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Pathology of pituitary neuroendocrine tumours: present status, modern diagnostic approach, controversies and future perspectives from a neuropathological and clinical standpoint

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Abstract

Neuroendocrine tumours of the adenohypophysis have traditionally been designated pituitary adenomas to underline their usually indolent growth and lack of metastatic potential. However, they may demonstrate a huge spectrum of growth patterns and endocrine disturbances, some of them significantly affecting health and quality of life. To predict tumour growth, risk of postoperative recurrence and response to medical therapy in patients with pituitary neuroendocrine tumours is challenging. A thorough histopathological and immunohistochemical diagnostic workup is an obligatory part of a multidisciplinary effort to precisely define the tumour type and assess prognostic and predictive factors on an individual basis. In this review, we have summarised the current status of the pathology of pituitary neuroendocrine tumours based on the selection of references from the PubMed database. We have presented possible diagnostic approaches according to the current pituitary cell lineage-based classification. The importance of recognising histological subtypes with potentially aggressive behaviour and identification of prognostic and predictive tissue biomarkers have been highlighted. Controversies related to particular subtypes of pituitary tumours and a still limited prognostic impact of the current classification indicate the need for further refinement. Multidisciplinary approach including clinical, pathological and molecular genetic characterisation will be essential for improved personalised therapy and the search for novel therapeutic targets in patients with pituitary neuroendocrine tumours.

Introduction

Pituitary neuroendocrine tumours are a heterogenous group of tumours in their origin, growth patterns and biological behaviours. They are traditionally designated pituitary adenomas, underlining their usually slow growth and exceptional metastatic potential. However, the highly variable clinical impact of these tumours, morbidity caused by invasive growth, metabolic dysfunction and/or insufficient response to medical and other treatments in a proportion of patients have led to a proposal for a change in terminology to pituitary neuroendocrine tumour (PitNET) [1]. Accordingly, we will use the term PitNET throughout this review.

Over the last two decades, we have witnessed a revolutionary transformation in the field of neuropathology. This has been driven by progress in molecular biology, which has provided better characterisation and classification of brain tumours [2]. A multidisciplinary approach based on extensive collaboration between clinicians, basic researchers, molecular biologists, and neuropathologists has enabled the selection of important prognostic and predictive markers. Proposals for standardised diagnostic algorithms have been published by authorities within the field [3], making it easier for institutions responsible for patient care to introduce methods and techniques required to fulfil modern diagnostic needs. In neuroendocrine oncopathology, a uniform terminology for neuroendocrine tumours (NET) arising in different organs, clearly defined criteria for evaluation of tumour cell proliferation, and new genomic insights are expected to further improve the tumour classification and diagnostic approach [4,5,6,7].

As pituitary neuroendocrine tumours may have significant impact on patients' health, pituitary tumour pathology needs to be updated to meet the demands of modern, integrated medicine. Multidisciplinary collaboration in the field of pituitary oncology is suboptimal, partly due to the lack of dedicated pathologists and molecular biologists, and partly due to the insufficient integration of molecular and tissue analyses into routine clinical practice, even when their prognostic and/or predictive value has been proven. Nonetheless, a substantial amount of data has been accumulated, enabling improved classification of PitNETs and more precise assessment of the receptors targeted by pharmacological therapies. In addition, intensive research is ongoing to provide further molecular profiling of PitNETs and identify new therapeutic targets.

In this review, we will a) summarise the present status of the pathology of sporadic pituitary neuroendocrine tumours, b) present possible diagnostic approaches that can be used in order to meet current classification criteria, c) discuss the controversies in the new classification of PitNETs and d) present the future perspectives and a research agenda within the field of pituitary tumour pathology from a multidisciplinary clinical-pathological viewpoint.

Present status in the pathology of pituitary neuroendocrine tumours

Epidemiology of PitNETs

Even though once considered rare, pituitary tumours represent more than 15% of intracranial tumours [8]. The incidence has increased during the past two decades, in parallel with the availability of modern imaging techniques that enable early and incidental diagnosis of PitNETs while still clinically asymptomatic [9].

Pituitary cell lineage-based classification of PitNETs

The current World Health Organisation (WHO) classification of pituitary neuroendocrine tumours or pituitary adenomas is based on pituitary cell lineages defined by the immunohistochemical expression of anterior pituitary hormones and pituitary specific transcription factors (TFs) in the tumour cells [4].

The transcription factors regulating differentiation of the hormone producing adenohypophysial cells have been known for a long time [10,11,12,13,14,15]. However, their practical immunohistochemical application has only recently been introduced [4], enabling more precise classification of the PitNETs with sparse or no hormone expression. Pituitary transcription factor 1 (Pit-1) governs the differentiation of somatotroph, lactotroph and thyrotroph cells [10] and is preserved in tumours derived from these cell types [16]. T-box family member TBX19 (T-Pit) acts as a TF for corticotroph cells [14,17] and is a marker of corticotroph pituitary tumours [18,19]. Steroidogenic factor 1 (SF-1) determines development of gonadotroph cells [12,13], and is also expressed in gonadotroph PitNETs [11]. In contrast to Pit-1 and T-Pit, which seem to be specific for the anterior pituitary, SF-1 is also active in the adrenal glands and reproductive system [20,21]. Estrogen receptor alpha (ERα) and GATA-2 are other TFs not limited to pituitary gland that are involved in the differentiation of

gonadothroph, lactotroph and thyrotroph cells and are expressed in pituitary tumours originating from these cells [22,23].

Based on the expression of adenohypophysial hormones and pituitary-related transcription factors, foremost Pit-1, SF-1 and T-Pit, PitNETs can be divided into six main categories (somatotroph, lactotroph, thyrotroph, corticotroph, gonadotroph and plurihormonal tumours). Each of the tumours may cause metabolic disorders due to hypersecretion of anterior pituitary hormones or behave as non-functioning or hormonally silent tumours, causing pituitary insufficiency and/or other symptoms related to the intrasellar tumour mass. However, approximately 80% of the clinically non-functioning or silent pituitary tumours are of gonadotroph type, expressing FSH and/or LH by using immunohistochemistry, followed by corticotroph tumours, and rarely other hormonal subtypes [19,24]. Rare hormonally inactive sellar tumours negative for both anterior pituitary hormones and pituitary-specific TFs are designated null cell adenomas [4,25]. Exceptionally, pituitary tumours are composed of two or even three distinct components, consistent with double or triple adenomas [4,26]. (Table 1). Representative tumours of the three pituitary cell lineages are illustrated in Figure 1A-C.

Histological prognostic factors in PitNETs
Cell proliferation and tumour invasion as prognostic factors in PitNETs
The general histological markers of proliferation, mitotic count and the Ki67 (MIB1) index, are important prognostic factors in NETs in general [4]. The role of Ki67 as a marker predicting growth, invasiveness and recurrence rate in PitNETs has been controversial, largely due to different cut off levels and assessment methods used in different studies [27,28,29,30,31]. The 2017 WHO classification of PitNETs does not define the cut-off for mitotic rate or Ki67 level for considering a pituitary tumour as proliferative. However, the presence of more than 2 mitoses per 10 microscopic high-power fields (HPF) and a Ki67 index of ≥ 3% are usually interpreted as signs of increased proliferation [31,32,33,34,35]. In cases with identifiable hotspots, the 2017 WHO classification recommends the assessment of the Ki67 in the hotspots [4].

Although p53 immunoreactivity, suggestive of mutations in the tumour suppressor gene *TP53*, is a potential marker of aggressive behaviour in many human tumours, its prognostic importance in PitNETs is still controversial [4]. *TP53* gene inactivation is frequent in human cancers [36]. However, it occurs very rarely in PitNETs, suggesting that this pathway may

not be important for the initiation of pituitary tumour development, although it may play a role in promoting tumour growth [37]. While some studies support the prognostic utility of definite p53 immunolabelling in PitNETs [31,34,38], others could not demonstrate the same [29,33,39].

Invasion of the cavernous or sphenoid sinus can be confirmed by preoperative MRI and during surgery in more than 40% of patients with PitNETs [34,40]. The combined use of pituitary tumour invasion and proliferation, as assessed by mitotic count and the Ki67 index, resulted in less than 3% of pituitary tumours being classified as atypical adenomas according to the 2004 WHO classification [41]. Criticism was directed towards the previous classification for not including invasion as a prognostic marker [42]. A case-control study on more than 400 patients with eight years follow-up by Trouillas and the French group, *Hypopronos*, convincingly demonstrated that taking into account both cell proliferation, defined as a combination of Ki67 index, mitotic count and p53 expression, and tumour invasion strongly predicted postoperative disease-free and recurrence/progression-free status in patients operated on for PitNETs [34]. The prognostic value of the Trouillas´ five-tiered classification has been confirmed in three large studies: two retrospective and one prospective [43,44,45].

The recent 2017 WHO classification introduced the term "high-risk pituitary adenoma" for PitNETs demonstrating rapid growth, radiological invasion and a high Ki67 proliferation index [4]. However, it lacks a precise definition of any of the criteria on which this category is based. Metastatic tumour deposits within or outside the central nervous system is still a criterion for diagnosis of the rare pituitary carcinoma [4].

Potentially aggressive histological subtypes of PitNETs
Certain subtypes of the PitNETs have emerged as potentially aggressive regardless of their histological grading, presumably due to their intrinsic biology [4,25]. Although the mechanisms underlying the potentially aggressive behaviour of these particular types are frequently unclear, these types should be identified by pathologists and specified in histopathological reports, as the patients bearing these tumours may need closer follow-up.

Sparsely granulated somatotroph tumours are characterised by a perinuclear dot-like pattern of cytokeratin/Cam 5.2 immunolabeling in the majority of tumour cells, tend to be larger, more invasive and occur in younger patients than densely granulated somatotroph tumours [46,47,48,49,50,51,52]. This sparsely granulated cytokeratin pattern in somatotroph tumours may also predict a poorer response to the somatostatin analogue octreotide [46,48,49,50,51,52,53], which seems to be related to the lower expression of somatostatin receptor 2 in this tumour subtype [49,51,53]. Sparsely granulated somatotroph tumours may occasionally demonstrate only sparse immunoreactivity for GH, emphasising the need for immunohistochemistry with an antibody towards Pit-1 TF. Sparsely and densely granulated somatotroph tumours differ also in their MRI characteristics, which potentially enables prediction of the response to somatostatin analogues, even before surgery and assessment of somatostatin receptors on the tumour cells [54,55,56,57,58,59].

Lactotroph tumours in men, especially younger men, are frequently invasive macroadenomas resistant to dopamine analogues [60,61], probably related to the lower expression of estrogen receptor alpha in the tumour cells [62].

Silent corticotroph tumours represent about 15% of all hormone non-secreting tumours, thus, being the second largest type of NF-PitNETs, after silent gonadotroph tumours [19]. Expression of ACTH in silent corticotroph tumours may be sparse, and immunohistochemical detection of T-Pit in these cases is crucial for diagnosis [19,24]. Due to their increased tendency for invasive growth, apoplexy and recurrence, silent corticotroph tumours are categorised as potentially more aggressive in the latest WHO classification [4,63,64,65,66].

Crooke cell adenoma is a rare subtype of corticotroph tumour with less than 100 cases described in the literature of which some are cited here with George *et al.* reporting the largest number of 36 cases [67,68,69,70,71]. It is composed of tumour cells demonstrating Crooke hyaline change, a phenomenon well described in pituitary corticotroph cells exposed to hypercortisolaemia [72]. Based on a limited number of cases, this exceptional type of tumour has been categorised as potentially aggressive in the current WHO classification [4].

Plurihormonal Pit-1 positive adenoma, previously called silent subtype 3 [73,74,75] has also recently been categorised as a potentially aggressive subtype [4]. Although considered silent, a significant proportion of patients have laboratory and/or clinical signs of growth hormone (GH), prolactin (PRL) or thyrotroph hormone (TSH) hypersecretion. In addition, these neoplasms frequently show residual disease after surgery and may show rapid progression of disease [76]. In the silent cases with sparse expression of Pit-1 cell lineage hormones, immunohistochemistry for transcription factor Pit-1 may be necessary for the diagnosis.

Other histological prognostic factors in PitNETs

Although still far from being included in routine pathological work-up, several markers have emerged as potentially useful prognostic tissue markers in PitNETs.

Estrogen receptor alpha (ERα). In addition to correlating with overall poor prognosis and early recurrence in men with lactotroph tumours [62], decreased expression of *ERα* in combination with younger age correlated with tumour recurrence in male patients with gonadotroph NF-PitNETs [77].

E- Cadherin. Reduced expression of membrane E-Cadherin, frequently with translocation of the intracellular E-cadherin domain to the nuclear compartment, correlates with growth and invasiveness in different types of PitNETs [78,79,80]. In gonadotroph tumours, however, nuclear expression following reduction of intracellular E-cadherin domain correlates with lower rates of postoperative recurrence and surgical reintervention [81].

O6-methylguanine DNA methyltransferase (MGMT). Better known as a marker of response to temozolomide, MGMT seems to have even prognostic importance as its lower expression has been shown to correlate with invasiveness and early recurrence both in functioning and non-functioning PitNETs [82,83,84,85,86,87].

Aryl hydrocarbon receptor interacting protein (AIP). Germline loss of function mutations in *AIP* gene cause development of somatotroph and less frequently other types of PitNET in a subset of patients with familial pituitary tumours [88]. In sporadic somatotroph tumours, AIP down-regulation has been shown to correlate with larger size and invasive tumour growth [89].

Predictive markers in PitNETs

Medical therapy is important in pre- or post-surgical management of patients with hormone producing pituitary tumours. The first-generation somatostatin analogues (SA), octreotide and lanreotide, targeting mainly somatostatin receptor (SSTR) type 2, are standard, usually postoperative, medical treatments of acromegaly [90]. Whereas, dopamine agonists are first-line treatment in patients with lactotroph tumours [91]. Pasireotide, a new generation of SAs, with high affinity to SSTR5, but also SSTR2 and 3, is a therapeutic option in patients with acromegaly who are resistant to octreotide or lanreotide [90], and in patients with persistent Cushing disease after surgery for corticotroph tumour or in whom tumour resection is not possible [92]. Temozolomide has been used for the last few years for the treatment of aggressive and metastasising pituitary tumours [35]. For the first time, immunohistochemical assessment of selected predictive tissue markers has been recommended in the WHO classification of PitNETs [4].

Somatostatin receptors (SSTRs). Acute and long-term response to SA octreotide in patients with acromegaly correlates with immunohistochemical expression of its main target SSTR2A in tumour cells of somatotroph tumours [93,94,95,96]. However, the results are conflicting when it comes to correlation between SSTRs expression and response to pasireotide in acromegaly. In a relatively small cohort of patients resistant to the first-generation SAs, response to pasireotide correlated with SSTR5 expression [97]. Very recently, however, it has been demonstrated, again on a relatively small cohort, that SSTR2A is a main driver of pasireotide effects in patients partially responsive to first-generation SA [98]. This discrepancy could be explained, at least partly, by the selection of patients in the two cohorts. Namely, the patients in Iacovazzo's report were resistant to the first-generation SAs and had a lower expression of SSTR2 in tumour cells, thus, making SSTR5 the main target for the pasireotide. The previously shown correlation between the granulation pattern in somatotroph tumours and response to SAs is also most probably related to SSTRs. Sparsely granulated somatotroph tumours demonstrate lower expression of SSTR2 and poorer response to octreotide [51,53,79]. Response to pasireotide seems, however, to be better in patients with sparsely granulated somatotroph tumours, probably related to SSTR5 expression, at least in patients resistant to first-generation SAs [97]. Thus far, there have been no reports on the correlation between immunohistochemical expression of SSTRs and response to pasireotide in patients with Cushing disease caused by corticotroph pituitary tumour. Although no patients should be denied somatostatin analogue treatment due to low or negative

immunolabeling of somatostatin receptors on tumour cells, information on the SSTR status may help endocrinologists to stratify patients for optimal targeted therapy instead of using a trial-and-error approach. Moreover, it could facilitate an early switch to alternative SAs, when the first-line treatment does not give satisfactory results [99].

Estrogen receptor alpha (ERα). In addition to being a negative prognostic factor in lactotroph tumours in men and in a subset of patients with NF-PitNETs, low ERα expression seems to have negative predictive value underlying resistance to dopamine agonists [62].

Aryl hydrocarbon receptor interacting protein (AIP). Down-regulation of AIP in sporadic somatotroph tumours assessed by using immunohistochemistry correlates not only with larger size and invasiveness of somatotroph tumours, as previously mentioned, but may also predict a poorer response to first-generation SAs [89, 97,100]. In patients treated with pasireotide, however, AIP status has not correlated with the treatment response [97]. Notably, somatotroph tumours with low AIP expression also had a lower SSTR2 score, while no differences in the SSTR5 score were observed related to AIP immunohistochemistry, which could at least partly explain the differences in responsiveness to different SAs [97].

O6-methylguanine DNA methyltransferase (MGMT). Temozolomide is an alkylating agent widely used in the treatment of glioblastoma [101]. MGMT is a DNA repair protein that removes methyl groups from the nucleotides and in that way counteracts temozolomide. In glioblastoma, low MGMT content in the tumour cells resulting from the methylation of the *MGMT* promotor correlates with the better response to temozolomide [102]. Temozolomide has been used in the treatment of pituitary carcinoma and aggressive pituitary tumours unresponsive to conventional therapy since 2006 [103,104,105], improving the overall survival rate of the patients [106]. In pituitary tumours, *MGMT* promotor methylation status does not correlate with MGMT content in tumour cells assessed by immunohistochemistry [84]. An association between *MGMT* promotor methylation status and temozolomide effect in patients with malignant pituitary tumours could not be clearly demonstrated [107,108,109]. Results regarding the correlation between MGMT as assessed by immunohistochemistry and response to temozolomide are somewhat discrepant. However, overall, they support a correlation between low MGMT expression and responsiveness [35,109,110,111].

DNA mismatch repair (MMR) proteins may have impact on cytotoxic effect of temozolomide. However, studies on some of the MMR proteins (MSH2, MSH6, MLH1, PMS2) could not demonstrate their predictive value in patients with aggressive pituitary tumours treated with temozolomide [111,112].

Receptors targeted by immune checkpoint inhibitors: Protein death ligand 1 (PDL-1); Cytotoxic T-lymphocyte antigen 4 (CTLA-4). Hypophysitis is a frequent side-effect of anticancer treatment with immune checkpoint inhibitors targeting CTLA-4 (e.g. Ipilimumab) or, less frequently PD-L1 (e.g. Novilumab) [113,114,115,116,117]. CTLA-4 expression has been demonstrated on pituitary endocrine cells [118], and PDL-1 transcript and protein have been demonstrated in different subtypes of PitNETs [119,120]. A recent case report demonstrated a significant response to immune checkpoint inhibitors, ipilimumab and nivolumab, in a patient with a hypermutated temozolomide-resistant corticotroph carcinoma [121]. Whether immunohistochemical expression of CTLA-4 and PDL-1 may be used as a predictor of response to anticancer immunotherapy in patients with aggressive pituitary tumours remains to be clarified in future studies.

Dopamine receptor type 2 (DR2). Reduced expression of dopamine receptor type 2 is one of the proposed mechanisms of resistance to dopamine agonists in patients with prolactinomas [122]. However, a lack of a reliable antibody hampers routine immunohistochemical analysis of DR2 as a predictive marker in patients with lactotroph tumours.

A modern approach in the histopathological diagnostics of pituitary neuroendocrine tumours

As in tumour pathology in general, the role of a pathologist examining the surgical specimen from a pituitary tumour is to make a correct diagnosis, to assess prognostic tissue markers and to evaluate the expression of biomarkers that can predict the response to postsurgical pharmacological therapy. Microscopic examination of the routine haematoxylin and eosin (HE) stained slide from a pituitary tumour surgical specimen is still the basis for recognition of cell morphology, assessment of mitotic activity, and, in some cases, invasiveness.
In cases lacking typical cell morphology, the neuroendocrine origin of the tumour can be confirmed by immunolabelling of the tumour cells by synaptophysin as a general neuroendocrine marker [123]. Chromogranin A is also useful as a general marker of

neuroendocrine cells and tumours [124], typically being strongly expressed in gonadotroph tumours and showing variable expression in other subtypes of PitNETs [124,125].

A thorough immunohistochemical analysis with a broad panel of antibodies towards anterior pituitary hormones and pituitary-specific transcription factors is optimal for a precise pituitary cell-lineage based classification of PitNET. Moreover, it may be necessary for characterisation of tumours with sparse or no hormone expression, cases with unusual combinations of hormone immunoreactivity or identification of rare so-called null cell adenomas. For all practical purposes, especially in laboratories with high workload that need to meet strict time- and cost-saving requirements, satisfactory classification in a majority of typical cases can be obtained by initially using a limited number of antibodies towards anterior pituitary hormones and transcription factors selected on the basis of clinical and laboratory information. Even an approach based primarily on the use of the three pituitary transcription factors as a starting point has been proposed [126]. Thus, there are three possible approaches to classify PitNETS according to the current WHO classification:

1. Use a broad panel of antibodies towards adenohypophysial hormones and transcription factors.

2. Use a limited number of antibodies towards anterior pituitary hormones selected on the basis of the clinical and laboratory findings and complete with antibodies towards the transcription factors when required, e.g. in hormone-negative cases.

3. Use immunohistochemistry for the pituitary transcription factors as a primary screening step in the tumour characterisation.

Our opinion is that each laboratory diagnosing pituitary tumours needs to choose its own diagnostic approach depending on what level of the diagnostic accuracy it aims for and on the local conditions such as workload and the economic situation.

Experience with the use of antibodies towards the pituitary TFs is still limited and laboratories may face difficulties, as illustrated in Figure 2, when introducing and optimising the immunohistochemical protocols. However, a combined use of antibodies toward anterior pituitary hormones and transcription factors results in a precise classification of PitNETs in the vast majority of cases.

An important part of the precise classification of PitNETs is identification of histological subtypes that may behave aggressively. This may require additional immunohistochemical analysis beyond pituitary hormones and transcription factors. Immunohistochemistry with antibodies towards cytokeratin, usually Cam 5.2, is necessary to identify the sparsely-granulated variant of somatotroph tumour and should be performed in all somatotroph tumours removed during surgery for acromegaly, as well as in rare silent somatotroph tumours. Cam 5.2 may be a helpful complement to ACTH and T-Pit in the diagnosis of ACTH microadenoma, enabling identification of small groups of neoplastic cells, usually strongly stained with Cam 5.2.

A subset of silent corticotroph tumours may demonstrate sparse or no ACTH immunoreactivity. The latter cannot be diagnosed without application of T-Pit immunohistochemistry (IHC). In this context, Cam5.2 can also be useful as it usually shows strong cytoplasmic expression in corticotroph tumours, in contrast to gonadotroph silent tumours that are typically Cam 5.2 negative. Similarly, plurihormonal Pit-1 positive tumours may be clinically silent with sparse hormone expression requiring Pit-1 IHC.

The Ki67 (MIB1) proliferation index complements mitotic count and needs to be assessed in all PitNETs. Ki67 assessment is quick and easy in the great majority of cases with a low Ki67 index not reaching 3% and in the rare cases with a clearly increased index exceeding 5%. Borderline cases may require more precise quantification such as precise manual counting of the percentage of positive cells [34] or application of digital image analysis [33]. According to the current WHO classification, the Ki67 index should be assessed in hotspots. We would like to stress however that Ki67 positive tumour cells are usually evenly distributed throughout the pituitary tumour specimen. Moreover, Ki67 can be increased in areas of bleeding, necrosis or inflammation in the tumour. It is thus important to avoid an overestimation of Ki67 index in these areas.

p53 is no longer routinely recommended as a relevant marker in PitNETs by the WHO classification. However, studies on its prognostic importance show conflicting results. Moreover, being available in almost all pathology laboratories, this marker can be useful as a complement to mitotic count and the Ki67 index [34,43,44,45].

We recommend that immunohistochemical evaluation of somatotroph and corticotroph tumours includes SSTRs in tumours from patients with acromegaly and Cushing disease who are potential candidates for postoperative treatment with somatostatin analogues. High-performance monoclonal anti-SSTRs antibodies are available, and immunohistochemical protocols are well-established [94, 95,127]. Although different methods for quantification of SSTRs have been used, results from the two largest studies comparing SSTR2 expression with response to first-generation SAs indicate that a good response can be expected in patients harbouring somatotroph tumours with distinct, moderate to strong membranous SSTR2 expression in more than 50% of the tumour cells [94, 95]. Immunohistochemistry for SSTRs should be a standard method in any pathology laboratory connected to a pituitary centre of excellence.

Studies described in an earlier section also encourage routine assessment of ER alpha in lactotroph tumours [62] and AIP in somatotroph tumours [89,97,100] as they can be of prognostic and predictive value.

Importantly, identification of multiple biomarkers strengthens their prognostic and predictive significance and is a justification for using a broad panel of immunohistochemical markers to provide a comprehensive evaluation of PitNET in a particular patient. Optimally, the spectrum of diagnostic methods relevant for individualised treatment and follow-up should be discussed at multidisciplinary conferences.

As sporadic PitNETs are rare in younger patients, genetic screening to search for germ-line mutations (*AIP*, *MEN1*, *CDKN1B*, *PRKAR1A*, *SDHx*) underlying familial predisposition for development of pituitary and/or other NETs should be considered in early onset PitNETs (age at diagnosis < 30 years) [128].

Moreover, pathology laboratories should be encouraged to save fresh frozen tissue from pituitary tumours, whenever possible, in order to facilitate future molecular genetic studies on pituitary tumours.

Controversies in the pathology of pituitary neuroendocrine tumours

"Null cell adenomas" – do they really exist?

Traditionally, "null cell adenomas" have been defined as clinically non-functioning pituitary neuroendocrine tumours without immunohistochemical or ultrastructural markers specific for adenohypophysial cell differentiation [32]. In the past, their frequency varied from a few percentages to more than 30% of all surgically removed PitNETs, partly due to sparse hormone expression in a subset of PitNETs and partly due to insufficient immunohistochemical protocols for identification of anterior pituitary hormones [24]. With the complementary use of antibodies towards the anterior pituitary hormones and pituitary TFs, the proportion of "null cell adenomas" have been reduced to less than 3% of all PitNETs [19].

As currently defined, "null cell adenomas" do not demonstrate pituitary-specific tissue markers by immunohistochemistry or electron microscopy [4]. Neuroendocrine tumours of different origin usually have similar morphology. Although a higher cell proliferation rate strengthens the suspicion of a pituitary metastasis from a distant malignant NET, it should be kept in mind that even low-proliferative and slowly growing NETs may have a metastatic potential [129]. For that reason, when well-known NETs occurring in the sellar region, such as paraganglioma, are excluded, these rare cases of "null cell adenomas" may require a broad panel of immunohistochemistry in order to exclude a sellar metastasis of a NET from another organ. Several markers, including TTF1, serotonin, ATRX, DAXX and CDX2 may be useful in the differential diagnosis between a PitNET and a metastatic NET in the sellar region [130,131,132]. However, more studies on larger and well-characterised cohorts are needed to define the set of biomarkers that can be reliable in the differential diagnosis between PitNETs and metastatic sellar NETs.

Additionally, in the rare patients with "null cell adenoma", an imaging work-up such as a positron emission tomography (PET) with concomitant intravenous contrast-enhanced computed tomography (CT) [133] should be considered. The vast majority of neuroendocrine tumours show high somatostatin receptor expression; therefore, PET/CT with a gallium-68-labelled somatostatin analogue is used, most commonly 68Ga-DOTATOC, 68Ga-DOTATATE or 68Ga-DOTANOC [133]. A small fraction of neuroendocrine tumours are

high grade and clinically aggressive, and for these patients PET/CT with 18F-fluoro-deoxy-glucose (FDG) may be an option [134].

Even after a thorough work-up, there is still a theoretical possibility that a small subset of the "null cell adenomas" may represent metastases from NETs from unknown primary locations or possibly primary sellar NETs of non-pituitary origin, similar to the primary NETs reported in the central nervous system [135,136,137,138].

It should be expected that a complementary use of immunohistochemistry for anterior pituitary hormones and pituitary specific transcription factors, in combination with additional organ-specific neuroendocrine markers and imaging work-up in order to search for NETs in other organs, will result in such a low frequency of "null cell adenomas" that their existence needs to be seriously questioned. As previously speculated, this tumour category might potentially only reflect the methodological problems in the diagnostics of PitNETs [24]. Future molecular genetic studies could generate valuable information regarding the origin and genesis of sellar tumours with "null cell adenoma" characteristics. A systematic collection of the fresh frozen tissue from pituitary tumours is important to enable these studies.

Thus, a revision of the concept of "null cell adenomas" should be considered in the next WHO classification of pituitary neuroendocrine tumours. Until that time, neuropathologists and endocrine pathologists diagnosing pituitary tumours should be encouraged 1) to use immunohistochemistry for pituitary-specific transcription factors, at least in the PitNETs showing no or only sparse immunolabeling for anterior pituitary hormones, 2) to use an expanded set of neuroendocrine and organ-specific tissue markers in the diagnosis of hormone-negative and transcription factor-negative pituitary tumours, and 3) to recommend an imaging work-up in search for NETs in extrasellar locations, in order to avoid misdiagnosis of a phantom "null cell adenoma". Close transdisciplinary collaboration has an important role in improving the diagnostic accuracy of these unusual pituitary tumours.

In addition to a previously reported case [131], we briefly describe an additional case demonstrating the importance of a complex and comprehensive diagnostic approach for pituitary neuroendocrine tumours with no expression of anterior pituitary hormones and pituitary specific TFs. A 69-year-old male patient underwent trans-sphenoidal surgery for a

sellar tumour. Tumour cell morphology and synaptophysin expression were consistent with a neuroendocrine tumour; however, the tumour cells did not show immunolabelling for anterior pituitary hormones or transcription factors. An extended immunohistochemical panel revealed focal CDX2 expression in the tumour cells. As CDX2 is a marker of gastrointestinal differentiation not previously reported in pituitary tumours [132], a workup with 68-Ga-DOTATOC PET/CT followed and showed tracer uptake in the upper intestinal tract with suspect mesenteric metastasis. Two separate NETs with metastases into the mesenteric lymph nodes were confirmed in the surgical intestinal resection (Figure 3).

Plurihormonal Pit-1 tumours; where has the prefix "poorly-differentiated" gone?

Horvath *et al.* (1988) described 20 patients with unusual pituitary tumour having specific ultrastructural appearance and aggressive behaviour, particularly in younger women. The tumour was termed silent subtype 3 adenoma, although several of the patients with available clinical data had some signs of prolactin or, less frequently, GH hypersecretion. Two patients had multiple endocrine neoplasia type 1 (MEN1) syndrome. With respect to the methodological problems linked to early IHC studies, it is noteworthy that many tumours expressed GH, PRL and/or TSH, some even ACTH [73]. Later, they were recognised as a distinctive form of rare plurihormonal adenomas with specific ultrastructural features, cellular atypia and a variable expression of GH, PRL and/or TSH in a majority of [74,75]. Recently, Mete *et al.* reviewed clinical, biochemical, radiological, immunohistochemical and ultrastructural characteristics of 31 patients and demonstrated that the tumour type previously called silent subtype 3 adenoma represents rare, aggressive, monomorphous tumour of Pit-1 lineage, exhibiting nuclear atypia and nucleolar prominence in routine HE staining and variable, sometimes sparse or absent immunolabelling for GH, PRL and/or TSH. Frequent association with acromegaly, hyperthyroidism, or galactorrhoea and amenorrhea in a significant proportion argued against the term "silent". Mete *et al.* proposed the new designation of "poorly-differentiated plurihormonal Pit-1 lineage tumour" [76].

Although separated from rare well-differentiated plurihormonal GH-PRL-TSH expressing tumours in the 2004 WHO classification, tumours originally described as "silent subtype 3 adenoma" and in Mete´s study cohort as "poorly-differentiated plurihormonal Pit-1 lineage tumours" have been synonymously designated as "plurihormonal Pit-1 positive adenomas" and characterised as an aggressive tumour variant in the 2017 WHO classification [4]. However, based on previously published data [139], our own experience and unreported

conversations with experts in pituitary pathology (see *acknowledgment*), we are not convinced that all Pit-1 positive plurihormonal PitNETS are poorly-differentiated and aggressive, which can be demonstrated through the two clinical cases presented here (Figure 4). A patient (upper part of Figure 4) was diagnosed incidentally with pituitary macroadenoma that corresponded histologically to a poorly-differentiated plurihormonal Pit-1 tumour. There were no clinical signs of GH, PRL or TSH hypersecretion and serum levels of pituitary hormones were within the normal range. Microscopic examination of the surgical specimen demonstrated a tumour with peculiar appearances composed of round-oval to spindle formed cells with pleomorphic nuclei and prominent nucleoli. There was patchy immunolabelling of GH, PRL and TSH. Multiple endocrine neoplasia type 1 and 4 as well as AIP gene defects were excluded.

Another patient with plurihormonal Pit-1 tumour, a 49-years-old male, presented with clinical and laboratory signs of GH and TSH hypersecretion before surgery. Microscopic examination of the tumour specimen (lower part of Figure 4) demonstrated a PitNET with typical cell morphology and extensive expression of GH, PRL and TSH. Thus, the two cases of plurihormonal Pit-1 positive tumour presented here differ strikingly in several clinical and histopathological aspects.

As plurihormonal Pit-1 positive tumours are uncommon, more studies with clinico-pathological correlations on larger number of cases are needed in order to determine which subset of these tumours are potentially aggressive. Molecular studies would be helpful in further refinement of this group of PitNETs.

Future perspectives and research agenda in the field of pathology of PitNETs
Need for further improvement of the classification of pituitary neuroendocrine tumours
The concept of the pituitary cell lineage has enabled more refined classification of pituitary neuroendocrine tumours and definition of the histological subtypes with potentially aggressive behaviour [4]. However, several aspects need further improvement. The term "pituitary neuroendocrine tumour, PitNET", similarly to the NET terminology in other organ systems [4], better emphasises the broad biological spectrum of these neoplasms and their unpredictable and occasionally aggressive behaviour compared to the traditional term "pituitary adenoma" [1]. Adoption of the term PitNET in the next WHO classification would clearly place these neoplasms within the context of endocrine oncology and encourage

further search for novel therapeutic strategies and elucidation of prognostic and predictive biomarkers.

Two tumour categories have been imprecisely characterised in the 2017 WHO classification. "Null cell adenomas", as currently defined, represent rare pituitary tumours with undetermined lineage. Future clinical, immunohistochemical and molecular studies are needed to demonstrate whether they really exist. Revision of the category "null cell adenoma" should be considered in the next classification in order to prevent under-diagnosis of neuroendocrine tumours of non-pituitary origin, some of which may represent metastases.

Moreover, further characterisation of plurihormonal Pit-1 positive tumours is needed in order to determine whether all of these tumours or only a subset previously designated as "silent subtype 3 adenomas" or "poorly differentiated Pit-1 positive tumours" should be considered as potentially aggressive.

Despite improvements, the WHO classification still lacks significant prognostic impact. Recent retrospective and prospective studies on large number of patients operated on for pituitary neuroendocrine tumours have demonstrated that combined use of proliferative markers and tumour invasion as a five-tiered score strongly predicts persistent tumour disease and early progression/recurrence after surgery [34,43,44,45]. Based on the published studies, it is to be expected that inclusion of both cell proliferation and tumour invasion, as previously proposed [34], in combination with the definition of potentially aggressive histological subtypes, would significantly improve prognostic and predictive value of the WHO classification of pituitary neuroendocrine tumours.

Need for standardisation of immunohistochemical protocols and assessment methods to facilitate personalised medicine

Progress has been made in characterisation of target receptors for pharmacological therapies and their predictive value, particularly in the field of hormone producing tumours, best exemplified through studies on somatostatin receptors in somatotroph tumours [93,94,95, 96,97,98]. However, different immunohistochemical protocols and assessment methods have been used in different studies. Methodological problems are also, at least partly, limiting factors for studies on dopamine receptors as markers of response to dopamine agonists in patients with lactotroph tumours and MGMT as a marker of response to Temozolomide in

patients with aggressive pituitary tumours. Thus, standardisation of the methodological approaches and validation of the prognostic value of the established biomarkers in large patient cohorts are prerequisites for their future application in routine work [99, 140].

Search for new therapeutic targets

Promising result of immunotherapy targeted PDL-1 in a patient with a corticotroph carcinoma [121] will hopefully encourage future studies addressing the immune checkpoint inhibitors as potential therapeutic targets in PitNETs.

The mTOR inhibitor Everolimus has been proposed as a potential new therapy for pituitary neuroendocrine tumours based on its efficacy in preclinical cell culture studies [141,142,143]. However, only a few patients with aggressive tumours resistant to other therapeutic options have been treated with limited effects [144,145]. Gene transcript analyses have demonstrated upregulation of the mTOR pathway in PitNETs [146]. However, the mTOR markers in tumour tissue have not been explored by using immunohistochemistry. More studies are needed to explore whether the drugs targeting the mTOR pathway could be useful in patients with PitNETs.

A sub-population of cells with stem cell characteristics has been demonstrated in the normal pituitary and in pituitary tumours [147,148,149]. Stem cells do play a role in pituitary homeostasis and tumorigenesis [150]. Future studies on well characterised tumour cohorts need to clarify whether cells with stem cell features may represent a target for anticancer therapy in patients with aggressive pituitary tumours, not responding to currently available pharmacological therapies.

Future molecular genetic studies on PitNETs

Few tumorigenic mutations occur in sporadic PitNETs. Activating mutations of the *GNAS* gene, coding for alpha-subunit of the stimulating G-protein linked to the growth hormone releasing hormone (GHRH) receptor have been reported in about 30% of somatotroph tumours [151]. Mutations in the deubiquitinase gene USP8 occur in corticotroph tumours in one-third of patients with Cushing disease [152]. Recent molecular genetic studies point toward epigenomic changes and genomic instability in different subclasses of the PitNETs [120, 153, 154]. Search for alternative oncogenic events and further molecular genetic

characterisation of PitNETs are essential for their precise classification, improved selection of prognostic and predictive markers and search for novel therapeutic targets.

Conclusion

Neuroendocrine tumours of the anterior pituitary are neoplasms with a broad spectrum of clinical manifestations, which may include severe endocrine disturbances and/or invasive tumour growth. Multidisciplinary approaches and thorough histological and immunohistochemical workup are required for diagnosis and assessment of the prognostic factors. Standardisation of the methods for the analysis and assessment of the prognostic and predictive tissue biomarkers is a pre-requisite for the use of personalised medicine in the treatment of patients with pituitary neuroendocrine tumours. Further improvement of the classification could be achieved by terminology change from pituitary adenoma to pituitary neuroendocrine tumour (PitNET), inclusion of both cell proliferation and tumour invasion in the classification criteria, and revision of some imprecisely defined tumour categories, including "null cell" and plurihormonal Pit-1 adenomas. The patients with aggressive and/or metastasising PitNETs benefit from temozolomide therapy and promising results have been obtained in a single patient with pituitary carcinoma treated with immune checkpoint inhibitors. Future molecular genetic studies may facilitate the search for novel therapeutic targets in patients with aggressive pituitary neuroendocrine tumours not responding to the available pharmacological treatments.

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Figure legend:

Table 1. Pituitary cell lineage-based classification of PitNETs.

Figure 1. Illustration of the main types of PitNETs belonging to the Pit-1 (Fig. 1A), T-Pit (Fig. 1B), and SF-1 pituitary cell lineages (Fig. 1C, upper row) classified on the basis of immunohistochemistry for anterior pituitary hormones and transcription factors. A "null cell adenoma" lacking signs of differentiation toward any pituitary cell lineage is presented in Fig. 1C, lower row.

Figure 2. Satisfactory immunohistochemical results with distinct nuclear immunolabeling can be obtained with available antibodies towards the pituitary transcription factors in the majority of cases (1a, 2a, 3a). However, cytoplasmic background staining is sometimes difficult to avoid when using Pit-1 and, more frequently SF-1 (1b, 3b). In a few cases, usually related to bleeding within the tumour, uneven staining with T-Pit may be observed, typically strongly positive in perivascular tumour cells and weakly positive or negative in tumour cells not surrounding the blood vessels (2b). A variable staining intensity with a proportion of the tumour cells lacking immunolabeling is a relatively frequent problem when using anti-SF-1 antibodies (3c). (Magnification x 200 for all microphotographs).

Figure 3. MR imaging of the sellar tumour demonstrating 1a) Coronal Gadolinium-enhanced T1-weighted image showing solid enhancing lesion filling the sella, with suprasellar extension above the optic chiasm. There is no discernible normal pituitary tissue. Corresponding sagittal image in 1b) shows enlarged sella and, in addition to the suprasellar,

also some anterior extension of the tumour. Cell morphology in 1c) routine haematoxylin and eosin staining and 1d) expression of synaptophysin were consistent with neuroendocrine differentiation. The tumour cells did not express pituitary hormones or transcription factors (1e). However, focally, the tumour cells expressed CDX2, a marker of gastrointestinal differentiation (1f). 68-Ga-DOTATOC-PET revealed two lesions with high tracer uptake (1g-h). A large lesion was found anterior to the right psoas muscle, interpreted as a mesenteric lymph node metastasis (1g) and a smaller lesion situated slightly anterior, which could represent the primary tumour (1h). In the surgical intestinal resection, two separated NETs were identified with transmural invasion and metastases in the mesenteric lymph nodes. Cell morphology (1i and 1j) as well as synaptophysin immunolabeling (1k) were consistent with NET. Despite invasive and metastasising growth, proliferative Ki67 index was < 1% (1l). The MRI and PET/CT images are kindly provided by Prof. Johan Wikström, Department of Radiology, Uppsala University Hospital.

Figure 4. Plurihormonal Pit-1 positive tumour. Upper part of the figure illustrates a plurihormonal Pit-1 positive tumour in a young male patient demonstrating marked cellular atypia and only patchy expression of GH, PRL and TSH, in consistence with poorl y-differentiated Pit-1 positive tumour. Lower part of the figure illustrates plurihormonal Pit-1 positive tumour in a middle-aged male patient demonstrating ordinary histological appearance and extensive expression of GH, PRL and TSH.

Table 1: Pituitary cell lineage-based classification of PitNETs

Pit-1 cell lineage tumours
Somatotroph tumour (GH +/- PRL)
Densely granulated somatotroph tumour (diffuse cytokeratin pattern)
Sparsely granulated somatotroph tumour (dot-like cytokeratin pattern)
Somato-lactotroph tumour (GH+PRL)
Lactotroph tumour (PRL)
Thyrotroph tumour (TSH)
Plurihormonal Pit-1 positive tumour (GH+PRL+TSH)
T-Pit cell lineage tumours
Corticotroph tumour (ACTH)
Crooke cell adenoma
SF-1 cell lineage tumours
Gonadotroph tumour (FSH and/or LH)
Tumours of undetermined cell lineage
Null cell adenoma (IHC negative for anterior pituitary hormones and TFs)
Tumours of the complex cell lineage differentiation
Double and triple PitNETs

Pit-1 cell lineage tumours
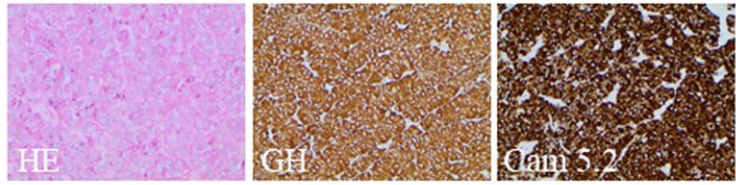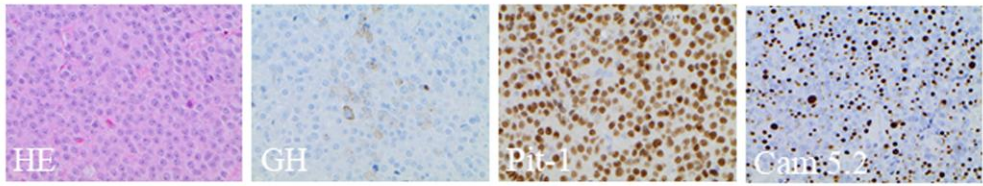
HE   GH   Cam 5.2

Densely granulated somatotroph tumour

HE   GH   Pit-1   Cam 5.2

Sparsely granulated somatotroph tumour

HE   PRL

Lactotroph tumour

HE   TSH

Thyrotroph tumour

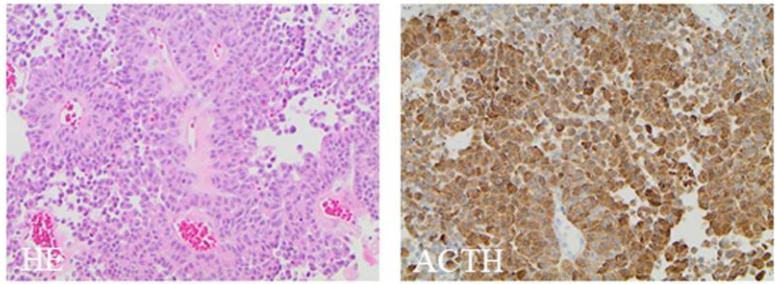HE   GH   PRL

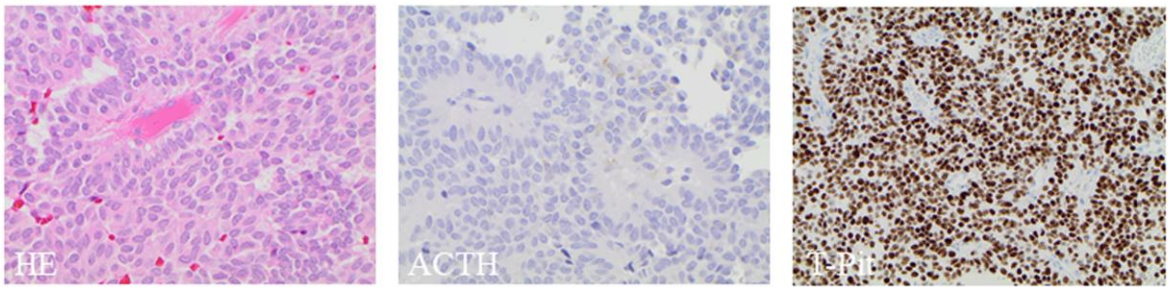Somato-lactotroph tumour

HE   GH   PRL   TSH   Pit-1
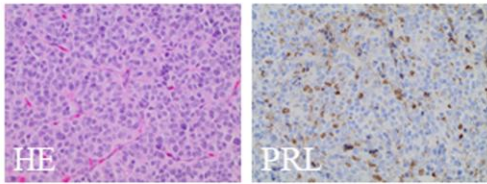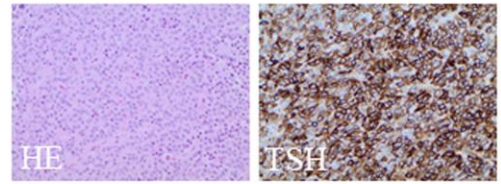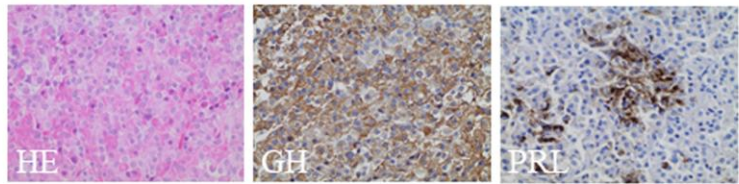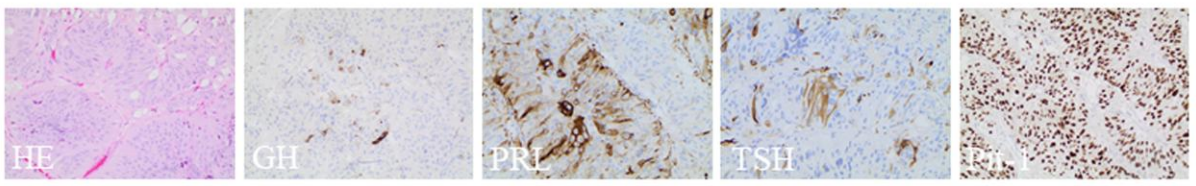
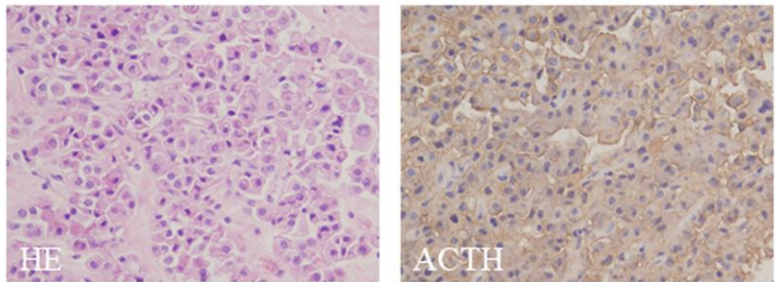Plurihormonal Pit-1 positive tumour

T-Pit cell lineage tumours

Corticotroph tumour

Corticotroph tumour (sparse or no ACTH expression)

Crooke cell adenoma