Pituitary (2015) 18:217–224
DOI 10.1007/s11102-014-0619-0

Current status on histological classification in Cushing's disease

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Published online: 14 December 2014

Abstract
Introduction Managing Cushing's disease remains a challenge. Surgery is the first option of treatment and it offers a high success rate. Even in cases where biochemical remission is not achieved, it is crucial to obtain surgical tissue for morphological diagnosis because the therapeutic approach can be modified according to the findings.
Materials and Methods A literature search was performed using PubMed for information regarding pathology and Cushing's disease.
Results The histopathological features found in the pituitary gland of patients with Cushing's disease are presented.
Conclusion Different subtypes of ACTH-producing pituitary tumors are recognized and characterized. The significance of finding a normal pituitary gland with or without Crooke's changes is also discussed.

Keywords Classification · Cushing's disease · Diagnosis · Pathology · Pituitary tumor

Introduction

Cushing described in [1] a peculiar pluriglandular syndrome associated with a basophilic adenoma of the pituitary. Despite many advances in terms of understanding its physiopathology, morphologic classification, therapeutic and treatment options, managing Cushing's disease remains a challenge [2–7].

Currently, the aims of treatment are: to control adrenocortical hypersecretion, to resect the tumor, to preserve anterior pituitary function and, whenever possible, to restore normal activity of the pituitary-adrenocortical axis [8]. Surgery is the first option of treatment and it offers a high success rate. Even in cases where biochemical remission is not achieved, it is crucial to obtain surgical tissue for morphological diagnosis because the therapeutic approach can be modified according to the findings [9, 10].

In patients with Cushing's disease, the obtained surgical specimen can show an adrenocorticotropic hormone (ACTH)-producing pituitary adenoma, a normal pituitary

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gland with or without changes due to long standing hypercortisolism, a Crooke's cell adenoma, or ACTH cell hyperplasia [11, 12]. In every case, the histological findings can serve as a guide for deciding the therapeutic approach, especially in cases with partial or absent biochemical remission after surgery.

Morphological investigation

A logical order of steps should be followed for studying the surgically obtained tissue of patients with Cushing's disease [9, 10, 13]. The first step is to establish if obtained material is tissue from a normal pituitary gland or a pituitary tumor. Then, immunohistochemical classification based on hormone production by the cells is accomplished and, eventually, prognostic information and treatment options can be determined by means of some biomarkers and by molecular/genetic/epigenetic investigation.

After initial evaluation using the hematoxylin and eosin (H&E) stain, the following step is to demonstrate reticulin fibers with silver stains. In the normal pituitary, the acinar reticular pattern is conserved; it can be enlarged but still intact in cases of hyperplasia and lost in tumors. This simple step should not be underestimated. Periodic Acid-Schiff (PAS) stain is used to recognize corticotrophs. Densely granulated corticotrophs are intensely PAS-positive because of the carbohydrate moiety present in pro-opiomelanocortin (POMC), the precursor of ACTH. Chromogranin and synaptophysin, both broad-spectrum neuroendocrine markers, can help to prove that the lesion is an endocrine tumor. Cam5.2, a low molecular weight keratin (LMWK), is of special value in ACTH-producing pituitary tumors and for recognizing normal or suppressed corticotrophs in the normal pituitary gland and will be discussed separately.

Classification of the tumor according to hormone production employs monoclonal antibodies against pituitary hormones: growth hormone (GH), prolactin (PRL), ACTH, thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), luteinizing hormone (LH) and α-subunit of the glycoprotein hormones (αSUB). In the absence of identifiable hormone content, pituitary transcription factors may help distinguish differentiation of tumor cells. Pituitary specific transcription factor 1 (Pit-1), t-box transcription factor (Tpit), steroidogenic factor (SF-1), estrogen receptor alpha (ER-α) and GATA binding protein 2 (GATA-2) can be employed. Tpit is expressed in corticotrophs; gonadotrophs express SF-1, ER-α and GATA-2; and Pit-1 defines cells producing GH, PRL and/or TSH. Lactotrophs co-express Pit-1 and ER-α whereas Pit-1 with GATA-2 are co-expressed in thyrotrophs [10].

G-protein-coupled somatostatin receptors 2 and 5 (SSTR2 and SSTR5) and dopamine D2 receptor are expressed in the majority of corticotroph adenomas [14, 15]. Examination of their expression and membrane density can help to classify the ACTH producing tumors [15].

Although many biomarkers have been investigated in tumors, there is no single biomarker currently available that predicts conclusively aggressive clinical behavior [16, 17]. In regards to ACTH-producing pituitary tumors, here we discuss only three of them: Ki-67, p53 and O^6-Methylguanine-DNA Methyltransferase (MGMT). Nuclear Ki-67, one of the most frequently used, is a cell division marker that is usually quantified to determine a cell proliferation index in various neoplasms. p53 is a tumor suppressor protein that plays an important role in cell proliferation, apoptosis, and genomic stability and MGMT, a DNA-repair enzyme, that appears to be commonly expressed in clinically aggressive macroadenomas of Cushing's disease [18–20]. If MGMT is negative, it may predict a favorable response to temozolomide as a therapeutic option [21–23].

The clinical behavior of pituitary tumors remains a challenge. The correct diagnosis of histological subtypes of pituitary tumors may predict clinical aggressive behavior in some cases [10]. A recent clinicopathological classification that takes into account tumor size, invasion to the sphenoid or cavernous sinuses confirmed by magnetic resonance imaging (MRI) or histology, hormonal immunoexpression profile and tumor cell proliferation markers (Ki-67 and p53) has been proposed [24]. Invasive tumors with high Ki-67 and p53 expression have been shown to have an increased probability of tumor persistence and/or progression as compared with non-invasive tumors. Correlation between imaging, as well as morphological findings and prognosis opens the possibility of personalized therapeutic strategies [25]. This novel classification system has yet to be validated in larger multi-center studies. The use of new biomarkers, as well as larger randomized studies, is needed to conclusively assess pituitary tumor behavior.

ACTH producing pituitary adenomas

ACTH-producing pituitary adenomas are found in the majority of patients with Cushing's disease. They are usually well demarcated microadenomas (85 % of the cases), some as small as 1–2 mm, and located in the central wedge of the anterior lobe. Due to their size they may be undetectable by MRI and difficult to find at surgery. In other cases, the adenomas are localized in the lateral wings, in the pars intermedia or in the neurohypophysis and, rarely, in the pituitary stalk. The cells of adenomas seen with Cushing's disease are basophilic or amphophilic,

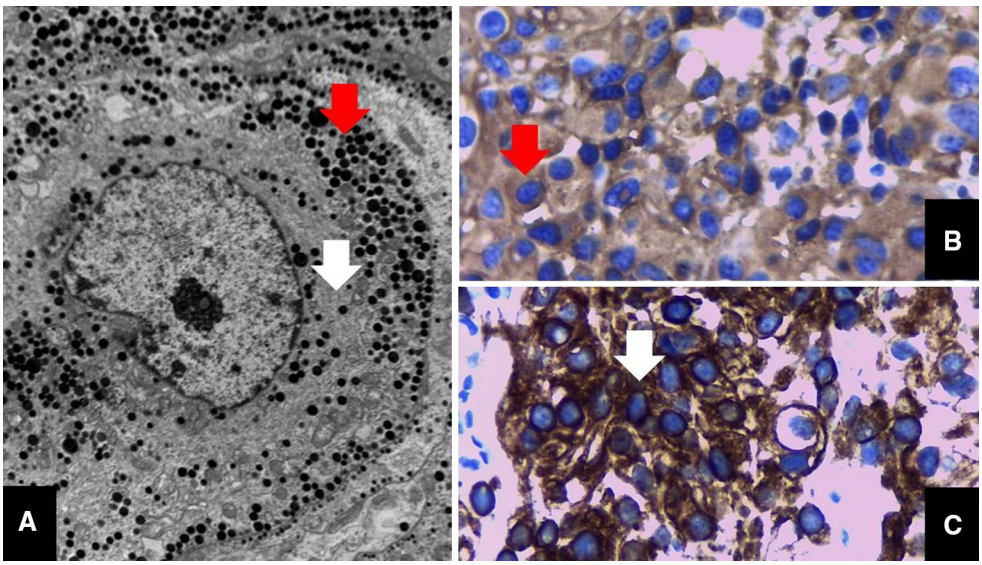

Fig. 1 Densely granulated ACTH—producing adenoma. **a** Electron microscopy reveals numerous secretory granules (*red arrow*) with bundles of perinuclear cytokeratin (*white arrow*). **b** Immunohistochemistry (IHC) for ACTH shows intense cytoplasmic immunoreactivity in the densely granulated cells (*red arrow*). **c** IHC for low molecular weight keratin (LMWK) demonstrates perinuclear ring-like keratin filaments (*white arrow*)

PAS-positive with strong immunopositivity for ACTH and LMWK in a perinuclear pattern. ACTH-producing pituitary adenomas are associated with the terminology *densely or sparsely granulated* which refer to the pattern of secretory granules as seen by electron microscopy (Fig. 1a–c). Cytoplasmic intensity of immunoreactivity for ACTH correlates with the number of secretory granules. Electron microscopic features include bundles of perinuclear ring-like keratin filaments around the nucleus, which can be demonstrated by LMWK immunostaining. The most frequent subtype is the densely granulated ACTH-producing pituitary adenoma (Fig. 1a–c). The sparsely granulated ACTH-producing pituitary adenoma is composed of chromophobic cells, LMWK is strongly positive and ACTH positivity is variable and usually weak (Table 1). This histological subtype has been suggested to have a more aggressive clinical behavior, although larger series and studies are required to confirm these data [10].

The World Health Organization (WHO) classifies pituitary tumors as typical and atypical adenomas (Table 1). Typical adenomas are tumors with monotonous cells, without high mitotic activity, Ki-67 <3 % and no p53 immunoreactivity. In contrast, atypical adenomas disclose elevated mitotic index, a Ki-67 labeling index >3 % and nuclear staining for the p53 protein. According to the clinical behavior, a pituitary tumor can be considered as aggressive or non-aggressive. Usually tumors that exhibit a high rate of recurrence, demand repeated surgeries and are resistant to conventional treatments are considered aggressive adenomas. Correlations between these classifications are not straightforward. Atypical adenomas do not always correlate with an aggressive clinical behavior and some typical adenomas (with Ki-67 <3 %) can have an aggressive clinical behavior with high rate of recurrence and resistance to medical and surgical treatments. Therefore, the terms typical and atypical adenoma should refer only to pathological features, invasive and non-invasive tumor to the anatomical patterns of invasiveness of the tumor towards the surrounding structures, and aggressive and non-aggressive pituitary adenomas to their clinical behavior [26].

Normal pituitary found at surgery

In some cases a normal pituitary gland is found solely in the surgical specimen, showing a normal acinar reticular pattern, as demonstrated by silver and reticulin stains. Immunohistochemical (IHC) staining techniques allow for the visualization of pituitary hormonal activity. The identification of Crooke's hyaline changes is of paramount importance. Crooke's changes, identified for the first time in 1935 [27], correspond to hyalinization of cytoplasm of adenohypophysial basophilic cells in patients with hypercortisolism with disappearance or displacement of the secretory granules to the periphery of the cell (Fig. 2a–c). The massive accumulation of perinuclear cytokeratin occurring in corticotrophs under the effect of glucocorticoid excess gives rise to the so-called "*Crooke's cells*". This accumulation can be demonstrated using LMWK

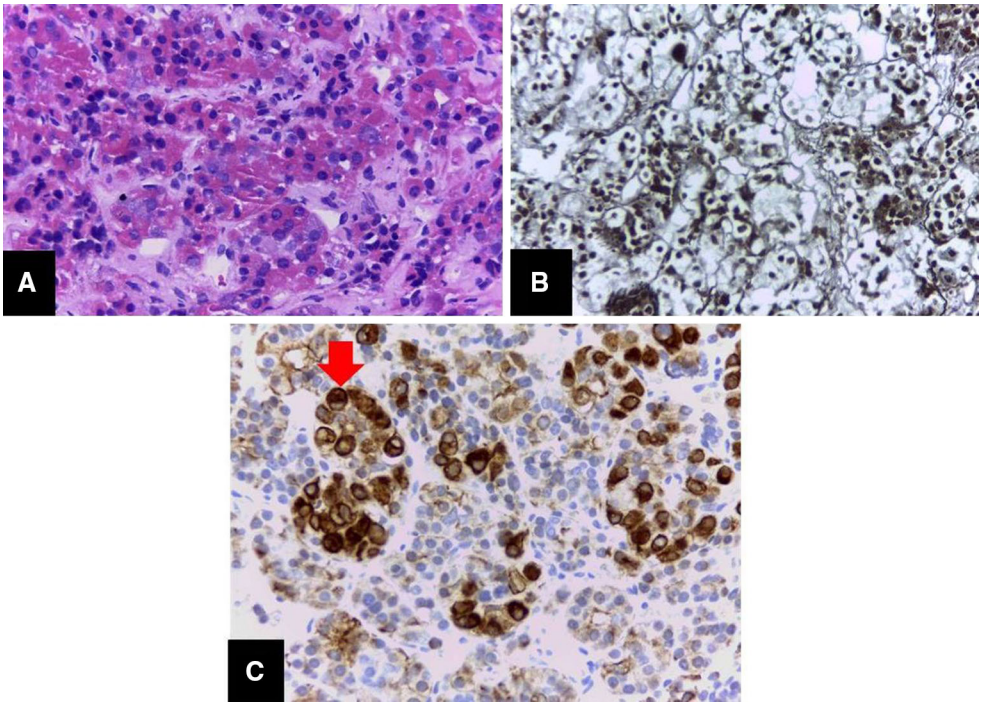

Fig. 2 Normal histology of the anterior pituitary gland. **a** Different cell types, eosinophilic, basophilic and cromophobic are demonstrated with hematoxylin—eosin stain. **b** The normal acinar reticular pattern is seen with reticulin stain. **c** Crooke's cells. Massive accumulation of perinuclear cytokeratin occurring in corticotrophs under the effect of glucocorticoid excess are demonstrated with low molecular weight keratin (LMWK) immunostaining as a strong ring-like pattern around the nucleus (*red arrow*)

immunostaining, which displays a strong ring-like pattern around the nucleus. Accumulation of these cytokeratin filaments causes a glassy hyaline appearance to the cytoplasm and displacement of PAS and ACTH-positive granules to the cell periphery. The identification of these cells in the normal pituitary tissue resected in patients with Cushing's disease confirms the state of hypercortisolemia. If Crooke's cells are present, the tumor may be adjacent in the pituitary gland, an ectopic ACTH-producing tumor may be the cause, or an adrenal lesion the source of the hypercortisolism. In cases of pseudo-Cushing, Crooke's cells will not be present in the tissue.

Crooke's cell adenoma

Earlier, Crooke's hyaline change was believed to occur only in non-tumorous corticotrophs, but recent evidence has conclusively demonstrated that massive hyaline changes are present in the cells of some ACTH-producing adenomas. These are identical to the Crooke's cells seen in the adenohypophysis of patients [28] with glucocorticoid excess described above (Fig. 3a–d). If this change affects more than 50 % of the cells, tumors are considered Crooke's cell adenomas (Table 1) [26, 28]. These types of tumors usually exhibit an aggressive clinical course, in terms of rate of recurrence and invasiveness. Crooke's cell tumors can produce ACTH causing Cushing's disease or can be endocrinologically inactive. The reason why the cells of Crooke's cell adenomas produce ACTH and at the same time display Crooke's hyaline changes as a response to elevated glucocorticoid excess is not well understood. In some cases, they present as silent ACTH tumors at the beginning but later change to functional tumors with hypercortisolism [29]. Due to their rarity, no clear indications exist in regards to their prognosis or optimal treatment.

Pituitary carcinoma

Pituitary carcinomas are defined as tumors arising in the pituitary gland exhibiting cerebrospinal and/or systemic metastasis (Table 1). They are rare, accounting for only 0.1–0.2 % of pituitary tumors. They are predominantly PRL or ACTH secreting tumors. Pituitary carcinomas develop mainly from invasive, relapsing adenomas [29, 30]. Morphologic and ultrastructural features of pituitary carcinomas are not distinctive from that of typical or atypical adenomas and the only difference is the presence of metastases, although not all cases show cytological features of malignancy.

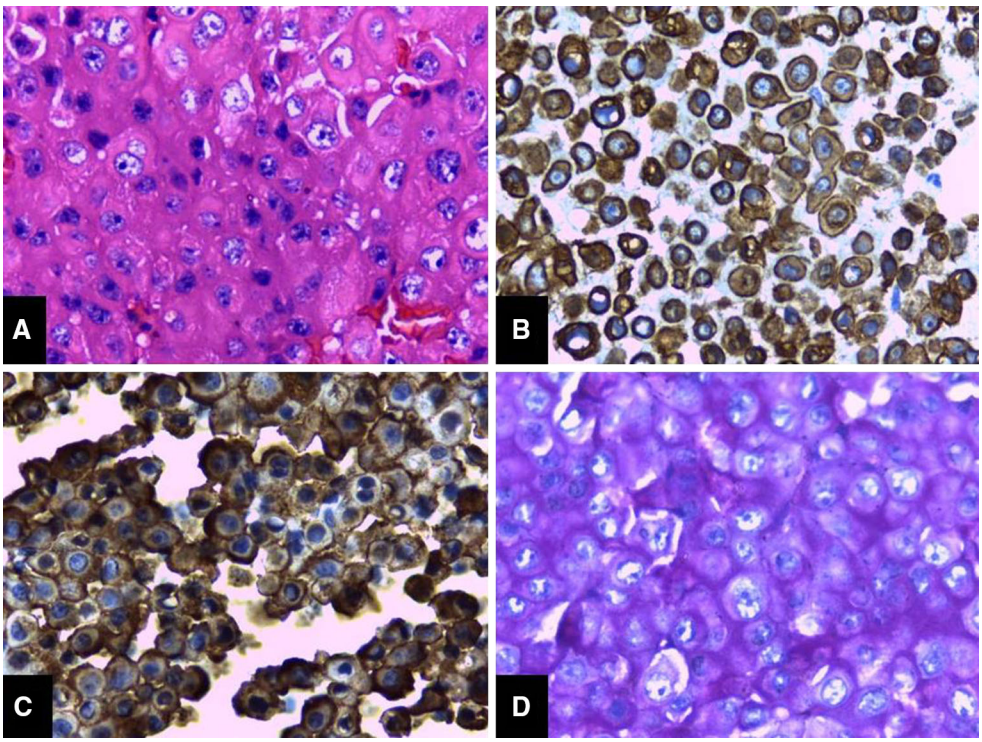

Fig. 3 Crooke's cell adenoma. **a** Hematoxylin eosin (H&E) staining, which is characterized by cells with massive hyaline changes present in the majority of the cells, identical to the Crooke's cells seen in the adenohypophysis of patients with glucocorticoid excess. **b** Low molecular weight keratin (LMWK) immunostaining displays a strong ring-like pattern around the nucleus. **c** Immunohistochemistry (IHC) for ACTH shows the cytoplasmic immunoreactivity of the secretory granules displaced to the periphery of the cell. **d** Periodic Acid-Schiff (PAS) stain. The cells show peripheral positivity with PAS staining

Pituitary hyperplasia

Non-neoplastic increase in the number of adenohypophysial corticotrophs, called corticotroph hyperplasia, may be due to reduced negative feedback effect of glucocorticoids or for the increased exposure to corticotropin-releasing hormone (CRH), a hypothalamic hormone which stimulates ACTH release. Corticotroph hyperplasia can be demonstrated in the pituitaries of patients with primary hypocorticism (Addison's disease) or enhanced secretion of CRH from a CRH producing extra-pituitary tumor. It can be focal or diffuse and localized to the anterior lobe corticotrophs. Basophil invasion in the posterior lobe, increase in the number of pars intermedia corticotrophs do not cause increased ACTH release. In autopsy obtained pituitaries of older patients, corticotroph hyperplasia may be demonstrated. This is an obscure finding and is not associated with increased ACTH release and Cushing's syndrome. In exceptional cases, corticotroph hyperplasia was found in the pituitaries of patients with Cushing's disease. Corticotroph hyperplasia is the probable cause of Cushing's disease in these patients.

Persistent hypercortisolism after surgery

Treating Cushing's disease remains a challenge. If pituitary surgery is chosen as a first line therapeutic approach, the post-operative status should be analyzed. If immediate post-operative biochemical status reveals persistent hypercortisolism, careful analysis between disciplines should be given to distinguish between different situations and their possible prognostic and therapeutic implications [2]. In every case, clinical and pathological data must be carefully considered to determine follow-up therapy (Table 2).

If pathological evaluation of the resected tissue reveals an ACTH—producing pituitary adenoma, several explanations must be systematically considered. A new post-operative MRI may clarify whether there was incomplete resection of the adenoma due to technical difficulties or if an ectopic adenoma is present above the sella or is invading the cavernous sinus. Such information is important for the neurosurgeon to determine the next course of therapy which may include a second surgery. If a second surgery is not feasible, the diagnosis of Cushing's disease is confirmed as the cause of hypercortisolism and the

Table 1 Immunohistochemical and electron microscopic criteria diagnosing pituitary tumors associated with Cushing's disease

Type of tumor	Immunohistochemical characteristics	Cam5.2 (LMWK)	Electron microscopy
Densely granulated corticotroph adenoma	Basophilic or amphophilic PAS positive ACTH diffusely positive Tpit positive	Strong, diffuse	Densely granulated Perinuclear keratin
Sparsely granulated corticotroph adenoma	Chromophobic PAS faintly positive Focal and weak ACTH positivity Tpit positive	Strong, diffuse	Sparsely granulated
Crooke's cell adenoma	Chromophobic or acidophilic Crooke's hyaline changes in more than 50 % of the cells ACTH positive at the cell periphery Tpit positive	Intense ring-like pattern	Dense perinuclear keratin
Typical adenoma	The majority of pituitary adenomas. They are typical with monotonous histological features and Ki-67 <3 %		
Atypical adenoma	According to the WHO, they disclose elevated mitotic index, a Ki-67 labeling index >3 % and nuclear staining for the p53 protein		
Pituitary carcinoma	By definition, tumors exhibiting cerebrospinal and/or systemic metastasis		

LMWK low molecular weight keratin, *PAS* periodic acid-schiff, *ACTH* adrenocorticotropic hormone; *Tpit* T-box transcription factor, *WHO* World Health Organization

References [9–12, 15]

Table 2 Possible causes of persistent postoperative hypercortisolism in relation to surgical findings in Cushing's disease

ACTH-producing pituitary adenoma
Tumor was not completely resected
Tumor located above the sella, the cavernous sinus or in the pituitary stalk
Normal pituitary gland with Crooke's cells
Tumor has been left behind by the surgeon
Misdiagnosis of Cushing's disease in a patient with Cushing's syndrome
Ectopic ACTH tumor
Adrenal source of hypercortisolism
Factitious Cushing syndrome
Normal pituitary gland without Crooke's cells
Peudo—Cushing state
Pituitary hyperplasia
Possible CRH producing tumor

ACTH adrenocorticotropic hormone, *CRH* corticotropin-releasing hormone

second line of therapy is started, directed to the pituitary source of ACTH. This is an important point that avoids further diagnostic testing.

Alternatively, if there is no evidence of an ACTH—producing adenoma at pathology, and normal pituitary gland is found, tissue must be analyzed to determine the presence of any Crooke's cell change in it. Its presence confirms the state of previous hypercortisolism. In this case the persistent postoperative hypercortisolism may have different explanations and is the worst scenario which clinicians are faced with. It may result from a small adenoma that was not visible and was left behind by the surgeon. Cushing's disease may also be misdiagnosed in cases whereby the exogenous sources of ACTH or cortisol are not located. Factitious Cushing syndrome is extremely rare but may mimic endogenous ACTH—dependent hypercortisolism during initial evaluation [31]. At this point a complete re-evaluation of the patient must be accomplished and a retrospective investigation of the evidence that led to the diagnosis of Cushing's disease must be analyzed and if necessary, all diagnostic testing should be repeated.

If Crooke's cell changes are not confirmed, a more plausible explanation is pseudo—Cushing in which no further testing is needed and an adequate therapy may be established [32].

If pituitary hyperplasia is found, a CRH producing tumor must be ruled out. There have been reports of ACTH—producing tumors which co-secrete corticotropin releasing hormone (CRH) along with ACTH. If pituitary hyperplasia is found, it is important to include CRH syndrome in the differential diagnosis of Cushing's disease [33].

Conclusion

Surgical pathology findings in Cushing's disease are of crucial importance to make a judicious clinical diagnosis

after surgery, especially in cases when biochemical remission is not achieved. In some instances the diagnosis of Cushing's disease must be systematically re-evaluated and an appropriate second line therapy initiated after a rational explanation of the incomplete biochemical remission of the hypercortisolism. Pituitary surgery is generally considered a first-line treatment since it can provide immediate relief from excess hormone secretion and decompression of adjacent neural structures. In cases of persistent hypercortisolism, obtaining a sample of tissue that is representative and is adequate in quality and quantity for proper pathological evaluation is critical to provide personalized care to patients with Cushing's disease.

Acknowledgments Authors are grateful to the Jarislowsky and Lloyd Carr-Harris Foundations for their generous support.

Conflict of interest The authors wish to declare that they have no conflict of interest and they have not received any financial compensation for this work.

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