


Genetics of Cushing's disease: an update

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Abstract

Introduction Cushing's disease (CD) results from uncontrolled hypercortisolism induced by ACTH-secreting corticotroph adenomas; accordingly, patients diagnosed with CD usually present several comorbidities and an increased risk of mortality. Hypothesis-driven screenings have led to identification of rare alterations in a low number of patients, although the genetic basis underlying CD has remained unclear until recently. Using whole-exome sequencing, recurrent mutations have been reported in the gene coding for the ubiquitin-specific protease 8 (*USP8*), a protein with deubiquitinase (DUB) activity that modulates the lysosomal turnover of the EGF receptor (EGFR) and other membrane proteins.

Methods In this review, we summarize the recent genetic findings and discuss the clinical and pathological implications of *USP8* deregulation in corticotroph adenomas.

Conclusions Mutations in *USP8* have been identified in 35–62 % of functional sporadic corticotroph adenomas causing Cushing's disease, but not in any other type of pituitary tumor. These mutations are found mostly in adult female patients and lead to an aberrant DUB activation by impairing the regulation of *USP8* by members of the 14-3-3 family of proteins. The consequence of this hyperactivation is a longer retention of EGFR at the plasma membrane which promotes an enhanced production of ACTH.

Keywords Cushing's disease · Pituitary tumor · *USP8* · EGFR · ACTH

Abbreviations

CD	Cushing's disease
ACTH	Adrenocorticotrophic hormone
POMC	Pro-opiomelanocortin
USP8	Ubiquitin-specific protease 8
DUB	Deubiquitinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

Introduction

Endogenous Cushing's syndrome is the consequence of the chronic exposure to high levels of cortisol produced by the adrenal glands. Most cases of Cushing's syndrome are caused by hypersecretion of adrenocorticotrophic hormone (ACTH) from corticotroph adenomas of the anterior pituitary, known as Cushing's disease (CD). Thus, the clinical manifestations of CD correspond to the typical signs and symptoms of hypercortisolism, such as accumulation of central fat, moon face, muscle weakness, skin lesions, bone fractures, impairment of immune function and the development of hyperlipidemia, hypertension and diabetes. When untreated, patients with Cushing's syndrome have an increased risk of mortality, mainly due to their susceptibility to infections, cardiovascular failures and stroke [1, 2].

Pituitary tumors are relatively common lesions, as revealed by epidemiologic studies based on imaging techniques and autopsy examinations [3], despite most of them are silent and clinically irrelevant. Corticotroph adenomas account for ~10 % of them, although the prevalence might be higher due to the difficulties in the detection that

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frequently implies a delay in the diagnosis of CD. The incidence of Cushing's disease varies with sex and age. Female patients are three to four times more likely to develop CD; however, the sex ratio is equal in children, with a male preponderance under the age of ten [4, 5] that turns into a female preponderance during the adolescence and becomes much more accentuated in the adulthood [6, 7].

Genetics of Cushing's disease

Pituitary adenomas are considered benign lesions, most of them sporadic, that arise from the clonal expansion of a single cell containing one or few mutations that confer particular adaptive advantages [8–10]. Based on that premise, multiple efforts have been done to identify causative mutations in pituitary adenomas, including functional ACTH-producing corticotroph tumors.

Screening for known mutations associated with other endocrine pathologies and cancers has been useful to detect uncommon variants of CD but was unsuccessful for identifying recurrent mutations. Rarely, CD can appear as part of a familial disorder, such as the multiple endocrine neoplasia type 1, an autosomal dominant disease caused by germline inactivating mutations in the gene *MEN1*. Pituitary adenomas occur in ~40 % of cases and represent predominantly prolactinomas; corticotroph adenomas, conversely, are very infrequent in this context. In a cohort of 78 pediatric patients with CD, three cases with history of familial disease (two harboring *MEN1* mutations and one with *TSC2* mutations) were positive for germline mutations, but none of the sporadic cases [11]. Regarding the related syndrome of *MEN4*, a very rare condition caused by mutations in *CDKN1B*, so far only one patient with ACTH-producing corticotroph adenoma has been reported [12]. Another candidate gene has been the aryl hydrocarbon receptor interacting protein *AIP*, which has been found mutated in ~20 % of familial isolated pituitary adenomas predisposing to acromegaly and gigantism. In pituitary adenomas with sporadic origin, however, *AIP* mutations are rare and do not seem to be involved in CD [13]. In a large, prospective study including 443 patients with sporadic pituitary adenomas and without familial history, *AIP* germline mutations were found only in 3.6 % of all cases including three of 44 patients with CD, one pediatric male and two adult female patients [14]. Similarly, 3 % of mutations in *AIP* have been described in another study with 127 sporadic patients, but none of them had a corticotroph adenoma causing CD [15].

Somatic mosaicism for gain-of-function of the $G\alpha$ subunit *GNAS* is associated with McCune–Albright syndrome. Several recurrent mutations in *GNAS1* have been found in ~40 % of sporadic somatotroph adenomas and occasionally other phenotypes, but so far only two patients from a series of 32 corticotroph adenomas had a somatic mutation

in the *GNAS1* gene [16]. In addition, other rare germline mutations found in CD include succinate dehydrogenase subunit [17], *DAX-1* mutations in the context of X-linked congenital adrenal hypoplasia [18] and *DICER-1* mutations in children presenting with pituitary blastoma [19]. Rare somatic events have been found in one patient with Nelson's syndrome and *TP53* mutations [20] and glucocorticoid receptor [21]. *PRKARIA*, *PDE11A*, *PDE8B* or the recently described *PRKACA* have been screened in corticotroph adenomas but no mutation has been found.

In summary, the identification of recurrent causative mutations has been unsuccessful for the vast majority of corticotroph tumors with sporadic origin and the genetic basis underlying CD has remained elusive for a long time.

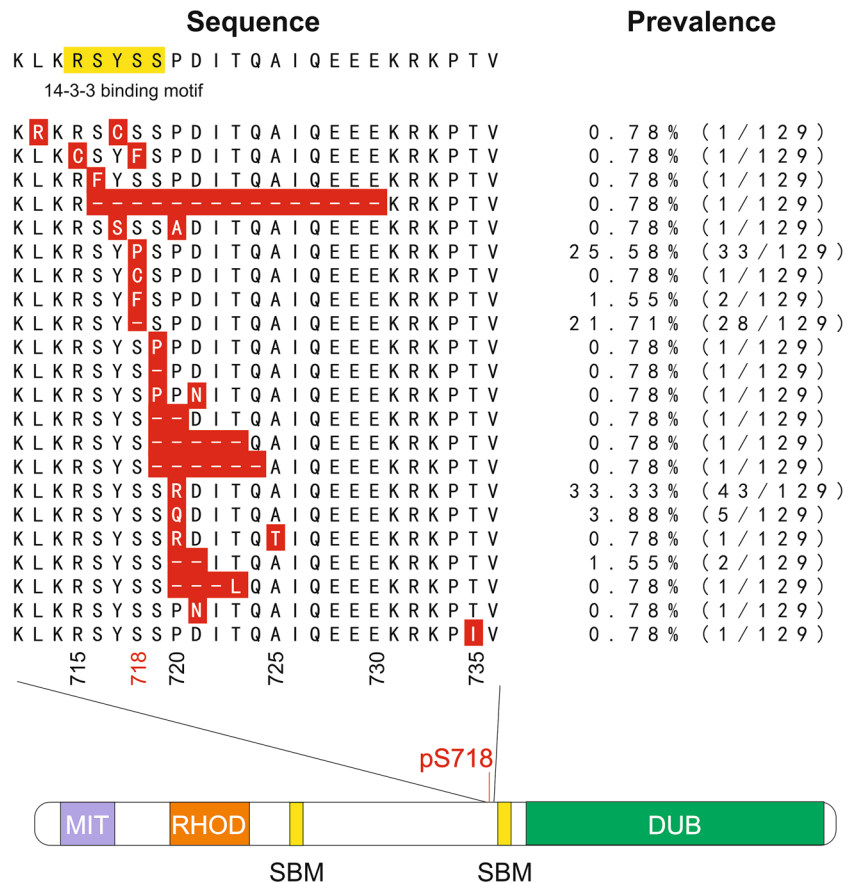
USP8 is recurrently mutated in Cushing's disease

Next-generation sequencing technologies are very useful tools to decipher the genetic events driving multiple diseases; for example, we have taken advantage of whole-exome sequencing to detect causative genetic alterations in different endocrine diseases, such as primary aldosteronism [22] or Cushing's syndrome produced by sporadic adrenal adenomas [23]. Using this approach, we have search for somatic mutations in corticotroph adenomas from patients with CD and we have identified recurrent somatic mutations in the gene encoding the ubiquitin-specific protease 8 (*USP8*) in four in an initial set of ten tumors. These mutations were validated in a small group of seven patients, with a final prevalence of 35 % (6/17 tumors) [24]. Of note, all of them were located in exon 14, defining a hotspot region that overlaps with the sequence that codes for the 14-3-3 binding motif, highly conserved between different species [24].

Subsequently, two different retrospective studies have analyzed the prevalence of *USP8* mutations in corticotroph adenomas in two large cohorts of patients with CD [25, 26]. We have designed a multicentric study to analyze the rate of *USP8* mutations including 134 patients with CD from seven different participating centers in Europe, Brazil and the United States. Similar to our first report, we have found a prevalence of 36 % (48/134) [25]. Ma and coworkers have reported a higher frequency of mutations using exome sequencing (8/12 corticotroph adenomas, 67 %) and Sanger sequencing in 108 additional adenomas from Chinese patients with CD, with a total prevalence of 62 % [26]. In addition to possible effects of ethnic diversity in the genetic background, more restrictive diagnostic and inclusion criteria could be an explanation for this difference.

Together with the high prevalence of *USP8* alterations, we should consider that (a) the average number of non-synonymous mutations per case was low in both exome-sequencing studies (median 7, range 3–23, reported in

Fig. 1 Mutations in USP8 identified in corticotroph adenomas. Diagram showing the hotspot region including the changes of the different mutations so far identified in the amino acid sequence (*white letters over red background*) in the context of the complete protein. On the *right*, the overall mutation frequency of the three series is shown. *Yellow box* amino acid sequence of the 14-3-3 binding motif. *MIT* microtubule-interacting and trafficking domain, *RHOD* rhodanese-like domain, *SBM* SH3-binding motif, *DUB* deubiquitinase catalytic domain



Reincke et al.; and median 5, range 1–9, reported in Ma et al.), (b) mutations in genes previously associated with adrenal Cushing’s syndrome were not identified, (c) recurrent mutations were detected only in *USP8*, (d) excluding synonymous changes, *USP8* was the only mutated gene in some adenomas [26], and (e) changes in *USP8* were absent in 11 silent (non-secreting) corticotroph adenomas [25] as well as 36 [24] and 150 [26] samples of other types of pituitary adenomas. Altogether, these observations strongly support the evidence that *USP8* plays a starring role in the development of the CD.

In total, 22 different *USP8* mutations have been reported in 129 functional corticotroph adenomas of 271 patients in these three studies (summarized in Fig. 1), comprising eight single missense, eight deletions and six different double mutations. All of them were in frame mutations found in heterozygosis and located in the exon 14, either in the hotspot previously described or very close to it. The analysis of available paired blood samples revealed that mutations were somatic. Strikingly, 94.5% (122/129) of all the events targeted only two residues recurrently, Ser718 and Pro720, with two substitutions (p.Pro720Arg and p.Ser718Pro) and one deletion (p.Ser718del) accounting for 85.2% of them (104/122).

The molecular effects as well as the clinical and pathologic implications of these mutations will be discussed in the next sections.

How does *USP8* promote Cushing’s disease?

USP8 regulates the recycling of EGFR

The gene *USP8* codes for an enzyme of ~130 kDa with deubiquitinase (DUB) activity, i.e., cleaves ubiquitin peptides from target proteins. This activity is tightly modulated by members of the 14-3-3 family of proteins [27], highly conserved proteins involved in many intracellular processes. The 14-3-3 binding motif RSYpSSP present in *USP8*—where pS is a phosphorylated serine corresponding to Ser718 in humans and Ser680 in mice—is critical for the recognition and interaction between both proteins. Phosphorylation of this residue enables binding and retains *USP8* inactive in the cytosol. On the contrary, its dephosphorylation leads to the release of 14-3-3 and the consequent activation of *USP8* [27]. Similarly, mutation of the 14-3-3 binding motif reduces or even abolishes the interaction between *USP8* and 14-3-3.

USP8 was originally postulated as a regulator of cell growth because its expression increases upon serum stimulation and the depletion of USP8 activity results in embryonic lethality in knock-out mice [28, 29]. Indeed, the pSer680 remains phosphorylated during interphase in mice cells and undergoes dephosphorylation in the M phase [27]. But the best known function of USP8 is the regulation of endosome sorting of different membrane proteins, mainly tyrosine-kinase receptors (RTKs). Several substrates of USP8 have been identified, including Nrdp1, c-Met, HER2 and the epidermal growth factor receptor (EGFR) [29–32]. EGFR and other RTKs are subject to fine negative regulatory mechanisms to prevent sustained activation that could lead to potentially damaging consequences [33]. Thus, in addition to trigger receptor activation, ligand binding induces EGFR internalization and polyubiquitination, a post-translational modification that targets the receptor to be degraded in the lysosome [34]. Conversely, USP8-mediated deubiquitination abrogates this process and directs EGFR back to the plasma membrane, where it can further contribute to signal transduction [35]. When hyperactivation of USP8 occurs by overexpression or mutation, ligand-induced degradation of EGFR is delayed and residence of potentially active receptor in the plasma membrane is prolonged [35].

EGFR promotes ACTH synthesis in corticotroph cells

Because EGFR is involved in cell growth and proliferation, and because uncontrolled EGFR activation is associated with different malignancies, several authors have investigated the presence of EGFR in normal pituitary gland, adenomas and pituitary carcinomas with the aim of finding a causative link between EGFR expression and tumorigenesis in the pituitary. EGFR is expressed in the normal pituitary, although at low levels [36–38]. Likewise, EGFR has been detected in ~60 % of adenomas at either the mRNA or the protein level. Its expression is not restricted to any specific tumor type and a variable immunoreactivity has been observed within each type [38] with the highest expression in corticotroph adenomas [37].

Evidence confirms the presence and functionality of EGFR in corticotroph cells. For example, sustained stimulation by EGF—that can be also be detected in ACTH-producing adenomas—induces the replication of corticotroph and mammothroph cells in primary cultures of mouse pituitary [39]. IHC staining using two antibodies against intracellular and extracellular epitopes of EGFR revealed that this receptor presents a moderate-to-high expression in corticotroph adenomas that also co-localizes with ACTH [37]. Moreover, Fukouka et al. demonstrated that EGFR can enhance POMC expression (the precursor of ACTH) and ACTH secretion in corticotroph tumors using canine and murine models. Likewise, treatment with gefitinib

abolished EGFR signaling and blocked POMC expression in corticotroph cells, reduced corticotroph tumor mass and reversed the signs of hypercortisolism in mice [40].

Completing the puzzle: USP8 increases ACTH synthesis in an EGFR-depending fashion

Functional assays have provided solid evidences on how mutated USP8 can be involved in the development of CD

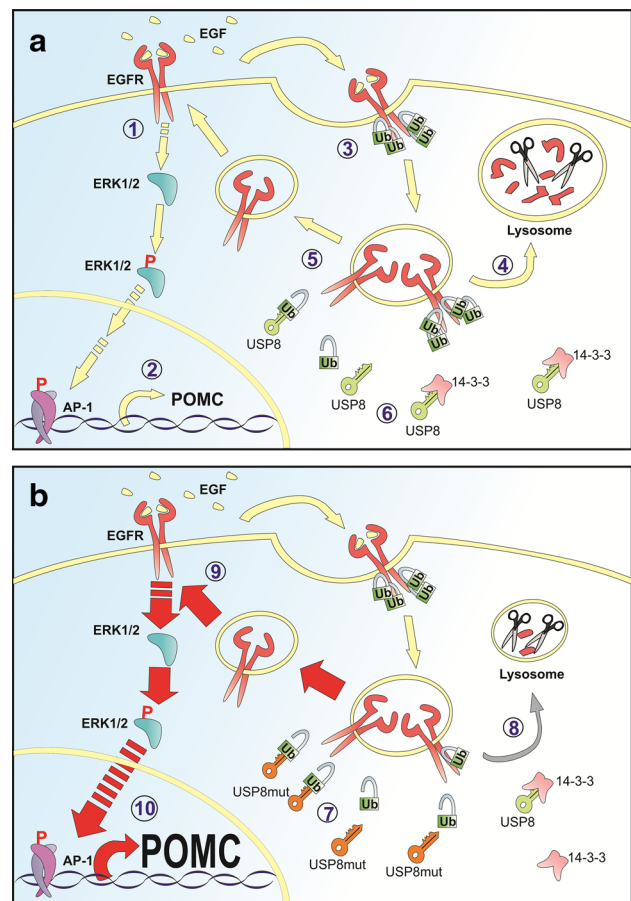


Fig. 2 USP8 modulates EGFR signaling and ACTH production. **a** In normal corticotroph cells, the stimulation of EGFR signaling (1) by EGFR ligands (such as EGF) promotes Erk1/2-mediated activation of transcription factors and the subsequent transcription of POMC (2), the precursor of ACTH. After activation, EGFR is internalized in endosome vesicles and covalently linked to ubiquitin chains (3); this ubiquitination targets EGFR to lysosomal degradation (4). USP8 regulates EGFR turnover by removing the chains of ubiquitin from the receptor; then, untargeted EGFR turns back to the plasma membrane via recycling endosomes (5). USP8 activity is controlled by the interaction with 14-3-3 proteins, which retain USP8 in an inactive state that is reversible (6). **b** Mutations found in corticotroph adenomas affect the binding of USP8 to 14-3-3. Thus, mutant USP8 proteins are constitutively active and reduce the amount of ubiquitin-ligated EGFR in the endosomes (7). Consequently, EGFR degradation is impaired (8) and the receptor is accumulated in the plasma membrane (9), increasing signal transduction and POMC transcription (10)

[24–26]. As mentioned before, all the mutations detected so far in the sequence of *USP8* by our group and by Ma and coworkers clustered in a hotspot region containing the highly conserved 14-3-3 binding motif (Fig. 2). Consequently, these mutants exhibit a reduced interaction with 14-3-3 proteins and higher DUB activity than the wild-type form, although at different degrees [24–26]. Likewise, EGFR deubiquitination is increased in cells expressing *USP8* mutants, resulting in recycling of activated EGFR from early endosomes and the accumulation of the receptor in the plasma membrane upon stimulation by EGF [24–26].

The mechanism underlying EGFR-dependent *POMC* transcription has been suggested by Fukuoka et al. and further supported by Ma et al. and our data. The stabilization of the receptor in the plasma membrane enhances the EGFR-dependent signal transduction in the presence of EGF, leading to the activation of downstream kinases, such as Erk1/2 and Akt. Experiments with kinase inhibitors have shown that *POMC* transcription is triggered by Erk1/2-mediated activation of transcription factors that bind to the AP-1 response element on the *POMC* promoter [24, 40]. The final evidence linking ACTH secretion to *USP8* activity has been provided by experiments in primary cultures of human corticotroph cells. Silencing *USP8* expression using shRNA significantly reduced the amount of secreted ACTH in cells originated from adenomas with mutated *USP8* but not in those from a wild-type adenoma [26]. In the same line, ACTH secretion is reduced after treatment with gefitinib only in corticotroph cells from mutated human adenomas and not from those with wild-type *USP8* [26]. These experiments demonstrate the dependence of corticotroph adenomas cells on *USP8* hyperactivation via EGFR signaling and might provide a rationale for personalized treatment strategies for these patients in the future.

From genotype to phenotype: clinical and pathologic implications

The development of CD is the logical consequence of sustained EGFR activation and ACTH production in the context of activating *USP8* mutations in corticotroph cells. To better understand the pathophysiological effects of these mutations, we will summarize the most relevant associations reported in the three series of patients published so far. Nevertheless, one should be aware that the retrospective nature of the studies, the lack of complete registries of pathologic and hormonal data, the differences in the diagnostic protocols and postoperative management, and intrinsic features of the study cohorts constitute important limitations.

Some observations are strongly consistent among the three publications. For example, the frequency of mutations in corticotroph adenomas is surprisingly high in all series.

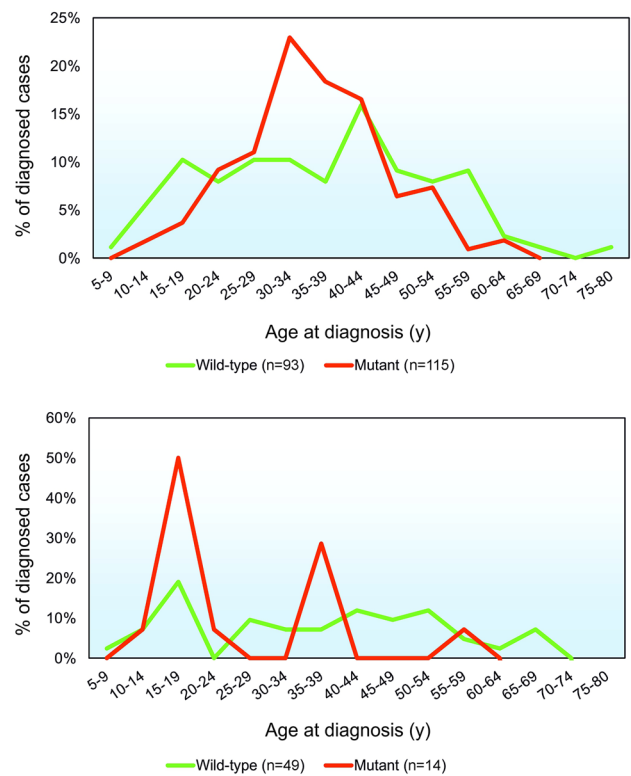


Fig. 3 Sex and age prevalence of *USP8* mutations in patients diagnosed with CD. Distribution of cases according to sex (females, *top graph*; males, *bottom graph*) and age at diagnosis. Data are represented as % of cases diagnosed in each category in 5-year intervals and have been obtained from Perez-Rivas et al. [25] and the supplementary tables of Reincke et al. [24] and Ma et al. [26]

Moreover, these mutations have only been detected in functional ACTH-producing corticotroph adenomas, but not in any other pituitary tumor (in total, $n = 197$), including silent corticotroph adenomas [24–26]. These facts clearly demonstrate the relevance of *USP8* mutations, which constitute a specific trait of CD.

Patients who develop tumors with *USP8* mutations are mainly female. The analysis of the data from the three series shows that the risk of having an *USP8*-mutant adenoma is two times higher in females: 55.3 % (115/208) in female vs. 22.2 % (14/63) in male patients. In addition, most mutations in females were found at the ages of 20–40 years old, while tumors in children and adults diagnosed >50 years were mostly wild type (Fig. 3). It is tempting to speculate that tumors harboring *USP8* mutations could be more sensitive to the effect of sex hormones, but this aspect requires further research.

Average tumor size in each series was different and it is not clear whether adenomas with mutations in *USP8* are smaller or larger than those with wild-type sequence. In our series, tumors in females with *USP8* mutations were larger than their wild-type counterpart (8 vs. 10 mm, respectively)

[25], while Ma and coworkers showed a predominance of mutations in smaller adenomas (<5 mm) [26]. However, large tumors (>20 mm) in both series were found mostly wild type, suggesting that USP8 hyperactivation influences hormonal production rather than tumor growth. In line with this hypothesis, we have observed that the proliferative effects of USP8 in AtT-20 mouse cells coexpressing EGFR are not further enhanced by the mutant proteins [24]. Even though the involvement of USP8 in this process is apparent—cell growth is increased after USP8 transfection in AtT-20 expressing EGFR, and the lack of USP8 activity leads to cell cycle arrest and a strong reduction of EGFR levels [24, 28, 29]—the indirect upregulation of EGFR signaling by altering a modulator protein might result in less mitogenic activity than direct alterations of the receptor by either overexpression or aggressive EGFR-activating mutations commonly found in other types of tumors. This hypothesis could feasibly explain the low mitotic index of most corticotroph adenomas despite exhibiting aberrant EGFR signaling, and the lack of oncogenic mutations in these tumors, including those affecting genes downstream to EGFR, usually related to a malignant phenotype.

Regarding hormonal features, few associations have been reported and none of them has been corroborated by the other studies. Basal plasma ACTH and morning serum cortisol do not seem to correlate with USP8 mutations, even though we found lower levels of ACTH in our discovery study [24]. Ma and coworkers have shown that USP8 tumors have a higher ratio of ACTH/tumor size, suggesting that these tumors are hormonally more active [26]. In our last study, we have reported that postoperative 24 h urinary-free cortisol levels are higher in patients that had adenomas with mutations in *USP8* and are less likely to develop adrenal insufficiency than those with wild-type adenomas. Because postoperative hypocortisolemia is considered a predictive marker of long-term remission, we hypothesize that the former patients could be on a higher risk of recurrence [25]. However, all these observations still need to be validated by further prospective, controlled studies.

Conclusions

In summary, recurrent somatic hotspot mutations in the gene of the ubiquitin-specific protease 8 (*USP8*) are frequently observed in corticotroph adenomas causing CD, but not in any other pituitary tumor entity. Mutations usually appear in young adult females and only occasionally in men, but further studies are needed to clarify their clinical implication. All the mutations described so far target the 14-3-3 binding domain of USP8 and generate proteins with constitutive DUB activity leading to EGFR stabilization in the plasma membrane. In consequence, mutant USP8 enhances EGFR

signaling, which promotes ACTH synthesis in corticotroph cells. Over time, this mechanism could lead to the development of hormonal active adenomas causing CD.

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Compliance with ethical standards

Conflict of interest The authors have nothing to disclose.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

Informed consent For this type of study formal consent is not required.

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