# Heterogeneity of TPIT expression in ACTH-secreting extra-pituitary neuroendocrine tumors (NETs) supports the existence of different cellular programs in pancreatic and pulmonary NETs

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# Abstract

Extra-pituitary ACTH secretion is associated with a variety of neoplastic conditions and may cause the so-called ectopic ACTH-dependent Cushing syndrome (CS). The clarification of the mechanisms of extra-pituitary ACTH expression would provide potential therapeutic targets for this complex and severe disease. In the adenohypophysis, the transcription factor TPIT, co-operating with other molecules, induces POMC expression and ACTH production. However, no data is currently available on the presence and role of TPIT expression in extra-pituitary ACTH-producing neoplasms. This study was designed to explore TPIT expression in a series of pulmonary and pancreatic ACTH-producing tumors, either CS-associated or not.

41 extra-pituitary ACTH-producing neuroendocrine tumors (NETs) were included in the study, encompassing 32 NETs of the lung (LuNETs), 7 of the pancreas (PanNETs) and 2 pheochromocytomas. Of these, 9 LuNETs, all PanNETs and the two pheochromocytomas were CS-associated. For comparison, 6 NETs the pituitary gland (PitNETs), and 35 ACTH-negative NETs (15 Lu-NETs and 20 PanNETs) were analyzed. Immunohistochemistry with specific anti-TPIT antibodies and quantitative real time PCR (qRT-PCR) were performed using standard protocols.

TPIT expression was completely absent (protein and mRNA) in PanNETs, pheochromocytomas, and all ACTH-negative NETs. In contrast, it was expressed in 16/32 LuNETs, although with lower levels than in PitNETs. No definite relationship was found between immunohistochemistry TPIT expression and NET grade or the presence of Cushing syndrome.

This study further highlights the clinical and biological heterogeneity of extrapituitary ACTH secretion and suggests that the differences between ACTH-secreting PanNETs and LuNETs may mirror distinct molecular mechanisms underlying POMC expression. Our results point towards the recognition of a real corticotroph-like phenotype of ACTH-producing LuNETs, that is not a feature of ACTH-producing PanNETs.


# Introduction

TPIT is a pituitary T-box transcription factor, encoded by *TBX19* gene, which is selectively expressed in corticotroph cells of the anterior pituitary that produce adrenocorticotrophic hormone (ACTH) and other peptides derived from the proteolytic cleavage of the precursor pro-opiomelanocortin (POMC) [1]. TPIT, in cooperation with other transcription factors (among which PITX1 and NEUROD1 are the most important), is essential for POMC gene expression and for the terminal differentiation of POMC-producing cell lineage [1]. The availability of anti-TPIT specific antibodies has provided the practicing pathologist with a robust marker for the identification of both normal and neoplastic corticotroph cells in the anterior pituitary [2], which is now included in routine diagnostics and classification of pituitary neuroendocrine tumors (PitNET)/adenomas [3-5].

Corticotroph PitNET accounts for less than 20% of PitNETs and its most frequent clinical manifestation is Cushing disease [5]. This latter is the most frequent form of ACTH-dependent Cushing syndrome (CS), accounting for 70-80% of the total cases, although an excess of circulating ACTH may also be related to ectopic secretion by extra-pituitary malignancies, mostly including neuroendocrine neoplasms of the lung,

pancreas, and thymus [6]. Notably, both pituitary and extra-pituitary ACTH-producing neoplasms may be clinically silent, despite immunohistochemistry reveals ACTH expression in tumor cells [7, 8]. Ectopic CS represents a diagnostic and therapeutic challenge, as the identification of the source of ACTH excess requires extensive and time-consuming work up, during which a treatment aimed to the normalization of hypercortisolism is crucial [Young 2020]. The definitive diagnosis is reached with the histopathological examination of the extra-pituitary tumor mass suspected to be the source of ACTH secretion and immunohistochemical demonstration of ACTH expression in tumor cells [6].

The mechanisms leading to extra-pituitary ACTH secretion are not completely understood. Interestingly, a TPIT-independent E2F1-mediated transcriptional mechanism of POMC expression has been demonstrated *in vitro* in tumor cells and cell lines derived from patients with ectopic CS, suggesting the possibility of a specific therapeutic target in these cases [9]. However, no data about the expression of TPIT in clinical series of ACTH-producing extra-pituitary neoplasms, whether or not associated with CS, are available. Thus, the aim of our study was to investigate the expression of TPIT at both the protein and the RNA level in a series of functioning and non-functioning ACTH-producing neuroendocrine neoplasms (NENs) of the lung and pancreas already characterized in two previous papers from our group [8, 10], as well as in two ACTH-producing pheochromocytomas associated with CS.

# Materials And Methods

### *Case series*

A total of 41 extra-pituitary ACTH-producing neuroendocrine neoplasms (NENs) were included in the study, encompassing 32 neuroendocrine tumors / carcinoids of the lung (LuNETs), 7 neuroendocrine tumors of the pancreas (PanNETs), and 2 adrenal pheochromocytomas (PHEOs). I detail, the 32 LuNETs included 24 typical carcinoids (TC, G1 LuNET) and 8 atypical carcinoids (AC, G2 LuNET); of these, 9 cases (8 TC and 1 AC) were associated with CS and the remaining 23 were non-functioning. The non-functioning cases were selected by screening with ACTH immunohistochemistry a total of 169 LuNETs diagnosed in the University Hospitals of Varese (68 cases) and Orbassano (101 cases), as detailed in our previous paper [8]. PanNETs included 3 G1 and 4 G2 NETs, all associated with CS, and were reported in a previous paper [10]. The two ACTH-producing PHEOs were associated with CS.

For comparison, 6 neuroendocrine tumors / adenomas of the pituitary gland (PitNETs) (3 corticotrophs – 2 functioning and 1 silent –, 1 somatotroph, 1 lactotroph, and 1 gonadotroph), 20 ACTH-negative PanNETs and 20 ACTH-negative LuNETs were pulled from the archives of the Pathology Service of University Hospital in Varese and included in the study.

### *Immunohistochemical study*

Immunohistochemical analyses were performed on 3mm-thick slides obtained from formalin fixed and paraffin embedded representative samples for each case, using manual or automated standard diagnostic protocols described elsewhere [10]. The minimum immunohistochemical panel included synaptophysin, chromogranin A, and ACTH [10]. TPIT was detected using an affinity purified polyclonal antibody raised

against recombinant Protein Epitope Signature Tag (PrEST) antigen sequence: EVHASTPGAFLLGNPAVTSPPSVLSTQAPTSAGVEVLGEPSLTSIAVSTWTAVASHPFAGWGGPGAGGHHSPSSLDG (Atlas Antibodies, Stockholm, Sweden). Cases with positive immunostain for the polyclonal antibody were stained also with a monoclonal anti-TPIT antibody (clone CL6251, Novus Biologicals, Centennial, CO, USA). Immunostains for ACTH and TPIT were evaluated semi-quantitatively, and both the percentage of positive neoplastic cell over the total neoplastic population and the intensity score (1+, faint; 2+, moderate; 3+, strong) were recorded for each case. In addition, a global score (GS) obtaining by multiplying the percentage of positive neoplastic cells with the intensity score was calculated. Positive and negative controls for ACTH and TPIT were represented by normal pituitary samples, in which corticotroph cells were positive and other cell types were negative.

### *Molecular analysis*

RNA was obtained from three representative formalin-fixed and paraffin-embedded sections (FFPE, 8µm) for each sample. Total RNA was extracted from FFPE tissues after microdissection by using Maxwell® RNA FFPE Kit and Maxwell 16 system (Promega, Madison, USA) according to the recommendations of the manufacturer. RNA was quantified using Qubit™ RNA XR Assay Kit (Invitrogen - Thermo Fisher Scientific, Whaltam, USA). Reverse transcription (RT) was carried out on 800ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Whaltam, USA) according to the manufacturer's protocol. The mRNAs expression levels of TPIT in neuroendocrine neoplasms and control hypophysis samples were determined by quantitative (q) PCR by using a Quantstudio 6/7 real-time PCR system (Thermofisher). qPCR reactions were prepared using reverse-transcribed RNA, TaqMan Gene Expression Assays for human TPIT (ThermoFisher, Hs00193027) and Beta-2 microglobulin (ThermoFisher, Hs00187842), and 1× TaqMan universal PCR Mastermix (Thermo Fisher scientific). TPIT expression was normalized to Beta-2 microglobulin expression and quantified using the ΔΔCT method.

### *Statistical analyses*

Comparisons between discrete variables were performed using Student's t test and Fisher's exact test. A p value less than 0.05 was considered as statistically significant. The degree of association between two variables was expressed using Spearman rank correlation. All analyses were performed using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, CA, USA ww.graphpad.com).

## Results

Our series of extra-pituitary ACTH-producing NENs showed variable immunohistochemical expression of ACTH, in terms of both the percentage of positive cells and of the global score, and such heterogeneity was evident both in CS-associated tumors (range 5-70% and 5-210) and in non-functioning ones (range 5-40%, 5-120) (table 1). However, when CS-associated and nonfunctioning tumors were compared, the former showed a significantly higher expression of ACTH than the latter, considering both the percentage of positive neoplastic cells (mean 29.4% Vs 7.8%, p=0.007) and the global score (mean 60.55 Vs 19.2, p=0.03) (table 1), independently of the site of origin of the tumor. The significantly higher ACTH expression in CS-associated

tumors than in non-functioning ones was also confirmed in the subgroup of LuNETs (mean percentage of neoplastic cells: 25,5% Vs 7.8%, p=0,05; mean global score: 54.4 Vs 19.13, p=0.04) (Table 1).

When TPIT immunostain was considered in ACTH-producing extra-pituitary tumors, we observed that the expression of this transcription factor was limited to a subset of LuNETs, whereas it was completely absent in PanNENs, as well as in pheochromocytomas (table 1, figure 1). The application of the monoclonal anti-TPIT antibody gave overlapping results with those obtained with the polyclonal one. Among LuNETs, TPIT expression was observed in a total of 16 out of 32 cases (50%) and the positive cases showed only a small fraction of immunoreactive neoplastic cells. In fact, only three cases showed more than 5% positive cells, yet the intensity of the immunoreaction was moderate to strong (2+ to 3+) in the majority of cases, comparable to that of corticotroph PitNETs, with only three cases with faint intensity (table 1, figure 1). When cases were subdivided according to tumor grade (G1 Vs G2) or for endocrine activity (Cushing Vs non-functioning), no significant difference was demonstrated in TPIT expression along the different groups, in terms of either the number of positive cases, or of the intensity of immunostain, or of the percentage of positive cells (table 1, figure 1). Finally, no correlation between TPIT expression and intensity or extent of ACTH immunostain was observed (Spearman correlation coefficient (r): 0.098 for LuNETs). In PitNETs, TPIT expression was limited to corticotroph tumors, as expected, with strong intensity in all tumor cells and no differences between the two functioning cases and the non-functioning one. Finally, ACTH-negative PanNETs and LuNETs were completely negative for TPIT.

With the aim of further exploring TPIT expression in extra-pituitary ACTH-producing NENs, and of validating the immunohistochemical result, we decided to perform a Real Time RT-PCR analysis for TPIT mRNA (table 1, supplementary table 1). All but one pulmonary and all pancreatic tumor samples were available for molecular analysis, as well as the ACTH-producing PHEOs and 5 of the 6 PitNETs (3 corticotrophs, 1 gonadotroph and 1 lactotroph). When results were compared, we observed, as expected, the highest expression levels of TPIT mRNA in corticotroph PitNETs (with no differences between the two functioning cases and the non-functioning one), whereas the gonadotroph and lactotroph tumors presented negligible TPIT expression. As for extra-pituitary tumors, molecular results confirmed the lowest expression levels of TPIT mRNA in PanNETs and PHEOs, comparable with those observed in non-corticotroph PitNETs. In contrast, a subset of LuNETs showed significantly higher relative expression levels than non-corticotroph PitNETs and PanNETs (p=0.004), even if in no case the expression levels were comparable with those of corticotroph PitNETs. When LuNETs were analyzed considering an arbitrary cut off of ten times the highest relative expression value of non-corticotroph PitNETs (0.029*10 = 0.29), 17 out of 31 evaluable cases (55%), showed TPIT expression levels above this threshold (table 1, supplementary table 1, figure 2). However, when we went back to immunohistochemical results in LuNETs, we found poor correlation between protein expression and mRNA levels (table 1). In addition, TPIT mRNA expression levels where not even correlated to the presence or absence of CS. The exception was represented by case LuNET 7, in which CS was present and TPIT expression was very high both at the protein and at the mRNA level.

# Discussion

Extra-pituitary ACTH secretion is a well-known phenomenon that represents the biological basis for the so-called ACTH-dependent ectopic Cushing syndrome (CS) and is associated with a variety of neoplastic conditions [6]. The molecular and cellular pathways leading to ACTH expression outside the pituitary gland are matter of ongoing debate, considering that their clarification would allow to identify potential therapeutic targets, useful to treat a complex and severe disease. In the last years, the identification of transcription factors driving cell lineage differentiation in the pituitary gland has elucidated the mechanisms underlying hormone secretion in normal and neoplastic cells, besides providing robust tools for the pathological diagnosis of pituitary diseases [4]. In pituitary corticotroph cells and tumors, the transcription factor TPIT seems to be essential in inducing POMC expression and, consequently, ACTH production [1]. This mechanism, however, does not explain why a subset of corticotroph PitNETs, which are indeed ACTH and TPIT-positive by immunohistochemistry, do not cause an excess of circulating ACTH and, ultimately, are not related to CS [11]. In turn, to our knowledge, no data is currently available on the presence and role of TPIT in extra-pituitary ACTH-producing neoplasms.

This study was designed with the aim to explore TPIT expression, at the protein and the mRNA level, in a series of extra-pituitary ACTH-producing tumors, either CS-associated or not, mainly represented by pulmonary and pancreatic NETs. Our results show complete absence of TPIT expression in PanNETs, which were all CS-associated, as well as in the pheochromocytomas. In contrast, half of the lung ACTH-producing tumors showed some TPIT expression, although, except for one case, no definite relationship was found either between immunohistochemistry and molecular analysis or between TPIT expression and the presence or absence of Cushing syndrome. To exclude the possible, albeit remote, event of a non-specific immunostaining for TPIT in ACTH-secreting Lu-NETs, we decided to perform the immunohistochemical study also on a comparable number of ACTH-negative LuNETs and PanNETs and we did not find any positive case, supporting the reliability of our data.

Our results suggest, as a whole, that ACTH-secreting PanNETs are TPIT-negative. In contrast, LuNETs seem to show, at least partially, some TPIT expression, suggesting that a “corticotroph-like” transcriptional program may be active in such tumors. The lack of complete overlap between protein and mRNA expression may be explained in several different ways, including technical and biological reasons, as we have already discussed elsewhere [12]. However, the analysis of all other tumor types that we have explored (ACTH-positive and ACTH-negative PanNETs and ACTH-negative LuNETs) supports that TPIT expression may be really limited to LuNETs and that ACTH secretion in these tumors may be, at least in part, related to TPIT activity. In PanNETs, other mechanisms of ACTH production may be active. In fact, TPIT function seems not to be the only driver of ACTH production and secretion even in corticotroph PitNETs, where TPIT is always expressed but the amount and functionality of ACTH secretion is variable. Indeed, the clinical spectrum of these PitNETs is wide, not only in terms of endocrinological function, but also of oncological aggressiveness [4, 11]. In this regard, Araki and co-workers have demonstrated the existence of two distinct POMC gene promoter regions, one of which is bound by TPIT and the other by E2F1 [9, 13, 14]. Interestingly, the first promoter is demethylated in all POMC-expressing cells and is highly demethylated only in pituitary ACTH-secreting tumors harboring the ubiquitin-specific protease 8 (USP8) mutation, whereas the second promoter region is highly methylated in non-functioning corticotroph PitNETs, partially demethylated in normal corticotrophs, and highly demethylated in pituitary and ectopic ACTH-secreting tumors [13]. In addition, it has been shown

that the demethylation of the second promoter was related to markers of aggressive phenotype (large size, invasion, Crooke's changes) in corticotroph PitNETs [13]. Moreover, the same authors were able to show, in vitro, that POMC transcription in human extra-pituitary ACTH-producing cell lines is independent of *TPIT*/*PITX1* function and is *E2F1*-mediated [9]. These findings are particularly interesting in the context of our study, if we consider that ACTH-producing PanNETs (which are TPIT-negative and, conceivably, should show high demethylation of the second POMC promoter) are, in general, oncologically aggressive neoplasms and are always related to signs and symptoms of CS [10]. In contrast, ACTH-producing LuNETs (which are TPIT-positive, at least in part, and in which POMC expression may be related to the demethylation of the first promoter) do not show a different course compared with non-ACTH-producing grade- and stage-matched LuNETs and may be non-functioning [8], whether or not they express TPIT.

In conclusion, this study further highlights the clinical and biological heterogeneity of extra-pituitary ACTH secretion and ectopic CS and supports the idea that the differences between ACTH-secreting PanNETs and LuNETs mirror distinct molecular mechanisms underlying POMC expression. In fact, our results point towards the recognition of a real corticotroph-like phenotype of ACTH-producing LuNETs, that is not a feature of ACTH-producing PanNETs.

# Declarations

**Ethical approval:** This study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethical Committee of the ASST dei Sette Laghi, Varese, Italy.

**Competing interests:** the authors declare that they do not have financial or non-financial competing interests

**Ethical responsibility of authors:** the authors declare that the submitted work is original and has not been published or submitted elsewhere in any form or language (partially or in full)

**Authors' contributions:** SU designed and coordinated the study, interpreted the results, and drafted the manuscript; EL designed the study, performed and interpreted the experiments and revised the manuscript; SK, RM, AV and LL performed and interpreted the experiments and revised the manuscript; MLT provided clinical data about the patients and revised the manuscript; MV andDD interpreted the results and revised the manuscript; SLR designed the study, interpreted the results and revised the manuscript. All Authors approved the final version of the manuscript.

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# Table 1

Table 1 is available in Supplementary Files section.

# Figures

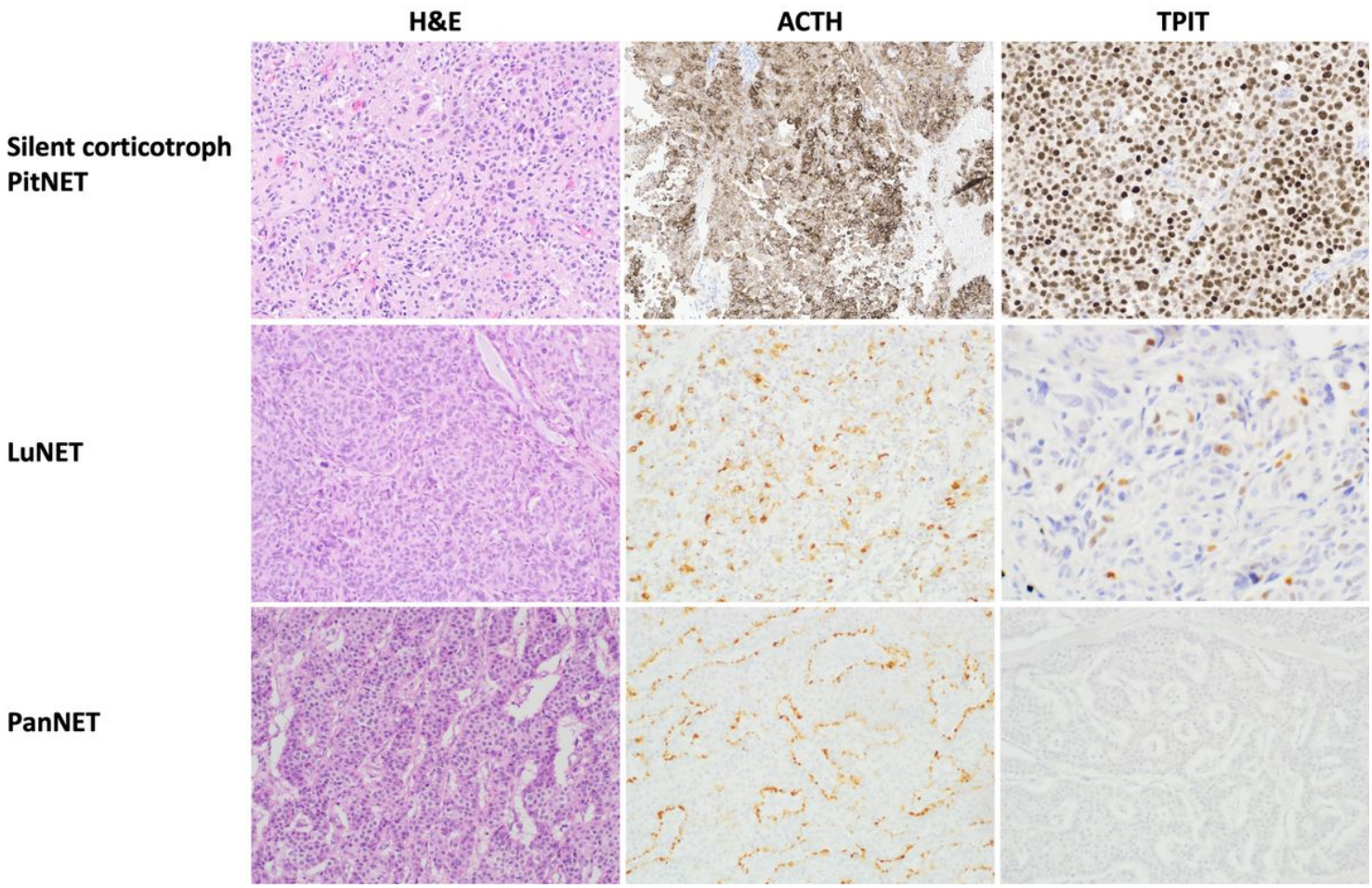


**Figure 1**

Immunohistochemical expression of ACTH and TPIT in corticotroph PitNET, and in ACTH-secreting LuNETs and PanNETs. Corticotroph PitNET shows the expected strong and diffuse expression of both ACTH and TPIT. Lung NET shows ACTH and TPIT expression in a lower number of neoplastic cells with a less intense immunostain. Pancreatic NET shows immunoreactivity for ACTH, but it is totally negative for TPIT.

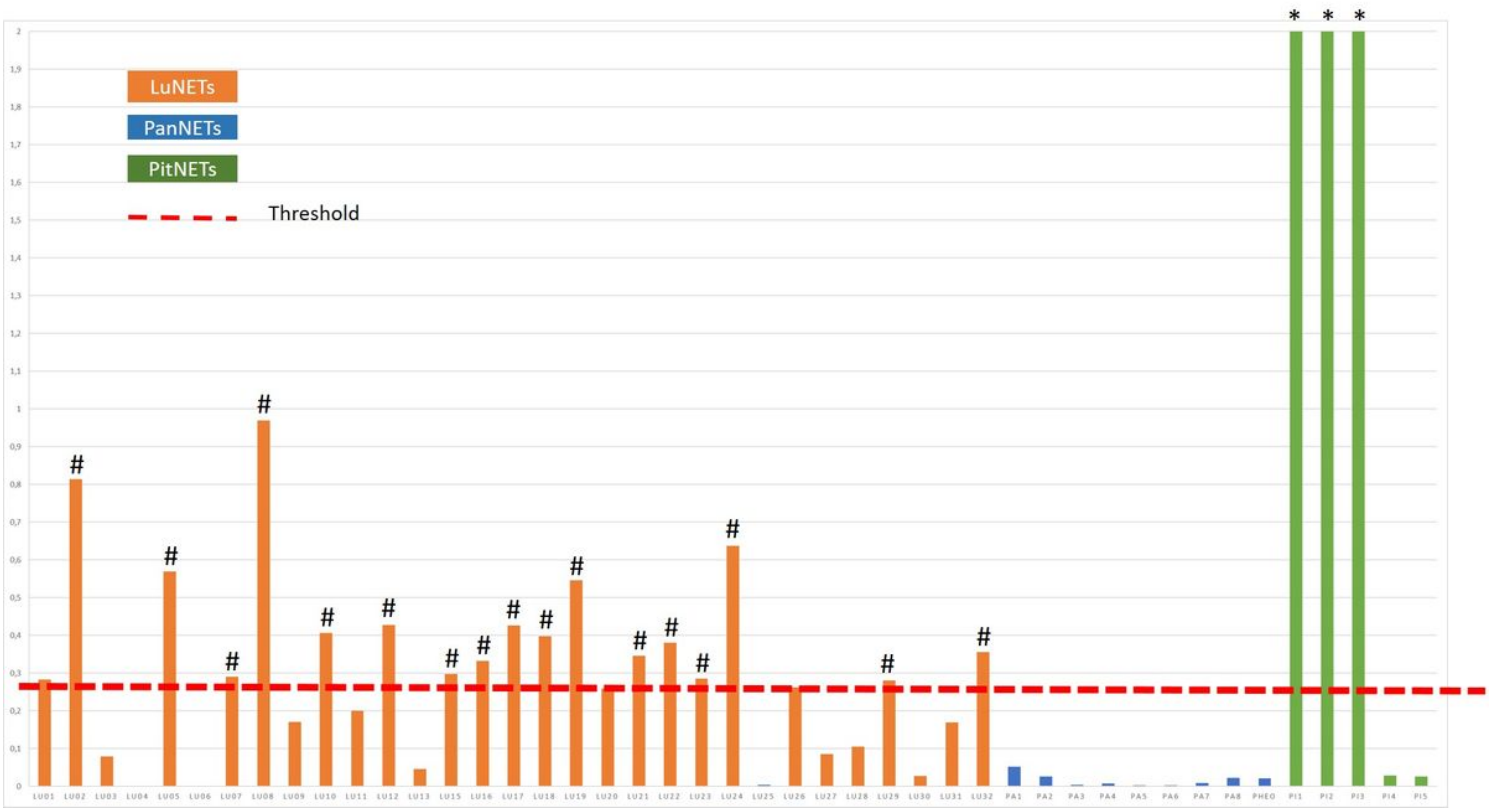


**Figure 2**

TPIT mRNA levels in ACTH-secreting lung NET, ACTH-secreting pancreatic NETs, and in control PitNETs. Threshold: arbitrary cut off of ten times the highest relative expression value of non-corticotroph PitNETs. #, cases above the threshold; *, in corticothroph PitNETs, TPIT expression levels fall off the scale.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.doc
- Supplementarytable1.xlsx