

ORIGINAL ARTICLE

Calcium-stimulated calcitonin test for the diagnosis of medullary thyroid cancer: results of a multicenter study and comparison between different assays

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ABSTRACT

BACKGROUND: A basal serum calcitonin (Ct) increase >100 pg/mL in patients with a thyroid nodule is consistent with the diagnosis of medullary thyroid cancer (MTC). In cases where the CT test have a slight to moderate increase, the calcium gluconate stimulation test is helpful to increase diagnostic accuracy. However, reliable cut-offs for calcium-stimulated Ct are still lacking. The aim of this study was to evaluate the sex-specific calcium-stimulated Ct cutoffs for the diagnosis of MTC in a multicenter series. A comparison between different Ct assays has been also performed.

METHODS: 90 subjects undergone calcium-stimulated Ct for a suspected MTC in 5 Endocrine Units between 2010-2021 were retrospectively analyzed. Serum Ct concentrations were assessed by immunoradiometric (IRMA) or chemiluminescence (CLIA) assays.

RESULTS: MTC was diagnosed in 37 (41.1%) and excluded in 53 (58.9%) patients. The best calcium-stimulated Ct cut-off to identify MTC was 611 pg/mL in males (AUC =0.90, 95% CI (0.76;1) and 445 pg/mL in females (AUC=0.79, 95% CI (0.66;0.91). Logistic regression analysis showed that both basal (OR 1.01, P=0.003) and peak Ct after stimulation (OR 1.07, P=0.007) were significantly associated with MTC, together with sex (OR=0.06, P<0.001). The "Ct assay" variable was also considered in the logistic regression model, but it was not significantly associated with MTC (OR=0.93, P=0.919).

CONCLUSIONS: This study indicates that calcium test could be helpful to identify patients with early-stage MTC and those without MTC. A Ct value of 611 pg/mL in males and 445 pg/mL in females are proposed as the optimal Ct cut-offs at the stimulation test.

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KEY WORDS: Thyroid cancer, medullary; Calcitonin; Diagnosis; Hyperplasia.

Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor originating from the parafollicular C cells of the thyroid.¹ Up to 35% of the patients with MTC with a palpable thyroid nodule have cervical lymph node metastases and up to 13% have distant metastases.² Thus, an early detection is mandatory to improve the surgical cure rate. High levels of serum calcitonin (Ct) are strongly suggestive for MTC, although false positive Ct increase is a frequent condition.³ In fact, several diseases including kidney failure, hyperparathyroidism, extra-thyroid neuroendocrine neoplasms and non-neuroendocrine tumors as well as some drugs can cause an increase in serum Ct levels.³

Routine measurement of serum Ct in the diagnostic work-up of solid thyroid nodules could precociously identify MTC at an early stage, with a relevant impact on patient's outcome.⁴ However, current American Thyroid Association (ATA)⁵ and American Association of Clinical Endocrinologists (AACE)/American College of Endocrinology (ACE)/Associazione Medici Endocrinologi (AME) guidelines do not recommend either for or against routine measurement of serum Ct in patients with thyroid nodules.⁶ On the other hand, a joint consensus of six Italian scientific societies recommends measurement of serum Ct only before surgery.⁷

Basal Ct assessment is consistent with MTC diagnosis when this parameter is >100 pg/mL.⁸ A mild-moderate basal Ct increase (from the upper limit of normal range to 100 pg/mL) has to be interpreted cautiously. The calcium gluconate stimulation test represents a confirmatory assay performed after the finding of a <100 pg/mL serum Ct increase at baseline, to increase the sensitivity of Ct testing and to avoid unnecessary thyroidectomies.^{6, 8} The test could be useful for the differential diagnosis between MTC and other nonthyroidal neuroendocrine neoplasms,⁸ and to discriminate between MTC and C-cell hyperplasia (CCH), a precancerous lesion potentially progressing to MTC. However, at now a relationship between CCH and cancer progression has been demonstrated only for hereditary MTC.^{9, 10}

Several studies investigated patients with high serum Ct concentrations to find sex-specific Ct thresholds predictive of MTC.⁹⁻¹³ However, uni-

versal, and reliable cut-offs for either basal or calcium-stimulated Ct are still lacking.

The aim of this study was to evaluate the sex-specific stimulated-Ct cutoff to achieve the diagnosis of MTC in a large multicenter series. A comparison between different Ct assays has been also performed.

Materials and methods

Patients

All patients, aged 18-80 years, undergone between 2010-2021 a calcium-stimulated test for Ct for a suspected MTC, based on ≥ 1 cm ultrasonographically-evident solid thyroid nodule associated to serum Ct levels more than the upper limits of the normality range and less than 100 pg/mL. Confounding factors for hypercalcitoninemia were checked, as previously reported [3]. In case of therapy with proton pump inhibitors these drugs were withdrawn for at least four weeks before Ct assessment. All other confounding factors have been excluded in this population.

Exclusion criteria were subjects <18 and >80 years of age, diagnosis of MEN2 syndrome. An excel database was built collecting data from 5 different Endocrine Units (Sant'Andrea University Hospital of Rome, Umberto I University Hospital of Rome, Federico II University Hospital of Naples, Cardarelli Hospital of Naples, San Martino University Hospital of Genoa). These centers share many research protocols on endocrine tumors as well as the same calcium test procedure and the same criteria for selecting the subjects to be testing. All patients provided written informed consent to data collection. The study was approved by the local review board at the Sant'Andrea University Hospital of Rome (Reference number CE6988/2022) and conducted in accordance with the Declaration of Helsinki.

Data collection

For all subjects, the following parameters were collected: age at diagnosis, sex, year of diagnosis, Body Mass Index (BMI), basal and calcium-stimulated serum Ct concentrations, serum Ct assay. For those who underwent surgery, histology was also recorded.

Methods

Serum Ct concentrations were assessed by a commercially available Ct assay: 1) immunoradiometric assay (IRMA); or 2) chemiluminescence assay (CLIA):

- two high affinity monoclonal antibodies in an IRMA system (Institute of Isotopes, Budapest, Ref: RK-83CT). Analytic sensitivity was 0.5 pg/mL. Normal values were 0.4-13 pg/mL (male subjects) and 0.0-5.8 pg/mL (female subjects). The intrassay coefficients of variation were 3.2% and 1.6%, respectively, at 25.3 pg/mL and 161.2 pg/mL. The interassay coefficients of variation were 5.1% and 6.2%, respectively, at 24.9 pg/mL and 170.6 pg/mL. The KIT has no “high-dose hook” effect with Ct levels up to 300 pg/mL;

- two-site sandwich-type CLIA (DiaSorin Inc., Stillwater, MN, USA). Normal values were 0.4-18.9 pg/mL (male subjects) and 0.0-5.5 pg/mL (female subjects). Analytic sensitivity was 1 pg/mL or less. The intrassay and interassay coefficients of variation were 3% and 4%, respectively, at 70.4 pg/mL and 1 and 2%, respectively, at 179 pg/mL. The Ct assay run time was 15 min. No hook effect was observed until a Ct concentration of 500,000 pg/mL.

Calcium gluconate was administrated intravenously at 5 mL/min, after a 4-hour fast at a dose of 25 mg or 2.3 mg of elemental calcium/kg of adequate body weight (www.manuelsweb.com/IBW.htm for ideal body weight and adjusted body weight calculator). Ct was assessed before and 2, 5, and 10 minutes after calcium gluconate injection, as previously described.⁶ Serum calcium levels were assessed before the test in order to exclude pre-existing hypercalcemia. Electrocardiography was performed before the calcium test as well and cardiopathic patients underwent further cardiologic examination. No major adverse effects were observed during calcium gluconate infusion test in the patients involved in the study.

Statistical analysis

This study used a convenience sample with no priori power analysis due to its retrospective exploratory nature. Quantitative variables were

summarized using mean (\pm standard deviation) and/or median (interquartile range) and Student's *t*-test or Mann-Whitney Test, as appropriate, was used to evaluate any statistically significant differences between the MTC patient group and the group of subjects free from thyroid tumor (NT). Qualitative variables were synthesized as absolute and percentage frequencies, and the comparison between the two groups was made using the χ^2 test.

ROC curve analysis, and the corresponding AUC, was used to evaluate the accuracy of calcium-stimulated Ct levels to discriminate between patients with MTC and NT, dividing by gender. The optimal cut-off was identified using the Youden index and the corresponding measures of sensitivity, specificity, positive predictive value and negative predictive value were provided. The same analysis was performed considering baseline Ct levels.

Multiple logistic regression analysis was performed to evaluate the association between MTC and the various predictive factors (calcium-stimulated Ct levels, baseline Ct levels and Ct assay), adjusting also for gender.

Patients were then divided into the three groups: MTC, NT and C-cell hyperplasia (CCH) and the ROC surface analysis,¹⁴ an extension of the ROC curves for classification problems with three classes, was performed to assess the accuracy of the calcium-stimulated Ct level in discriminating between the 3 groups of patients. The volume under the surface ROC (VUS) was calculated and then the optimal cut-offs were identified. All statistical analyses were performed using the R statistical software. The significance level for all statistical tests was set at 5%.

Data availability

The data associated with the paper are not publicly available but are available from the corresponding author on reasonable request.

Results

Patients' characteristics

A total amount of 105 patients were collected in the five centers participating in the study. Eleven

TABLE I.—Comparison between Ct assays.

	CLIA N.=30	IRMA N.=60	P value
MTC, N. (%)	15 (50%)	22 (36.7%)	0.325
Basal serum Ct pg/mL (mean±SD)	29.3±22.1	22.5±23	0.202
Stimulated serum Ct peak pg/mL (mean±SD)	401±487	429±372	0.761

MTC: medullary thyroid cancer.

TABLE II.—Calcium at baseline in no-tumor and MTC subjects.

	N.	NT		MTC			P value
		Mean (±SD)	Me (IQR)	N.	Mean (±SD)	Me (IQR)	
Males	31	16.2 (±11)	13 (7.8;22.2)	9	43.6 (±30.8)	40.8 (11.5;66.2)	<0.001
Females	22	13.2 (±7.2)	11 (8;15.2)	28	36 (±26.5)	30.4 (17;46.6)	<0.001

NT: no-tumor, MTC: medullary thyroid cancer.

TABLE III.—Calcium stimulated serum Ct peak in no-tumor and MTC subjects.

	N.	NT		MTC			P value
		Mean (±SD)	Me (IQR)	N.	Mean (±SD)	Me (IQR)	
Males	31	291.3 (±154.8)	240 (162.6;410)	9	668 (±275.7)	645.9 (417;851.9)	<0.001
Females	22	204 (±142.2)	169.7 (81.4;330.3)	28	660.3 (±603.6)	472.5 (191.8;748.7)	<0.001

Ct peak more than 2000 pg/mL are levelled at this cut-off.

NT: no-tumor; MTC: medullary thyroid cancer.

patients were excluded because affected with MEN2 syndrome, 4 patients were excluded because of incomplete data. The final study population serving for the statistical analysis included 90 subjects. Ct assay resulted to be IRMA in 60 subjects and CLIA in 30. There was no significant difference in terms of rate of MTC as well as serum Ct levels at baseline and calcium test peak between the two assays (Table I).

Diagnostic performance of basal and calcium-stimulated Ct peak

The study population included 40 males and 50 females. The histological examination showed a MTC in 37/90 (41.1%), with 9 MTC in males (22.5%) and 28 in females (56%). Basal and calcium-stimulated Ct peak were significantly different between NT (including CCH) and MTC both in males and females ($P<0.001$) (Table II, III).

Using the ROC curve, the optimal calcium-stimulated Ct cut-off optimally discriminating NT and MTC was 611 pg/mL in males (AUC=0.90, 95% CI [0.76;1], sensitivity=0.67, Specificity=1, Ppv=1, NPV=0.91) and 445 pg/mL

in females (AUC=0.79, 95% CI [0.66;0.91] sensitivity=0.54, specificity=1, PPV=1, NPV=0.63 (Figure 1).

Regarding basal Ct, the optimal cut-off discriminating NT and MTC was 39 pg/mL in males (AUC = 0.76, 95% CI [0.56;0.96], sensitivity=0.67, specificity=0.97, PPV=0.86, NPV=0.91) and 19.2 pg/mL in females (AUC=0.80, 95% CI [0.68;0.92] sensitivity=0.75, specificity=0.82, PPV=0.84, NPV=0.72).

Logistic regression analysis showed that both basal (OR 1.01, $P=0.003$) and peak Ct after stimulation (OR 1.07, $P=0.007$) were significantly associated with MTC, together with sex (OR=0.06, $P<0.001$, female as reference category). The "Ct assay" variable was also considered in the logistic regression model, but it was not significantly associated with MTC (OR=0.93, $P=0.919$).

If CCH was considered separately from NT, the study population included 19 NT, 12 CCH and 9 MTC in males and 10 NT, 12 CCH and 28 MTC in females. In males, the calcium-stimulated Ct optimal cut-off between NT and CCH identified by ROC surface analysis was 328.3 pg/mL, while between CCH and MTC was 615 pg/

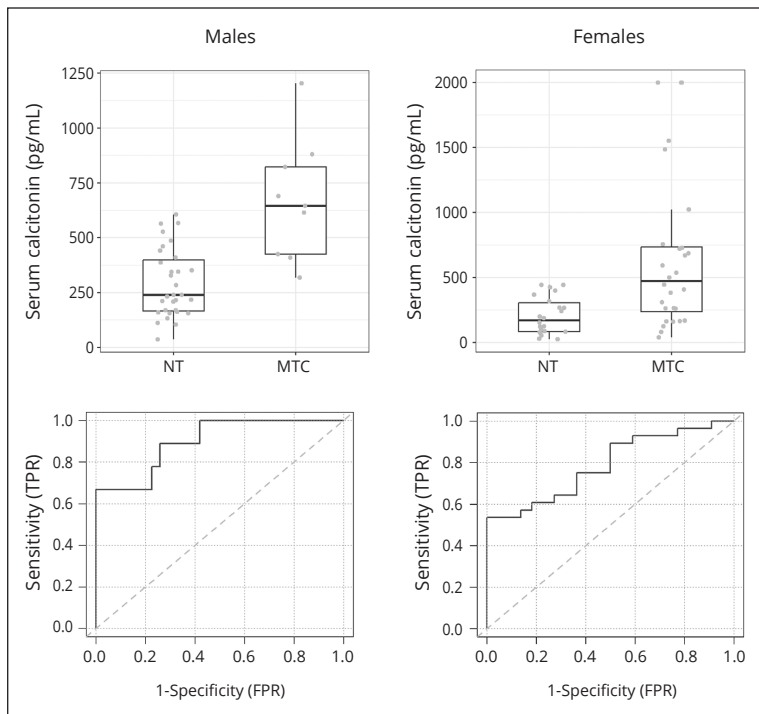


Figure 1.—Calcium stimulated serum Ct peak in no-tumor and MTC subjects according to sex.

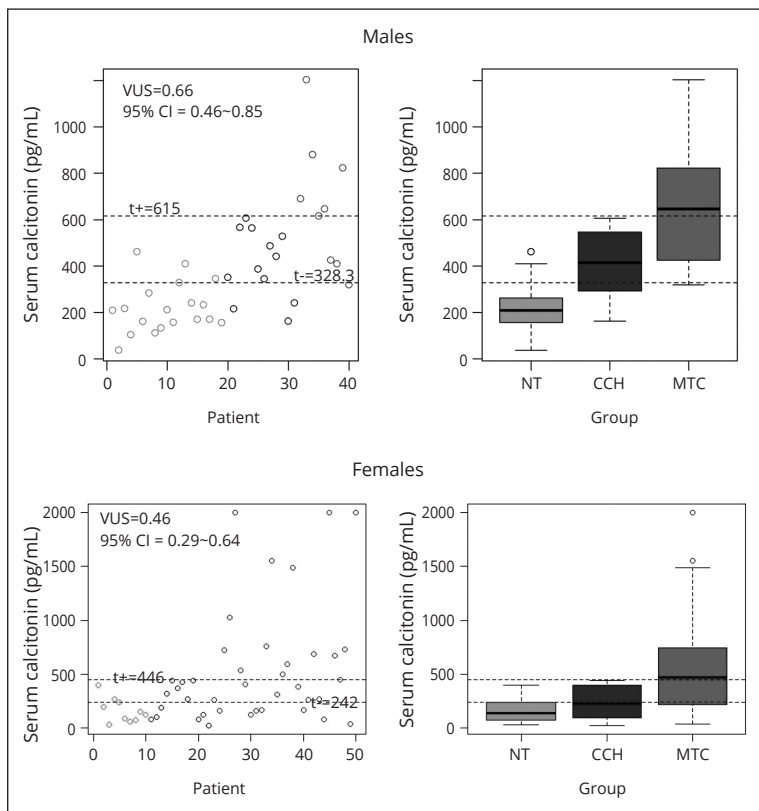


Figure 2.—Calcium stimulated serum Ct peak in no-tumor, C-cell hyperplasia and MTC subjects, in males (top graphs) and females (bottom graphs).

mL (VUS= 0.66). In females, the calcium-stimulated Ct cut-off between NT and CCH was 242 pg/mL, while between CCH and MTC was 446 pg/mL (VUS=0.46) (Figure 2).

Regarding basal Ct, the optimal cut-off was only evaluable in males, being 10.1 pg/mL between NT and CCH and 39 pg/mL between CCH and MTC (VUS 0.44), because in females the order of the three groups (*i.e.* Ct in MTC \geq Ct in CCH \geq Ct in NT) was not respected.

Discussion

Stimulated Ct shows an elevated diagnostic performance, which represents a helpful tool in the borderline cases of thyroid nodule with serum Ct levels between the upper limit of the normal range and 100 pg/mL. Procalcitonin has been proposed as another marker of MTC, particularly in cases where calcitonin levels are not significantly elevated. Albeit procalcitonin can be elevated in MTC, it does not represent a specific marker of MTC since its elevation can be observed in other types of cancer and inflammatory conditions too.¹⁵ The pentagastrin test has been well established as reference test in this attempt with well-defined cut-off to recognize MTC subjects.^{16, 17} Unfortunately, this test has been no longer performed due to unavailability of pentagastrin. For this reason, in recent years the calcium test has become the main procedure for cases with suspected MTC but basal Ct less than 100 pg/mL. As previously discussed, different cut-offs have been proposed by different studies, resulting in a fixed point represented by the statement of higher value in males than females but high variability between one to another.⁹⁻¹³ The present series on 90 patients undergone the calcium test identified a cut-off of 611 pg/mL in males and 445 pg/mL in females, with a specificity of 100% in both, which is guarantee of no false positive diagnoses and inappropriate thyroid surgery. Considering the significant sensitivity and the specificity of 100%, the calcium-stimulated Ct test represents the best method to precociously identify non-clinically evident MTC in the most of cases, with a consequent positive impact on prognosis, at the same time avoiding further investigations and treatment in normal subjects. These results

are still more relevant because based on a multi-center database. Finally, the comparison between two different Ct assays did not show significant differences. Ct assay was not a confounding factor in the relationship between MTC and Ct peak because it did not improve the prediction of MTC compared to the model without the “Ct assay” variable. In other words, if to experience a highly performing Ct assay method is a priority for each center dedicated to MTC, on the other hand the calcium-stimulated Ct seems to be equally effective regardless the assay employed.

Almost all patients were well characterized so providing an adequate study population for the purpose of the study. To further increase the homogeneity and avoid confounding factors, all *RET* positive subjects were excluded. MTCs are genetically inherited in about 30% of cases. These MEN2-related MTCs develop early from CCH to overt tumor and their detection is guided by the *RET* gene mutational analysis, regardless from Ct assessment.^{18, 19} A Ct increase in this setting is a time point to perform surgery, regardless from the finding of CCH rather of MTC at histology, as these genetically-determined tumors are expected to progress in all cases. For this reason, calcium-stimulated Ct cut-offs in MEN2-MTC should be evaluated separately for this specific population.

Finally, the homogeneity of this study was ensured even in terms of calcium test procedure which was the same in all centers.

Compared to other studies, the present one included only subjects with baseline Ct concentrations above the upper limit of the normality range. Other authors also included subjects with normal baseline Ct values, likely to increase the statistical power of the study. However, it has to be remarked that, in clinical practice, Ct stimulation needs to be performed only in subjects with elevated baseline Ct values. Accordingly, this study population carefully reproduces real world. This aspect represents a strength of this study.

The possibility to achieve a surgical cure is related to tumor stage at diagnosis. Both in sporadic and genetic tumors, an early diagnosis is consistent with an increased rate of surgical cure and a decreased rate of tumor relapsing. For this

purpose, serum Ct is an optimal marker of early diagnosis. When basal serum Ct is >100 pg/mL this is strongly suggestive of MTC occurrence. However, when a mild Ct increase is found in a subject with a solid thyroid nodule, this could be related to an early stage MTC but also to many confounding factors of hypercalcitoninemia.³ Finally, a variable rate of subjects with Ct concentrations above the normal and without any confounding factors are not affected with MTC. To go in deep in the differential diagnosis, the calcium-stimulated Ct test is a reliable tool. In the last ten years some studies proved to identify the Ct threshold after calcium stimulation.

In 2012 Colombo *et al.* proposed a basal Ct threshold of 18.7 pg/mL and a stimulated Ct threshold of 184 pg/mL for females and of 68 pg/mL and 1620 pg/mL for males, which were able to distinguish healthy controls and CCH from MTCs.¹¹ Afterwards, in a larger population, Mian *et al.* identified a cutoff of 26 and 68 pg/mL for basal Ct and of 79 and 544 pg/mL for stimulated Ct in females and males, respectively.⁹ More recently, Niederle *et al.* have demonstrated that a basal Ct >23 pg/mL or stimulated Ct >780 pg/mL in females and basal Ct >43 pg/mL or stimulated Ct >1500 pg/mL in males were able to early detect MTC and to predict the presence of cervical lymph node metastasis.^{12, 13} Finally, Fugazzola *et al.* reported that the best basal Ct cutoff values able to discriminate non-MTC from MTC patients were >30 pg/mL for females and >34 pg/mL for males, whereas the best stimulated-Ct thresholds were >79 pg/mL for females and >466 pg/mL for males.¹⁰ These findings reveal a great variability in the proposed Ct thresholds between different studies and populations. The present data confirm higher calcium-stimulated Ct cut-offs in males than females, suggesting a cut-off of 611 pg/mL in males and of 445 pg/mL in females to achieve the diagnosis of MTC.

In clinical practice, to discriminate MTC cases from non-MTC cases (NT and CCH), is a crucial point in the diagnostic work-up. CCH is frequently responsible for increased basal Ct and has been found in various thyroid diseases other than MTC and MEN2 syndrome, such as multinodular goiter, Hashimoto's thyroiditis, papillary thyroid carcinoma.^{20, 21} Surgery could be avoided

in patients with CCH, outside the condition of MEN2 syndrome. On the contrary, in patients with preoperative diagnosis of MTC, total thyroidectomy should be combined with prophylactic dissection of central compartment lymph nodes, in all cases of no evidence of neck lymph node metastases and no evidence of distant metastases.⁸

As highlighted in the current study, the prevalence of CCH is quite relevant, being 26.7% of all subjects with Ct levels above the normal and 45.3% of non-MTC cases. Furthermore, both basal and calcium-stimulated Ct levels were higher in CCH than in NT group, so making CCH an issue to be faced in clinical practice.

In this meaning, a cut-off of 615 pg/mL in males and 446 pg/mL in females is reliable to achieve the diagnosis of MTC and exclude CCH. On the other hand, if both basal and peak Ct after stimulation significantly distinguished between MTC and normal subjects, however basal Ct values were less reliable to distinguish CCH and MTC.

Conclusions

The present study supports the role of calcium test in the diagnostic work-up of MTC, improving the capacity of Ct to achieve the diagnosis of MTC and avoiding an unhelpful thyroid surgery both in normal and CCH subjects. Future prospective studies on larger series are needed to define the reference limits of the calcium gluconate test and improve the diagnostic work-up of MTC.

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Conflicts of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Authors' contributions

Conceptualization: Antongiulio Faggiano; methodology: Pasquale Dolce, Antongiulio Faggiano; formal analysis: Pasquale Dolce; data curation: Manuela Albertelli, Livia Barba, Maria G. Deiana, Ruggero Martinelli, Roberta Modica, Cecilia Motta, Luca Patti, Francesco Scavuzzo, Virginia Zamponi; writing—original draft preparation: Antongiulio Faggiano, Elisa Giannetta, Franz Sesti, Virginia Zamponi; supervision: Annamaria Colao, Salvatore Monti.

History

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