presented round or oval nuclei, rare nucleoli with low pleomorphism and some mitotic figures; extensive areas of edema were also found; there were no histological signs of capsular or vascular invasion and the peripheral areas showed normal adrenal gland histology. Supplemental data was obtained by an immunohistochemistry (IHC) panel of monoclonal antibodies, used either for staining neuroendocrine tumors or to differentiate PCC from several other types of neoplasia. Tumor cells showed positivity for neuroendocrine neoplasm markers: anti-synaptophysin (DAK-SYNAP, DAKO clone) and anti-chromogranin A (DAK-A3, DAKO clone), and were negative for S100 protein (used to reveal the sustentacular cells from paragangliomas). Several other markers were applied for differential diagnosis with local or neighboring carcinomas: Epithelial Membrane Antigen (EMA) (E29, DAKO clone), cytokeratin (MN 116, DAKO clone), inhibin (R1, DAKO clone), CD10 (56C6, DAKO clone), or for evidence of possible vascular invasion and presence of emboli: CD31 (JC70A, DAKO clone) and CD34 (QBEnd10 DAKO clone), or tumors of vascular origin, but their expression was negative.

Due to bilateral PCC, serum CT, as well as parathormone (PTH) levels were also investigated; elevated CT basal levels (227 pg/mL) were found (normal level <13 pg/mL), highly suggestive of MTC, but PTH levels were within normal limits (53.6 pg/mL vs. normal range 15–65 pg/mL).

In order to confirm the diagnosis of MTC, fine-needle aspiration (FNA) cytology from the left lobe bigger nodule and one enlarged lymph node were obtained. The smears were stained with Papanicolaou and HE (Figure 3, a and b). On a hematic background, clusters as well as single dispersed cells of a slightly more pleomorphic population have been observed; plasmacytoid type cells, with lots of cytoplasm and eccentric nuclei and spindle shaped cells, with moderate nuclear pleomorphism, were intermingled. Based on these cytological features, a suggestive diagnosis of plasmacytoid type MTC was rendered, allowing for definite surgery. Smears from cervical lymph nodes showed infiltration of lymphoid tissue with inflammatory cells.

Total thyroidectomy with neck lymph node dissection was performed three weeks after the adrenalectomy. Grossly, the thyroidectomy piece (7/4.5/1 cm diameter) showed a white nodule of 5 cm diameter in the left thyroid lobe, while the right thyroid lobe presented a white nodule of 1/0.7 cm diameter; only the thyroid isthmus had a homogeneous appearance.

The microscopic HE sections established that the
two white nodules corresponded to foci of medullary carcinoma composed of small and medium-size cells with atypical hyperchromic nuclei and mitoses; tumor cells were grouped as solid nests in a stroma with extracellular hyaline (amyloid); no intralymphatic or intravenous tumor emboli were identified and tumor proliferation was not infiltrating the thyroid capsule and isthmus; C-cell hyperplasia hotbeds were noticed in the surrounding thyroid parenchyma. IHC showed tumor cells strongly and diffusely positive with anti-calcitonin (SP17, Zeta clone), anti-carcinoembryonic antigen (CEA, II-7 DAKO clone) and anti-thyroid transcription factor-1 (TTF-1, 8G7G3/1 DAKO clone) monoclonal antibodies, which are specific markers for MTC (Figure 4, a–f).

As a result, the three children of the patient underwent clinical, imaging and laboratory screening. One of the daughters, age 26, showed no anomalies. The other daughter, age 31, was diagnosed with asymptomatic MTC, due to multinodular goiter on ultrasound examination and increased basal CT levels (208 pg/mL vs. normal ≤13 pg/mL). She underwent total thyroidectomy and MTC was histopathologically confirmed.

![Figure 3](image1.png)  
**Figure 3** – Cytological MTC diagnosis by fine-needle aspiration (FNA), Papanicolau staining: (a) 200×; (b) 400×.

![Figure 4](image2.png)  
**Figure 4** – Histopathological MTC diagnosis – total thyroidectomy piece [bifocal MTC of both lobes: stage pT(m) Ib/Nx/Mx]. HE staining: (a) 100×; (b) 200×; (c) 400×. Immunohistochemistry: (d) Anti-calcitonin, 200×.
Examination of the 37-year-old son revealed at first a 3 mm hypo-echoic thyroid micro-nodule and slightly increased basal CT levels (19 pg/mL). In absence of CT stimulation tests, dynamics of serum CT levels showed persisting slightly increased levels (16.7 pg/mL in April 2013 and 19.1 pg/mL in June 2013). However, six months after the first screening (i.e., in June 2013) increased urinary and free plasma metanephrines were also detected as follows: total urinary metanephrines 1.51 mg/24 h (normal range 0–1 mg/24 h), free plasma metanephrines 97.856 pg/mL (normal <90 pg/mL) and free plasma normetanephrines 138.042 pg/mL (normal <180 pg/mL). Concomitantly, an 11/13 mm left adrenal gland tumor suggesting PCC was diagnosed at abdominal computer tomography scan. Serum iCa and intact PTH levels remained normal (calcium 4 mg/dL vs. normal range 3.82–4.82 mg/dL and PTH 45.7 pg/mL vs. normal range 15–65 pg/mL). The patient was sent for adrenalectomy and total thyroidectomy was also programmed.

The mandatory genetic tests were performed on the genomic DNA isolated from peripheral blood samples on EDTA, according to standard protocols. RET gene exons 10 and 11 were screened for mutations by HRM analysis, using a Rotorgene 6500HRM real-time PCR instrument (Corbett Research, Sydney, Australia), comparing the melting curves of patient samples with a wild type control and a codon 634 positive-control mutant. We detected the c.1901G>T (p.Cys634Phe or C634F) heterozygous mutation, in three of the screened subjects (Figure 5), that is in the female patient and the affected daughter and son. The other daughter showed no mutation.

PCR products were also sequenced using a Beckman CEQ8000 genetic analyzer (Beckman-Coulter, High Wycombe, UK), following a previously published protocol [12]. HRM genotypes were concordant with sequencing results (Figure 6).

In the light of all these analyses, a final diagnostic of hereditary MEN2A syndrome was established (Figure 7).

The differential diagnosis with the other two hereditary syndromes implied criteria concordant with literature data. Since at genetic testing, MEN2A and FMTC share similar RET gene mutations (e.g., exon 11 – codon 634)
and because in our case, none of the more specific mutations [2, 6] were identified (e.g., exon 13 – codons 768, 790, 791; exon 14 – codon 804 and exon 15 – codon 891), the differentiating criterion used to exclude the diagnosis of FMTC was the presence of hereditary PCC. The difference between MEN2A and MEN2B syndromes was given by the absence of specific phenotypic MEN2B features, together with the late MTC clinical onset, its lack of aggressiveness and the absence of the typical RET mutations [6] (exon 16 – codon 918 or exon 15 – codon 883) at genetic testing.

Discussion

As it has previously been shown, MEN2A syndrome consists of several neuroendocrine origin abnormalities: MTC, PCC and HPT. Diagnosis of any of these tumors implies correlative analyses and undertakes further investigations for the detection of the other possible associated neoplasia [5].

MTC, arising from parafollicular thyroid C-cells, currently accounts for 5–10% of all thyroid cancers [5, 13]. Despite its extreme versatile morphology, MTC diagnosis benefits from the identification of specific markers, both genetic (somatic or germline mutations of RET proto-oncogene) and serological (C-cell secreted CT level) [1, 5, 7].

The majority of MTC cases are sporadic, but the hereditary ones (30%) are present in virtually all cases of MEN2 syndromes [1, 5]. MTC being diagnosed in more than 90% of carriers of different germline RET mutations. The strong genotype/phenotype correlation between MEN2 and germline RET mutations [1, 5, 7] defined the mutational screening as a mandatory test for affected families.

Genetic screening for RET mutations has certain advantages over the biochemical or clinical screening, allowing the early detection of C-cell hyperplasia and microscopic MTC, before any clinical or laboratory tests become positive; this means that C-cells are more sensitive to RET gene mutations than adrenal medullary or parathyroid cells [6, 14]. Prophylactic surgery should be performed only if RET gene analysis is positive; so this test should be offered to all patients and their relatives, therefore improving the cure rate after thyroidectomy and the long-term prognosis [15].

According to the literature data, RET gene has 21 exons spanning over 50 kb [16] and encodes a transmembrane tyrosine kinase receptor. Its endogenous ligand appears to be the glial cell-derived neurotrophic factor (GDNF) family, which is critical for normal enteric and renal nervous system development [3, 17]. The RET protein consists of three parts: the extracellular region with six domains (four cadherin-like, a calcium-binding site and a cysteine-rich domain), the transmembrane region, and the intracellular region with two tyrosine kinase domains [6]. Mutations of RET gene reported in MEN2A act in a “two-hit” model [18] and concern the cysteine-rich extracellular domain of the receptor, mostly in exons 10 and 11 (codons 609, 611, 618, 620 and 634, respectively). These missense mutations result in RET gain of function and activation of downstream signaling pathways [3]. Less frequently, exons 5, 8 and 13–16 may be also involved (codons 790, 791, 804 and 918) [1, 18, 19].

In hereditary syndromes, RET mutations were previously classified on a three-level risk scale. Recently, the American Thyroid Association (ATA) [11] categorized mutations on four levels (A, B, C, D) according to their risk for aggressive MTC: level D (codons 883, 918) – the highest risk; level B (codons 609, 611, 618, 620, 630, 631) and C (codon 634) – high risk and level A (codons 321, 515, 533, 600, 603, 606, 635, 649, 666, 768, 776, 790, 791, 804, 819, 833, 844, 861, 891, 912) – moderate risk [1, 6, 8]; apparently, mutations affecting the extracellular domains of RET cause the most severe phenotypes [20] and prophylactic thyroidectomy is recommended as early as possible, in many cases before the age of five years (level C) or even within the first six months of life (level D) [1, 5].

Approximately 98% of MEN2A patients have a RET mutation in either exons 10 or 11 [1]. Most frequently (more than 80%), mutations involve codon 634 [1] affecting one of the six Cys residues from the extracellular domain; the result is a permanent activation of the receptor through homodimerization [3]. In 50% of MEN2A patients, there is a specific point mutation replacing the Cys residue with Arg (C634R) [20]; these patients present more distant metastases at diagnosis, nodal metastases being reported even at the age of 5 [10] and also HPT is more common [6].

In cases when the molecular screening of common exons (10, 11, 13, and 14) is negative, RET gene sequencing [9] or analysis of microsatellites is recommended [5].

For carriers of p.Cys634Arg/Gly/Phe/Ser/Trp/Tyr mutation, there is a high age-related risk for aggressive MTC (level C) [4], which can become malignant in very young children (even at one year of age) [10], distant metastases, bilateral PCC and HPT [6, 21]. In such cases, prophylactic total thyroidectomy (with or without central node dissection) is recommended, whenever possible, before the age of 5 [5] and screening for PCC and HPT should be started as soon as possible [4, 6]. In cases when surgery is not possible at a younger age, the cure rates depend on the lymph node metastases (i.e., less than 50% for 1–10 affected nodes to less than 4% when more than 10 nodes are involved) [18].

Level D RET mutations in codons 918 or 883 (exons 16 and 15, respectively) are associated with MEN2B, so the genetic analysis is very important for the differential diagnosis from MEN2A [6]. In case of FMTC, mutations span the entire RET gene, especially in exons 10 and 11 (codons 618, 620, 634), 13 (codons 768, 790, 791), 14 (codon 804) and 15 (codon 891) [2, 6]. Since MEN2A and FMTC share similar RET gene mutations, the differential diagnosis can be difficult in the absence of PCC or HPT [6]; recently, it was proposed that FMTC should be considered as a subtype of MEN2A [22].

In our study, patients presented level C RET mutation (C634F), notably not the most frequent one (C634R). According to general consensus, total thyroidectomy should be performed prior to the age of five years (in case of early diagnosis); in our case, due to late
diagnosis, it was indicated to all RET mutation carriers. Although the identified RET mutation indicated a possible aggressive evolution of MTC, in our case there was a slow development and no distal metastases in all three patients. Since the recurrence risk for MEN2A is 50% [13, 23, 24], there is a high risk for hereditary transmission but in our case only two of the children inherited the RET C634F mutation, the 26-year-old daughter being free of this mutation.

Usually, the clinical picture in MTC is not a reliable diagnostic element. The clinical course is rather discrete in the beginning (with irrelevant diffuse neck pain), frequently being masked or altered by emerging signs from an associated PCC or HPT [4], as it was found in our case. Moreover, in our study, both mother and daughter presented with overlapping of other thyroid related symptoms and morphological signs (chronic autoimmune thyroiditis, multinodular goiter), which completely hid the tumor features. Only the son had a 3 mm hypoechoic thyroid micronodule, which was, up to a point, suggestive for the diagnosis. Anyway, in his case the diagnosis confirmation came from the correlations with increased basal CT levels in the context of hereditary transmission. Yet, according to literature data [1, 4–6, 9], hoarseness, dysphagia or respiratory distress may appear in cases of posterior region tumors, compressing or invading local structures. Weight loss, neuroendocrine features such as flushing or diarrhea or in rare cases skin lesions or intestinal problems (i.e., cutaneous lichen amyloidosis – correlated with exon 11 RET gene mutations, codon 634 or Hirschsprung’s disease – correlated with exon 10 RET gene mutations, codons 609, 611, 618, 620) are linked to poor prognosis. Unlike sporadic MTC, which is considered a slowly developing tumor, hereditary syndromes are characterized by clinically aggressive MTC associated with a high mortality rate. The tumor onset can be very early (sometimes before 5-year-old and generally prior the age of 35) with multifocci and bilateralism [5], but in the case of our patients the onset mimicked the time of diagnosis for sporadic MTC. Local cervical lymph nodes or even distal metastases in a reduced number of cases (mediastinal lymph nodes, liver, bones, lungs) can occur in the fifth or sixth decade of life [4, 5].

In most occasions, the diagnosis of MTC is primarily suggested by ultrasound neck examinations that can evidentiate thyroid nodules, which are sometimes bilateral. The ultrasound can also be used for diagnosis of cervical adenopathy or for detecting tumor recurrences after thyroidectomy [5]. Part of the metastatic evaluation of a patient with an initial diagnosis of MTC, a contrast-enhanced computed tomography of the chest, mediastinum and abdomen can also be recommended [5].

Following the imaging techniques, possible biochemical abnormalities (such as increased serum CT or CEA levels) are verified parallel to a FNA of the thyroid nodules [5, 13].

In our study, the female patient underwent an ultrasound neck examination that confirmed the presence of chronic autoimmune thyroiditis. The thyroid parenchyma was globally inhomogeneous, hypoechoic and multinodular, initially masking the MTC. The diagnosis was reconsidered only after endocrine evaluation (elevated serum CT values) and FNA.

Serum CT, a hallmark of this tumor, is generally used for MTC early detection (highlighting the precursor lesion, C-cell hyperplasia) and diagnosis, but also for prognosis and follow-up after surgical resection [5, 13, 23, 24]. Unlike sporadic MTC, where C-cell hyperplasia appears only in small foci, in hereditary cases this precursor lesion is important, enabling the prevention of the tumor [4]. High serum CT levels (>100 pg/mL) are well correlated with tumor presence. Slightly elevated CT levels (10–40 pg/mL vs. normal <10 pg/mL) characterize the local metastases, while levels between 150–1000 pg/mL or more can be a sign for distant metastases [5, 13, 23, 24]. An elevated postoperative CT level implies a careful metastatic evaluation, which is compulsory prior to a new surgical exploration. Cases with severe symptoms due to CT excess may be treated with somatostatin analogues or by cytoreductive paleative surgery [5, 13]. In our case, the mother and the daughter serum CT values (227 pg/mL and 208 pg/mL, respectively) were highly suggestive for a MTC, but interestingly the son presented only slightly (still persisting) increased levels (16.7–19.1 pg/mL) in the absence of local metastases.

The literature data [5, 13, 23, 24] confirm the efficiency of CEA as another serological marker; its levels are elevated in more than 50% of patients with MTC and values higher than 100 ng/mL define extensive local adenopathy and distant metastases. Increased CEA levels in the presence of stable CT values (as a sign for tumor dedifferentiation) frequently associate with poor prognosis.

Even if literature data states that FNA is the first line test for early evaluation (diagnosis and classification) of thyroid nodules, it still does not substitute for the conventional surgical histopathology [5, 13, 23, 25]; it is also of particular value in patient screening for subsequent surgery [1, 5, 26]. According to Bethesda 2010 System [27], the MTC diagnostic criteria consist in the absence of thyroid follicles, frequent plasmacytoid cell pattern and small clumps of amorphous, amyloid-like, glassy and eosinophilic background material. In our case, the FNA cytological aspects fall into the plasmacytoid cell pattern, thus being highly suggestive for a MTC.

Despite the representative tumor morphology, the detection of amyloid (altered CT molecules) by Congo Red staining or IHC (by CT or CEA) can be necessary for differential diagnosis [5, 13, 28]. Recently, new extremely sensitive techniques based either on CT levels in FNA washout fluid or on the in vivo fluorescence of the tumor cells, are being introduced for the problematic diagnostic cases [5, 13]. Anyway, the specific cytomorphology of the tumor is the first element to be taken into consideration for differential diagnosis, because MTC small cell pattern must be differentiated from malignant lymphoma, poorly differentiated carcinoma or metastatic small cell carcinoma and MTC spindle cell variant may mimic a sarcoma or a melanoma [5, 23–25].

Finally, it is important to remember that FNA presumptive diagnosis allows for definite surgery, such as in our case. FNA was also valuable in differentiating
between the inflammatory and the metastatic lymph nodes.

The histopathological examination after the tumor ablation still remains the most reliable diagnostic procedure. Grossly, MTC may be white or gray, generally with foci of necrosis and hemorrhage. Usually firm, the tumor is multifocal and bilateral in case of hereditary syndromes (sporadic neoplasms being unilateral). Microscopically, MTC mimics the histology of other neuroendocrine cell tumors: nests of uniform round or polygonal cells are separated by a characteristic fibrovascular, amyloid containing stroma [5, 23, 24, 29]. Literature data confirm eleven subtypes of MTC [5, 13, 24, 30], the most frequent being the classical, the carcinoid-like, the papillary, the giant-cell type or with squamous differentiation. Mucus production and melanin pigmentation are rarely seen [13, 31]. There is no pre-existing adenoma, but C-cell hyperplasia (defined as more than six C-cells per follicle or more than 50 C-cells per low power field) is associated with malignancy in hereditary disease [5, 13, 23, 24]. IHC is strongly indicated for all cases of solid tumors without typical features of papillary or follicular carcinoma [13, 23, 24]. Metastatic neuroendocrine tumor, thyroid paraganglioma or hyalinizing trabecular adenoma are typical differential diagnoses [13, 23, 29]. In our case, specific IHC panel for CT, CEA and TTF-1 created a synergic, strongly diffuse staining for tumor cells, highly suggestive for MTC [13, 23, 24].

PCC represents the second highly penetrant tumor in MEN2 syndrome, being found in up to 50% of patients. Its presence is especially important for differentiating MEN2A from FMTC. The association of PCC with hereditary diseases (such as MEN2 or neurofibromatosis type 1, von Hippel–Lindau disease) raises the issue of a powerful neoplastic stimulus that affects neuroendocrine cells family [13, 29]. For the moment, 10 different susceptibility genes have been taken into consideration for the pathogenesis of PCC, but it is estimated that their number will keep growing, along with the number of these genetically inherited tumors [32, 33]. In MEN2 syndrome, PCC is caused by RET gene mutation, codon 634 being the most incriminated (there is a strong correlation between the position of the RET mutation and the clinical phenotype) [32, 34].

PCC accounts for 6.5% of incidentally discovered adrenal tumors [13, 29, 35], but only 10–20% of cases are part of familial syndromes and have a strong tendency to bilateralism; 80–90% of patients present sporadic and solitary neoplasms [35]. In MEN2 syndromes, the diffuse or nodular adrenal medulla hyperplasia is considered to be an earlier stage in tumor development, before the onset of an overt neoplasm (usually a benign form [36]), but the two entities may occasionally be correlated [29].

As reported in our study, the tumor can be the first clinical manifestation in a quarter of MEN2A cases [15]. Interestingly, there was a late onset for bilateral PCC: at 37 years of age (the first) and then at 55 years of age (the second; concomitant with the MTC). In case of the son, the onset of PCC was after MTC onset.

PCC is a chromaffin cell tumor and therefore the excess of catecholamine (usually epinephrine) secretion is responsible for the well-recognized clinical pattern (e.g., episodic paroxysmal hypertension); unlike MTC, PCC symptoms have great impact for establishing a presumptive diagnosis [32, 34].

Generally linked to clinical symptoms, a tumor biochemical-screening test is compulsory. Elevated values of plasma free metanephrine (normal >90 pg/mL) and normetanephrine (normal <180 pg/mL), as well as measurement of fractionated urinary metanephrines (normal range 0–1 mg/24 h), correlate well with PCC presence [13, 29, 37]. A four-time increase of catecholamine plasma concentration (above the upper reference limit) is not seen in almost any patient without PCC, but occurs in 70–80% of patients with the tumor [32].

For the patients with inconclusive biochemistry, the Clonidine suppression test must be done (by inhibition of centrally mediated stimulatory adrenergic influences, Clonidine decreases only resting plasma catecholamines and does not suppress catecholamine release in PCC) [38]. Measured by ELISA test, normetanephrine response to Clonidine (elevated in 96% of patients, three hours post Clonidine) enables to reliably exclude PCC. Another sensitive test (83–89%) is to measure the plasma levels of chromogranin A (acidic protein stored with catecholamine in secretory granules and co-released with them), marker of neuroendocrine neoplasms. High plasma levels correlate with tumor mass and frequency, with malignancy; its values are also used in gauging the tumor response and relapse [32].

Imaging techniques (abdominal ultrasound, computed tomography or magnetic resonance imaging) are also required before (initial evaluation of patients) or after (locate the tumor for surgical resection) biochemical measurements. In our study, the second PCC diagnosis of the mother was established by the strong correlation between symptoms (210/110 mmHg paroxysmal blood pressure values, headache, tachycardia, previous ischemic stroke), biochemical tests (elevated plasma metanephrines levels of 275.8 pg/mL and normetanephrines levels of 220.3 pg/mL) and ultrasound examination, the latter confirming the presence of an adrenal mass (44/34 mm in diameter). Among the three children, only the son was presenting PCC, the diagnosis being established by biochemical screening and abdominal computer tomography scan. Elevated catecholamines levels were detected both in serum: free plasma metanephrines 97.856 pg/mL and normetanephrines 138.042 pg/mL, and in urine: total metanephrines 151.5 mg/24 h. A left adrenal gland mass (11/13 mm in diameter) was also evidenced.

Recent advances confirm the diagnostic importance of new nuclear medicine methods: [123I]-Metaiodobenzylguanidine (MIBG) scintigraphy as first choice specific functional imaging test and [123I]-MIBG scintigraphy as second choice. When MIBG scintigraphy is negative but there is a strong suspicion of PCC, there are other newer specific tests such as positron emission tomography (PET) with [18F]-F-Fluorodopamine (F-DA) and [18F]-F-Dihydroxyphenylalanine (DOPA) [37].

After the ablation of the tumor, the histopathological diagnosis usually reconfirms the clinical hypothesis, being the most reliable diagnostic procedure.

Grossly, PCCs are encapsulated masses, but
occasionally show invasion into surrounding tissues. Their cut surface is gray or white, with hemorrhage foci, calcification or cystic changes. The small number (10–15%) of tumors located in extra-adrenal chromaffin tissues form the paragangliomas [29] (they share overlapping characteristics with PCCs – molecular pathobiology, epidemiology, histopathology; but are also different in terms of behavior – more aggressive, dopamine secreting tumors and augmented metastatic potential) [32].

The macroscopic examination delivers data that can be well correlated with clinical symptoms. Generally, an average tumor weight over 100 g is associated with high blood pressure values. Moreover, the elevated catecholamine content of fresh tissue on cut sections changes their color when exposed to the air. Biochemical or histochemical techniques can also be used for the evaluation of increased catecholamines level in fresh tumor tissue samples [29].

At microscopic examination, PCC expresses a mixed alveolar and trabecular pattern; a spindle cell component is rarely found. In general, cells resemble normal pheochromocytes (mature cells with basophile, granular cytoplasm which contain catecholamine), but neoplastic ganglion cells and neuroblasts may be found, too; only some of these neoplasms are characterized by increased cellularity and nuclear pleomorphism [13, 29]. Hyaline globules and melanin-like pigment are also found frequently. Cytoplasmic clearing, so-called ‘lipid degeneration’ may mimic an adrenal cortical tumor. Stromal sclerosis may be marked and amyloid has been described. The vascular component is often prominent [13, 29].

The histopathological examination can also provide valuable information about the tumor secretion and prognosis. Yet, it should be noted that none of these features can be completely investigated by tumor microscopy alone, since malignant and benign PCC may have an identical histological appearance [29, 32, 34].

Moreover, the functional activity of a neoplasm cannot be judged by the abundance of cellular catecholamine granules, since actively secreting neoplasm may contain only few [29].

The only malignancy criterion in PCC is distant metastasis (10% of cases) that occurs in the related lymph nodes, liver, lungs and bones [29, 32, 34]. Mitotic figures (which are rare), vascular emboli (observed in benign tumors, too) or local invasion and recurrence do not always imply malignancy [29, 35]. An index of tumor differentiation, the epinephrine to epinephrine plus norepinephrine ratio, is lower in malignant than benign tumors [32]. There are few markers discussed as correlated with malignancy: N-cadherin (considered to heighten tumors [32]), vascular emboli or neoplasm (CD31 and CD34). HPT, as well as PCC, is actually more frequently associated with RET gene, codon 634 mutations (genotype/phenotype correlation), as part of a MEN syndrome; however association with more rare mutations has also been reported [39].

HPT (primary form) occurs when one or more parathyroid glands become enlarged and overactive. The excess of released PTH generally leads to high levels of iCa in the blood. This can cause various symptoms, commonly tiredness, nausea, vomiting, kidney stones and bone pains [40, 41]. PTH levels will be either elevated or ‘inappropriately normal’ in the presence of elevated calcium. Typically, they vary greatly over time in the affected patient and (similar to iCa values) must be restested several times to see the pattern. Hyperfunction of the parathyroid glands is due to hyperplasia, adenoma or carcinoma of the parathyroid glands [41, 42].

Diagnosis is made by parathyroid immunoassay, measuring serum iCa and intact PTH (intended to detect only relatively intact and biologically active PTH molecules) levels, which are usually increased after the third decade of life [1]. Generally, the level of serum phosphate is also low in the context [40–42]. Screening for HPT should begin by the age of 8 in carriers of exon 10 and 11 mutations, and by the age of 20 in carriers of other exon mutations [39].

In our study, HPT was absent both in mother and children. The biochemical markers (iCa and intact PTH levels) ranged within normal parameters (3.82–4.82 mg/dL and 15–65 pg/mL, respectively) both for the woman (53.6 pg/mL) and her son (4 mg/dL and 45.7 pg/mL, respectively).

Differential diagnosis must be primarily done with the other forms of HPT [40–42], by parathyroid immunoassay (primary HPT form: PTH and iCa values are both elevated; secondary HPT form: PTH values are high, iCa level is low), neck ultrasound, CT or Technetium scan (the imaging techniques showing the possibly enlarged parathyroid glands and being indicated if surgery is considered as a treatment option). A biopsy using scanning may help to exclude parathyroid carcinoma [40]. Asymptomatic HPT may be taken into discussion and it is characterized by the following: 24-hours urinary Ca >400 mg; serum Ca >1 mg/dL above upper limit of normal; creatinine clearance >30% below normal for patient’s age; bone density >2.5 standard deviations for below peak (i.e., T-score of -2.5); patient age <50 years [42].

The therapeutic scheme in MEN2A syndrome includes
individual therapy of each tumor. Programs of patient follow-up are compulsory after surgical procedures.

The current curative treatment for MTC is surgery [27] – total thyroidectomy with neck lymph node dissection, procedure applied both in case of the mother and the affected children.

However, there is literature debate over the timing of the surgical procedure, since clinically apparent disease is rarely reported before the age of 10; some surgeons suggest late intervention [5]. Preoperative ultrasound examination and tumor markers, such as serum CT and CEA levels are useful to assess neck lymphadenopathy.

Since early diagnosis for MTC became possible in hereditary cases, recent studies have demonstrated that 75–85% of patients were disease free 10 years after thyroideectomy. Overall, the prognosis for patients with MTC is good. In more advanced neoplasm (i.e., locally invasive tumors, extension beyond the capsule, distant metastasis or recurrences), the mean survival rate is approximately five years [5].

In case of positive RET mutations patients or negative initial PCC screening tests [4] long-term biochemical screening (measure of CT levels on regular basis [4]) is recommended.

Efforts are made towards finding effective molecular targeted therapy (e.g., inhibition of the RET pathway) [43, 44]. Recent evidence demonstrates that RET is involved in multiple downstream pathways such as RAS/MEK/ERK controlling the cell cycle progression or PI3K/AKT/NF-kB controlling the cell motility, cell cycle progression and survival [3]. RET also acts by stimulating p38, MAPK, JAK/STAT and protein kinase C. In view of this, many clinical trials are evaluating specific targeted therapies for MTC [36]. The RET receptor tyrosine kinase inhibitors (TKI) are tested both in vitro and in vivo for MTC cure. Among these inhibitors, Vandetanib shows promising results by blocking the phosphorylation and the RET receptor, and so inhibiting tumor cell proliferation [3]. Another possible direction is the use of Epidermal Growth Factor Receptors (EGFR) inhibitors such as Gefitinib and Erlotinib, which show beneficial effects in refractory or metastatic MTC. Axitinib is a very potent inhibitor of vascular endothelial growth factor (VEGF) receptors successfully used in clinical trials for MTC therapy, considering the elevated levels of VEGF reported recently in thyroid cancer patients [36]. Inhibition of the glycogen synthase kinase-3b (GSK-3b) through phosphorylation because of Raf-1 activation, results in MTC growth inhibition, making this another possible target for MTC [5]. Activation of the Notch1 gene in tumor cells reduces cell proliferation and CT levels in a dose-dependent manner. The proteasome inhibitor Bortezomid can inhibit the in vitro growth of MTC cell lines at low concentrations by blocking the PI3K pathway [3].

Other tumor suppressor genes (e.g., p53) and oncogenes (c-myc, c-jun, c-fus nuclear proto-oncogenes) have been generally related to thyroid cancers [9], but there is no suggestive evidence of their link to MTC. Some studies have proved that aggressive types of thyroid cancers have abnormal expression of p53 gene and as a result, high risk of recurrence [36]. Although no specific mutations were identified for the nuclear proto-oncogenes, both high mRNA expression levels (for c-myc and c-jun) and high frequency allelic loss of c-myc were observed; although, high levels of c-myc expression apparently indicate less differentiated tumors [36].

Evidence emerges about immunological-based therapy with dendritic cell vaccination for reducing tumor mass [44].

Even if all these therapeutic options are still limited and under evaluation there is much hope for MTC cure as molecular techniques are developing continuously.

In PCC, successful surgery (either by open laparotomy or laparoscopy) ensures a survival rate similar to that of the general population. After adequate alpha-adrenergic blockade (Phenoxybenzamine, Doxazosin, Prazosin, etc. – minimizing the possibility of severe intraoperative hypertension) laparoscopic surgery is the primary method of treatment [34, 45]. Patients with hereditary adrenal PCC may undergo adrenal cortical sparing surgery (balance tumor removal with preservation of adrenocortical functions) [32]. Before the surgical intervention it is usually advised to “salt load” patients, either by direct salt replacement (consumption of high salt food) or through the administration of intravenous saline solution, in order to prevent severe hypotension [37, 45]. Guidelines recommend that in MEN2 syndromes, the PCC excision should be performed before the thyroidectomy in order to prevent secondary complications related to catecholamine crisis [36]. The same protocol was followed in our case, for both mother and son.

A good prognosis results from chemotherapy in cases of inoperable disease [34].

Genetic tests are under research: about 40–50% of patients with malignant PCC will have mutations in genes encoding for the B and D subunits of succinate dehydrogenase complex and von Hippel–Lindau tumor suppressor gene and hence should be offered testing for at least these three genes [32, 37]. Anyway, the acknowledgement that germline mutations of the RET gene in MEN2A or von Hippel–Lindau, neurofibromatosis type 1 tumor suppressor genes and succinate dehydrogenase genes are important in the pathogenesis and clinical presentation of patients with PCC confirms genetic testing as part of patient management [32, 34]. Other guiding factors for the sequence in which genes must be tested are represented by the family history and radiological and histopathological features of the tumor [32].

Treatment of HPT consists in medication (vitamin D and calcium therapy: bisphosphonates) [5, 36]. In mild primary HPT (mildly raised calcium level and few symptoms), a regular monitoring approach (considered controversial) may be indicated; the monitoring procedure usually includes blood tests for calcium level and kidney function, regular blood pressure checks and dual-energy X-ray absorptiometry bone scanning [40, 42]. For the management of HPT in MEN syndromes, one important issue is to decide the number of glands involved in the disease and the best surgical procedure to be followed: total parathyroidectomy, subtotal parathyroidectomy or single gland removal [39]. Recent advances suggest a new surgery technique in order to prevent hypopara-
thyroidism [5, 39]. Parathyroid tissue (fresh or cryopreserved) can be auto transplanted to the non-dominant forearm or the sternocleidomastoid muscle, which are the best locations for subsequent control and management. Usually, these glands do not become functional until after one or two months, so patients receive drug treatment [5]; calcium level will need close monitoring after surgery to ensure that it returns to normal and does not drop too low.

Sometimes hormonal replacement therapy may be suggested in postmenopausal women who also have menopausal symptoms that they wish treatment for [40]. Yet the prognosis after surgery is excellent [40, 42]. Yearly checking is optimal as the calcium estimation is an inexpensive and simple blood test [39].

Conclusions

MEN2 is a hereditary autosomal dominant transmitted cancer syndrome that benefits from a plurifactorial diagnosis through genetic screening tests, imaging techniques, biochemical markers and morphologic examinations. Early diagnosis ensures a high cure rate, improving the duration and quality of life. Specific genetic testing (RET germline mutations detection especially on exons 10 and 11) is offered nowadays to every individual attaining the age and their first and second-degree relatives, because of the complete penetrance and very strong genotype–phenotype correlation and therefore provides precise information on the clinical evolution and prognosis of the illness. Based on the presented data, the particularities of our case reside in the delayed onset of bilateral PCC – suggestive mark of a MEN syndrome – and the masked clinical signs of MTC in the mother and son. Although the identified RET mutation (C634F level C) indicated a possible aggressive evolution of this tumor, there was a slow development and no distal metastases in all patients. The 50% recurrence risk for MEN2A implies a high-risk for hereditary transmission but as it was discussed, only two of the three children inherited the mutation.

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