Octreotide and pasireotide effects on medullary thyroid carcinoma (MTC) cells growth, migration and invasion

E. Giardino, R. Catalano, F. Mangili, A.M. Barbieri, D. Treppiedi, F.M. Elli, A. Dolci, A. Contarino, A. Spada, M. Arosio, G. Mantovani, E. Peverelli

PII: S0303-7207(20)30394-4

DOI: https://doi.org/10.1016/j.mce.2020.111092

Reference: MCE 111092

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 9 June 2020

Revised Date: 18 November 2020

Accepted Date: 21 November 2020

Please cite this article as: Giardino, E, Catalano, R, Mangili, F, Barbieri, A., Treppiedi, D, Elli, F., Dolci, A, Contarino, A, Spada, A, Arosio, M, Mantovani, G, Peverelli, E, Octreotide and pasireotide effects on medullary thyroid carcinoma (MTC) cells growth, migration and invasion, *Molecular and Cellular Endocrinology*, https://doi.org/10.1016/j.mce.2020.111092.

CRediT author statement

Giardino E: investigation, methodology, Formal analysis, writing - Original Draft, Visualization

Catalano R: investigation, validation, Formal analysis, Visualization

Mangili F: investigation,

Barbieri AM: investigation

Treppiedi D: investigation

Elli FM: investigation

Dolci A: resources

Contarino A: resources

Spada A: Writing – Review & Editing

Arosio M: Writing – Review & Editing, Supervision

Mantovani G: Conceptualization, Validation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

Peverelli E: Conceptualization, Methodology, Validation, Formal analysis, writing - Original Draft– Review & Editing, Supervision, Visualization, Project administration, Funding acquisition.

Octreotide and pasireotide effects on medullary thyroid carcinoma (MTC) cells growth, migration and invasion

Giardino E[1], Catalano R[1,2], Mangili F[1], Barbieri AM[1], Treppiedi D[1], Elli FM[3], Dolci A[3], Contarino A[3], Spada A[1], Arosio M[1,3], Mantovani G[1,3], Peverelli E[1]

[1]Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy

[2]PhD Program in Endocrinological Sciences, Sapienza University of Rome, Rome, Italy

[3]Endocrinology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico Milan, Italy

Correspondence should be addressed to Giovanna Mantovani: giovanna.mantovani@unimi.it

Giovanna Mantovani, MD, PhD

Endocrinology Unit

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico - Pad. Zonda

Via Lamarmora, 5

20122-Milan, Italy

Phone +39 02 50320613

Fax +39 02 50320605

E-mail: giovanna.mantovani@unimi.it

Short title: Octreotide and pasireotide in MTC cells

Keywords: somatostatin, medullary thyroid cancer, cell proliferation, cell migration

Declaration of interest: none

Word count: 4713

Abstract

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine neoplasm of the parafollicular thyroid C cells. Although somatostatin receptors are expressed by MTCs, treatment with octreotide has shown poor efficacy, whereas recently pasireotide has demonstrated antiproliferative effects in persistent postoperative MTCs.

Aim of this study was to test the effects of octreotide and pasireotide on MTC cells proliferation, cell cycle proteins expression, MAPK activation, apoptosis, calcitonin secretion, migration and invasion in TT cell line as well as in primary MTC cultured cells. Our results showed that both octreotide and pasireotide reduced TT cell proliferation (-35.2±12.1%, $p<0.001$, and -25.3±24.8%, $p<0.05$, at 10^{-8} M, respectively), with concomitant inhibition of ERK phosphorylation and cyclin D1 expression. This cytostatic effect was accompanied by a proapoptotic action, with an increase of caspase3/7 activity of 1.5-fold. Moreover, both octreotide and pasireotide inhibited cell migration (-50.9±11.3%, $p<0.01$, and -40.5±17%, $p<0.05$, respectively) and invasion (-61.3±35.1%, $p<0.05$, and -49.7±18%, $p<0.01$, respectively). No effect was observed on calcitonin secretion. We then tried to extend these observations to primary cultures (n=5). Octreotide and/or pasireotide were effective in reducing cells proliferation in 3 out of 5 tumors, and to induce cell apoptosis in 1 out of 3 MTCs. Both octreotide and pasireotide were able to reduce cell migration in all MTC tested. SST2, SST3 and SST5 were expressed in all MTC, with a tendency to increased expression of SST2 in RET mutated vs wild type MTCs. In agreement, inhibition of mutated RET in TT cells reduced SST2 expression.

In conclusion, we demonstrated that octreotide and pasireotide inhibited cell proliferation and invasiveness in a subset of MTC, supporting their potential use in the control of tumor growth.

1. Introduction

MTC is a rare neuroendocrine neoplasm (NEN), representing 5% of thyroid malignancies, that results from the malignant transformation of neural crest derived, calcitonin-secreting, parafollicular

thyroid C-cells (Roman *et al*. 2006). Activating mutations of RET (REarranged during Transfection) proto-oncogene are the cause of 98% of hereditary MTC and of 56% of the sporadic tumors (Ciampi *et al*. 2019), with a striking genotype/phenotype correlation. MTC displays a highly variable biological behavior, with mean survival rate of 65% after 5 years (Elisei *et al*. 2012). The treatment of choice is complete surgical resection. Tyrosine kinase inhibitors (TKI) showed only moderate effects and their use is restricted to patients with significant tumor burden and well-established tumor progression (Wells *et al*. 2012, Elisei *et al*. 2013).

Somatostatin (SS) analogs (SSAs) are used in the therapy of other NENs, and the expression of SS receptors (SSTs) subtype 1, 2, 3 and 5 (Mato *et al*. 1998, Kendler *et al*. 2017) in MTCs has suggested the possibility to treat these tumors with SSAs. However, first-generation SSAs octreotide and lanreotide, specific for SST2, demonstrated poor antiproliferative effects in MTC patients. In particular, Modigliani and coworkers found that in 4/14 thyroidectomized MTC patients with persistently elevated plasma calcitonin levels, octreotide treatment reduced calcitonin, but did not induced morphological improvement (Modigliani *et al.* 1992). Another study in 7 patients showed a remission in 2 patients after octreotide treatment (Frank-Raue *et al*. 1995). Disease stabilization, minor tumor regression and calcitonin reduction were induced by lanreotide in combination with interferon-alpha2β in 3/7, 2/7 and 6/7 patients, respectively (Vitale *et al*. 2000). Octreotide effects in inducing subjective and biological partial remission (Vainas *et al*. 2004) and in reducing calcitonin (Mahler *et al*. 1990, Cano *et al*. 2017) have been reported.

A recent study demonstrated that pasireotide, a second generation, multi-receptor targeted SSA with preferential binding to SST5, exerted antiproliferative effects in persistent postoperative MTC (Faggiano *et al*. 2018).

In vitro data have demonstrated that human MTC cells TT differentially responded to SSAs selective for different SST subtypes, being responsive to the antiproliferative effects of SST2 and SST1 but not SST5-specific analogs (Zatelli *et al.* 2001, 2002*a*). Moreover, a selective analog for SST1, but not for SST2 and 5, was able to reduce calcitonin secretion in TT cells (Zatelli *et al.*

2002*a*,*b*), and only a subset of primary cultured MTC cells was responsive to SSAs antisecretory activity (Zatelli *et al.* 2006). Notably, SSA antiproliferative and antisecretory effects were not correlated (Zatelli *et al.* 2002*b*, 2006). However, no data are available on the effects of octreotide and pasireotide in MTC cells.

Overall, these results underline the need for a better knowledge of the molecular mechanisms specifically activated by the different clinically available SSAs, acting through different SSTs subtypes in MTC cells.

In addition to antimitotic effects, SSAs have shown proapoptotic effects (Ferrante *et al.* 2006) and more recently anti-invasive effects in pituitary (Peverelli *et al.* 2018*a*) and pancreatic (Vitali *et al.* 2016) NENs, but no data are available in MTC.

In the present study the effects of octreotide and pasireotide on MTC cells proliferation, cell cycle proteins expression, MAPK activation, apoptosis, calcitonin secretion, migration and invasion have been tested in TT cell line, the most widely used cell model for MTC, harboring the RET mutation C634W, as well as in primary MTC cultured cells.

2. Material and methods

2.1 Cell cultures

Human TT cells were obtained from ATCC (CCL-82.1), tested and authenticated by genetic profiling using polymorphic short tandem repeat (STR) loci with PowerPlex Fusion system (Promega, BMR Genomics Cell Profile service, Italy), that allows to amplify 23 loci STR (D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, DYS391, D8S1179, D12S391, D19S433, FGA, D22S1045) and Amelogenin, AMEL, for sex determination. Results were compared to reference cell line databases (ATCC, DSMZ, JCRB and RIKEN).

TT cells were cultured in F12K medium, 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotics (Gibco, Invitrogen, Life Technologies Inc., Carlsbad, CA, USA).

Human MTC tissue samples were obtained at the time of surgery, and immediately dissected by the pathologist under sterile conditions. Fresh specimens (n=5) were partly stored at -80°C for nucleic acids and proteins extraction and partly dissociated to obtain primary cell cultures as previously described (Giardino *et al.* 2019). Briefly, tissues were digested in Dulbecco's modified Eagle's medium (DMEM) with 3.5 mg/mL collagenase (Sigma Aldrich, St. Louis, MO, USA) at 37°C for 90 min, passed on a 100 μm filter (nylon cell strainer, BD Transduction Laboratories, Lexington, UK) and cultured in TT medium.

2.2 SSTs expression level analysis

Analysis of SSTs protein expression levels was performed on frozen samples of MTC tissues (n=5) and TT cells by western blotting. TT cells were incubated when indicated with RPI-1 (Cayman Chemical Company, Michigan, USA) for 24h. Total proteins were extracted with lysis buffer (Cell Signaling, Danvers, MA, USA), and 30 μg were separated on sodium dodecyl sulfate (SDS)/polyacrylamide gels and transferred to a nitrocellulose filter. To detect the total levels of receptor proteins, we used specific antibodies against SST1 (Abcam, Cambridge, UK) (1:1000), SST2 (Santa Cruz Biotechnology, Dallas, TX, USA) (1:200), SST3 (Abcam, Cambridge, UK) (1:1000), SST5 (Abcam, Cambridge, UK) (1:1000). Secondary anti-mouse and anti-rabbit horseradish peroxidase-linked antibodies (Cell Signaling, Danvers, MA, USA) were used (1:2000). GAPDH was used as housekeeping gene (antibody from Ambion, Austin, TX, USA). Chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Upland, CA, USA) and the resulting bands were analyzed with the image analysis program NIH ImageJ software.

2.3 Genetic analysis

To perform RET genetic analysis, DNA was extracted from MTC tissue samples with Gentra Puregene Tissue Kit (QIAGEN, Hilden, Germany) and from peripheral blood lymphocytes with

FlexiGene DNA Kit (QIAGEN, Hilden, Germany). RET (GenBank accession no. NG_007489.1) exons 10, 11, 12, 13, 14, 15, and 16 were amplified by PCR. Direct sequencing of the amplified exons was then performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA USA). Primers are shown in Suppl. Tab.1.

2.4 Cell proliferation assay

Cell proliferation was assessed by colorimetric measurement of 5-bromo-2-deoxyuridine (BrdU) incorporation in newly synthesized DNA according to the manufacturer's instructions (GE Healthcare, Life Science, Buckinghamshire, UK) (Giardino *et al*. 2019). Cells were plated at $3x10^4$ cells/well and treated with octreotide or pasireotide (both from Novartis Pharma AG, Basel, Switzerland) at different concentrations for 48h or 72h. Cells were then incubated with BrdU for 2 h (TT cells) or 24h (primary cultured MTC cells) at 37°C. All experiments were repeated at least 3 times and each determination was done in triplicate.

2.5 Western blot analysis

For cyclin D1 expression analysis, cells were seeded in 6-well plate at a cell density of $4x10^5$ cells/well, starved 18 h at 37°C and incubated with or without octreotide and pasireotide for 3h, 8h, 18h or 24h in complete medium. ERK1/2 phosphorylation status was evaluated after starvation followed by 10 min octreotide and pasireotide incubation in complete medium. Total proteins were extracted from cells, quantified by BCA assay, separated by SDS-polyacrylamide gels, and transferred to a nitrocellulose filter. Cyclin D1 antibody (Cell Signaling, Danvers, MA, USA) was used at 1:1000 dilution. To detect phosphorylated ERK1/2, 1:1000 dilution of anti-phospho p42/44 antibody (Immunological Science, Italy) was used. The presence of total ERK1/2 was analyzed by stripping and reprobing with anti-total p42/44 antibody (1:1000 dilution, Immunological Science, Italy). Secondary HRP-linked antibodies were used (1:2000 dilution, Cell Signaling, Danvers, MA, USA). GAPDH was used as housekeeping gene (antibody from Ambion, Austin, TX, USA).

Chemiluminescence was detected using the ChemiDoc-IT Imaging System (UVP, Upland, CA, USA) and the resulting bands were analyzed with ImageJ software. Experiments were repeated 3 times.

2.6 Caspase-3/7 activity

To test cell apoptosis, caspase-3/7 enzymatic activity was measured using Apo-ONE Homogenous Caspase-3/7 assay (Promega, Madison, WI, USA) as previously reported (Peverelli *et al*. 2018*b*). Cells were seeded at a cell density of 3×10^4 cells/well in 96-well plate and incubated with octreotide or pasireotide 10^{-8} and 10^{-6} M for 48 h at 37°C. The amount of fluorescent product generated from a profluorescent caspase-3/7 consensus substrate is representative of the amount of active caspase-3/7 present in the sample. Each determination was done in quintuple. Experiments were repeated 3 times.

2.7 Calcitonin assay

Calcitonin was measured in culture medium by a specific chemiluminescence immunoassays (Immulite 2000 Calcitonin, Siemens Healthcare GmbH, Erlangen, Germany), according to the manufacturer's instructions.

2.8 Transwell migration and invasion assays

Cell migration was tested by transwell assays as previously described (Giardino *et al.* 2019). Briefly, $3x10^4$ cells were suspended in 200 µl of serum-free F12K medium and seeded into the upper chamber of a nanoporous transwell insert (InoMATRIX, Inocure, Czech Republic), whereas complete medium was added in the lower chamber. The 3D structure of InoMATRIX allows cell cultures to develop into tissues closely resembling native tissue. Cells were incubated at 37°C for 18 h, and migrated cells were stained with crystal violet, extracted with 10% acetic acid, and

measured using a plate reader at a wavelength of 560 nm. A negative control with serum-free medium in lower chamber was used in each experiment.

For cell invasion experiments, cell suspension ($3x10^5$ cells/insert) was seeded in the upper chamber of a transwell insert porous polycarbonate membrane (pore diameter: 8μm) (Merck Millipore, Darmstadt, Germany) coated with Matrigel (0.3 mg/ml, diluted in DMEM) (Corning, BD Transduction Laboratories, Lexington, U.K.), as previously described (Giardino *et al.* 2019). Cells that invaded on the lower surface of the membrane were quantified as described above.

2.9 Statistical analysis

The results are expressed as the mean ± SD. A paired two-tailed Student's t test was used to assess the significance between two series of data. SSTs densitometric data were analyzed by the non-parametric Mann-Whitney U test. In all analyses, a probability value $p<0.05$ was accepted as statistically significant. Data were analyzed by using GraphPad Prism 5.0.

3. Results

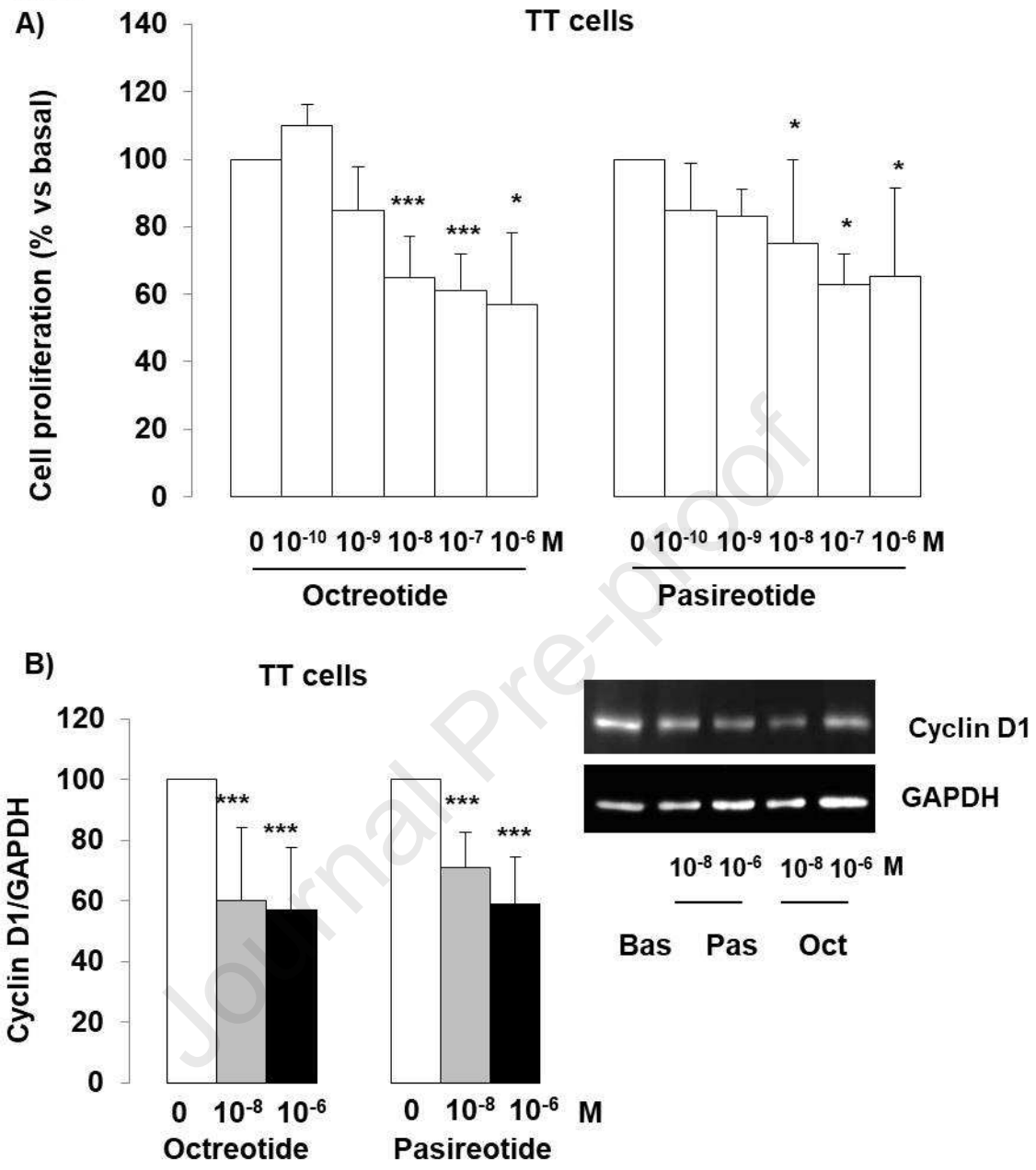
3.1 Octreotide and pasireotide effects on cell proliferation in TT and primary cultured MTC cells

We first assessed the ability of octreotide and pasireotide to affect TT cell proliferation, by measuring BrdU incorporation in newly synthesized DNA. After 24h starvation, TT cells were incubated in medium containing 10% FBS with or without octreotide or pasireotide at increasing concentrations (from 10^{-10} to 10^{-6} M). Our results (Fig. 1A) showed that both octreotide and pasireotide were able to reduce BrdU incorporation in newly synthesized DNA after 48 h incubation (-35.2±12.1%, $p<0.001$, and -25.3±24.8%, $p<0.05$, at 10^{-8} M, respectively), without significant differences between the two drugs. A sustained reduction of cell proliferation was observed at longer times of incubation (72h), although to a lesser extent (data not shown).

These effects were accompanied by a reduced expression of cyclin D1, as revealed by western blot analysis performed in TT cells stimulated for 3 h with octreotide or pasireotide (Fig. 1B). We observed a -40±24% reduction of cyclin D1 (CD1) in cells stimulated with octreotide 10^{-8} M ($p<0.01$) and -29±12% with pasireotide 10^{-8} M ($p<0.01$). This effect was maintained after 8h incubation, whereas no change in CD1 level was measured after 18h and 24h incubation (data not shown).

Accordingly, both octreotide and pasireotide reduced ERK phosphorylation (-35.4±25.2%, $p<0.01$ vs basal, and -20.9±9.6%, $p<0.001$ vs basal, at 10^{-8} M, respectively) (Fig. 1C).

Fig.1



214

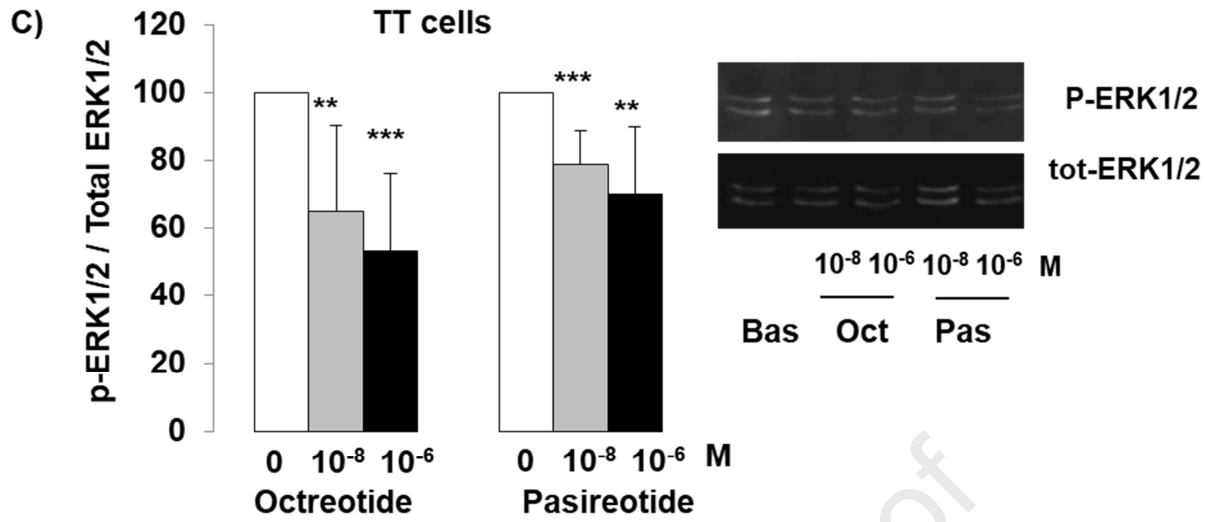


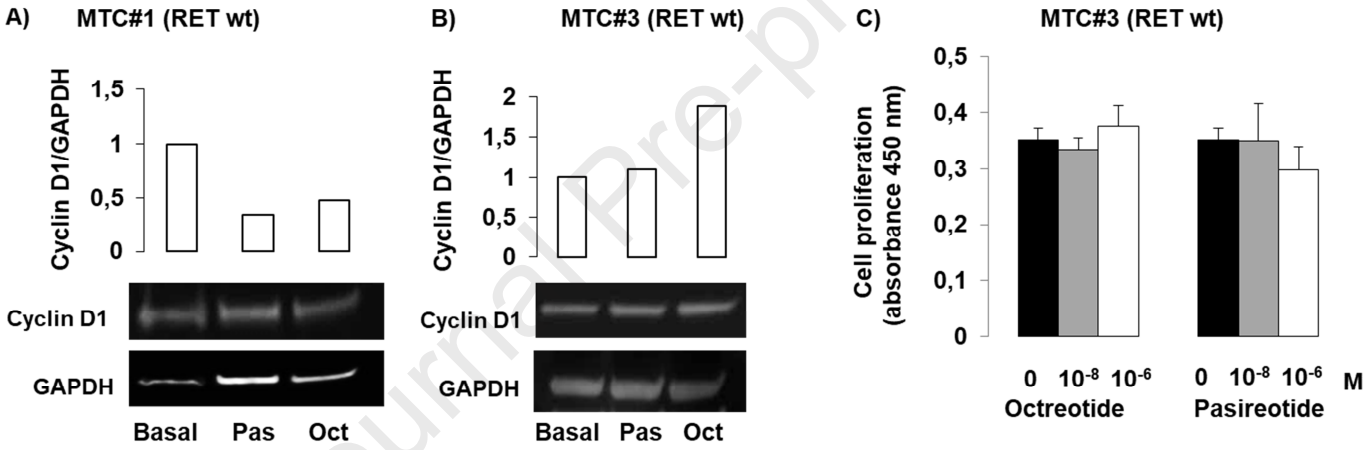
Figure 1. Octreotide and pasireotide treatment effects on cell proliferation in TT cells. A) Proliferation assay. TT cells were incubated with octreotide or pasireotide at the indicated concentrations for 48 h, and BrdU incorporation in newly synthesized DNA was measured. Experiments were repeated 3 times. Each determination was done in triplicate. B) Cyclin D1 assay. Cell were incubated 3h with octreotide or pasireotide 10 nM or 1 μM. The graph shows the quantification of cyclin D1 normalized to GAPDH (mean value ± S.D. from 3 independent experiments). Representative immunoblots are shown. C) ERK phosphorylation assay. Cells were incubated with octreotide or pasireotide 10 nM or 1 μM for 10 min. The graph shows the quantification of p-ERK1/2 normalized to total ERK1/2 (mean value ± S.D. from 3 independent experiments). Representative immunoblots are shown. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ vs corresponding basal.

The effects of octreotide or pasireotide on cell proliferation have also been tested in primary MTC cultured cells. We used primary cells derived from 5 MTCs, 3 bearing wild type RET gene (#1-3), and 2 bearing mutated RET (sample #4: somatic mutation M918T; #5: germinal mutation C611F). Due to the limited and variable number of cells derived from each tumor, we couldn't perform the complete set of dose-response experiments for all MTCs.

To test cell proliferation, we decided to first analyze CD1 expression, when possible accompanied by the BrdU incorporation assay, and we chose to test 10^{-6} M as single dose, since lower concentrations were not effective in preliminary experiments.

We found a reduction in cyclin D1 after 24 h incubation with octreotide (-52.6%) or pasireotide (-66.1%) in cells derived from one wild type tumor (MTC#1, Fig.2A), but not from wild type MTC #2 (data not shown) and #3, accordingly to BrdU incorporation assay (Fig.2B,C). In RET mutated tumor MTC#4, no effect was observed on CD1 expression (Fig.2D), whereas a slight but significant inhibitory effect of pasireotide on BrdU incorporation was found (-16.2±4%, $p<0.05$) (Fig.2E). This discrepancy might be due to the higher sensitivity of the proliferation assay. Similarly, in mutated MTC#5, only pasireotide, but not octreotide, reduced CD1 levels (-36%) and cell proliferation (-27.9±10%, $p<0.05$) (Fig. 2F,G).

Fig.2



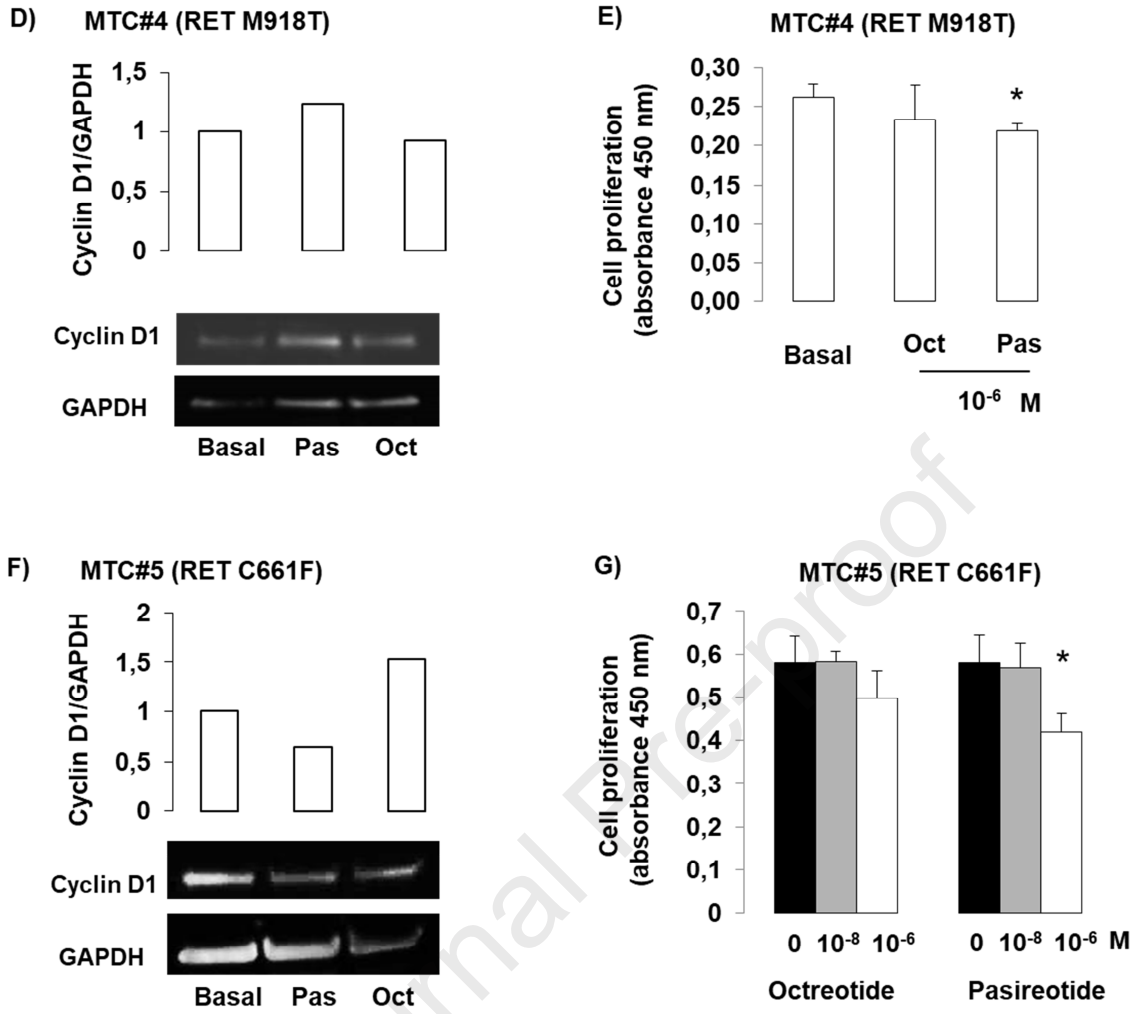


Figure 2. Octreotide and pasireotide treatment effects on cell proliferation in primary cultured MTC cells. A,B,D,F) CD1 assay. Cells were treated with 1 μM octreotide or pasireotide. The graphs show the quantification of CD1 normalized to GAPDH. Representative immunoblots are shown. C,E,G) Proliferation assays. Cells were treated with octreotide or pasireotide 10 nM or 1 μM at 37°C for 48 h, and BrdU incorporation in newly synthesized DNA was measured. Each determination was done in triplicate. * = $p < 0.05$ vs corresponding basal.

3.2 Octreotide and pasireotide induced apoptosis in TT and primary cultured MTC cells

We then tested the proapoptotic effects of octreotide and pasireotide. Cells were incubated with octreotide or pasireotide for 48 h and caspase-3 and -7 activity was measured.

In TT cells, both drugs were able to increase cell apoptosis. Octreotide induced an increase of caspase 3/7 activity of 136±10% ($p<0.001$ vs basal, 10^{-8} M), and pasireotide of 149.2±20% ($p<0.001$ vs basal, 10^{-8} M) (Fig. 3A).

In primary cultured cells from wild type MTC#3 and mutated MTC#4 (RET M918T), no effect was observed after octreotide or pasireotide incubation, whereas in MTC#5 (RET C611F), octreotide induced an increase of cell apoptosis of 220±7% ($p<0.001$ vs basal, 10^{-6} M), and pasireotide of 182±19% ($p<0.05$ vs basal, 10^{-6} M) (Fig. 3B-D).

Fig.3

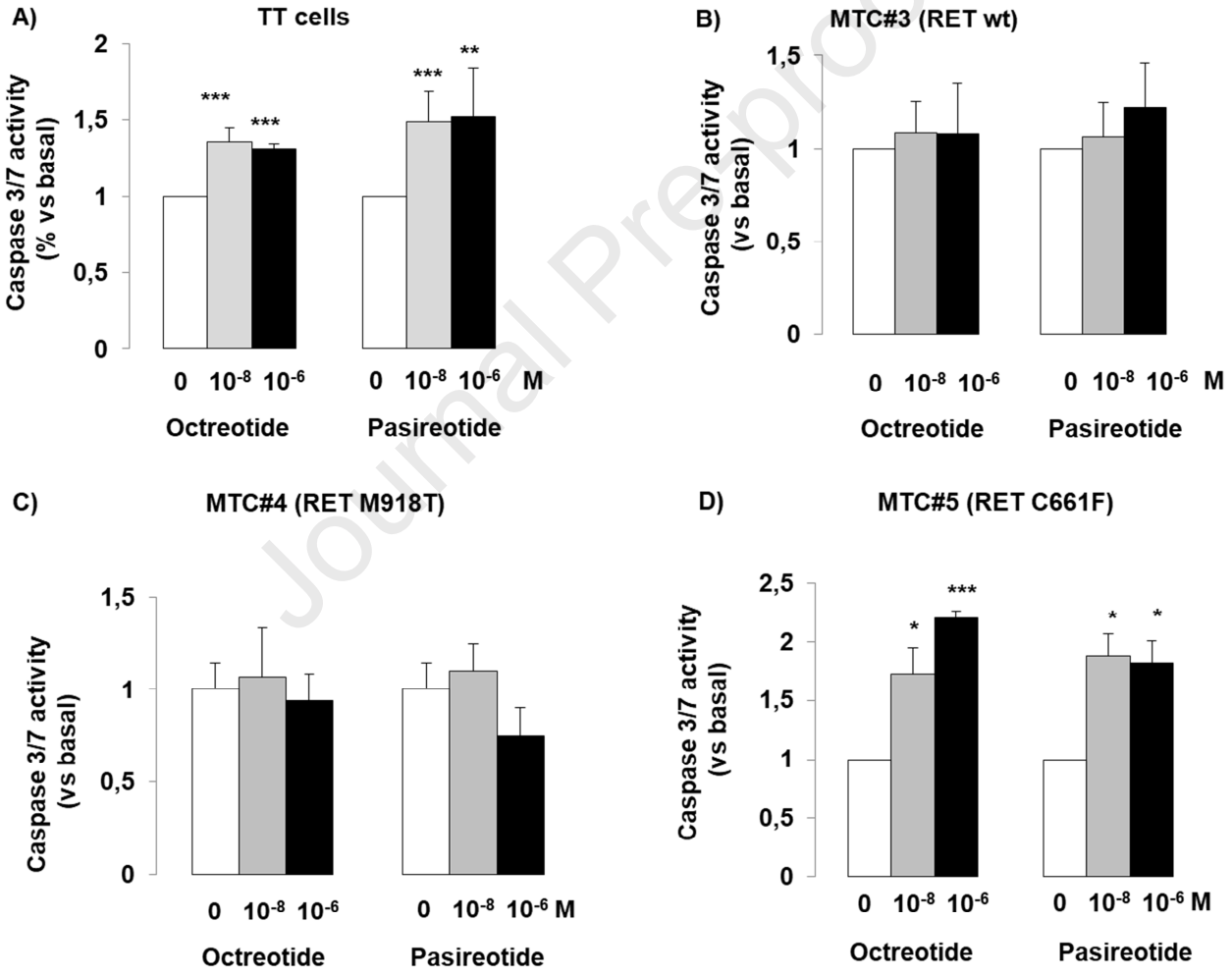


Figure 3. Octreotide and pasireotide treatment induced cell apoptosis in TT cells and in MTC primary cultures. TT cells (A) and a primary cultured MTC cells (B-D) were incubated with octreotide or pasireotide 10 nM or 1 μM at 37°C for 48 h. Caspase-3/7 enzymatic activity was measured. Graphs show the percentage of caspase-3/7 enzymatic activity vs basal (mean value ± S.D. from 3 independent experiments for TT cells

and mean ± S.D. of an independent experiment for MTC primary cultures). Each determination was done in quintuple. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ vs corresponding basal.

3.3 Octreotide and pasireotide did not affect calcitonin secretion in TT and primary cultured MTC cells

To test the effects of octreotide and pasireotide on calcitonin secretion, cells were incubated with the SSAs and cell media were collected for calcitonin assay.

No changes in calcitonin secretion were observed after incubation with octreotide or pasireotide in TT cells after 3 h (Fig. 4A) or 24 h (data not shown) nor in primary cultured MTC cells from wild type (MTC#3) (Fig. 4B) and mutated tumors (MTC#4 and #5) (data not shown), after 24 h incubation.

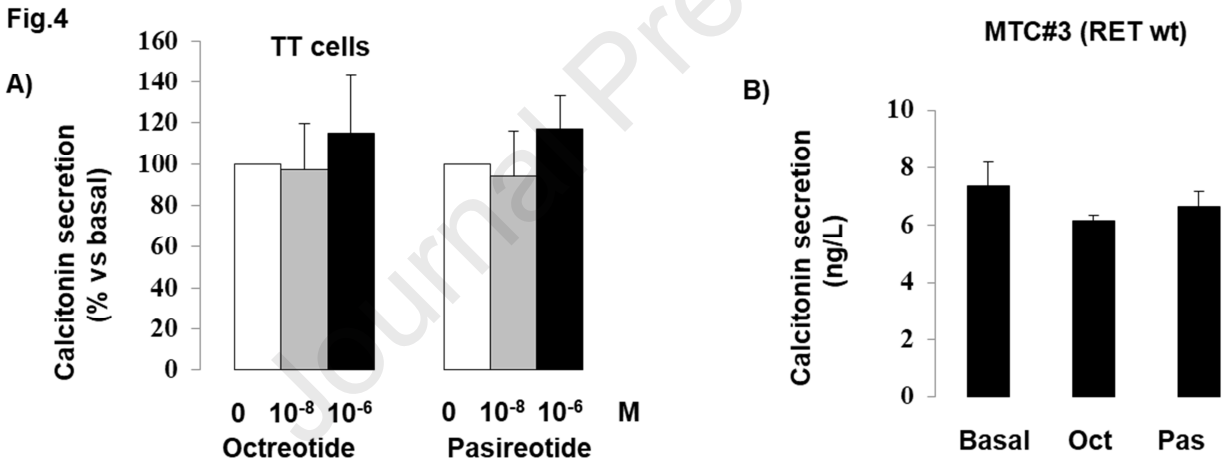


Figure 4. Octreotide and pasireotide treatment did not affect calcitonin secretion in TT and primary cultured MTC cells. TT cells (A) and a primary cultured MTC cells (B) were incubated with octreotide or pasireotide 10 nM or 1 µM at 37°C for 3 h (A) or 24 h (B). Calcitonin secretion in cell media was measured. Graphs show the percentage of calcitonin secretion versus each basal (A) (mean value ± S.D. from 3 independent experiments in TT cells) and the concentration of calcitonin in cell media of primary cultured MTC cells (ng/L) (B). Each determination was done in triplicate.

3.4 Octreotide and pasireotide inhibited migration and invasion of MTC cells

To test the effects of SSAs on cell motility, we performed transwell assays. In TT cells, FBS-induced chemotactic migration was strongly reduced after 18 h of octreotide or pasireotide

incubation (-50.9±11.3%, $p<0.01$ vs basal, and -40.5±17%, $p<0.05$ vs basal, respectively, at 10^{-8} M (Fig. 5A). Moreover, octreotide or pasireotide incubation significantly inhibited TT cell invasion through Matrigel (-61.3±35.1%, $p<0.05$ vs basal, and -49.7±18%, $p<0.01$ vs basal, respectively, at 10^{-8} M) (Fig. 5B). Remarkably, octreotide reached 81% inhibition of cell invasion at 10^{-6} M ($p<0.001$ vs basal).

This strong antimigratory effect of octreotide was confirmed in all primary cultured MTC cells, both from RET wild type (MTC#3) or mutated (MTC#4, #5) tumors (Fig. 5C-E), reaching an inhibition of 94.6%, at a concentration of 10^{-8} M in MTC#5. Pasireotide efficiently reduced cell migration in MTC#4 and #5, but was ineffective in MTC#3.

Fig.5

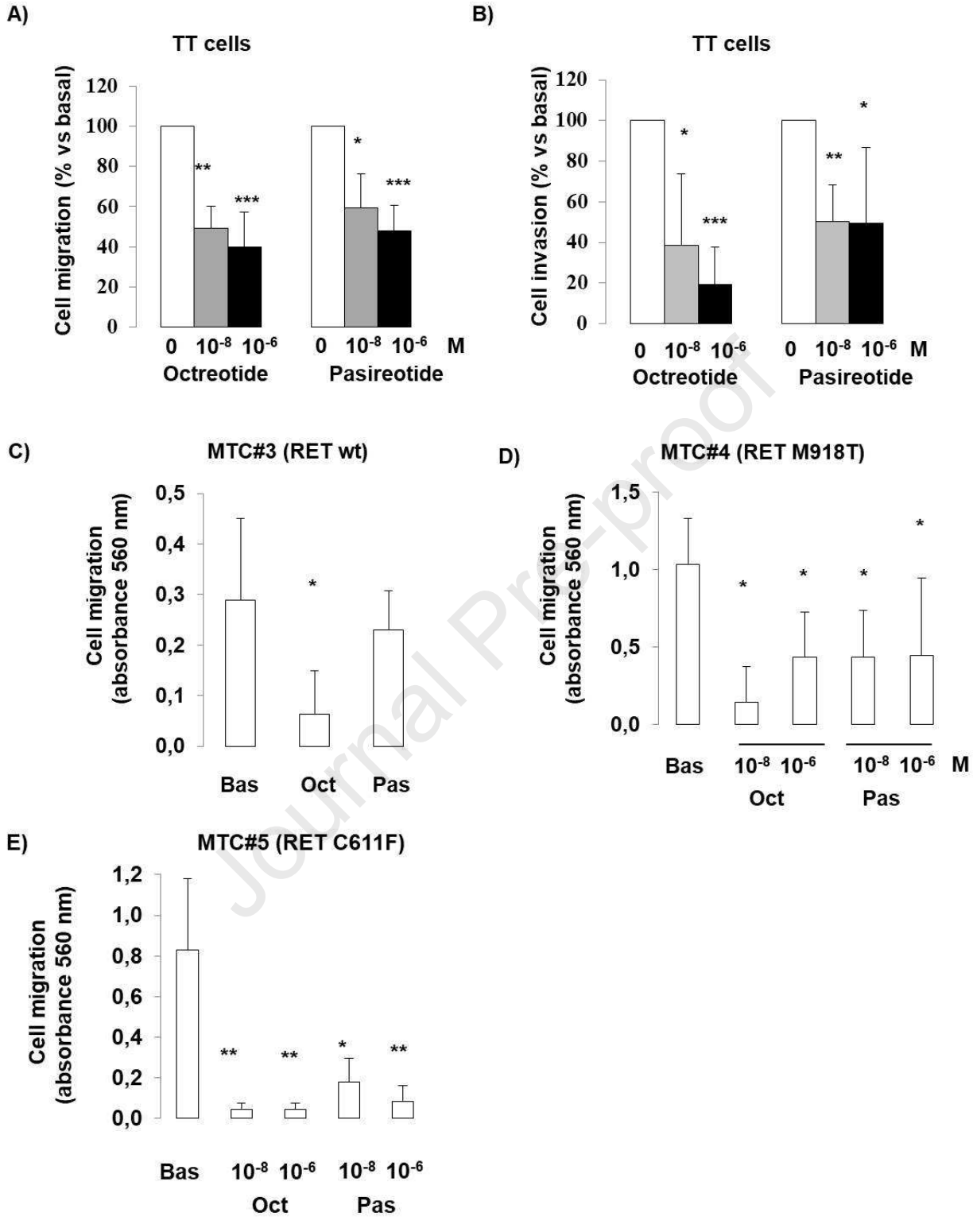


Figure 5. Octreotide and pasireotide treatment inhibited cell migration and invasion in TT and primary cultured MTC cells. TT cells (A, B) and primary cultures (C-E) were incubated with octreotide or pasireotide 10 nM or 1 µM at 37°C for 18 h. Transwell migration (A, C-E) or invasion on matrigel (B) were performed. Graphs show the quantification of migration or invasion (A-B, percentage vs corresponding basal, mean value ± S.D. from 3 independent experiments on TT cells; C-E, mean ± S.D. of independent

experiments on 3 MTC primary cultures). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ vs corresponding basal.

3.5 SSTs expression in MTC tissues: correlation with RET mutations and SSA responsiveness

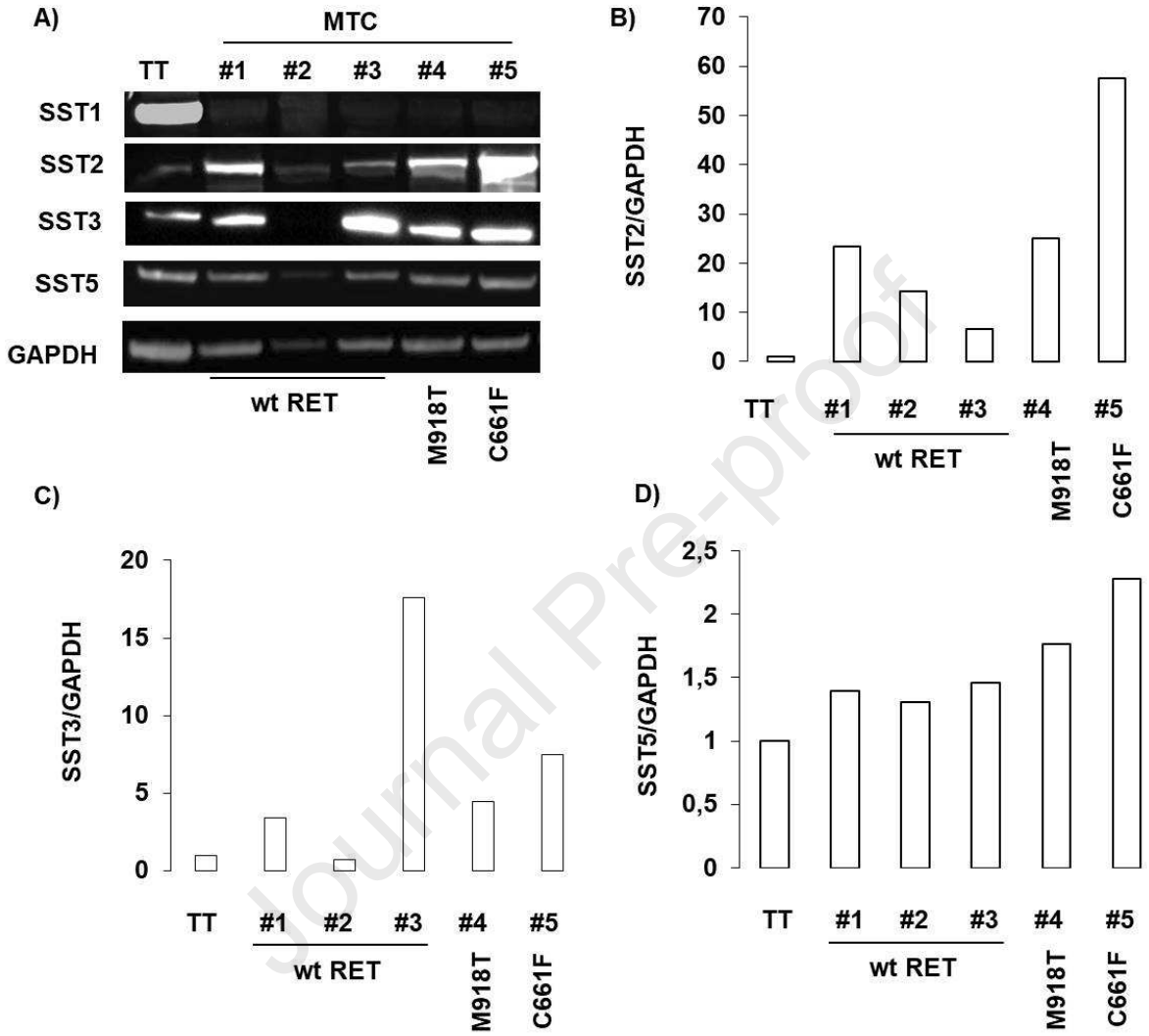
By western blot analysis, we tested the expression of SST1,2,3 and 5 (Fig.6A-D). We found that SST2, SST3 and SST5 were expressed in all samples analyzed. SST1 was highly expressed in TT cells, but nearly undetectable in all MTC samples analyzed. SST2 expression was higher in MTC tissues than in TT cells, and highly variable among different samples. An high variability between MTC samples was also observed for SST3 expression. SST5 was expressed at comparable levels in MTCs with respect to TT cells.

Despite the low sample size, we tried to correlate SSTs expression to RET mutational status. Although mean expression of SST2, SST3 and SST5 was not significantly different in the group of the MTCs with wild type RET vs the group with RET mutations, we observed a tendency to increased SST2 expression in mutated vs wild type MTCs (mean SST2/GAPDH ratio, normalized on TT cells, 14.7±8 vs 41.3±22, respectively) (Fig.6E-G). To test whether oncogenic RET might affect SST2s expression, we blocked the constitutively active C634W RET endogenously expressed by TT cells using the RET inhibitor RPI-1, as previously described (Giardino *et al.*, 2009). RPI-1 is an indolinone-based selective inhibitor of RET kinase activity (Lanzi *et al.*, 2000), that inhibited RET tyrosine phosphorylation, expression, and signaling in TT cells (Cuccuru *et al.*, 2004). Our results showed that RPI-1 incubation reduced SST2 expression (-32±10%, $p<0.01$ at 50 nM) (Fig.6H), but not SST3 and SST5 (data not shown).

Regarding the *in vitro* responsiveness to octreotide and pasireotide, we found a tendency to a lower SST2 expression in MTC unresponsive to pasireotide (n=2, mean SST2/GAPDH 2.06±1.09) with respect to responsive ones (n=3, mean SST2/GAPDH 7.03±3.82), although we didn't find a statistical significance probably due to the low number of samples (Fig.6I). In contrast, mean expression level of SST5 was nearly identical between the MTC responsive or not to pasireotide.

333 Interestingly, the only MTC responsive to proapototic effect of both octreotide and pasireotide
334 (MTC#5) showed the highest expression of both SST2 and SST5, but not SST3 (Fig.6A-D).

Fig.6



335

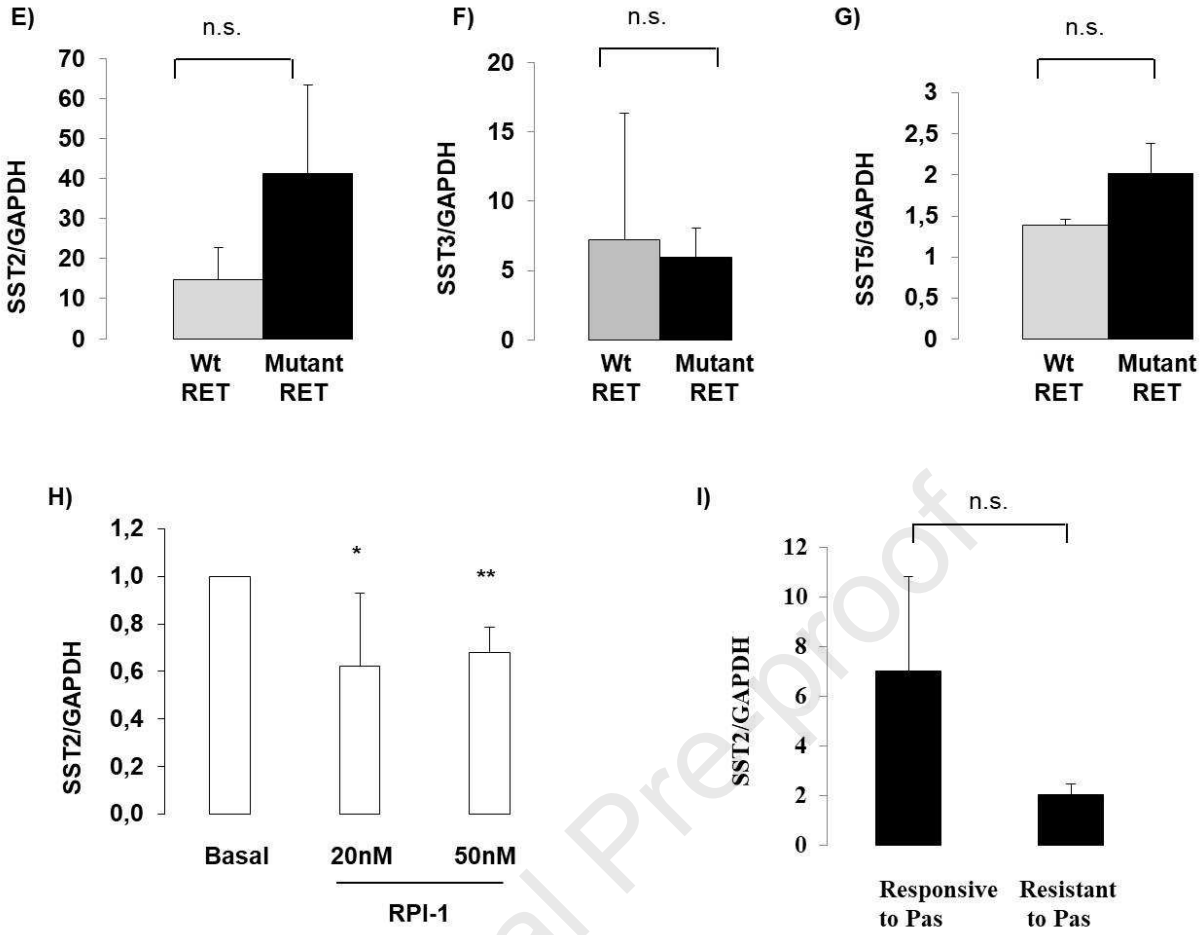


Figure 6. SSTs protein expression in MTC. A) SST1,2,3 and 5 expression levels were analyzed by western blot in TT and 5 MTC samples. RET mutational status is indicated. Representative immunoblots are shown. B-G) The graphs show SST2 (B), SST3 (C) or SST5 (D) expression levels normalized to GAPDH, and mean SST2/GAPH (E), SST3/GAPDH (F) or SST5/GAPDH (G) levels in RET wild type (n=3) vs mutated MTCs (n=2). H) TT cells were treated with increasing concentrations of RPI-1, a selective inhibitor of RET kinase activity, for 24h. The graph shows the quantification of SST2 normalized to GAPDH vs untreated cells. Data are plotted as mean ± SD of 3 independent experiments. *, $p < 0.05$, **, $p < 0.01$ vs untreated cells. I) The graph shows SST2 expression normalized to GAPDH (mean±S.D.) in pasireotide responsive (n=3) vs resistant MTCs (n=2).

4. Discussion

The expression of SSTs in MTC represents the rationale for the therapy with SSAs, but contrasting data are present in literature on the antiproliferative effects of first-generation (octreotide) and second-generation (pasireotide) SSAs in MTC patients.

In the present work, we analyzed the antitumoral effects of octreotide and pasireotide in TT cells and in primary cultured cells obtained from human sporadic or hereditary MTCs.

Regarding the antimitotic effects of octreotide and pasireotide in TT cells, we found that both drugs were able to inhibit cell proliferation to a similar extent.

This antimitotic effect is accompanied by a decrease of ERK1/2 phosphorylation and a reduction of cyclin D1 levels, as previously demonstrated by Zatelli *et al.* with SS and SST2-selective agonist BIM23120 (Zatelli *et al.* 2005, 2006), although the molecular mechanism involved has not been dissected. Zatelli and co-authors found that in TT cells, only SST2- and SST1- but not SST5-selective agonists exerted an antiproliferative effect (Zatelli *et al.* 2001, 2002*a*). Since pasireotide, compared with octreotide, displays a 40-, 5- and 30-fold higher binding affinity for SST5, SST3 and SST1, respectively, and 2.5-fold lower affinity for SST2 (Bruns *et al.* 2002), we can hypothesize that the resulting inhibitory effects on cell proliferation, MAPK activation and cyclin D1 expression derive from the simultaneous activation of different SST subtypes, which are all expressed in TT cells (Zatelli *et al.* 2001) rather than from the higher SST5 activation. In the present work, we decided to use octreotide and pasireotide being these two drugs already commonly used in clinical practice.

Although MTC are very rare tumors, we were able to test the effects of SSAs in a small group of surgically removed human MTC. In primary cultured MTC cells, we found variable efficacy of SSAs in reducing cell proliferation, being pasireotide effective in 3/5 tumors, and octreotide in 1/5 tumors. Interestingly, pasireotide exerted an antiproliferative effect in both mutated tumors, that were resistant to octreotide. However, pasireotide was also effective in one wild type tumor, with an effect similar to octreotide. It is worth noting that the inhibitory effect on cell

proliferation in primary cultured MTC was reached at high concentrations of SSAs, whereas TT cells were responsive starting from 10^{-8} M.

Despite the small sample size, we found a tendency to a higher SST2 expression in MTC responsive to pasireotide with respect to resistant ones, but further experiments are required to test a possible correlation between SSAs efficacy and the expression of SST2 and SST5.

Beside SSTs expression, other molecular factors might influence MTC responsiveness to SSAs. In the past years, several studies have investigated the molecular determinants possibly involved in the resistance to SSAs. For example, our group has found that in pituitary NENs the expression and activity of cytoskeleton actin binding protein filamin A is crucial for an efficient SST2 intracellular signal transduction (Peverelli *et al.* 2014, 2018*b*). In MTC, it has been found that the expression of the truncated isoform of SST5 (sst5TMD4) prevented SST2-agonist antiproliferative effects (Molè *et al.* 2015). Further studies in a high number of MTCs are required to identify post-receptor alterations inducing resistance to SSAs.

The present study first demonstrated that in MTC cells octreotide and pasireotide exerted proapoptotic effects. It is well known that SST3 (Sharma *et al.* 1996, War *et al.* 2011) and SST2 (Ferrante *et al.* 2006) are the SST subtypes mainly involved in SS-induced cell death. The apoptotic effect of pasireotide, that was comparable to that of octreotide, might be mediated by SST3 activation, as suggested in ACTH-secreting pituitary tumors (Treppiedi *et al.* 2019). However, SSTs expression data revealed that the MTC with the highest SST3 expression level was unresponsive to proapoptotic effects of both octreotide and pasireotide, and that the only MTC responsive to proapototic effect of both octreotide and pasireotide showed the highest expression of both SST2 and SST5, suggesting that the latter two might be the main mediators of apoptosis in MTC cells.

Calcitonin secretion was not affected by octreotide neither by pasireotide in TT cells and primary cultured MTC cells, regardless their responsiveness to SSAs antiproliferative effects. These data are in agreement with the previously observed dissociation between antimitotic and antisecretive effects of SSAs in TT cells (Zatelli *et al.* 2001, 2002*b*) and in primary cultured MTC

cells (Zatelli *et al.* 2006). Accordingly, Faggiano *et al.* found no concordance between serum marker and radiological changes and concluded that neither calcitonin nor CEA serum concentrations can be reliable predictor of MTC responsiveness to pasireotide (Faggiano *et al.* 2018).

Our data provide new evidence for a strong inhibitory effect of SSAs on MTC chemotactic cell migration and invasion. The ability of the SST2 agonist BIM23120 to inhibit cell motility has been previously demonstrated both in pituitary (Peverelli *et al.* 2018*a*) and pancreatic (Vitali *et al.* 2016) NENs, whereas no data are available about the effects of SSAs in MTC. In TT cells, we found a stronger inhibition of cell migration and invasion with octreotide than pasireotide, that may be explained by the higher octreotide affinity for SST2 (Bruns *et al.* 2002). Similarly, octreotide was effective in inhibiting cell migration in all MTC primary cultured cells tested, both RET mutated and RET wild type.

Interestingly, in MTC#3, SSAs reduced cell migration but not cell proliferation, suggesting that the proliferative and migratory responses are dissociated and involves distinct cellular pathways, although further experiments will be required to confirm this hypothesis.

Since the molecular mechanism by which SST2 inhibits cell migration in pituitary tumors involves cytoskeleton protein cofilin (Peverelli *et al.* 2018*a*), crucial in mediating RET-promoted MTC cells migration (Giardino *et al.* 2019), we can hypothesize the involvement of this cytoskeleton protein in modulating the intracellular response to SSAs in MTC cells.

The analysis of the SSTs expression in MTC tissues revealed a tendency to increased expression of SST2 in mutated vs wild type MTCs. This observation is in agreement with the results obtained in TT cells, showing that RET inhibitor RPI-1 induced a reduction of SST2 expression. These data suggest that oncogenic mutant RET exerted a positive effect on SST2 expression. Further experiments are required to test in a large cohort of patients an association between RET mutations and SST2 expression, as well as the molecular mechanism involved.

Overall, our data demonstrated a similar efficacy of octreotide and pasireotide in reducing cell growth, by both cytostatic and cytotoxic effects, and cell invasiveness in TT cell line. In contrast, only a subset of primary cultured cells derived from MTC patients were responsive to antiproliferative effects of octreotide and pasireotide, suggesting the need for new biomarkers useful to stratify patients for therapeutic response to SSAs. RET mutated tumors seems to be more responsive to pasireotide than to octreotide, although further studies with a large number of tumors are required to establish a correlation between SSAs responsivity and RET mutational status.

In addition, we first reported antiinvasive properties of octreotide and pasireotide that may suggest the use of these drugs at an early stage of disease to prevent the subsequent development of distant metastases.

Declaration of interest: none

Funding

This work was supported by AIRC (Associazione Italiana Ricerca Cancro) grant to GM (IG 2017-20594), Italian Ministry of Health grant to GM (PE-2016-02361797), Ricerca Corrente Funds from the Italian Ministry of Health and Progetti di Ricerca di Interesse Nazionale (PRIN) grant to E.P. (2017N8CK4K).

References

Bruns C, Lewis I, Briner U, Meno-Tetang G & Weckbecker G 2002 SOM230: a novel somatostatin peptidomimetic with broad somatotropin release inhibiting factor (SRIF) receptor binding and a unique antisecretory profile. *European Journal of Endocrinology* **146** 707–716. (https://doi.org/10.1530/eje.0.1460707)

Cano JM, Galán R & López R 2017 Recurrent metastatic medullary thyroid carcinoma: a case of sustained response to prolonged treatment with somatostatin analogues. *Thyroid* **27** 1450–1455. (https://doi.org/10.1089/thy.2016.0540)

Ciampi R, Romei C, Ramone T, Prete A, Tacito A, Cappagli V, Bottici V, Viola D, Torregrossa L, Ugolini C, *et al.* 2019 Genetic landscape of somatic mutations in a large cohort of sporadic medullary thyroid carcinomas studied by next-generation targeted sequencing. *iScience* **20** 324–336. (https://doi.org/10.1016/j.isci.2019.09.030)

Cuccuru G, Lanzi C, Cassinelli G, Pratesi G, Tortoreto M, Petrangolini G, Seregni E, Martinetti A, Laccabue D, Zanchi C, *et al.* 2004. Cellular effects and antitumor activity of RET inhibitor RPI-1 on MEN2A-associated medullary thyroid carcinoma. *J Natl Cancer Inst.* **96** 1006-14. (https://doi.org/10.1093/jnci/djh184)

Elisei R & Pinchera A 2012 Advances in the follow-up of differentiated or medullary thyroid cancer. *Nature Reviews Endocrinology* **8** 466–475. (https://doi.org/10.1038/nrendo.2012.38)

Elisei R, Schlumberger MJ, Müller SP, Schöffski P, Brose MS, Shah MH, Licitra L, Jarzab B, Medvedev V, Kreissl MC, *et al.* 2013 Cabozantinib in progressive medullary thyroid cancer. *Journal of Clinical Oncology* **31** 3639–3646. (https://ascopubs.org/doi/10.1200/JCO.2012.48.4659)

Faggiano A, Modica R, Severino R, Camera L, Fonti R, Del Prete M, Chiofalo MG, Aria M, Ferolla P, Vitale G, *et al.* 2018 The antiproliferative effect of pasireotide LAR alone and in combination with everolimus in patients with medullary thyroid cancer: a single-center, open-label, phase II, proof-of-concept study. *Endocrine* **62** 46–56. (https://doi.org/10.1007/s12020-018-1583-7)

Ferrante E, Pellegrini C, Bondioni S, Peverelli E, Locatelli M, Gelmini P, Luciani P, Peri A, Mantovani G, Bosari S, *et al*. 2006 Octreotide promotes apoptosis in human somatotroph tumor cells by activating somatostatin receptor type 2. *Endocrine-Related Cancer* 2006 **13** 955–962. (https://doi.org/10.1677/erc.1.01191)

Frank-Raue K, Raue F & Ziegler R 1995 Therapie des metastasierten medullären Schilddrüsenkarzinoms mit dem Somatostatinanalogon Octreotide [Therapy of metastatic medullary thyroid gland carcinoma with the somatostatin analog octreotide]. *Medizinische Klinik* **90** 63-66. PMID: 7708002

Giardino E, Catalano R, Barbieri AM, Treppiedi D, Mangili F, Spada A, Arosio M, Mantovani G & Peverelli E 2019 Cofilin is a mediator of RET-promoted medullary thyroid carcinoma cell migration, invasion and proliferation. *Molecular and Cellular Endocrinology* **495** 110519. (https://doi.org/10.1016/j.mce.2019.110519)

Kendler DB, Araújo ML Jr, Alencar R, de Souza Accioly MT, Bulzico DA, de Noronha Pessoa CC, Accioly FA, de Farias TP, Lopes FPPL, Corbo R *et al.* 2017 Somatostatin receptor subtype 1 might be a predictor of better response to therapy in medullary thyroid carcinoma. *Endocrine* **58** 474–480. (https://doi: 10.1007/s12020-017-1424-0)

Lanzi C, Cassinelli G, Pensa T, Cassinis M, Gambetta RA, Borrello MG, Menta E, Pierotti MA, Zunino F. 2000 Inhibition of transforming activity of the ret/ptc1 oncoprotein by a 2-indolinone derivative. *Int J Cancer.* **85** 384-90. (https://doi.org/10.1002/(SICI)1097-0215(20000201)85:3<384::AID-IJC15>3.0.CO;2-Y)

Mahler C, Verhelst J, de Longueville M & Harris A 1990 Long-term treatment of metastatic medullary thyroid carcinoma with the somatostatin analogue octreotide. *Clinical Endocrinology* **33** 261–269. (https://doi.org/10.1111/j.1365-2265.1990.tb00490.x)

Mato E, Matías-Guiu X, Chico A, Webb SM, Cabezas R, Berná L & De Leiva A 1998 Somatostatin and somatostatin receptor subtype gene expression in medullary thyroid carcinoma. *The Journal of Clinical Endocrinology & Metabolism* **83** 2417–2420. (https://doi.org/10.1210/jcem.83.7.4955)

Modigliani E, Cohen R, Joannidis S, Siame-Mourot C, Guliana JM, Charpentier G, Cassuto D, Bentata Pessayre M, Tabarin A, Roger P *et al*. 1992 Results of long-term continuous subcutaneous

496 octreotide administration in 14 patients with medullary thyroid carcinoma. *Clinical Endocrinology*
497 **36** 183–186. (https://doi.org/10.1111/j.1365-2265.1992.tb00955.x)

498 Molè D, Gentilin E, Ibañez-Costa A, Gagliano T, Gahete MD, Tagliati F, Rossi R, Pelizzo MR,
499 Pansini G, Luque RM, *et al*. 2015 The expression of the truncated isoform of somatostatin receptor
500 subtype 5 associates with aggressiveness in medullary thyroid carcinoma cells. *Endocrine* **50** 442–
501 452. (https://doi.org/10.1007/s12020-015-0594-x)

502 Peverelli E, Giardino E, Treppiedi D, Vitali E, Cambiaghi V, Locatelli M, Lasio GB, Spada A,
503 Lania AG & Mantovani G 2014 Filamin A (FLNA) plays an essential role in somatostatin receptor
504 2 (SST2) signaling and stabilization after agonist stimulation in human and rat somatotroph tumor
505 cells. *Endocrinology* **155** 2932–2941. (https://doi.org/10.1210/en.2014-1063)

506 Peverelli E, Giardino E, Treppiedi D, Catalano R, Mangili F, Locatelli M, Lania AG, Arosio M,
507 Spada A & Mantovani G 2018*a* A novel pathway activated by somatostatin receptor type 2 (SST2):
508 Inhibition of pituitary tumor cell migration and invasion through cytoskeleton protein recruitment.
509 *International Journal of Cancer* **142** 1842–1852. (https://doi.org/10.1002/ijc.31205)

510 Peverelli E, Giardino E, Mangili F, Treppiedi D, Catalano R, Ferrante E, Sala E, Locatelli M, Lania
511 AG, Arosio M *et al*. 2018*b* cAMP/PKA-induced filamin A (FLNA) phosphorylation inhibits SST2
512 signal transduction in GH-secreting pituitary tumor cells. *Cancer Letters* **435** 101–109.
513 (https://doi.org/10.1016/j.canlet.2018.08.002)

514 Roman S, Lin R & Sosa JA 2006 Prognosis of medullary thyroid carcinoma: demographic, clinical,
515 and pathologic predictors of survival in 1252 cases. *Cancer* **107** 2134–2142.
516 (https://doi.org/10.1002/cncr.22244)

517 Sharma K, Patel YC & Srikant CB 1996 Subtype-selective induction of wild-type p53 and
518 apoptosis, but not cell cycle arrest, by human somatostatin receptor 3. *Molecular Endocrinology* **10**
519 1688–1696. (https://doi.org/10.1002/cncr.22244)

Tagliati F, Zatelli MC, Bottoni A, Piccin D, Luchin A, Culler MD & Degli Uberti EC 2006 Role of complex cyclin D1/Cdk4 in somatostatin subtype 2 receptor-mediated inhibition of cell proliferation of a medullary thyroid carcinoma cell line in vitro. *Endocrinology* **147** 3530–3538. (https://doi.org/10.1210/en.2005-1479)

Treppiedi D, Giardino E, Catalano R, Mangili F, Vercesi P, Sala E, Locatelli M, Arosio M, Spada A, Mantovani G *et al*. 2019 Somatostatin analogs regulate tumor corticotrophs growth by reducing ERK1/2 activity. *Molecular and Cellular Endocrinology* **483** 31–38. (https://doi.org/10.1016/j.mce.2018.12.022)

Vainas I, Koussis Ch, Pazaitou-Panayiotou K, Drimonitis A, Chrisoulidou A, Iakovou I, Boudina M, Kaprara A & Maladaki A 2004 Somatostatin receptor expression in vivo and response to somatostatin analog therapy with or without other antineoplastic treatments in advanced medullary thyroid carcinoma. *Journal of Experimental & Clinical Cancer Research*. 2004 **23** 549–559. PMID: 15743023

Vitale G, Tagliaferri P, Caraglia M, Rampone E, Ciccarelli A, Bianco AR, Abbruzzese A & Lupoli G 2000 Slow release lanreotide in combination with interferon-alpha2b in the treatment of symptomatic advanced medullary thyroid carcinoma. *The Journal of Clinical Endocrinology & Metabolism* **85** 983–988. (https://doi.org/10.1210/jcem.85.3.6435)

Vitali E, Cambiaghi V, Zerbi A, Carnaghi C, Colombo P, Peverelli E, Spada A, Mantovani G & Lania AG 2016 Filamin-A is required to mediate SST2 effects in pancreatic neuroendocrine tumours. *Endocrine-Related Cancer* **23** 181–190. (https://doi.org/10.1530/ERC-15-0358)

War SA, Somvanshi RK & Kumar U 2011 Somatostatin receptor-3 mediated intracellular signaling and apoptosis is regulated by its cytoplasmic terminal. *Biochimica et Biophysica Acta* **1813** 390–402. (https://doi.org/10.1016/j.bbamcr.2010.12.015)

543 Wells SA Jr, Robinson BG, Gagel RF, Dralle H, Fagin JA, Santoro M, Baudin E, Elisei R, Jarzab
544 B, Vasselli JR *et al*. 2012 Vandetanib in patients with locally advanced or metastatic medullary
545 thyroid cancer: a randomized, double-blind phase III trial. *Journal of Clinical Oncology* **30** 134–
546 141. (https://doi.org/10.1200/JCO.2011.35.5040)

547 Zatelli MC, Tagliati F, Taylor JE, Rossi R, Culler MD & degli Uberti EC 2001 Somatostatin
548 receptor subtypes 2 and 5 differentially affect proliferation in vitro of the human medullary thyroid
549 carcinoma cell line TT. *The Journal of Clinical Endocrinology & Metabolism* **86** 2161–2169.
550 (https://doi.org/10.1210/jcem.86.5.7489)

551 Zatelli MC, Tagliati F, Piccin D, Taylor JE, Culler MD, Bondanelli M & degli Uberti EC 2002*a*
552 Somatostatin receptor subtype 1-selective activation reduces cell growth and calcitonin secretion in
553 a human medullary thyroid carcinoma cell line. *Biochemical and Biophysical Research*
554 *Communications* **297** 828–834. (https://doi.org/10.1016/S0006-291X(02)02307-0)

555 Zatelli MC, Tagliati F, Taylor JE, Piccin D, Culler MD & degli Uberti EC 2002*b* Somatostatin, but
556 not somatostatin receptor subtypes 2 and 5 selective agonists, inhibits calcitonin secretion and gene
557 expression in the human medullary thyroid carcinoma cell line, TT. *Hormone and Metabolic*
558 *Research* **34** 229–233. (DOI: 10.1055/s-2002-32134)

559 Zatelli MC, Piccin D, Tagliati F, Bottoni A, Luchin A & degli Uberti EC 2005 Src homology-2-
560 containing protein tyrosine phosphatase-1 restrains cell proliferation in human medullary thyroid
561 carcinoma. *Endocrinology* **146** 2692–2698. (https://doi.org/10.1210/en.2005-0001)

562 Zatelli MC, Piccin D, Tagliati F, Bottoni A, Luchin A, Vignali C, Margutti A, Bondanelli M,
563 Pansini GC, Pelizzo MR *et al*. 2006 Selective activation of somatostatin receptor subtypes
564 differentially modulates secretion and viability in human medullary thyroid carcinoma primary
565 cultures: potential clinical perspectives. *The Journal of Clinical Endocrinology & Metabolism* **91**
566 2218–2224. (https://doi.org/10.1210/jc.2006-0334)

Highlights

- Octreotide and pasireotide reduced with similar efficacy TT cell growth
- Only a subset of primary MTC cells were responsive to octreotide and pasireotide
- Octreotide and pasireotide exerted cytostatic and cytotoxic effects in MTC cells
- We first showed antiinvasive properties of octreotide and pasireotide in MTC cells
- Oncogenic mutant RET induced an increase of SST2 expression.