F. Calcitonin (CT) and RET Proto-oncogene

Medullary carcinoma of the thyroid (MTC) arises from a malignant transformation of the parafollicular C cells of the thyroid and accounts for ~5-8% of all cases of thyroid cancer. Approximately 75% are sporadic in presentation and 25% are hereditary *(9,11,347)*. In a study of thyroid nodules, the prevalence of MTC is reported as 0.57% *(348)*. The behavior and management of MTC differs from that of well-differentiated follicular-derived thyroid carcinomas *(346)*. The inherited forms of MTC come under the heading of multiple endocrine neoplasia (MEN) types 2A and 2B. These are autosomal dominant inherited multiglandular syndromes with age-related penetrance and variable expression. Familial MTC (FMTC) is characterized by the occurrence of MTC without any associated endocrinopathy. In 1993, genetic mutations in the RET proto-oncogene were discovered *(349,350)*. The gene responsible for these diseases is known to be located on the chromosome sub-band 10q11.2. The phenotypic expressions of inherited MEN are summarized in Table 7.

1. Detection of MTC by Measuring Serum Calcitonin (CT)

(a) Calcitonin Biosynthesis

The *CALC-1* gene encoding human CT is located on the tip of the short arm of chromosome 11 (11p15.3-15.5). Although the parafollicular C cells of the thyroid gland are the dominant source of circulating mature CT, several other categories of neuroendocrine cells besides the thyroid normally contain and secrete CT.

Mature CT is a 32-amino-acid polypeptide with a disulfide bridge and a carboxyterminal proline amide that play functionally important roles in mature CT *(350)*. As shown in Figure 9, mature CT results from the post-translational modification of a larger 141 amino-acid precursor (preprocalcitonin) within the parafollicular C cells. Preprocalcitonin first undergoes cleavage of a signal peptide to form procalcitonin (proCT), a prohormone consisting of 116 amino-acid residues. At the proCT amino-terminus there is a 57-amino-acid peptide, called aminoproCT (or PAS-57), and at the carboxyl terminus, there is a 21 amino-acid peptide called calcitonin carboxyterminal peptide-1 (CCP-1 or Katacalcin). The immature CT peptide consisting of 33 aminoacids is located centrally within the ProCT molecule. The mature, active, 32 aminoacid CT (which includes an amidated proline at its carboxyterminus) is produced from immature CT by the enzyme peptidylglycine-amidating mono-oxidase (PAM).

(b) Calcitonin (CT) Methods

Until 1988, CT assay methods were primarily based on radioimmunoassay involving the use of polyclonal antibodies that recognised both the mature CT monomer and other circulating forms (precursors and degradation products). These earlier assays lacked specificity and sensitivity. Since 1988, improvements with new immunometric techniques based on the use of monoclonal antibodies (one of which recognises the N-terminal region and the other the C-terminal region) has allowed for the development of more specific and sensitive assays that detect the mature-32 amino-acid monomer of CT. Currently two-site immunometric assays detect CT in fasting plasma samples in 83% of healthy men and 46% of healthy women *(351-353)*. The CT values produced by different methods can differ, however leading to difficulties in the interpretation of CT results. It is important for physicians to recognize that inter-method differences do exist and can play a role in the proper interpretation and use of CT for the diagnosis and management of MCT.

(c) Basal Calcitonin (CT) Values

Basal CT values were found to be a diagnostically useful marker for MTC in 1968 *(354)*. Currently two-site IMAs, specific to mature CT, typically report CT levels below 10 ng/L (pg/ml) for all normal healthy controls and 90 % of patients with thyroid abnormalities, other than MTC *(348,355-357)*.

Table 7. MEN Disease Phenotypes

PHENOTYPE	CLINICAL FEATURES	
MEN2A (60%)	Medullary thyroid Carcinoma (MTC) Pheochromocytoma Hyperparathyroidism Notalgia	100% 8-60% 5-20% <5%
MEN2B (5%)	MTC Pheochromocytoma Marfanoid Habitus Mucosal neuromas and ganglionneuromatosis of the gut	100% 50% 100% 100%
FMTC (35%)	MTC	100%

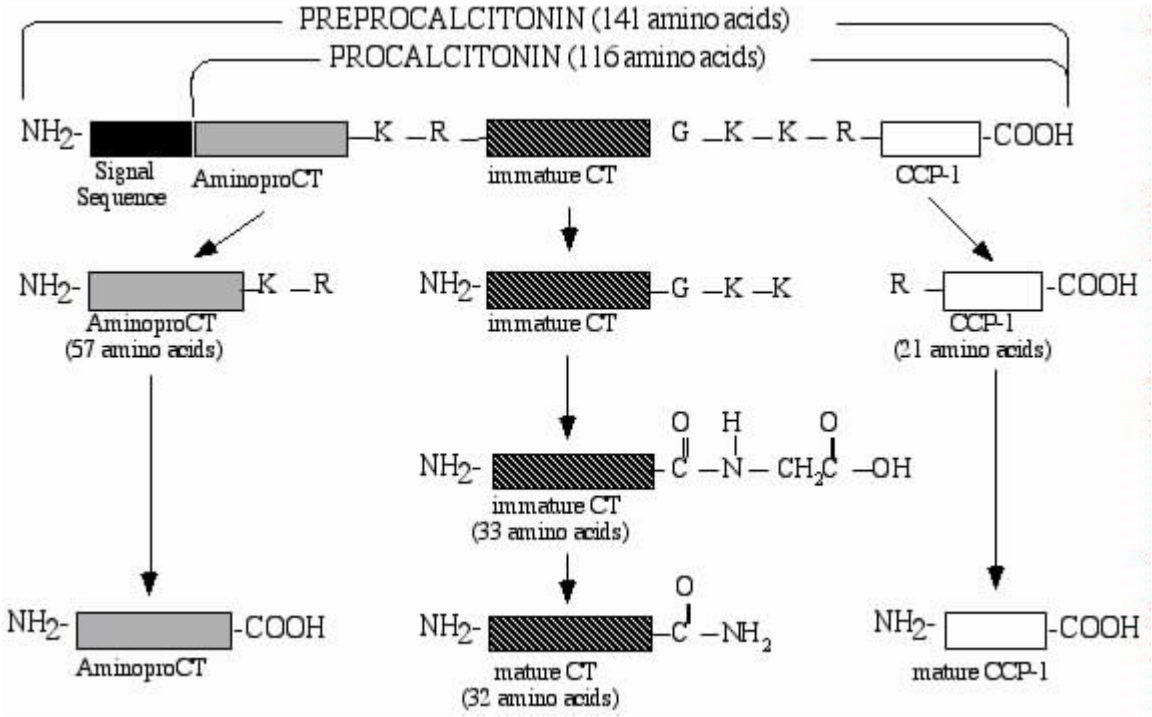

Fig 9. Post Transcriptional Calcitonin Maturation

Guideline 51. Calcitonin (CT) Assays

- Mature (32 amino acid) CT is the principal tumor marker for MTC.
- CT measurements used for the diagnosis of MTC and for monitoring purposes should be performed using two-site immunometric assays that are specific for the mature 32 amino acid monomer of CT.
- Currently, the lower normal threshold for CT is generally accepted as being under 10 pg/ml (ng/L).
- As new, more sensitive CT kits become available, the lower CT threshold should be redefined.

Patients with micro or macro forms of MTC (sporadic or familial form) have elevated CT levels that correlate with the tumor mass *(358)*. Hyperplasia of the C cells (HCC) is the earliest histologic finding prior to the development of a microcarcinoma, when patients present with MEN2. HCC appears soon after birth, and at this stage in the disease, the basal CT levels can be normal. A normal CT result therefore cannot rule out C-cell pathology at its earliest stages.

(d) Provocative Calcitonin-Stimulation Tests Used for Diagnosing MTC

Provocative stimuli, such as calcium and pentagastrin (Pg) and when Pg is unavailable, omeprazole, have been used to expose C-cell abnormalities, since they induce an increase in the CT level at all stages of MTC *(359-364)*). One advantage of these tests is that they are able to detect HCC before MTC appears in earnest. In countries like the United States where genetic testing is readily available, surgery for gene carriers is based on genetic testing alone and provocative tests are rarely used. In some countries Pg has become difficult to obtain and the majority of surgeries are now performed based on genetic testing alone. Provocative tests are usually employed:

- To confirm the diagnosis of MTC preoperatively when basal CT levels are only mildly elevated (less than 100 pg/ml).
- To detect C-cell disease in *RET*-positive gene carriers
- For pre-surgical monitoring of *RET*-positive children
- For post-operative monitoring for tumor recurrence
  When genetic testing is not readily available

Guideline 52. Clinical Utility of Serum CT Measurements for Diagnosing MTC

- Calcitonin (CT) measurements are method-dependent. This can impact the interpretation of CT results.
- Increased levels of calcitonin in the serum can be seen for patients with autoimmune thyroid diseases (Hashimoto's thyroiditis or Graves' disease).
- Hyperplasia of the C cells (HCC) is the earliest histological finding prior to the development of a microcarcinoma. A non-elevated CT may be seen with HCC in the earliest stages of developing MTC.
- A rise in serum CT levels above 10 pg/ml (ng/L) suggests early MTC at the microcarcinoma stage
- There is a positive correlation between CT levels and tumor mass.

(i) Pentagastrin (Pg) Stimulation Test

The Pg stimulation test has been widely used for the diagnosis of MTC but is not readily available in many countries *(359,365)*. The Pg test consists of an I.V. infusion of Pg (0.5μg/kg/body weight) over 5 seconds. Slow administration of Pg reduces transient side effects (nausea, vomiting, substernal tightness, flushing, and tingling of the extremities) and improves patient tolerance of the test. Blood samples are drawn at baseline and 1, 2, 5 and sometimes 10 minutes after starting the infusion.

The results and interpretation of Pg-stimulated CT values are tabulated in Table 8. The Pg-stimulated peak in CT is typically less than 10 ng/L (pg/ml) in 80% of healthy adult volunteers, and under 30 ng/L (pg/ml) for 95% of the general population. Normal men exhibit higher values than women. A positive test [CT peak response greater than 100 ng/L (pg/ml)] suggests the presence of MTC. When patients have the familial mutation responsible for MEN 2, a peak between 30 and 100 ng/L (pg/ml) is typically seen and suggests HCC or a microcarcinoma. Although a Pg-induced increase in CT of less than 100 ng/L (pg/ml) is known to occur in

adults with thyroid abnormalities other than MTC (see Table 9) no such results have ever been obtained in children under 12 years of age not bearing the *RET* mutation *(366)*. The absence of an increase in CT in a young individual bearing the *RET* mutation does not rule out the possibility that MTC can occur at an older age.

Table 8. Interpretation of the Pentagastrin (Pg) Test

#	CT ng/L (pg/mL)	Interpretation
1	CT Peak < 10	Normal (80% of adults)
3	CT Peak >30 <50	5% of normal adults
4	CT Peak >50 <100	Possible CMT or other thyroid pathologies
5	CT Peak >100	Probable CMT
6	Basal or post Pg CT value > 10 pg/ml	C-cell pathology or residual tissue in MEN 2 patients and MTC after surgery

The best age to test for a C cell pathology in children bearing the *RET* mutation for MEN 2 with the Pg stimulation test has not yet been established. It varies with the type of mutation and the type of MEN 2 present in these families *(367,368)*. Hence, young carriers of the mutation with normal basal levels of CT should have genetic or stimulation testing performed as early as possible post-natally for MEN 2B, and at 2 years of age for MEN 2A. However, it should be stressed that high CT levels are normally found in neonates followed by an age-related decline from birth to about one year of age; no data is yet available on this age-group as far as stimulation testing is concerned *(369)*. This test should be repeated at least once a year until it becomes positive, at which time a total thyroidectomy should be performed. Given the prognosis of MTC, the low tolerance to a Pg test, and the psychological implications for the family, some physicians prefer not to repeat the Pg test until it becomes positive and opt to perform a thyroidectomy on all 4 to 5 year old carriers of the *RET* mutation.

(ii) Calcium Stimulation Test

This test consists of administering 2.5 mg/kg of calcium gluconate intravenously over 30 seconds. Blood samples are drawn at baseline and again at 1, 2 and 5 minutes after the calcium infusion. C-cell hyperplasia is suspected if the plasma CT level rises above 100 ng/L. No important adverse effects have been observed with this test, with the exception of a mild and transient generalized sensation of warmth. Calcium infusions have been reported to be less sensitive than the Pg test for the diagnosis of MTC *(370-372)*. Furthermore, this test has not been evaluated using a CT assay specific for the mature CT monomer, and thus needs to be re-evaluated. It has been reported that calcium infusion combined with Pg test enhances the sensitivity of the Pg test *(359)*.

(e) Basal and Post-Stimulated CT Levels in the Follow-Up of Surgery Patients

After a thyroidectomy, serum CT measurements are the accepted tumor marker for detecting residual thyroid tissue or metastases. A detectable basal or post Pg stimulated CT level constitutes proof that there is some residual tumor tissue present *(373,374)*.

Guideline 53. Postoperative Follow-up of MTC

- Serum CT and CEA should be measured just prior to, and 6 months after, surgery for MTC. Serum CT levels fall slowly in some patients. The first post-operative CT measurement should not be made until 2 weeks after surgery.
- The presence of residual tissue or a recurrence of MTC can only be ruled out if both basal and post pentagastrin or calcium-stimulated CT levels are undetectable.

In view of the variations in the rate of disappearance of serum calcitonin, the first post-operative control sample should be taken at least 2 weeks after surgery *(375)*. It should be noted that carcino-embryonic antigen (CEA) is also measured along with CT to detect the recurrence of MTC. In addition, CEA appears to be a useful marker of MTC de-differentiation, and is indicative of a poor prognosis.

(f) Elevated Calcitonin Levels in Conditions other than MTC

As shown in Table 9, elevated calcitonin levels have also been observed in other pathologies besides MTC and neuroendocrine tumors. Increased serum calcitonin release occurs with autoimmune thyroid diseases (Hashimoto's thyroiditis or Graves' disease) *(376-378)*. Non-thyroid conditions where elevated CT has been noted include severe renal insufficiency, hypercalcemia and hypergastrinemia, acute pulmonary inflammatory conditions and other local or general forms of sepsis (Biermer's disease, iatrogenic disorders, etc.) *(379-381)*.

Since, in some cases the elevated CT levels were detected by polyclonal RIA, these reports require confirmation using the current monoclonal antibody based assays that are more specific for mature CT. Studies using specific antiserum raised against ProCT, CT and CCP-1, in conjunction with HPLC and gel filtration, have shown that patients with an elevated calcitonin associated with a non-thyroid condition have markedly increased serum levels of intact ProCT, and to a lesser extent the un-cleaved form, CT-CCP-1. These patients usually have normal or only minimally elevated levels of mature CT. Using epitope-specific antiserum and isolation techniques, it has been shown that tumors other than MTC can secrete large amounts of mature CT and various CT precursors *(382)*. This can be seen with various neuroendocrine tumors, especially small cell lung cancer and bronchial carcinoid. However, only a slight increase in the CT level, if any, is observed after the Pg test when patients with these neuroendocrine tumors are tested *(383)*. C-cell hyperplasia occurs in lymphocytic thyroiditis and some patients with differentiated thyroid cancer *(384-386)*. This HCC may be responsible for a slightly elevated mature CT level and for the increased CT response observed with the Pg test.

Table 9. Conditions with Elevated Calcitonin other than MTC

Neuroendocrine tumors	Lung small cell carcinoma, bronchial and intestinal carcinoid, all neuroendocrine tumors
Benign C-cell Hyperplasia (HCC)	Autoimmune thyroid diseases Differentiated thyroid cancer
Other diseases	Kidney disease Hypergastrinemia Hypercalcemia

2. Detection of MTC by Measuring the *RET* Proto-oncogene

Until 1987, the only method available for detecting subjects at risk for MTC was to perform repeated stimulated CT measurements on family members of MTC patients. The subsequent identification of the locus 10q11.2 responsible for MEN 2 on chromosome 10 then made it possible to detect at-risk subjects by genetic screening *(378)*. It has now been established that several types of mutations on chromosome 10 can activate the proto-oncogene *RET*, that is responsible for MEN 2 *(349,350)*. This now allows physicians to screen for the condition before the first biological signs appear. Currently in many developed countries, genetic studies are the first line approach for this diagnosis. For accurate disease prediction however, it is necessary that positive genetic screening results be followed with an exhaustive survey of both the healthy and affected members of the family.

The *RET* gene is a 21 exon gene that encodes a membrane tyrosine kinase receptor. This membrane-associated receptor is characterized by a cadherin-like region in the extra-cellular domain, a cysteine-rich region immediately external to the membrane and an intracellular tyrosine kinase domain. As shown in Figure 10, the mutations described so far in MEN2 are located in exons 8, 10, 11, 13, 14, 15 and 16 *(368,387-391)*.

(a) Genetic Screening for MEN 2 Diagnosis

MEN2 is an autosomal, dominant familial disease, caused by the activation of missense mutations in the *RET* proto-oncogene *(349)*. Approximately 75% of all MTCs are sporadic and solitary in origin. In 44% of such tumors, a somatic mutation at codon 918 is present *(392)*. Screening must be performed on all collateral family members, ancestors and descendants of the index case, and then all of the descendants of members known to be affected. The screening is based on the identification of a proto-oncogene *RET* genomic mutation using genomic DNA sequence analysis of the index case and on a systematic search for this mutation in all the potentially affected members of the family (Figure 11) *(393,394)*.

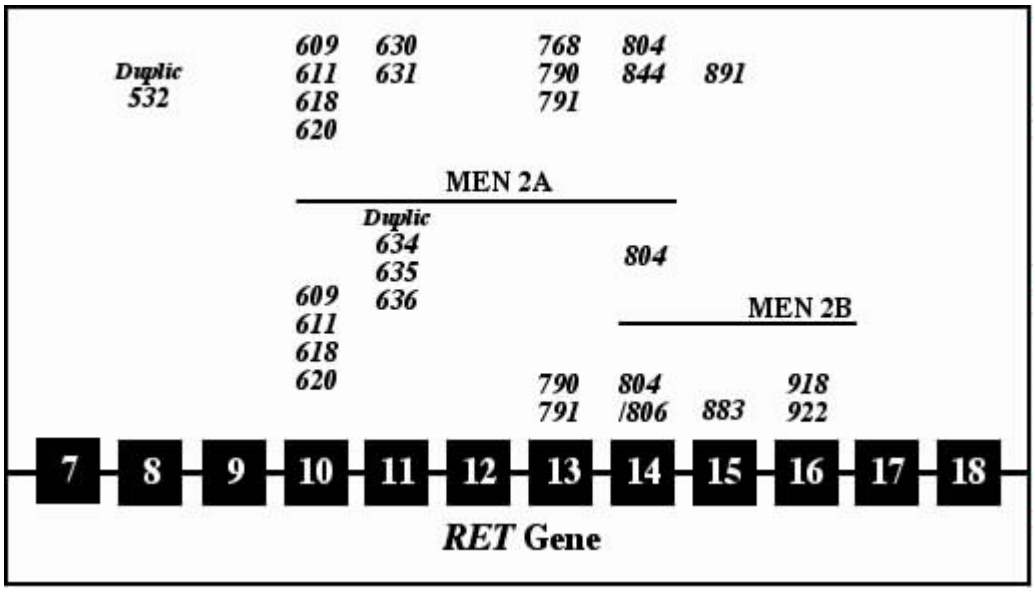

Fig 10. Most common RET proto-oncogene mutations

To date, five mutations of the *RET* gene are present in 97% of all cases of MEN2 (Figure 10). Mutations responsible for MEN 2A mainly affect the cysteine-rich extracellular domain, each converting a cysteine into another amino acid. Mutations also occur in the cysteine codons 609, 611, 618 and 620 of exon 10 and cysteine codon 634 *(368,378)*. Familial Medullary Thyroid Carcinoma (FMTC) is most frequently associated with mutations in the cysteine codons in exon 10 as well as codons 768 and 804 in exons 13 and 14 *(368)*. Most (87 %) of the mutations in codon 634 in exon 11 are associated with the multiple organ manifestations of MEN 2A (MTC, pheochromocytoma, and hyperparathyroidism) *(9,378)*.

MEN 2B-associated tumors are caused by mutations in the intracellular TK2 domain. Most (97%) MEN 2B cases involve amino acid 918 in exon 16 with a methionine converted into a threonine. These often occur as new (de novo) germline mutations *(395)*. A minority (5%) of MEN2B mutations affect amino acid 883 in exon 15 or 922 *(378,394)*. A correlation between phenotype and genotype suggests that in FMTC patients with non-cysteine *RET* mutations, the onset of C-cell disease is delayed to later in life compared to patients with classical *RET* mutations in exon 10 *(368,396)*.

Guideline 54. Genetic Risk of MTC

- In MEN 2 kindred 50% of the family members are potentially affected by the disease.
- Almost all patients bearing *RET* mutations will develop MTC. (Note: inactivating mutations of the *ret* gene also cause Hirschsprung's disease).
- 5-10% of sporadic MTC have been found to carry germline *RET* mutations. Therefore *RET* analysis is justified in all patients with apparently sporadic MTC.

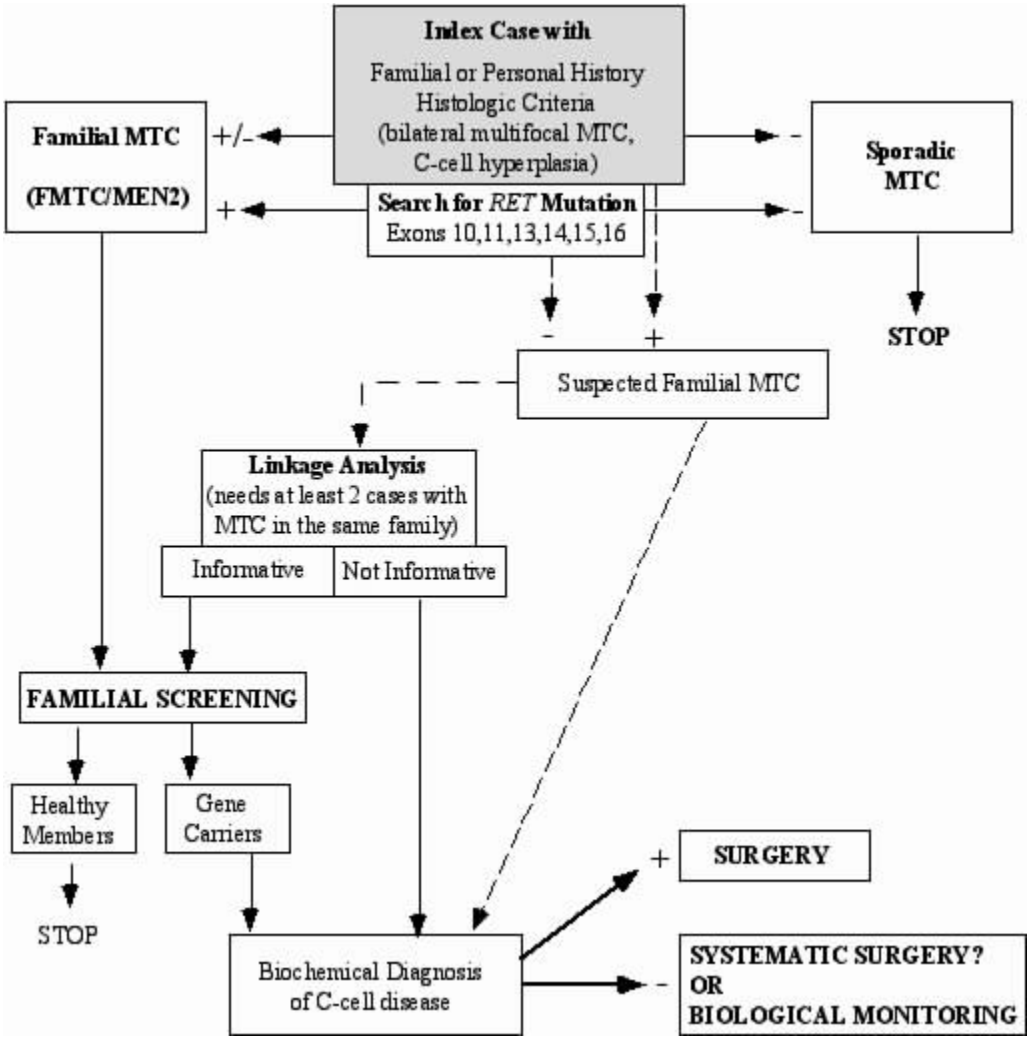

Figure 11. Diagnosis/Treatment Algorithm for MTC

When a mutation has been identified in a family, one can be certain that family members and their descendants not bearing the mutation are free of the pathology. Conversely, the subjects bearing the mutation have the pathology and will require surgical treatment to manage, or prevent the development of disease (Figure 11). If no genomic mutation is identified in the index case, as is the case in less than 3% of MEN 2A and 5% of FMTC, linkage analysis can be used to predict the risk level for the family members. If no predictions of this kind are possible because of the genealogy of the family, the detection of disease will have to be carried out by repeated clinical studies and specific biological tests at appropriate intervals.