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Somatic candidate gene mutations in the ubiquitin system as a cause of Cushing's disease – a multicentric study

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To my dear grandmother Luise Pielmeier

IV

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1 Introduction

1.1 The pituitary gland

The pituitary gland is an endocrine organ that holds an essential role in the regulation of the hormonal balance in humans. It is a superordinate hormonal gland and important neuroendocrine interface between the central nervous system (CNS) and the peripheral endocrine system. Therefore, the pituitary gland acts as final common path for central neuronal modulation processes of the endocrine system, regulating a vast number of important homeostatic mechanisms such as metabolism, growth and reproduction (Pape, Kurtz, & Silbernagl, 2014).

Those two characteristics are reflected in its anatomical structure as well as in its integration into the hierarchical structured regulatory circuits of the hormonal system. A unique anatomical composition is needed to ensure morphologic and functional interlinkage between nervous and hormonal mechanisms (Frotscher & Kahle, 2013).

1.1.1 Anatomy and physiology

The pituitary gland is an approximately hazelnut-sized organ that rests upon the so-called sella turcica of the sphenoid bone and is located immediately adjacent to the hypothalamus. Based on its embryonic origin, it can be subdivided into two functionally different parts, the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary).

The anterior pituitary derives from the Rathke's pouch during embryological development and comprises about three quarters of the total pituitary gland (Asa & Kovacs, 1984). It is a typical hormonal gland consisting of reticular tissue, ramified strands of adenocytes (gland cells) and numerous sinusoids that are connected to the portal system.

The adenohypophysis produces four glandotropic hormones (ACTH, TSH, FSH and LH) and two non-glandotropic hormones (GH and prolactin). Each hormone is produced by a different cell type.

The posterior pituitary has a neural origin. It does not synthesise hormones by itself, but they are produced in the hypothalamus. The supraoptic nucleus of the hypothalamus is responsible for the production of the antidiuretic hormone (ADH) and the paraventricular nucleus for the production of oxytocin. The secretion of both of these hormones is initiated by action potentials that trigger exocytosis and therefore the release of hormones from the posterior pituitary into the blood (Welsch, Deller, & Kummer, 2014a).

1.1.2 Pituitary tumours

Pituitary tumours are among the most common intracranial neoplasms in adults (McNeill, 2016). A distinction is made between pituitary adenomas und carcinomas. While the term “pituitary adenoma” describes a slowly growing entity, the term “pituitary carcinoma” is merely used when cerebrospinal and/or systemic metastases are detected. Compared to pituitary adenomas, primary pituitary carcinomas are very rare, representing only 0.2% in surgical series (Kontogeorgos, 2005). Recently some authors have recommended the generic term “pituitary tumour” to highlight the fact that many of those lesions have devastating short and long-term consequences for the patient due to an active hormone secretion and, in some cases, to invasive features and a lack of response to treatments, which leads to a high disease burden and a substantially diminished quality of life (Asa et al., 2017; Ho et al., 2019).

As revealed by epidemiologic studies based on histologic examinations of autopsy specimens and imaging techniques, pituitary adenomas are frequently encountered, mostly benign, intracranial neoplasms and the most common cause of pituitary hormone hypersecretion and hyposecretion syndromes (functioning tumours), even though most of them are silent and clinically irrelevant (non-functioning tumours) (Ezzat et al., 2004). The 2017 WHO classification classifies pituitary tumours based on their adenohypophyseal cell lineage (Lopes, 2017). Moreover, a distinction can be made between hormonally active pituitary tumours and hormonally inactive ones, so-called incidentalomas (Paschou, Vryonidou, & Goulis, 2016; Scangas & Laws, 2014). Hormonally active pituitary tumours are characterized by an autonomous hormone secretion and the overstimulation of the peripheral target organ (Melmed, 2011). Functioning pituitary tumours arise

from one of the five cell types of the anterior pituitary. Accordingly, there are tumours arising from corticotroph (ACTH), lactotroph (PRL), somatotroph (GH), thyrotroph (TSH) or gonadotroph (LH, FSH) cells that hypersecrete their respective hormones. The clinical phenotype depends on the cell type from which they are derived. Additionally, plurihormonal tumours that express combinations of ACTH, PRL, GH and/or TSH can be found (Mete & Lopes, 2017; Saeger et al., 2007).

Clinical manifestations stretch from signs caused by excessive hormone secretion to symptoms related to tumour mass expansion, which results in the compression and decreased function of surrounding structures, such as the normal pituitary gland, the optic chiasm or cranial nerves (Arafah & Nasrallah, 2001).

According to their maximal diameter size, pituitary tumours are traditionally classified as microadenomas (<10 mm) and macroadenomas (≥10 mm). Despite the fact that the size cut-off is arbitrary, microadenomas do not extend beyond the sella turcica, while macroadenomas are often associated with extrasellar extension and a worse disease outcome. Such tumours frequently extend into the sphenoid sinus or into the suprasellar space, compressing the optic chiasm, or grow laterally into the cavernous sinuses on either side (Asa & Ezzat, 2002; Ezzat et al., 2004; Gsponer et al., 1999).

1.1.3 The hypothalamic–pituitary–adrenal (HPA) axis

The HPA axis is a complex set of feedback loops regulating the secretion of hormones among three endocrine glands: the hypothalamus, the pituitary gland and the adrenal glands. Three hormones act as the primary signals of the HPA axis: corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and the glucocorticoid hormone cortisol. A functioning HPA axis is the foundation of an adequate and dynamic regulation of the stress hormone cortisol, which plays an important role in a number of metabolic processes and also in states of stress, such as physical work, emotional distress or illness (Bonfiglio et al., 2011; S. M. Smith & Vale, 2006; Spencer & Deak, 2017; Tsigos & Chrousos, 2002).

The HPA axis is a modular, closed-loop circuit with a negative feedback effect exerted by cortisol blood levels [Figure 1] (J. P. Herman, McKlveen, Solomon, Carvalho-Netto, & Myers, 2012; M. Keller-Wood, 2015; Watts, 2005).

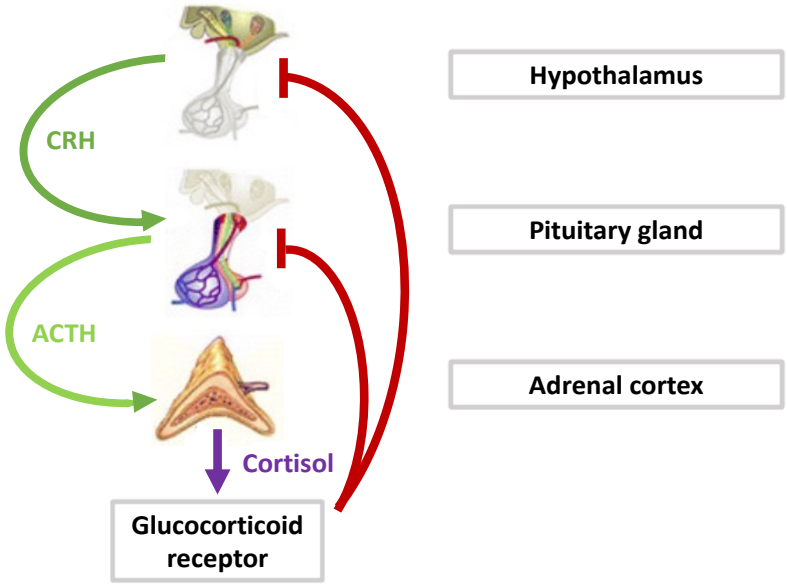


Figure 1. Regulation of the hypothalamic-pituitary-adrenal axis (Eckstein, Haas, Hass, & Pfeifer, 2014).
CRH – corticotropin-releasing hormone; ACTH – adrenocorticotropic hormone;

The paraventricular nucleus of the hypothalamus comprises neuroendocrine neurons. These neuroendocrine neurons synthesize corticotropin-releasing hormone (CRH) and vasopressin (Vale, Spiess, Rivier, & Rivier, 1981). When secreted from neurosecretory nerve terminals, CRH is transported to the anterior pituitary through the portal blood vessel system of the hypophyseal stalk while vasopressin is conveyed to the posterior pituitary by axonal transport. At pituitary level, CRH and vasopressin have a synergistic effect (Antoni, 1993; Rivier & Vale, 1983; Whitnall, 1993) and stimulate the secretion of the precursor hormone of ACTH, pro-opiomelanocortin (POMC), and also have a direct influence on its synthesis by activating the *POMC* gene (Jenks, 2009). The POMC peptide is cleaved to give rise to multiple peptide hormones including ACTH (Raffin-Sanson, de Keyzer, & Bertagna, 2003). In the anterior pituitary, POMC is processed to ACTH by the prohormone convertase 1 and is stored in secretory

granules until secretion, once stimulated by CRH and vasopressin (Stevens & White, 2010). ACTH then reaches the cortex of the adrenal gland via blood where it directly stimulates the biosynthesis of corticosteroids such as cortisol from cholesterol (Arnett, Muglia, Laryea, & Muglia, 2016; Simpson & Waterman, 1988). Glucocorticoids cannot be pre-synthesized and stored in the adrenal glands because of their lipophilic nature. Thus, they have to be rapidly synthesized upon ACTH stimulation (Ramamoorthy & Cidlowski, 2016).

To inhibit further release of CRH and vasopressin and reduce the cleavage of POMC into ACTH, the feed-forward mechanism within the HPA axis is balanced by negative feedback of glucocorticoids acting at both, the anterior pituitary and the hypothalamus [Figure 1] (J. P. Herman et al., 2012; M. E. Keller-Wood & Dallman, 1984). In addition to the classic genomic effects, rapid, non-genomic feedback inhibition of hypothalamic hormone secretion can be mediated by glucocorticoids (Di, Malcher-Lopes, Halmos, & Tasker, 2003). Furthermore, modelling results suggest that a rapid autoregulation of glucocorticoid synthesis may exist within the adrenal gland itself (Spiga et al., 2017; Walker et al., 2015). As a rule, concentrations of glucocorticoids in the nanomolar range lead to genomic effects within a time frame of hours or days, while significantly higher concentration ranges provoke non-genomic effects, that occur within seconds or minutes (Stahn & Buttgereit, 2008; Stahn, Lowenberg, Hommes, & Buttgereit, 2007).

Under standard conditions, the secretion of cortisol follows a stable, circadian rhythm. In humans, serum cortisol concentrations peak during the morning hours, anticipating wakening and the activity cycle (Krieger, Allen, Rizzo, & Krieger, 1971; Lightman et al., 2000; Weitzman et al., 1971). Circadian rhythm is coordinated by outputs of a central clock in the suprachiasmatic nucleus of the hypothalamus (Maywood, O'Neill, Chesham, & Hastings, 2007; Reppert & Weaver, 2002). This rhythm overlays a more dynamic ultradian, pulsatile pattern for both ACTH and glucocorticoid secretion (Spiga, Walker, Terry, & Lightman, 2014). Ultradian rhythmicity originates in the interaction between an intrinsically oscillating pituitary-adrenal network and the release of hypothalamic hormones in a dynamic fashion. Thus, a pattern of pulses is created. These pulses vary in amplitude during the course of the day. The release of glucocorticoids into the blood in that highly dynamic fashion allows humans to anticipate regular daily

changes in their environment (Dickmeis, Weger, & Weger, 2013; Walker et al., 2012).

1.2 The glucocorticoid cortisol

The adrenal cortex comprises three functionally different zones that can be appreciated at microscopic level. Each zone can be distinguished from one another based on its structural and histological characteristics. The zona glomerulosa is the main site for the production of mineralocorticoids (mostly aldosterone), the zona fasciculata is responsible for the production of glucocorticoids (in humans primarily cortisol) and in the zona reticularis the production of androgens takes place (Welsch, Deller, & Kummer, 2014b).

The ACTH receptor (MC2R) is primarily found in the zona fasciculata. Binding of the MC2R by ACTH stimulates the production of glucocorticoids (Fridmanis, Roga, & Klovins, 2017). Glucocorticoids are derived from cholesterol. The process of converting cholesterol into biologically active steroid hormones is called steroidogenesis and requires many complex and tightly regulated steps that are catalysed by various enzymes and cofactors. Progestogens are the precursors of glucocorticoids and therefore cholesterol must first be converted into pregnenolone. This conversion is the rate-limiting step of steroid synthesis and takes place in mitochondria (John & Buckingham, 2003; Miller & Auchus, 2011).

Only approximately 5% of systemic glucocorticoids are free and bioactive. The other 95% of circulating glucocorticoids are bound either to corticosteroid binding globulin (80 – 90%) or to albumin (5 – 15%) and therefore remain inactive (Breuner & Orchinik, 2002; Ramamoorthy & Cidlowski, 2016).

The inability of humans to survive without glucocorticoids indicates the importance of glucocorticoids in physiology. Cortisol is a systemic regulator due to the wide expression of the glucocorticoid receptor (Bamberger, Schulte, & Chrousos, 1996; Gustafsson et al., 1987; Munck, Guyre, & Holbrook, 1984). It influences the metabolisms of lipids, carbohydrates and proteins and regulates diverse cellular functions including homeostasis, development and growth, cardiovascular function, immune function, inflammation and functions within

reproductive physiology and neurobiology (Charmandari, Tsigos, & Chrousos, 2005; McEwen, 2008; McEwen et al., 1997; Ramamoorthy & Cidlowski, 2016; Sapolsky, Romero, & Munck, 2000). Cortisol stimulates gluconeogenesis in the liver and the inhibition of glucose transport and glucose utilisation (Munck et al., 1984), which lead to elevated blood sugar levels and therefore may trigger diabetes. Furthermore, cortisol triggers catabolic processes of the muscles, the lymphatic tissue, the skin and the bones (Bodine & Furlow, 2015; Pufall, 2015; Schoepe, Schacke, May, & Asadullah, 2006; Warriner & Saag, 2013). The amino acids thus released are used for gluconeogenesis in the liver. Fatty acids are released through the lipolytic activity of cortisol while it also inhibits lipogenesis by blocking the incorporation of glucose into fat cells (Gathercole et al., 2011; Geer, Islam, & Buettner, 2014).

Glucocorticoids repress a number of immune processes. Not only do they lead to a reduction in thymus and lymph node tissue but also reduce the number of lymphocytes and are required for immunological fitness (J. J. Cohen, 1992; Mittelstadt, Monteiro, & Ashwell, 2012; Pufall, 2015; L. K. Smith & Cidlowski, 2010). Moreover, glucocorticoids inhibit the release and effects of most cytokines, regulate granulocyte apoptosis and therefore also play a major role in the inhibition of inflammatory processes (Caramori & Adcock, 2005; Heasman et al., 2003; Rhen & Cidlowski, 2005).

Glucocorticoids also have effects on the CNS that exceed their part in the feedback mechanism of the neuroendocrine system. In addition they increase the effects of some endogenous signalling molecules, which is called ‚permissive effect' (Joels & Baram, 2009; Sapolsky et al., 2000).

1.3 Hypercortisolism – Cushing's syndrome

1.3.1 Definition

The term Cushing's syndrome refers to a collection of clinical signs and symptoms due to prolonged exposure to excessive concentrations of circulating free cortisol. One distinguishes between exogenous and endogenous Cushing's syndrome. Exogenous Cushing's syndrome is the most common form and is caused by lengthy and extensive therapeutic administration of glucocorticoids.

Endogenous Cushing's syndrome, however, results from an aberrant function of the HPA axis and can once again be divided into ACTH-dependent and ACTH-independent Cushing's syndrome (Heinrich M. Schulte & Kamphausen, 2010).

1.3.2 Endogenous causes of hypercortisolism

Endogenous Cushing's syndrome is considered a rare disorder with population-based studies showing an incidence between 0.2 – 5.0 cases per million people per year and an overall prevalence of 39 – 79 per million (Ambrosi, Bochicchio, Ferrario, Colombo, & Faglia, 1990; Bolland et al., 2011; Etxabe & Vazquez, 1994; Lindholm et al., 2001; Steffensen, Bak, Rubeck, & Jorgensen, 2010; Valassi et al., 2011). The causes of endogenous Cushing's syndrome can be either ACTH-dependent or ACTH-independent [Table 1].

ACTH-dependent causes account for 70 – 80% of cases, the vast majority of them caused by pituitary tumours (Cushing's disease), outnumbering ectopic ACTH syndrome by about seven-to-one (Arnaldi et al., 2003; Biller et al., 2008; Lacroix, Feelders, Stratakis, & Nieman, 2015; Newell-Price, Bertagna, Grossman, & Nieman, 2006). Ectopic ACTH secretion is produced by a variety of endocrine and non-endocrine tumours that are located outside the pituitary gland, most commonly small-cell lung cancer tumours or bronchial carcinoids (Isidori et al., 2006; Lacroix et al., 2015; Newell-Price et al., 2006). Rarely, an excess of ACTH secretion by the pituitary gland is caused by tumours that are able to produce CRH ectopically (Arnaldi et al., 2003; Biller et al., 2008; Lacroix et al., 2015; Newell-Price et al., 2006).

About 20 – 30% of Cushing's syndrome cases are ACTH-independent, most of them caused by a unilateral tumour: 60% of these cases are adrenal adenomas and 40% are adrenal carcinomas. Very rare causes of corticotropin-independent Cushing's syndrome include McCune-Albright syndrome, primary bilateral macronodular adrenal hyperplasia and primary bilateral micronodular adrenal hyperplasia, like primary pigmented nodular adrenocortical disease (Lacroix, 2009; Newell-Price et al., 2006; Stratakis, 2008).

Endogenous Cushing's syndrome is more frequent in women than in men, except for the ectopic ACTH syndrome, which is equally distributed between both sexes

(Lacroix et al., 2015; Lindholm et al., 2001; Newell-Price et al., 2006; Steffensen et al., 2010). However, the sex ratio of children suffering from Cushing's disease is equal, with a male preponderance under the age of ten (Libuit et al., 2015; Storr et al., 2004) contrasting with a female preponderance during the adolescence, which becomes even more considerable in adulthood (Lonser et al., 2013; Storr et al., 2011).

Aetiology	**Proportion (%)**	**Female:male ratio**
ACTH-dependent	70 – 80	
Cushing's disease	60 – 70	3 – 5 : 1
Ectopic corticotropin syndrome	5 – 10	0.6 – 1 : 1
Unknown source of ACTH*	5	5 : 1
Ectopic CRH	Very rare	–
ACTH-independent	20 – 30	
Unilateral adrenal adenoma	10 – 22	4 – 8 : 1
Unilateral adrenal carcinoma	5 – 7	1.5 – 3 : 1
Bilateral macronodular adrenal hyperplasia	<2	2 – 3 : 1
Bilateral micronodular adrenal hyperplasia	<2	0.5 – 2 : 1
McCune-Albright syndrome	Rare	1 : 1
Bilateral adenomas or carcinomas	Rare	2 – 4 : 1

Table 1. Aetiology of Cushing's syndrome.
Adapted from (Lacroix et al., 2015; Newell-Price et al., 2006).
**Patients might ultimately prove to have Cushing's disease*

1.3.3 Clinical features and comorbidities

Uncontrolled hypercortisolaemia is associated with metabolic, cognitive, psychological and cardiovascular alterations. Manifestations vary among patients and range from subclinical or mild to rapid onset, severe variants. Symptoms may also fluctuate, making Cushing's syndrome difficult to diagnose in many cases

(Arnaldi et al., 2003; Biller et al., 2008; Newell-Price et al., 2006; Nieman et al., 2008; Pappachan, Hariman, Edavalath, Waldron, & Hanna, 2017).

There are no pathognomonic signs, but the most reliable ones for distinguishing Cushing's syndrome from simple obesity are those of protein wasting – proximal myopathy, wide purple striae distensae, thin skin and easy bruising (Newell-Price, 2008; Nieman, 2015; Nieman et al., 2008). However, as Cushing's syndrome tends to progress over time, these signs might not be noted early on. As relevant features accumulate over time, diagnosis might be established more easily in advanced cases (Nieman, 2015).

Symptoms are caused by the metabolic effects of hypersecreted cortisol on the body. Table 2 summarises the most important clinical features of Cushing's syndrome. The most common symptoms and clinical signs are (in decreasing number of frequency): truncal obesity, moon face, decreased libido, facial plethora, thin skin, menstrual disorders, hirsutism, hypertension, lethargy and psychic changes, ecchymotic haemorrhages, striae rubrae distensae and proximal muscle weakness (Newell-Price, 2008; Nieman, 2015). Less common clinical symptoms include ECG abnormalities or atherosclerosis, dorsal fat pad, ankle oedemas, abnormal glucose tolerance/diabetes, osteopenia or bone fractures, kidney stones, headache, backache, recurrent infections, abdominal pain, acne, female balding, polyuria and polydipsia (Faggiano et al., 2003; Nieman, 2015).

Weight gain mostly affects only the trunk, while extremities often stay slim because of muscular atrophy (Nieman, 2015).

As glucocorticoids have a suppressive effect on the immune system, patients may suffer from frequent infections (Aucott, 1994; McEwen et al., 1997). Furthermore, hypercortisolism predisposes to abnormalities like hypertension, glucose intolerance/diabetes and other manifestations that lead to an adverse metabolic profile, putting patients at a higher cardiovascular risk, which might not fully return to normal after remission (De Leo et al., 2010; Isidori et al., 2015; Mancini, Kola, Mantero, Boscaro, & Arnaldi, 2004; Pivonello et al., 2016; Terzolo et al., 2014). Cushing's syndrome is not only associated with an increased prevalence of cardiovascular events, but also with a hypercoagulable state,

reflected in an increased incidence of venous thromboembolisms, including ischemic strokes (van der Pas, Leebeek, Hofland, de Herder, & Feelders, 2013).

Clinical feature	**Proportion (%)**
Obesity or weight gain	95 (100 in children)
Facial plethora	90
Rounded face	90
Decreased libido	90
Thin skin	85
Decreased linear growth in children	70 – 80
Menstrual irregularity	80
Hypertension	75
Hirsutism	75
Depression/emotional lability	70
Easy bruising	65
Glucose intolerance	60
Proximal myopathy	60
Osteopenia or fracture	50
Nephrolithiasis	50

Table 2. Clinical features of Cushing's syndrome.
Adapted from (Faggiano et al., 2003; Newell-Price, 2008; Pecori Giraldi, Moro, & Cavagnini, 2003; Savage, Lienhardt, et al., 2001).
The most discriminating features are presented in italics.

Neuropsychiatric manifestations have long been recognized as important symptoms of Cushing's syndrome (Starkman & Schteingart, 1981), with labile mood, irritability, depression, mania, anxiety and neurocognitive impairment being the most important clinical abnormalities. Deficits in short-term memory and cognition are common and reverse only slowly after correction of

hypercortisolaemia, due to the loss of brain volume that at least partially persists (Bourdeau et al., 2005; Forget, Lacroix, & Cohen, 2002; Pivonello, Simeoli, et al., 2015).

Clinical features differ in children, with obesity and decreased linear growth being especially evident (Davies et al., 2005; Magiakou et al., 1994; Savage, Lebrethon, et al., 2001; Savage, Lienhardt, et al., 2001; Stratakis, 2012). A difference in clinical presentation can also be seen between sexes. While muscle atrophy, purple striae distensae, osteoporosis and kidney stones are more frequent in men, no single symptom seems to be more common in female patients (Pecori Giraldi et al., 2003). Gonadal dysfunction is common in men as well as in women (Newell-Price et al., 2006).

The comorbidities associated with Cushing's syndrome contribute to impaired quality of life, which only partially resolves after treatment (Heald et al., 2004; Lindsay, Nansel, Baid, Gumowski, & Nieman, 2006; van Aken et al., 2005). Mortality is increased in Cushing's syndrome, with cardiovascular events being the most common cause of death, followed by infection/sepsis (Bolland et al., 2011; Clayton, Raskauskiene, Reulen, & Jones, 2011; Dekkers et al., 2013; Graversen, Vestergaard, Stochholm, Gravholt, & Jorgensen, 2012; Hassan-Smith et al., 2012; Lindholm et al., 2001; Ntali et al., 2013). Several studies indicate that early diagnosis, treatment that involves aggressive management of comorbidities in a multidisciplinary setting, and long-term follow-up are important to reduce morbidity and mortality (Hammer et al., 2004; Sharma, Nieman, & Feelders, 2015; Swearingen et al., 1999). Patients with persistent Cushing's disease after pituitary surgery, however, are associated with an even higher standard mortality rate (Bolland et al., 2011; Dekkers et al., 2013; Graversen et al., 2012; Lindholm et al., 2001).

1.3.4 The diagnosis and treatment of Cushing's disease

Cushing's disease is the ACTH dependent form of Cushing's syndrome caused by pituitary corticotroph tumours hypersecreting ACTH. Corticotroph tumours account for 4 – 8% of hormonally active tumours of the anterior pituitary (Pivonello, De Leo, Cozzolino, & Colao, 2015). Cushing's disease was first

described in 1932 by the neurosurgeon Harvey Cushing (Cushing, 1994; Ellis, 2012).

Due to variability in clinical presentation, establishing the diagnosis and treatment of Cushing's disease is frequently a complex process, that needs the cross-sectoral cooperation of general practitioners, endocrinologists, chemical pathologists, radiologists and surgeons (Arnaldi et al., 2003; Biller et al., 2008; Loriaux, 2017; Nieman et al., 2008; Yorke, Atiase, Akpalu, & Sarfo-Kantanka, 2017). A set of hormonal tests are required for a definitive diagnosis. Given a clinical suspicion, a set of hormonal tests need to be performed to establish the diagnosis of hypercortisolaemia. For establishing the diagnosis of hypercortisolaemia four tests are in common use: the low-dose dexamethasone-suppression testing, the 24-hour urinary free cortisol, the midnight plasma cortisol and the late-night salivary cortisol. There are two different ways of performing the low-dose dexamethasone-suppression test. Firstly, the overnight dexamethasone-suppression test in which 1 mg of dexamethasone is administered at 11 p.m. and serum cortisol measured the next day at 8 – 9 a.m. Secondly, the 48-hour test in which 0.5 mg dexamethasone is administered every six hours for two days in a row, at 9 a.m., 3 p.m., 9 p.m. and 3 a.m. with measurements of serum cortisol at 9 a.m. at the beginning and the end of the test. Following either test, the serum cortisol levels should be below 50 nmol/l to exclude Cushing's syndrome (Newell-Price, Trainer, Besser, & Grossman, 1998). Noteworthy are the 3 – 8% of patients with Cushing's disease that show a false negative result with suppression of serum cortisol to below 50 nmol/l (Findling, Raff, & Aron, 2004; Isidori et al., 2003). 24-hour urinary free cortisol (UFC) is the least sensitive test. Three 24-hour collections are needed in order to avoid missing mild or cyclical forms of disease. Values four-fold greater than the upper limit of normal hint at Cushing's syndrome, but can also be due to other causes of hypercortisolaemia. An incomplete collection or renal impairment can lead to falsely low values of UFC (Arnaldi et al., 2003; Newell-Price et al., 2006). The normal circadian rhythm of cortisol secretion is impaired in patients with Cushing's syndrome. A single sleeping midnight plasma cortisol below 50 nmol/l excludes Cushing's syndrome (Newell-Price et al., 1995), while an awake midnight plasma cortisol of over 207 nmol/l is consistent with Cushing's syndrome, but may miss 7% of mild forms of disease (Papanicolaou, Yanovski,

Cutler, Chrousos, & Nieman, 1998). Salivary cortisol reflects free circulating cortisol and elevated levels of late-night salivary cortisol point to Cushing's syndrome (Findling & Raff, 2005). In case of continuously high clinical suspicion, repeated tests and further investigations are in order. Once the diagnosis of hypercortisolaemia is established, the cause of Cushing's syndrome needs to be determined. The first step is to measure plasma ACTH. Plasma ACTH levels consistently below 5 pg/ml point to ACTH-independent Cushing's syndrome while ACTH levels persistently above 15 pg/ml indicate ACTH-dependent Cushing's syndrome. If ACTH-independent Cushing's syndrome is suspected, adrenal imaging with computed tomography is required to differentiate between the different causes of ACTH-independent Cushing's syndrome (Newell-Price, 2008). If ACTH-dependent Cushing's syndrome is suspected, magnetic resonance imaging of the pituitary needs to be performed for, however, the pituitary is normal in 40% of patients with Cushing's disease and 10% of the general population were found to have pituitary incidentalomas. Therefore, to differentiate between pituitary and non-pituitary sources, biochemical evaluation is crucial (Newell-Price et al., 1998). The high-dose dexamethasone-suppression test has a low sensitivity for the diagnosis of Cushing's disease and is therefore no longer recommended in centres where bilateral inferior petrosal sinus sampling (BIPSS) is available. For BIPSS catheters are placed in both inferior petrosal sinuses to achieve a corticotropin gradient sample. A basal central to peripheral ratio of over 2:1, or a ratio of above 3:1 when CRH is administered, is indicative of Cushing's disease. BIPSS has a high sensitivity and specificity of 94% and has been the gold standard for distinguishing between Cushing's disease and ectopic corticotropin syndrome (Lindsay & Nieman, 2005; Newell-Price et al., 2006).

In order to prevent the development and/or worsening of the manifold comorbidities and clinical complications, prompt and effective treatment is crucial, as they are the cause for increased mortality (Pivonello, De Leo, et al., 2015). First line therapy for Cushing's disease is selective transsphenoidal surgery (Biller et al., 2008; Lacroix et al., 2015; Nieman et al., 2015; Pivonello, De Leo, et al., 2015). Effective surgery by a trained neurosurgeon results in an approximate remission rate of 80% (Honegger & Grimm, 2018). Remission is characterized by a gradual resolution of the signs derived from hypercortisolism and a slow

recovery of the hypothalamic-pituitary-adrenal axis function over the period of one year or more (Lacroix et al., 2015). The review of literature shows broad ranges of remission between 42.0 and 96.6% and recurrence rates as high as 47.4% (Dimopoulou et al., 2014; Petersenn et al., 2015).

Second line therapy is required to minimize the harmful consequences of hypercortisolism whenever Cushing's disease persists or recurs after surgery (Bertagna & Guignat, 2013; Nieman et al., 2015; Pivonello, De Leo, et al., 2015). Second pituitary surgery has proven to be favourable when the residual tumour is visible and resectable or to debulk if surrounding structures are compressed (Arnaldi et al., 2003; Bertagna & Guignat, 2013; Biller et al., 2008; Tritos, Biller, & Swearingen, 2011). When pituitary surgery is not recommended or has definitely failed, radiotherapy, medical therapy or bilateral adrenalectomy (BA) may be used to lower cortisol levels (Bertagna & Guignat, 2013). Firstly, persisting hypercortisolism can be treated with pituitary radiotherapy. The major side effect of pituitary radiotherapy is hypopituitarism, which means the deficiency of one or more hormones (Darzy & Shalet, 2009; Feigl, Bonelli, Berghold, & Mokry, 2002; Feigl, Pistracher, Berghold, & Mokry, 2010; Minniti & Brada, 2007). Secondly, medical therapy is used to lower cortisol levels. There are three classes of drugs available for medical treatment: anticorticotroph drugs (pasireotide, cabergoline), antiadrenocortical drugs (ketoconazole, metyrapone, mitotane or in rare cases etomidate or lysodren) and the antiglucocorticoid mifepristone (Bertagna & Guignat, 2013; Cuevas-Ramos, Lim, & Fleseriu, 2016; Pivonello, De Leo, et al., 2015). A combination of drugs might be necessary to reach eucortisolism (Kamenicky et al., 2011). Medical therapy is rarely a good long-term solution and is mainly used as adjunctive treatment to other modalities such as surgery and pituitary radiotherapy or as first-line treatment in patients with surgical contraindications (Cuevas-Ramos et al., 2016; Pivonello, De Leo, et al., 2015). Indications for medical treatment also include acute complications of hypercortisolism, such as infection and psychosis (Lacroix et al., 2015). BA is the definitive treatment for Cushing's syndrome and may be required to achieve adequate control of cortisol levels when other therapies have failed or when rapid eucortisolism is crucial (Lacroix et al., 2015; Ritzel et al., 2013; Thompson et al., 2007). Due to the many severe consequences of BA, like live-long adrenal insufficiency with mandatory hormone replacement, the consequent risk of

adrenal crisis and the development of Nelson's syndrome, it is used as an ultima ratio. It is only conducted as an emergency treatment in patients with very severe, life-threatening ACTH-dependent disease that cannot be promptly and sufficiently controlled by other medical measures (Reincke, Ritzel, et al., 2015). Patients undergoing BA have a significant risk of developing the so-called Nelson's syndrome (hyperpigmentation and macroscopic (>1 cm) enlargement of the tumour), as the primary corticotroph adenoma remains in situ after adrenalectomy. Therefore a regular evaluation for corticotroph tumour progression using ACTH levels and pituitary magnetic resonance imaging in patients with Cushing's disease is crucial (Nieman et al., 2015).

1.4 The genetics of Cushing's disease

1.4.1 The long road to decoding the genetic basis of Cushing's disease

In humans, most pituitary tumours originate from the sporadic clonal expansion of a single cell containing one or few mutations that give them particular adaptive advantages and are considered overwhelmingly non-familial and benign (Biller et al., 1992; Gicquel, Le Bouc, Luton, Girard, & Bertagna, 1992; Heaney, 2011; V. Herman, Fagin, Gonsky, Kovacs, & Melmed, 1990; Melmed, 2011; H. M. Schulte et al., 1991). Only rarely Cushing's disease can be observed as a manifestation in the context of genetic tumour syndromes, such as multiple endocrine neoplasia type 1 or type 4, familial isolated pituitary adenoma, McCune-Albright syndrome, Carney complex and tuberous sclerosis complex (Dahia et al., 1998; Georgitsi, Raitila, Karhu, Tuppurainen, et al., 2007; Georgitsi, Raitila, Karhu, van der Luijt, et al., 2007; Hernandez-Ramirez et al., 2017; Igreja et al., 2009; Kasturi et al., 2017; Kiefer et al., 2017; Melmed, 2011; Nandagopal, Vortmeyer, Oldfield, Keil, & Stratakis, 2007; Naziat et al., 2013; Riminucci et al., 2002; Simonds, Varghese, Marx, & Nieman, 2012; Stratakis et al., 2010; Thakker et al., 2012; Tigas et al., 2005; Verges et al., 2002; Williamson, Ince, Harrison, Kendall-Taylor, & Harris, 1995). Screening for known mutations associated with other endocrine pathologies that predispose individuals to familial pituitary tumours has been useful in identifying uncommon germline mutations, such as AIP, MEN1, PRKAR1A and x chromosome microduplications. Very rarely germline mutations where found in DICER1, CDKN1B and SDH (Gadelha, Trivellin, Hernandez

Ramirez, & Korbonits, 2013; Lecoq, Kamenicky, Guiochon-Mantel, & Chanson, 2015; Stratakis et al., 2010). AIP germline mutations could also be found in isolated, sporadic variants of Cushing's disease without familial history (Cazabat et al., 2012; Stratakis et al., 2010). Somatic mutations could be identified in the GNAS1 gene in somatotroph tumours that account for McCune-Albright syndrome (Landis et al., 1989; Weinstein et al., 1991), however, the identification of recurrent, somatic mutations in corticotroph tumours was largely unsuccessful for a long time (Dworakowska & Grossman, 2012).

Multiple efforts have been made to identify mutations causing ACTH-producing corticotroph tumours. Previous studies have shown different gene expression patterns and dysregulated methylation in different types of pituitary tumours (Melmed, 2011), but the search for somatic mutations in candidate genes remained largely unsuccessful. Despite the fact that CRHR1 and the vasopressin receptor V1B or V3R are expressed abundantly in corticotroph tumours, no mutations in the respective coding regions have been reported (Dahia et al., 1996; de Keyzer, Rene, Beldjord, Lenne, & Bertagna, 1998; Luque et al., 2013).

Thanks to next-generation sequencing technologies like whole-exome sequencing, Reincke and co-workers were the first to identify recurrent somatic mutations in the gene encoding the ubiquitin-specific protease 8 (*USP8*) in six out of seventeen patients (35 %) (Reincke, Sbiera, et al., 2015).

1.4.2 The ubiquitin-specific protease 8 (USP8) and its role in the recycling of the epidermal growth factor receptor (EGFR)

The ubiquitin-specific protease 8 (*USP8)* gene codes for an enzyme of ~130 kDa with deubiquitinase (DUB) activity, i.e., it cleaves ubiquitin peptides from target proteins, many of them membrane receptors that control cell growth and proliferation (Naviglio et al., 1998). In most cases, ubiquitin is related to proteolytic cleavage by either the proteasome or lysosomes and therefore USP8 action reduces the degradation and increases the recycling rate of its target protein (Hochstrasser, 1995; Komada, 2008).

USP8 activity is tightly regulated. USP8 remains catalytically inactive through its interaction with members of the 14-3-3 family of proteins, a class of small (25 – 30 kDa) adapter proteins, composed of seven isoforms in humans (Mhawech, 2005). The interaction is mediated by a specific sequence, the 14-3-3 binding motif (RSXpS/TXP, RSYpS(718)SP in *USP8*), which is recognized by 14-3-3 proteins when the Ser718 is phosphorylated (Mizuno, Kitamura, & Komada, 2007; Yaffe et al., 1997). *USP8* mutations found by Reincke et al. in corticotroph tumours target the 14-3-3 binding motif and thus drastically reduce the interaction and specific regulation of USP8 by 14-3-3. Therefore, mutant USP8 remains constitutively active in the cell (Reincke, Sbiera, et al., 2015).

Among other functions, USP8 is involved in the lysosomal trafficking of the epidermal growth factor receptor (EGFR) (Komada, 2008). The EGFR belongs to the family of receptor tyrosine kinases, that consists of 20 functionally similar but structurally different forms. Four closely related receptors are comprised by the epidermal growth factor subfamily: EGFR (or HER1 or ErbB1), HER2 (or ErbB2/neu), HER3 (or ErbB3) and HER4 (or ErbB4) (Hackel, Zwick, Prenzel, & Ullrich, 1999). EGFR is overexpressed in a wide range of tumours and therefore poses a target for cancer treatment (Kanematsu, Yano, Uehara, Bando, & Sone, 2003; Klijn, Berns, Schmitz, & Foekens, 1992; Sugawa, Ekstrand, James, & Collins, 1990; Tang, Gong, Moscatello, Wong, & Lippman, 2000; Xu, Richert, Ito, Merlino, & Pastan, 1984), including corticotroph tumours. In normal pituitary tissue, EGFR is ubiquitously expressed, although at low levels (Kontogeorgos, Stefaneanu, Kovacs, & Cheng, 1996; LeRiche, Asa, & Ezzat, 1996; Onguru et al., 2004; Theodoropoulou et al., 2004). Its expression is not restricted to any specific tumour type (LeRiche et al., 1996; Onguru et al., 2004; Theodoropoulou et al., 2004). Theodoropoulou et al. demonstrated that EGFR expression is more common in the hormonally active pituitary tumours than in the non-functional ones and is the highest in corticotroph tumours (Theodoropoulou et al., 2004). Earlier studies suggest that EGFR expression was higher in non-functional than in hormonally active pituitary tumours (LeRiche et al., 1996; Onguru et al., 2004), however, a much larger number of non-functional pituitary tumours was used for immunohistochemical analysis by Theodoropoulou et al.

EGFR expression has also been reported to correlate with tumour invasiveness (Jaffrain-Rea et al., 1998; LeRiche et al., 1996).

Fukuoka et al. were able to show that EGFR plays an important role in pituitary tumours. They demonstrated that the activation of EGFR induces *POMC* transcription via mitogen-activated protein kinase (MAPK) dependent pathways and therefore induces the secretion of ACTH. Furthermore, they showed that blocking EGFR activity in corticotroph tumours with gefitinib, an EGFR tyrosine kinase inhibitor, attenuated the expression of POMC, the precursor of ACTH, inhibited the proliferation of corticotroph tumour cells and induced apoptosis (Fukuoka et al., 2011).

Under normal conditions, USP8 interacts with polyubiquitinated EGFR. Ubiquitin cleavage prevents the lysosomal degradation of the EGFR and enables EGFR recycling back to the plasma membrane (Mizuno et al., 2005; Naviglio et al., 1998). The current mechanism proposed for Cushing's disease implies a constitutive activity of USP8 that reduces EGFR degradation and therefore leads to higher EGFR stability, which enhances EGFR-induced *POMC* transcription and therefore ACTH secretion in corticotroph cells (Reincke, Sbiera, et al., 2015).

Figure 2 shows the mechanism how USP8 modulates EGFR signalling and therefore the secretion of ACTH in a normal corticotroph cell. EGFR signalling is stimulated by EGFR ligands, such as EGF, that promote Erk1/2-mediated activation of transcription factors and subsequently lead to the transcription of *POMC*. Afterwards, EGFR is linked to ubiquitin chains. The ubiquitination marks the receptor for lysosomal degradation. USP8 regulates EGFR turnover by removing the ubiquitin chains from the receptor. Deubiquitinated EGFR gets recycled and therefore is moved back to the plasma membrane, where it again activates signalling cascades, such as the Erk1/2-pathway. USP8 activity is strictly controlled by 14-3-3 proteins, that withhold USP8 in an inactive state that is reversible (Theodoropoulou, Reincke, Fassnacht, & Komada, 2015).

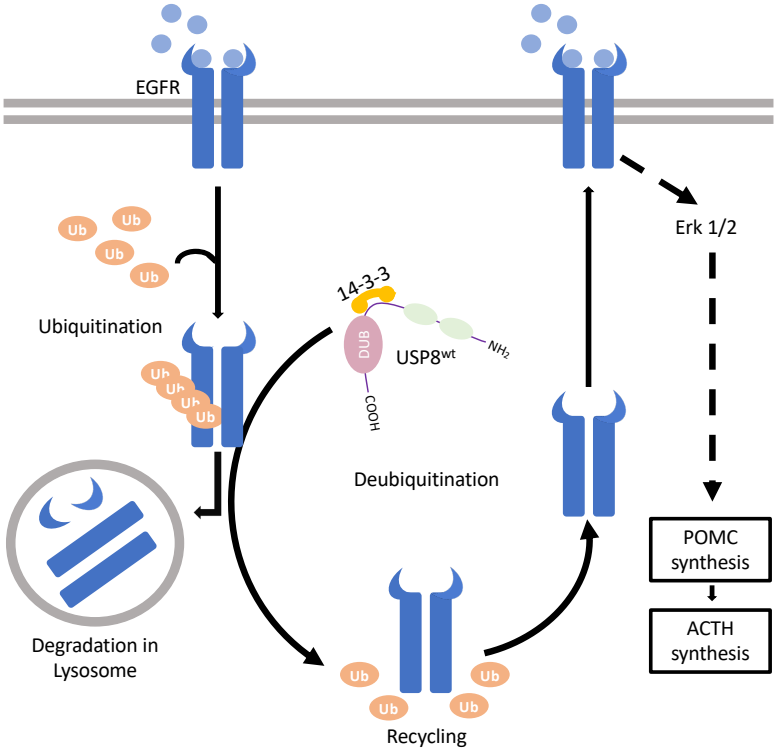
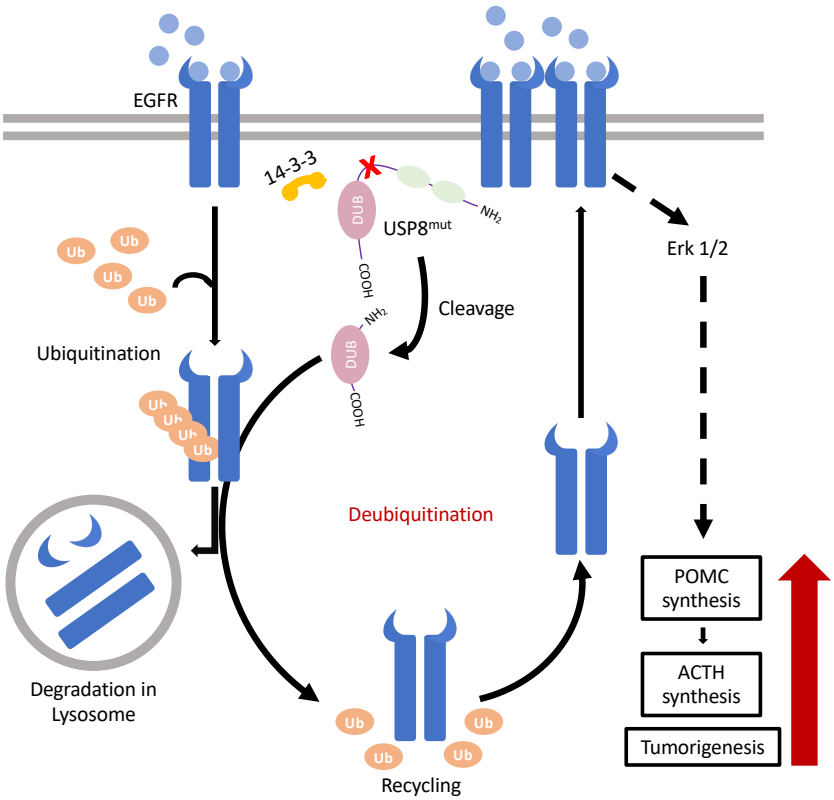


Figure 2. Schematic representation of how USP8 regulates EGFR trafficking and signalling and therefore ACTH secretion in a normal corticotroph cell. *Adapted from (Reincke, Sbiera, et al., 2015).*

Figure 3 illustrates the impact *USP8* mutations have on the binding of USP8 to 14-3-3 and therefore on EGFR trafficking and signalling. The cleaved catalytic domain of the USP8 mutants provokes the constitutive activation of USP8 proteins. With its higher DUB activity USP8 mutants effectively deubiquitinate EGFR and consequently impair EGFR degradation. Thus, EGFR accumulates in the plasma membrane and increases Erk 1/2-mediated transcription of POMC and therefore leads to a rise of plasma ACTH levels. Hence, USP8 mutants act concomitantly via the EGFR on a substantial increase in ACTH secretion as well as on corticotroph tumorigenesis (Reincke, Sbiera, et al., 2015).



Corticotroph adenoma cell with USP8 mutation

Figure 3. Schematic representation of the impact of USP8 mutations on EGFR trafficking and signalling and therefore on ACTH secretion in corticotroph tumour cells.

Adapted from (Reincke, Sbiera, et al., 2015).

2 Objectives of this study

After numerous studies trying to gain a deeper insight into the development of Cushing's disease and its underlying molecular mechanisms, Reincke et al. succeeded in identifying recurrent somatic mutations in the *USP8* gene. These mutations induce hypersecretion of ACTH via deregulation of EGFR signalling and may lead to different clinical phenotypes, i.e. different clinical presentation. However, only a small number of cases was analysed (Reincke, Sbiera, et al., 2015). This study aims to analyse the *USP8* status in a large series of 145 ACTH-positive pituitary tumours.

This study has two major objectives:

- To determine the prevalence of *USP8* mutations in a representative multicentric cohort of patients diagnosed with Cushing's disease.
- To investigate the genotype-phenotype correlation between the *USP8* mutational status of the tumour and different clinical features and biochemical test results.

These experiments could not only clarify the prevalence of somatic mutations in the *USP8* gene in patients suffering from Cushing's disease, but could also potentially reveal the clinical relevance of those genetic variants.

3 Material and Methods

3.1 Patient cohort and samples

During 1998 – 2013, patients with pituitary tumours were recruited through seven different centres from Europe and America, more precisely through the different departments of endocrinology presented in Table 3.

The number and proportion of patients recruited by each centre and their proportion of the total cohort can be retraced in Figure 4.

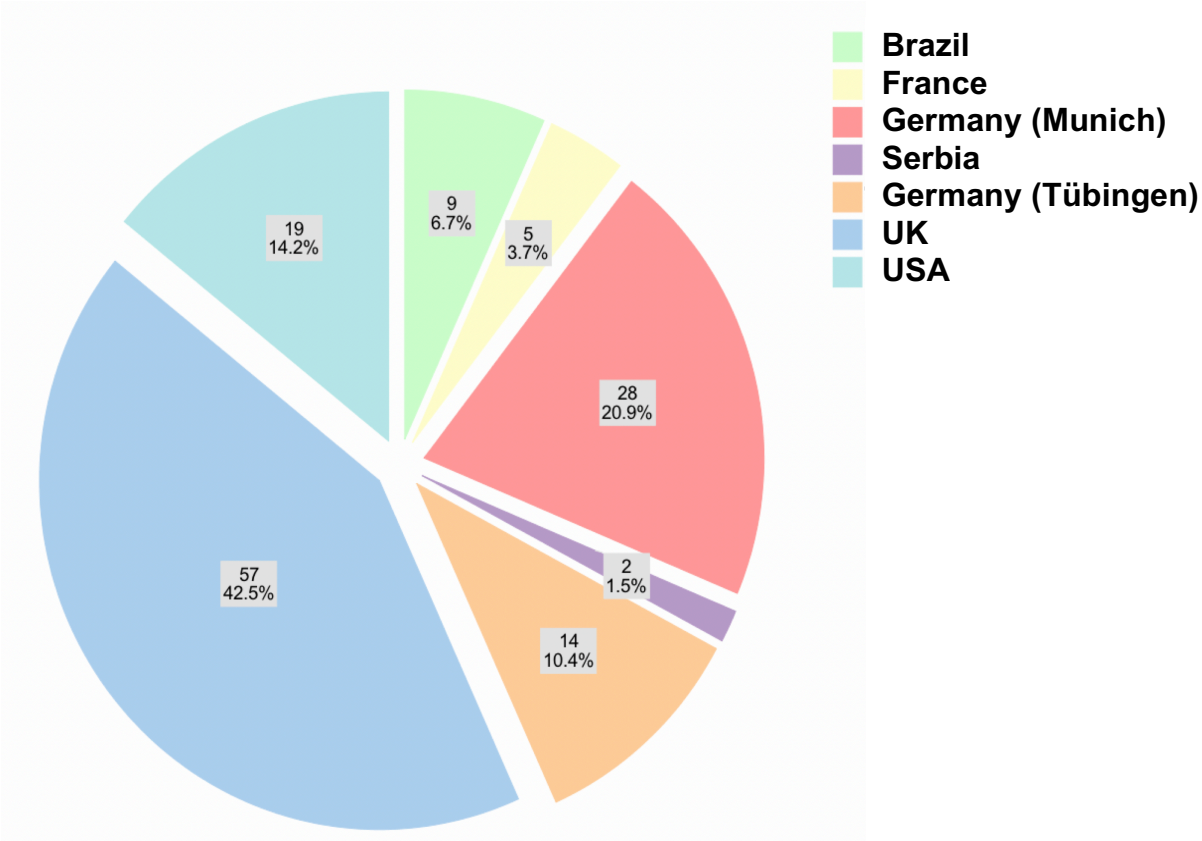


Figure 4. Distribution of patients diagnosed with Cushing's disease according to their centre of origin (n=134).

Origin	No. of patients
Brazil Universidade Federal do Rio de Janeiro	9
France Department of Endocrinology of the CHU Paris Centre-Hôpital Cochin	5
Germany Department of Endocrinology, Max Planck Institute of Psychiatry (MPI), Munich	28
Germany Department of Neurosurgery of the University Hospital Tübingen	14
Serbia Neuroendocrine Department, Pituitary Tumours and Endocrine Diseases of the University of Belgrade	2
United Kingdom William Harvey Research Institute, Barts and The London, Queen Mary's School of Medicine and Dentistry, London	57
United States of America Section on Endocrinology & Genetics, Program on Developmental Endocrinology & Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland	19*

Table 3. Overview of the seven participating centres.
** paediatric patients only*

In this work, 145 patients with corticotroph tumours were included. 134 of these were diagnosed with Cushing's disease, 105 adults (≥18 years old) and 29

children. 11 adult patients with silent corticotroph macroadenomas were included. In all cases, the diagnosis was histologically confirmed by an expert after surgical resection. Written informed consent was obtained from all patients or – whenever needed – from their respective surrogates. The study was approved by the ethics committee of each individual institution. All clinical investigations were conducted according to the ethical standards laid down in the ‚Declaration of Helsinki'.

3.2 Clinical data and diagnosis

The diagnosis of ACTH-dependent Cushing's syndrome was based on the combination of typical clinical signs and symptoms of hypercortisolism (recent weight gain, truncal obesity, buffalo hump, moon face, muscle weakness, striae rubrae distensae, easy bruising, parchment skin, hirsutism, acne, easy bruising, low-impact bone fractures, irregular menstruation, loss of libido, infertility, impotency and mood changes) and biochemical hallmarks of hypercortisolism, such as increased late-night salivary or serum cortisol levels, elevated urinary excretion of free cortisol and non-suppressible serum cortisol after 1 mg overnight or 2 mg per day (48 hours) dexamethasone test (>1,8 µg/dl; >50 nmol/l). In addition, the patients showed inadequately high or elevated plasma ACTH levels. ACTH dependency was confirmed through levels of basal plasma ACTH >2.2 pmol/l (10 pg/mg), >50% suppression of serum cortisol during high-dose (8 mg) dexamethasone tests and through cortisol response to corticotropin releasing hormone. Moreover, patients underwent additional procedures, such as magnetic resonance imaging of the pituitary and inferior petrosal sinus catheterization to confirm the pituitary dependent Cushing's syndrome.

All patients underwent transsphenoidal surgery. The presence of an ACTH-producing pituitary tumour was confirmed histologically by experts after resection. Fresh tumour tissue was frozen immediately using liquid nitrogen. Data collected at the time of surgery included the actual tumour size, postoperative ACTH levels, serum cortisol levels and 24-hour urinary free cortisol.

Adrenal insufficiency was determined as concentrations of morning serum cortisol <5 µg/dl (138 nmol/l).

Silent ACTH tumours were defined as clinically non-functioning pituitary tumours with positive immunoreactivity for ACTH on histological examination.

The clinical data requested for each patient included in this study can be found in Table 4. Collection of relevant clinical data, including clinical signs and biochemical hallmarks of hypercortisolism, was performed by means of a preconceived Excel sheet provided by us. Not available data could be marked as ‘unknown’ by the participating centres.

Collected clinical data
Sex
Age at diagnosis (years)
Clinical diagnosis (Cushing's disease or silent ACTH tumour)
Maximum tumour size (mm) Microadenoma (smaller than 10 mm) Macroadenoma (exceeding 10 mm)
Clinical catabolic signs (yes/no)
Hypertension (yes/no)
Diabetes (yes/no)
BMI (kg/m^2)
Preoperative variables Basal plasma ACTH (pg/ml) Basal serum cortisol (µg/dl) Urinary free cortisol (µg/24 h) Late-night salivary cortisol (ng/ml) Serum cortisol after 1 or 2 mg (low dose) DMX (µg/dl) Serum cortisol after 8 mg (high dose) DMX (µg/dl) Basal cortisol during CRH testing (µg/dl) Peak cortisol during CRH testing (µg/dl) Basal ACTH during CRH testing (pg/ml) Peak ACTH during CRH testing (pg/ml)

Postoperative variables
Basal levels of plasma ACTH after surgery (pg/ml)
Minimum serum cortisol after surgery (µg/dl)
Urinary free cortisol after surgery (µg/24 h)
Late-night salivary cortisol after surgery (ng/ml)
Serum cortisol after 1 or 2 mg (low dose) DMX after surgery (µg/dl)
Adrenal insufficiency (yes/no)
Prior treatments (yes/no)
Kind of prior treatments
Number of pituitary surgeries to date
Clinical remission of Cushing's syndrome

Table 4. Overview of clinical data attempted to obtain from every patient participating in this study.
Units or response options shown in brackets as appropriate. Clinical data could be entered freely unless stated otherwise in brackets.

3.3 DNA extraction, PCR amplification and Sanger sequencing

Genomic DNA was extracted from 122 fresh frozen tumours using the Maxwell 16 Instrument (Promega) with the Maxwell Tissue DNA Purification Kit (Promega). DNA from peripheral blood leukocytes of 54 patients (25 adults and 29 paediatric patients) was prepared with the same instrument and the Maxwell Blood DNA Purification Kit. RNA was extracted from 23 fresh frozen tumours by means of the RNeasy Mini Kit (Qiagen) and converted to cDNA using the M-MLV reverse transcriptase (Invitrogen).

DNA was amplified with the aid of a GoTaq DNA polymerase (Promega) and specifically designed primers. The list of primers used for the amplification by polymerase chain reaction (PCR) and for sequencing are listed in Table 5 (only the USP8 hotspot sequence) and Table 6 (the whole coding sequence of USP8) respectively.

Orientation	Function	Sequence
Forward	PCR	5'-GCAGAATACTTTGGAGTGATTTCTT-3'
Reverse	PCR	5'-TCCAACTCCCTGACACTAACA-3'
Forward	PCR	5'-CTTGACCCAATCACTGGAAC-3'
Reverse	PCR	5'-CAGCACATTATTTTAGTTCTAGGAGTT-3'

Table 5. List of primers used for amplification of the USP8 hotspot sequence.

Orientiation	Function	Sequence
Forward	2	5’-TCACTTGTTTTATTGTGAATGAGGA-3’
Reverse	2	5’-TCATCTAACTTTAATATGGAAACGAA-3’
Forward	3	5’-GCCGTGAACCAGTACCAATC-3’
Reverse	3	5’-TCATGCTGCATATAATTTGAGCTAC-3’
Forward	4	5’-AAGCACCATGATTTTAATGATTTTA-3’
Reverse	4	5’-CGCGAGACTCTGTCTCAAAA-3’
Forward	5	5’-TGGTGGAGGGAGAAAGCATA-3’
Reverse	5	5’-TCATCCTTGTTGCCTAAAAGAAC-3’
Forward	6	5’-AAAAGGCCAGTACTCTGCAC-3’
Reverse	6	5’-AAACCTGATGCTTATTCTGATTAAAAG-3’
Forward	7	5’-TGGAGTAGTAAATATGTGGCATCC-3’
Reverse	7	5’-CCACCACCCACACACATAAA-3’
Forward	8	5’-TGGTGTGGTAAAGACTGTGGA-3’
Reverse	8	5’-AACATGCCTTTCTAACAACCAGA-3’
Forward	9-10*	5’-TTTTGTCCTTAAGGGAACAACTTT-3’
Reverse	9-10*	5’-GATTACAGGCGTGAGGCACT-3’
Forward	11	5’-GATGTTGTCTCCACAAAGTGACA-3’
Reverse	11	5’-ATGGAATGCCACTGGTGTTT-3’
Forward	12	5’-TCTCATAGATTCGGTTGTGTTAGC-3’
Reverse	12	5’-GCAACGATCCCCTTACACTG-3’
Forward	13-14*	5’-AAATATGAAGGGCAGCCAAG-3’
Reverse	13-14*	5’-CCATCCACAGAAATTTCCAA-3’
Forward	14 ⁜	5’-CTTGACCCAATCACTGGAAC-3’

Reverse	14 #	5'-TTACTGTTGGCTTCCTCTTCTC-3'
Forward	15	5'-TGCTACAGTTTGCTGCCATT-3'
Reverse	15	5'-GCAGCAGAAAACTAATGGACTAAG-3'
Forward	16	5'-GGTGGTGAGCCTGCAAATAA-3'
Reverse	16	5'-AAACAAGCACTGAACTATCACCAA-3'
Forward	17	5'-TGTTTGTATTCACTTTTATTCTTTCAA-3'
Reverse	17	5'-GACCAGAAATTTACATCTCTATATCCA-3'
Forward	18	5'-GGTGCTCTCTGACATTATTGAAG-3'
Reverse	18	5'-TGGCAGGCAGTACAACAAGT-3'
Forward	19-20*	5'-CTGGCTGTTTGACCTTAGGC-3'
Reverse	19-20*	5'-CACAGCTCCCACTGTCCTAGA-3'

Table 6. List of primers used for amplification and sequencing of the coding region of human USP8.

**Exons where amplified and sequenced together.*

#Exon 14 was sequenced with specific primers.

PCR quality and performance were evaluated on agarose gel electrophoresis. PCR products were cleaned up from salts, proteins and primers using the Wizard SV Gel and PCR Clean-up System (Promega). Purified DNA was quantified by means of a Nanodrop (ThermoScientific). Sanger sequencing of PCR products was performed using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

3.4 Statistical analysis

Continuous variables were compared between paired groups using the parametric Student's t-test or the non-parametric Mann-Whitney U. Multiple comparisons were performed using ANOVA or Kruskal-Wallis tests, and P-values were corrected as applicable and appropriate. Categorical variables were

compared using the Fisher's exact test. Binary logistic regression in a backward-stepwise fashion was used for multivariate analysis.

Measurements are documented as means with their respective standard deviations (SD) or medians with their respective interquartile ranges (IQR). An exact, two-tailed significance level of $p<0.05$ was considered the threshold to being statistically significant. The software package SPSS, version 25.0 (IBM SPSS Statistics) was used for statistical analysis.

4 Outcome

4.1 Overview over the data collected

This retrospective multicentric study included 134 patients diagnosed with Cushing's disease, 105 adults (>18 years old) and 29 paediatric cases. Furthermore, 11 adults with silent corticotroph macroadenomas were considered in this analysis. All 145 patients were recruited from the seven participating European and American centres (see section 3.1).

Clinical and hormonal data of the study population are summarised in Table 7.

	Cushing's disease tumours (n=134)	**Silent ACTH tumours (n=11)**
Age at diagnosis, years (range), n	34 (7-76), 134	45 (27-66), 11
Sex, n (%)		
Male	36 (26.9)	6 (54.5)
Female	98 (73.1)	5 (45.5)
Maximum tumour size in mm, n	12.2, 110	19.3, 6
Tumour size, n (%)		
Microadenoma	69 (51.5)	–
Macroadenoma	65 (48.5)	11 (100)
Body Mass Index in kg/m², n	31.1, 106	25.1, 2
Clinical catabolic signs, n (%)		
No	16 (28.1)	3 (60.0)
Yes	82 (71.9)	2 (40.0)
Diabetes, n (%)		
No	71 (65.7)	–
Yes	37 (34.3)	4 (100)

Basal levels of plasma ACTH in pg/ml, n	112.4, 108	–
Basal levels of plasma ACTH in pg/ml, n	25.5, 100	–
24-hour urinary free cortisol in µg/24 h, n	539.7, 49	–
Serum cortisol after 1/2 mg dexamethasone test in µg/dl, n	18.6, 76	–
Serum cortisol after 8 mg dexamethasone test in µg/dl, n	8.4, 51	–
Postoperative levels of ACTH in pg/ml, n	24.9, 56	–
Postoperative minimal cortisol levels in µg/dl, n	6.9, 77	–
Postoperative levels of 24-hour urinary free cortisol in µg/24 h, n	56.5, 30	–
Postoperative levels of cortisol after 1/2 mg dexamethasone test in µg/dl, n	5.3, 42	–
Prior treatments, n (%)		
No	39 (58.2)	–
Yes	28 (41.8)	–
Postoperative adrenal insufficiency, n (%)		
No	40 (37.7)	–
Yes	66 (62.3)	–

Table 7. Clinical features of the study cohort (n=145).

As broadly reported for Cushing's disease (Lacroix et al., 2015; Newell-Price et al., 2006), females were more represented than males (98 vs. 36 cases, respectively). A quite similar number of macro- and microadenomas were included into this study (65 and 69, respectively).

4.2 Mutations in *USP8* are common in pituitary tumours causing Cushing's disease

We analysed the complete coding region of *USP8* in tumour samples of 19 patients and the hotspot region identified by Reincke et al. in the exon 14 of *USP8* (Reincke, Sbiera, et al., 2015) in another 126 samples, including the 11 silent corticotroph tumours.

In this study *USP8* variants in a total of 48 corticotroph tumours (35.8%) were identified. All variants appeared in heterozygosity and clustered into a hotspot region overlapping with the 14-3-3 binding motif of *USP8* [Figure 5]. None of them were present in the paired blood samples of 54 patients, indicating that they had a somatic origin. In addition, no somatic variant was detected in any other exon of *USP8*.

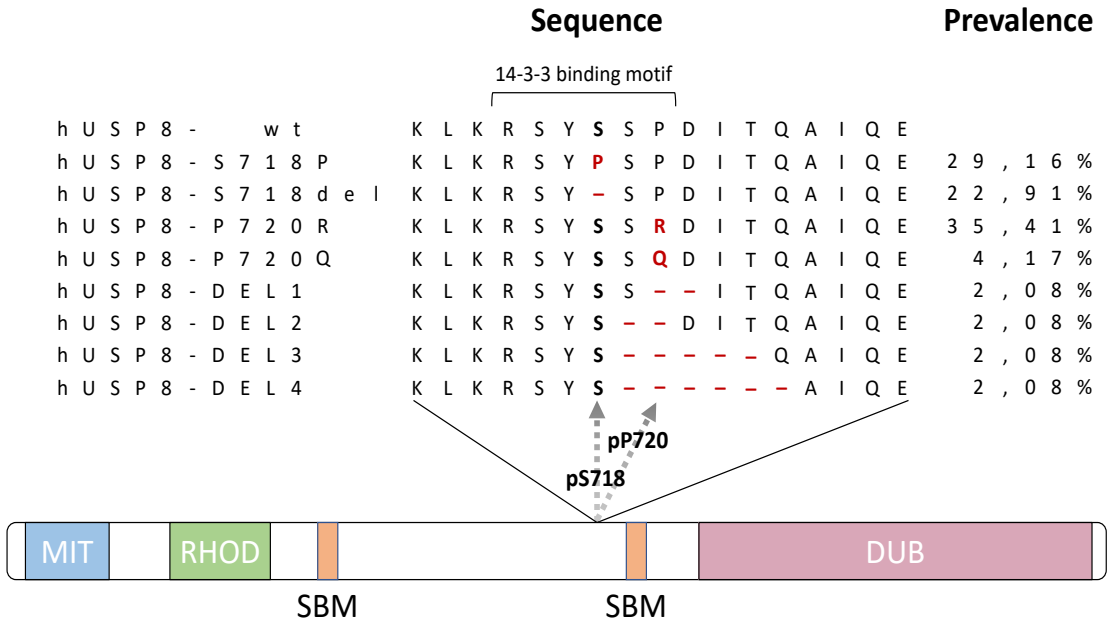
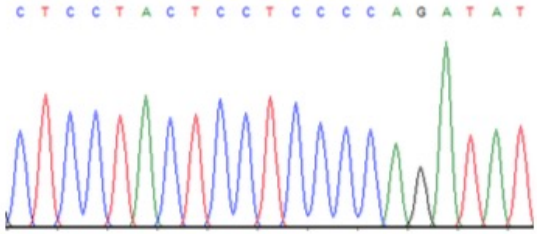


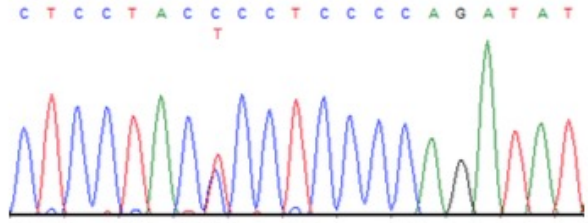
Figure 5. Diagram summarising the location of the different somatic mutations in USP8.

All the mutations found in corticotroph tumours are clustered into the region of USP8 coding for the 14-3-3 binding motif. This hotspot region is shown in the context of the complete protein. DUB – deubiquitinase catalytic domain; MIT – microtubule-interacting and trafficking domain; RHOD – rhodanese-like domain; SBM – SH3-binding motif. On the right the overall mutation frequency is shown (prevalence = x/48).

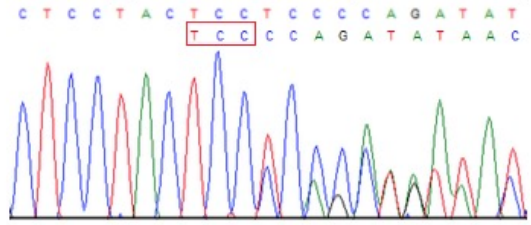
Wild-type USP8



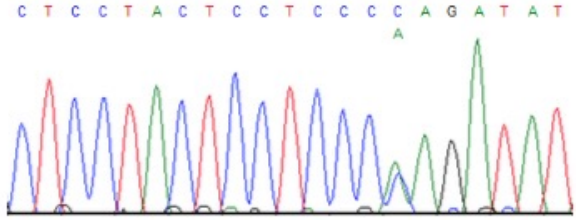
c.2152T>C (p.718Ser>Pro)



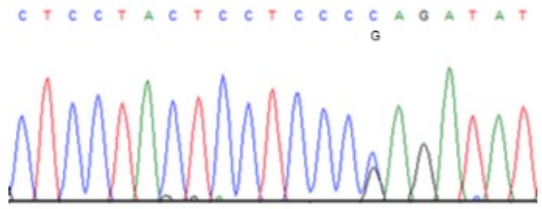
c.2155_2157delTCC (Ser718del)



c.2159C>G (p.720Pro>Arg)



c.2159C>A (p.720Pro>Gln)



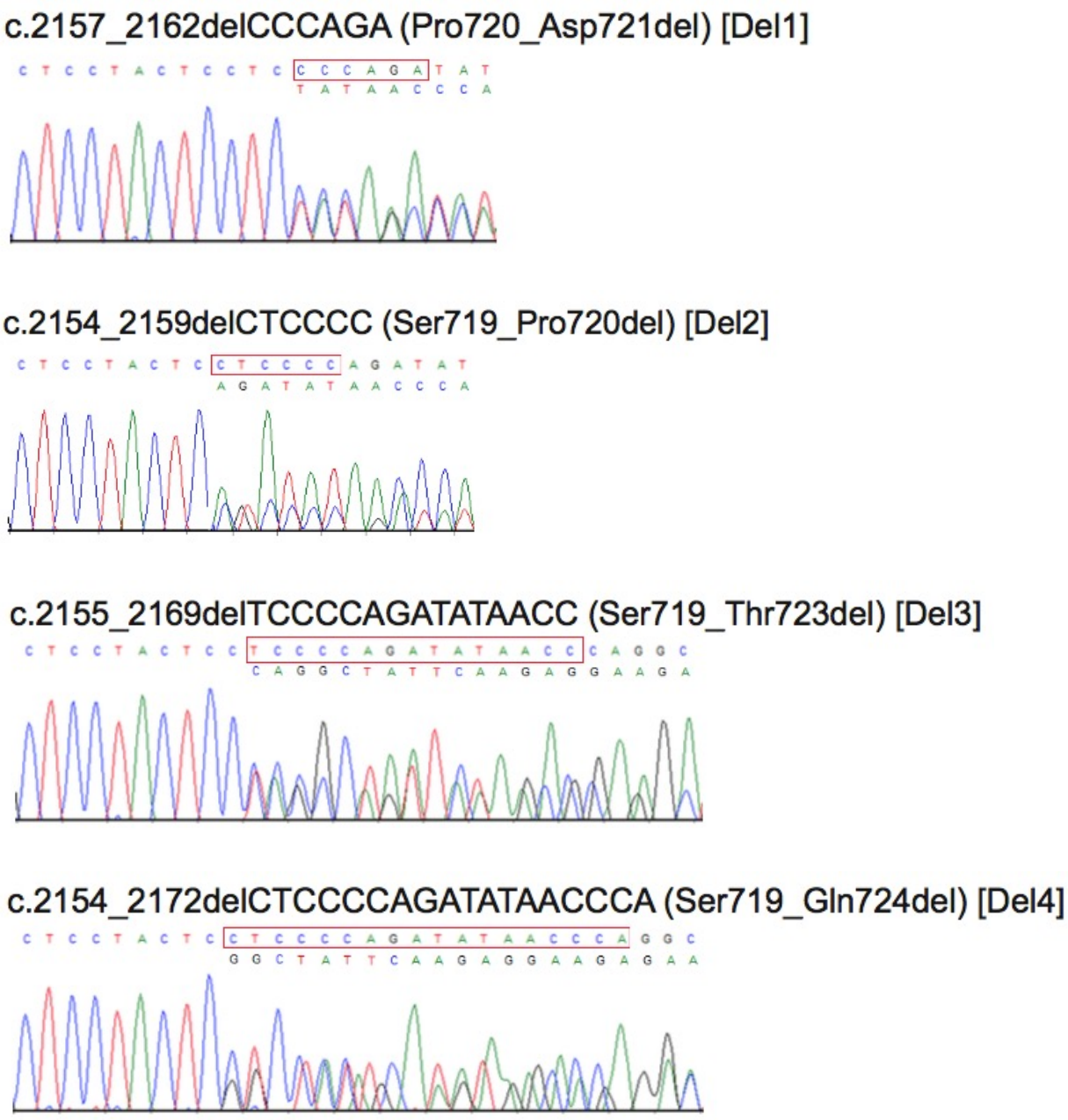


Figure 6. Mutations identified in corticotroph tumours causing Cushing's disease.

Chromatograms showing the different mutations identified in this study. The wild type nucleotide sequence is shown on top, the mutated sequences (point mutations and deletions) are shown below. The red boxes indicate the deleted residues.

In addition to mutations discovered before by Reincke et al. – Ser718Pro (S718P), Pro720Arg (P720R) and Ser718del (S718del) (Reincke, Sbiera, et al., 2015) – five new mutations could be identified [Figure 5 and Figure 6, Table 8]: The substitution Pro720Gln (P720Q) in two unrelated patients and four short in-

frame deletions of 6 – 18 nucleotides (affecting either two, five or six amino acids) in four different patients (referred to as Del1 – Del4).

Regarding the relative prevalence of mutations [Table 8], 52% of all mutations found affected the residue of Ser718, while 48% targeted the residue of Pro720. More precisely, concerning the residue of Ser718, 29% of all mutations identified where missense mutations and 22% where deletions of a single amino acid. As regards the residue of Pro720, 40% of all mutations discovered were missense mutations, while 8% accounted for in-frame deletions.

Mutations	**n**	**(%)**
Mutations in Ser718	25	(52.1)
c.2152T>C (p.718Ser>Pro) [S718P]	14	(28.6)
c.2155_2157delTCC (Ser718del) [S718del]	11	(22.4)
Mutations in Pro720	23	(47.9)
Missense mutations	19	(39.6)
c.2159C>G (p.720Pro>Arg) [P720R]	17	(35.4)
c.2159C>A (p.720Pro>Gln) [P720Q]	2	(4.1)
Deletions	4	(8.2)
c.2157_2162delCCCAGA (Pro720_Asp721del) [Del1]	1	(2.1)
c.2154_2159delCTCCCC (Ser719_Pro720del) [Del2]	1	(2.1)
c.2155_2169delTCCCCAGATATAACC (Ser719_Thr723del) [Del3]	1	(2.1)
c.2154_2172delCTCCCCAGATATAACCCA (Ser719_Gln724del) [Del4]	1	(2.1)

Table 8. Type and frequency of the mutations identified in corticotroph tumours from patients with Cushing's disease.

Figure 7 features the mutation status and gives an overview over type and frequency of all mutations identified in this study. The most common mutation found in this study was the substitution P720R (35%), followed by the substitution S718P (29%) and the deletion S718del (22%). The substitution P720Q and the

different short in-frame deletions (Del1 – Del4) were less common (4% and 2% each, respectively).

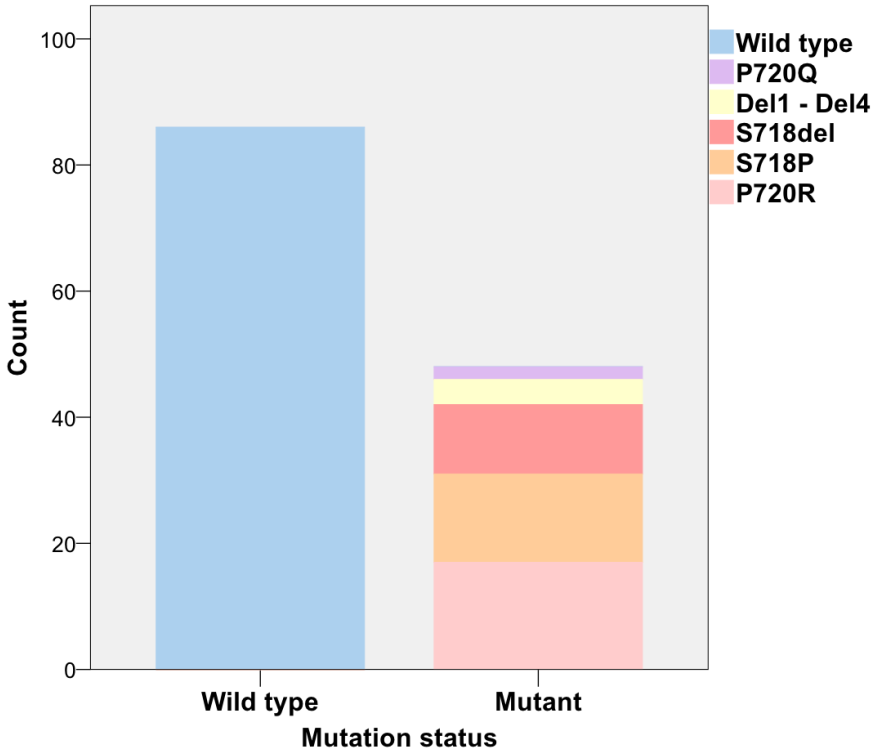


Figure 7. Mutation status and different types of mutations in proportion to all mutations found in this study.

In the different series of different origins, distinct mutation rates could be detected [Figure 8]. The highest mutation rate was found in the series from Brazil (5/9 cases, 56%), while the lowest mutation rate of 0% was found in the series from Serbia, though only two samples from Serbia were studied.

A low mutation rate of 11% could also be identified in the series from the United States of America, but these samples solely included paediatric cases.

Importantly, alterations in the *USP8* gene were not detected in any of the silent corticotroph tumours.

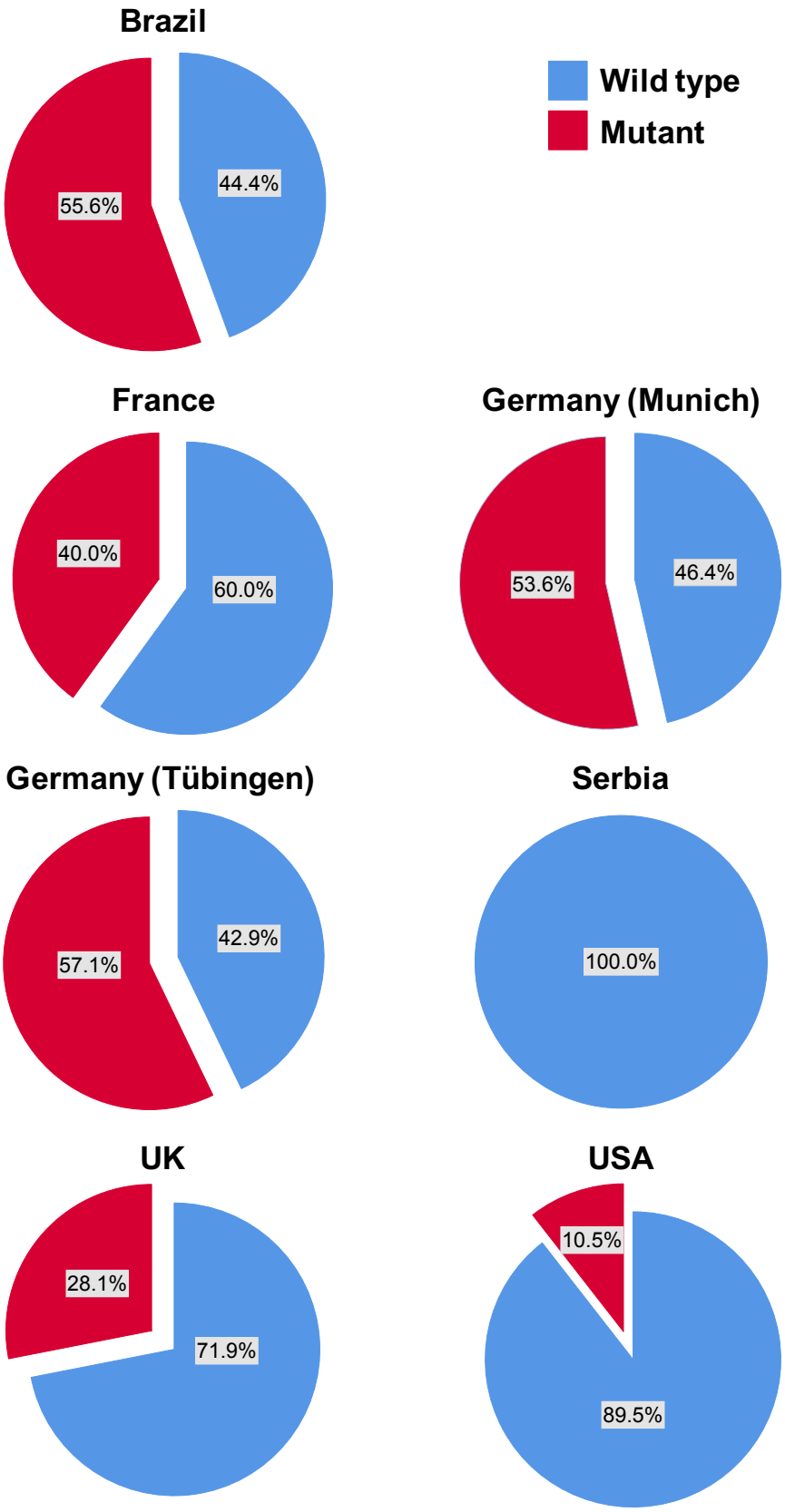


Figure 8. Mutation rates in series according to origin.
Pie charts showing the different proportions of wild type (blue) and mutated (red) USP8 according to origin. Size of the different series: Brazil – n=9, France – n=5, Germany (Munich) – n=28, Serbia – n=2, Germany (Tübingen) – n=14, UK – n=57; USA – n=19 (only paediatric cases).

4.3 Sex and age distribution

In a total of 19 cases we searched for somatic mutations in the complete coding region of *USP8* and analysed the exon 14 of *USP8* in another 126 samples, including the 11 silent corticotroph tumours.

	Wild type	**Mutated**	***p* value**
Patients, n (%)	86 (64.2)	48 (35.8)	
Pediatric cases	24 (82.8)	5 (17.2)	0.03
Adult cases	62 (59.0)	43 (41.0)	
Age at diagnosis, years (mean, SD)	35.7, 17.4	33.75, 1.7	0.49
Age of pediatric cases	14.6, 2.2	15.2, 2.6	0.62
Age of adult cases	43.8, 13.3	35.9, 10.3	0.001
Sex, n (%)			
Males	30 (83.3)	6 (16.7)	0.005
Females	56 (57.1)	42 (42.9)	
Max. tumour size, mm (median, IQR)	8.5, 12.0	10.0, 8.0	0.32
Microadenomas, n (%)	46 (65.2)	23 (34.8)	
Size, mm (median, IQR)	6.0, 3.0	8.0, 3.0	0.01
Macroadenomas, n (%)	40 (61.5)	25 (38.5)	
Size, mm (median, IQR)	18.0, 13.0	16.0, 9	0.09
Body mass index, kg/m² (mean, SD)	30, 6.6	32.7, 6.6	0.04

Table 9. Clinical features in patients with wild type versus USP8 mutated tumours.

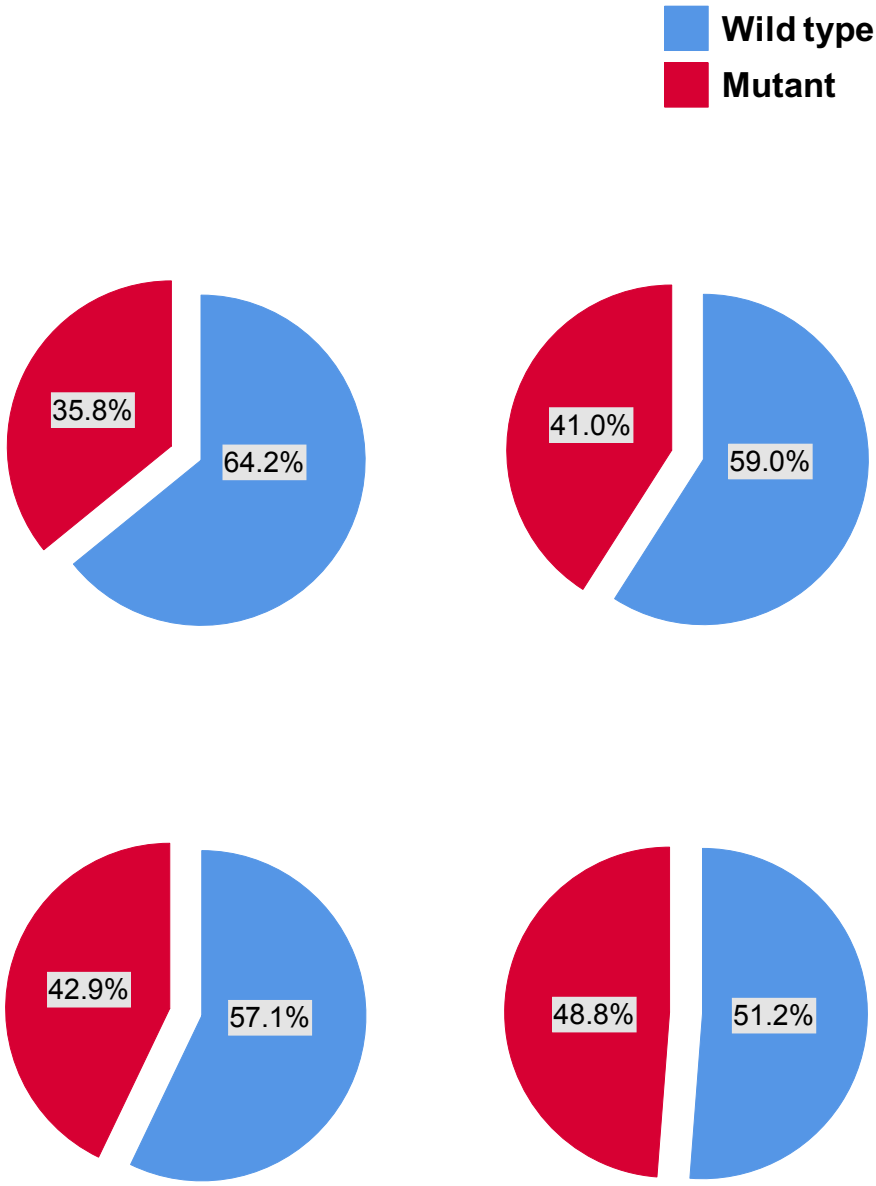


Figure 9. Prevalence of mutations in the USP8 gene in different cohorts.
Pie charts showing the different proportions of wild type (blue) and mutated (red) USP8 in different cohorts. Top left – prevalence of USP8 mutations in the total cohort (n=134); top right – prevalence of USP8 mutations in adults (n=105); bottom left – prevalence of USP8 mutations in females (n=98); bottom right – prevalence of USP8 mutations in female adults (n=82).

The prevalence of mutations in the entire cohort was 35.8%, but varied depending on age and sex [Table 9, Figure 9], being much more frequent in adults than in paediatric cases (41% vs. 17%; p=0.027) and more common in females than in males (43% vs. 17%; p=0.005). Considering only the cohort of adult patients, patients with *USP8* mutant tumours were associated with a younger age at diagnosis compared to those with wild type tumours (36 ± 10 years of age vs. 44 ± 13 years of age; p=0.001).

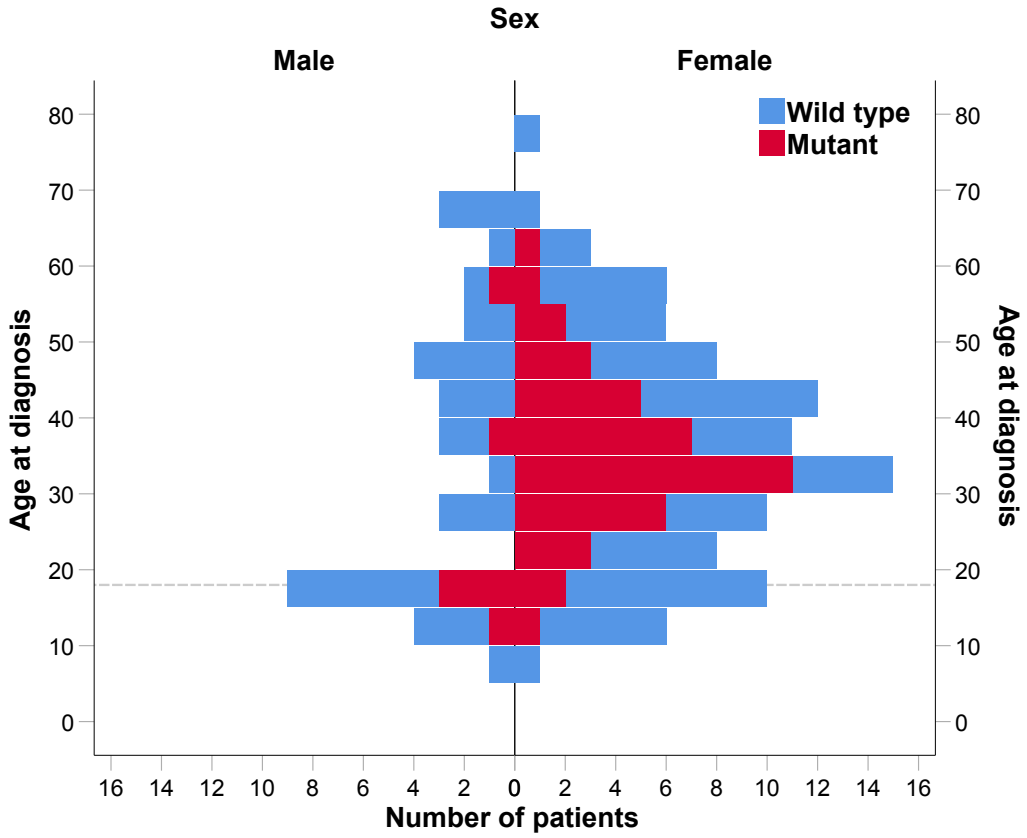


Figure 10. Age at diagnosis as influenced by USP8 mutations in male versus female patients.
The histograms represent the distribution of cases with either wild type (blue) or mutated (red) USP8 in males and females at different ages at diagnosis. Each bar represents five years. The dashed line is drawn at the age of 18, meaning that the bars above that line represent adult patients while the bars below that line represent paediatric patients.

Sex specific prevalence was also linked to age [Figure 10, Figure 11]. While *USP8* mutations were rather seldom in female paediatric patients (2/16 cases, 13%), they were indeed commonly detected in adult women (40/82 cases, 49%). These adult women were also found to be younger than those with wild type tumours (36 ±10 vs. 42 ±14 years old; p=0.01; Figure 11). While most mutations in female patients were found between the ages of 25 – 44 years, tumours in children as well as tumours diagnosed in adults >50 years of age were usually wild type. Concerning the male patients, tumours with an *USP8* mutation were predominantly found in teenagers between 15 – 19 years of age.

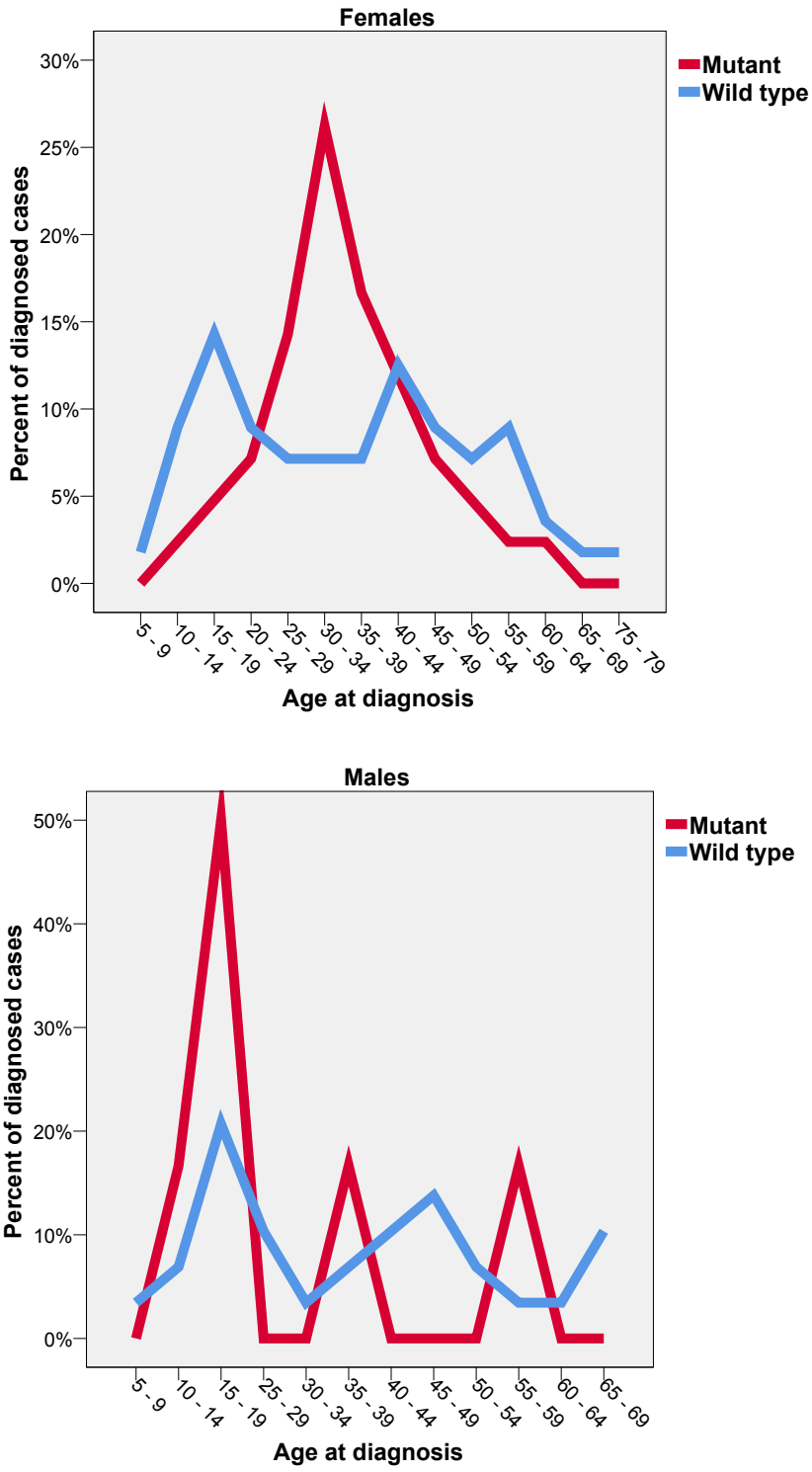


Figure 11. Prevalence of USP8 mutations in patients diagnosed with CD at different ages.

Distribution of cases according to age at diagnosis. Data are represented in 5-year intervals and as percent of cases diagnosed in each age category. Top graph – female patients; bottom graph– male patients.

4.4 Differences in size

The maximum tumour size was not significantly different between tumours with a mutant *USP8* gene and those with a wild type sequence. The maximum tumour size median for *USP8* mutated tumours was 10 mm while it was 8.5 mm for wild type tumours (IQR 8 vs. 12 respectively) [Table 9]. Over 50% of *USP8* mutated tumours had a size ranging between 8 and 16 mm. When analysing microadenomas separately, tumours with mutant *USP8* turned out to be significantly larger (p=0.013). Whereas when analysing macroadenomas independently, mutated macroadenomas tended to be smaller, although the difference was not statistically significant (p=0.089). In female patients, tumours with *USP8* mutations were found to be slightly larger than wild type tumours (median maximum diameter of 10 vs. 8 mm, respectively; IQR 7 vs. 9, respectively; p=0.048). No significant difference could be shown between male patients with and without *USP8* mutations, as the median maximum diameter for both was 12 mm (IQR 17 vs. 20 respectively) [Figure 12].

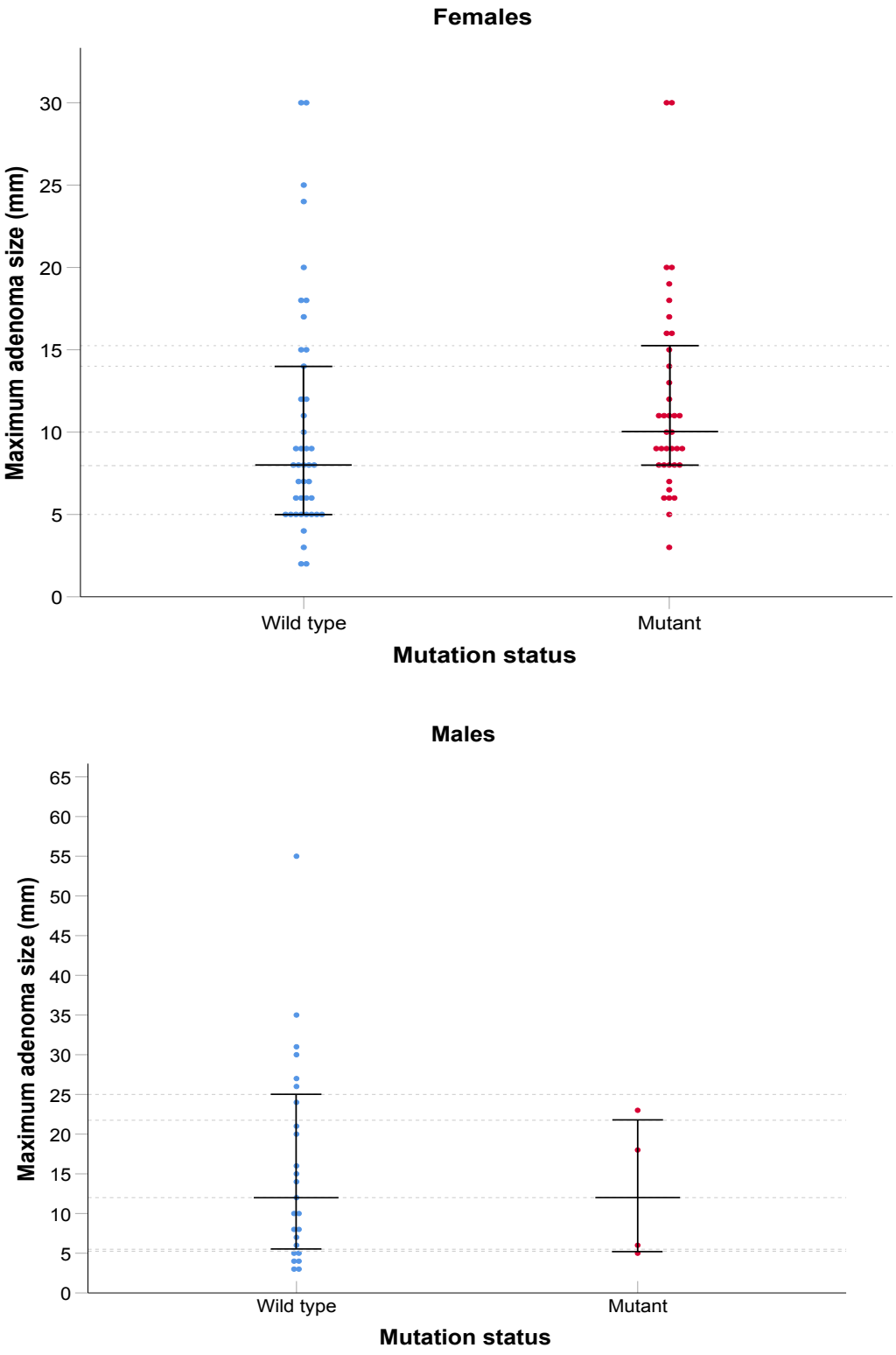


Figure 12. Distribution of tumour size in female versus male patients without and with USP8 mutations.

The dot plots represent the distribution of tumour size in female (top graph) and male (bottom graph) patients without and with USP8 mutations. The bars mark the first, second (the median tumour size) and third quartiles as weighted averages and therefore define the middle 50% of tumour sizes studied, the interquartile range. The difference in tumour size in females with wild type versus mutant USP8 reached significance ($p<0.05$).

4.5 Differences in hormonal status

	Wild type	Mutant	*P* value
Preoperative variables			
Basal plasma ACTH, pg/ml (median, IQR)	74.0, 67.7	67.0, 57.0	0.76
Basal serum cortisol, µg/dl (median, IQR)	24.1, 14.8	21.6, 9.2	0.41
Urinary free cortisol, µg/24 h (median, IQR)	379.6, 415.0	370.0, 490.1	0.62
Serum cortisol after 1/2 mg DMX, µg/dl (median, IQR)	14.7, 14.1	17.2, 16.1	0.60
Serum cortisol after 8 mg DMX, µg/dl (median, IQR)	5.2, 6.8	2.5, 2.5	0.01
Postoperative variables			
Basal levels of plasma ACTH after OP, pg/ml (median, IQR)	8.3, 12.3	14.0, 30.0	0.12
Minimum serum cortisol after OP, µg/dl (median, IQR)	2.5, 7.0	3.3, 7.9	0.72
Urinary free cortisol after OP, µg/24 h (median, IQR)	2.5, 6.0	22.5, 241.3	0.007
Adrenal insufficiency, n (%)			
No	19 (29.2)	21 (51.2)	
Yes	46 (70.8)	20 (48.8)	0.03

Table 10. Hormonal status in patients with wild type versus USP8 mutated tumours.

Regarding preoperative hormonal parameters, levels of cortisol after the high-dose (8 mg) dexamethasone test were significantly lower in patients with *USP8* mutations (p=0.01). Differences in basal plasma ACTH, basal serum cortisol, 24-hour urinary free cortisol, and cortisol levels after 1 mg overnight and low dose

(2 mg) dexamethasone test did not reach significance. Patients with wild type tumours were significantly more likely to develop adrenal insufficiency post-operatively than those with *USP8* mutations (71% vs. 49%, respectively; p=0.026), independently of other factors (p=0.028 corrected for age and tumour size). These differences in adrenal insufficiency were even more distinct in patients of a younger age (below the age of 36, 82% vs. 49%; p=0.014).

In addition, postoperative 24-hour urinary free cortisol levels were significantly higher in patients with *USP8* mutant tumours (p=0.007), but the number of cases with available data regarding the postoperative urinary free cortisol levels was limited (n=30). Other differences in postoperative hormonal parameters, like in basal levels of plasma ACTH or minimum serum cortisol, did not reach statistical significance.

All recorded differences in hormonal status are listed in Table 10.

5 Discussion

5.1 Summary of aims and findings

ACTH-secreting pituitary tumours are sporadic tumours of monoclonal origin, whose tumourigenesis has been presumed to originate from a genetic cause (Biller et al., 1992; V. Herman et al., 1990). Even though several hypothesis-driven studies have tried to gain a deeper insight into the development of Cushing's disease and its underlying molecular mechanisms, the genetic basis underlying Cushing's disease has remained unclear until recently. Thanks to next-generation sequencing technologies, recurrent mutations were reported in the gene encoding the ubiquitin-specific protease 8 (*USP8*), but only in a small cohort of patients suffering from Cushing's disease (Reincke, Sbiera, et al., 2015). This gene codes for a protein with deubiquitinase activity that modulates the lysosomal turnover of the EGF-receptor among other proteins (Komada, 2008; Naviglio et al., 1998).

The objectives of this study were to determine the prevalence of *USP8* mutations in a representative cohort and also to explore the genotype-phenotype correlation in a large series of patients diagnosed with Cushing's disease. With these purposes we have retrospectively analysed the *USP8* status in a multicentric cohort of 134 functioning and 11 silent corticotroph tumours by means of Sanger sequencing and investigated possible associations with different biochemical and clinical features.

In brief, mutations in the *USP8* gene were identified in 36% of functional sporadic corticotroph tumours causing Cushing's disease, but in none of the silent corticotroph tumours. These mutations were found mostly in female adult patients. The patients harbouring a *USP8* mutation were diagnosed at an earlier age than those with wild type lesions (36 vs. 44 years of age). *USP8* mutations were predominantly found in tumours measuring 10 ± 7 mm. Their presence was inversely associated with the development of postoperative adrenal insufficiency. All the mutations found in the *USP8* gene in this study affected the residues Ser718 or Pro720, including all five newly identified mutations.

5.2 Strengths and weaknesses of a retrospective, multicentric study

Unlike very clearly defined study designs that are used in prospective, controlled, clinical studies, in which all relevant information can be gathered within the framework of specific, clearly structured, standardised questionnaires, only the data collected during time of diagnosis and treatment can be used in a retrospective setting. The lack of uniformity of diagnostic protocols and postoperative management poses another limitation of a multicentric study. As different protocols were used in each of the seven participating centres and diagnostics as well as postoperative management were not conducted considering a certain scientific interest, an inconsistent and non-uniform approach needs to be factored in. Moreover, the number of different researchers involved in documentation in each participating centre needs to be considered. Additionally, many patients were not diagnosed and treated in the same centre, as cross-sectoral cooperation of different medical specialties is crucial for the diagnosis and treatment of Cushing's disease. Furthermore, the time span between collection of data and begin of this study was up to 16 years. All in all, this leads to heterogeneous and in some cases even incomplete documentation.

Results of diagnostic testing for hypercortisolism were only sparsely documented [Table 11]. Especially postoperative values of hormone tests and results of late-night serum cortisol measurement were not completed thoroughly.

	Valid	Missing
Preoperative variables		
Basal plasma ACTH, n (%)	108 (81)	26 (19)
Basal serum cortisol, n (%)	100 (75)	34 (25)
24-hour urinary free cortisol, n (%)	49 (37)	85 (63)
Late-night salivary cortisol, n (%)	11 (8)	123 (92)
Serum cortisol after low dose DMX, n (%)	76 (57)	58 (43)
Serum cortisol after high dose DMX, n (%)	51 (38)	83 (62)
Basal cortisol during CRH testing, n (%)	40 (30)	94 (70)
Peak cortisol during CRH testing, n (%)	39 (29)	95 (71)
Basal ACTH during CRH testing, n (%)	44 (33)	90 (67)
Peak ACTH during CRH testing, n (%)	43 (32)	91 (68)
Postoperative variables		
Basal levels of plasma ACTH after surgery, n (%)	56 (42)	78 (58)
Minimum serum cortisol after surgery, n (%)	77 (57)	57 (43)
24-hour urinary free cortisol after surgery, n (%)	30 (22)	104 (78)
Late-night salivary cortisol after surgery, n (%)	6 (4)	128 (96)
Serum cortisol after low dose DMX after surgery, n (%)	42 (31)	96 (69)

Table 11. Frequency of data available to different biochemical tests of hypercortisolism, pre- and postoperatively.

Another problem is the use of different assays for the different hormone tests. But not only the assays were different, also the methods of testing were sometimes different. For example, the test for suppression of serum cortisol was carried out using different quantities for the low dose dexamethasone test (1 mg vs. 2 mg) as well as the high dose dexamethasone test (8 mg vs. 16 mg), making the comparison of outcomes even more challenging.

Advantages of retrospective studies include the fact, that no additional efforts need to be shouldered by patients. Furthermore, these studies can be conducted at comparatively low financial expense. Diagnostic testing and therapy are already completed and only the findings need to be collected, which can be realised at relatively low cost. Retrospective studies also mostly do not raise any ethical concerns.

On the whole, given the relatively low prevalence of Cushing's disease (Ambrosi et al., 1990; Bolland et al., 2011; Etxabe & Vazquez, 1994; Lindholm et al., 2001; Steffensen et al., 2010; Valassi et al., 2011), retrospective, multicentric studies are the only realistically feasible study design, even if the exact impact of *USP8* status on the outcome of Cushing's disease is difficult to assess and quantify.

5.3 Discussion of findings

5.3.1 The prevalence of mutations in the *USP8* gene in ACTH-producing tumours

In this study, the prevalence of *USP8* mutations can be reported in a representative cohort of patients suffering from Cushing's disease. In the overall cohort, a prevalence of 36% was found, but it varied significantly depending on age and sex. Viewed on its own, the adults show a prevalence of 41% and when considering the female adults only, the prevalence rose to as high as 49%. In their initial report Reincke et al. reported a prevalence of 35% (6/17 corticotroph tumours) (Reincke, Sbiera, et al., 2015). In an analysis of a total of 120 patients suffering from Cushing's disease Ma et al. reported a higher prevalence of *USP8* mutations in Cushing's disease cases (75/120 corticotroph tumours; 62%) (Ma et al., 2015). Unlike the patients in this study, Ma et al. examined a cohort of primarily Chinese patients, and therefore ethnic diversity in the genetic background could pose as an explanation for the difference in prevalence. Interestingly enough, the highest mutation rate in our series was found in the samples from Brazil (5/9 cases, 56%). This finding could stress the theory of different mutation rates in different ethnicities. Thus, more research needs to be done to discover possible ethnic diversities in the prevalence of *USP8* mutations in corticotroph tumours.

All in all, subsequent multicentric studies showed a prevalence of *USP8* mutations between 31 – 40%, in line with the prevalence discovered in this study (Faucz et al., 2017; Hayashi et al., 2016; Ma et al., 2015; Reincke, Sbiera, et al., 2015; Song et al., 2016). Like Ma et al., also Song et al. showed a higher prevalence rate (55%) in a primarily Chinese cohort, albeit the cohort of Song et al. overlapped in parts with Ma and co-workers' cohort (Song et al., 2016). In contrast, Ballman et al. and Losa et al. both reported lower prevalence rates with 21.4% and 23.9% respectively (Ballmann et al., 2018; Losa et al., 2019). The lower overall *USP8* mutation frequency could be due to the fact that a high number of very small tumours were included (38% ≤ 5 mm in size) in the study conducted by Ballman and co-workers.

In general, a difference in ethnic background and the handling of diagnostics and inclusion criteria cannot be ruled out. Only further, larger studies with more varied series of patients suffering from Cushing's disease will be able to shed light on this apparent discrepancy.

5.3.2 Different types of *USP8* mutations

All the mutations in the *USP8* gene identified in this study were heterozygous and clustered into a hotspot region overlapping with the 14-3-3 binding motif. Eight different mutation types were found, none of them in the germline. To date, only one patient with a de novo germline *USP8* mutation has been reported. Cohen et al. described the case of a 16-year-old female with recurrent Cushing's disease besides multiple other severe medical problems, including developmental delay, hyperglycemia, dilated cardiomyopathy with congestive heart failure, chronic lung disease, chronic kidney disease, dysmorphic features, ichthyosiform hyperkeratosis and a previous history of hyperinsulinism and partial GH deficiency (M. Cohen et al., 2019). *USP8* knockout has been found to result in embryonic lethality. Conditional knockout adult mice die from liver failure, resulting from a major reduction or the absence of several growth factor tyrosine kinases, like the epidermal growth factor receptor (Niendorf et al., 2007). These findings hint towards a yet to be discovered multitude of signalling events that USP8 is involved in and emphasise the important role USP8 plays in EGFR signalling.

All of the mutations found in this study targeted either the Ser718 or the Pro720. Three highly prevalent mutations could be identified (the substitutions Pro720Arg and Ser718Pro as well as the deletion Ser718del), which account for the vast majority of mutated cases (87,5 %) [Figure 5]. Taking together the data from all studies focusing on *USP8* mutations in corticotroph adenomas, all *USP8* mutations so far described were somatic, heterozygous single point mutations, clustered in the mutational hotspot that is located in exon 14, with the only exception of the paediatric case with a germline mutation described by Cohen and co-workers (M. Cohen et al., 2019). It wards USP8 off 14-3-3 proteins that prevent the cleavage to a highly active C-terminal fragment (Reincke, Sbiera, et al., 2015). The substitutions Pro720Arg and Ser718Pro together with the deletion Ser718del accounted for the majority of all mutations. These data clearly identify the scope of USP8 mutations.

USP8-mediated deubiquitination prevents EGFR from lysosomal degradation by cleaving ubiquitin peptides from it and directing it back to the plasma membrane, where it can again play a role in signal transduction (Mizuno et al., 2005). Overexpression or mutation of *USP8* can lead to its hyperactivation, which reduces the ubiquitination level of EGFR and delays its degradation and therefore negatively regulates the rate of its down-regulation (Mizuno et al., 2005). EGFR is plentifully present in corticotroph tumours and as a major mitogenic factor, it is of special significance in corticotroph pathophysiology (Theodoropoulou et al., 2015). Interestingly, *in vitro* and *in vivo* studies showed that small molecule inhibitors displayed strong antisecretory and antiproliferative action and therefore mitigated Cushing's disease in animal models (Fukuoka et al., 2011; Kontogeorgos et al., 1996; Theodoropoulou et al., 2004). Thus, the development of Cushing's disease is the obvious corollary of prolonged EGFR activation and the consequential ACTH production as a result of activating *USP8* mutations in corticotroph cells. Corticotroph tumours harbouring a *USP8* mutation showed higher POMC transcription levels compared to their wild type counterparts (Hayashi et al., 2016). It is still an issue of controversy if *USP8* mutated corticotroph tumours also display higher EGFR levels (Hayashi et al., 2016; Ma et al., 2015).

Notwithstanding, functional assays have provided strong evidence on how the development of Cushing's disease can be traced back to mutations in the *USP8* gene (Ma et al., 2015; Perez-Rivas et al., 2015; Reincke, Sbiera, et al., 2015).

5.3.3 Age- and sex-related distribution of *USP8* mutations

In consensus with the initial report (Reincke, Sbiera, et al., 2015), mutations were predominantly found in female patients and, within the adult population, patients with *USP8* mutant tumours were younger than those with wild type lesions. Subsequent studies on *USP8* mutations in adrenocorticotropic pituitary tumours report a higher prevalence of *USP8* mutations in female patients suffering from Cushing's disease (Ballmann et al., 2018; Hayashi et al., 2016; Losa et al., 2019). The study conducted by Ma et al., that studied the prevalence of *USP8* mutations in different pituitary tumours in a primarily Chinese cohort (n of corticotroph ACTH-secreting tumours = 108), came to a similar conclusion (Ma et al., 2015). Although the difference in age between patients with and without *USP8* mutations did not reach significance in their study, a trend could be made out towards patients with *USP8* mutations being diagnosed at a younger age than patients with wild type tumours (36 vs. 40 years of age, respectively; p=0.08).

Even though purely speculative, a potentially growth-stimulating effect of oestrogens on *USP8* mutant corticotroph cells in the development of Cushing's disease could pose as an explanation for these observations (Zilio et al., 2014). The potential influence of sex steroids in the development of the disease is further implied by the different epidemiology of the pathology in children. A balanced sex ratio has been reported in unselected paediatric patients (Libuit et al., 2015), whereas a higher prevalence of Cushing's disease is reported among prepubertal male patients under the age of 10 years (Storr et al., 2004), while the percentage of female patients gradually increases during adolescence (Lonser et al., 2013; Storr et al., 2011), reaching a preponderance in post pubertal age. This may be seen in line with hormonal change, as females produce increasingly more oestrogens during and after puberty. Interestingly enough, ACTH producing tumours express oestrogen receptors (Chaidarun, Swearingen, & Alexander, 1998; Manoranjan et al., 2010). Furthermore, at least in vitro, oestradiol can

stimulate corticotroph proliferation, an effect which is conveyed by EGFR signalling (Oomizu et al., 2000).

The outcome of this study suggests a possibly lower frequency of *USP8* mutations in paediatric patients (17%). However, Faucz et al. enlarged the paediatric cohort, including a subgroup of twenty-four subjects that were part of the cohort of this study. In this larger paediatric cohort a frequency of somatic mutations in the *USP8* gene of 31% was identified and is therefore similar to the frequency shown in the adult population. Paediatric patients harbouring somatic *USP8* mutations were older at diagnosis and had a lower BMI compared to those without wild type *USP8*. Interestingly, recurrence of disease occurred only in patients harbouring *USP8* mutations (Faucz et al., 2017).

Nevertheless, further efforts need to be undertaken to properly identify the factors causing the sex- and age-related dispersal of *USP8* mutations in patients suffering from Cushing's disease.

5.3.4 *USP8* mutations and tumour size

Commonly, tumour size is associated with clinical remission. Patients with microadenomas are generally more likely to go into remission than those with macroadenomas (Cannavo et al., 2003; Colao, Boscaro, Ferone, & Casanueva, 2014; Dimopoulou et al., 2014; Esposito et al., 2006; Valassi et al., 2010). On the other hand, however, patients presenting with clinically and biochemically confirmed Cushing's disease with no visible lesion in preoperative magnetic resonance imaging, have lower remission rates than patients with visible microadenomas (Alexandraki et al., 2013; Esposito et al., 2006; Yamada et al., 2012).

In this series, histologically confirmed corticotroph tumours that were not visible in preoperative imaging and also large macroadenomas were both mostly wild type. Over 50% of tumours with mutations in the *USP8* gene had a size ranging between 8 and 16 mm. The tumours in female patients with *USP8* mutations tended to be larger than their wild type counterparts (8 vs. 10 mm, respectively). However, when compared to the other series conducted, average tumour size differed in each series and no tendency can be seen whether tumours with

mutations in the *USP8* gene are smaller or larger than the wild type ones. In contrast to this series, Ma et al. discovered a predominance of mutations in smaller tumours (<5 mm) (Ma et al., 2015). However, in line with this series, Ma et al. showed that in large tumours (>20 mm) wild type adenomas prevailed the *USP8* mutated tumours. Also the tumours harbouring *USP8* mutations discovered by Song and co-workers were significantly smaller in size than those with wild type *USP8* (Song et al., 2016). Similar to these primarily Chinese cohorts, Hayashi et al. showed that *USP8* mutated tumours were significantly smaller than wild type tumours in a primarily Japanese cohort (Hayashi et al., 2016). Therefore, the ethnic differences of the subjects may once again be a reason for the conflicting data on tumour size. In paediatric patients with varied ethnicity, but primarily Caucasian ethnicity, the tumour size in the two groups was similar. However, the results may have been biased, as the available samples were usually from larger tumours (Faucz et al., 2017).

All in all, these findings indicate that tumour growth is not affected by mutations in the *USP8* gene. This is in line with the findings of Reincke at al., who observed no further enhancement of the proliferative effects of USP8 in AtT-20 mouse cells when the *USP8* gene was mutated (Reincke, Sbiera, et al., 2015).

5.3.5 *USP8* mutations and biochemical hallmarks of hypercortisolism

As differences in preoperative hormonal parameters mostly did not reach significance, the focus is on the impact *USP8* mutations seem to have on the postoperative outcomes. In the small number of cases with available data (n = 30), the 24-hour urinary cortisol levels were postoperatively higher in patients harbouring a *USP8* mutation. In accordance with this finding, an inverse association between the development of adrenal insufficiency and the presence of *USP8* mutations could be observed, that was even more evident in younger patients. Postoperative hypocortisolism is a known indicator for long-term remission (Dimopoulou et al., 2014; Lodish, Dunn, Sinaii, Keil, & Stratakis, 2012; Pereira et al., 2003), although long-term biochemical and clinical follow-up is needed to detect late recurrences after initially successful transsphenoidal surgery (Patil et al., 2008; Yap, Turner, Adams, & Wass, 2002). These data may hint towards a worse outcome of transsphenoidal surgery in patients with *USP8*

mutant tumours compared to patients with wild type tumours, as patients with *USP8* mutant tumours could run a higher risk of recurrence. In line with these findings, Faucz et al. reported higher recurrence rates in paediatric *USP8* variant carriers, although the follow up was considerably short (median 17 months) (Faucz et al., 2017). Also in line with our findings, Albani et al. showed a significantly higher risk of recurrence after initial remission in *USP8* mutants (58% vs. 18 %) and a significantly earlier recurrence in this group (months 70, 44-97 95% CI vs. 102, 86-119 95% CI; P = 0.019) (Albani et al., 2018). Another study suggested a shorter mean recurrence period in *USP8* variant carriers (29 vs. 48 months) in a subset of patients suffering from Cushing's disease that suffered a recurrence. However, patients with only very short follow-up were included (Ma et al., 2015).

Conversely, compared to the late surgical outcome, the early surgical outcome seems to be similar in wild type and mutant *USP8* carriers or even more favourable in *USP8* variant carriers. Together with other multicentre studies, this study shows superimposable remission rates in the two groups (Albani et al., 2018; Ma et al., 2015). Losa et al. as well as Hayashi et al. even reported a higher likelihood of surgical remission in *USP8* variant carriers (Hayashi et al., 2016; Losa et al., 2019).

It is still uncertain in which way the *USP8* mutational status may have an effect on tumour recurrence. Higher postoperative 24-hour urinary cortisol levels and higher ACTH levels observed by Ma et al. in *USP8* variant tumours could be the reason for a more clinical overt syndrome and could lead to a quicker diagnosis of Cushing's disease (Ma et al., 2015). Indeed, results of in vitro studies indicated that *USP8* mutants trigger *POMC* transcription and therefore ACTH secretion more strongly than the wild type forms (Reincke, Sbiera, et al., 2015). Moreover, human corticotroph tumours harbouring *USP8* mutations showed higher levels of *POMC* transcription (Ma et al., 2015). The hypothesis of a more blatant clinical presentation is also consistent with the fact that a trend could be made out towards *USP8* mutated patients being diagnosed at a younger age than patients with wild type tumours (36 vs. 40 years of age, respectively; p=0.08). Also, Albani and co-workers found that patients with *USP8* variant corticotroph tumours were diagnosed significantly earlier (mean ± SD 46 ± 10 years vs. 53 ± 11 years; p=0.028). A subgroup (n=9) of the patients studied by Albani at al. (n=48),

however, were already included in this study (Albani et al., 2018). This supports the hypothesis that due to activating *USP8* mutations the tumours are more hormonally active and therefore remnant tumour tissue after transphenoidal surgery regains its hypersecretory function earlier than wild type *USP8* tumour remnants.

Taking these findings into account, it would be of crucial importance to monitor patients with *USP8* mutant tumours more closely during the first years after transphenoidal surgery in order to detect recurrences as early as possible.

5.3.6 Mutations in the *USP8* gene – a specific trait of ACTH-secreting tumours?

In this study, mutations in the *USP8* gene were found in 36% of the ACTH-secreting tumours, but were absent in the 11 silent (non-secreting) corticotroph tumours. No mutations in the *USP8* gene could be found in 2 Nelson's tumours, 14 somatotroph tumours, 10 lactotroph and 10 non-functioning tumours studied by Reincke at al. (Reincke, Sbiera, et al., 2015) and also Ma et al. did only find mutations in the *USP8* gene in ACTH-secreting pituitary tumours and none in 50 somatotroph, 50 lactotroph and 50 non-functioning pituitary tumours (Ma et al., 2015). Interestingly, Perez-Rivas et al. reported a similar prevalence of *USP8* mutations in Nelson's tumours (Perez-Rivas et al., 2018). Another study conducted by Perez-Rivas et al. shows that in contrary to the pituitary ACTH-secreting tumours, *USP8* mutations do not occur in ectopic ACTH-secreting tumours (Perez-Rivas et al., 2017). However, a recent study suggests that mutations in the *USP8* gene are not a specific trait of ACTH-secreting tumours, as Bujko and co-workers show that *USP8* mutations do not only occur in functioning but also in silent corticotroph tumours, albeit with low frequency (Bujko et al., 2019). These findings highlight the pleiotropic effects of *USP8*, as it is involved in a variety of molecular processes and not only in EGFR signalling (Dar, Wu, Lee, Shibata, & Dutta, 2014; Kim et al., 2018; Mukai et al., 2010; G. A. Smith et al., 2016; Wu, Yen, Irwin, Sweeney, & Carraway, 2004; Xia, Jia, Fan, Liu, & Jia, 2012).

Besides, mutations in genes formerly identified in the context of adrenal Cushing's syndrome were not described in any study focusing on *USP8*

mutations in pituitary ACTH-secreting tumours and so far, apart from mutated *USP8*, no other recurring mutations could be detected in corticotroph tumours (Song et al., 2016; Uzilov et al., 2017; Xiong & Ge, 2016).

Of note, mutations in the *USP8* gene seem to be a primate-specific trait of ACTH-secreting tumours, as *USP8* mutations could not be detected in tumours from a large cohort of dogs suffering from Cushing's disease (Sbiera et al., 2016).

All in all, these observations strongly suggest that *USP8* plays a starring role in the development of Cushing's disease.

5.3.7 *USP8* mutations and their clinical and pathological implications

As surgery alone often does not fully cure Cushing's disease, new therapeutic concepts are needed to specifically target the ACTH-secreting tumours and suppress ACTH production (Biller et al., 2008; Colao et al., 2014). The findings in this study together with the findings of Ma et al. clearly suggest that inhibiting the catalytic activity of USP8 might be a promising therapeutic approach for patients with Cushing's disease harbouring *USP8* mutations. This might be especially relevant for patients suffering from either residual or recurrent tumours.

Even before *USP8* mutations were discovered in ACTH-producing pituitary tumours, EGFR-targeted therapies were tested as a treatment for Cushing's disease. In 2011, Fukuoka et al. speculated that the EGFR could be a new target for the therapy of Cushing's disease, and consequently tested EGFR signalling in ACTH-secreting pituitary tumours. Gefitinib, a drug that is also effective in other cancers that overexpress EGFR, like pulmonary adenocarcinoma, is a tyrosine kinase inhibitor targeting the EGFR. In their study, Fukuoka et al. show, that treatment with gefitinib decreased both tumour size and corticosterone levels and therefore concluded that inhibiting EGFR signalling might be a novel strategy for treating Cushing's disease (Fukuoka et al., 2011). As there is strong evidence that mutations in the *USP8* gene lead to Cushing's disease through activation of EGFR signalling (Reincke, Sbiera, et al., 2015), anti-EGFR therapy poses a potential therapeutic approach for the treatment of Cushing's disease. Ma et al. succeeded in demonstrating that treatment with gefitinib significantly reduces ACTH secretion in primary *USP8* mutated corticotroph tumour cells, but not in

wild type cells (Ma et al., 2015) and therefore prove that the inhibition of USP8 and EGFR are promising therapeutic strategies for treating patients with Cushing's disease, harbouring a *USP8* mutation.

Deubiquitinase inhibitors are in early development. Jian et al. presented a small molecule USP8 inhibitor that displayed anti-secretory and anti-proliferative action and even induced apoptosis in immortalized murine corticotroph cells (Jian et al., 2016). USP8 inhibitor DUBs-IN-2 (9-oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile) was found to have potent effects on ACTH production and cell proliferation in mouse corticotroph tumour cells (Kageyama, Asari, Sugimoto, Niioka, & Daimon, 2020). These findings emphasise that USP8 could pose as a novel, promising pharmaceutical target.

Somatostatin receptor 5 (SSTR5) and dopamine receptor D2 (DRD2) can be found in the majority of corticotroph tumours. Treatment with dopamine agonists and somatostatin analogues decreases ACTH secretion in vitro (de Bruin et al., 2009; Hofland et al., 2005; Pivonello et al., 2004). Another auspicious observation is that *USP8* mutant corticotroph tumours displayed higher levels of SSTR5 and O6-methylguanine DNA methyltransferase (MGMT) (Hayashi et al., 2016). This could also yield potential therapeutic implications, as these tumours could respond favourably to SSTR5- targeting somatostatin analogues and temozolomide. Treatment with the somatostatin analogue pasireotide alone or in combination with the DRD2 agonist cabergoline led to reduced cortisol levels and is already approved for the treatment of Cushing's disease (Feelders et al., 2010).

Collectively, these data will contribute to the development of innovative, new therapies for Cushing's disease. Therapeutic strategies targeting USP8 and/or EGFR might even become the new first-line treatment for *USP8* mutated ACTH-producing tumours, particularly for the ones that remain clinically active despite surgical therapy or for disease recurrences.

6 Abstract

6.1 Abstract in English

Context: Somatic mutations in the ubiquitin-specific protease *USP8* gene have recently been reported in a small series of tumours of patients with Cushing's disease (Reincke, Sbiera, et al., 2015).

Objective: To identify not only the prevalence of *USP8* mutations, but also the genotype-phenotype correlation in an extensive series of patients diagnosed with Cushing's disease.

Design: A multicentric, retrospective, genetic analysis of 134 functioning and 11 silent corticotroph tumours was conducted by means of Sanger sequencing. Clinical and biochemical features were gathered and analysed within the context of the mutation status of *USP8*.

Patient cohort: 145 patients who underwent transsphenoidal surgery for an ACTH-producing or a silent pituitary tumour.

Results: Somatic mutations in the *USP8* gene were discovered in 48 (36%) of the pituitary tumours of patients with Cushing's disease, but in none of the 11 silent corticotroph tumours. The prevalence in adults was higher than in paediatric cases (41% vs. 17%) and likewise higher in females than in males (43% vs. 17%). Adults with *USP8*-mutated tumours were diagnosed at an earlier age than those with wild type lesions (36 vs. 44 years). Mutations were particularly found in tumours of 10 ±7 mm in size and were inversely associated with the development of postoperative adrenal insufficiency. All of the mutations detected, affected the residues Ser718 or Pro720, including five newly discovered alterations.

Conclusions: Mutations in the *USP8* gene can be frequently found in pituitary tumours causing Cushing's disease, in particular in those of female adult patients diagnosed at a younger age.

6.2 Abstract in German

Kontext: Unlängst wurden somatische Mutationen im Ubiquitin-spezifischen Protease (*USP8*)-Gen in corticotropen Hypophysentumoren entdeckt. Jedoch war nur eine kleine Anzahl an Patienten mit Morbus Cushing untersucht worden (Reincke, Sbiera, et al., 2015).

Zielsetzung: In einer umfangreicheren Kohorte von Patienten mit Morbus Cushing soll nicht nur die Prävalenz von *USP8*-Mutationen ermittelt, sondern auch die Genotyp-Phänotyp-Korrelation näher untersucht werden.

Studiendesign: Es wurde eine multizentrische, retrospektive, genetische Analyse von 134 hormonaktiven und 11 hormoninaktiven corticotropen Tumoren mittels Sanger–Sequenzierung durchgeführt. Informationen über klinische und biochemische Merkmale wurden zusammengetragen und im Kontext des Mutationsstatus von *USP8* analysiert.

Patientenkohorte: 145 Patienten, die sich einer transsphenoidalen Operation wegen eines ACTH-produzierenden oder endokrin inaktiven Hypophysentumors unterzogen hatten.

Ergebnisse: Somatische Mutationen im *USP8*-Gen wurden bei 48 (36%) der Hypophysentumoren von Patienten mit Morbus Cushing entdeckt, jedoch bei keinem der 11 hormoninaktiven corticotropen Tumoren. Die Prävalenz bei Erwachsenen war höher als bei pädiatrischen Fällen (41% gegenüber 17%) und bei Frauen höher als bei Männern (43% gegenüber 17%). Morbus Cushing wurde bei Erwachsenen mit *USP8*-mutierten Tumoren in einem jüngeren Alter diagnostiziert als bei Erwachsenen mit Wildtyp-Läsionen (36 gegenüber 44 Jahre). Mutationen wurden insbesondere bei Tumoren mit einer Größe von 10 ± 7 mm gefunden und stehen in einem umgekehrten Zusammenhang mit der Entwicklung einer postoperativen Nebenniereninsuffizienz. Alle nachgewiesenen Mutationen, einschließlich fünf neu entdeckter Mutationen, betrafen Ser718 oder Pro720.

Schlussfolgerung: Mutationen im *USP8*-Gen finden sich häufig bei Hypophysentumoren, die Morbus Cushing verursachen, insbesondere bei erwachsenen Patientinnen, bei denen diese Diagnose in einem jüngeren Alter gestellt wurde.

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8 List of Abbreviations and Acronyms

ACTH	adrenocorticotropic hormone
ADH	antidiuretic hormone
BA	bilateral adrenalectomy
BIPSS	bilateral inferior petrosal sinus sampling
BMI	body mass index, body mass (kg)/ (body height (m))²
CD	Cushing's disease
CI	confidence interval
CNS	central nervous system
CRH	corticotropin-releasing hormone
CS	Cushing's syndrome
DMX	dexamethasone
DNA	deoxyribunucleic acid
DRD2	dopamine receptor D2
DUB	deubiquitinase
DUBs-IN-2	(9-oxo-9H-indeno[1,2-b]pyrazine-2,3- dicarbonitrile)
ECG	electrocardiogram
FSH	follicle-stimulating hormone
GH	Growth hormone
HPA	hypothalamic-pituitary-adrenal axis
IQR	interquartile range
LH	luteinizing hormone
MAPK	mitogen-activated protein kinase
MC2R	melanocortin receptor 2
MGMT	O6-methylguanine DNA methyltransferase
MIT	microtubule-interacting and trafficking domain

MRI	magnetic resonance imaging
PCR	polymerase chain reaction
POMC	pro-opiomelanocortin
RHOD	rhodanese-like domain
SBM	SH3-binding motif
SD	standard deviation
SSTR5	somatostatin receptor 5
TSH	thyroid-stimulating hormone
UFC	urinary free cortisol
USP8	ubiquitin-specific protease 8

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