Universitat Autònoma de Barcelona

Pituitary disease in the context of unusual situations

Francisca Caimari Palou

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DOCTORAL THESIS

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ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
AIP	Aryl hydrocarbon receptor interacting protein
AhR	Aryl hydrocarbon receptor
AP	Anterior pituitary
cAMP	Cyclic adenosine monophosphate
cAMP/PKA	cAMP-dependent protein kinase A
CBG	Corticosteroid-binding globulin
CD	Cushing´s disease
CI	Confidence Interval
CRH	Corticotropin-releasing hormone
CS	Cushing's syndrome
CT	Computed tomography
DHEAS	Dehydroepiandrosterone sulphate
DM	Diabetes Mellitus
FIPA	Familial Isolated Pituitary Adenoma
FSH	Follicle-stimulating hormone
GDM	Gestational Diabetes Mellitus
Gαi	G inhibitory protein
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
HPA	Hypothalamus-pituitary-adrenal
HT	Hypertension
IGF-1	Insulin-like growth factor
LH	Luteinizing hormone
MEN1	Multiple endocrine neoplasia type 1
MEN4	Multiple endocrine neoplasia type 4
MRI	Magnetic resonance imaging

NFPA	Non-functioning pituitary adenoma
OR	Odds ratio
PA	Pituitary adenoma
PDE4	Type 4 phosphodiesterase
PKA	Protein kinase A
PitNET	Pituitary neuroendocrine tumor
PRL	Prolactin
SDH	Succinate dehydrogenase
SSA	Somatostatin analog
SSTR2	Somatostatin receptor subtype 2
TCDD	Tetrachlorodibenzo-p-dioxin
TPR	Tetratricopeptide repeat
TSH	Thyrotropin
TSS	Transsphenoidal surgery
UFC	Urinary free cortisol
XLAG	X-linked acrogigantism

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SUMMARY

Cushing's syndrome (CS) and familial isolated pituitary adenomas (FIPA) are rare diseases, present in less than 1-9 cases/100.000 of the general population. Pregnancy in women with a diagnosis of CS is an extremely rare event and its diagnosis and treatment are a real medical challenge. The difficulties in diagnosis are related to the resemblance of symptoms of CS and those of pregnancy, and to the complex interpretation of the screening tests. Importantly, the etiology of CS in pregnancy differs from non-pregnant status as the adrenal origin is the most frequent in up to 60% of the cases. There is no consensus as to the most effective treatment in these circumstances in terms of improving maternal and fetal outcomes, as there are no studies comparing the different modalities of treatment for CS in pregnancy.

On the other hand, clinically relevant pituitary tumors during childhood are also a rare medical condition. These cases can be related to germline mutations predisposing to pituitary tumorigenesis, often in a familial setting, including classical tumor predisposition syndromes such as multiple endocrine neoplasia type 1 or 4 syndromes, as well as FIPA, a heterogeneous condition of patients with unknown genetic cause, patients with mutation in *AIP* and X-linked acrogigantism, often leading to pituitary gigantism.

This thesis is composed of two studies. The first study aimed to investigate whether the etiology of CS in pregnancy determined a different impact on the fetal/newborn and maternal outcomes. A systematic review of cases published in the literature was performed from January 1952 to April 2015 including the words "Cushing AND pregnancy". Two-hundred and sixty-three pregnancies with active CS during pregnancy and with a history of CS, but treated and cured hypercortisolism at the time of gestation, were included in the study. Adrenal adenoma was the main cause of active CS during pregnancy (44.1%). Women with active CS had more pregnancy-related complications like gestational diabetes mellitus, gestational hypertension and preeclampsia, than those with cured disease. The proportion of fetal loss in active CS was higher than in cured CS (23.7 vs 8.5%, $p=0.021$), as well as global fetal morbidity (33.6 vs 4.9%, $p<0.001$). Patients with active CS, especially in pregnancy-induced CS, experienced more problems in pregnancy and had the worst fetal prognosis in comparison to other causes. Diagnosis

of CS during pregnancy was also associated with worse overall fetal morbimortality. Finally, both medical treatment and surgery during pregnancy appeared to be protective in avoiding fetal loss.

The second study aimed to develop and validate a reliable risk category system for *AIP* mutations in patients with pituitary adenomas (PA). An international cohort of 2227 subjects were consecutively recruited between 2007 and 2016, including patients with PAs (familial and sporadic) and their relatives. 1405 patients had a pituitary tumor, of which 43% had a positive family history, 55.5% had somatotropinomas and 81.5% presented with macroadenoma. Overall, 134 patients had an *AIP* mutation (9.5%). Four independent predictors for the presence of an *AIP* mutation were identified and used to develop the risk category system: age of onset, family history, growth hormone excess tumor type and large tumor size. The risk category system classified patients into low-risk (<5% risk of *AIP* mutation), moderate- (5-19%) and high-risk (>20% risk). Excellent discrimination (c-statistic=0.87) and internal validation were achieved, indicating it can reliably estimate the individual risk of carrying an *AIP* mutation for a given patient.

RESUMEN

El síndrome de Cushing (SC) y los adenomas hipofisarios aislados familiares (FIPA, del inglés familial isolated pituitary adenomas), son enfermedades raras que afectan a menos de 1-9 casos/100.000 habitantes. El embarazo en mujeres que han sido diagnosticadas con SC es extremadamente infrecuente y, tanto su diagnóstico como el tratamiento, suponen un verdadero reto médico. La dificultad del diagnóstico recae principalmente en la similitud de los síntomas del SC y del embarazo y en la complejidad de la interpretación de las pruebas diagnósticas en este contexto. La etiología del SC en estas pacientes difiere de aquellos pacientes con SC en la población general, pues en el primer grupo el orígen suprarrenal es la causa más frecuente que se da en hasta el 60% de los casos. No existe un consenso en cuanto al tratamiento más efectivo para mejorar el pronóstico materno y fetal, ya que hasta el momento no existen estudios que comparen las diferentes modalidades de tratamiento del SC durante el embarazo.

Por otro lado, los tumores hipofisarios clínicamente relevantes, diagnosticados en la infancia, son también una condición médica infrecuente. Estos casos a menudo se dan junto con mutaciones germinales que predisponen al desarrollo de tumores hipofisarios, habitualmente en un marco familiar, como, por ejemplo, ocurre en los síndromes clásicos como la neoplasia endocrina múltiple tipo 1 o 4, así como FIPA, una condición heterogénea de pacientes que incluye a aquellos sin causa genética conocida, a pacientes con mutaciones en *AIP* y al acrogigantismo ligado al cromosoma X, todas ellas siendo una causa frecuente de gigantismo de origen hipofisario.

Esta tesis comprende dos estudios. El primero tiene el objetivo de investigar si la etiología del SC durante el embarazo constituye un impacto diferente en el pronóstico fetal y materno. Para ello se realizó una revisión sistemática de los casos publicados en la literatura entre enero de 1952 y abril de 2015, incluyendo las palabras “Cushing AND pregnancy”. Se incluyeron doscientos sesenta y tres embarazos de pacientes con SC activo durante el embarazo y pacientes con historia de SC curadas en el momento de la gestación. La causa principal de SC activo durante el embarazo fue el adenoma suprarrenal (44.1%). Aquellas mujeres con SC activo presentaron más complicaciones durante el embarazo en comparación con aquellas con SC curado, tales como diabetes

gestacional, hipertensión y preeclampsia. El porcentaje de pérdida fetal fue mayor en aquellas pacientes con SC activo, en comparación con las curadas (23.7 vs 8.5%, p=0.021), así como la morbilidad global fetal (33.6 vs 4.9%, p<0.001). En comparación con otras causas de SC, las pacientes con diagnóstico de SC inducido por el embarazo presentaron más probemas durante la gestación y tuvieron peor pronóstico fetal. El diagnóstico de SC durante el embarazo también se asoció con mayor morbi-mortalidad fetal. Finalmente, tanto el tratamiento médico como el quirúrgico, demostraron ser efectivos frente a la mortalidad fetal.

El segundo estudio tiene como objetivo desarrollar y validar una escala de riesgo para detectar pacientes con tumores hipofisarios portadores de mutaciones en el gen *AIP*. Se incluyeron de forma consecutiva una cohorte internacional de 2227 sujetos entre el año 2007 y 2016, incluyendo pacientes con tumores hipofisarios (familiares y esporádicos) y sus familiares. 1405 pacientes tenían un tumor hipofisario, de los cuales un 43% con historia familiar, 55.5% eran somatotropinomas y 81.5% macroadenomas. Se detectaron mutaciones en *AIP* en 134 pacientes (9.5%). Se identificaron cuatro predictores independientes para la presencia de mutaciones en *AIP,* los cuales se utilizaron para el desarrollo de la escala de riesgo: la edad de aparición de síntomas, la historia familiar, los tumores hipofisarios secretores de hormona de crecimiento y la presencia de macroadenoma. Esta escala de riesgo clasifica a los pacientes en bajo riesgo (<5% riesgo de mutación en *AIP*), moderado- (5-19%) y alto riesgo (>20%). El estadístico 'c' obtenido (0.87) indica una excelente discriminación del modelo, el cual se evaluó mediante el método de validación interna, indicando la fiabilidad de la estimación del riesgo individual de portar una mutación en *AIP*.

BACKGROUND

1. Pituitary gland

The pituitary gland is an endocrine organ located in the pituitary fossa or sella turcica, a bony depression of the sphenoid bone. It weights approximately 400-900 mg and measures 12 mm in transverse and 8 mm in anterior-posterior diameter (1,2), although physiologic hyperplasia occurs in pregnancy and lactation (3).

Various structures surround the endocrine gland: anteriorly the optic chiasma, posteriorly, the mammillary bodies, superiorly, the diaphragma sellae, inferiorly the sphenoid sinuses, and laterally the cavernous sinuses. A fold of dura matter covers the pituitary and has an opening to allow for the infundibulum or pituitary stalk to pass through, allowing a connection between the pituitary and the median eminence of the hypothalamus (4,5).

The pituitary gland is formed by an anterior and a posterior lobe, which have a different embryonic origin. The anterior pituitary (AP) comprises the anterior and intermediate lobe, and derives from the Rathkes's pouch, an invagination of the oral ectoderm. The posterior pituitary has a neural origin and with the pituitary stalk both derive from the ventral diencephalon (1).

After embryogenesis, the AP is formed by two main cell types, the folliculostellate cells and the hormone-secreting cells. These hormone-secreting cells will release growth hormone (GH), prolactin (PRL), adrenocorticotropin (ACTH), thyrotropin (TSH) and gonadotropin hormones (luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) (2).

On the other hand, the posterior pituitary gland is a neural tissue composed of the hypothalamic magnocellular neuron distal axons. The posterior pituitary secretes antidiuretic and oxytocin hormones which are synthesized in the hypothalamus and released into the neurohypophyseal capillaries which surround the gland. Antidiuretic hormone is synthesized in the supraoptic nuclei of the hypothalamus while oxytocin is synthesized in the paraventricular nuclei of the hypothalamus (2,6,7).

2. Pituitary adenomas

2.1. Definition and classification

Pituitary adenomas (PAs) are benign lesions arising from the AP. Most studies suggest they are monoclonal neoplasms in origin (8).

Typically, they are classified according to their size as micro and macroadenomas. Microadenomas are tumors less than 1 cm in diameter, mostly restricted to the sella turcica. Around 40% of all PAs are macroadenomas, often compressing the optic chiasm and the pituitary stalk, as well as invading areas around the pituitary gland such as the cavernous sinus, the suprasellar area or the sphenoid sinus (9).

PAs can be classified according to their hormonal production; however, the 2017 World Health Organization (WHO) classification proposed to classify adenomas according to their pituitary linages using the essential transcription factors for cell differentiation of the AP: PIT-1, leading differentiation for somatotroph, lactotroph and thyrotroph cells, SF-1, for gonadotroph cell and T-PIT, driving the corticotroph cell differentiation (10). With this new concept, PAs are classified as somatotroph adenomas, corticotroph adenomas, lactotroph adenomas, thyrotroph adenomas and gonadotroph adenomas. Most gonadotroph adenomas are clinically non-functioning tumors that lack hormone overproduction. Null-cell adenomas, representing a tiny proportion of pituitary adenomas, defined as that tumors that exhibit immunoreactivity neither for pituitary hormones, nor for transcription factors and clinically they present as non-functioning adenomas (10,11). Plurihormonal adenomas, defined as tumors that produced more than one pituitary hormone, with the exception of the combination of GH/PRL and LH/FSH.

Patients with PAs have a high disease burden as they can present symptoms due to hormonal disturbances due to over but also underproduction of hormones, and compression symptoms secondary to local invasion that can lead to hypopituitarism and visual field defects, with a potential to lead to severe long-lasting consequences.

PAs generally present as slowly growing lesions with low mitotic rate and Ki-67 labelling index (12). They are usually resistant to malignant transformation and display variable

propensities for proliferative and invasive behavior (13) that may be not entirely benign and can cause significant morbidity, even when they are not metastatic. Hence, it has been recently proposed a new terminology for pituitary tumors, pituitary neuroendocrine tumor (PitNET), which recognizes the highly variable impact of these tumors on patients (14).

Although in the majority of the sporadic cases, the exact molecular pathogenesis remains unknown, a number of different molecular mechanisms leading to PAs have been identified (15). Pituitary tumor formation is thought to be a multi-step process, whereby cells transform as the result of somatic or inherited mutations, such as aberrant loss of tumor suppressors, overexpression of oncogenes such as activating *GNAS* mutations or *USP8* gene causing activation of the EGF signaling pathway (13,16), results of epigenetic changes as dysregulation of cell cycle or altered growth factors and promotion of cellular proliferation and abnormal intrapituitary microenvironment (17,18).

2.2. Epidemiology

Although most of PAs are small lesions and frequently incidental findings (19), clinically relevant PAs account for 1:1000 in the general population (20,21). Data derived from autopsies and radiologic imaging studies have shown that PAs are estimated to be present in 17% of the general population and account for 10-15% of all intracranial tumors (19,22), representing the third most-frequent intracranial tumor type after meningiomas and gliomas (21).

PAs are more frequent in women with a ratio women:men of 2.5:1 (23); however, females have a lower proportion of macroadenomas than males (23). The median age at diagnosis in different studies is around 40 years old, and the lactotroph adenomas are the most frequent tumors, followed by non-functioning pituitary adenomas (NFPA) (mostly silent gonadotroph but also silent corticotroph adenomas), somatotropinomas and corticotropinomas, while thyrotropinomas, clinically active gonadotropinomas and null-cell adenomas are rarer (11,20,23,24).

3. Pituitary disease in unusual situations: pregnancy and childhood

3.1. Pregnancy

Fertility is often impaired in women with PAs as the gonadotrophic axis is frequently compromised, either due to the mass effect of macroadenomas or as a result of abnormal secretion of hormones irrespective of tumor size (25). In summary, hyperprolactinemia can cause galactorrhea, oligo-amenorrhea, anovulation and, consequently, infertility (26). Patients with GH excess can present with gonadotropin deficiency or hyperprolactinemia, insulin resistance and polycystic ovary syndrome (27) and cortisol excess in patients with Cushing's syndrome can impair pituitary gonadotropin secretion, causing anovulation and abnormal menstrual periods (28), hence impairing fertility.

3.2. Childhood

Although PAs are common, secretory adenomas like ACTH or GH secreting are rare (19,21). Moreover, PAs are typically presenting at median age and their presence during childhood is unusual, representing 3% of all intracranial neoplasms in children, and 5% of all PAs (29).

Nevertheless, the frequency of different types of PAs varies according to age and sex (30). Children present predominantly secreting tumors such as prolactinomas, ACTH- and GH- secreting tumors (29,31). ACTH-secreting adenomas are most common before puberty, and prolactinomas during and after puberty (30,32). The median age of presentation in children is 15 years, being more frequent in female children up to 2:1 (29,31). The majority of patients presents with headaches, visual disturbances and menstrual dysfunction in females (31). Importantly, an association with germline aryl hydrocarbon receptor interacting protein gene (*AIP)* mutations or genetic syndromes such as multiple endocrine neoplasia type 1 (MEN1), have to be considered in early onset PAs, as in this context the age of presentation is much lower than in sporadic cases and they can be resistant to conventional therapy (33–35).

The following sections will focus on two clinically unusual situations of PAs: 1) Cushing´s syndrome in the course of pregnancy and 2) PAs in the context of germline *AIP* mutations, predisposing to tumors mostly arising during childhood.

4. Pituitary disease in unusual situations (I): Cushing's syndrome in pregnancy

Cushing's syndrome (CS) is a disorder caused by prolonged exposure to cortisol excess. CS is more common in women (36) and the incidence ranges from 1.7 to 2.4 per million population per year (37,38). CS is considered a rare disease, but recent studies have suggested a somewhat higher prevalence in specific, at-risk populations including patients with type 2 diabetes mellitus (DM), hypertension, and osteoporosis (39). The diagnosis of CS can be often challenging, since other conditions like pregnancy or the metabolic syndrome can present with some of the Cushing symptoms (40). Importantly, pregnancy is a physiological cause of hypercortisolism and the simultaneous presence of CS and pregnancy is an extremely rare situation (41).

4.1. The physiology of the hypothalamus-pituitary-adrenal axis during pregnancy

During normal gestation, the maternal hypothalamic-pituitary-adrenal (HPA) axis is significantly altered. There is a gradual increase of the total plasma cortisol and free cortisol (42), explained due to the secretion of corticotropin-releasing hormone (CRH) by the placenta, which acts as a neuroendocrine organ (43), and due to the increase of the estrogens in pregnancy that stimulates the production of hepatic corticosteroid-binding globulin (CBG), resulting in a rise in total cortisol levels (44,45).

The HPA axis is mainly composed by the action of the CRH, ACTH and cortisol, leading the stress-response. In physiologically non-pregnant conditions, CRH is secreted by the paraventricular nucleus in the hypothalamus and stimulates the secretion of ACTH from the pituitary gland. ACTH also stimulates the secretion of cortisol from the adrenal gland, which regulates the hypothalamus and pituitary secretion by negative feedback (**Figure 1**).

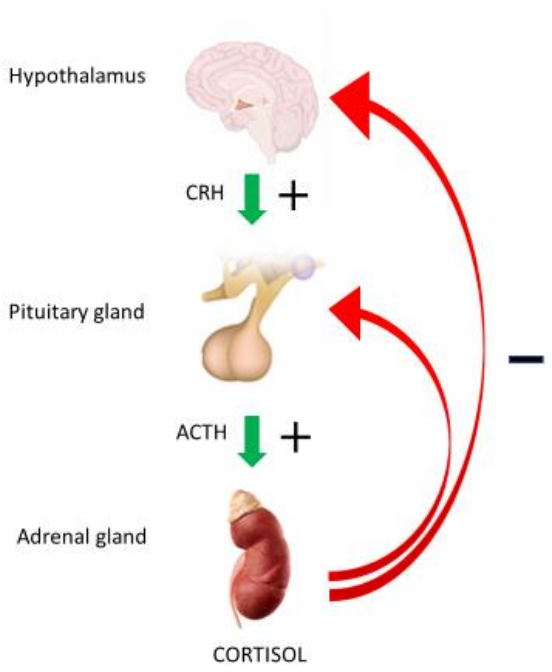

Figure 1. Hypothalamic-pituitary-adrenal axis.

Corticotropin-releasing hormone (CRH) is released from paraventricular neurons as well as supraoptic and arcuate nuclei and limbic system. CRH stimulate the secretion of corticotropin (ACTH) from the anterior pituitary gland. ACTH in turns acts on the adrenal cortex, which produces glucocorticoid hormones (mainly cortisol) in response to stimulation by ACTH. Cortisol in turn act back on the hypothalamus and pituitary (to suppress CRH and ACTH production) in a negative feedback cycle.

In pregnancy, the placenta also secretes CRH which is involved in the implantation of fertilized egg, maternal tolerance to the fetus, initiation of parturition (46–48), lung maturation and brain development (28,49–52). Plasma CRH increases progressively during pregnancy and stimulates both the maternal pituitary and adrenal gland, increasing the cortisol production. On the other hand, CRH receptors are also found in the uterus, fetal pituitary and fetal adrenal glands (51). CRH acts on the fetal adrenal to produce dehydroepiandrosterone sulphate (DHEAS), which will be converted to estrogen by the placenta, and also increases the release cortisol through stimulation of the fetal pituitary gland (50). Closing the loop, placental CRH expression is increased by the cortisol produced from the fetal and maternal adrenal gland, generating a positive feedback system that ends in increasing placental production of estrogen via conversion of DHEAS (42) (**Figure 2**).

Differently to non-pregnant status, plasma ACTH levels are not subject to normal feedback control regardless of the rising levels of cortisol, estrogen and progesterone (53), as its secretion from the placenta is induced by CRH in a dose-dependent fashion (54). Consequently, total plasma cortisol, CBG and 24-hour urinary free cortisol (UFC) continuously rise during gestation, with maximum levels at the third trimester. Importantly, despite of all changes in HPA axis in pregnancy, the diurnal rhythm of cortisol secretion is maintained throughout pregnancy (44,45,55).

Cortisol is transferred from the mother to the fetus via the placenta. Although the glucocorticoids levels are 5-10 times higher in the mother than in the fetus (56,57), the latter is protected from the high levels of cortisol due to the action of the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), that converts cortisol to an inactive form of glucocorticoid (cortisone) (44,58). In late gestation, there is a reversal of this reaction in the uterus, and cortisol can be converted from inactive cortisone by the enzyme HSD11B1 in the chorion trophoblasts and amnion epithelium (57,59). Additionally, cortisol can be produced by the fetal adrenal cortex, which produces cortisol de novo using cholesterol from around week 30 of pregnancy and previously using progesterone as a precursor (60).

Postnatally, the function of the HPA axis gradually returns to its pre-pregnant state (44). CRH levels, estriol and progesterone fall rapidly up to day 6 after delivery, whereas cortisol levels fall modestly. ACTH concentrations decline up to day 3 post-delivery and increase thereafter up to day 6. The insensitivity of plasma cortisol to glucocorticoid inhibition persists beyond normal pregnancy in a significant proportion of healthy women for two to three weeks after birth and returns to normal responsiveness usually by the 5th postnatal week (61). Plasma ACTH response to iv bolus of CRH is abnormally low at 3 and 6 weeks postpartum, but returns to normal by 12 weeks postpartum, whereas the mean plasma cortisol response to CRH is at the upper limit of normal at until 12 weeks postpartum (62).

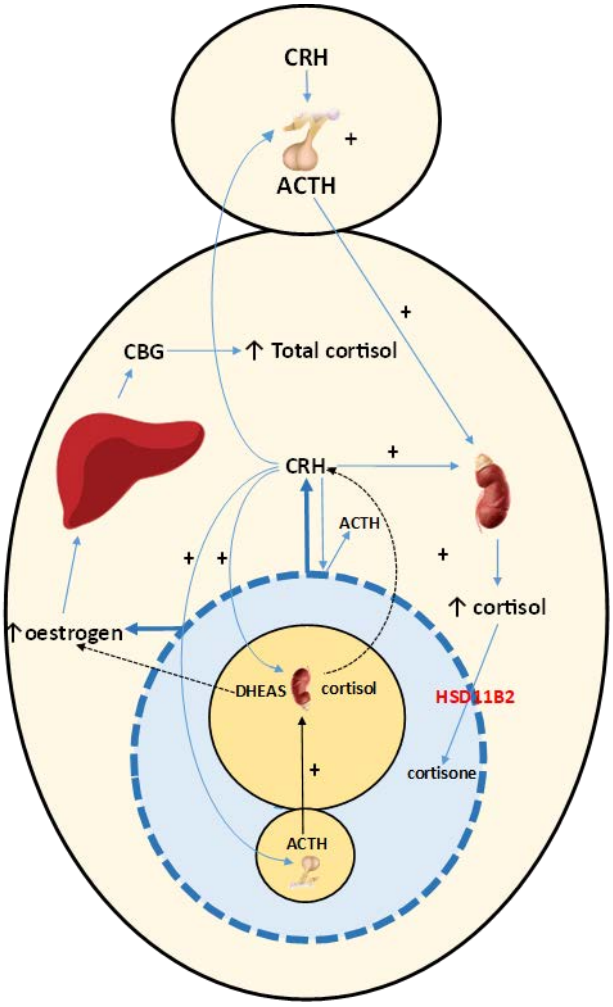

Figure 2. Hypothalamic–pituitary–adrenal axis during normal pregnancy.

The placental works as an endocrine organ secreting estrogen, CRH and ACTH. The placental production of estrogen and the conversion from fetal DHEAS, lead to the increased of the CBG with a rise in total cortisol levels. Placental CRH increases the plasma cortisol though the stimulation of the maternal and fetal pituitary gland and maternal and fetal adrenal gland. In consequence, the levels of total plasma cortisol and plasma free cortisol increase significantly throughout gestation. The placental enzyme HSD11B2 protects the fetus from excess of cortisol due to the inactivation of cortisol to cortisone. The placenta is represented by a staggered blue line. Black arrows indicate fetal origin and blue arrows maternal origin.

4.2. Etiology of Cushing's syndrome in pregnancy

CS can be divided in ACTH-dependent, due to pituitary or non-pituitary ACTH-secreting tumors, or ACTH-independent, due to an adrenal source. Rarely ectopic CRH secretion by a neuroendocrine tumor can also cause CS. In non-pregnant patients, 85% of CS are caused by ACTH-dependent causes, and the other 15% are caused by ACTH-independent adrenal disorders (**Table 1**) (63–65), although the most common cause of CS is

exogenous glucocorticoids (iatrogenic CS), due to prolonged use of supraphysiological doses of corticosteroids due to another medical condition (65).

Table 1. Etiology of the endogenous Cushing's syndrome

ACTH-dependent (85%)
• Cushing's disease (corticotroph pituitary adenoma) (70%) • Ectopic ACTH syndrome (10%) • Unknown source of ACTH (5%)
ACTH-independent (15%)
• Adrenal adenomas (10%) • Adrenal carcinoma (5%) • Other rarer conditions (bilateral macronodular adrenal hyperplasia, McCune-Albright syndrome and primary pigmented nodular adrenal disease*)*

In contrast, the proportion of patients with primary adrenal causes of CS is increased in pregnancy (66), accounting for approximately 50% of cases (mainly benign adenomas). Therefore, Cushing's disease (CD) is less common in pregnancy compared to general population (30% vs 70%, respectively) (41,67).

Ectopic ACTH-secretion in the setting of pregnancy has been reported in the literature including so far pheochromocytoma (68–70), ACTH-secreting islet cell tumor (71), thymic neuroendocrine carcinoma (72) and small cell carcinoma of the uterine cervix (73). A unique cause of CS occurring during pregnancy is the associated with the aberrant expression of LHCG receptor on primary adrenocortical tumor or hyperplasia. These group of patients are characterized for a spontaneous remission of CS after pregnancy and the absence of lesions in the pituitary or adrenal gland (74–80). A patient with CS exacerbation during pregnancy secondary to a different mechanism resulting from the placental-derived ACTH stimulation of MC2 receptors on the adrenocortical adenoma has recently been described (81).

The increased incidence of adrenal CS in pregnancy is not well understood. It is plausible that patients with adrenal cause are less androgenic than CD, as it has been suggested that adrenal adenomas mainly produce excess of cortisol, but CD produce both cortisol and androgens where anovulation may be more prevalent (66,82). Of note, pregnancy is a rare event in any form of CS, as patients with CS present with high incidence of oligo-amenorrhea (70-85%) (36) due to prolonged excess of cortisol excess impairs the action of gonadotropins on the gonads and the release from the hypothalamus of gonadotropin-releasing hormone (GnRH) (36).

4.3. The diagnosis of Cushing's syndrome in pregnancy

The diagnosis of CS in pregnancy is a medical challenge as there are resemblances of clinical features between CS and normal pregnancy. Additionally, diagnostic tests used in the screening and confirmation of CS can be misread due to the alteration of the HPA axis during normal pregnancy.

Patients with CS in the context of pregnancy usually presents symptoms of DM, hypertension, weight gain and striae, although these clinical features can also be present in normal pregnancy. However, the presence of osteoporosis, deep purple striae, dorsocervical fat pad and muscular weakness are more specific of CS rather than normal pregnancy, especially if the clinical context is suggestive of CS (83,84).

The diagnostic value of the laboratory screening tests for CS during pregnancy are summarized in **Table 2**. Two tests are used as confirmatory tests in the majority of patients reported in the literature with CD: the CRH test with the expected ACTH and cortisol response and the high-dose dexamethasone suppression test with a 50% reduction of cortisol (28).

Pituitary magnetic resonance imaging (MRI) with gadolinium contrast is the preferable imaging-test used to identify pituitary tumors; however, the safety of contrast during pregnancy is questionable. Importantly, small microadenomas may not be identified in non-contrast MRI and physiological enlargement of the pituitary gland in pregnancy should be considered in the differential diagnosis (85,86). To identify adrenal tumors,

abdominal ultrasound could be used as the sensitivity of the test is fairly good in these type of tumors (86). In contrast, computed tomography (CT) scans should be avoided as the radiation is a potential risk to the fetus and MRI without contrast would be preferable, if needed (85). Nevertheless, it is only necessary to request an imaging test when surgery is planned prior to birth.

Table 2. Screening test values for Cushing's syndrome and ACTH levels

Test	Normal pregnancy	Diagnostic value in pregnancy
24-hour UFC	UFC excretion is normal in the first trimester*. 24-h UFC excretion increases up to three times the normal upper limit during the 2nd and 3rd trimesters (66).	Values in the 2nd and 3rd trimester > 3 times the upper limit of normal suggestive of CS (41).
LDDST	Elevated CBG levels in pregnancy lead to high total cortisol levels.	Suppression of cortisol is blunted when compared to the non-pregnant state (28).
Salivary cortisol	Salivary cortisol levels rise two- to threefold during pregnancy.* Diurnal variation of cortisol is preserved in pregnancy (87,88).	Suggested cut-off values of the three trimesters in the pregnancy groups are respectively: 7.0 nmol/L, 7.2 nmol/L, and 7.9 nmol/L (89).
Serum cortisol	Elevated CBG levels in pregnancy lead to high total cortisol levels. Cortisol diurnal variation is preserved in pregnancy (41).	Cortisol diurnal variation is lost in patients with CS who are pregnant, although there are no stablished cut-off levels (66).
ACTH levels	ACTH is not suppressed under physiological circumstances of pregnancy (53).	Suppressed ACTH strongly suggests a CS of adrenal origin, but detectable ACTH does not discriminate between adrenal or extra-adrenal origin.

*Using the reference values established in non-pregnant subjects.
ACTH: corticotropin; CBG: corticosteroid-binding-globulin; CS: Cushing's syndrome; UFC: urinary free cortisol; LDDST: Low dose dexamethasone suppression test.

5. Pituitary disease in unusual situations (II): *AIP* mutations and pituitary tumors

5.1. Familial isolated pituitary adenoma

Pituitary adenomas are increasingly recognized in a familial setting in approximately 5% of the cases. The largest group is the familial isolated pituitary adenoma (FIPA), defined by the presence of PA in two or more related members with no other associated manifestations and in the absence of known genetic causes such as MEN1 and MEN4, Carney complex or tumors related to mutations in the succinate dehydrogenase (*SDH*) genes (90) (**Figure 3**).

The frequencies of the various different tumor types in all FIPA patients (with and without recognized mutation) are somatotropinoma or somatolactotropinoma (40%), prolactinoma (38%), NFPA (15%), and rarely gonadotropinoma, Cushing's disease and thyrotropinoma (remainder 7%) (91). Approximately 50% of the FIPA families are affected by the same PA subtype (homogeneous families), most of them prolactinomas or somatotroph adenomas, and the other half have a combination with other tumor types (heterogeneous families) (35).

FIPA is an heterogeneous condition which includes patients with unknown genetic cause, patients with mutation in *AIP* gene (approximately 15-30%) (92) and X-linked acrogigantism (X-LAG), due to microduplications in the Xq26.3 region (93). However, *AIP* has been found implicated not only in the context of FIPA, but also in sporadically diagnosed PAs, probably explained by the low penetrance of the disease (approximately 20%) (35,94), rather than by de novo mutations (95,96).

More than 100 different germline mutations have been described , including nonsense, missense, in frame deletion/insertion, large genomic deletion, intronic, frameshift, promoter, start codon, and splice-site mutations, while somatic *AIP* mutations have not been recognized (35).

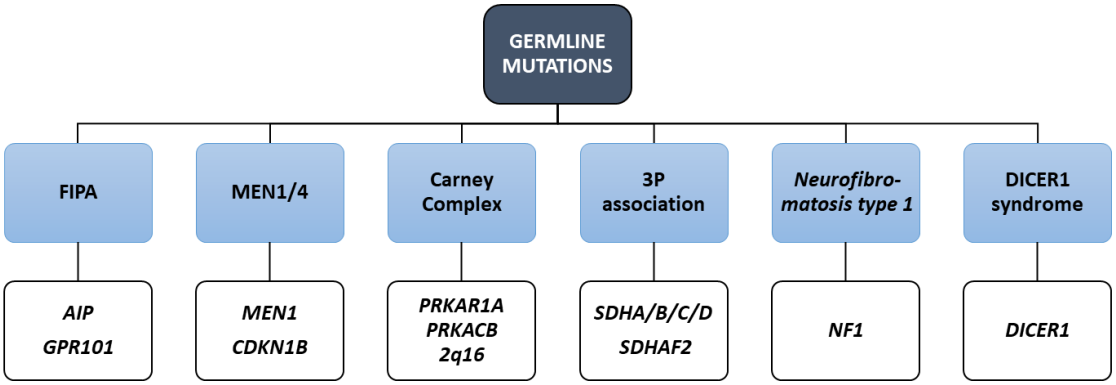

Figure 3. Germline mutations identified in familial and sporadic pituitary adenoma.

FIPA, familial isolated pituitary adenoma; MEN, multiple endocrine neoplasia; 3P association, paraganglioma/ pheochromocytoma/pituitary adenoma; NF1, neurofibromatosis type 1.

5.2. AIP gene and protein

AIP is a gene located in chromosome 11q13, formed by 6 exons which encodes a 330 amino acid protein (37 kDa), acting as a tumor suppressor gene. AIP is ubiquitously expressed but its expression levels vary considerably among different tissues (97). In normal pituitary, the AIP protein is expressed in somatotrophs and lactotrophs cells, where it associates with cytoplasmic secretory vesicles (98).

The AIP protein has a peptidyl-prolyl cis-trans isomerase-like domain (PPIase-like domain) at the N-terminal, three tetratricopeptide repeat domain (TPR domains) at the C-terminal part and a terminal α-7 helix. The TPR domain is necessary to mediate the binding between AIP and its partners (99).

AIP is a co-chaperone and numerous partners have been identified as heat-shock proteins and the aryl hydrocarbon receptor (AhR), through AIP is involved in the xenobiotic signaling (100). The best-known function of AIP is the interaction with the AhR in the cytoplasm forming the complex AIP/AhR/Hsp90, preventing AhR-mediated transcription and protecting from ubiquitin–proteasome-mediated degradation. This interaction requires the integrity of last 5 amino acids in the C-terminus of AIP, being this relevant so 70% of all known mutations are truncated mutations and cause a disruption in this region (35). When AhR binds to one of its ligand, the environmental toxin (98)

2,3,7,8-tetrachlorodibenzo-p-dioxin(TCDD), the AhR/AIP/Hsp90 complex is translocated to the nucleus, where AhR detaches from the complex following a conformational change. Into the nucleus, AhR forms a dimeric complex with the AhR nuclear translator (ARNT, also known as HIF-1b), which regulates the transcription of xenobiotic metabolizing enzymes (detoxification enzymes) by binding to the xenobiotic response elements. AIP also interacts with phosphodiesterase 4A4/5 (PDE4A4/5), which regulates the cyclic adenosine monophosphate (cAMP) pathway (100–102). There is also a possible role of AIP in cytoskeletal organization, cell motility/adhesion and oxidative stress responses (103).

The idea that *AIP* acts as a tumor suppressor is supported from the findings that the heterozygous inactivating germline mutations are frequently associated with loss of heterozygosity due to a second somatic mutation ("second hit") of the other allele at the level of tumor DNA, causing the disease (92,98). In addition, most of the *AIP* mutations result with a truncated protein or in highly unstable protein with reduced half-life (104).

The exact molecular mechanisms by which loss of function of AIP leads a development to PA remains to be elucidated; however, the cAMP-dependent protein kinase A (cAMP/PKA) pathway is likely to be involved, via defective G inhibitory protein signalling, altered interaction with phosphodiesterases and AIP interaction with members of the PKA complex.

The cAMP/PKA pathway is involved in the regulation of GH expression and proliferation of somatotroph cells (105). Importantly, deregulation of the cAMP signalling pathway has been reported to be a common occurrence in pituitary tumorigenesis such as the *GNAS* mutation in sporadic somatotroph adenomas and McCune-Albright syndrome, mutations in *PRKAR1A* and *PRKACB* in Carney complex and duplication of the cAMP-coupled orphan receptor *GPR101* in the X-LAG.

Evidence suggests that AIP deficiency causes a dysfunction of cAMP signalling, increasing the accumulation in cAMP through defective Gαi signalling and reduction of the G inhibitory protein Gαi-2, resulting in constitutive activation of cAMP synthesis and to the subsequent activation of PKA (106,107). The phosphorylation of the cAMP

response element-binding protein (CREB) has a central role in the activation of the GH promoter (108). Additionally, cAMP/PKA pathway can be altered as AIP physically interacts with components of the PKA pathway, including the main PKA regulatory (PRKAR1A) and catalytic subunit (PRKACA) and functionally with PDE4-dependent PKA activation (103,105). Moreover, AIP has been shown to interact with members of the PDE4 family, involved in the degradation of cAMP, suggesting that reduced expression of PDE4 enzymes might contribute to the enhanced cAMP signalling observed as a consequence of the loss of *AIP* (109).

5.3. Clinical features in *AIP* mutated patients

Mutations in *AIP* gene predispose to PAs and it has not been associated with other kinds of tumors (35). *AIP*-mutated patients (familial and sporadic) have distinctives characteristics in comparison with patients with non-mutated *AIP* gen. The first remarkable difference is the age of onset and age of diagnosis, as mutations in the *AIP* gene predispose to childhood or young-onset disease, and in most of the cases, patients are less than 30 years old at diagnosis. The different types of mutations seems to have a clinical influence, as patients with truncating mutations were significantly younger at disease onset in comparison with other *AIP* mutations (35) (**Figure 4A, 4D).**

The gender distribution has been a subject of discussion as some studies described a male preponderance (110), but in contrast, this difference was not clear in other studies (35). An ascertainment bias could play a role in this difference, as gigantism is more commonly seen in male patients.

One of the most important characteristics is that patients with *AIP* mutation present predominantly somatotropinomas or somatolactotropinomas, which account around 80% of the cases, with prolactinomas and clinically NFPA with positive GH and/or PRL staining also well described. ACTH or TSH secreting adenomas or gonadotrophin positive or null cell NFPAs are very rare (110). Of note, at least half of the cases of affected somatotropinoma presents with gigantism (35) (**Figure 4B)**.

Importantly, the *AIP* mutated patients have in up to 90% of the cases macroadenomas and also a larger size and extrasellar extension in comparison with non-mutated patients (35). Several cases of double adenomas have been described in *AIP*-mutation positive patients (101) while pituitary hyperplasia associated with GH excess is rare (98,111). Since these tumors are frequently large and rapidly growing adenomas, they have a higher predisposition to present pituitary apoplexy as the first expression of the disease, being more than 6 times more frequent in comparison with non-mutated patients (35) (**Figure 4C**).

The *AIP*-associated tumors have a more aggressive profile, supported by the higher proliferation rates and also that they are typically sparsely granulated adenomas, known to be more invasive and respond less well to somatostatin analog (SSA) therapy (98).

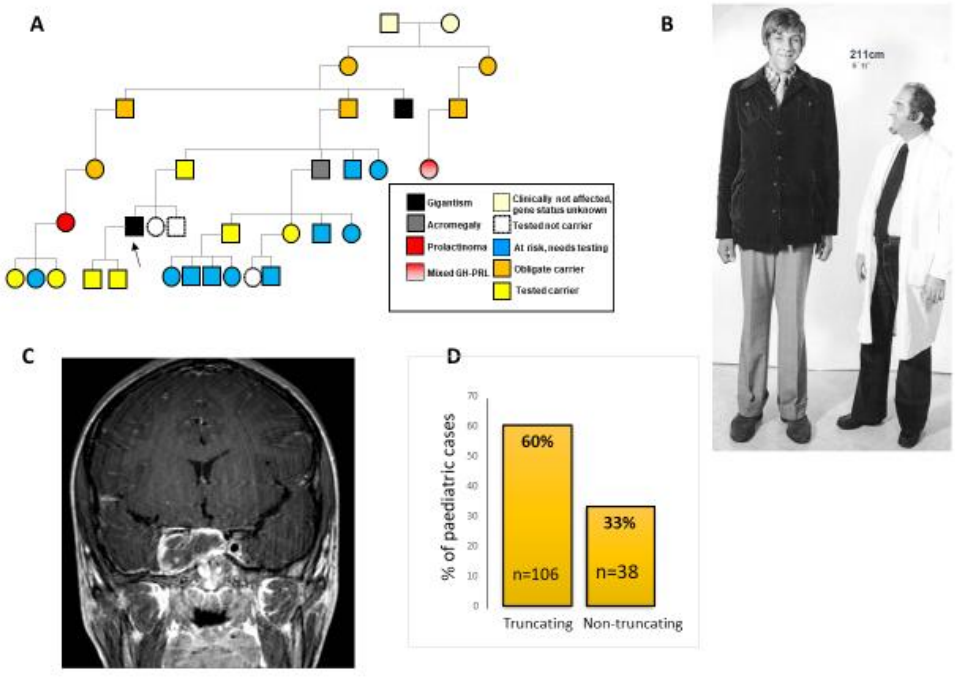

Figure 4. Clinical aspects of AIP mutations (15).

A) Family tree of patient with a complex insertion-deletion AIP mutation showing 5 other family members affected by pituitary adenomas. B) He was successfully operated at the age of 15 years by Dr Jules Hardy in 1975 and he has normal pituitary function since. C) MRI of a young female patient with a truncating AIP mutation presenting at the age of 6 years with acute severe headache and ptosis on the right side. D) Patients with truncation AIP mutations show earlier disease onset and are more common in pediatric cases.

5.4. Treatment particularities

AIP-mutated patients characteristically need multimodal therapies and usually require a combination of repeat surgery, external pituitary irradiation, and medical therapy to achieve the best possible hormonal control (35).

The histology characteristics are very important to define the aggressiveness and to predict the treatment response of PAs. Histological and inmunohistochemical parameters are typically used with a prognosis purpose to define atypical PAs. These are the Ki-67 labelling index greater than 3%, the invasive tumor growth, the elevated mitotic activity and the extensive nuclear staining for p53 (112). Interestingly, AIP has been suggested to be a better marker of invasiveness in somatotropinomas than Ki-67 and p53 even in *AIP*-mutation negative adenomas (113).

Generally, patients with *AIP* mutations have a relatively resistance to the effect of SSAs with a poor reduction in hormone levels and tumor size. Also, the efficacy observed with dopamine agonists is relatively poor in patients with prolactinomas. It is well studied that the somatostatin receptor subtype 2 (SSTR2) expression is a predictor for patient responses to SSA therapy (33,34) in addition to MRI signal, as a hypointense T2-weighted MRI signal is associated with a better response to SSA treatment (114). Of note, low expression of AIP has been associated with reduced SSTR2 in comparison with tumor with conserved AIP expression and, subsequently they present decreased responsiveness to first-generation SSAs (33). AIP is likely to be involved in the regulation of the action of SSA via the ZAC1 pathway, a presumed tumor suppressor gene involved in the anti-proliferative and anti-secretory effects of SSAs , as AIP knockdown was found to reduce the mRNA expression of ZAC1 (115).

Long-term pasireotide LAR therapy can be beneficial in some patients with *AIP*-mutations and acromegaly resistant to first-generation SSAs, as reported in some cases (116). Tumors with low AIP expression do not have difference in SSTR5 expression compared to conserved AIP expression tumors. Moreover, in a study of 39 patients treated post-operatively with SSAs, tumors with low AIP presented the same degree of response

to pasireotide than those with conserved AIP, suggesting that AIP deficient adenomas may benefit from pasireotide treatment (33).

There is not enough evidence on the response to pegvisomant therapy in AIP mutated tumors, although isolated case reports showed that pegvisomant can successfully normalize IGF-I levels (117).

AIP mutated patients need more surgical interventions than non-AIP counterparts, with lower chance to control the disease after the surgery (110). In case of prolactinomas, they appear to have more aggressive characteristics than sporadic adenomas, being significantly more frequently invasive and extending toward the optic chiasm. They also appear to be relatively resistant to dopamine agonists, frequently requiring surgery or radiotherapy (110).

Although the current treatment is far from being ideal, there are ongoing studies aiming to identify factors and molecular pathways to predict tumor behavior and identify novel therapeutic targets. Nevertheless, PAs associated to *AIP* mutations should be managed according to current guidelines for PAs.

HYPOTHESIS

1. Women with Cushing's syndrome (CS) who become pregnant are at higher risk of developing gestational diabetes mellitus, hypertension, preeclampsia, and risk of death in comparison to healthy pregnancies. CS in pregnancy also affect the fetus, which is at higher risk of fetal death, preterm birth, and several other complications including infections, hypoglycemia or respiratory distress. The impact of the different causes of CS on pregnancy has not been formally addressed. We therefore hypothesize that the etiology of CS in pregnancy might determine a different impact on the fetal/new-born and maternal outcome, in terms of mortality and complications.

2. The main goal of genetic screening for *AIP* germline mutations is to identify those at risk of potentially aggressive pituitary adenomas and facilitate early diagnosis of adenomas at a non-invasive stage, where treatment is more likely to be effective or curative and, importantly, to avoid excessive height in those with growth hormone secreting adenomas. Screening in unselected pituitary adenomas populations is not justifiable and currently, the clinical decision for screening is based on expert recommendation as there are no formal guidelines defining the criteria for genetic screening of pituitary adenoma patients for *AIP* mutations. We therefore hypothesize that a reduced number of clinical features can predict the risk of *AIP* mutation in patients with pituitary adenomas.

OBJECTIVES

1. Main aim

To identify risk factors for predicting clinical outcomes and to estimate the probability of a rare pituitary disease.

2. Secondary aims

1. To identify predictors of fetal morbidity and mortality in patients with active Cushing's syndrome and their association with the etiology.

2. To assess the maternal morbidity and mortality in pregnant patients with active and cured Cushing's syndrome.

3. To describe the risk factors of carrying an *AIP* mutation and quantify the predictive value for each risk factor adjusted by the others.

4. To develop and validate a risk category system to estimate the individual risk of carrying an *AIP* mutation for a given patient.

PUBLICATIONS

1. Paper I

Caimari F, Valassi E, Garbayo P, Steffensen C, Santos A, Corcoy R, Webb SM. Cushing's syndrome and pregnancy outcomes: a systematic review of published cases. *Endocrine* 2017;55(2):555–563.

2. Paper II

Caimari F, Hernández-Ramírez LC, Dang MN, Gabrovska P, Iacovazzo D, Stals K, Ellard S, Korbonits M. Risk category system to identify pituitary adenoma patients with *AIP* mutations. *J Med Genet* 2018;55(4):254–260.

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ORIGINAL ARTICLE

Cushing's syndrome and pregnancy outcomes: a systematic review of published cases

Francisca Caimari[1] · Elena Valassi[1,2,3] · Patricia Garbayo[4] · Charlotte Steffensen[5] · Alicia Santos[1,2,3] · Rosa Corcoy[1,3,6] · Susan M. Webb[1,2,3]

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Abstract Pregnancy in Cushing's syndrome (CS) is extremely rare due to the influence of hypercortisolism on the reproductive axis. Purpose of this study is to investigate whether the etiology of CS in pregnancy determines a different impact on the fetal/newborn and maternal outcomes. We performed a systematic review of cases published in the literature from January 1952 to April 2015 including the words "Cushing AND pregnancy". We included 168 manuscripts containing 220 patients and 263 pregnancies with active CS during pregnancy and with a history of CS but treated and cured hypercortisolism at the time of gestation. Adrenal adenoma was the main cause of active CS during pregnancy (44.1 %). Women with active CS had more gestational diabetes mellitus (36.9 vs. 2.3 %, $p = 0.003$), gestational hypertension (40.5 vs. 2.3 %, $p < 0.001$) and preeclampsia (26.3 vs. 2.3 %, $p = 0.001$) than those with cured disease. The proportion of fetal loss in active CS was higher than in cured CS (23.6 vs. 8.5 %, $p = 0.021$), as well as global fetal morbidity (33.3 vs. 4.9 %, $p < 0.001$). The predictors of fetal loss in active CS were etiology of hypercortisolism [Odds Ratio –OR—for pregnancy-induced CS 4.7 (95 % Confidence Interval–CI 1.16–18.96), $p = 0.03$], publication period [OR for "1975–1994" 0.10 (95 % CI 0.03–0.40), $p = 0.001$] and treatment during gestation ($p = 0.037$, [OR medical treatment 0.25 (95 % CI 0.06–1.02), $p = 0.052$], [OR surgical treatment 0.34 (95 % CI 0.11–1.06), $p = 0.063$]). The period of diagnosis of CS (before, during or after pregnancy) was the only predictor of overall fetal morbimortality [OR for diagnosis during pregnancy 4.66 (95 % CI 1.37–15.83), $p = 0.014$]. Patients with active CS, especially in pregnancy-induced CS, experienced more problems in pregnancy and had the worst fetal prognosis in comparison to other causes. Diagnosis of CS during pregnancy was also associated with worse overall fetal morbimortality. Both medical treatment and surgery during pregnancy appeared to be protective in avoiding fetal loss.

Keywords Cushing's syndrome · Pregnancy · Fetal outcomes · Maternal mortality

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✉ Susan M. Webb
swebb@santpau.cat

[1] Endocrinology/Medicine Departments, Hospital Sant Pau, Barcelona, Spain

[2] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER, Unidad 747), IIB-Sant Pau, Instituto de Salud Carlos III, Madrid, Spain

[3] Universitat Autònoma de Barcelona (UAB), Barcelona, Spain

[4] Teaching Unit Hospital Sant Pau, UAB, Barcelona, Spain

[5] Department of Medicine, Aarhus University Hospital, Aarhus, Denmark

[6] CIBER Bioengineering, Biomaterials and Nanotechnology, Instituto de Salud Carlos III, Zaragoza, Spain

Introduction

Cushing's syndrome (CS) is a rare disease with an incidence in general population of about 2–25 per million/year [1, 2]. Moreover, it is extremely rare for pregnancy to occur in CS, due to the influence of hypercortisolism on the reproductive axis; excess cortisol leads to hypogonadism

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and infertility secondary to an impairment of follicular development and ovulation [3–6]. Additionally, the etiology of CS in women who become pregnant is most frequently adrenal in origin (50–60 % of the cases), while in non-pregnant population pituitary-dependent Cushing's disease (CD) is responsible for 70 % of the cases [4, 7–10]. The reason for this difference is not known, however, it has been suggested that in CD there is hypersecretion of both cortisol and androgens, impairing fertility, while in CS of adrenal origin hypersecretion is almost exclusively of cortisol with minimal androgen production [11].

Women with CS who become pregnant are at much higher risk of developing gestational diabetes mellitus (GDM), hypertension (HT), preeclampsia, and risk of death in comparison to healthy pregnancies [12]. Risks also affect the fetus, which is at higher risk of fetal death, preterm birth, and several other complications including infections, hypoglycemia or respiratory distress [13].

The diagnosis of this condition during pregnancy is challenging due to similarities of some features of normal pregnancy, which is accompanied by physiological hypercortisolism [13]. Moreover, there is no consensus on the management of CS during pregnancy, due to the rarity of this condition that complicates the treatment of these patients.

The impact of the different causes of CS on pregnancy has not been formally addressed. We aimed to investigate this by performing a systematic review of cases published in the literature. We hypothesized that the etiology of CS in pregnancy might determine a different impact on the fetal/newborn and maternal outcome, in terms of mortality and complications.

Patients and methods

We performed a quantitative systematic review of the literature using MEDLINE/PubMed and EMBASE including the words "Cushing AND pregnancy" from January 1952 to April 2015. Inclusion of articles was restricted to articles in English or Spanish. All articles were independently reviewed by two authors and entered in a database. Discordances between the authors were discussed and agreed upon with a third author. From 552 initial references, 168 manuscripts describing one or more case reports were included in the database (Supplemental Bibliography). Articles which did not include case reports or which included cases but unrelated with the pregnancy period were discarded.

Criteria for classification of CS in relation to pregnancy

We classified the reports into two groups: patients with active CS during pregnancy and patients with a history of CS but treated and cured at the time of gestation. Pregnancies with delivery taking place within the 12 months prior to CS diagnosis were empirically considered as pregnancies in the context of active CS, due the high probability of concomitant hypercortisolism during the pregnancy period. Pregnancies that had occurred more than 12 months prior to the diagnosis of CS were considered as previous obstetric history in relation to CS and pregnancy (Fig. 1). We included more than one pregnancy per patient if all the pregnancies had taken place during the active phase of CS; however, for the analysis of prepregnancy maternal characteristics we considered only the total number of patients rather than number of pregnancies.

Definition of variables

We collected all CS patients reported in the literature. We accepted the diagnostic criteria of CS published by the authors, which changed over 63 years included in this review. The etiology was classified as Cushing's disease (CD), adrenal adenoma, adrenal carcinoma, ectopic CS, iatrogenic CS, micronodular hyperplasia, Carney complex syndrome, and pregnancy-induced hypercortisolism, described as transitory CS probably due to aberrant LH/hCG-receptors in the adrenal gland, without any other identifiable pathology, that resolved postpartum [14–16]. For statistical analysis, we grouped together micronodular hyperplasia and Carney Complex syndrome under the name of "bilateral hyperplasia". The severity of hypercortisolism was assessed by evaluating the fold increase of 24-hour urinary free cortisol (UFC) above the normal range, as specified by the authors, and by the presence of severe hypokalemia (<3 mmol/L).

As to maternal characteristics and outcomes, we collected information on maternal type 2 diabetes mellitus (DM) and chronic HT, GDM, pregnancy-induced HT and preeclampsia, based on the diagnostic criteria used by the authors. For previous pregnancies, we collected information on maternal age and outcome of pregnancy, including miscarriage, fetal loss and preterm birth, as defined by the authors.

As far as treatment for CS, patients were classified as not treated during pregnancy, only medically treated, only surgically treated or undergoing both medical and surgical therapy during pregnancy.

To address a temporal trend, we arbitrarily divided the manuscripts into three 20-year periods: 1953 to 1973, 1974 to 1994 and 1995 to 2015. We analyzed fetal outcome in terms of mortality and morbidity.

As to fetal loss, considered outcomes were spontaneous abortion (if gestation ended before week 22), ectopic pregnancy, induced abortion, intrauterine fetal death (if taking place antenatally, at 22 weeks or later) and neonatal

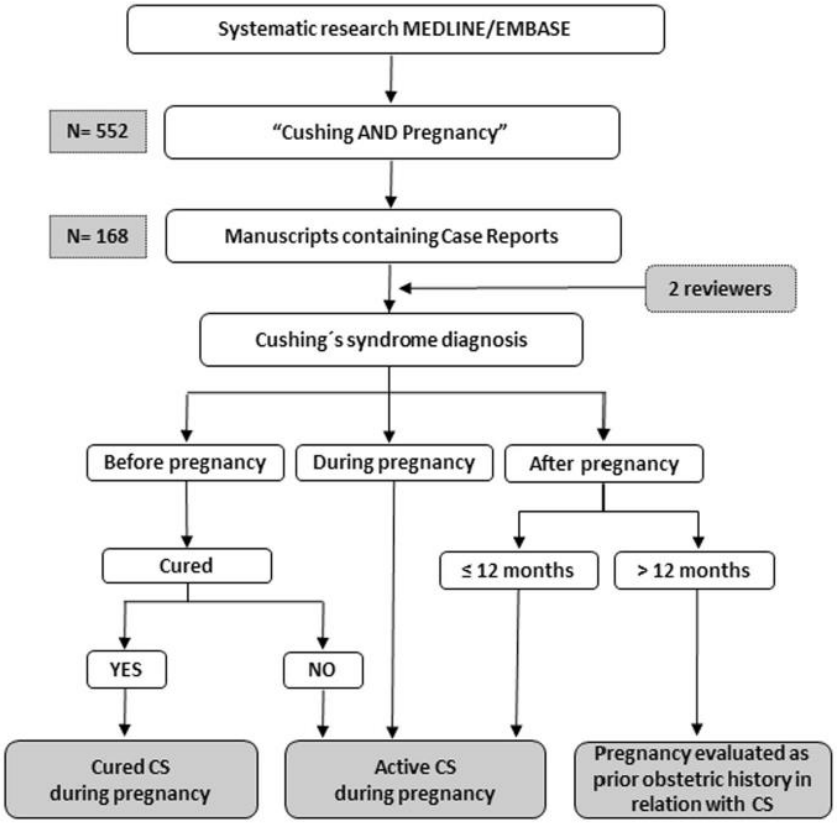

Fig. 1 Flow chart of articles through the selection process and classification of Cushing's syndrome (CS) in relation to pregnancy

death (if the newborn died within seven days of delivery). Intrauterine fetal and neonatal deaths were referred to as perinatal death. As to fetal morbidity, we included in the database the following fetal complications: prematurity (birth before week 37), intrauterine growth restriction, low birth weight (lower than 2500 gr at any gestational age), adrenal insufficiency, hypoglycemia, respiratory distress, sepsis, jaundice, tracheomalacia, fetal distress, neonatal virilization, left ventricle hypertrophy, and minor or major malformations. Definitions of all complications were those provided by the authors unless otherwise specified. We considered overall fetal morbimortality as any complication or any kind of fetal loss described above. Finally, we calculated the maternal mortality ratio (number of maternal deaths per 100,000 livebirths up to 42 days after delivery) and reported it by etiology of CS.

Statistical analysis

STATA version 13 was used for statistical analysis. Shapiro-Wilks test was performed to test the normal distribution of the quantitative variables. Quantitative variables with a normal distribution were expressed as mean and standard deviation (SD) and compared with a Student *t*-test. Non-normal distribution variables were expressed as median and P25-P75 range and analyzed with a Mann-Whitney U-test or Kruskall Wallis test if more than two groups were compared. Qualitative variables were expressed as percentages, and Chi-square test was performed to compare two or more groups. For multivariable analysis, a multinomial regression was performed to test predictors for different categories of fetal loss, and logistic regression to test predictors for overall fetal loss, preterm birth, low birth weight for gestational age and fetal morbimortality, in patients with active CS. In all the logistic regression analyses we used the same potential predictors as defined above: publication period, period of diagnosis of CS (before, during or after pregnancy), maternal age, etiology of CS, and treatment during pregnancy. Due to the low number of cases in some categories of the multivariate analysis, some OR and 95 % CI could not be calculated, and are identified in the tables as *not computed* (NC).

Results

Two-hundred and twenty patients were included in the analysis with 263 pregnancies. Fifty-one pregnancies were published in the period 1953–1973, 88 in 1974–1994, and 124 in 1995–2015. Two-hundred and fourteen (81.4 %)

gestations were in the context of active CS and 49 (18.6 %) after cure of CS. The age at diagnosis was 28.4 ± 5.2 years. In the group of active CS, the diagnosis was prior to gestation in 30 patients (14 %), during gestation in 138 (64.5 %) and after gestation in 46 women (21.5 %). The main cause of CS in active hypercortisolism was an adrenal adenoma (44.1 %), while in the cured group it was pituitary CD (73.5 %, $p < 0.001$). The causes of CS by groups are described in Table 1. Patients with pregnancy-induced hypercortisolism ($n = 11$) had different types of adrenal lesions: 8 had no adrenal lesions, 2 had bilateral adrenal hyperplasia and 1 bilateral adrenal nodular hyperplasia.

Prepregnancy maternal characteristics

Seventeen (9.3 %) patients had DM and 56 (30.1 %) chronic HT. Sixty nine patients (31.4 %) referred prior pregnancies at a mean age of 26.0 ± 4.0 years. Of these patients, 24 (34.8 %) had a past history of spontaneous abortion, 7 (10.1 %) other types of fetal loss, 9 (13 %) preterm birth, 8 (11.6 %) GDM and 9 (13 %) preeclampsia.

Severity of hypercortisolism in active CS

Twenty-four hour UFC levels were only available in 44.4 % of the patients. No differences were observed for the various etiologies. Severe hypokalemia was present in more than 50 % of the patients with ectopic CS, pregnancy-induced CS and adrenal carcinoma ($p = 0.019$), but this data was only reported in 39 % of the cases.

Table 1 Etiology of Cushing´s syndrome during pregnancy in patients with active or cured hypercortisolism, $p < 0.001$

Etiology of CS	Active CS (%) N = 213	Cured CS (%) $N = 49$
Cushing's disease	28.2	73.5
Adrenal adenoma	44.1	16.3
Adrenal carcinoma	9.4	6.1
Ectopic	3.8	0
Pregnancy-induced	13.2	0
Iatrogenic	0.5	0
Bilateral hyperplasia	0.9	4.1

Treatment during pregnancy in active CS

One hundred and twenty eight (60.1 %) of the patients with active CS during pregnancy received no treatment during gestation. Of these, 14.1 % were diagnosed before pregnancy, 50 % during pregnancy and 35.9 % after pregnancy. Twenty-four (11.3 %) women only received medical treatment, 5 of them starting before the pregnancy period; fifty-one (23.9 %) had only surgery and 10 (4.7 %) surgery and medical treatment. The drugs used were metyrapone in 69.7 %, ketoconazole in 15.2 %, aminoglutethimide in 3 %, cyproheptadine in 6.1 %, cabergoline in 3 %, and mitotane in 3 % (stopped at 6 weeks of pregnancy [17]). Fetal outcomes for each drug for patients who only received medical treatment during pregnancy are described in Supplementary Table 1.

From the total of 61 cases who underwent surgery, 11 had transsphenoidal surgery (TSS), 44 unilateral adrenalectomy and 6 bilateral adrenalectomy. Surgery during pregnancy was performed at a gestational age of 21 [17–26] weeks. Fetal outcomes by type of treatment are described in Table 2. Forty-seven patients attained remission after surgery (7 TSS, 35 unilateral adrenalectomy and 6 bilateral adrenalectomy), 7 were still active (4 TSS and 3 unilateral adrenalectomy) and in 6 cases the information was not available. Comparing CS with or without remission after surgery, 6.7 % vs. 28.6 % ended with fetal loss (p 0.067) respectively, 56.1 % vs. 80 % had a preterm birth (p ns) and 70.6 % vs. 100 % had low birth weight (p ns).

Description of the course of pregnancy

Women with active CS became pregnant at an age of 28.9 ± 5.2 in comparison to those with cured CS whose age was 30.4 ± 5.6 ($p = 0.075$). Patients with active CS delivered at an earlier gestational age than those with cured CS (34 [30, 37] vs. 39 [38, 40] weeks, $p < 0.001$) and had a higher rate of Cesarean sections (51.7 vs. 21.9 %, $p = 0.003$). They also presented more pregnancy-related complications like GDM (36.9 vs. 2.3 %, $p < 0.001$), gestational HT (40.5 vs. 2.3 %, $p < 0.001$) and preeclampsia (26.3 vs. 2.3 %, $p = 0.001$). Maternal diabetes and hypertensive disorders according to the etiology of CS are described in Fig. 2.

Table 2 Fetal outcome by treatment in women with active Cushing's syndrome during pregnancy

	No treatment $N = 128$	Medical treatment $N = 24$	Surgical treatment $N = 49$	Medical and surgical treatment $N = 10$	p-value
Overall fetal loss (%)	30.6	20.8	12.5	0	0.021
Preterm birth (%)	66.3	76.2	56.1	80	0.304
Low birth weight (%)	68.3	68.8	73.3	80	0.883

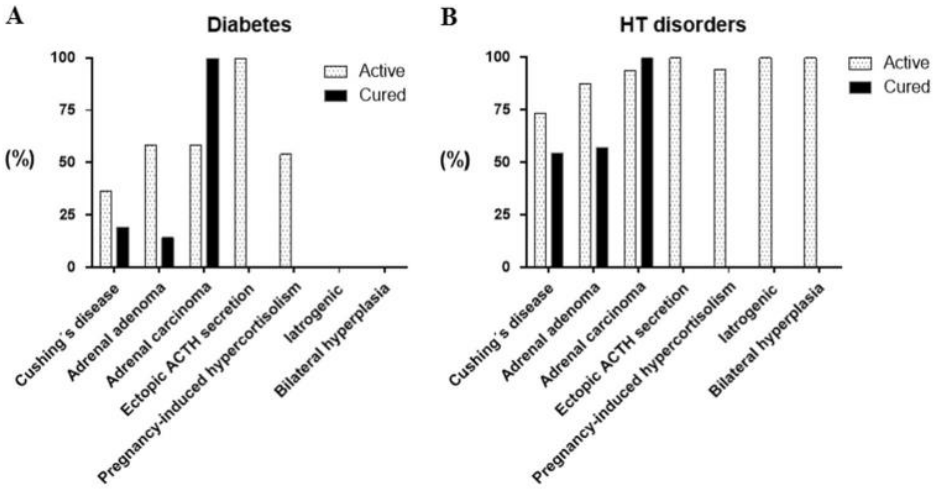

Fig. 2 Maternal complications during pregnancy in women with Cushing´s syndrome according to etiology. **a** Type 2 Diabetes Mellitus or Gestational Diabetes Mellitus (active vs. cured, $p < 0.001$); **b** chronic hypertension, gestational hypertension or preeclampsia (active vs. cured, $p < 0.001$)

As to fetal outcomes, no differences in newborn sex were observed between both groups (active 54.9 vs. cured 57.6 % male newborns, NS). The proportion of overall fetal loss in active CS was higher than in cured CS (23.6 vs. 8.5 %, $p = 0.021$), as well as global fetal morbidity (33.3 vs. 4.9 %, $p < 0.001$). Details on fetal outcomes are shown in Table 3.

Predictors of fetal loss in patients with active CS

The independent variables identified as predictors of fetal loss (Table 4) were etiology of hypercortisolism [OR for pregnancy-induced CS vs. CD 4.70 (95 % CI 1.16–18.96), $p = 0.03$], publication period [OR for "1974–1994" vs. "1953–1973" 0.10 (95 % CI 0.03–0.40), $p = 0.001$] and treatment during gestation [OR for medical treatment vs. no treatment 0.25 (95 % CI 0.06–1.02), $p = 0.053$], [OR for surgical treatment vs. no treatment 0.34 (95 % CI 0.11–1.06), $p = 0.063$, overall p 0.037].

The predictors of specific types of fetal loss among women with active CS are shown in Supplementary Table 2. Predictors of spontaneous abortion were publication period with a decreased rate for the period "1974–1994" vs. "1953–1973" [OR 0.09 (95 % CI 0.01–0.72), $p = 0.023$] and borderline significance for etiology of hypercortisolism vs. CD, [OR for pregnancy-induced CS 17.08 (95 % CI 1.77–164.6), $p = 0.014$, OR for adrenal carcinoma 10.09 (95 % CI 1.04–97.43), $p = 0.046$], overall $p = 0.075$). For induced abortion, the only predictor was maternal age with lower rates with increasing maternal age [OR 0.71 (95 % CI 0.52–0.97), $p = 0.03$] and for perinatal death, the publication period with a decreased rate for "1974–1994" vs. "1953–1973" [OR 0.08 (95 % CI 0.01–0.43), $p = 0.004$]. None of the variables analyzed were significant predictors for ectopic pregnancy.

Predictors of fetal morbidity in patients with active CS (Supplementary Table 3)

The period of diagnosis was a predictor for low birth weight with a higher rate for diagnosis during pregnancy vs. before pregnancy [OR 7.01 (95 % CI 1.12–44, $p = 0.038$]. None of the other variables included in the analyses (publication period, maternal age, etiology of CS, treatment during pregnancy) were significant predictors of fetal morbidity.

Predictors of fetal morbimortality in patients with active CS (Supplementary Table 4)

The period of diagnosis of CS was the only predictor of fetal morbimortality with higher rates for diagnosis during pregnancy vs. before pregnancy [OR 4.66 (95 % CI 1.37–15.83), $p = 0.014$].

Maternal mortality in patients with active CS

Two patients with active CS died in the postpartum period, within the first two weeks after delivery. The first, an adrenal adenoma, died after adrenalectomy complicated by gastric ulcers, pulmonary edema and pneumonia [18]. The second had an ectopic ACTH secreting tumor of the pancreatic tail. Her pregnancy ended spontaneously at 27 weeks and was complicated with GDM, pre-eclampsia, and severe hypokalemia. She died 2 weeks post-delivery after progressive deterioration [19]. The maternal mortality ratio was 1257/100,000 livebirths. No maternal mortality was described in patients with CD, bilateral hyperplasia, adrenal carcinoma, iatrogenic CS, and pregnancy-induced CS.

Table 3 Fetal outcome in women with active and cured Cushing's syndrome during pregnancy

Fetal outcomes	Active, $N = 214$ N (%)	Cured, $N = 49$ N (%)	p-value
Overall fetal loss	48 (23.6)	4 (8.51)	0.021
Spontaneous abortion	22 (10.84)	3 (6.38)	0.359
Induced abortion	6 (2.96)	0	0.233
Ectopic pregnancy	1 (0.5)	0	0.630
Intrauterine fetal death	11 (6.32)	1 (2.27)	0.293
Neonatal death	8 (4.91)	0	0.138
Perinatal death	19 (10.91)	1 (2.27)	0.076
Fetal distress	14 (9.66)	0	0.036
Preterm birth	106 (65.8)	1 (2.56)	<0.001
Intrauterine growth restriction	26 (15.03)	2 (4.88)	0.083
Low birth weight	69 (71.1)	5 (16.13)	<0.001
Adrenal insufficiency	8 (5.6)	1 (2.3)	0.377
Hypoglycemia	10 (704)	0	0.081
Respiratory distress	19 (13.77)	1 (2.44)	0.043
Sepsis	4 (2.82)	0	0.277
Jaundice	9 (6.47)	1 (2.4)	0.309
Tracheomalacia	1 (0.72)	0	0.581
Neonatal virilization	1 (0.56)	0	0.613
Left ventricle hypertrophy	2 (1.37)	0	0.446
Congenital malformations	5 (2.91)	0	0.247

Table 4 Predictors of overall fetal loss in women with active Cushing's syndrome after multivariate logistic regression analysis

Variable	OR[a]	95 % CI[b]	p-value
Publication period			**0.001**
1953–1973	1	–	–
1974–1994	0.10	0.03–0.40	**0.001**
1995–2015	0.41	0.13–1.29	0.126
Diagnosis of Cushing's syndrome			0.101
Pre-pregnancy	1	–	–
During pregnancy	2.15	0.51–9.09	0.297
Post-pregnancy	0.61	0.11–3.15	0.558
Maternal age (years)	1.01	0.94–1.09	0.850
Etiology of Cushing's syndrome			**0.003**
Cushing's disease	1	–	–
Pregnancy-induced	4.7	1.16–18.96	**0.030**
Adrenal adenoma	0.58	0.19–1.78	0.342
Ectopic ACTH secretion	3.64	0.54–24.71	0.186
Adrenal carcinoma	2.72	0.62–11.88	0.184
Bilateral hyperplasia	1	NC[c]	NC[c]
Iatrogenic	1	NC[c]	NC[c]
Treatment during gestation			**0.037**
No treatment	1	–	–
Medical	0.25	0.06–1.02	0.052
Surgical	0.34	0.11–1.06	0.063
Medical and surgical	1	NC[c]	NC[c]

[a] OR: Odds Ratio

[b] CI: Confidence Interval

[c] NC: not computed

Discussion

We have systematically reviewed the literature and analyzed a large group of 220 patients diagnosed of CS in whom 263 pregnancies occurred, including 214 gestations in active CS. Although there are published case reports and reviews, knowledge in this field is very limited, given the rarity of CS itself and the even rarer occurrence of pregnancy with concomitant hypercortisolism. Thus, large cohort studies in this field are not feasible. This is the first time that the impact of the etiology of CS on fetal morbimortality has been investigated in a large group of patients, recruited systematically from reports published in the literature.

We confirmed the previous observation that the etiology of CS in women with active CS and pregnancy is different from that seen in non-pregnant CS patients; namely, the adrenal origin was the most frequent etiology in active CS [11, 20]. It has been suggested that in CD there is hypersecretion of both cortisol and androgens, which interferes with the gonadal axis producing amenorrhea in over 70 % of the cases [21], while in adrenal causes hypersecretion is almost purely of cortisol [7, 13].

These women had a higher frequency of prior history of Type 2 DM (9.3 %) and chronic HT (30.1 %) in comparison with the prevalence in healthy women of the same age [22, 23]. Interestingly, we have observed that the past obstetrical history was clearly worse in comparison to the general population, as well as the frequency of spontaneous abortion (34.8 %) and fetal loss (10.1 %), respectively 2 and 10 fold higher than corresponding figures in healthy women [24]. A past history of GDM (11.6 %) was also 2–3 times higher than in women without CS (4 %) [25] as well as that of preeclampsia (13 %), which was 6 times higher than in healthy women (2.2 %) [26]. All these findings seem to indicate that hypercortisolism was present prior to pregnancy, and was associated with worse obstetrical history. This would parallel other endocrinological disorders such as GDM [27] or hyperparathyroidism [28] where past obstetric history is worse than in the general population. Similarly, worse prognosis in the three years prior to diagnosis of CS in non-pregnant subjects has also been observed in a large epidemiological study in Denmark where more venous thromboembolism, stroke, peptic ulcers, fractures, and

infections were observed in 343 CS patients diagnosed over 30 years, compared to 34,300 matched controls from the general Danish population, also pointing to undiagnosed hypercortisolism as the reason for this worse outcome [29].

Patients with active CS showed a high incidence of preterm deliveries, probably due to more frequent complications during pregnancy such as GDM, HT or preeclampsia that can also lead to higher rates of Cesarean section in comparison to cured CS (51.7 vs. 21.9 %). The prevalence of GDM (36.9 %), gestational HT (40.5 %) and preeclampsia (26.5 %) in active cases was higher than in cured CS (2.3, 2.3, and 2.3 % respectively), where prevalence was similar to that observed in the general population [25, 26]. Fetal loss was almost three times higher in active CS than in cured CS (24 vs. 8.5 %) and interestingly, cured women had a similar fetal mortality to that expected in healthy women [24, 30]. Altogether, these findings indicate that patients with cured CS normalized both maternal and fetal risks, while active CS had a markedly negative impact on the prognosis of pregnancy.

Pregnancy-induced CS was shown to have the worst impact on overall fetal loss (≅5 times higher than for CD) and spontaneous abortion (≅17 times higher than for CD), compared to the other causes of CS. This might be due to a higher degree of hypercortisolism, but cannot be evidenced with the limited data available. This special situation of pregnancy-induced CS is difficult to identify, especially in a first pregnancy, so that therapy is delayed and hypercortisolism appears early in pregnancy stimulated by the rise of beta-HCG. Furthermore, there is no definitive treatment for this entity (except perhaps bilateral adrenalectomy, an aggressive procedure, followed by irreversible adrenal insufficiency); it is therefore not surprising, that it is associated with the worst fetal outcome. Adrenal carcinoma was also associated with a borderline significant increase in spontaneous abortion and we hypothesized that this could be due to more severe hormonal abnormalities in these patients and the higher rate of hypokalemia would support this. Finally, maternal age was a negative independent predictor of induced abortion, less common as maternal age increased (OR 0.71) following the same trend as in the general population [31].

The limited number of cases described precludes any definite conclusion as to the best management for CS during pregnancy, which is usually individualized for each patient, depending on the cause, the stage of pregnancy and the severity of hypercortisolism. Nevertheless, untreated CS is associated with significantly more maternal and fetal morbidity [11, 12]. Surgery has been recommended as a first choice treatment [12, 21], and is safer in the second trimester due to the lower risk of fetal and maternal complications [7, 32]. Surgical treatment reduces perinatal mortality and maternal morbidity rates, but does not affect the occurrence of preterm birth and intrauterine growth restriction [33]. On the other hand, medical treatment with different drugs has been used in a considerable numbers of patients without any apparent adverse consequences, metyrapone being the drug most commonly used. It has been used at different stages of gestation [18, 34–36], when surgery was contraindicated, or initially after diagnosis for symptomatic control. We observed that receiving medical or surgical treatment decreased the risk of overall fetal loss between 3 and 4 times, but did not protect from prematurity, as previously described [11].

Diagnosis during pregnancy as compared to diagnosis before pregnancy was shown to increase the risk of low birth weight 7-fold. It also increased 5-fold the overall risk of fetal morbimortality, compared with diagnosis before pregnancy. This suggests that a longer delay in diagnosis during pregnancy is likely to impair fetal outcome, given the increased difficulty in diagnosing hypercortisolism during gestation.

Finally, we report a very high maternal mortality in patients with active CS compared with the worldwide maternal mortality ratio, which was reported in 2013 to be 209.1 per 100,000 livebirths; in fact it was above the highest reported maternal mortality of 956.8 seen in South Sudan [37], indicating an increased risk of pregnancy-related mortality in CS.

This study has some limitations. First, available data suggests a publication bias, as the proportion of etiologies includes a considerable number of rare cases, with favorable outcomes especially in the period 1974–1994. Second, the patients included are from a wide historical period, as we collected cases since 1952, when treatments and diagnosis were very different from recent publications. This is the reason why we decided not to analyze the different treatment options separately, given their heterogeneity. Moreover, the criteria to define maternal and fetal complications could be different, as the patients came from different centers with their own criteria. Finally, biochemical data to evaluate the degree of hypercortisolism were limited. However, since there is no large series of CS and pregnancy from a single center, we believe that this review of data has highlighted several issues, which contribute to the knowledge on prognostic factors in this rare but challenging clinical situation.

In conclusion, we have confirmed that the most common etiology in women diagnosed of CS who became pregnant, is a benign adrenal cause, rather than pituitary-dependent CD, most commonly found in non-pregnant patients. Second, our findings suggest that the prior obstetric history of these patients was clearly worse than in the general population suggesting that the negative effect of hypercortisolism had started long before the diagnosis of CS. Finally, we have confirmed the departing hypothesis that fetal outcomes

are different depending on the cause of CS, as patients with pregnancy-induced CS (presumably due to aberrant LH or beta-hCG receptors on the adrenal) and adrenal carcinoma had the worst fetal prognosis in comparison to other causes. Additionally, diagnosis of CS during pregnancy was also associated to worse overall fetal morbimortality and both medical treatment and surgery during pregnancy appeared to be protective in avoiding fetal loss. However, pregnancy should be avoided in the presence of hypercortisolism, given the increased incidence of both maternal and fetal complications.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Risk category system to identify pituitary adenoma patients with *AIP* mutations

Francisca Caimari,[1,2] Laura Cristina Hernández-Ramírez,[1,3] Mary N Dang,[1] Plamena Gabrovska,[1] Donato Iacovazzo,[1] Karen Stals,[4] Sian Ellard,[4] Márta Korbonits,[1] on behalf of The International FIPA consortium

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[1]Centre of Endocrinology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK
[2]Department of Endocrinology, Universitat Autònoma de Barcelona (UAB), Barcelona, Spain
[3]Section of Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, Maryland, USA
[4]Department of Molecular Genetics, Royal Devon and Exeter NHS Foundation Trust, Exeter, UK

Correspondence to
Professor Márta Korbonits, Centre of Endocrinology, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London, London EC1M 6BQ, UK; m.korbonits@qmul.ac.uk

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ABSTRACT

Background Predictive tools to identify patients at risk for gene mutations related to pituitary adenomas are very helpful in clinical practice. We therefore aimed to develop and validate a reliable risk category system for aryl hydrocarbon receptor-interacting protein (*AIP*) mutations in patients with pituitary adenomas.

Methods An international cohort of 2227 subjects were consecutively recruited between 2007 and 2016, including patients with pituitary adenomas (familial and sporadic) and their relatives. All probands (n=1429) were screened for *AIP* mutations, and those diagnosed with a pituitary adenoma prospectively, as part of their clinical screening (n=24), were excluded from the analysis. Univariate analysis was performed comparing patients with and without *AIP* mutations. Based on a multivariate logistic regression model, six potential factors were identified for the development of a risk category system, classifying the individual risk into low-risk, moderate-risk and high-risk categories. An internal cross-validation test was used to validate the system.

Results 1405 patients had a pituitary tumour, of which 43% had a positive family history, 55.5% had somatotrophinomas and 81.5% presented with macroadenoma. Overall, 134 patients had an *AIP* mutation (9.5%). We identified four independent predictors for the presence of an *AIP* mutation: age of onset providing an odds ratio (OR) of 14.34 for age 0-18 years, family history (OR 10.85), growth hormone excess (OR 9.74) and large tumour size (OR 4.49). In our cohort, 71% of patients were identified as low risk (<5% risk of *AIP* mutation), 9.2% as moderate risk and 20% as high risk (≥20% risk). Excellent discrimination (c-statistic=0.87) and internal validation were achieved.

Conclusion We propose a user-friendly risk categorisation system that can reliably group patients into high-risk, moderate-risk and low-risk groups for the presence of *AIP* mutations, thus providing guidance in identifying patients at high risk of carrying an *AIP* mutation. This risk score is based on a cohort with high prevalence of *AIP* mutations and should be applied cautiously in other populations.

INTRODUCTION

Pituitary adenomas are relatively common lesions, present in approximately 17% of the general population,[1] although clinically relevant disease is identified in only around 1:1000 subjects.[2 3] Most pituitary adenomas are sporadic; however, familial cases are increasingly recognised, representing some 5% of all patients presenting with pituitary adenomas.[4] Mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene predispose to the development of pituitary adenomas but with a low penetrance (20%–23%).[5–8] *AIP* mutations can be identified either in the context of familial isolated pituitary adenomas (FIPA), defined by the presence of pituitary adenomas in two or more family members with no other syndromic features, or as simplex cases with a germline mutation but no known family history of the disease. The prevalence of *AIP* mutations is around 20% in FIPA kindreds,[7 8] while in sporadic pituitary adenomas, the prevalence ranges between 3.6% and 20%,[9 10] depending on the age group studied.

More than 100 different 'pathogenic' or 'likely pathogenic'[11] germline *AIP* variants have been described (non-sense, missense, in frame deletion/insertion, segmental duplication, large genomic deletion, frameshift, promoter, start codon and splice-site mutations[8]), while several variants are currently considered as having 'unknown significance'.[12]

Although all types of pituitary adenomas have been described with germline *AIP* mutations, patients with such mutations typically have young-onset growth hormone (GH)-secreting or GH-secreting and prolactin-secreting tumours with generally poor responsivity to conventional treatment, and aggressive behaviour compared with those with no recognised mutation,[7 8] often requiring repeated surgery and radiotherapy and therefore needing close surveillance.[7 13 14]

Risk assessment for an *AIP* mutation has important clinical implications, and the genetic screening of family members allows for the identification of those at risk of developing aggressive pituitary adenomas.[8 15 16] Early diagnosis at a non-invasive stage can potentially lead to a higher chance of effective or curative treatment.[8 15]

There are no formal guidelines defining the criteria for genetic screening pituitary adenoma patients for *AIP* mutations, and currently the clinical decision for screening is based on expert recommendation.[16 17] Identification of *AIP* mutation-positive patients can lead to the detection of carriers with otherwise unrecognised disease,[8 15] potentially leading to a better prognosis. Our study aimed to develop and validate a risk category system to stratify patients with isolated pituitary adenomas for their risk of carrying *AIP* mutations. This risk

category system was designed to serve as an effective tool to aid clinical decision making and the identification of *AIP* mutation carriers in clinical practice.

MATERIALS AND METHODS

Two thousand two hundred and twenty-seven subjects were included in our database from February 2007 to June 2016, including 1429 affected subjects with pituitary tumours and 798 unaffected relatives. Subjects were recruited via the FIPA consortium, an international research group. The data collected from medical records related to each individual patient were sent to our group, and the information was checked to confirm that all patients met the inclusion criteria. All subjects included signed the informed consent approved by the local ethics committee.

We included patients who presented with FIPA and patients diagnosed with apparently sporadic pituitary adenomas with disease onset at ≤30 years of age. In addition, we also included referred patients with sporadic adenomas and an age of onset >30 years. Patients with X-linked acrogigantism syndrome (XLAG)[18 19] and patients who presented with other recognised syndromes such as multiple endocrine neoplasia type 1 or type 4, Carney complex and DICER1 syndrome, were excluded.[20] These conditions were excluded on the basis of clinical, biochemical and, in some cases, genetic testing, as appropriate. Although patients with XLAG also belong to the FIPA group, their clinical characteristics are so distinct that we felt that they should not be included in this risk prediction analysis. Patients diagnosed prospectively as a result of familial screening of known *AIP* mutations were also excluded from the analysis. Genetic screening for *AIP* mutations was performed using Sanger sequencing and multiplex ligation-dependent probe amplification, as previously described.[8] Genomic DNA was obtained from blood or saliva samples. The pathogenicity of the detected variants was assessed using the Mutation Taster (http://www.mutationtaster.org/), Anovar[21] and Variant Effect Predictor (VEP)[22] in silico prediction programmes. We also included published clinical and experimental data on the previously reported variants. Only pathogenic or likely pathogenic variants were considered as mutations.[11]

Definition of variables

Patients were identified as affected if they had either (1) a pituitary tumour or (2) pituitary hyperplasia associated with hormone hypersecretion. FIPA was defined by the presence of pituitary adenomas in two or more members of a family with no other associated clinical features. The family history included assessment of all known 'blood relatives'. The diagnoses were categorised as GH excess (including acromegaly and gigantism, with or without prolactin cosecretion), non-functioning pituitary adenoma (NFPA), prolactinoma, Cushing's disease and other diagnoses (any other type of functioning pituitary tumour). Macroadenomas were defined by tumour size ≥10mm. Age of onset was defined as the age at presentation of the first symptom. Pituitary apoplexy was defined by a clinical history of haemorrhage and/or infarction of a pituitary adenoma.

Statistical analysis

The Shapiro-Wilks test was used to assess Gaussian distribution for continuous variables. Normally distributed variables were expressed as mean and SD and were analysed with the Student's t-test. Median and IQR were used to describe non-normally distributed variables. These variables were analysed with the Mann-Whitney U test. Qualitative variables were expressed as percentage and analysed with the χ^2 test to compare two or more groups; $P<0.05$ was taken as significant.

The following clinically relevant variables were included to generate the model: family history of pituitary tumours, gender, age of onset (categorised as ≤18 years, 19–30 years and >30 years old), adenoma type (categorised as tumours secreting GH vs others), adenoma size (categorised as macroadenoma vs microadenoma or hyperplasia) and history of pituitary apoplexy. Interactions between all the studied variables were assessed with the likelihood ratio test. Variable selection was carried out through all possible equations methods, where every potential combination of the independent variables were computed and subsequently evaluated to assess the performance of the possible models.[23] We selected the final model based on the Akaike information criteria, area under the receiver operating characteristic (ROC) curve and the Hosmer-Lemeshow tests. A logistic regression with the selected variables was performed, and results were expressed with an odds ratio (OR) and its 95% confidence interval (CI).

We arbitrarily categorised the risk of *AIP* mutation into low-risk (<5%), moderate-risk (5%–19%) and high-risk (≥20%) groups. Discrimination of the model was assessed with the c-statistic, and its calibration was assessed comparing observed versus model-derived *AIP* mutation risk and with the Hosmer-Lemeshow test. Finally, we assessed internal validity with a cross-validation procedure for a realistic estimation of the performance of the prediction model. Performance measures included R^2 (explained variation of the model). Considering the size of our cohort, we randomly divided the sample in five equal-sized parts, and we calculated the difference between our model and the resampling average. STATA software V.13.1 was used for statistical analysis.

RESULTS

Out of the 1429 pituitary adenoma patients, 153 carried an *AIP* mutation (10.7%). Out of the 343 relatives of patients with *AIP* mutations, 165 were carriers of an *AIP* mutation (48.1%). The clinical characteristics of the whole cohort are detailed in table 1.

Twenty-four family members were prospectively diagnosed with a pituitary adenoma, 19 of these carried an *AIP* mutation (clinical characteristics are included in online supplementary table 1), while five belonged to *AIP* mutation-negative

Table 1 Clinical characteristics of the whole cohort

Clinical characteristic	n=1405*
AIP mutation, n (%)	134 (9.5)
Familial, n (%)	607 (43.2)
Gender, n (% male)	680 (48.5)
Diagnosis, n (%)	
GH excess	767 (55.5)
NFPA	185 (13.4)
Prolactinoma	344 (24.9)
Cushing's disease	74 (5.4)
Other diagnosis	11 (0.8)
Age of onset (years)	27.1±13.1
Age at diagnosis (years)	30.8±13.4
Macroadenoma, n (%)	977 (81.5)
Extrasellar extension, n (%)	446 (60.1)
Pituitary apoplexy, n (%)	48 (3.9)

*Prospectively diagnosed patients excluded.
NFPA, non-functioning pituitary adenoma.

Table 2 Novel *AIP* mutations not previously reported. gnomAD: http://gnomad.broadinstitute.org/

AIP mutation	MAF in gnomAD	Variant type	In silico prediction*	Probability score†	Gender	Familial versus simplex	Diagnosis‡	Age at diagnosis	Age at onset
c.240_241delinsTG (p.M80_R81delinsIG)	Not reported	Insertion deletion	High	Disease causing 1	M	Simplex	Gigantism	8	5
c.333delC (p.K112Rfs*44)	Not reported	Frameshift	High	Disease causing 1	F	Simplex	Gigantism	9	7
c.376_377delCA (p.Q126Dfs*3)	Not reported	Frameshift	High	Disease causing 1	F	Simplex	Gigantism	13	10
c.605A>G (p.Y202C) §	Not reported	Missense	High	Disease causing 0.99	M	Simplex	Gigantism	10	10
c.645+1G>C (p.?)	Not reported	Splicing	High	Disease causing 1	M	Simplex	Acromegaly	33	24
c.991T>C (p.331Rext91)	Not reported	Missense	High	Polymorphism 0.99	M	Simplex	Gigantism	16	12

*In silico prediction of probability of damaging mutation by Variant Effect Predictor and Anovar.
†Probability of pathogenic mutation by Mutation Taster.
‡All patients had macroadenoma, and none of them presented with pituitary apoplexy.
§This missense variant affects position 22 in the first tetratricopeptide domain of AIP, a well-conserved position in various tetratricopeptide domain proteins.[32 38]
AIP, aryl hydrocarbon receptor-interacting protein; MAF, minor allele frequency.

families. Prospectively diagnosed patients were excluded from the analysis.

Six novel *AIP* mutations were found in one patient each, their characteristics are detailed in table 2. All *AIP* mutations identified are listed in online supplementary table 2.

The age of onset was significantly lower in *AIP*-positive versus *AIP*-negative patients (16 (14–24) (IQR) vs 25 (19-33) years, P<0.001), as was the age at diagnosis (21 (16–31) vs 29 (22–38) years, P<0.001). Table 3 contains the comparison of clinical characteristics of *AIP* mutation-positive and *AIP*-negative patients.

The likelihood ratio test to evaluate interaction terms was non-significant (P=0.149), hence interaction terms were excluded from the model. The variable selection process suggested that pituitary apoplexy and gender should be removed from the model, as they did not add predictive power. A markedly increased risk of an *AIP* mutation was associated with having a family history, a GH-excess adenoma, macroadenomas and young age of onset. The variables included in the predictive model are listed in table 4 in the order of their statistical strength for prediction. Good discriminative power was achieved with the area under the curve (AUC), reaching a value of 0.87 (95% CI 0.84 to 0.90) (figure 1). We stratified the risk of having an *AIP* mutation into low risk (<5%), moderate risk (5%–19%) and high risk (≥20%), based on our predictive model. Figure 2 shows stratified risks according to age, family history, tumour type and tumour size. In our cohort, enriched with familial, GH-secreting adenomas and young-onset cases, 70.8% of patients were identified as low risk, 9.2% as intermediate risk, while 20% were at high risk (risk ≥20%). Calibration results, comparing observed and model-predicted *AIP* mutation risk across the three risk groups, are depicted in the online supplementary figure 1. The Hosmer-Lemeshow test was non-significant (P=0.213), suggesting that the model is well calibrated.

Finally, the model showed good internal validation, as tested by the cross-validation technique, as the R^2 shrinkage was <10% in absolute terms (from 0.294 to 0.223).

Table 3 Clinical characteristics comparing *AIP*-positive and *AIP*-negative patients

Clinical characteristic	*AIP* positive	*AIP* negative	P value
Familial, n (%)	89 (66.4)	518 (40.8)	<0.001
Gender, n (% male)	83 (61.9)	597 (47.1)	0.001
Diagnosis, n (%)			<0.001
GH excess	119 (88.8)	648 (52)	
NFPA	4 (3)	181 (14.5)	
Prolactinoma	11 (8.2)	333 (26.7)	
Cushing's disease	0	74 (5.9)	
Other diagnosis	0	11 (0.9)	
Age of onset (years and percentages)			<0.001
0–18	79 (60.3)	259 (23.6)	
19–30	33 (25.2)	506 (46)	
>30	19 (14.5)	336 (30.5)	
Age at diagnosis (years and percentages)			<0.001
0–18	53 (40.5)	163 (14.1)	
19–30	44 (33.6)	497 (43)	
>30	34 (26)	495 (42.9)	
Maximum diameter (mm)*	16 (10.7–25)	20 (11–30)	0.518
Macroadenoma, n (%)	112 (93.3)	865 (80.2)	<0.001
Extrasellar extension, n (%)	52 (81.3)	394 (58.1)	<0.001
Pituitary apoplexy, n (%)	12 (9.5)	36 (3.3)	0.001
Number of treatments*	2 (1–3)	1(1–2)	0.055

*Median and IQR.
NFPA, non-functioning pituitary adenoma.

Table 4 Logistic regression to generate a predictive model for *AIP* mutations*

Variable	OR (95% CI)	P value
Age of onset		
>30	1	–
0–18	14.34 (7.41 to 29.31)	<0.001
19–30	2.26 (1.17 to 4.35)	0.015
Positive family history	10.85 (6.48 to 18.16)	<0.001
Diagnosis		
Others	1	–
GH excess	9.74 (5.12 to 18.52)	<0.001
Size (macroadenoma)	4.49 (1.91 to 10.59)	0.001

*Variables are listed in the order of their statistical strength for prediction and each OR is adjusted for all the other variables.
AIP, aryl hydrocarbon receptor-interacting protein; GH, growth hormone.

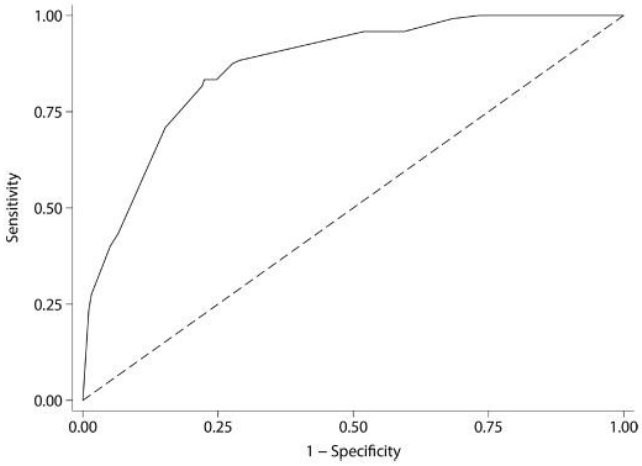

Figure 1 Area under the receiver operating characteristic curve of the *AIP* mutation risk category system is 0.87 (95% CI 0.84 to 0.90), indicating an excellent discriminating power.

Age of onset ≤ 18

	Familial		Simplex	
	GH excess		GH excess	
	No	Yes	No	Yes
Macro	25%	76%	3%	23%
Micro	7%	42%	1%	6%

Age of onset 19-30

	Familial		Simplex	
	GH excess		GH excess	
	No	Yes	No	Yes
Macro	5%	34%	1%	4%
Micro	1%	10%	<1%	1%

Age of onset ≥ 31

	Familial		Simplex	
	GH excess		GH excess	
	No	Yes	No	Yes
Macro	2%	18%	<1%	2%
Micro	1%	5%	<1%	1%

Figure 2 Risk stratification for *AIP* mutations, classified as low risk (<5%), moderate risk (5%–19%) or high risk (≥20%). Red: risk of *AIP* mutation ≥20%; orange: risk of *AIP* mutation between 5% and 19%; green: risk of *AIP* mutation <5%. GH, growth hormone; macro, macroadenoma; micro, microadenoma; simplex, patients with no known family history.

DISCUSSION

Using state-of-the-art statistical methods applied to a large cohort of patients, we have identified four significant predictors for the presence of carrying an *AIP* mutation, and these are immediately accessible for routine clinical practice. Our data suggest that a positive family history, young age of onset, somatotroph tumour type and large tumour size can predict the risk of an *AIP* mutation according to our risk model, which has been validated in a large series of patients. Once a mutation carrier is identified, genetic testing can be performed for family members. The overall risk category of a kindred should be based on the risk score of the family member with the highest risk.

Despite the increasing number of genes associated with FIPA,[5 18] formal guidelines do not currently include recommendations for screening for *AIP* mutations,[24 25] and therefore such screening is usually performed on the basis of expert recommendations.[16 17 26 27] These recommendations include patients who have (1) a family history of pituitary adenoma, (2) childhood-onset pituitary adenoma or (3) a pituitary somatotroph or lactotroph macroadenoma diagnosed before the age of 30 years; however, no data stratifying the different risks between these groups have been provided.[9 10 16 17] Here we provide risk stratification for *AIP* mutation positive patients, using a combination of clinical variables, all of which should be easily available at the time of diagnosis.

Not surprisingly, all the variables included in our *AIP* risk category system have been repeatedly reported as typical clinical features of *AIP* mutation-positive patients. The age of disease onset is the strongest predictive factor, with a maximum risk for an *AIP* mutation present in those patients who presented with an adenoma during childhood (OR 14.3 (95% CI 7.4 to 27.7), P<0.001). This result was expected, as the prevalence of *AIP* mutations in paediatric cases has been reported to be between 6% and 23%.[9 10 28 29] An age between 19 and 30 years (OR 2.3 (95% CI 1.2 to 4.4), P=0.015) is also a strong predictor. In a previous study, among subjects with sporadic macroadenomas diagnosed before the age of 30 years, an *AIP* mutation was found in 11.7% overall, with a positive finding in 13.3% of patients with somatotrophinomas, 11.5% of those with prolactinomas and 6.3% of those with NFPAs.[28] Taking the historical 10% risk cut-off for genetic screening,[30] patients with familial GH-secreting macrodenoma, ≤30-year-old patients with familial GH-secreting microadenomas and sporadic childhood-onset GH-secreting macroadenomas are all above this threshold.

The second strongest predictive variable was positive family history of pituitary adenomas (OR 10.85 (95% CI 6.48 to 18.16), P<0.001). Although the majority of FIPA families have not yet had the causative gene identified, the largest available cohorts found that about 20% of FIPA kindreds harbour a heterozygous germline mutation in the *AIP* gene,[7 8] with the overall rate being slightly higher in homogeneous versus heterogeneous kindreds (22.8% and 16.7%, respectively).[16]

GH excess is also a good independent predictor for *AIP* mutations in our model (OR 9.74 (95% CI 5.12 to 18.52), P<0.001). One of the most important characteristics of patients with *AIP* mutations is the predominance of somatotrophinomas or somatolactotrophinomas, which account for around 80% of the cases. Prolactinomas and clinically NFPAs with positive GH and/or PRL immunostaining are also well described, while ACTH-secreting or TSH-secreting adenomas or gonadotrophin positive or null cell NFPAs are very rare.[31]

In addition, we demonstrate that patients with macroadenomas have more than four times the risk of harbouring an *AIP* mutation compared with those with microadenomas (OR

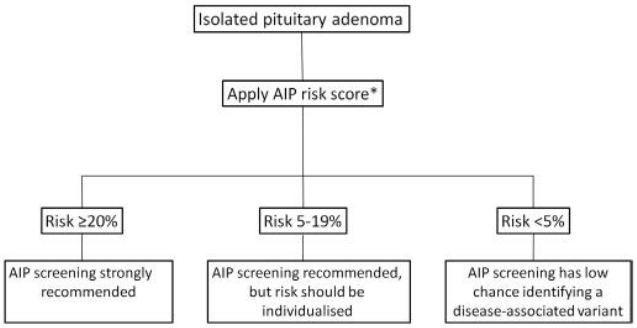

Figure 3 AIP screening algorithm based on the proposed risk category system. The overall risk category of a kindred should be based on the risk score of the family member with the highest risk. AIP, aryl hydrocarbon receptor-interacting protein. *See figure 2.

4.49 (95% CI 1.91 to 10.59), P=0.001). Patients with *AIP* mutations have macroadenomas in up to 90% of the cases, and their tumours are significantly larger and more frequently show an extrasellar extension compared with non-mutated familial[8] and sporadic cases.[6–8] Cases of double adenomas have also been described among *AIP* mutation positive patients,[32] while pituitary hyperplasia associated with GH excess[33 34] is rare.

We also evaluated other clinical characteristics as possible predictors of *AIP* mutations. For instance, *AIP* mutation-positive patients frequently have a history of pituitary apoplexy, which is often the presenting feature.[8 33 35] It is unclear whether this is due to the fact that these tumours are large and rapidly growing adenomas or whether an additional molecular mechanism renders these adenomas prone to apoplexy. In our study, pituitary apoplexy was significantly more frequent in *AIP* mutated tumours than in negative cases (9.5% vs 3.3%, P=0.001); however, this variable did not add any predictive power to the risk model when we adjusted for the other variables.

Gender distribution was statistically significant in the univariate analysis (61.9% vs 47.1% males, P=0.001), but not when adjusted for the other variables. There is no clear consensus in the published literature about the gender distribution of *AIP* mutation-positive patients. While several studies reported an increased prevalence of male patients, ascertainment bias probably plays a role, as in large *AIP* mutation positive families the percentage of affected male patients was lower compared with sporadic *AIP* mutation-positive cases.[8]

Using the described model, we were able to stratify the risk of *AIP* mutation into low, moderate and high categories, and we believe this system can be an easy-to-use tool in clinical practice. The model performs well in terms of discrimination, calibration and internal validation. AUC was 0.87 (95% CI 0.84 to 0.90), where 0.5 represents no discrimination and 1 represents perfect discrimination, indicating that our model achieved an excellent discriminatory power.[36] Additionally, there were no obvious differences between observed and model-predicted *AIP*-positive patients. The Hosmer-Lemeshow test showed adequate goodness of fit of our model.[36] We have validated our model using an internal cross-validation procedure, one of the preferable methods when external validation is not feasible.[36] The performance of the model was evaluated comparing the explained variation of the model (R^2) in each of the five equal samples of the data and the total sample, achieving a reduction of $R^2 < 10\%$.[36] To the best of our knowledge, there is no other available *AIP* risk category system assessment in the literature for comparison.

Using our risk stratification model, we are able to: (1) describe the risk factors of carrying an *AIP* mutation; (2) quantify the predictive value for each risk factor adjusted by the others; and (3) estimate the individual risk of carrying an *AIP* mutation for a given patient. We expect this tool to be valuable for clinicians to improve the decision-making process of referring patients for genetic screening based on the individual risk of *AIP* mutation.

A screening algorithm based on the results of our risk category system is shown in figure 3. We need to emphasise that we used age of onset and not age of diagnosis for this analysis. This parameter is often more subjective and needs careful history taking, reviewing parents' height and available photographic evidence of change of features. Patients with pituitary gigantism should be considered to have childhood-onset disease and offered screening.

There are some limitations inherent to our risk model. First, our risk score is based on a cohort enriched with familial, young-onset patients and GH-excess tumours as the number of *AIP* mutated patients in unselected cohorts is low.[9 37] Although all possible diagnostic groups have some representation in our study, caution should be taken when extrapolating these results to a population with significantly different prevalence of *AIP* mutations than the one found in our cohort; this score ideally estimates the risk in patients where the mutation is already suspected. Second, the determination of age of onset can be subjective and subject to patient recall. Nevertheless, when comparing the model using the age of onset with the one produced using age of diagnosis, the AUC was significantly better using age of onset rather than age at diagnosis; this might be explained due to the well-documented delay of diagnosis in patients with acromegaly. To minimise subjectivity, we categorised the variable age of onset into three broad groups. Finally, it was not possible to perform an external validation of the model due to the relatively low number of cases with *AIP* mutations in our cohort (although it is the largest *AIP* mutation positive series so far described), which precluded splitting the sample into a derivation and validation group. However, we have successfully validated the model using an internal cross-validation method.

In summary, the risk category system we have developed has the potential for widespread use as it includes readily available predictors. We believe this tool, ideally used in patients where the mutation is already suspected, can facilitate the most effective use of genetic screening, which we believe is currently clearly underused, allowing the identification of patients who carry *AIP* mutations and providing the opportunity of early diagnosis in at-risk relatives.

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Collaborators Members of the FIPA consortium are listed at http://www.fipapatients.org/fipaconsortium/.

Contributors FC and MK planned the study. FC, LCH-R, MND, PG and DI collected the data. FC performed the statistical analysis. KS and SE performed the genetic analysis. FC and MK wrote the manuscript. All authors reviewed and approved the manuscript.

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RESULTS

1. Cushing's syndrome and pregnancy outcomes: a systematic review of published cases

Two-hundred and twenty patients were included in the analysis with a total of 263 pregnancies: 81.4% gestations in the context of active CS and 18.6% after cured CS. In the group of active CS, the diagnosis was preceding gestation in 30 patients (14 %), during gestation in 138 (64.5%) and after gestation in 46 women (21.5%).

1.1. Etiology of CS during pregnancy in patients with active or cured hypercortisolism

Adrenal adenomas were the main cause of CS in the context of pregnancy (44.1%), in contrast to the cured group, where pituitary CS was predominant (73.5%, p<0.001). The rest of causes of CS divided by groups is described in **Table 3**.

Table 3. Etiology of Cushing's syndrome during pregnancy in patients with active or cured hypercortisolism

Etiology of CS	**Active CS (%) N=213**	**Cured CS (%) N=49**
Cushing's disease	28.2	73.5
Adrenal adenoma	44.1	16.3
Adrenal carcinoma	9.4	6.1
Ectopic ACTH secretion	3.8	0
Pregnancy-induced	13.2	0
Iatrogenic	0.5	0
Bilateral hyperplasia	0.9	4.1

1.2. Pre-pregnancy maternal characteristics

Chronic hypertension affected 56 (30.1%) patients and diabetes mellitus (DM) was present in 17 (9.3%) patients. The percentage of patients with prior pregnancies was 31.4%, at a mean age of 26.0±4 years. Of these patients, 8 (11.6%) had a past history of gestational DM (GDM) and 9 (13%) preeclampsia. Past history of spontaneous abortion was positive in 24 (34.8%) patients, other types of fetal loss in 7 (10.1%) and preterm birth in 9 (13%).

1.3. Severity of hypercortisolism in active CS

Twenty-four hour UFC levels were only available in 44.4% of the patients. No differences were observed for the various etiologies. Severe hypokalemia was present in more than 50% of the patients with ectopic CS, pregnancy-induced CS and adrenal carcinoma (p=0.019), but this data was only reported in 39% of the cases.

1.4. Treatment during pregnancy in active Cushing's syndrome

Eighty-three patients (39.1%) were actively treated during pregnancy: 24 (11.3%) only received medical treatment, 5 of them starting before pregnancy period; 51 (23.9%) had only surgery and 10 (4.7%) surgery and medical treatment.

The drug that was used more frequently was metyrapone in 69.7%, followed by ketoconazole in 15.2%, cyproheptadine in 6.1%, aminoglutethimide in 3%, cabergoline in 3% and mitotane in 3%, the last one stopped at 6 weeks of pregnancy (118). Fetal outcomes for each drug for patients who only received medical treatment during pregnancy are described in **Table 4**.

Table 4. Fetal outcomes by drugs used during pregnancy

Drug	N	Overall fetal loss N=24	Preterm birth N=21	Low birth weight N=16
Ketoconazole	2	0	1	1
Ketoconazole & cabergoline	1	0	0	1
Metyrapone	16	4	13	9
Metyrapone & ketoconazole	1	0	0	0
Mitotane	1	1	NA	NA
Cyproheptadine	2	0	2	0
Cabergoline	1	0	0	0

NA: not applicable

A total of 61 patients underwent surgery, 11 had transsphenoidal surgery (TSS), 44 unilateral adrenalectomies and 6 bilateral adrenalectomies. Surgery during pregnancy was performed at a median gestational age of 21 [17-26] weeks.

One hundred and twenty-eight patients (60.1%) received no treatment during gestation. Of these, 14.1% were diagnosed before pregnancy, 50% during pregnancy and 35.9% after pregnancy. Fetal outcomes by type of treatment are described in **Table 5**.

Table 5. Fetal outcome by treatment in women with active Cushing's syndrome during pregnancy

	No tx N=128	Medical tx N=24	Surgical tx N=49	Medical & surgical N=10	p-value*
Overall fetal loss (%)	30.6	20.8	12.5	0	0.021
Preterm birth (%)	66.3	76.2	56.1	80	0.304
Low birth weight (%)	68.3	68.8	73.3	80	0.883

*p-value for the comparison of each fetal outcome by treatment categories.
tx: Treatment

Forty-seven patients attained remission after surgery (7 TSS, 35 unilateral adrenalectomies and 6 bilateral adrenalectomies), 7 were still active (4 TSS and 3 unilateral adrenalectomies) and in 6 cases the information was not available. Comparing CS with or without remission after surgery, 6.7% vs 28.6% ended with fetal loss respectively (p=0.067), 56.1% vs 80% had a preterm birth (p NS) and 70.6% vs 100% had low birth weight (p NS).

1.5. Description of the course of pregnancy

There was no difference in the age of pregnancy between women with active CS vs cured CS (28.9±5.2 years vs 30.4± 5.6 years (p=0.075)). The gestational age at delivery was lower in patients with active CS than those with cured CS (34 [30, 37] vs 39 [38, 40] weeks, p< 0.001) and had a higher rate of Caesarean sections (51.7 vs 21.9%, p=0.003).

Pregnancy-related complications were more frequent in the active CS vs cured CS, such as GDM (36.9 vs 2.3%, p< 0.001), gestational hypertension (40.5 vs 2.3%, p<0.001) and preeclampsia (26.3 vs 2.3%, p=0.001). Maternal DM and hypertensive disorders according to the etiology of CS is illustrated in **Figure 5**.

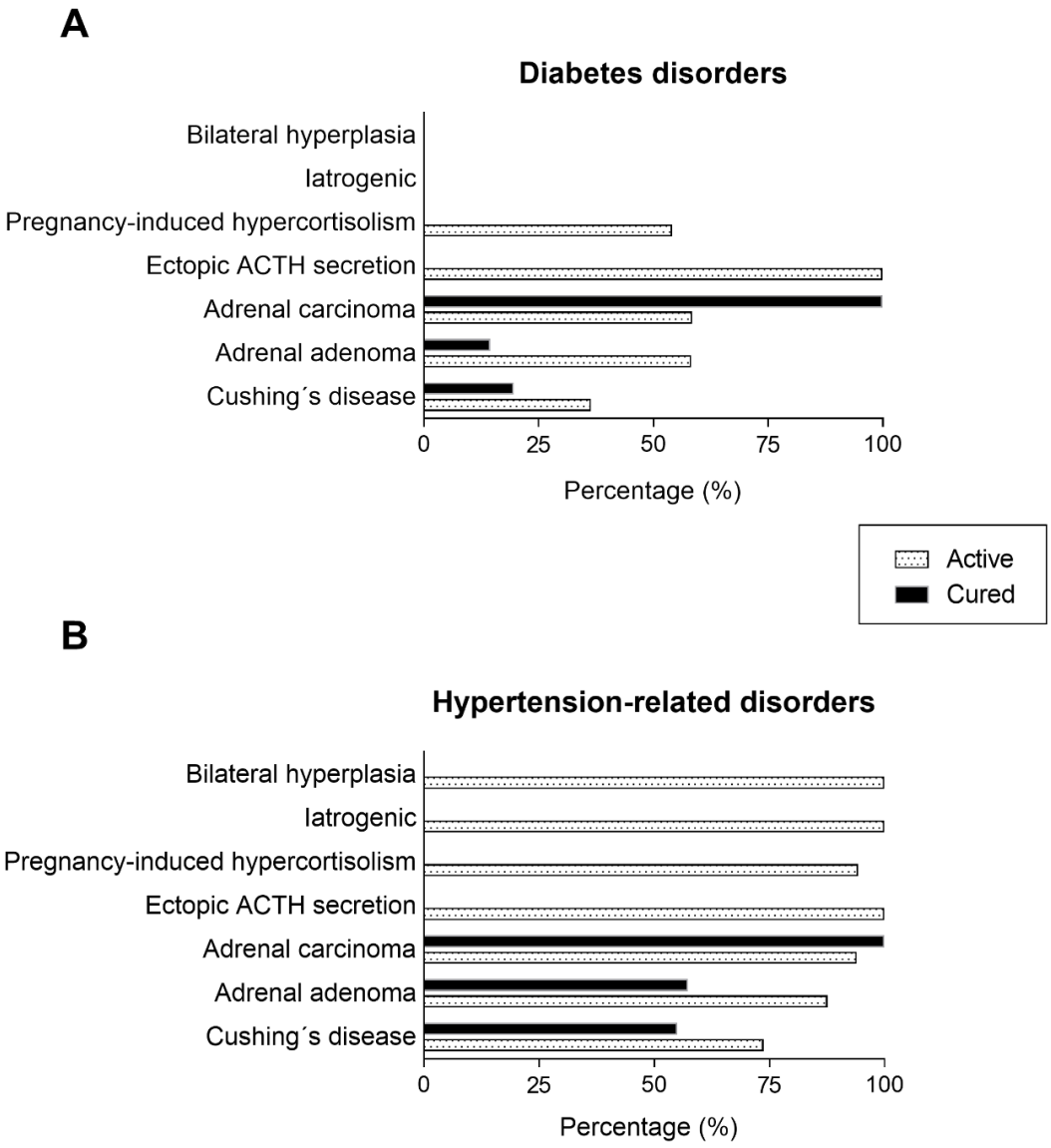

Figure 5. Maternal complications during pregnancy in women with Cushing's syndrome according to etiology.

A) Type 2 Diabetes Mellitus or Gestational Diabetes Mellitus (active vs cured, p<0.001); B) Chronic hypertension, gestational hypertension or preeclampsia (active vs cured, p<0.001)

1.6. Fetal outcomes

No differences in newborn sex were observed between both groups (active 54.9 vs cured 57.6% male newborns, NS). The proportion of overall fetal loss in active CS was higher than in cured CS (23.7 vs 8.5%, p=0.021), as well as global fetal morbidity (33.3 vs 4.9%, p<0.001). Details on fetal outcomes are shown in **Table 6.**

Table 6. Fetal outcome in women with active and cured Cushing´s syndrome during pregnancy

Fetal outcomes	**Active, N=214** **N (%)**	**Cured, N=49** **N (%)**	**p-value**
Overall fetal loss	48 (23.6)	4 (8.51)	0.021
Spontaneous abortion	22 (10.84)	3 (6.38)	0.359
Induced abortion	6 (2.96)	0	0.233
Ectopic pregnancy	1 (0.5)	0	0.630
Intrauterine fetal death	11 (6.32)	1 (2.27)	0.293
Neonatal death	8 (4.91)	0	0.138
Perinatal death	19 (10.91)	1 (2.27)	0.076
Fetal distress	14 (9.66)	0	0.036
Preterm birth	106 (65.8)	1 (2.56)	<0.001
Intrauterine growth restriction	26 (15.03)	2 (4.88)	0.083
Low birth weight	69 (71.1)	5 (16.13)	<0.001
Adrenal insufficiency	8 (5.6)	1 (2.3)	0.377
Hypoglycemia	10 (704)	0	0.081
Respiratory distress	19 (13.77)	1 (2.44)	0.043
Sepsis	4 (2.82)	0	0.277
Jaundice	9 (6.47)	1 (2.4)	0.309
Tracheomalacia	1 (0.72)	0	0.581
Neonatal virilization	1 (0.56)	0	0.613
Left ventricle hypertrophy	2 (1.37)	0	0.446
Congenital malformations	5 (2.91)	0	0.247

1.7. Predictors of fetal loss in patients with active Cushing's syndrome

Three variables were identified as predictors of fetal loss: etiology of hypercortisolism [odds ratio (OR) for pregnancy-induced CS vs CD 4.70 (95% CI 1.16-18.96), p=0.03], publication period [OR for "1974-1994" vs "1953-1973" 0.10 (95% CI 0.03-0.40), p=0.001] and treatment during gestation [OR for medical treatment vs no treatment 0.25 (95% CI 0.06-1.02), p=0.053], [OR for surgical treatment vs no treatment 0.34 (95% CI 0.11-1.06), p=0.063, overall p=0.037] (**Table 7**).

Table 7. Predictors of overall fetal loss in women with active Cushing's syndrome after multivariate logistic regression analysis

Variable	OR	95% CI	p-value
Publication Period			**0.001**
1953-1973	1	-	-
1974-1994	0.10	0.03-0.40	**0.001**
1995-2015	0.41	0.13-1.29	0.126
Diagnosis of CS		-	0.101
Pre-pregnancy	1	0.51- 9.09	-
During pregnancy	2.15	0.11- 3.15	0.297
Post-pregnancy	0.61		0.558
Maternal age (years)	1.01	0.94 - 1.09	0.850
Etiology of CS			**0.003**
Cushing's disease	1	-	-
Pregnancy-induced	4.7	1.16 – 18.96	**0.030**
Adrenal adenoma	0.58	0.19 – 1.78	0.342
Ectopic ACTH secretion	3.64	0.54 -24.71	0.186
Adrenal carcinoma	2.72	0.62- 11.88	0.184
Bilateral hyperplasia	1	NC	NC
Iatrogenic	1	NC	NC
Treatment during gestation			**0.037**
No treatment	1	-	-
Medical	0.25	0.06-1.02	**0.052**
Surgical	0.34	0.11-1.06	**0.063**
Medical and Surgical	1	NC	NC

OR: Odds Ratio; CI: Confidence Interval; NC: not computed, CS: Cushing's syndrome

Predictors for specific types of fetal loss including spontaneous and induced abortion, ectopic pregnancy and perinatal death was also studied. Predictors of spontaneous abortion were publication period with a decreased rate for the period "1974-1994" vs "1953-1973" [OR 0.09 (95% CI 0.01-0.72), p=0.023] and borderline significance for etiology of hypercortisolism [OR for pregnancy-induced CS 17.03 (95% CI 1.77-164.6), p=0.014, OR for adrenal carcinoma 10.09 (95% CI 1.04-97.43), p=0.046], overall p=0.075). For induced abortion, the only predictor was maternal age with lower rates with increasing maternal age [OR 0.71 (95% CI 0.52-0.97), p=0.03] and for perinatal death, the publication period with a decreased rate for "1974-1994" vs "1953-1973" [OR 0.08 (95% CI 0.01-0.43), p=0.004]. None of the variables studied were significant predictors for ectopic pregnancy. See **Table 8.**

Table 8. Predictors of specific types of fetal loss in women with active Cushing's syndrome after multinomial logistic regression analysis

Variable		**Spontaneous abortion**			**Ectopic pregnancy**			**Induced abortion**			**Perinatal death**		
	global p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value	OR	95% CI	p-value
Publication Period	**0.032**												
1953-1973		1	-	-	1	-	-	1	-	-	1	-	-
1974-1994		0.09	0.01-0.72	**0.023**	5.0	NC	1	$8.44*10^{9}$	NC	0.999	0.08	0.01-0.43	**0.004**
1995-2015		0.42	0.09- 2.08	0.291	$6.95*10^{7}$	NC	0.998	$4.11*10^{10}$	NC	0.999	0.25	0.06-1.05	0.058
Diagnosis of Cushing's syndrome	0.092												
Pre-pregnancy		1	-	-	1	-	-	1	-	-	1	-	-
During pregnancy		1.39	0.15- 12.73	0.766	$2.26*10^{-8}$	NC	0.998	23.14	NC	1	4.16	0.58-29.68	0.155
Post-pregnancy		0.28	0.02-3.41	0.316	$6.41*10^{-9}$	NC	0.999	$2.18*10^{-13}$	NC	0.999	2.64	0.30-23.07	0.379
Maternal age (years)	**0.032**	1.07	0.95- 1.20	0.287	1.03	0.73- 1.46	0.849	0.71	0.52-0.97	**0.03**	1	0.90- 1.11	0.974
Etiology of Cushing's syndrome	**0.075**												
Cushing's disease		1	-	-	1	-	-	1	-	-	1	-	-
Pregnancy-induced		17.08	1.77- 164.60	**0.014**	0.24	NC	1	$2.47*10^{8}$	NC	0.995	1.27	0.21-7.80	0.796
Adrenal adenoma		2.20	0.29-16.51	0.451	0.35	NC	1	4589633	NC	0.996	0.29	0.07-1.13	0.074
Ectopic ACTH secretion		9.10	0.45- 184.72	0.150	0.25	NC	1	1.01	NC	1	2.78	0.35-22.25	0.336
Adrenal carcinoma		10.09	1.04- 97.43	**0.046**	0.67	NC	1	$2.77*10^{14}$	NC	0.993	0.50	0.05-5.39	0.570
Bilateral hyperplasia		$1.08*10^{-7}$	NC	0.999	0.20	NC	1	$3.57*10^{12}$	NC	0.999	$9.36*10^{-9}$	NC	0.999
Iatrogenic		$9.43*10^{-8}$	NC	0.999	2614459	NC	1	1.56	NC	1	$1.87*10^{-8}$	NC	0.999
Treatment during gestation	**0.071**												
No treatment		1	-	-	1	-	-	1	-	-	1	-	-
Medical		0.26	0.04- 1.71	0.161	$3.10*10^{-8}$	NC	0.998	$2.75*10^{-14}$	NC	0.993	0.60	0.09-3.90	0.593
Surgical		0.24	0.04- 1.39	0.111	$4.60*10^{-8}$	NC	0.998	$3.52*10^{-7}$	NC	0.993	0.50	0.11-	0.367
Medical and Surgical		$1.03*10^{-8}$	NC	0.998	$5.15*10^{-8}$	NC	0.999	$1.71*10^{-13}$	NC	0.997	$1.59*10^{-8}$	2.329NC	0.998

OR: Odds Ratio; CI: confidence interval; NC: not computed

1.8. Predictors of fetal morbidity in patients with active Cushing's syndrome

Predictors for prematurity and low birth weight were also studied. The period of diagnosis was a predictor for low birth weight with a higher rate for diagnosis during pregnancy vs before pregnancy [OR 7.01 (95% CI 1.12-44), p=0.038]. None of the other variables included in the analyses (publication period, maternal age, etiology of CS, treatment during pregnancy) were significant predictors of fetal morbidity (**Table 9**).

Table 9. Predictors of preterm birth and low birth weight in women with active Cushing's syndrome

Variable	Prematurity			Low birth weight		
	OR	**95% CI**	**p-value**	**OR**	**95% CI**	**p-value**
Publication Period			0.660			0.975
1953-1973	1	-	-	1	-	-
1974-1994	0.51	0.12-2.25	0.374	0.94	0.16-5.53	0.944
1995-2015	0.59	0.14-2.53	0.487	1.07	0.16-6.98	0.941
Diagnosis of CS			0.114			**0.016**
Pre-pregnancy	1	-	-	1	-	-
During pregnancy	3.63	1.05- 12.56	0.042	7.01	1.12-44	**0.038**
Post-pregnancy	3	0.69-13.1	0.145	1.11	0.19-12.75	0.933
Maternal age (years)	1.02	0.95-1.10	0.621	1.08	0.97-1.2	0.169
Etiology of CS			0.275			0.051
Cushing's disease	1	-	-	1	-	-
Pregnancy-induced	6.16	0.64- 59	0.115	4.1	0.29-58.64	0.299
Adrenal adenoma	1.59	0.64- 4	0.321	0.30	0.07-1.39	0.124
Ectopic ACTH secretion	2.33	0.2- 26.8	0.497	NC	NC	NC
Adrenal carcinoma	3.39	0.73- 15.6	0.117	1.76	0.11-28.1	0.688
Bilateral hyperplasia	1	NC	NC	1	NC	NC
Iatrogenic	1	NC	NC	NC	NC	NC
Treatment during gestation			0.161			0.331
No treatment	1	-	-	1	-	-
Medical	1.23	0.31-4.84	0.769	0.24	0.04-1.44	0.118
Surgery	0.43	0.16-1.15	0.093	0.74	0.19-2.85	0.659
Medical and Surgery	2.09	0.33-13.11	0.433	1.55	0.18-13.51	0.693

OR: Odds Ratio; CI: confidence interval; NC: not computed

1.9. Predictors of fetal morbidity and mortality in patients with active Cushing's syndrome

The period of diagnosis of CS was the only predictor of fetal morbidity and mortality with higher rates for diagnosis during pregnancy vs before pregnancy [OR 4.66 (95% CI 1.37-15.83), p=0.014] (**Table 10**).

Table 10. Global predictors of fetal morbidity and mortality in women with active Cushing's syndrome

Variable	OR	95% CI	p-value
Publication Period (year)			0.390
1953-1973	1	-	-
1974-1994	0.42	0.10-1.68	0.218
1995-2015	0.61	0.15-2.43	0.483
Diagnosis of CS			**0.040**
Pre-pregnancy	1	-	-
During pregnancy	4.66	1.37-15.83	**0.014**
Post-pregnancy	2.83	0.67-11.96	0.157
Maternal age (years)	1.03	0.96-1.12	0.4
Etiology of CS			0.151
Cushing's disease	1	-	-
Pregnancy-induced	5.93	0.63-55.46	0.119
Adrenal adenoma	1.45	0.57 - 3.68	0.431
Ectopic ACTH secretion	1.84	0.18- 18.82	0.606
Adrenal carcinoma	4.87	0.92- 25.8	0.063
Bilateral hyperplasia	1	NC	NC
Iatrogenic	1	NC	NC
Treatment during gestation			0.273
No treatment	1	-	-
Medical	0.99	0.23-4.23	0.986
Surgery	0.39	0.14-1.08	0.071
Medical and Surgery	1.09	0.16-7.33	0.931

OR: Odds Ratio; CI: confidence interval; NC: not computed

1.10. Maternal mortality in patients with active Cushing's syndrome

Two patients with active CS died in the postpartum period, within the first two weeks after delivery. The first was diagnosed with an adrenal adenoma and died from surgical complications after adrenalectomy (119). The second case had an ectopic ACTH secreting tumor of the pancreatic tail. Her pregnancy ended spontaneously at 27 weeks and was complicated with GDM, preeclampsia and severe hypokalemia. She died 2 weeks post-delivery after progressive deterioration (71). No maternal mortality was described in patients with CD, bilateral hyperplasia, adrenal carcinoma, iatrogenic CS and pregnancy-induced CS. The maternal mortality ratio was 1,257/100,000 livebirths.

2. Risk category system to identify pituitary adenoma patients with *AIP* mutations

2.1. Clinical characteristics of the cohort

Out of the 1429 PAs patients, 153 carried an *AIP* mutation (10.7%). Out of the 343 relatives of patients with *AIP* mutations, 165 were carriers of an *AIP* mutation (48.1%). The clinical characteristics of the patients with PAs are described in **Table 11**.

Twenty-four family members were prospectively diagnosed with a PA, 19 of these carried an *AIP* mutation, while five belonged to *AIP* mutation-negative families. Prospectively diagnosed patients were excluded from the analysis and their characteristics are described in **Table 12**.

Table 11. Clinical characteristics of the patients with pituitary adenomas

Clinical characteristic	**N=1405***
AIP mutation, n (%)	134 (9.5%)
Familial, n (%)	607 (43.2%)
Gender, n (% male)	680 (48.5%)
Diagnosis, n (%)	
GH excess	767 (55.5%)
NFPA	185 (13.4%)
Prolactinoma	344 (24.9%)
Cushing's disease	74 (5.4%)
Other diagnosis	11 (0.8%)
Age of onset (years) †	27.1±13.1
Age at diagnosis (years) †	30.8±13.4
Macroadenoma, n (%)	977 (81.5%)
Extrasellar extension, n (%)	446 (60.1%)
Pituitary apoplexy, n (%)	48 (3.9%)

*Prospectively diagnosed patients excluded; †Median and interquartile range

Table 12. Clinical characteristics of the *AIP* positive prospectively diagnosed patients

Clinical characteristic	**N=19**
Familial, n (%)	19 (100%)
Gender, n (% male)	12 (63.2%)
Diagnosis, n (%)	
GH excess	9 (47.4%)
NFPA	10 (52.6%)
Age at diagnosis (years)	30 [19-37]
Maximum diameter (mm)*	6 [4.9-10]
Macroadenoma, n (%)	6 (31.6 %)
Extrasellar extension, n (%)	2 (11.8%)
Pituitary apoplexy, n (%)	0 (0%)
Number of treatments*	0 [0-2]

NFPA: non-functioning pituitary adenoma
*Median and interquartile range

Six novel *AIP* mutations were found in one patient each, five with a diagnosis of gigantism and one with acromegaly. Their characteristics are detailed in **Table 13**. All AIP mutations identified are listed in **Table 14**. Patients with the c.911G>A (p.R304Q) and c.100-18C>T variants were excluded from this study, as recent data suggest that these might represent variants of unknown significance.

Table 13. Novel *AIP* mutations not previously reported

***AIP* mutation**	**MAF in GnomAD**	**Variant type**	***In silico* prediction§**	**Probability score#**	**Gender**	**Familial vs simplex**	**Diagnosis***	**Age at diagnosis**	**Age at onset**
c.240_241delinsTG (p.M80_R81delinsIG)	not reported	Insertion deletion	High	Disease causing 1	M	Simplex	Gigantism	8	5
c.333delC (p.K112Rfs*44)	not reported	frameshift	High	Disease causing 1	F	Simplex	Gigantism	9	7
c.376_377delCA (p.Q126Dfs*3)	not reported	frameshift	High	Disease causing 1	F	Simplex	Gigantism	13	10
c.605A>G (p.Y202C) †	not reported	missense	High	Disease causing 0.99	M	Simplex	Gigantism	10	10
c.645+1G>C (p.?)	not reported	splicing	High	Disease causing 1	M	Simplex	Acromegaly	33	24
c.991T>C (p.331Rext91)	not reported	missense	High	Polymorphism 0.99	M	Simplex	Gigantism	16	12

gnomAD: http://gnomad.broadinstitute.org/
§ In silico prediction of probability of damaging mutation by VEP and Anovar.
Probability of pathogenic mutation by Mutation taster.
*All patients had macroadenoma and none of them presented with pituitary apoplexy.
† This missense variant affects position 22 in the first tetratricopeptide domain of AIP, a well-conserved position in various tetratricopeptide domain proteins (101,120).

Table 14. List of *AIP* mutations in our cohort divided into truncating and non-truncating

Truncating mutations	**Non-truncating mutations †**
g.4856_4857CG>AA(98,101,121)	c.140_163del (p.G47_R54del)(122)
c.1-?_993+?del- (whole gene deletion)(101)	c.469-2A>G (p.E158_Q184del)(123–125)
c.100-1025_279+357del (p.A34_K93del) (exon 2 deletion)(126)	c.562C>T(p.R188W)(104)
c.240_241delinsTG (p.M80_R81delinsIG)	**c.605A>G (p.Y202C)**
c.241C>T (p.R81*)(98,121,127–129)	c.713G>A (p.C238Y)(98,130)
c.249G>T (p.G83Afs*15)(101)	c.760T>C (p.C254R)(104)
c.333delC (p.K112Rfs*44)	c.762C>G (p.C254W)(104)
c.338_341dupACCC (p.L115Pfs*16)(35,95)	c.805_825dup (p.F269_H275dup)(98,121,124)
c.376_377delCA (p.Q126Dfs*3)	c.807C>T (p.(=))(98,101,126,131–133)
c.3G>A (p.?)(134)	c.811C>T p.R271W(101,122,132,135)
c.40C>T (p.Q14*)(35,92,136,137)	c.815G>G (p.G272D)(134,138)
c.427C>T (p.Q143*)(35)	c.872_877delTGCTGG (p.V291_L292del)(96)
c.490C>T (p.Q164*)(101)	**c.991T>C(p.331Rext91)**
c.570C>G (p.Y190*)(35)	
c.645+1G>C (p.?)	
c.662dupC (p.E222*)(101)	
c.70G>T (p.E24*)(98,130)	
c.74_81delins7 (p.L25Pfs*130)(101,139)	
c.783C>G (p.Y261*)(35,124,140)	
c.787+9C>T(35)	
c.804C>A (p.Y268*)(35,127,141)	
c.816delC (p.K273Rfs*30)(35)	
c.868A>T (p.K290*)(35)	
c.910C>T (p.R304*)(92,98,122–124,132,140,142)	
c.967delC (p.R323Gfs*39)(35)	
c.976_977insC (p.G326Afs*?)(35)	
c.978dupG (p.I327Dfs*?)(35)	

Mutations in bold are novel mutations not previously described.

† Patients with the c.911G>A (p.R304Q) and c.100-18C>T variants were excluded from this study, as recent data suggest that these might represent variants of unknown significance.

2.2. Clinical characteristics comparing *AIP* positive and *AIP* negative patients

The age of onset was significantly lower in *AIP* positive vs *AIP* negative patients (16 [14-24] (interquartile range) vs 25 [19-33] years, p<0.001), as was the age at diagnosis (21 [16-31] vs 29 [22-38] years, p<0.001). **Table 15** contains the comparison of clinical characteristics of *AIP* mutation positive and *AIP* negative patients.

Table 15. Clinical characteristics comparing *AIP* positive and *AIP* negative patients

Clinical characteristic	***AIP* positive**	***AIP* negative**	**p-value**
Familial, n (%)	89 (66.4%)	518 (40.8%)	<0.001
Gender, n (% male)	83 (61.9%)	597 (47.1%)	0.001
Diagnosis, n (%)			<0.001
GH excess	119 (88.8%)	648 (52%)	
NFPA*	4 (3%)	181 (14.5%)	
Prolactinoma	11 (8.2%)	333 (26.7%)	
Cushing's disease	0	74 (5.9%)	
Other diagnosis	0	11 (0.9%)	
Age of onset (years)			<0.001
0-18	79 (60.3%)	259 (23.6%)	
19-30	33 (25.2%)	506 (46%)	
>30	19 (14.5%)	336 (30.5%)	
Age at diagnosis (years)			<0.001
0-18	53 (40.5%)	163 (14.1%)	
19-30	44 (33.6%)	497 (43%)	
>30	34 (26%)	495 (42.9%)	
Maximum diameter (mm) †	16.0 [10.7-25]	20 [11-30]	0.518
Macroadenoma, n (%)	112 (93.3%)	865 (80.2%)	<0.001
Extrasellar extension, n (%)	52 (81.3%)	394 (58.1%)	<0.001
Pituitary apoplexy, n (%)	12 (9.5%)	36 (3.3%)	0.001
Number of treatments †	2 [1-3]	1 [1-2]	0.055

NFPA: non-functioning pituitary adenoma
†Median and interquartile range

2.3. Building a risk category system

The likelihood-ratio test to evaluate interaction terms of the previous statistically significant variables was non-significant (p=0.149), hence interaction terms were excluded from the model. The variable selection process suggested that pituitary apoplexy and gender should be removed from the model, as they did not add predictive power. A markedly increased risk of an *AIP* mutation was associated with having a family history, a GH-excess adenoma, macroadenomas and young age of onset. The variables included in the predictive model are listed in **Table 16**.

Table 16. Logistic regression to generate a predictive model for *AIP* mutations

Variable	OR [95% CI]	p-value
Age of onset		
>30	1	-
0-18	14.34 [7.41-29.31]	<0.001
19-30	2.26 [1.17-4.35]	0.015
Positive family history	10.85 [6.48-18.16]	<0.001
Diagnosis		
Others	1	-
GH excess	9.74 [5.12-18.52]	<0.001
Size (macroadenoma)	4.49 [1.91-10.59]	0.001

Variables are listed in the order of their statistical strength for prediction and each odds ratio (OR) is adjusted for all the other variables.

We stratified the risk of having an *AIP* mutation into low (<5%), moderate (5-19%) and high risk (>20%), based on our predictive model. **Figure 6** shows stratified risks according to age, family history, tumor type and tumor size.

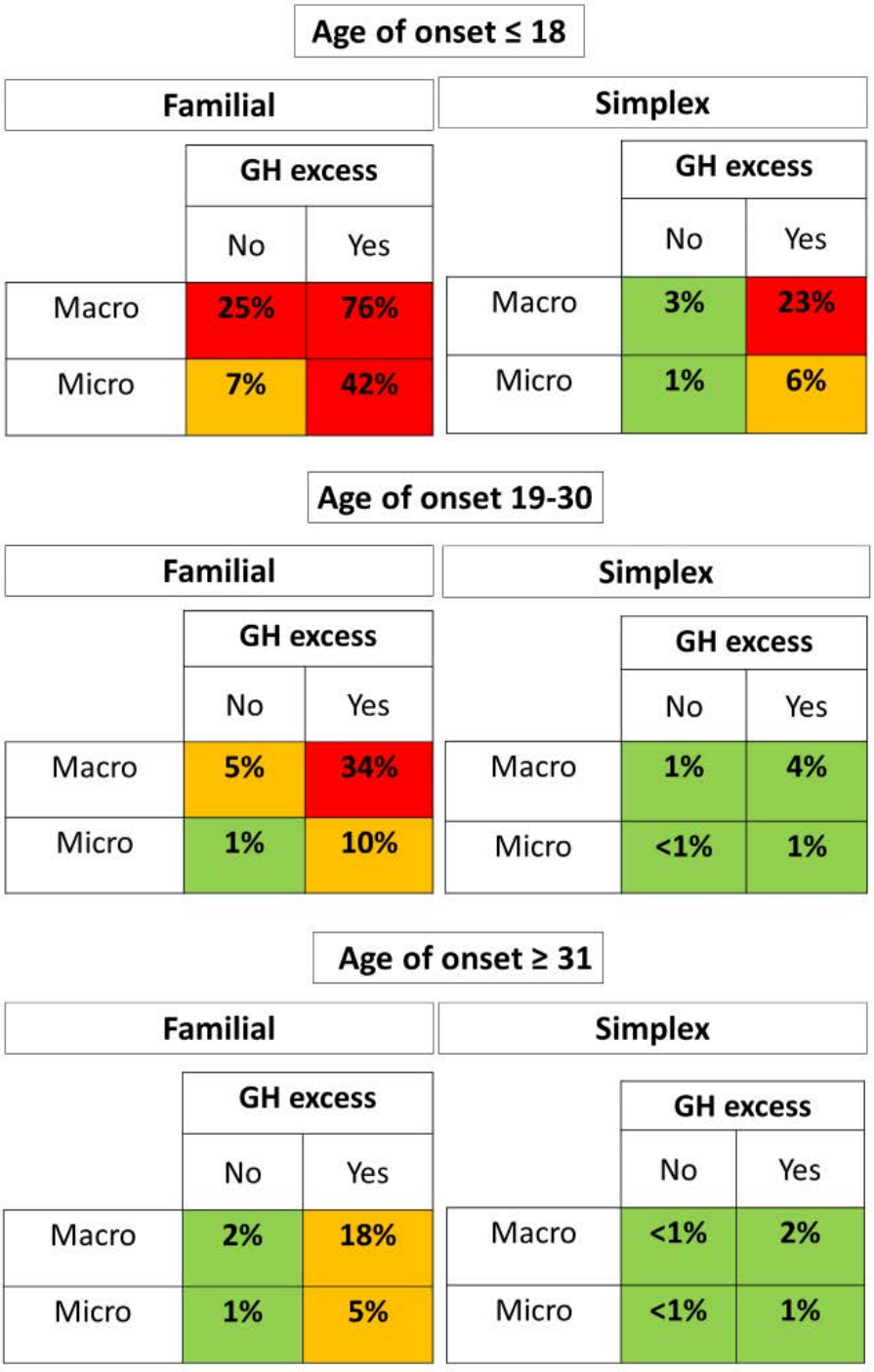

Figure 6. Risk stratification for *AIP* mutations, classified as low (<5%), moderate (5-20%) or high risk (>20%).

Red: risk of AIP mutation >20%; orange: risk of AIP mutation between 5 and 20%; green: risk of AIP mutation <5%. Micro: microadenoma; Macro: macroadenoma. Simplex: patients with no family history or sporadic tumors.

2.4. Performance and internal validation of the model

In our cohort, 70.8% of patients were identified as low-risk, 9.2% as intermediate risk, while 20% were at high risk (risk >20%).

Good discriminative power was achieved with the AUC, reaching a value of 0.87 (95% CI 0.84-0.90) (**Figure 7).**

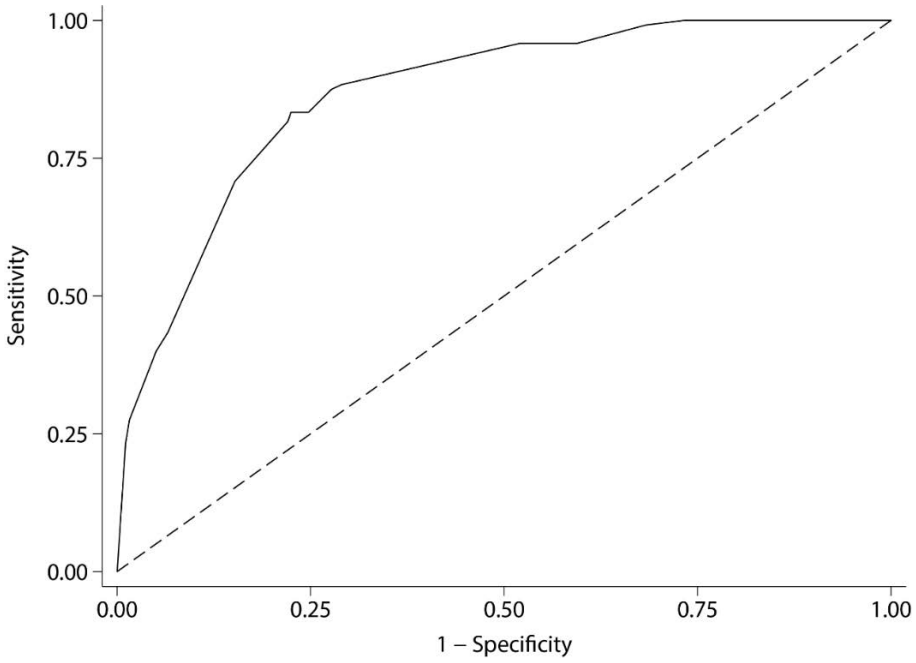

Figure 7. Area under the ROC curve of the *AIP* mutation risk category system.

Area under the ROC (receiver operating characteristic) curve of the AIP mutation risk category system is 0.87 (95% CI 0.84-0.90), indicating an excellent discriminating power.

Calibration results, comparing observed and model-predicted *AIP* mutation risk across the three risk groups, are depicted in **Figure 8**. The similar probabilities for estimated and observed risk indicates a good calibration of the model. The Hosmer-Lemeshow test was non-significant (p=0.213), suggesting that the model is well calibrated.

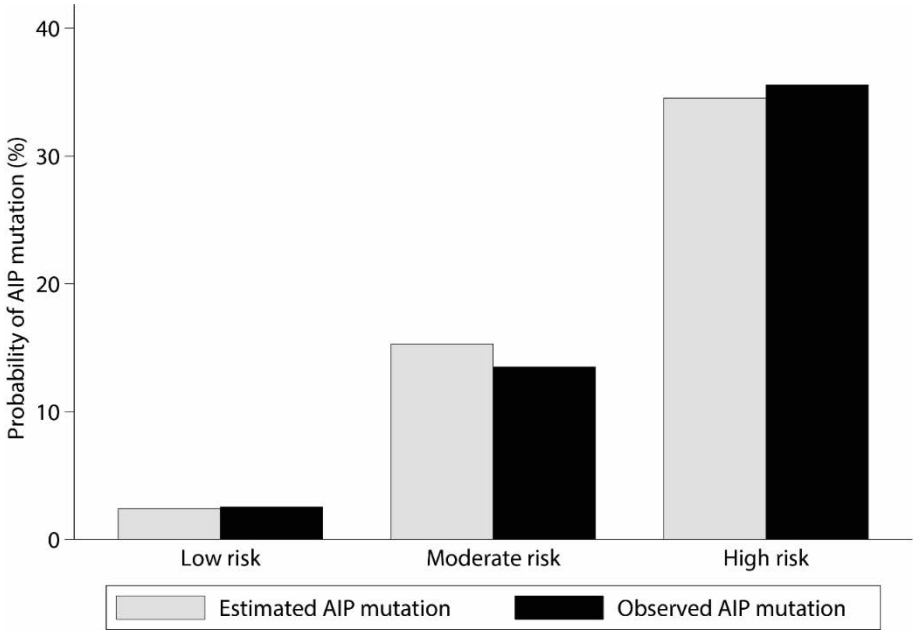

Figure 8. Observed versus model-derived *AIP* mutation risk model with low (<5%), moderate (5-19%) and high risk (≥20%) categories.

Finally, the model showed good internal validation, as tested by the cross-validation technique, as the R^2 shrinkage was <10% in absolute terms (from 0.294 to 0.223).

DISCUSSION

A rare disease is a health condition affecting a small number of people compared with other prevalent diseases in the general population (less than 50 cases/100,000 people, in the European Union) (143). There has been increasing attention on rare diseases over the last several decades as a result of scientific and technological advances providing a broader understanding of their genetic, molecular, and biochemical basis. This is also encouraged by legislation to intend to facilitate patient access to effective treatments (143).

Cushing's syndrome (CS) and familial isolated pituitary adenomas (FIPA) are considered rare diseases as their prevalence are about 1-9/100,000 people. The number of cases further decreases significantly for both pathologies in unusual situation such as pregnancy and childhood. In this thesis I addressed two rare conditions: 1) the causes and consequences of CS in pregnancy, and 2) the prediction of *AIP* mutations in pituitary adenoma patients.

1. Causes and consequences of Cushing's syndrome in pregnancy

A large group of 220 patients diagnosed of CS in whom 263 pregnancies occurred were systematically reviewed in the literature.

The observation that the etiology of CS in pregnant women is different from non-pregnant patients was confirmed with this study. For instance, the adrenal origin was the most frequent etiology in active CS during pregnancy (82,144), likely to be due to the highest degree of impairment of the gonadal axis in hypercortisolemia secondary to a pituitary source, as it has been suggested that adrenal adenomas mainly produce excess of cortisol and in CD there is both cortisol and androgen hypersecretion (145–147).

Pre-pregnancy maternal characteristics are described in this study, including past medical history of Type 2 diabetes mellitus (DM) and chronic hypertension as well as past obstetric history. The frequency of Type 2 DM was 9.3% and chronic hypertension 30.1%, both much higher than the prevalence observed in healthy women of the same

group of age (148,149). Pointing in the same direction, the past obstetrical history was worse in comparison to general population, being the past history of GDM (11.6%) 2-3 times higher than in women without CS (4%) (150) as well as preeclampsia (13%), which was 6 times higher than in healthy women (2.2%) (151). Moreover, the frequency of spontaneous abortion (34.8%) and fetal loss (10.1%), was respectively 2 and 10 fold higher than in healthy women (152). These findings seem to reflect that excess of cortisol was present prior to pregnancy, and was associated with a worse obstetrical history. Interestingly, other endocrinological disorders such as GDM (153) or hyperparathyroidism (154) have shown worse past obstetric history than in the general population. Similarly, a population-based cohort study in Denmark studied the morbidity and mortality in CS in non-pregnant subjects before and after treatment. They observed a poorer prognosis in the three years prior to diagnosis of CS, where more venous thromboembolism, stroke, peptic ulcers, fractures and infections were observed in 343 CS patients diagnosed over 30 years, compared to 34300 matched controls, also suggesting that undiagnosed hypercortisolism was the reason for this worse outcome (155).

Pregnancy outcomes were also addressed in this study. The incidence of preterm deliveries was higher in patients with active CS, likely related to more frequent complications during pregnancy such as GDM, hypertension or preeclampsia, increasing the rates of Caesarean section when compared to cured CS (51.7 vs 21.9%). Patients with active CS had higher prevalence of GDM (36.9%), gestational hypertension (40.5%) and preeclampsia (26.5%) than in cured CS (2.3, 2.3 and 2.3% respectively). In this last group, prevalence was similar to that observed in the general population (150,151). Fetal mortality occurred in 24% of the pregnancies with active CS, compared to cured CS (8.5%) that had similar fetal mortality to that expected in healthy women (152,156). These results suggest that patients with cured CS normalized both maternal and fetal risks, while active CS had a notably negative impact on the prognosis of pregnancy.

We aimed to study the predictors for fetal morbidity and mortality in this cohort. The results indicated that pregnancy-induced CS had the highest impact on overall fetal loss (≅5 times higher than for CD) and spontaneous abortion (≅ 17 times higher than for CD), when compared to other causes of CS. This association to a higher risk for fetal loss is

not surprising, as in pregnancy-induced CS the hypercortisolism appears at an early stage of pregnancy stimulated by the rise of beta-HCG, which might be difficult to diagnose and therefore treatment is often delayed. Moreover, the aim for the treatment in these cases is to control hypercortisolemia, as definitive treatment such as bilateral adrenalectomy, would involve irreversible adrenal insufficiency and therefore avoided in this reversible cause of CS. Adrenal carcinoma was also associated with a borderline significant increase in spontaneous abortion. The severity of hypercortisolism was not studied; however, it is plausible that patients with adrenal carcinoma had higher degree of hormonal abnormalities as they presented with higher rate of hypokalemia that would support this. On the other hand, maternal age was negatively associated with induced abortion, less common as maternal age increased (OR 0.71) consistent with the general population (157).

Only 39.1% of the patients (83) received any kind of treatment during pregnancy (medical, surgical or both), and up to 7 combinations of drugs were used in 24 patients, including ketoconazole, metyrapone, cabergoline, mitotane and cyproheptadine (**Table 4**). Therefore, no definite conclusion can be reached as to the optimal management for CS during pregnancy due to the low number of cases for each treatment modality. Despite this, receiving any medical or surgical treatment decreased the risk of overall fetal loss between 3 and 4 times, but did not protect from prematurity, as previously described (82). Untreated CS is associated with significantly more maternal and fetal morbidity as described in previous reports (41,82). The first choice of treatment is surgical (41,145), which is safer in the second trimester due to the lower risk of fetal and maternal complications (146,158). It reduces perinatal mortality and maternal morbidity rates, but does not affect the occurrence of preterm birth and intrauterine growth restriction (159). Otherwise, different medical drugs have been used in a significant number of patients without any apparent adverse consequences. The most commonly used is metyrapone followed by ketoconazole. Medical treatment has been used at different times of pregnancy (18, 28–30), for symptomatic control after diagnosis or when surgery was contraindicated.

Those patients diagnosed of CS during pregnancy, compared to those diagnosed before pregnancy, presented with a 7-fold increased the risk of low birth weight and also a 5-fold increased the overall risk of fetal morbidity and mortality. These findings indicate

that a longer delay in diagnosis during pregnancy is likely to impair fetal outcome, which can be explained due to the difficulty in diagnosing hypercortisolism during gestation.

Importantly, the maternal mortality ratio was 1,257/100,000 livebirths in patients with active CS. This ratio is 6 times higher than the worldwide maternal mortality ratio (239 per 100,000 livebirths and 12 per 100,000 livebirths in developing and developed countries in 2015, respectively), and even higher than the highest reported maternal mortality of 956.8 seen in South Sudan (163), indicating a significant risk of pregnancy related mortality in CS.

Several limitations have to be considered in this study. First, the proportion of etiologies includes a considerable number of rare cases, with favorable outcomes especially in the period 1974-1994, suggesting a publication bias. Second, the patients included are from a wide historical period starting in 1952, when treatments and diagnosis were very different from recent publications. Third, the criteria to define maternal and fetal complications might differ across centers, as the patients came from different centers with their own criteria. Finally, biochemical data to evaluate the degree of hypercortisolism were limited, and therefore it could not be included in the multivariate analysis.

2. Risk category system to identify pituitary adenoma patients with *AIP* mutations

Despite of the number of genes associated with FIPA is increasing (92,93), formal guidelines do not currently include recommendations for screening for *AIP* mutations (164). Nowadays, screening for *AIP* mutations in patients with PAs is performed based on the clinician judgment and following expert recommendations, including: a) family history of PA, b) childhood-onset PA, or c) pituitary somatotroph or lactotroph macroadenoma diagnosed before the age of 30 years. (91,132,165). Regardless of these recommendations, patients do not receive an individual risk depending on their particular clinical characteristics (91,124,140,166).

In this study we identified four significant predictors for the presence of carrying an *AIP* mutation, all of them readily available at the time of diagnosis: 1) positive family history, 2) young age of onset, 3) somatotroph tumor type and 4) large tumor size. A risk score was generated using the combination of these clinical features, which reliably predict the risk of carrying an *AIP* mutation. Once a mutation carrier is identified, genetic counselling and genetic testing should be offered to family members.

As expected, several studies in the literature have highlighted the influence of the aforementioned predicting factors as typical clinical features of *AIP* mutation positive patients. They are discussed in next paragraphs in the order of their statistical strength for prediction.

The strongest predictor identified was the age of onset, being the group of patients with maximum risk for an *AIP* mutation those who presented with a PA during childhood (OR 14.3 (95% CI 7.4-27.7), $p<0.001$). This is in the line with previous publications that reported a prevalence of *AIP* mutations in pediatric cases in the range 6 of 23% (124,132,140,167). An age of onset comprised between 19 and 30 years was also a strong predictor (OR 2.3 (95% CI 1.2-4.4), $p=0.015$), also in agreement with previous epidemiological studies that identified a relative high frequency of patients with *AIP* mutation in sporadic macroadenomas diagnosed before the age of 30 years (11.7%), being

this percentage up to 13.3% of patients with somatotropinomas, 11.5% of prolactinomas and 6.3% of those with non-functioning pituitary adenomas (NFPA) (132).

A positive family history of PA was the second strongest predictive variable (OR 10.85 (95% CI 6.48-18.16), P<0.001), not surprising as PA in the context of *AIP* mutations is an autosomal dominant disease, although its penetrance is incomplete in about 20% (35,110).

It is well known that somatotropinomas or somato-lactotropinomas account for around 80% of the patients with *AIP* mutations, although prolactinomas and clinically NFPAs with positive GH and/or PRL immunostaining are also well described. In contrast, ACTH, TSH-secreting adenomas or gonadotrophin positive or null cell NFPAs are rarely described (168). In line with these findings, GH excess tumor type was also a good independent predictor for *AIP* mutations in our model (OR 9.74 (95% CI 5.12-18.52), P<0.001).

PAs in the context of *AIP* mutations are significantly larger, being macroadenomas in up to 90% of the cases, and more frequently show an extrasellar extension compared to non-mutated familial and sporadic cases (35,122). Not surprisingly, the last independent predictor factor in our model was the tumor size, and patients with macroadenomas had more than four times the risk of harboring an *AIP* mutation compared to those with microadenomas (OR 4.49 (95% CI 1.91-10.59), p=0.001).

Other clinical features widely available in the clinic were also considered for the risk category system, such as pituitary apoplexy and gender distribution. It is not uncommon a past history of pituitary apoplexy in the context of patients with positive mutation in *AIP* (35,98,111). This type of tumors prone to apoplexy are perhaps secondary to the rapidly growing adenomas or perhaps due to underlying molecular mechanism affecting vascularization. In our cohort, pituitary apoplexy was almost 3 times more frequent in *AIP* positive patients than in *AIP* negative (9.5% vs 3.3%, p=0.001). This candidate predictor was not included in the risk model as its inclusion did not add any predictive power when adjusted for the other variables (AUC was 0.869 *vs* 0.868 with and without pituitary apoplexy, respectively).

Although gender distribution in favor of male gender was different in the univariate analysis (61.9 % vs 47.1 %, *AIP* mutation positive vs negative, respectively, p=0.001), it was not statistically significant after adjusting for the other variables and it did not add any predictive value to the model (AUC 0.869 vs 0.868, with and without gender distribution, respectively). The gender of distribution of *AIP* mutation positive patients has been debated as there are contradictory results published in the literature. Nevertheless, the observed trend to increase prevalence of male patients is likely an ascertainment bias and disappeared when adjusted for other co-variates.

Other variables could be a potential predictor for *AIP* mutations as for example SSA resistance or immunohistochemically features characteristics such as sparsely granulated subtype. In a multicenter study, 50 patients with resistance to first generation SSAs were investigated for *AIP* mutations. In 8% of them, mutations or variations of unknown significance were found (133). Although this is highly informative, the responsiveness of the treatment cannot be assessed before completing 6 months of treatment using the maximum usual dose of the drug (octreotide, lanreotide). Even though at the moment acromegaly guidelines do not recommend different treatments in patients with *AIP* mutations, some studies suggest that pasireotide could be a better option as a first-line treatment in these patients (33,116). In fact, one of the potential interests of this risk score is to identify those patients with *AIP* mutations even before starting the treatment, as in the future it would be a useful tool to select the appropriate modality of treatment for each patient (i.e. pasireotide vs first generation SSAs). Moreover, resistance to SSAs would be only of interest in somatotropinomas, as other tumor types such as prolactinomas or NFPA would not be included as their treatments are different to SSAs. On the other hand, the immunohistochemical features of the tumor other than the typical hormonal markers are not always available for the clinicians, therefore the risk score would be of less usefulness if these data were missing.

A risk category system was generated using the four identified predictors, stratifying the risk of *AIP* mutation into low (<5%), moderate (5-19%) and high categories (≥20%) (screening algorithm in **Figure 6**). These cut-offs were arbitrarily decided, as we believed that a risk <5% was low enough from a clinical perspective as to not recommend genetic screening. However, family membership should be into taken into account. Within the

same family, members could have different risk depending on age and tumor type, but the overall risk category of a kindred should be based on the risk score of the family member with the highest risk. Given that the outcome predicted is a germline mutation, it should be expected that if one member is likely harboring an *AIP* mutation (i.e. high risk), the rest of the members should have the same probability to be affected despite they might be categorized in a different risk category (i.e. low risk). Nevertheless, phenocopy patients also should be considered in this equation.

Calibration is reported graphically and using the Hosmer-Lemeshow test, which showed adequate calibration. Moreover, **Figure 8** showed an excellent goodness-of-fit of our model as there were no obvious differences between observed and model-predicted *AIP* positive patients. The model achieved an excellent discriminatory power as the AUC was 0.87 (95% CI 0.84-0.90) (169). Finally, the model was internally validated using a cross-validation method. The performance of the model was evaluated comparing the explained variation of the model (R2) in each of the five equal samples of the data and the total sample, achieving a reduction of R2 <10% (170).

A screening algorithm based on the results of the risk category system is depicted in **Figure 9**. Of note, age of onset was used to develop the risk category system, therefore it requires careful history taking, reviewing parents' height and available photographic evidence of change of features. Patients with pituitary gigantism should be considered to have childhood-onset disease and offered screening.

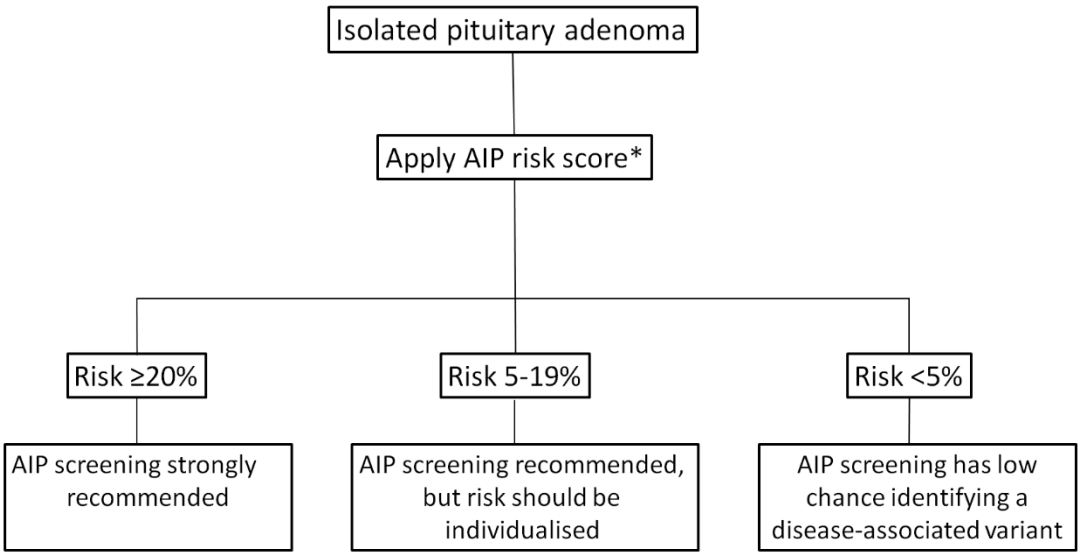

Figure 9. AIP screening algorithm based on the proposed risk category system.

The overall risk category of a kindred should be based on the risk score of the family member with the highest risk. AIP, aryl hydrocarbon receptor-interacting protein. *See **Figure 6**.

This study has several limitations. First, this risk score was based on a cohort enriched with familial, young-onset patients and GH-excess tumors as the number of *AIP* mutated patients in unselected cohorts is low. Although all possible diagnostic groups had some representation in the study, caution should be taken when extrapolating these results to a population with significantly different prevalence of *AIP* mutations than the one found in this cohort. Second, the determination of age of onset can be subjective and rely on patient recall. Nevertheless, when comparing the model using the age of onset with the one produced using age of diagnosis, the AUC was significantly better using age of onset rather than age at diagnosis, this might be explained due to the well-documented delay of diagnosis in patients with acromegaly. Third, an external validation of the model was not performed due to the relatively low number of cases with *AIP* mutations in the cohort, which precluded splitting the sample into a derivation and validation group. However, internal validation, the preferable method when external validation is not feasible, was successfully carried out.

3. Methodology challenges

Both CS and FIPA are considered rare diseases and their study required innovative and unconventional approaches.

Firstly, getting the study cohort was challenging due to the nature of the disease. This challenge was addressed in two different ways: conducting a systematic review of published cases and using an international collaboration to obtain a significant cohort of patients for both conditions. Despite of the available published case reports and reviews in CS and pregnancy, its knowledge is very limited, given the rarity of CS itself and the even rarer occurrence of pregnancy with concomitant hypercortisolism. Thus, large cohort studies in this field are not feasible and hence the importance of a systematic review in this context. We therefore collected patients with CS and pregnancy using all case reports from the literature. On the other hand, *AIP* mutations are present in about 20% of all FIPA patients, which in turn represents around 3-4% of PAs. The outcome of the study – the presence or not of *AIP* mutations – required a significant number of patients harboring an *AIP* mutation, as to perform a multivariate analysis, a minimum of 10 patients per predictor was required to avoid overfitting the statistical model. Therefore, the collective effort from the FIPA consortium was crucial to recruit a large cohort of FIPA patients in order to identify a large number of patients with *AIP* mutations.

Secondly, to perform this study, we faced with several statistical challenges. Although meta-analysis and systematic reviews of data coming from randomized clinical trials and observational studies are common, systematic collection of published case reports is a novel approach. The number of patients collected through this approach was sufficient to go beyond the univariate analysis that is typically conducted in cases reports series. Using a large cohort of patients gave us the opportunity to overcome the potential confounding provided by univariate analysis and allowed us to adjust for potential confounders in multivariate analyses in order to obtain a less biased predictors for the outcomes of pregnancy in CS. As a limitation of using this approach, we had to pay the toll of collecting patients from a large frame of time and with a significant number of rare cases, which might incur in a potential temporal bias.

Regarding the development of a risk category system to identify patients with PAs and *AIP* mutations, we faced several decisions, such as the decision of the cut-off values for risk categorization and the assessment of the robustness of the prediction model.

In our study, risk groups were labelled as low, intermediate or high-risk groups using the probabilities of the multivariate prediction model with the aim to aid clinical decision making (i.e. it is unlikely that the screening for *AIP* mutations would be worthwhile in low risk patients, whereas it should be strongly recommended in high risk patients). However, there is no consensus on how to create risk groups (169,171), though this decision may be taken considering the clinical background. Our three groups classified patients into low, intermediate and high risk based on the probabilities of having the *AIP* mutation (<5%, 5-19% and ≥20%, respectively). The rationale behind this method was to rule out the necessity for the screening, hence the low risk contained the vast majority of the patients of the cohort (71%) under the assumption that we might misdiagnose *AIP* mutation in 1 out of 20 patients (<5% risk), whereas in the high-risk group we select a sensible percentage of patients to whom the screening could be applied (20% of the cohort) assuming that 1 out of 5 patients (≥20%) would really have the mutation. In a way, we tried to sacrifice a bit of "positive predictive value" in exchange for having a higher "negative predictive value" (in our risk score, it is more likely for a low risk patient to not have the outcome than for a high-risk patient to have the outcome). We also tried to avoid the "grey zone" of the intermediate category for which the clinical-decision making recommendation was more difficult to establish (this the reason why only 9% of our cohort was grouped in this category). Despite the underlying rationale for the selection of these cut-offs was a trade-off between what is sensible and feasible in clinical practice (i.e. not all patients can be screened, but we need a tool to have certain guarantees that we rule out those patients with low probabilities of having the mutation), it must be acknowledged that other risk categorization can be done and would be equally valid. For instance, we might have chosen higher cut-off values for the high-risk category (i.e. 30% rather than 20%) if we were in a situation where we were lacking economical resources and had the intention to be sure that the selected population is really likely to have the mutation). Likewise, we might reduce the cut-off values for the low-risk category (i.e. from 5% to 2.5%) if we wanted to have a higher negative predictive value in exchange of having more patients undergoing the screening process.

The robustness of a prognostic model can be assessed through its performance measures (calibration and discrimination). Like it happened in the risk category system, the final decision of including one predictor instead of another, as well as in which form they are included (continuous vs categorical age) is a trade-off between pragmatism (the model has to be easy to use for clinicians) and reliability (the model has to predict well). The performance (reliability in layman' terms) of the model can be measured in several dimensions. Calibration reflects the agreement between predictions from the model and observed outcomes, whilst discrimination relates to the ability of a prediction model to differentiate between those who do or do not experience the outcome event. Whereas the *AIP* risk model is well calibrated, perhaps its most relevant feature is its ability to discriminate across patients. In this risk score, despite the reduced number of predictor variables included, it has an excellent discriminatory power, with an AUC (or c-statistic) of 0.87 (95% CI 0.84 to 0.90). A model has perfect discrimination if the predicted risks for all individuals who develop the outcome (*AIP* mutation) are higher than those for all individuals who do not experience the outcome (lack of *AIP* mutation). The AUC reflects the probability that for any randomly selected pair of individuals, one with and one without the outcome, the model assigns a higher probability to the individual with the outcome. It ranges from 0.5 (no discrimination) to 1 (perfect discrimination). In other words, the c-statistic is the probability that the predicted risk is higher for a case than for a non-case (172). In medicine, a c-statistic of 0.87 is unusually high and relatively uncommon. The reasons behind this high c-statistic are not only the fact that the predictors are strongly associated with the outcome, but also that there might be some degree of selection bias in our cohort (i.e. high percentage of acromegaly). Therefore, having a high c-statistics is sometimes a double-edged sword, given that it may translate a potential lack of generalizability to other populations. In view of the fact that selection bias is inherent to all observational studies and that we have a large international cohort representing worldwide clinical practice, we believe that this potential selection bias does not jeopardize the generalizability of our findings and that the *AIP* risk score can be performed in any given outpatient clinic.

4. Clinical implications

In both studies, we performed a prognostic model that allowed us to better identify patients under risk of fetal loss or harboring an *AIP* mutation, respectively. In the era of personalized medicine, the identification of predictors is crucial to adapt the clinical management to each case in particular.

In CS and pregnancy, predictors for fetal loss are the etiology of CS, specifically the pregnancy-induced CS and adrenal carcinoma had worse prognosis compared to the rest of causes. Importantly, although we cannot preclude any definitive conclusion about the best management of the patients, we confirmed that the treatment during gestation, either medical or surgical treatment, seems to reduce the risk for fetal loss. Altogether, these predictors can be useful for clinicians to better estimate the prognosis of the pregnancy, and therefore a better decision-making process could be done considering them.

Regarding genetic screening for *AIP* mutations, the use of genetic screening is currently clearly underused and there is an unmet clinical need for predictive tools to identify patients at risk for gene mutations related to PAs. While one indicator cannot achieve good discrimination, a risk score based on several indicators is essential for reliable prediction. This risk prediction tool, developed with the help of a large international cohort of patients, uses items routinely available in clinical setting and could be used routinely by clinicians to support the decision-making process for referral individuals to genetic tests. This risk score can help identifying patients with *AIP* mutations and equally important, to recognize those at risk of potentially aggressive PA. The identification of carriers could permit an early diagnosis of adenomas at a non-invasive stage, where treatment is more likely to be effective or curative and, importantly, to avoid excessive height in those with GH-secreting adenomas.

CONCLUSIONS

In this thesis, predictors have been reported for two unusual situations in the context of rare diseases, as summarized below.

1. The fetal outcomes are different depending on the cause of CS, as patients with pregnancy-induced CS (presumably due to aberrant LH or beta-hCG receptors on the adrenal) and adrenal carcinoma had the worst fetal prognosis in comparison to other causes.

2. The diagnosis of CS during pregnancy is associated to poorer overall fetal outcome, with increased morbidity and mortality, presumably related to the delay in diagnosis during pregnancy.

3. Both medical treatment and surgery during pregnancy appear to be protective in avoiding fetal loss.

4. A positive family history, young age of onset, somatotroph tumor type and large tumor size can reliably predict the risk of an *AIP* mutation in patients with pituitary adenomas.

5. Although the frequency of pituitary apoplexy and gender distribution in favor of male gender is increased in the *AIP* positive group, these variables do not add any predictive value when adjusted for the rest of variables.

6. The risk category system to identify patients with *AIP* mutation is a tool that can facilitate the clinical decision making for genetic screening, allowing the identification of patients who carry *AIP* mutations and providing the opportunity of early diagnosis in at-risk relatives.

FUTURE RESEARCH

Regarding CS it would be of interest to create a prospective European registry for patients with CS and pregnancy, with special emphasis on the diagnosis and treatment. The diagnosis is difficult, as there are many clinical and biochemical resemblances between CS and pregnancy, therefore, there is a need to develop validated cut-off levels for screening tests to diagnose CS in the context of pregnancy, as this would be of great value for clinical decision making. Additionally, there is a need for identifying the best strategy for CS treatment in the context of pregnancy. An international prospective registry would be useful as a single center would not be able to recruit the necessary number of patients in order to perform these studies.

Regarding the *AIP* predictive model, an external validation of the model using a different cohort would be of great value for the use of the *AIP* risk category system. Moreover, other possible predictors to improve discriminatory power of the risk category system could be studied, as for example medical treatment resistance or histology findings. In addition, in those prospectively diagnosed, it would be interesting to study which factors, other than genetic, are involved in the appearance of tumors, as for example smoking habit or environmental factors. Furthermore, it would be of interest to evaluate the impact on prognosis in those patients with an earlier diagnosis. Finally, there is a pressing need to identify novel therapies for patients who respond poorly to somatostatin analogues, therefore future research should also focus on identifying direct cellular and molecular targets for *AIP* that will lead to identification of targeted therapies and lead to better management of this condition.

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ANNEX I

In addition to the originals papers included in this thesis, the following two reviews have been written by the PhD candidate.

1. Review I

Caimari F, Corcoy R, Webb SM. Cushing's disease: major difficulties in diagnosis and management during pregnancy. Minerva Endocrinol. 2018 Dec;43(4):435-445.

2. Review II

Caimari F, Korbonits M. Novel Genetic Causes of Pituitary Adenomas. *Clin. Cancer Res.* 2016;22(20):5030–5042.

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REVIEW

HOW TO HANDLE PITUITARY DISEASE DURING PREGNANCY

Cushing's disease: major difficulties in diagnosis and management during pregnancy

Francisca CAIMARI [1, 2], Rosa CORCOY [1, 2, 3, 4], Susan M. WEBB [1, 2, 4, 5] *

[1]Department of Endocrinology, Sant Pau Hospital, Barcelona, Spain; [2]Autonomous University of Barcelona, Barcelona, Spain; [3]Department of Bioengineering, Biomaterials and Nanotechnology, Carlos III Institute for Health, Madrid, Spain; [4]Teaching Unit, Sant Pau Hospital, Autonomous University of Barcelona, Barcelona, Spain; [5]Unit 747, Center for Biomedical Investigation of Rare Diseases (CIBERER), Sant Pau Hospital, Carlos III Institute for Health, Madrid, Spain

*Corresponding author: Susan M. Webb, Department of Endocrinology, Sant Pau Hospital, Pare Claret 167, 08025 Barcelona, Spain. E-mail: swebb@santpau.cat

ABSTRACT

Pregnancy in women with a diagnosis of Cushing' syndrome (CS) is an extremely rare event and its diagnosis and treatment are a real medical challenge. During pregnancy, the hypothalamus-pituitary-adrenal axis undergoes major changes leading to a significant increase in plasma cortisol levels throughout gestation. The difficulties in diagnosis are related to the resemblance of symptoms of CS and those of pregnancy, and to the complex interpretation of the screening tests. Moreover, the diagnostic work up in the postnatal period may be difficult in the first weeks postpartum. Importantly, the etiology of CS in pregnancy differs from non-pregnant status. In pregnancy, the adrenal origin is the most frequent in up to 60% of the cases, in contrast to ACTH-secreting corticotroph adenomas of the pituitary gland, which account for 70% of the cases outside pregnancy. Nevertheless, maternal and fetal outcomes are severely affected in the context of CS whichever the etiology is, with high rates of maternal and fetal morbimortality, and with a rate of overall fetal loss of about 25% of the pregnancies. There is no consensus as to the most effective treatment in these circumstances in terms of improving maternal and fetal outcomes, as there are no studies comparing the different modalities of treatment for CS in pregnancy. However, evidence suggests that patients receiving treatment during pregnancy achieve better fetal outcomes than those who do not receive treatment. We aim to summarize in this review the major diagnostic and management difficulties during pregnancy.

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KEY WORDS: Cushing Syndrome - Pregnancy - Fetal death - Pituitary ACTH hypersecretion.

The hypothalamus-pituitary-adrenal (HPA) axis mediates the stress response of glucocorticoids. Corticotrophin-releasing hormone (CRH) is secreted by the hypothalamus from the paraventricular nucleus and stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH also stimulates the secretion of cortisol from the adrenal gland, which regulates the hypothalamus and pituitary secretion by negative feedback. Of note, the expression of CRH and its related peptides is widespread in peripheral tissue, with important functions in the immune system, energy metabolism, and female reproduction. CRH is involved in the implantation of fertilized ovum and in maternal tolerance to the fetus.[1-3]

Endogenous Cushing Syndrome (CS) is a rare disorder resulting from cortisol excess. The incidence ranges from 1.7 to 2.4 per million population per year,[4, 5] and is more common in women than in men.[6] The diagnosis of CS can be often challenging, since other conditions like for ex-

ample pregnancy or the metabolic syndrome can resemble some of the Cushing symptoms.

Pregnancy is a physiological cause of hypercortisolism and the simultaneity of CS and pregnancy is an extremely rare situation. An increasing number of cases has been reported in the literature,[7, 8] confirming the difficulties in the diagnosis and treatment in these circumstances. This review summarizes these diagnostic and management difficulties in CS in the event of pregnancy.

The hypothalamus-pituitary-adrenal axis during pregnancy and early postpartum

During pregnancy, the HPA undergoes major changes and the levels of total plasma cortisol and plasma free cortisol increase significantly throughout gestation.[9] This is explained in part by the estrogen stimulation of corticosteroid-binding globulin (CBG) with a rise in total cortisol levels,[10-12] and in part due to the placenta acting as a neuroendocrine organ controlling some of the adaptive phenomena by autocrine, paracrine, and endocrine mechanisms.[13] For instance, the placenta secretes CRH, which is involved in the timing of birth by modulating signaling systems that control the contractile properties of the myometrium [14, 15] and it is equally importantly involved in fetal lung maturation, brain development and other fetal organs.[16, 17]

CRH, ACTH and cortisol are secreted in a pulsatile fashion.[18] The secretion of placental CRH stimulates both the maternal pituitary and adrenal, leading to an increase in cortisol production. Plasma CRH increases progressively throughout pregnancy and correlates well with the weeks of gestation [9] and maternal cortisol levels,[18] but not with the ACTH levels.[18] This may be explained through a direct action on the CRH receptors present in maternal adrenal glands.[18] Importantly, CRH receptors are found in uterine tissue, fetal pituitary and human fetal adrenal gland.[2]

Morphologically, the fetal adrenal gland is comprised by 3 zones: the outer, definitive zone; a large, inner fetal zone; and an intermediate transitional zone. The fetal adrenals undergo rapid growth throughout gestation, and at term, they are significantly larger, relative to body weight, than adult adrenals.[19] In the fetus, CRH stimulates the inner fetal adrenal zone, to produce dehydroepiandrosterone sulphate (DHEAS), which is converted to estrogen by the placenta, and the transitional zone to release cortisol, both directly and through stimulation of the fetal pituitary gland.[1] Cortisol induced from the fetal and maternal adrenal glands by placental CRH induces further placental CRH expression, forming a positive feedback system that results in increasing placental production of estrogen *via* conversion of DHEAS.[1, 9]

Plasma ACTH levels are not suppressed despite the increasing levels of plasma cortisol, estrogen and progesterone [20] due to CRH stimulates the secretion of peptides containing the ACTH sequence in the placenta in a dose-dependent manner, as it does in the pituitary.[21] This ACTH-induced cell proliferation during pregnancy gradually leads to maternal adrenal gland hypertrophy (Figure 1).

There is a progressive rise in total plasma cortisol, CBG, and 24-hour urinary free cortisol (UFC) during pregnancy, peaking during the third trimester. CBG concentrations are 3-fold higher in the third trimester and remain elevated 2-3 months postpartum. Plasma free cortisol concentration has been found 1.6-fold elevated during the third trimester and the 24-hour UFC excretion rates are up by 1.7-, 2.4-, and 3.1-fold during the first, second, and third trimesters, respectively, compared with non-pregnant women.[22] Despite the increasing circulating levels of cortisol, the diurnal rhythm of cortisol secretion is maintained throughout pregnancy.[10, 11, 18] As pregnancy progresses, the increased circulating cortisol downregulates hypothalamic production of CRH and thus the responsiveness of the HPA axis to both physiological and psychological stress is attenuated during late pregnancy.[10]

Maternal cortisol levels are 5 to 10 times higher than those of the fetus.[19, 23, 24] Nevertheless, the fetus is protected from these high levels of maternal glucocorticoids by the action of the placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2). This enzyme converts cortisol (an active glucocorticoid) to cortisone (an inactive glucocorticoid), thus protecting the fetus from excessive glucocorticoid exposure.[10, 25] In addition to the transplacental transfer from the moth-

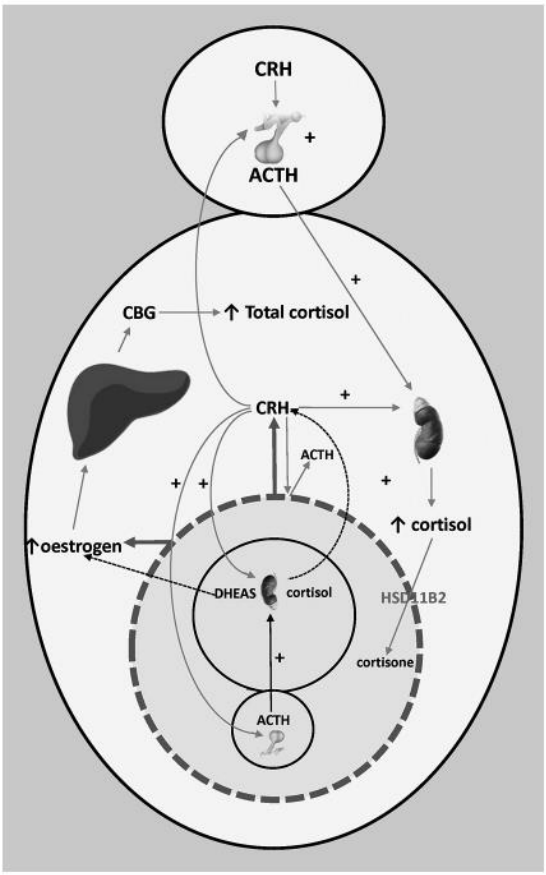

Figure 1.—Hypothalamic-pituitary-adrenal axis during normal pregnancy. The placental works as an endocrine organ secreting estrogen, CRH and ACTH. The placental production of estrogen and the conversion from foetal DHEAS, lead to the increased of the corticosteroid-binding globulin (CBG) with a rise in total cortisol levels. Placental CRH increases the plasma cortisol though the stimulation of the maternal and fetal pituitary gland and maternal and fetal adrenal gland. In consequence, the levels of total plasma cortisol and plasma free cortisol increase significantly throughout gestation. The placental enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) protects fetus from excess of cortisol due to the inactivation of cortisol to cortisone. The placenta is represented by a discontinue grey line. Black arrows indicate fetal origin and gray arrows maternal origin.

er, cortisol in the human fetus can derive from the human fetal adrenal cortex, and from local conversion from inactive cortisone by HSD11B1 activity within the chorion trophoblasts and amnion epithelium.[19] Data suggests that the human fetal adrenal cortex does not produce cortisol de novo from cholesterol until around week 30 of gestation; however, in early pregnancy cortisol in the fetus is produced using progesterone as precursor.[26]

In the postpartum period, maternal plasma cortisol levels fall and the function of the HPA axis gradually returns to its pre-pregnant state.[10] Estriol, progesterone and CRH levels fall rapidly up to day 6 after delivery, whereas cortisol levels fall modestly. ACTH concentrations decline up to day 3 post-delivery and increase thereafter up to day 6.[27]

Etiology of Cushing's syndrome in pregnancy

Cushing syndrome can be categorized as ACTH or non-ACTH dependent. In non-pregnant status, 85% of CS are caused by ACTH-dependent causes, which includes ACTH-secreting corticotroph adenoma of the pituitary gland (Cushing's disease, CD) in 70% of the cases, non-pituitary tumor ectopically secreting ACTH in 10%, and unknown source of ACTH in 5% of the cases. The other 15% are caused by ACTH-independent adrenal disorders, mostly adrenal adenomas (10%), adrenal carcinomas (5%) and other rare conditions, including bilateral macronodular adrenal hyperplasia, McCune-Albright syndrome and primary pigmented nodular adrenal disease.[28-30] Nevertheless, the most common cause of CS in the general population is iatrogenic or exogenous, caused by the prolonged external use of supraphysiological doses of corticosteroids, for a variety of other medical conditions.[30]

In contrast, the etiology of CS in women who become pregnant is most frequently adrenal in origin (50-60%). Two hundred and thirteen pregnancies were analyzed in a recent systematic review of all published cases of CS and pregnancy. From those, only 28.2% had CD in contrast to 53.5% who had an adrenal cause of hypercortisolism (adenoma 44.1%, adrenal carcinoma 9.4%). Additionally, 3.8% had tumors ectopically secreting ACTH and 13.2% developed pregnancy-induced CS,[8] presumably due to aberrant LH or beta-hCG receptors on the adrenal. These percentages were similar to those reported by Murakami *et al*. in 1998.[31]

The incidence of amenorrhea or oligomenorrhoea in CS is around 70-85%, which explains the rare incidence of pregnancy in CS.[6] Patients with CS frequently present with fertility issues

due to anovulation. The mechanisms responsible for this alteration are still debated but are presumed to be related to the excess of androgens and to the cortisol excess itself, that suppresses the gonadal axis acting at the hypothalamic level.[6] Of note, chronic hypercortisolemia blocks both the action of gonadotropins on the gonads and the secretion from the hypothalamus of GnRH, and serum cortisol levels inversely correlated with serum estradiol, but not with serum androgens.[6]

The difference in the etiology of CS in pregnancy has been suggested to be due to adrenal adenomas mainly producing excess of cortisol, compared to CD, where both cortisol and androgens are secreted,[32] potentially affecting the gonadal axis more significantly.

Pregnancies in cases of CS due to ectopic ACTH secretion published so far include pheochromocytomas,[33, 34] mixed phechromocytoma and corticomedullary tumor,[35] small cell carcinoma of the uterine cervix,[36] thymic neuroendocrine carcinoma [37] and adrenocorticotropin secreting islet cell tumor.[38] Interestingly, several cases have been reported where CS spontaneously resolved after pregnancy. Data suggest that these patients presumably with pregnancy-induced CS present aberrant LH/hCG receptors on the adrenal gland. A spontaneous remission of CS after giving birth and the lack of lesions in the adrenal or pituitary gland suggests this diagnosis.[39-45]

Diagnosis during pregnancy

Because of the changes in HPA during gestation, diagnosis of CS in pregnancy can be challenging. Symptoms of CS may resemble those of pregnancy and the results of the screening tests are difficult to interpret. We summarize here the evidence available on the diagnosis of CS in pregnancy.

Signs and symptoms

Most of the reported cases of CS during pregnancy describe the typical signs and symptoms, including central obesity, thin skin, easy bruising, striae, hypertension and diabetes. Of note, gestational diabetes mellitus (GDM) has been described in 25% to 37% of reported cases, gestational hypertension (HT) in 41-68% and preeclampsia 14-26%.[8, 46] Commonly, these patients have a worse obstetrical history when compared to the general population, as well as a high frequency of fetal loss. In addition, some patients have a prior diagnosis of diabetes or hypertension, suggesting that undiagnosed hypercortisolism was present prior to pregnancy.[8]

However, CS can also present with subtle symptoms and it may be underdiagnosed in pregnancy. Pregnancy itself often exhibits some of the clinical features seen in CS such as hypertension, diabetes mellitus, weight gain and striae. The striae of CS are typically purplish, depressed and wide (often 0.5-2.0 cm), may also involve the axilla, thighs, and breasts in addition to the abdomen, when compared to pregnant women, who usually present white striae. Other signs and symptoms that can predict CS with great discriminatory value are osteoporosis, dorsocervical fat pad, and muscular atrophy.[47] Other catabolic features, such as easy bruising and proximal weakness, are suggestive of CS, especially if accompanied by HT and GDM.[48]

Biochemically, although moderate hypokalemia can be normal in the context of pregnancy, severe hypokalemia should increase the suspicion of CS if the clinical picture also suggests the diagnosis. Severe hypokalemia was present in more than 50% of the patients with ectopic CS, pregnancy-induced CS and adrenal carcinoma, but these data were reported in only 39% of the cases.[8]

Twenty-four-hour urinary free cortisol

In a normal pregnancy, the 24-hour urinary free cortisol (UFC) excretion increases up to three times the normal upper limit during the second and third trimesters, resulting in a high false-positive rate (using the reference values outside pregnancy).[48] Lindsay *et al*. reviewed 136 cases with CS and found in 34 patients a mean 8-fold elevation in UFC (range 2-22). In addition, we analyzed all published cases with CS and pregnancy published between 1952 and 2015. Twenty-four-hour UFC levels were only available in 44% of the patients, but was increased 4-fold in CD, 7-fold in adrenal adenoma and carcinoma, 52-fold in ectopic ACTH secreting tumors and

7-fold in pregnancy-induced CS. Given the wide disparity of results, however, no differences were observed between the various etiologies (unpublished data). Thus, although there is no well-established cut-off, it is accepted that only UFC values in the second and third trimester greater than 3 times the upper limit of normal can be taken as suggestive of CS.[46]

Overnight dexamethasone suppression test

As mentioned before, elevated CBG levels in pregnancy lead to high total cortisol levels. Starting from a higher baseline means that even a normally suppressible HPA axis will not show the levels of suppression achieved in normal circumstances. Therefore, the suppression of cortisol after a dexamethasone suppression test is attenuated when compared to the non-pregnant state [16] and thus, there are more false-positive tests (using the reference values outside pregnancy).[43]

In pregnant women, the reduction of cortisol after 1 mg of dexamethasone overnight has been globally reported to be of 40%, 83% reduction in the first trimester, 44% in the second trimester and 37% in the third trimester.[49] In another series of 17 patients, most failed to suppress cortisol after 1 mg of dexamethasone or after a 48-hour low-dose dexamethasone suppression test, with a range of cortisol levels between 152 and 1173 nmol/L (the accepted cut-off to exclude CS for non-pregnant patients is less than 50 nmol/L after 1 mg of dexamethasone).[46] Altogether, these results seem to indicate that the overnight dexamethasone suppression test can exclude CS when cortisol is suppressed; however, when cortisol suppression fails, the test lacks specificity.

Diurnal variation: salivary and serum cortisol

Salivary cortisol levels rise two- to threefold during pregnancy, although they usually remain below those observed in CS.[48] Of note, the diurnal variation of cortisol is preserved in pregnancy.[50, 51] In non-pregnant populations, the combination of clinical variables and late-night salivary cortisol improved the diagnostic performance of CS in a high-risk population in which the diagnosis of CS is particularly challenging.[47] Although there are no data in pregnancy, these results could perhaps be extrapolated to other challenging populations as pregnant women.

In parallel, the plasma cortisol diurnal variation is preserved in pregnancy but is lost in patients with CS who are pregnant. This was demonstrated in an extensive review of patients with CS and pregnancy, where the diurnal variation in serum cortisol was absent in 46 patients, with morning values of 1040 nmol/L; and evening values of 994 nmol/L.[46]

The diagnostic thresholds for evening serum or salivary cortisol in pregnant patients have not been clearly established,[48] but a lack of diurnal variation strongly supports the diagnosis of CS.

ACTH levels

In non-pregnant patients, ACTH levels are used to distinguish between ACTH-dependent and -independent CS in the majority part of the cases. But ACTH is not suppressed under physiological circumstances of pregnancy. However, in CS patients who become pregnant only 50% with adrenal disease have undetectable ACTH possibly because of the lack of suppression of placental CRH production; additionally, some patients with CD may present a suppressed ACTH.[43, 46] Furthermore, stimulation with exogenous CRH during pregnancy fails to increase ACTH and cortisol but recovers in few weeks after delivery. However, higher doses of CRH can induce an increase in ACTH and cortisol during the third trimester.[52] Thus, a suppressed ACTH strongly suggests a CS of adrenal origin, but detectable ACTH does not discriminate between adrenal or extra-adrenal origin.

Inferior petrosal sinus sampling

Inferior petrosal sinus sampling (IPSS) has been used in some patients with CS during pregnancy, to differentiate between an ectopic and a pituitary source of ACTH. In all reported cases, IPSS was performed early in the second trimester. In two cases, the IPSS failed to demonstrate a central to peripheral gradient, the first one despite later being proven to have CD.[46] The delivery was induced at week 34 due to severe preeclampsia and intrauterine growth retardation. The second case was found to have adrenal aberrant estrogen

receptors in whom all ACTH values were at the lower limit of detectability.[53] Her child was born at 33 weeks with several malformations; however, the patient also suffered from hypertension, so the causality of the malformations were difficult to establish.[54] In another two cases a pituitary origin was confirmed,[46, 55] although one had a stillbirth at 33 weeks of gestation, despite of normal fetal grown during follow-up.[46]

Since both the patient and fetus would receive radiation with the procedure, a jugular access rather than a femoral vein access has been proposed to reduce radiation exposure. Nevertheless, the indication for IPSS should be very limited during pregnancy and only performed if the patient is going to undergo surgical treatment.

Postnatal period

The characteristic insensitivity of the pituitary-adrenal system in pregnant women to feedback inhibition persists for at least four days postnatally. The insensitivity of plasma cortisol to glucocorticoid inhibition persists beyond normal pregnancy in a significant proportion of healthy women for two to three weeks and is absent by the 5th postnatal week;[56] suppression of cortisol after 1 mg dexamethasone may not occur in the first two weeks but normalizes by the 35th day after delivery.[56] On the other hand, the plasma ACTH response to iv bolus of CRH is abnormally low at 3 and 6 weeks, but normal at 12 weeks postpartum, whereas the mean plasma cortisol response to CRH is at the upper limit of normal at all 3 times.[57] These results indicate that the diagnostic work up in the postnatal period may be difficult up to 12 weeks postpartum.

Imaging

Imaging should be used only when the clinical diagnosis of CS is confirmed and there is a plan for surgery during the course of pregnancy.

When CD is suspected, pituitary magnetic resonance imaging (MRI) should be performed. The administration of gadolinium has not been proven to be safe in pregnancy and should be avoided, at least during the first trimester. Nevertheless, during pregnancy the pituitary gland physiologically enlarges and may trigger a false-positive diagnosis, especially in microadenomas.[58] Of interest, pituitary macroadenomas has been reported in about half of the patients, significantly overrepresented, compared with non-pregnant cases of CD, where microadenomas are more frequent.[46] Therefore, regular visual field examination is recommendable in patients with CS, especially when pituitary imaging has not been performed.

In cases with suppressed plasma ACTH concentrations or suspected adrenal tumors, abdominal ultrasound is preferred to localize adrenal lesions because of its greater safety. Ultrasound has reasonably good sensitivity for the adrenal tumors associated with CS, but it is more operator-dependent than CT scan or MRI and has limited sensitivity for small tumors.[58] Ultrasound was informative in 11 of 15 cases leading to successful surgical localization in cases reported in the literature.[46] When ultrasound is non-diagnostic, non-gadolinium-contrasted MRI could be used,[48] but adrenal CT scans should be avoided due to the risks associated with ionizing radiation.[16, 59]

Treatment during pregnancy

There are no studies comparing the different modalities of treatment for CS in pregnancy. Therefore, data are limited, and treatment should be individualized for each patient, depending on the cause, the stage of pregnancy and the severity of hypercortisolism.

Three studies that analyzed the published literature suggest that patients receiving treatment during pregnancy achieve better fetal outcomes than those who do not receive treatment.[8, 32, 46]

Surgery has been recommended as a first choice treatment [46, 60] and is considered safer in the second trimester due to the lower risk of fetal and maternal complications.[60] Transsphenoidal resection, unilateral and bilateral adrenalectomy have been performed successfully in pregnant women, and CS remission has been described in a significant number of patients. Importantly, those that achieved remission fetal loss rate was 4 times lower compared with non-treated patients.

Medical treatment with different drugs has been used in a considerable number of patients without any apparent adverse consequences, metyrapone being the drug most commonly used

followed by ketoconazole. Cyproheptadine, aminoglutethimide and cabergoline have also been used in a few number of patients. Mitotane in pregnancy has been used in two patients with ACC.[61, 62] In one of them it was stopped at 6 weeks of pregnancy, where the fetus developed normally, however pregnancy was terminated at gestation week 21 due to ACC recurrence.[61] The second was treated with mitotane throughout the entire pregnancy and the baby was born at week 31 without malformation and with a normal adrenal gland function.[62] Nevertheless, it seems reasonable to avoid mitotane during pregnancy due to its potential teratogenic effect.

All drugs were used at different stages of gestation, but generally beginning during the second and third trimesters,[46] and manly when surgery was contraindicated, or initially after diagnosis, for symptomatic control. Medical management appears effective in controlling hypercortisolemia during pregnancy with strict monitoring of blood pressure and fetal surveillance and remains the only active management in the setting of pregnancy-induced CS.[63]

The limited number of cases described precludes any definitive conclusion as to the best management for CS during pregnancy. Some authors suggested that surgical treatment reduced perinatal mortality and maternal morbidity rates but did not affect the occurrence of preterm birth and intrauterine growth restriction.[64] In a systematic review we have previously described that receiving medical or surgical treatment decreased the risk of overall fetal loss by 3 to 4 times, but it did not protect from prematurity.[8]

Pregnancy outcomes

Maternal and fetal outcomes are severely affected in the context of CS and pregnancy, with high rates of maternal and fetal mortality.

Maternal outcomes

The most common maternal complications described in pregnant CS are hypertension, preeclampsia and diabetes; other complications typical of CS have also been described including hypokalemia, osteoporosis and fractures, cardiac failure, psychiatric disorders, gastric ulcers, pneumonia, wound infection [46] and spontaneous postpartum uterine rupture.[65] The so-called HELLP syndrome (hemolytic anemia, elevated liver enzymes, low platelet count) has also been reported in a small number of cases.[62, 66] In terms of delivery, patients with active CS showed higher rates of cesarean section in comparison to healthy normal population, which could be attributed to maternal and fetal reasons. Importantly, maternal mortality has been described in two cases, giving a maternal mortality ratio of 1257/100,000 livebirths,[8] this updated maternal mortality being much higher than the one reported in 2013 (209.1 per 100,000 livebirths). Both women died within the first two weeks after delivery, one with CS due to an adrenal adenoma, who developed HELLP syndrome and had an emergency cesarean section plus unilateral adrenalectomy. The postoperative period was complicated with a wound infection, followed by hematemesis due to gastric ulcers requiring antrectomy, complicated with pulmonary edema, pneumonia and recurrent bleeding.[66] The other patient had a disseminated ectopic ACTH secreting tumor of the pancreatic tail.[38]

The patients with cured CS have similar prevalence of GDM, gestational HT and preeclampsia to that observed in the general population.[8, 67, 68] Cured women also had fetal mortality similar to that expected in healthy women,[69] indicating that patients with cured CS normalized both maternal and fetal risks.[8]

Fetal outcomes

In contrast to other endocrine entities that have a marked influence on sex ratio at birth,[70-75] no differences have been reported in the context of CS.[8, 46] Low birth weight and prematurity are the most common complications in live births (43-65%) followed by intrauterine growth restriction (15-20%). Patients diagnosed during pregnancy have up to 7 times more risk of low birth weight in comparison of those diagnosed before pregnancy.[8] The rate of overall fetal loss has been described in 1 out of 4 pregnancies, the majority being due to spontaneous abortion (5-10%) and perinatal death (in 11% of all reported pregnancies).[8, 46] The list of reported complications is provided in Table I.[53, 66, 76-81]

These complications are likely to be due to

more frequent presentation during pregnancy of GDM, HT or preeclampsia in addition to the effect of cortisol itself. There are no data available on the long-term outcome after prenatal cortisol exposure in the context of CS, in those babies who apparently did not present with complications at birth. However, evidence suggests that glucocorticoids play a key role during intrauterine development on cellular growth, differentiation and programming. The exposure to inappropriate concentrations of glucocorticoids during developmental periods has been suggested to affect the childhood risk of obesity, insulin resistance and abnormal immune function in the offspring once they are adults.[73-75] Additionally, human studies have shown that maternal stress or anxiety during pregnancy is associated with an increased risk of disturbance in the offspring's neurodevelopment and behavior, which is thought to be related to the increased fetal exposure to glucocorticoids. Therefore, we hypothesize that these data could perhaps be extrapolated to those subjects who were exposed to maternal excess of corticosteroids as a consequence of CS.

Importantly, patients who received treatment during gestation (medical or surgical) presented lower rates of fetal loss, but the long-term effect on these subjects is unknown. Of note, the etiology of the CS does affect pregnancy outcome differently and is a predictor for fetal loss. Pregnancy-induced CS has the worst prognosis, with an overall fetal loss and incidence of spontaneous abortion 5 and 17 times higher respectively, when compared to CD. This is probably due to the severity of hypercortisolism and the longer delay in correctly diagnosing and treating this group of patients.[8]

Conclusions

In summary, the diagnosis of CS during pregnancy can be very difficult, especially in those pregnant patients with subtle symptoms, due to the physiological changes of the HPA during pregnancy. Although, the gestational age at diagnosis and the severity of symptoms are important to establish how urgent and what best treatment is required, prompt management is essential in reducing the fetal and maternal morbimortality.

TABLE I.—*List of fetal complications reported in all published pregnancies (N.=223) with active Cushing' syndrome between 1952 and 2017.*[53, 66, 76-81]

Complications	N. of pregnancies (N.=223)
Overall fetal loss	48 (21.5%)
Spontaneous abortion	22 (9.9%)
Induced abortion	6 (2.7%)
Ectopic pregnancy [76]	1 (0.4%)
Intrauterine fetal death	11 (4.9%)
Neonatal death	8 (3.6%)
Perinatal death	19 (8.5%)
Fetal distress	14 (6.3%)
Preterm birth	108 (46.4%)
Intrauterine growth restriction	27 (12.1%)
Low birth weight	70 (31.4%)
Adrenal insufficiency	8 (3.6%)
Hypoglycemia	10 (4.5%)
Respiratory distress	19 (8.5%)
Sepsis	4 (1.8%)
Jaundice	9 (4%)
Tracheomalacia [77]	1 (0.4%)
Neonatal virilization [78]	1 (0.4%)
Left ventricle hypertrophy [79]	1 (0.4%)
Ductus arteriosus [80]	1 (0.4%)
Cleft palate [81]	1 (0.4%)
Several congenital malformations (not specified) [53]	1 (0.4%)
Bronchopulmonary dysplasia [66]	1 (0.4%)
Intraventricular hemorrhage [66]	2 (postpartum) (0.9%)

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Novel Genetic Causes of Pituitary Adenomas

Francisca Caimari and Márta Korbonits

Abstract

Recently, a number of novel genetic alterations have been identified that predispose individuals to pituitary adenomas. Clinically relevant pituitary adenomas are relatively common, present in 0.1% of the general population. They are mostly benign monoclonal neoplasms that arise from any of the five hormone-secreting cell types of the anterior lobe of the pituitary gland, and cause disease due to hormonal alterations and local space-occupying effects. The pathomechanism of pituitary adenomas includes alterations in cell-cycle regulation and growth factor signaling, which are mostly due to epigenetic changes; somatic and especially germline mutations occur more rarely. A significant proportion of growth hormone- and adrenocorticotrophin-secreting adenomas have activating somatic mutations in the *GNAS* and *USP8* genes, respectively. Rarely, germline mutations predispose to pituitary tumorigenesis, often in a familial setting. Classical tumor predisposition syndromes include multiple endocrine neoplasia type 1 (MEN1) and type 4 (MEN4) syndromes, Carney complex, and McCune-Albright syndrome. Pituitary tumors have also been described in association with neurofibromatosis type 1, *DICER1* syndrome, and *SDHx* mutations. Pituitary adenomas with no other associated tumors have been described as familial isolated pituitary adenomas. Patients with *AIP* or *GPR101* mutations often present with pituitary gigantism either in a familial or simplex setting. *GNAS* and *GPR101* mutations that arise in early embryonic age can lead to somatic mosaicism involving the pituitary gland and resulting in growth hormone excess. Senescence has been suggested as the key mechanism protecting pituitary adenomas turning malignant in the overwhelming majority of cases. Here we briefly summarize the genetic background of pituitary adenomas, with an emphasis on the recent developments in this field. *Clin Cancer Res; 22(20); 5030–42. ©2016 AACR.*

See all articles in this *CCR Focus* section, "Endocrine Cancers: Revising Paradigms."

Introduction

The pituitary gland consists of an anterior lobe of epithelial origin and a posterior lobe of neuronal origin. The main cell types of the anterior lobe are the hormone-secreting cells [growth hormone (GH), prolactin, adrenocorticotrophin (ACTH), thyrotropin (TSH), or gonadotrophin (LH and FSH)] and the folliculostellate cells. The term "pituitary adenoma" is attributed to the usually benign tumors arising from the hormone-secreting cells of the anterior pituitary. Typically, pituitary adenomas are classified as either functioning pituitary adenomas with characteristic clinical symptoms, such as acromegaly or Cushing disease, or clinically nonfunctioning pituitary adenomas (NFPA), usually arising from cells secreting LH and FSH. These adenomas generally present as slowly growing lesions with low mitotic rate and Ki-67 labeling index (1). Symptoms are present due to hormonal disturbances, hypersecretion or lack of pituitary hormones, and compression symptoms that are secondary to local invasion and lead to hypopituitarism and visual field defects.

Centre for Endocrinology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, United Kingdom.

Corresponding Author: Márta Korbonits, Centre for Endocrinology, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London EC1M 6BQ, United Kingdom. Phone: 4420-7882-6238; Fax: 4420-7882-6197; E-mail: m.korbonits@qmul.ac.uk

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Data derived from autopsies and radiologic imaging studies have shown that pituitary adenomas are relatively common, estimated to be present overall in 17% of the general population (2, 3). Although most of these small lesions are incidental findings, with no obvious clinical impact (3), clinically relevant pituitary adenomas are present in 0.1% of the general population, and they represent the third most-frequent intracranial tumor type after meningiomas and gliomas (4).

Pituitary adenomas are monoclonal neoplasms in origin (5). A number of different molecular mechanisms that lead to pituitary adenomas have been identified, although in the majority of the sporadic cases, the exact molecular pathogenesis remains unknown. Factors hypothesized to contribute to pituitary neoplasia initiation and proliferation include altered growth factors and cell-cycle regulators that are the result of epigenetic changes (6), abnormal hormonal milieu, abnormal intrapituitary microenvironment (7), and inherited or somatic mutations (Fig. 1). The role of environmental factors remains questionable (8–10). In the following brief overview of the underlying pathomechanisms, we will concentrate on germline and somatic mutations that lead to pituitary adenomas.

Germline Mutations

Familial pituitary adenomas can be divided into (i) an isolated group, in which no other organs are involved in addition to the pituitary gland, and (ii) a syndromic group, which includes multiple endocrine neoplasia type 1 (MEN1), MEN4, Carney complex, DICER1 syndrome, *SDHx* gene–associated syndromes, and neurofibromatosis type 1 syndrome (Fig. 1; Table 1). Familial

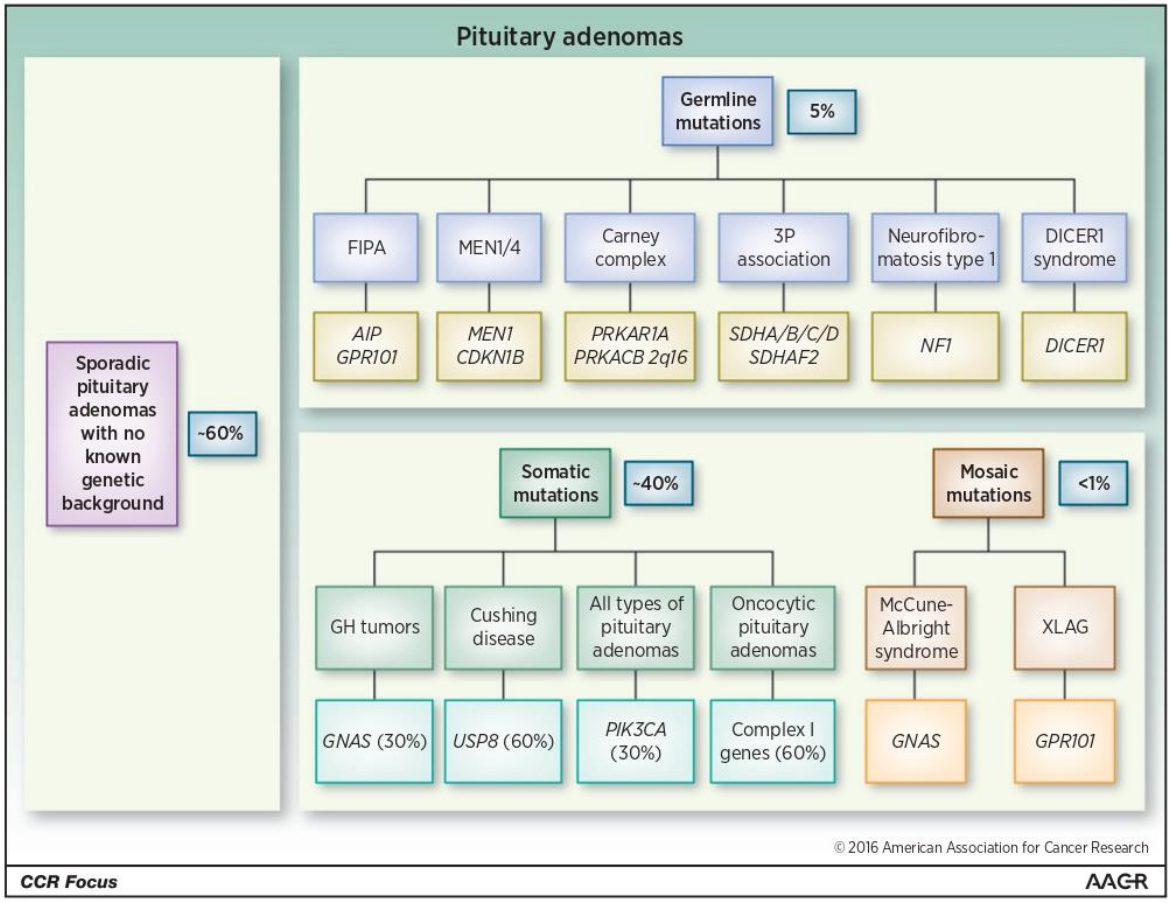

Figure 1.
Germline, somatic, and mosaic mutations identified in familial and sporadic pituitary adenomas. Mosaic mutations denote the presence of two or more populations of cells with different genotypes in one individual who has developed from a single fertilized egg. 3P association, paraganglioma/pheochromocytome/pituitary adenoma; FIPA, familial isolated pituitary adenoma; MEN, multiple endocrine neoplasia; NF1, neurofibromatosis type 1.

isolated pituitary adenoma (FIPA) is the most common type followed by MEN1, which together represent 5% to 7% of patients with pituitary adenomas (11).

Isolated pituitary adenomas

FIPA is defined by the presence of pituitary adenomas in two or more family members with no other syndromic features present (12). FIPA is a heterogeneous condition that includes patients with mutations in the aryl hydrocarbon receptor–interacting protein (*AIP*) gene (13), patients with X-linked acrogigantism (XLAG) due to duplication of *GPR101* (14), and patients with a family history of pituitary adenomas with no known genetic cause. Patients with *AIP* or *GPR101* mutations or with *AIP* and *GPR101*-negative FIPA do not present with other types of tumors, hence the name "isolated" pituitary adenomas. Not all cases grouped under this category have a known family history, either due to low penetrance (such as in *AIP* mutation–positive simplex cases) or due to *de novo* mutations (most cases of XLAG).

AIP mutations. The prevalence of *AIP* mutations in FIPA families is 17% to 20% (15, 16), whereas in sporadic cases, it ranges between 3.6% (unselected pituitary adenoma patients) and 10% to 20% (pediatric pituitary adenoma cases; refs. 17, 18). About 50% of *AIP* mutation–positive probands have a positive family history (16), whereas mutations in the other half are found as a germline mutation in sporadically diagnosed pituitary adenoma patients, so-called "simplex cases." The lack of apparent family history in the latter group is due to low penetrance, as *de novo* mutations have only been found in two cases (19, 20). Penetrance in *AIP*-mutated families is incomplete (Fig. 2A): only every fifth mutation carrier manifests the disease. The age of onset is also characteristic; the disease usually manifests in the second decade of life and almost all cases are diagnosed before the age of 30 years (15, 16, 21, 22).

AIP encodes a 330 amino acid protein acting as a tumor suppressor. It has a wide tissue distribution; in the normal human pituitary, it is expressed in GH cells (somatotroph cells) and prolactin-secreting cells (23). Lack of interaction with cell type–

Table 1. Germline and mosaic mutations predisposing to pituitary adenomas

Syndrome	Genetic alteration (inheritance pattern)	Function	Location	Penetrance	Prevalence[c]	Main clinical characteristics
Carney complex	*PRKAR1A* (AD)	TSG	17q24.2	>95% overall, 80% for GH excess	Unknown	Skin pigmentation; myxomas; thyroid, testis and adrenal tumors, as well as somatotroph hyperplasia or adenomas
	2p16 locus (unknown gene)	—	2p16	Unknown	Unknown	Less severe Carney complex phenotype, mostly in sporadic cases
	PRKACB	Oncogene	1p31.1	Unknown	One case described	Described in one case with Carney complex (31)
DICER1	*DICER1* (AD)	TSG	14q32.13	Unknown, <1% for pituitary	Unknown	Pituitary ACTH-secreting blastomas
FIPA	*AIP* (AD)	TSG	11q13.2	30%	2.5%	Young-onset GH adenomas and prolactinomas, rarely other pituitary adenoma type. Twenty percent of FIPA and 4%–20% of sporadic pituitary adenomas have *AIP* mutations
	GPR101 (X-chromosome linked)[a]	Oncogene	Xq26.3	100%	Unknown (very low, 10% of pituitary gigantism cases)	Gigantism due to pituitary hyperplasia or adenoma
McCune-Albright[b]	*GNAS* (mosaic postzygotic mutation)	Oncogene	20q13.32	10–20% for pituitary	1:100,000–1:1,000,000	Polyostotic fibrous dysplasia, café-au-lait spots, and precocious puberty with GH/prolactin excess in 10%–20%
MEN1	*MEN1* (AD)	TSG	11q13.1	>95% overall, 30–40% for pituitary	1:30,000	Pancreatic, pituitary (typically prolactinomas), and parathyroid gland tumors with other tumors as well
MEN4	*CDKN1B* (AD)	TSG	12p13.1	Unknown overall, high for pituitary	Unknown (very low)	MEN1 like, typically somatotroph adenomas
Neurofibromatosis type 1	*NF1* (AD)	TSG	17q11.2	>95% overall, very low for pituitary	1:4,000	Café-au-lait spots, Lisch nodules, neurofibromas, optic pathway gliomas, and pheochromocytoma. Unclear whether pituitary adenomas caused by *NF1* mutations
Paraganglioma/ phaeochromocytoma and pituitary adenomas—3P association	*SDHA* (AD)	TSG	5p15.33	Low penetrance	Unknown	Familial paraganglioma type 5 (PGL and PHEO)
	SDHB (AD)	TSG	1p36.13	~50% overall, very low for pituitary	Unknown	Familial paraganglioma type 4 (PGL with increased malignant potential)
	SDHC (AD)	TSG	1q23.3	Low penetrance overall, very low for pituitary	Unknown	Familial paraganglioma type 3 (head and neck PGL predominance, no pheochromocytoma, associated with GIST)
	SDHD (AD)	TSG	11q23.1	Up to 80% overall, very low for pituitary	Unknown	Familial paraganglioma type 1 (head and neck PGL but also pheochromocytoma)
	SDHAF2 (AD)	TSG	11q12.2	Low penetrance, very low for pituitary	Unknown	Familial paraganglioma type 2 (head and neck PGL)

Abbreviations: AD, autosomal dominant; GIST, gastrointestinal stromal tumor; PGL, paraganglioma; PHEO, pheochromocytoma; TSG, tumor suppressor gene.
[a]Germline or somatic mosaicism.
[b]Somatic mosaicism.
[c]In patients with pituitary adenomas.

specific molecular partners might explain the specific clinical phenotype of AIP deficiency. AIP has numerous interacting partners (12). It is co-chaperone to several heat shock proteins and nuclear receptors including the aryl hydrocarbon receptor, and it interacts with phosphodiesterase 4A4/5, which regulates the cAMP pathway (24–26); however, lack of AIP leads to reduced inhibitory G protein Gαi-2 expression. The latter two aspects could be important to the tumorigenic role of AIP (27), as activation of the cAMP pathway is important in somatotroph tumorigenesis (Fig. 3): (i) activating mutations of the stimulatory G protein *GNAS* are present in approximately 30% of sporadic somatotroph (GH-secreting) adenomas and in McCune-Albright syndrome (28, 29), (ii) mutations in *PRKAR1A* and *PRKACB* lead to increased protein kinase A activity in Carney complex (30, 31), and (iii) the duplication of the cAMP-coupled orphan receptor *GPR101* causes XLAG (14).

AIP mutations predispose individuals to childhood or young adult-onset disease with large, aggressive, poorly responsive, mostly GH or prolactin-secreting tumors, leading to gigantism in 40% of the cases (Fig. 2B and C; refs. 15, 16, 23). There is a phenotype–genotype correlation, as patients with truncating mutations are significantly younger at disease onset in comparison with patients with nontruncating *AIP* mutations (Fig. 2C; ref. 16). Patients with *AIP* mutations have an increased risk for pituitary apoplexy (Fig. 2B) compared with *AIP* mutation–negative young GH excess patients (16, 23, 32). Classical

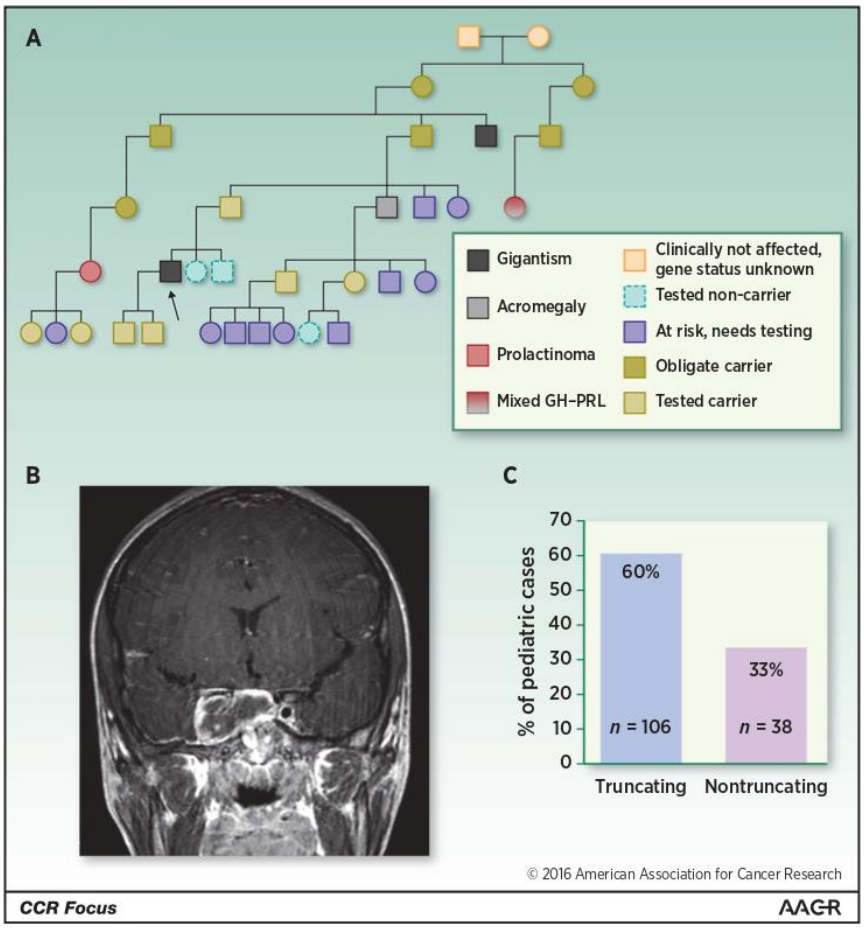

Figure 2.
Clinical and biological aspects of AIP mutations. **A,** family tree of patient (arrow) with heterozygous *AIP* mutation showing four other family members affected by pituitary adenomas, representing various phenotypes and incomplete penetrance (16). **B,** MRI of a 6-year-old female *AIP* mutation–positive patient (p.R304*) presenting with acute severe headache and ptosis on the right side due to pituitary apoplexy of a large pituitary adenoma (courtesy of Dr. Miles Levy, Leicester, United Kingdom). **C,** patients with truncating *AIP* mutations show significantly earlier disease onset than those with nontruncating mutations. There is a significantly higher number of pediatric cases among truncating *AIP* mutation–positive patients compared with nontruncating cases, suggesting a genotype–phenotype correlation (16).

pituitary apoplexy refers to a clinical syndrome that is characterized by sudden onset of headache, vomiting, visual impairment, and decreased consciousness that evolves over hours or days and is caused by hemorrhage and/or infarction of a pituitary adenoma. Patients with *AIP* mutations often need repeated surgery and external pituitary irradiation, and they are relatively resistant to treatment with somatostatin analogues [(SSA) i.e., poor reduction in hormone levels and tumor size (15)]. The mechanism of somatostatin resistance could involve the somatostatin receptor type 2 (SSTR2)–ZAC1 pathway (33). AIP is upregulated by SSA (34, 35) and, in turn, AIP can upregulate *ZAC1* mRNA expression (35, 36). Another possible mechanism explaining the somatostatin resistance of these patients is the reduced expression of the inhibitory G protein subtype Gαi-2. SSTR2 is known to regulate cAMP levels via inhibitory G proteins (37), and deficiency of Gαi-2 could play a role in the SSA resistance of *AIP* mutation–positive samples (ref. 35; Fig. 3).

In sporadic adenomas that do not harbor *AIP* mutations, low AIP protein expression is associated with reduced SSTR2 expression, which predicts a reduced responsiveness to SSA therapy (38). Tumors pretreated with SSA before surgery show an increase in AIP expression (34, 35). It has been suggested that AIP might be a better marker of invasiveness in somatotrophinomas (GH-secreting adenomas) than Ki-67 and p53, even in *AIP* mutation–negative adenomas (39).

Genetic screening and clinical follow-up of family members of *AIP* mutation–positive probands revealed numerous cases of prospectively identified patients, where the early diagnosis led to earlier treatment and potential avoidance of significant complications (16). This suggests that patients with gigantism or young-onset acromegaly and their families could benefit from genetic screening and clinical follow-up.

GPR101–X-linked acrogigantism. XLAG is a novel genetic cause of GH excess. It usually presents at a very early age as a sporadic disease due to a *de novo* microduplication on the X chromosome involving the *GPR101* gene in patients with gigantism (40–42). The majority of the cases are females with germline microduplication (14, 40, 42). Two familial cases have been described with transmission from affected mother to an affected son and show full penetrance (14). Somatic mosaic mutation cases have also been described in males where the mutation was identified in the pituitary tissue and/or at low level in germline (18, 41, 42). Although the originally identified Xq26.3 duplicated area involves four genes (14), only one of these, the *GPR101* gene,

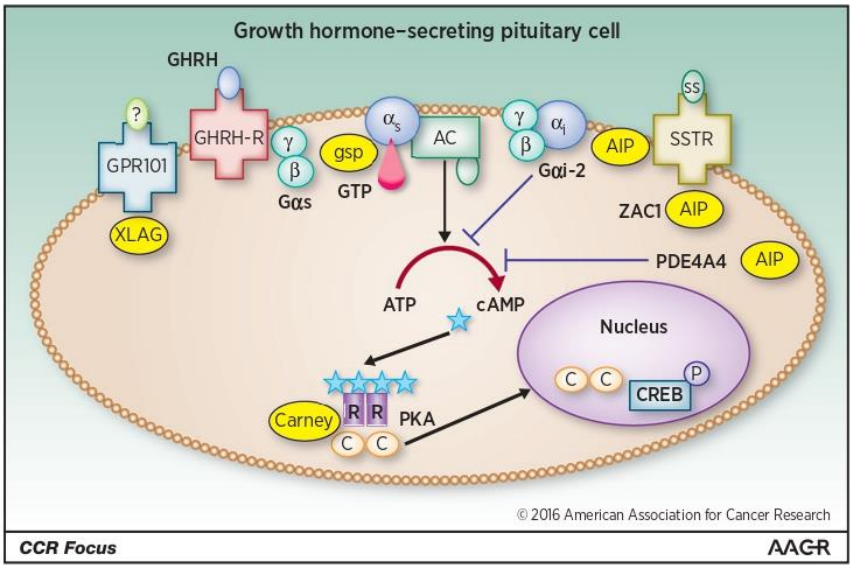

Figure 3.
cAMP pathway alterations in somatotroph adenomas. Several mechanisms leading to somatotroph adenomas involve the cAMP pathway. AIP, aryl hydrocarbon receptor interacting protein; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; Carney, site of mutations in Carney complex; CREB, cAMP response element; Gαs, G protein stimulatory alpha subunit; Gαi-2, G protein inhibitory alpha subunit type 2; GHRH, growth hormone–releasing hormone; GHRH-R, GHRH receptor; gsp, mutated Gαs; GTP, guanine triphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; SSTR, somatostatin receptor; ZAC1, zinc finger protein PLAGL1; PDE4A4, phosphodiesterase type 4A4; PKA, protein kinase A; R, PKA, regulatory subunit; C, PKA, catalytic subunit; XLAG, X-linked acrogigantism.

has been found upregulated at the mRNA level in pituitary tissue. We have recently identified a patient with XLAG whose duplicated area includes only the *GPR101* gene, but not the other three genes, indicating the pathogenic role of *GPR101* (14, 42). Activation of GPR101, an orphan Gs protein–coupled receptor, leads to an increase in cAMP levels (refs. 43, 44; Fig. 3). GPR101 is expressed in the caudate putamen, nucleus accumbens, and hypothalamus (45). The endogenous ligand and the exact function of GPR101 remain unknown. It has been suggested, in a uterine cancer cell line, that a fragment of the gonadotrophin-releasing hormone [GnRH-(1–5)] indirectly stimulated GPR101 to induce the release of EGF and subsequent phosphorylation of the EGFR (46), leading to enhanced cellular migration. However, (i) GnRH-(1–5) does not change cAMP levels, which are important for somatotroph tumorigenesis, and (ii) an upregulated EGFR pathway seems to lead to corticotroph (i.e., ACTH-secreting) adenomas (see section on USP8), but not somatotroph adenomas. Therefore, the mechanism by which mutant *GPR101* contributes to increased GH secretion is still unclear.

Patients with XLAG have significant GH excess leading invariably to gigantism due to their particularly early age of onset, even in comparison with other pituitary gigantism cases, occurring before the age of 5 years, with growth curves significantly exceeding the 97th percentile (14, 40–42, 47). Most of these patients secrete increased amounts of both GH and prolactin due to a somatomammotroph adenoma and mixed cell hyperplasia. In the adenomas, the Ki-67 index is low or moderate (except one case; ref. 48), SSTR expression is preserved, and AIP expression is moderate or high; GHRH is not expressed, whereas there is increased expression of GPR101 and the GHRH receptor (40, 42). Patients with XLAG provide a particular challenge in management due to the very young age of onset, the fact that some have hyperplasia rather than an adenoma, and due to partial or complete resistance to SSA and dopamine agonist therapy. Radical pituitary surgery or radiotherapy could be effective but often leads to hypopituitarism (40). The GH receptor antagonist pegvisomant in combination with somatostatin and dopamine agonists has been shown to control IGF-1 and excessive growth (40, 41).

Familial isolated pituitary adenoma with no known gene mutation. Approximately 80% of FIPA cases do not have an identified gene mutation. While *AIP*- and *GPR101*-related disease starts in young or very young patients, in this group, the age of diagnosis is often (>60%) after the third decade of life and only 2% of the cases have gigantism (16). In our cohort, the most frequent diagnosis was acromegaly followed by prolactinoma, while in another large cohort, macroprolactinomas were the most common (49). Fifty-eight percent of our families presented with the same pituitary adenoma type (homogeneous FIPA families), whereas different adenoma types were present in the same family in 42% of the cases. The penetrance seems to be lower than in *AIP* mutation–positive families, as approximately 80% of the families have only two affected family members. We cannot exclude the possibility that, given the prevalence of pituitary adenomas in the general population (1:1,000), some of these adenomas coexist in a family as a coincidence rather than due to an existing underlying germline mutation.

Syndromic pituitary adenomas

MEN1 syndrome. MEN1 is characterized by the presence of the classical triad of hyperparathyroidism (in almost all patients by the age of 50 years), pituitary adenomas (in about 30%–40% of cases), and neuroendocrine tumors [in about 60% of cases; further data on neuroendocrine tumors can be found in the *CCR Focus* article by Maxwell and colleagues (50)]. In addition, other tumor types have also been associated with this syndrome, such as facial angiofibromas (85%), collagenomas (72%), adrenal cortical adenomas (40%), lipomata (30%), meningiomas (8%), pheochromocytomas (rarely). Breast cancer (6%) has also been suggested more recently.

The *MEN1* gene encodes a ubiquitously expressed transcriptional cofactor that regulates cell-cycle proteins such as p27 (51) and cyclin-dependent kinase subunit 4 (CDK4; ref. 52), factors also found to be altered in sporadic pituitary adenomas (see the following section). The interaction with p27 is especially interesting, as mutations in the gene encoding p27 (*CDKN1B*) are also associated with a MEN1 syndrome–like phenotype (see the following section). MENIN also has a role in G_1–S checkpoint regulation, response to DNA damage and apoptosis, regulation of histone deacetylation and methyl transferase complexes, interaction with transcription factors and nuclear receptors, as well as transport of β-catenin (53).

MEN1-related pituitary disease is dominated by prolactinoma cases; however, systematic screening reveals similar numbers of small nonfunctioning pituitary adenomas (42%), followed by somatotrophinomas (7%) and a smaller percentage of the other pituitary adenoma types (54). Nonfunctioning adenomas were more often microadenomas detected during screening. Pituitary adenomas could be the first manifestation of MEN1 syndrome in 15% to 20% of the cases, and the current guidelines suggest that pituitary screening should start at the age of 5 years in mutation carriers (55).

MEN4 syndrome. Not all patients with MEN1 syndrome harbor a mutation in the *MEN1* gene. Loss-of-function mutations have been identified in *CDKN1B*, coding for p27, in a subset of these patients, and the phenotype was named MEN4 syndrome (56). The most common pituitary tumor type is somatotrophinoma, and a childhood-onset case has also been described (57). Other tumors include parathyroid adenomas, adrenal tumors, renal angiomyolipomas, uterine fibroids, gastrinomas, neuroendocrine cervical carcinomas, bronchial carcinoids, papillary thyroid carcinomas, and gastric carcinomas. Interestingly, in addition to the usual mutation types (nonsense, frameshift, and missense), alterations in the 5′ open reading frame were also shown to disrupt the function of the p27 protein (57, 58). In a few MEN1-like syndrome cases, mutations in other cell-cycle inhibitors, such as p15 (*CDKN2B*), p18 (*CDKN2C*), and p21 (*CDKN1A*), have also been described (59). These cases, together with MEN1-related pituitary adenomas and with the gene expression data of sporadic pituitary adenomas (see below), support the hypothesis that cell-cycle dysregulation is an important factor in pituitary adenoma development.

Carney complex. Carney complex is characterized by the presence of endocrine and nonendocrine tumors with spotty skin pigmentation, as well as cardiac and cutaneous myxomas (60). More than two-thirds of patients present asymptomatic elevation of IGF-1, GH, and prolactin due to pituitary hyperplasia, and 10% of the patients present with adenomas and symptomatic acromegaly (61). The age of onset is usually after the third decade; however, a few cases of gigantism have also been described. Carney complex is caused in 70% of the cases by inactivating mutations in the regulatory subunit of protein kinase A (*PRKAR1A*), which leads to excessive cAMP signaling (ref. 62; Fig. 3). Large deletions within the gene lead to a more severe phenotype. Another genetic locus at 2p16 has also been shown to be associated with Carney complex (63). More recently, a single case was described of an activating mutation (gene duplication) of the catalytic subunit of protein kinase A (*PRKACB*; ref. 31). It has been shown that *PRKAR1A* haploinsufficiency leads to a dysregulated WNT signaling pathway, in addition to cell-cycle abnormalities (64). Interestingly, embryonic mutations in β-catenin (*CTNNB1*), a crucial element of the classical WNT pathway, leads to craniopharyngiomas, a nonhormone–secreting pituitary tumor (65).

DICER1 syndrome. Pituitary blastoma is a novel aspect of the DICER1 syndrome. Germline mutations in *DICER1* lead to infant pleuropulmonary blastoma, differentiated thyroid carcinoma, multinodular goiter, nasal chondromesenchymal hamartoma, ovarian sex cord stromal tumor, Sertoli-Leydig cell tumor, pineoblastoma, and pituitary blastoma. Pituitary blastomas develop before the age of 2 years and secrete ACTH, causing severe Cushing disease (66–68). The penetrance of pituitary blastoma is low (~1%; ref. 66). Approximately half of the children with confirmed pituitary blastoma die of the disease within months of diagnosis (66–68).

DICER1 is a cytoplasmic endoribonuclease that processes hairpin precursor miRNAs into short, functional miRNAs that downregulate targeted mRNAs, thereby modulating cellular protein production (69). In the more than 50 reported *DICER1* mutation kindreds, germline mutations usually led to truncated proteins (67), whereas the "second hit" somatic mutations were typically in the metal-binding sites of the catalytic RNase IIIa and b domains. The exact mechanism explaining this special scenario or why *DICER1* mutations in general lead to tumorigenesis remains unclear.

Succinate dehydrogenase mutations (SDHx): paraganglioma, pheochromocytoma, and pituitary adenoma association. Pheochromocytomas and/or paragangliomas (PGL) have rarely been associated with pituitary adenomas (70–74). Patient with these tumors harbor mutations in the *SDHA-D* or *SDHA2F* genes. Loss of heterozygosity at the SDH locus in the pituitary adenomas, as well as data from animal studies, supports the causality between these genes and pituitary adenoma tumorigenesis (71, 73, 75).

SDH, a multimeric enzyme bound to the inner membrane of mitochondria, has two essential roles: (i) it is an important member of the Krebs cycle, and (ii) it plays a role in oxidative phosphorylation and controls activation of hypoxia-inducible factor 1a (HIF1α), leading to VEGF upregulation (75). SDH protein complex is formed by the subunits A, B, C, and D with its associated assembly factor (SDHAF2). Several mechanisms have been described to explain the tumorigenic effect of SDH mutations: (i) succinate accumulation that inhibits HIFα-prolyl hydroxylases, leading to activation of HIF1α and resulting in a state of tissue pseudohypoxia (76) and reactive oxygen species accumulation, and (ii) inhibition of histone demethylases that leads to epigenetic changes [ref. 77; see further details in the article by Jochmanova and Pacak in this *CCR Focus* (78)]. Interestingly, the HIF1α–VEGF pathway is upregulated by *RSUME*, a gene with increased expression in sporadic pituitary adenomas (79). The upregulated VEGF pathway in SDH-related pituitary adenomas may play a role in their invasive and aggressive behavior (80).

The majority of the described SDH-related pituitary adenoma cases are prolactinomas, followed by NFPA- and GH-secreting adenomas. The age of onset of pituitary adenomas is comparable with that of sporadic tumors without germline mutations (~40–50 years), and the vast majority of them present as invasive macroadenomas with a unique vacuolated histologic phenotype (71). The penetrance of pituitary adenomas in patients with *SDH* mutations is low; whether imaging of the pituitary fossa should be added to at least the first MRI screening of the neck and skull base area, especially in *SDHB* mutation–positive cases, remains to be determined.

Somatic Mutations

Sporadic pituitary adenomas harbor a lower somatic mutation rate in comparison with malignant tumors (81, 82), which is consistent with their typically low proliferation rate and benign phenotype.

GNAS

The most frequently observed (up to 40%) genetic change in somatotroph adenomas is the somatic heterozygous gain-of-function mutation of the *GNAS* gene coding for the Gsα subunit (11, 83, 84). The mutations, known as *gsp* mutations, affecting codon 201 or 227 destroy the GTPase activity of the protein (Fig. 3). Prolonged adenylyl cyclase stimulation and increased cAMP synthesis result in increased cell proliferation and GH secretion. *GNAS* is an imprinted gene in the pituitary; mutations are always located on the maternal allele (85). Some, but not all, studies found *gsp*-positive somatothropinomas to be more responsive to SSA treatment and mostly to be densely granulated somatotroph adenomas (6, 36). No other specific recurrent somatic mutations have been identified in somatotroph adenomas (82).

USP8

In ACTH-secreting adenomas, novel somatic gain-of-function mutations have recently been identified in the *USP8* gene (86, 87). Although USP8 is expressed in all anterior pituitary cell types (88), *USP8* mutations have only been identified in corticotroph adenomas (87). The pathomechanism leading to corticotroph adenomas has been linked to EGFR. *USP8* encodes a deubiquitinase enzyme that can protect EGFR from degradation by removing lysosome targeting ubiquitin tags, allowing recycling of the receptor to the cell surface (ref. 89; Fig. 4). EGFR is known to be expressed in corticotrophinomas, where it leads to increased POMC levels, ACTH synthesis, and corticotroph cell proliferation, and its presence correlates with invasiveness (86, 87, 90–92). All the identified *USP8* mutations are located in the 14-3-3 protein-binding motif of the protein, a domain highly conserved across species, suggesting that lack of 14-3-3 binding leads to increased cleavage and, therefore, increased catalytic activity of the shortened USP8 protein. Gain-of-function mutations in *USP8* increase deubiquitination of EGFR, which inhibits its degradations and leads to activation of EGFR signaling. Corticotroph adenomas with mutated *USP8* are more frequently found in females (67% vs. 38%; ref. 87), are smaller tumors, have higher ACTH production, and show better prognosis (87, 90). Not all studies found a correlation between EGFR expression and *USP8* mutation status (93). *USP8*-mutated tumors express significantly higher levels of proopiomelanocortin (the gene encoding ACTH) and SSTR5. An important clinical question is whether these adenomas, treated preoperatively or after unsuccessful surgery, respond better to

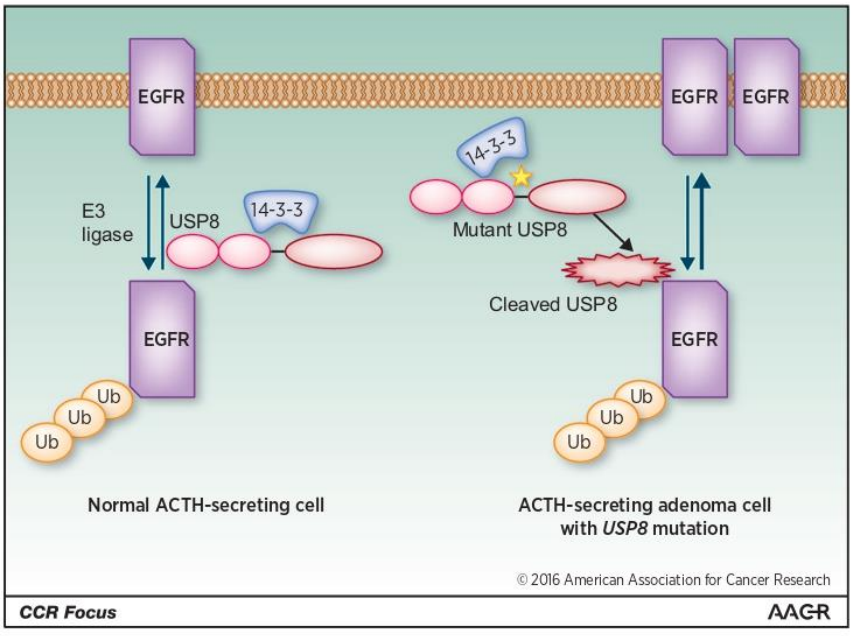

Figure 4.
Mechanism of USP8 induced tumorigenesis. In the normal pituitary, there is a balance between EGFR ubiquitination and degradation and deubiquitination by USP8 leading to EGFR recycling. Mutation in the 14-3-3 protein–binding site of USP8 (yellow star) results in excessive cleavage and higher deubiquitination activity, leading to increased recycling of EGFR.

multiligand somatostatin analogue pasireotide than *USP8* mutation–negative adenomas. The USP8-related tumorigenesis also opens up the possibility of EGFR-directed therapy for corticotrophinomas; indeed, this concept has already been tested in various animal models using EGFR inhibitor gefitinib (94).

Prolactinomas and NFPAs

Some prolactinomas show loss of chromosome 11 (95) and trisomy of chromosomes 5, 8, and 12, whereas no specific recurrent single-nucleotide mutations have been identified to date.

No recurrent specific somatic mutations have been identified in NFPAs using exome sequencing (81); however, intronic mutations, copy-number variations, or other genetic mechanism, not detected with this method, could play a role.

All pituitary adenoma types

Point mutations and increased copy number of the gene coding for the catalytic subunit of phosphoinositide 3-kinase catalytic subunit PI3K (phosphoinositide 3-kinase catalytic subunit *PIK3CA*) have been identified in 20% to 40% of various types of pituitary adenomas (96–98), but patients with Cowden syndrome who harbor germline mutations in the PI3K–PTEN–AKT pathway genes do not present with pituitary adenomas (99), suggesting that *PIK3CA* amplification could be a permissive phenomenon.

Sixty percent of oncocytic pituitary adenomas show mutations in mitochondrial DNA genes coding for various respiratory complex I components (*MTND1,2,4,5*, *MTTL2*, *MTTM*, *MTCYB*, and *MTRNR2*) leading to the disruption of respiratory complex I (100). It has been suggested that this leads to lack of HIF1α stabilization and, therefore, the relatively benign nature of these adenomas (101).

Mosaic Mutations

GNAS

Patients with somatic mosaicism with *GNAS* mutations have McCune-Albright syndrome (Fig. 3). One of the characteristic manifestations of this disease is pituitary hyperplasia or tumor resulting in increased GH and prolactin levels in addition to the classical triad of polyostotic fibrous dysplasia, café-au-lait spots, and precocious puberty (102). Other endocrine dysfunctions include testicular lesions, hyperthyroidism, phosphate wasting, and hypercortisolism. GH-secreting tumors (20%–30% of patients) usually present

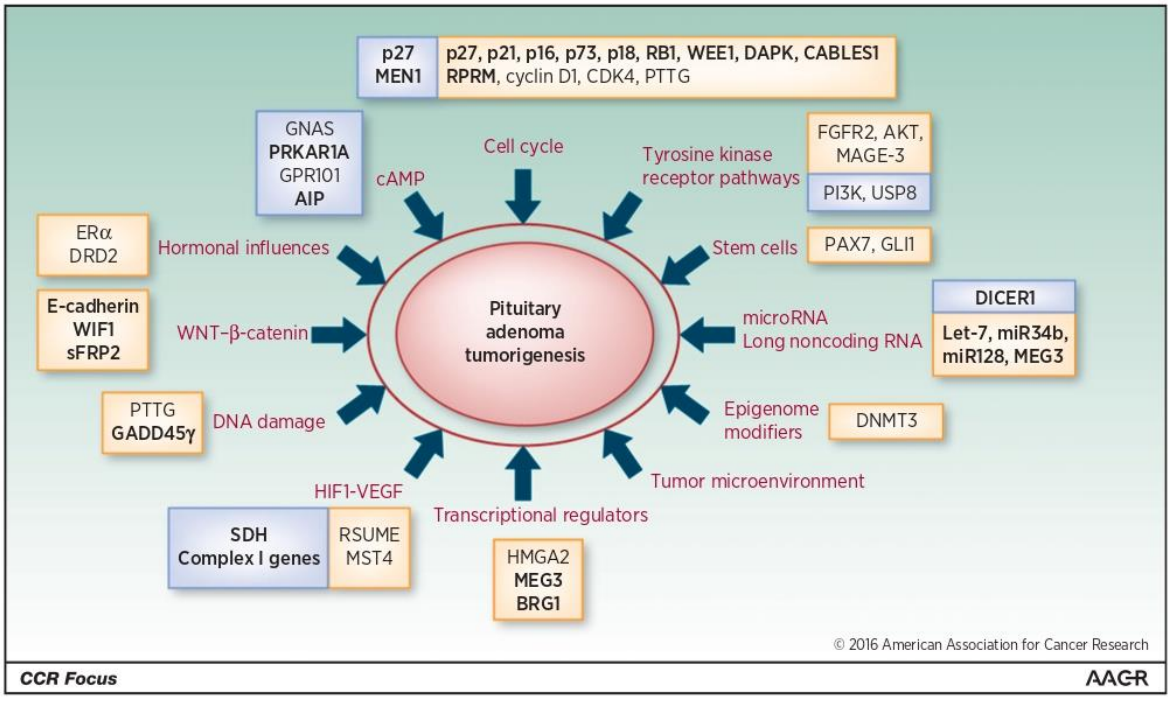

Figure 5.
Multiple pathways to pituitary tumorigenesis. Pathways, with representative examples, suggested to be involved in pituitary adenoma tumorigenesis either due to mutations (blue boxes; bold gene names represent tumor suppressor genes, gene names that are not bold represent oncogenes) or altered gene expression (orange boxes). Pituitary adenomas have significantly lower somatic mutation burden compared with other neoplasms, and simultaneous mutational events with representative mutational gene sets have not been described. Altered gene expression, however, has been identified in various pathways—the most important being the regulation of the cell cycle [80% of pituitary adenomas have altered gene expression resulting in a dysregulated cell cycle (110)].

before 20 years of age, although the diagnosis can be delayed due to craniofacial fibrous dysplasia resembling features of acromegaly (103–106). This is one of very few diseases that can result in both abnormally short stature, due to precocious puberty, or gigantism, due to young-onset GH excess. Patients with GH excess and craniofacial fibrous dysplasia present a surgical challenge due to skull-base thickening and obliteration of the sphenoid sinus, and treatment often requires total hypophysectomy due to diffuse involvement of the pituitary (104). Somatostatin analogues, especially in combination with GH receptor antagonist treatment, can effectively reduce IGF-1 levels (103, 105).

GPR101

In addition to germline mutations, *GPR101* duplication can occur as somatic mosaicism (41, 107). The clinical phenotype of patients with mosaic *GPR101* duplication, only described in male patients until now, does not differ from the germline cases. *GPR101* sequence variants, apart from gene duplication, do not play a role in pituitary tumorigenesis (42, 108).

Gene Expression in Pituitary Adenomas

The pathogenesis of sporadic pituitary adenomas is influenced by multiple factors (Fig. 5), and we refer to other reviews for detailed discussion of these elements (84, 109–111). The number of somatic mutations identified are small compared with other neoplastic conditions, and no data are available for simultaneous multiple mutated gene sets that are so characteristic of many other tumor types (112, 113). Abnormalities in cell-cycle regulation are considered a crucial event in the formation of pituitary adenomas. Loss of CDK inhibitors, such as p16 or p27 (114, 115), or overexpression of CDKs, such as cyclin D (116), are involved in tumor development (109). It has been estimated that 80% of human pituitary adenomas display alterations in at least one cell-cycle regulator (110). A systematic review of epigenetic regulation of pituitary adenomas identified altered expression of tumor suppressor genes [including genes coding for p16, p21, p27, p14, death associated protein kinase (*DAPK*), growth arrest, and DNA damage-inducible protein (GADD45γ), p73, retinoblastoma protein, BMP-4], oncogenes (*PTTG* and *MAGEA3*), imprinted genes (*GNAS, NNAT, MEG3*), epigenome modifiers (DNMT3b), and transcription regulators (HMGA2). More recent examples of proteins involved in pituitary tumorigenesis are the mammalian sterile-20-like kinase (MST4) and CABLES1. MST4, which was found to be upregulated in NFPAs, stimulates p38, AKT, and HIF1—known factors in pituitary tumorigenesis—whereas an MST4 inhibitor showed promising results in *in vitro* studies (117, 118). Another recently identified protein is CABLES1, which was lost in 55% of human corticotroph adenomas, and its levels correlated with loss of p27. The feedback effect of glucocorticoids following upregulation of CABLES1 might link the tumorigenic process with glucocorticoid-regulated cell-cycle progression (119). Hormonal factors could also have a role as prolactinomas with lower expression of estrogen receptor α have a higher tumor grade, greater resistance to treatment, and worse prognosis (120).

Pituitary Carcinomas

Pituitary carcinomas are extremely rare. They are diagnosed when distant metastases are detected, and survival is usually less than 2 years after diagnosis, which is similar to some other malignancies such as adrenal carcinoma [see article by Payabyab and colleagues in this *CCR Focus* (121)]. The majority of the cases arise from prolactin- and ACTH-secreting cells, and more rarely GH- or LH/FSH-secreting cells. It is currently unclear whether they develop as *de novo* carcinomas or pituitary adenomas that gradually gain malignant features. Somatic changes in tumor suppressor genes (e.g., *TP53* and *RB1*) and oncogenes (e.g., *HRAS* and *MYC*) commonly present in other neoplasia, have been identified in only a few cases of pituitary carcinoma (122). A few *MEN1*-related pituitary carcinomas (123, 124) and one *SDH*-related (80) pituitary carcinoma have been described in patients with pituitary tumor–predisposing syndromes.

The precise mechanism of why pituitary adenomas do not turn cancerous, even after many years of disease, remains unknown; however, it has been suggested that oncogene-induced senescence plays an important role (125, 126). Senescence is characterized by a signal transduction program leading to irreversible cell-cycle arrest and represents an important protective mechanism against malignancy. Senescence restrains proliferation, but allows the cell to remain viable and perform its physiologic function. This process can be activated by DNA damage, telomere shortening, lysosomal or oxidative stress, chromosomal instability, aneuploidy, loss of tumor-suppressive signaling, or oncogenic activity (110, 127). These events trigger the activation of different cell-cycle regulators, such as p53 and pRb, to upregulate the senescence pathways. *PTTG*, an oncogene often overexpressed in pituitary adenomas, promotes chromosomal instability and aneuploidy, and lack of *PTTG* results in pituitary-specific senescent features (127). IL6 is a cytokine involved in pituitary tumor progression, but it is also required for induction and maintenance of oncogene-induced senescence via an autocrine mechanism (128). Senescence markers have been shown to be present in human adenomas where subtype-specific senescence induction pathways could play an important role (125, 129).

In summary, several new pituitary adenoma–predisposing genes have been identified in the last few years: germline mutations, causing isolated pituitary adenomas or syndromic disease, as well as somatic or mosaic mutations. The clinical characterization of patients with sporadic disease or genetic predisposition for pituitary adenomas, and the elucidation of the exact molecular mechanisms will contribute to the identification of novel physiologic pathways, leading to better understanding of tumorigenesis as well as novel therapies.

Disclosure of Potential Conflicts of Interest

M. Korbonits reports receiving commercial research grants from Novartis and Pfizer, and is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other author.

Authors' Contributions

Conception and design: F. Caimari, M. Korbonits
Writing, review, and/or revision of the manuscript: F. Caimari, M. Korbonits

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Caimari, M. Korbonits
Study supervision: M. Korbonits

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ANNEX II

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