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Genetic characterization of a series of pituitary tumors

Adriana Filipa Oliveira da Silva

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Genetic characterization of a series of pituitary tumors

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Resumo

Os tumores neuroendócrinos hipofisários (PitNETs) são neoplasias intracranianas frequentes que, apesar da sua natureza maioritariamente benigna, podem causar morbilidade significativa devido à disfunção hormonal e aos efeitos locais de massa. Investigações recentes têm identificado mecanismos genéticos e epigenéticos fundamentais no desenvolvimento dos PitNETs; contudo, muitas características moleculares permanecem pouco definidas, sobretudo nos subtipos menos estudados.

Este estudo teve como objetivo investigar as alterações genéticas subjacentes a diferentes PitNETs, através do rastreio de mutações em genes candidatos frequentemente implicados na tumorigénese hipofisária. Foram analisados os genes *GNAS*, *USP8*, *BRAF* e *SF3B1*, conhecidos por influenciarem vias como a sinalização do cAMP, a regulação do EGFR, a ativação da via MAPK e o *splicing* do RNA. No total, foram estudadas 153 amostras tumorais fixadas em formol e incluídas em parafina. O DNA genómico foi extraído e submetido a amplificação por reação em cadeia da polimerase (PCR), purificação com ExoSAP e sequenciação de Sanger para deteção de mutações nas regiões alvo. Foram ainda integrados dados clínicos e anatomopatológicos, incluindo características demográficas, dimensões tumorais, invasão tumoral, perfil de secreção hormonal e índices proliferativos, para avaliar potenciais correlações genótipo-fenótipo.

Os resultados revelaram mutações recorrentes no gene *GNAS*, restritas a tumores somatotróficos, com uma prevalência (24.8%) consistente com a descrita na literatura. Foi também detetada a presença de mutações no gene *USP8*, em tumores corticotróficos (18.8%), enquanto alterações no gene *SF3B1* não foram identificadas em nenhum tumor lactotrofo e alterações do *BRAF* estiveram ausentes em toda a coorte. Observou-se uma associação estatisticamente significativa entre o estado mutacional e a remissão ao fim de um ano, não tendo sido observadas diferenças relevantes nas restantes características clínicas.

Através da integração da análise molecular com dados clínicos e anatomopatológicos, este estudo contribui para clarificar o perfil genético dos PitNETs e confirma que as mutações são frequentes e ocorrem de forma subtipo-específica, nomeadamente *GNAS* em tumores somatotróficos e *USP8* em tumores corticotróficos. Embora não tenham sido identificadas associações genótipo-fenótipo significativas, os resultados permitem uma melhor descrição da heterogeneidade dos PitNETs e constituem uma base para investigações futuras que explorem mais profundamente a sua relevância biológica.

Palavras-Chave: Endocrinologia; Tumores neuroendócrinos da hipófise; Genética molecular; Tumorigénese; Análise de mutações; Reação em Cadeia de Polimerase; Sequenciação de Sanger

Abstract

Pituitary neuroendocrine tumors (PitNETs) are common intracranial neoplasms that, despite their mostly benign nature, can cause significant morbidity due to hormone dysregulation and local mass effects. Recent research has revealed key genetic and epigenetic mechanisms driving PitNET development; however, many molecular features remain poorly defined, particularly across less-studied subtypes.

This study aimed to investigate the genetic alterations underlying diverse PitNETs by screening for mutations in candidate genes frequently implicated in pituitary tumorigenesis. Specifically, the study focused on *GNAS*, *USP8*, *BRAF*, and *SF3B1*, which are known to influence pathways such as cAMP signaling, EGFR regulation, MAPK activation, and RNA splicing. A total of 153 formalin-fixed, paraffin-embedded (FFPE) tumor samples representing multiple PitNET subtypes were analyzed is this study. Genomic DNA was extracted and subjected to polymerase chain reaction (PCR) amplification, ExoSAP-based purification, and Sanger sequencing to detect mutations in the target regions. Clinical and pathological data, including patient demographics, tumor size, invasiveness, hormone secretion profile, and proliferative indices, were integrated to assess potential genotype–phenotype correlations.

The results revealed recurrent *GNAS* mutations in somatotroph tumors, with a prevalence (24.8%) consistent with that in the previous literature. *USP8* mutations were present in corticotroph tumors (18.8%), whereas *SF3B1* alterations were absent in the lactotroph tumors, and *BRAF* in the entire cohort. A statistically significant association was observed between mutation status and remission at one year, while no relevant differences were found in the remaining clinical characteristics.

By combining molecular screening with clinicopathological data, this study helps to clarify the genetic profile of PitNETs and confirms that recurrent mutations are frequent and occur in a subtype-specific manner, particularly *GNAS* in somatotroph tumors and *USP8* in corticotroph tumors. Although no significant genotype-phenotype associations were found, the results contribute to a better description of PitNET heterogeneity and provide a basis for future work to further explore their biological relevance.

Keywords: Endocrinology; Pituitary neuroendocrine tumors (PitNETs); Molecular genetics; Tumorigenesis; Mutation analysis; Polymerase chain reaction (PCR); Sanger sequencing

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List of Abbreviations

ACTH- Adrenocorticotropin
ADP- Adenosine diphosphate
***BRAF*-** B-Raf proto-oncogene, serine/threonine kinase
***CADM1*-** Cell Adhesion Molecule 1
cAMP- Cyclic AMP
***CASP8*-** Caspase 8
***CDKN2A*-** Cyclin-dependent kinase inhibitor 2A
DGCT- Densely granulated corticotroph tumors
DGLT- Densely granulated lactotroph tumors
DGST- Densely granulated somatotroph tumors
DNA- Deoxyribonucleic acid
DNMT1- DNA methyltransferase 1
DNMT3A- DNA methyltransferase 3A
DNMT3B- DNA methyltransferase 3B
dNTP- Deoxynucleotide triphosphates
EGFR- Epidermal growth factor receptor
ERK- Extracellular signal-regulated kinase
F- Female
F1- Fragment 1
F2- Fragment 2
F3- Fragment 3
FFPE- Formalin-fixed, paraffin-embedded
FSH- Follicle-stimulating hormone
GH- Growth hormone
***GNAS*-** GNAS complex locus
GTP- Guanosine triphosphate
H3K14- Histone H3 lysine 14
H3K27- Histone H3 lysine 27
H3K4- Histone H3 lysine 4
H3K9- Histone H3 lysine 9
HAT- Histone acetyltransferases
HDAC- Histone deacetylase
HDM- Histone demethylases
IQR- Interquartile range
LH- Luteinizing hormone

M- Male

MAPK- Mitogen-activated protein kinase

MEK- MAPK/ERK kinase

***MEN1*-** Menin 1

MEN1- Multiple endocrine neoplasia type 1

miRNA- Micro RNA

mRNA- Messenger RNA

MSH- α-melanocyte-stimulating hormone

MUT- Mutated

NA- Not available

NR5A1- Nuclear receptor subfamily 5 group A member 1 (Alias symbol SF-1)

PCR- Polymerase chain reaction

Pit-1- Pituitary-specific transcription factor 1

PitNET- Pituitary neuroendocrine tumor

PKA- Protein kinase A

***POMC*-** Proopiomelanocortin

POUF1- POU domain, class 1, transcription factor 1

PRL- Prolactin

***PTAG*-** Pituitary tumor apoptosis gene

***RARB*-** Retinoic acid receptor beta

***RASSF1A*-** Ras association domain family member 1 isoform A

RNA- Ribonucleic acid

rpm- Rotations per minute

SD- Standard deviation

SF-1- Steroidogenic factor 1

***SF3B1*-** Splicing factor 3b subunit 1

SGCT- Sparsely granulated corticotroph tumors

SGLT- Sparsely granulated lactotroph tumors

SGST- Sparsely granulated somatotroph tumors

TBX19- T-box transcription factor 19

Tpit- T-box transcription factor expressed in pituitary

TSH- Thyroid-stimulating hormone

U- Enzymatic units

***USP8*-** Ubiquitin Specific Peptidase 8

VUS- Variant of uncertain significance

WT- Wild-type

1. Introduction

1.1. The pituitary gland: structure and function

The pituitary gland, often referred to as the "master gland" of the human body, is a small pea-sized organ of around 6 millimeters (mm) in diameter, located at the base of the brain, nestled within the sella turcica, a bone structure of the skull (1).

The pituitary gland is divided into two main parts, the anterior pituitary or adenohypophysis and the posterior pituitary or neurohypophysis, each with different functions. The adenohypophysis makes for about 80% of the pituitary and is composed of five distinct secretory cell types: somatotrophs, corticotrophs, lactotrophs, thyrotrophs, and gonadotrophs and has the capability to produce growth hormone (GH), adrenocorticotropin (ACTH), prolactin (PRL), thyroid-stimulating hormone (TSH), and gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), respectively (2). Between these two regions lies the *pars intermedia*, a narrow band of tissue that is rudimentary in humans but well-developed in other mammals (3). It is primarily composed of colloid-filled cysts and a small number of melanotrophs, which can produce α-melanocyte-stimulating hormone (MSH), although its physiological role in adult humans is limited (3). The neurohypophysis is composed of a set of supraoptic and paraventricular nuclei and comprises magnocellular neurons that store and secrete oxytocin and vasopressin, which play key roles in processes such as water balance, blood pressure regulation, and uterine contractions during childbirth (4).

The pituitary gland's activities are controlled by the hypothalamus, a region in the brain that signals by releasing stimulating and inhibiting hormones, and signals the pituitary gland to produce or suppress the secretion of specific hormones (5). This gland plays a crucial role in regulating various physiological processes, such as growth, metabolism, reproduction, and stress response, among other essential functions, through the secretion of different hormones, and helps maintain homeostasis. Therefore, the pituitary is a vital component of the endocrine system, controlling the release of hormones throughout the body whose intricate balance is essential for maintaining overall health and well-being (6).

1.2. Pituitary neuroendocrine tumors (PitNETs): classification and clinical impact

The emergence of pituitary neuroendocrine tumors (PitNETs), which arise from abnormal growth within the pituitary, can cause the dysregulation or dysfunction of the

pituitary gland leading to a variety of health issues, such as growth disorders, thyroid dysfunction, adrenal insufficiency, and reproductive abnormalities (7). PitNETs are mostly benign and relatively common, representing approximately 15% of all intracranial neoplasms (8). However, they can have a huge impact on patients' lives due to the compression of nearby structures and hormone imbalances (8).

Clinically, pituitary tumors can be classified in terms of size as microadenomas, if smaller than 10 mm in diameter, or macroadenomas, if equal to or larger than 10 mm in diameter (8). Furthermore, they can also be divided into functioning or non-functioning PitNETs. The non-functioning ones do not exhibit hormone hypersecretion or secrete hormones at a level that is insufficient to be detected, and often grow slowly, not causing noticeable symptoms until they start compressing nearby structures (9). On the other hand, functioning PitNETs produce excessive quantities of one or more hormones, creating hormonal imbalances and typical clinical syndromes, depending on the hormone(s) affected (10).

The anterior pituitary is composed of hormone-producing cells that arise from distinct embryological lineages (Fig.1), each governed by specific transcription factors that drive their differentiation and identity (11). Three principal lineages have been defined based on the expression of the transcription factors Pit-1 (POU1F1), Tpit (TBX19), and SF-1 (NR5A1) (7, 12). These transcription factors are critical during pituitary development, determining the fate of progenitor cells and restricting them to lineages committed to the synthesis of specific hormones. Pit-1 is essential for the development of somatotrophs, lactotrophs, and thyrotrophs; Tpit directs corticotroph lineage differentiation; and SF-1 is involved in gonadotroph specification (7).

The multiple differentiation programs of pituitary cells are mirrored by the various tumors comprised under the functioning PitNET umbrella term. These include corticotroph tumors, which arise in the anterior pituitary gland and are characterized by their ability to produce and secrete excessive amounts of ACTH, which stimulates the adrenal glands to produce cortisol and therefore causing Cushing’s syndrome (8, 9). Lactotroph tumors, which are the most common PitNETs, lead to excessive production of prolactin and cause hyperprolactinemia. Somatotroph tumors produce and secrete excessive amounts of GH and are associated with acromegaly (8, 9). Thyrotroph tumors are the ones that secrete too much TSH, leading to hyperthyroidism. Finally, gonadotroph tumors lead to deficient secretion of LH and/or FSH, leading to secondary hypogonadism (8).

The accurate identification of these lineages in adult pituitary tissue and tumors relies on immunohistochemical detection of lineage-specific transcription factors rather

than solely on hormone expression, which may be variable or absent in certain neoplastic contexts (11).

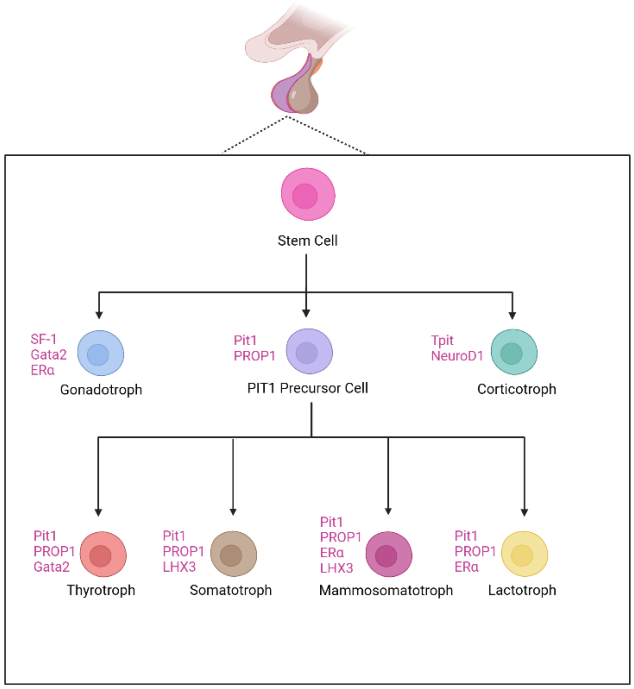


Figure 1. *Schematic representation of anterior pituitary cell lineage differentiation and associated transcription factors.*

Pituitary stem cells differentiate into distinct hormone-producing cell types through specific transcriptional programs. Tpit drives corticotroph differentiation, while Pit-1 is essential for the development of somatotrophs, lactotrophs, thyrotrophs, and mammosomatotrophs. This figure summarizes the major pathways of anterior pituitary development and the key transcription factors involved in lineage determination. Created in https://BioRender.com

1.2.1 Corticotroph tumors and Cushing's syndrome

Corticotroph PitNETs arise from cells of the Tpit lineage, more precisely from adenohypophyseal cells, and make up around 15% of all PitNETs (13). These tumors are divided into several subtypes based on their histological characteristics, hormone production, and clinical behavior. Densely granulated corticotroph tumors (DGCTs) are one subtype of tumors, marked by densely packed secretory granules and usually small in size. Typically, these tumors correlate with higher cortisol levels and consequently are more likely to produce clinical signs of Cushing's disease (11). Another subtype is sparsely granulated corticotroph tumors (SGCTs), which have fewer secretory granules and a less distinct granule structure but are larger in size. They are also associated with hypercortisolism to a lesser extent than densely granulated tumors, which translates into fewer and less pronounced signs of Cushing´s disease and often being mistaken for silent corticotroph tumors (13).

Additionally, silent corticotroph tumors represent a clinically important and distinct subset (14). Unlike their hormonally active counterparts, silent corticotroph tumors do not cause overt Cushing's syndrome despite expressing ACTH immunohistochemically (15-17). These tumors often present as non-functioning PitNETs and are typically diagnosed due to mass effects, such as headaches or visual disturbances (14). Silent corticotroph tumors are further divided into type 1: silent densely granulated corticotroph tumors, and type 2: silent sparsely granulated corticotroph tumors. Although silent, these tumors may demonstrate aggressive behavior, including a higher propensity for invasion and recurrence compared to other non-functioning tumors (16, 18). Clinically, silent corticotroph tumors pose diagnostic challenges and often require careful histopathological and immunohistochemical evaluation to ensure accurate identification (19).

Lastly, Crooke's Cell Tumors are the rarest subtype and are highly aggressive and proliferative. These tumors are defined by the gathering of cytokeratin filaments near the nucleus, which when stained with hematoxylin and eosin, present as very hyaline (13).

1.2.2 Somatotroph tumors and acromegaly

Somatotroph tumors are formed from Pit-1 lineage adenohypophyseal cells and constitute around 10-15% of PitNETs (13). Somatotroph tumors can be divided into densely granulated or sparsely granulated somatotroph tumors. Densely granulated somatotroph tumors (DGSTs) have large cells with acidophilic cytoplasm, and on immunohistochemical staining, these tumors show strong and diffuse positivity for growth hormone (20). On the other hand, sparsely granulated somatotroph tumors (SGSTs) are characterized by a low density of secretory granules within the somatotroph cells, they are made of chromophobic cells and typically show weaker or even negative reactivity for growth hormone. They may also exhibit a fibrous body within the cytoplasm, which is a distinguishing feature (20). Similarly to densely granulated tumors, these tumors lead to acromegaly, with symptoms such as enlarged extremities, facial changes such as protruding jaw and brow, and skin thickening. However, clinical manifestations might differ slightly due to variations in hormone secretion patterns (21). Sparsely granulated somatotroph tumors are generally more aggressive and less responsive to somatostatin therapy compared to their densely granulated counterparts (22). They tend to exhibit a higher proliferation index, increased invasiveness, and a greater likelihood of recurrence (13, 22, 23).

Mammosomatotroph tumors are a subtype of pituitary neuroendocrine tumors that arise from cells capable of producing both growth hormone and prolactin (24). These tumors originate from a common precursor cell and typically show co-expression of GH and prolactin on immunohistochemical analysis. Histologically, they resemble densely granulated somatotroph tumors but can be differentiated by their dual hormonal activity (11). Clinically, mammosomatotroph tumors may lead to acromegaly or hyperprolactinemia (25). Compared to pure somatotroph or lactotroph tumors, these tumors may present with a broader spectrum of symptoms due to the combined effects of GH and prolactin hypersecretion (25).

1.2.3 Lactotroph tumors and hyperprolactinemia

Lactotroph tumors are the most common pituitary neuroendocrine tumors composing 30-50% of PitNETs and arising from adenohypophyseal cells of the Pit-1 lineage (13). These tumors are the leading cause of non-pharmacological and pathologic hyperprolactinemia, which can lead to several serious problems, such as infertility, irregular menstrual cycles or amenorrhea, low bone mass, gynecomastia, sexual dysfunction, and, in men, low serum testosterone levels (26).

Similarly to somatotroph tumors, lactotroph tumors can also be classified as densely granulated or sparsely granulated lactotroph tumors (13). Densely granulated lactotroph tumors (DGLTs) are a very rare tumor subtype with a high production of prolactin (13). However, the hormone´s expression in the cytoplasm is scattered and tumor size is usually substantial. Sparsely granulated lactotroph tumors' (SGLTs) cells have an acidophilic cytoplasm and prolactin expression is found around the nucleus contrary to their densely granulated counterparts, making it a differentiation feature (11).

1.2.4 Thyrotroph tumors and hyperthyroidism

Thyrotroph PitNETs are rare and normally made up of spindle-shaped cells with cytoplasmatic positivity for TSH (11). TSH-secreting tumors result in increased or improperly regulated TSH levels, often accompanied by normal or elevated levels of thyroid hormones, which can consequently lead to hyperthyroidism (8).

Hyperthyroidism is a condition with several complications for patients, that include an enlarged thyroid gland (goiter), increased heart rate, hyperhidrosis, weight loss, irritability, anxiety, etc. (27).

1.2.5 Gonadotroph tumors and hypogonadism

Gonadotroph PitNETs are a common type of PitNETs, but they are rarely functioning tumors and are usually detected when patients complain about visual impairment or headaches caused by the size of the tumor (9). These tumors are distinguished by chromophobic cells that can gather around vascular channels in rosettes, sheets, or trabeculae (11).

In the rare cases where gonadotroph PitNETs are functioning, they may produce abnormally high baseline levels of intact FSH in the blood, while elevated levels of intact LH are observed less frequently. This can lead to symptoms such as menstrual irregularities, infertility, galactorrhea, and ovarian hyperstimulation syndrome in pre-menopausal women and testicular enlargement in men (28, 29). Patients can also present secondary hypogonadism, due to the compression the tumor exercises on the gonadotroph cells, which impairs LH secretion (29).

1.3. Pituitary tumorigenesis

The vast majority of pituitary tumors are benign. However, through the process of pituitary tumorigenesis, normal pituitary cells can transform into neoplastic pituitary cells. This process involves a complex interplay of genetic mutations, epigenetic alterations, disrupted cellular signaling pathways, and cell cycle disruption, which can promote abnormal cell proliferation and evasion of apoptosis (30). Therefore, understanding these mechanisms is crucial for developing targeted therapeutic strategies.

1.3.1 Molecular mechanisms of pituitary tumorigenesis

Genetic mutations are central to the initiation of pituitary tumors. Several genes have been identified as key players in the development of these adenomas (31).

Ubiquitin Specific Peptidase 8 (*USP8*) mutations have been identified as significant contributors to the pathogenesis of certain types of pituitary tumors, particularly corticotroph tumors occurring in about 35-60% of them (18, 32, 33). *USP8* encodes a deubiquitinating enzyme that plays a critical role in the regulation of various cellular processes, including signal transduction, protein trafficking, and receptor downregulation (34, 35). The enzyme functions by removing ubiquitin components from target proteins, which prevents their degradation by the proteasome (36). One of the key

substrates of *USP8* is the epidermal growth factor receptor (EGFR), a cell surface receptor that, upon activation, triggers downstream signaling pathways promoting cell growth and survival (34, 37).

Mutations in the *USP8* gene are usually located within the 14-3-3 binding motif of the protein, which is a crucial region for the regulation of *USP8* activity, as binding to 14-3-3 proteins inhibits *USP8*'s deubiquitinating function. Mutations in this region disrupt the interaction with 14-3-3 proteins, leading to constitutive activation of USP8 thereby increasing deubiquitination of some proteins, including EGFR (38). This prevents the receptor's degradation, resulting in prolonged EGFR signaling (39).

The *GNAS* gene, which encodes the Gs alpha subunit of the G protein involved in the cyclic AMP (cAMP) signaling pathway, is frequently mutated in somatotroph tumors (39).

Furthermore, enhanced EGFR signaling has been shown to upregulate proopiomelanocortin (*POMC*) gene expression, the precursor of ACTH which consequently leads to excessive ACTH production, a hallmark of corticotroph tumors, and directly contributes to the clinical manifestations of Cushing's disease (38).

Studies have shown that approximately 40% of somatotroph tumors harbor *GNAS* complex locus mutations, making it a critical factor in the pathogenesis of these tumors (40-42) This protein is implicated in the activation of adenylate cyclase, which converts ATP to cAMP, leading to the activation of protein kinase A (PKA), which then phosphorylates target proteins, leading to the regulation of gene expression, cell proliferation, and hormone hypersecretion (7, 39). Mutations in this gene lead to constitutive activation of the cAMP pathway, resulting in increased cell proliferation and growth hormone secretion (7, 39).

Mutations in the menin 1 (*MEN1*) gene, which encodes the tumor suppressor protein menin, have also been associated with PitNETs and involved in its tumorigenesis (39). These mutations occur in approximately 3.5% of pituitary tumors (43). Menin is involved in several crucial cellular processes, such as gene transcription regulation, where it interacts with different transcription factors and helps regulate the expression of genes involved in cell cycle control, DNA repair, and apoptosis (44, 45). Menin also plays a role in controlling the cell cycle through its interaction with proteins that control the transition between different phases of the cycle (45). Furthermore, menin is also involved in repairing DNA damage (45).

Mutations in this gene are associated with multiple endocrine neoplasia type 1 (MEN1) syndrome, which is a hereditary syndrome predominantly marked by the development of neoplasms in the parathyroid glands, pancreas, and the anterior pituitary (46). The loss of the tumor suppressive functions mentioned above predisposes

individuals to PitNETs, such as lactotroph tumors, somatotroph tumors, corticotroph tumors, and non-functioning tumors, as well as other endocrine tumors (45). Although earlier reports indicated that MEN1-related pituitary tumors tend to be larger and more treatment-resistant, recent evidence shows that many lesions, especially those detected through surveillance, behave similarly to sporadic pituitary tumors, often presenting as small, non-functioning tumors with stable clinical behavior (47, 48). In patients with MEN1, functioning pituitary tumors occur more often than non-functioning ones, although their overall prevalence is comparable to that seen in individuals without MEN1 (49). Furthermore, MEN1 patients can develop multiple pituitary tumors simultaneously, leading to unforeseen multi-hormonal secretion, which can complicate treatment and management, hormone hypersecretion (43).

Mutations in *SF3B1* (splicing factor 3b subunit 1), a key component of the spliceosome complex, have been increasingly recognized in various human cancers due to their role in aberrant RNA splicing (50). Although *SF3B1* mutations are well-established in hematologic malignancies and some solid tumors, recent genomic studies have identified their presence in a subset of PitNETs, particularly in lactotroph tumors, where the prevalence has been reported to be as high as 20% (50). *SF3B1* encodes a protein critical for the proper recognition of branch points during mRNA splicing, and mutations, particularly at codons such as K700E, lead to the use of cryptic 3' splice sites, generating abnormal transcripts (51).

In pituitary tumors, *SF3B1* mutations have been found primarily in prolactin-secreting tumors (lactotroph tumors) and appear to be associated with more aggressive clinical features, including increased tumor size and potential invasiveness (50). A study reported the prevalence of this mutation in around 20% of lactotroph PitNETs (51). These findings suggest a potential role for spliceosomal dysregulation in pituitary tumorigenesis. Importantly, *SF3B1*-mutant tumors may exhibit distinct molecular signatures and therapeutic vulnerabilities, which opens new avenues for targeted approaches in tumor subtypes traditionally managed with dopamine agonists (50).

Chen et al. (32) reported the presence of *BRAF* mutations in 16.4% of corticotroph tumors, which secrete adrenocorticotropic hormone and cause Cushing's disease. The frequency of *BRAF* mutations in these tumors does not translate to other PitNETs, but its detection in corticotroph tumors suggests that these mutations may contribute to the pathogenesis of a subset of pituitary tumors with distinct molecular profiles (32).

Mutations in the *BRAF* gene, which is well-known for its role in various cancers, have been investigated for their potential involvement in pituitary tumorigenesis, particularly the *BRAF* V600E mutation, which is a missense mutation resulting in a

valine-to-glutamic acid substitution at codon 600 in exon 15 (52) and leads to constitutive activation of the MAPK pathway (53). The *BRAF* gene encodes a protein called B-Raf, a serine/threonine-protein kinase that plays a critical role in the mitogen-activated protein kinase (MAPK) signaling pathway (53). This pathway is essential to the regulation of cell growth, differentiation, and survival (54). The MAPK pathway includes a cascade of phosphorylation events, where *BRAF* phosphorylates and activates MEK (MAPK/ERK kinase), which in turn activates ERK (extracellular signal-regulated kinase) that translocates to the nucleus and influences gene expression, cell proliferation, and survival (54).

In pituitary tumors with *BRAF* mutations, the persistent activation of the MAPK pathway leads to increased cellular proliferation and resistance to apoptosis, which is a hallmark of tumorigenesis and contributes to the development and progression of the tumor (55). This pathway also interacts with other signaling pathways that regulate cell growth and differentiation, such as crosstalk between the MAPK pathway and the PI3K/AKT pathway which can enhance tumor survival and resistance to therapy (56).

Table 1. Summary of clinical, molecular, and lineage characteristics of Pituitary Neuroendocrine Tumors

Tumor Type	Predominant Sex	Typical Age (years)	Common Mutation (% prevalence)	Function of Altered Gene	Cell Lineage	Altered Hormone	Associated Disease	References
Somatotroph (GH)	Females	30–50	*GNAS* (~40%)	Activates cAMP pathway → cell proliferation and GH hypersecretion	Pit-1	GH	Acromegaly	(7) (8) (57)
Lactotroph (PRL)	Females	20–30	*SF3B1* (in ~10–20%)	RNA splicing alterations	Pit-1	PRL	Hyperprolactinemia	(7) (26) (50) (57)
Corticotroph (ACTH)	Females	Any age	*USP8* (~35–60%) *BRAF* (up to 20%)	*USP8* → ↑ EGFR signaling; *BRAF* → MAPK pathway activation	Tpit	ACTH	Cushing's disease	(7) (8) (32) (38) (50) (57)
Gonadotroph (LH/FSH)	Males	40–70	Rare specific mutations	Limited data; typically non-functioning	SF-1	LH / FSH	Mostly non-functioning pituitary tumor	(7) (57) (58)
Thyrotroph (TSH)	No clear predominance	40–70	No somatic mutations associated	-	Pit-1	TSH	Central hyperthyroidism	(7) (57) (59) (60)
Crooke's Cell Tumor	Females	40–50	Unknown	-	Tpit	ACTH	Cushing's (aggressive form)	(7) (11)
Mammosomatotroph	No predominance	Any age but more common in young patients with gigantism	Unknown	-	Pit-1	GH + PRL	Acromegaly ± Hyperprolactinemia	(7) (18) (25)

In closing, understanding the molecular mechanisms underlying PitNETs continues to be a significant challenge in endocrine oncology. Much remains unclear about the full spectrum of genetic alterations present in PitNETs, especially in less common, mixed, or non-functional tumor types. This lack of comprehensive molecular data limits the precision of tumor classification, hampers accurate prediction of clinical behavior, and slows progress toward tailored treatment options. While the genetic profiles of corticotroph and somatotroph tumors have been explored in more detail, other subtypes, including lactotroph, thyrotroph, and gonadotroph tumors, have yet to be thoroughly studied at the molecular level.

1.3.2 Epigenetic modifications

In addition to genetic mutations, epigenetic changes play a significant role in pituitary tumorigenesis (61). Unlike DNA alterations, these modifications leave the underlying sequence intact but influence which genes are turned on or off (62).

A prime example is DNA methylation which is the addition of methyl groups, donated by S-adenosylmethionine, to cytosine residues (most often within CpG islands in gene promoters) condenses chromatin, limits access to transcriptional machinery, and silences gene expression (63). The enzymes responsible are DNA methyltransferases, DNMT3A and DNMT3B establish new methylation patterns, and DNMT1 maintains them through cell division (63).

In pituitary tumors, the promoters of numerous tumor suppressor genes are hypermethylated and transcriptionally silenced (64). For example, *CDKN2A*, which encodes the cell-cycle inhibitor p16, is hypermethylated in both functioning and non-functioning tumors (65). Loss of p16 expression leads to an increase in transcription factors, enabling unchecked proliferation (65). Similarly, the pituitary tumor apoptosis gene (*PTAG*) is inactivated by promoter methylation in many tumors, reducing programmed cell death (65). Other tumor suppressors, such as *RASSF1A* and *RARB*, also display frequent hypermethylation, particularly in larger or more invasive tumors (63).

Contrasting with focal hypermethylation, many pituitary tumors exhibit global DNA hypomethylation (61). DNA hypomethylation has been implicated in somatotroph tumors and methylation patterns can distinguish between different PitNETs types (7).

It is worth noting, however, that methylation and gene expression do not always correlate in direct ways. Some studies of pituitary tumors have failed to link promoter methylation with reduced transcript levels, and in certain cases, hypomethylation has

been associated paradoxically with downregulation of genes such as *CASP8* (Caspase 8) and *CADM1* (Cell Adhesion Molecule 1) (66).

In addition to DNA methylation, post-translational modifications of histones play a crucial role in determining chromatin accessibility and thus gene expression (67). In pituitary tumors, increased histone deacetylase activity is frequently detected, resulting in a tighter nucleosome structure and suppression of tumor suppressor loci (67).

More specifically, the attachment of acetyl groups to lysine residues on histone tails (for example, H3K9 and H3K14) by histone acetyltransferases (HATs) neutralizes positive charges, loosening DNA-histone interactions and facilitating transcription factor binding (68). Conversely, histone deacetylase (HDAC) complexes remove these acetyl marks, promoting chromatin compaction and gene silencing (68). Histone methylation adds another layer of complexity: depending on the particular lysine residue (e.g., H3K4 versus H3K27) and the number of methyl groups, the resulting mark can either activate or repress transcription (63). Histone demethylases (HDMs) can then erase these methyl modifications, allowing dynamic regulation of gene expression (63). Although phosphorylation, ubiquitination and ADP-ribosylation of histones have also been described, their roles in pituitary neoplasia remain less well characterized (69).

Together, these reversible histone marks coordinate with DNA methylation and non-coding RNA networks to shape the epigenetic landscape of pituitary tumors, an area of intense research as we seek epigenetic therapies with greater specificity and fewer off-target effects (70).

MicroRNAs (miRNAs) are short, non-coding RNAs that fine-tune gene expression after transcription (71). By pairing with complementary sequences in the 3′ untranslated region (or, in some cases, within coding regions) miRNAs recruit the RNA-induced silencing complex and trigger mRNA degradation or translational repression (70). As a result, targeted mRNAs are removed more rapidly, and protein synthesis is effectively silenced.

In pituitary tumors, disrupted miRNA profiles contribute to disease progression (61). Approximately two-thirds of the miRNAs found to be downregulated in these tumors normally act as tumor suppressors; their loss permits increased translation of oncogenic targets and promotes cell proliferation (61). Conversely, miRNAs that are overexpressed in pituitary neoplasms function as oncogenic drivers, repressing transcripts of key growth-inhibitory or apoptotic factors (61). Together, these imbalanced miRNA networks play a decisive role in pituitary tumorigenesis and represent both potential biomarkers and therapeutic targets (61).

Understanding the molecular mechanisms underlying pituitary neuroendocrine tumors continues to be a significant challenge in endocrine oncology. Much remains

unclear about the full spectrum of genetic alterations present in PitNETs. This lack of comprehensive molecular data limits the precision of tumor classification, hampers accurate prediction of clinical behavior, and slows progress toward tailored treatment options. While the genetic profiles of corticotroph and somatotroph tumors have been explored in more detail, other subtypes, including lactotroph, thyrotroph, and gonadotroph tumors, have yet to be thoroughly studied at the molecular level.

2. Objectives

As such, to build on current knowledge and fill existing gaps, this study was designed with the following aims:

1. To determine the prevalence of mutations in key candidate genes across different PitNET subtypes. The analysis focused on hotspot areas *GNAS* (exons 8 and 9), *USP8* (exon 14), *SF3B1* (exon 14), and *BRAF* (exon 15), selected for their reported or suspected roles in pituitary tumorigenesis.
2. To describe the spectrum of variants identified and assess their potential biological relevance.
3. To evaluate genotype–phenotype correlations within clinical subgroups. Associations between mutation status and demographic, pathological, and clinical variables (including tumor subtype, hormone secretion profile, Ki-67, remission at one year, and others) were to be explored. Particular attention was directed to disease-specific analyses in acromegaly, Cushing's disease, and prolactinomas.

By pursuing these aims, the study intended to expand current knowledge on the mutational landscape of PitNETs and to explore potential links between genetic alterations and clinical behavior, paving the way for future research into personalized diagnostics and therapies that could improve patient care.

3. Materials and Methods

3.1. Tumor samples

A total of 153 samples preserved as formalin-fixed, paraffin-embedded (FFPE) tissue blocks were obtained from the Pathology Department. The tumor series includes multiple subtypes, such as somatotroph (n=101), corticotroph (n=32), lactotroph (n=17), silent PitNETs (n=2), and pluri-hormonal tumors (n=1). All samples were anonymized and were accompanied by relevant clinical data, including patient sex, age, tumor size, invasiveness, hormone expression profile, and others. Ethical approval for the use of human material was obtained from the appropriate institutional review board, and all procedures were conducted in accordance with the Declaration of Helsinki.

3.2. DNA extraction

DNA was extracted from 10 µm-thick sections of FFPE tissue. The sections were first deparaffinized using xylene and subsequently rehydrated through a graded ethanol series. The tissue was then carefully collected using a scalpel and transferred into a microcentrifuge tube for further processing. DNA extraction was performed by using the Maxwell® RSC DNA FFPE Kit, following the manufacturer's instructions. DNA purity and concentration were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific), and DNA concentration, A260/A280 ratio, and A260/A230 were determined.

3.3. Polymerase Chain Reaction (PCR)

Targeted regions of candidate genes known to be frequently mutated in PitNETs, including *GNAS* (for somatotroph tumors), *USP8* (for corticotroph tumors), *SF3B1* (for lactotroph tumors), and *BRAF* (for all tumors) were amplified by PCR using gene-specific primers listed in Table 2. Primers were designed using NCBI Primer-BLAST and synthesized by Integrated DNA Technologies. Each reaction was performed in a total volume of 25 µL, containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 1 U of Taq DNA polymerase (Thermo Fisher Scientific), and 100 ng of genomic DNA. SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific) was used and, for *BRAF* thermal cycling conditions consisted of an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 20 seconds, with a final extension step at 72°C for 1 minute. For *GNAS*, *USP8* and *SF3B1* thermal cycling conditions were an initial denaturation cycle at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C

for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension step at 72°C for 10 minutes.

Table 2. Primer sequences for PCR amplification of candidate genes in PitNETs

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon size (bp)
GNAS8	TGTTTCGGTTGGCTTTGGTG	GGGACTGGGGTGAATGTCAA	186
GNAS9	ACATTCACCCCAGTCCCTCT	AGCGACCCTGATCCCTAACA	179
USP8			
F1	GCAGAATACTTTGGAGTGATTTCTT	TGAAGGAGCCATTTCCGGTG	172
F2	TTGACCCAATCACTGGAACCT	GAGGAGTAGGAGCGCTTCAG	181
F3	CTTCATCTGCACCTCCTTCC	TCAGAGTAGAAACTTTGAAATACAGCA	243
SF3B1			
F1	ACCAACTCATGACTGTCCTTTCT	GCACACAGCTTTTAAGAAGGGC	188
F2	CTTCATCTGCACCTCCTTCC	AGATGGCACAGCCCATAAGAA	232
F3	AGAAGTCCTGGCAAGCGAGA	AAACATGGACAGGCTGTGTGT	273
BRAF	TTCCTTTACTTACTACACCTCAG	CATCCACAAAATGGATCCAGAC	133

Primer sequences are presented in the 5′→3′ orientation. F1, F2, and F3 indicate fragments 1, 2, and 3 of the respective gene. Amplicon size is indicated in base pairs (bp).

3.4. PCR product validation and purification

Before purification, the integrity and specificity of the PCR products were verified by agarose gel electrophoresis. Amplicons were loaded on a 1.0% agarose gel, samples were then mixed with a nucleic acid dye (GelRed™) for staining and visualized under UV light to confirm the presence of a single band of the expected size and the absence of non-specific amplification.

To monitor for potential contamination, two negative controls (blanks) were included in each PCR batch: one at the beginning and one at the end of the sample plate. These blanks contained all PCR reagents except for DNA template and were used to detect possible reagent contamination and cross-contamination during sample handling. Only PCR reactions with clean blank controls and well-defined target bands proceeded to the purification step.

PCR products were purified to remove unincorporated primers and dNTPs prior to sequencing. For most samples, purification was performed using the ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems), following the manufacturer's instructions. Briefly, 10 µL of PCR product was treated with 2 µL of ExoSAP-IT™ and

incubated at 37°C for 30 minutes, followed by enzyme inactivation at 85°C for 15 minutes in the SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific).

For the last 60 samples in the series, purification was conducted using NZYtech ExoSAP (NZYTech, Portugal), based on exonuclease I and shrimp alkaline phosphatase digestion. Incubation was carried out at 37°C for 2 minutes followed by inactivation at 80°C for 1 minute, according to the manufacturer's protocol using the same machine as before. Both purification methods produced high-quality amplicons suitable for downstream Sanger sequencing.

3.5. Sanger sequencing

Purified PCR products were subjected to Sanger sequencing. The sequencing reaction mixture consisted of 0.4 µL of BigDye™ Terminator v3.1, 3.4 µL of 5× sequencing buffer, 3.5 µL of nuclease-free water, and 0.3 µL of the appropriate primer, per sample. Forward primers were used for sequencing *BRAF* and *SF3B1* amplicons, while reverse primers were used for *GNAS* and *USP8*.

The sequencing reactions were carried out in a SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 2 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 55 °C for 15 seconds, and extension at 60 °C for 3 minutes. A final extension step was performed at 60 °C for 10 minutes.

After amplification in the thermal cycler, the sequencing reaction products were purified using Sephadex™ column-based gel filtration to remove unincorporated dye terminators and primers. Initially, column preparation involved a washing step in which the columns were centrifuged with distilled water for 1 minute at 14,000 rpm using a Sorvall™ Legend Micro 21R centrifuge (Thermo Fisher Scientific). Subsequently, Sephadex matrix was added to the columns, which were then centrifuged for 4 minutes at 3,200 rpm to compact the gel and form the columns. The sequencing reaction mixture was then carefully pipetted onto the center of each column and centrifuged for an additional 4 minutes at 3,200 rpm. After this step, the columns were discarded. Finally, 15 µL of formamide was added to each purified sample under a laminar flow hood to denature the DNA prior to capillary electrophoresis and chromatogram generation.

The purified sequencing products were taken to the Sanger sequencing lab where they loaded into 3500xL Dx Genetic Analyzer (Applied Biosystems™), an automated capillary electrophoresis system, which separates DNA fragments based on size and fluorescently labeled terminators. The resulting electropherograms were

analyzed using dedicated software, enabling the identification of nucleotide sequences and the detection of point mutations within the target gene regions

3.6. Data analysis and genotype-phenotype correlation

Sequences were analyzed using Benchling [Biology Software] retrieved from https://benchling.com.

Statistical analyses were performed using IBM® SPSS® Statistics, version 30.0.0.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were calculated for all clinical and pathological variables. Continuous data were expressed as mean ± standard deviation (SD) when normally distributed, or as median with interquartile range (IQR) when not normally distributed. Categorical data were presented as absolute numbers and percentages.

Normality of continuous variables (age at diagnosis and number of surgeries) was tested using the Shapiro-Wilk test. Comparisons between wild-type and mutated cases were carried out with the independent samples t-test for normally distributed data and with the Mann-Whitney U test for non-normally distributed data.

Associations between mutation status and categorical variables (sex, hormone secretion profile, Ki-67 index, remission at one year, radiotherapy, number of surgeries, endocrine insufficiencies, and tumor subtype when available) were assessed using Pearson's Chi-square test. When expected cell counts were <5 or when variables contained more than two categories, Fisher's exact test (including the Freeman-Halton extension when appropriate) was applied.

Ki-67 was analyzed both as a continuous variable and after categorization using a clinically relevant threshold of ≥3% vs <3%. Analyses were conducted for the full cohort as well as within disease-specific subgroups: acromegaly (somatotroph adenomas), Cushing's disease (corticotroph adenomas), and prolactinomas (lactotroph adenomas).

Missing values were coded and excluded pairwise from each analysis; therefore, the number of valid cases varied across variables. A two-sided p-value<0.05 was considered statistically significant.

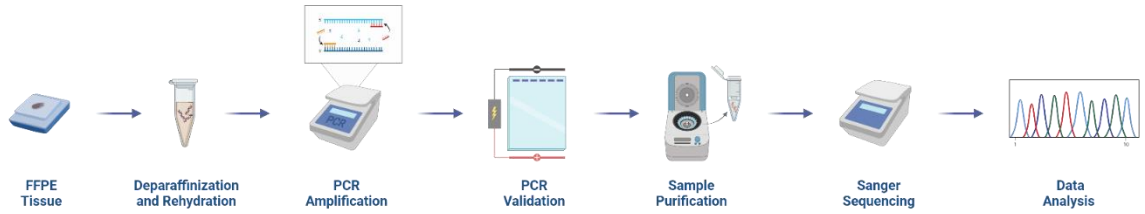


***Figure 2**. Workflow of molecular analysis.*

Overview of the experimental pipeline from FFPE tissue processing through PCR amplification, product validation, purification, and Sanger sequencing, culminating in data analysis for mutation detection. Created in https://BioRender.com

4. Results

4.1. Patient characteristics

A total of 153 patients were included in the study cohort. The mean age at diagnosis was 44.5 ± 14.4 years, with a range from young adulthood to late adulthood, and 65.1% of the patients were male. The median number of surgeries performed per patient was 1 [1–2]. With respect to hormonal secretion, GH-secreting tumors were the most frequent (49.3%), followed by ACTH-secreting tumors (19.6%) and PRL-secreting tumors (12.2%). A smaller proportion of cases showed mixed GH and PRL secretion (12.8%), while non-functioning tumors (4.7%) and PRL+TSH–secreting tumors (1.3%) were uncommon.

Tumor subtype classification was available for 72 cases. Among these, somatotroph adenomas represented the majority, including sparsely granulated (26.4%), densely granulated (23.6%), and somato-lactotroph adenomas (23.6%). Plurihormonal adenomas (6.9%), Pit-1 positive plurihormonal adenomas (2.8%), and null cell adenomas (1.4%) were rare. Radiotherapy had been administered in 12.3% of patients, and remission at 1 year was observed in 39.0%. Baseline endocrine insufficiencies were present in 45.9% of the cohort. Patient characteristics are summarized in Table 3.

Table 3. Patient characteristics of the study cohort

Variable	Category	n (%) or Mean ± SD / Median [IQR]
Age at diagnosis (years)		44.5 ± 14.4
Number of surgeries		1 [1–2]
Sex	Male	97 (65.1)
	Female	52 (34.9)
Hormone secretion pattern	Non-functioning	7 (4.7)
	GH	73 (49.3)
	GH + PRL	19 (12.8)
	ACTH	29 (19.6)
	PRL	18 (12.2)
	PRL + TSH	2 (1.3)
Tumor subtype	Null cell adenoma	1 (1.4)
	Plurihormonal adenoma	5 (6.9)
	Plurihormonal Pit-1+ adenoma	2 (2.8)
	Somatotroph adenoma	9 (12.5)
	Mixed somato-lactotroph adenoma	17 (23.6)
	Densely granulated somatotroph adenoma	17 (23.6)
	Sparsely granulated somatotroph adenoma	19 (26.4)
Radiotherapy	Yes	18 (12.3)
	No	128 (87.7)
Remission at 1 year	Yes	55 (39.0)
	No	86 (61.0)
Current insufficiencies	Yes	68 (45.9)
	No	80 (54.1)

Values are presented as mean ± standard deviation (SD), median [interquartile range, IQR], or number (percentage). Percentages are based on the total cohort of 153 patients unless otherwise specified. Missing values reflect unavailable clinical data. Abbreviations: GH, growth hormone; PRL, prolactin; ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone. Numbers may vary across variables due to missing values.

4.2. Genetic findings: *GNAS* and *USP8* mutations

4.2.1 *GNAS* mutations

Sequencing of *GNAS* exons 8 and 9 revealed several variants. The canonical hotspot mutations p.R201C and p.R201H were detected in multiple somatotroph tumors. Both substitutions affect codon 201 within the GTP-binding domain of the Gαs subunit, abolishing intrinsic GTPase activity and leading to constitutive adenylate cyclase stimulation with persistent cAMP/PKA signaling. These variants are well established in the literature as oncogenic drivers and are classified as pathogenic.

A synonymous variant, p.Q241Q, was also identified. Because this change does not alter the amino acid sequence and has not been linked to splicing alterations, it is considered a likely benign finding. However, no records of this variant were found in public databases; suggesting that it has not been previously reported in the literature.

In exon 9, additional variants were observed. p.Q227R is a missense substitution within the GTP-binding domain, previously described in endocrine tumors and generally regarded as likely pathogenic. Another variant, p.G225D, was not found in public databases; Franklin (Genoox) classifies it as likely pathogenic based on in silico predictions, although no functional data are available, so it remains a novel variant of uncertain significance (VUS). p.R228Q is another unreported missense change located in the G3 box of the GTP-binding domain. Because this residue is part of a critical structural motif, an effect on GTP binding or hydrolysis is possible, but in the absence of experimental evidence it should be regarded as a novel VUS. Finally, the frameshift variant p.(Q227HfsTer4) introduces a premature stop codon predicted to trigger nonsense-mediated decay or yield a truncated protein. Unlike the classic activating missense mutations, this alteration would be expected to result in loss of function. However, no reports of this variant were identified in public databases therefore, it was classified as a variant of uncertain significance (VUS), but it leads to a protein truncation.

4.2.2 *USP8* mutations

Several variants were identified in *USP8*: missense mutations clustered within or near the 14-3-3 binding motif.

The variant p.P720R, located within the 14-3-3 binding motif, directly disrupts the interaction with 14-3-3 proteins. This loss of regulation increases *USP8* deubiquitinase activity, stabilizes EGFR, and enhances downstream MAPK/ERK signaling, ultimately

promoting ACTH secretion. Variants at this site are recurrent and are regarded as pathogenic or likely pathogenic.

Two further substitutions, p.S718F and p.S719F, alter adjacent residues that are essential for 14-3-3 recognition. Functional studies have shown that S718F leads to increased *USP8* activity compared with the wild-type protein. Although S719F has not been as extensively investigated, its location within the same critical motif and its similarity to other pathogenic variants strongly suggest the same effect. Both are therefore considered pathogenic or likely pathogenic in the context of corticotroph tumors. An additional p.T739A substitution, which is classified as benign in ClinVar and is considered a likely polymorphism, was detected in 12 cases.

The identified *GNAS* and *USP8* variants are summarized in Table 4.

Table 4. Summary of *GNAS* and *USP8* variants identified in the study cohort

Mutation	Number of Patients	Codon	Domain	Clinical Significance
GNAS p.R201C	17	CGT> TGT	GTP-binding domain	Pathogenic
GNAS p.R201H	1	CGT> CAT	GTP-binding domain	Pathogenic
GNAS p.Q241Q	1	CAG>CAA	Outside hotspot	Likely benign
GNAS p.Q227R	3	CAG>CGG	GTP-binding domain	Likely pathogenic
GNAS p.G225D	1	GGT>GAT	Near GTP-binding domain	Likely pathogenic (in silico, Franklin) / VUS
GNAS p.R228Q	1	CGG>CAG	GTP-binding domain (G3 box)	VUS (novel)
GNAS p.(Q227HfsTer4)	1	Frameshift	Exon 9	VUS (novel)
USP8 p.T739A	12	ACA>GCA	Adjacent to 14-3-3 binding motif	Benign/Polimorphism (ClinVar)
USP8 p.P720R	3	CCA>CGA	14-3-3 binding motif	Pathogenic
USP8 p.S718F	1	TCC>TTC	14-3-3 binding motif	Pathogenic
USP8 p.S719F	1	TCC>TTC	14-3-3 binding motif	Likely pathogenic
USP8 p.T739A + p.P720R	1	p.T739A: ACA>GCA; p.P720R: CCA>CGA	p.T739A: adjacent to 14-3-3 motif; p.P720R: within 14-3-3 motif	p.T739A: Benign (ClinVar); p.P720R: Pathogenic / Likely pathogenic

Abbreviations: VUS, variant of uncertain significance. Number of patients refers to cases in the present cohort carrying the indicated variant. Codon annotations are based on NCBI RefSeq transcripts NM_000516.7 (*GNAS*) and NM_005154.6 (*USP8*). Clinical significance was assigned according to ClinVar, Franklin (*in silico* prediction), and published literature as of September 2025. One tumor harbored two concurrent *USP8* variants (p.T739A and p.P720R), which are presented together in the table.

4.3. Acromegaly

A total of 101 patients with acromegaly were included in this analysis, comprising 76 with *GNAS* wild-type (WT) tumors and 25 with *GNAS*-mutated (MUT) tumors, comprising a 24.8% prevalence. Somatotroph tumors were the most frequent subtype in both WT and MUT tumors. The average age at diagnosis was similar between groups (44.21 ± 14.05 years in WT vs. 46.91 ± 15.19 years in MUT; p=0.456). The number of surgeries was also comparable, with a median of one procedure in both groups (IQR 1-2).

Regarding categorical variables, there were no significant differences in tumor subtype (p=0.676), sex (p=0.545), Ki-67 index (p=0.379), current insufficiencies (p=0.105), or hormone secretion (p=1.000). For remission at 1 year, Pearson's chi-square showed a difference (p=0.038). Radiotherapy also showed a borderline association (p=0.107). The comparison of clinical and pathological characteristics between WT and MUT acromegaly cases is presented in Table 5.

Table 5. Clinical and pathological characteristics of acromegaly cases according to *GNAS* mutation status

Variable	WT (n=76)	MUT (n=25)	p-value
Age at diagnosis, mean ± SD	44.21 ± 14.05	46.91 ± 15.19	0.456
Number of surgeries, median (IQR)	1 (1-2)	1 (1-2)	1.000
Sex (M/F)	50/25	15/10	0.545
Ki-67 (≥3%/<3%)	11/57	2/19	0.379
Radiotherapy (Yes/No)	13/59	1/24	0.107
Current insufficiencies (Yes/No)	36/39	7/17	0.105
Remission at 1 year (Yes/No)	23/49	6/14	0.038*
Hormone secretion pattern	GH=54; GH+PRL=14; others rare	GH=19; GH+PRL=5; others rare	1.000

Abbreviations: WT, wild-type; MUT, mutated; M/F Male/Female. Values are presented as mean ± SD for normally distributed variables, median (IQR) for non-normally distributed variables, and absolute numbers for categorical variables. p-values were obtained using independent samples t-test, Mann-Whitney U test, Chi-square test, or Fisher's exact test, as appropriate. Numbers may vary slightly across variables due to missing data. *p<0.05 was considered statistically significant.

4.4. Cushing´s Disease

A total of 32 patients with Cushing's disease were included in this analysis, comprising 26 with *USP8* WT tumors and 6 with *USP8*-MUT tumors, corresponding to 18.8% prevalence. The median age at diagnosis was 43 years (IQR 30.5-55.5) in the

WT group and 59 years in the MUT group (IQR not available, as only one case had valid data) with the difference between groups not being statistically significant (p=0.444). The median number of surgeries was also similar between groups, with 2 (IQR 2-3) in WT and 1 (IQR 1-2) in MUT (p=0.416).

No significant differences were observed between WT and MUT cases in sex distribution (p=0.556), Ki-67 index (p=0.155), radiotherapy (p=0.580), current insufficiencies (p=0.157), or remission at one year (p=1.000). Hormone secretion patterns were also similar across groups (p=1.000). The comparison of clinical and pathological characteristics between WT and MUT Cushing´s disease cases is presented in Table 6.

Table 6. Clinical and pathological characteristics of Cushing´s Disease cases according to *USP8* mutation status

Variable	WT (n=26)	MUT (n=6)	p-value
Age at diagnosis, median (IQR)	43 (30.5-55.5)	59 (NA)	0.444
Number of surgeries, median (IQR)	2 (2-3)	1 (1-2)	0.416
Sex (M/F)	20/5	5/0	0.556
Ki-67 (≥3%/<3%)	2/19	2/3	0.155
Radiotherapy (Yes/No)	2/21	0/5	0.580
Current insufficiencies (Yes/No)	15/10	1/4	0.157
Remission at 1 year (Yes/No)	15/10	3/2	1.000
Hormone secretion pattern	ACTH=20; PRL=5	ACTH=5; PRL=1	1.000

Abbreviations: WT, wild-type; MUT, mutated; NA, not available; M/F, Male/Female. Values are presented as median (IQR) for continuous variables and as absolute numbers for categorical variables. P-values were derived using Mann-Whitney U test for continuous variables. Categorical variables were analyzed using Fisher's exact test (Freeman-Halton extension when >2 categories) due to small sample sizes and expected cell counts <5. For age at diagnosis in the USP8_MUT group, data were available for only one patient; therefore, the IQR could not be calculated and results should be interpreted with caution. Numbers may vary slightly across variables due to missing data.

4.5. Prolactinoma

A total of 17 patients with prolactinomas were included in this analysis, all of which were wild-type for *SF3B1*.

4.6. *BRAF*

All 153 tumor samples in the cohort were analyzed for hotspot mutations in *BRAF*, but no mutated cases were identified, and therefore, no further genotype-phenotype correlations could be established for this gene.

5. Discussion

In this study, the mutational status of selected candidate genes across different pituitary neuroendocrine tumor subtypes was investigated. The aim was to evaluate the prevalence of known pathogenic variants and to explore possible associations with clinical or pathological features. Because previous work has shown that the distribution of genetic drivers varies considerably between functional tumor types, the analysis focused on *GNAS* and *BRAF* in somatotroph tumors, *USP8* and *BRAF* in corticotroph tumors, and *SF3B1* and *BRAF* in lactotroph tumors.

5.1. Molecular profile of mutations identified

5.1.1 *GNAS* mutations

Most alterations involved the canonical hotspot codon 201, with p.R201C and p.R201H detected in somatotroph tumors. Both substitutions abolish the intrinsic GTPase activity of the Gαs subunit, locking the protein in an active state (40). Similarly, the p.Q227R mutation, which also lies within the GTP-binding domain, inhibits GTP hydrolysis and maintains Gαs in its constitutively active GTP-bound form (40). These alterations result in persistent stimulation of adenylate cyclase and continuous activation of the cAMP/PKA pathway, which drives hormone hypersecretion and tumorigenesis (40).

The frameshift mutation p.(Q227HfsTer4) shifts the reading frame and introduces a premature stop codon only four amino acids downstream. Frameshift mutations of this type usually have severe consequences for protein integrity, as they alter the downstream amino acid sequence and commonly generate unstable transcripts (72). Codon 227 lies within the GTP-binding domain of Gαs, and a truncation at this site would abolish motifs that are required for GTP binding, hydrolysis, and interaction with downstream effectors (73). This mechanism differs fundamentally from the well-established activating mutations at codon 201, which abolish GTPase activity and lead to constitutive stimulation of adenylate cyclase and persistent cAMP/PKA signaling (40). Instead, the predicted effect of p.(Q227HfsTer4) would be consistent with loss of function.

Two unreported substitutions, p.G225D and p.R228Q, were also detected and, although their functional impact is unknown, their location within the conserved G3 box raises the possibility of impaired GTP binding or hydrolysis (73, 74). In fact, using the *in silico* Franklin tool (for evaluations of biological impact of the genetic variants) these

previously unreported variants are classified as likely pathogenic/ VUS. Additional functional studies are needed to fully characterize those novel variants described in our study.

The synonymous variant p.Q241Q does not change the encoded amino acid, as glutamine is preserved in the protein sequence. Because the amino acid composition remains unaltered, synonymous substitutions are generally assumed not to impact protein structure or function. In variant databases such as ClinVar, synonymous changes are typically classified as likely benign unless there is supporting evidence that they interfere with splicing signals or regulatory motifs. For p.Q241Q specifically, no published reports have linked this variant to disease, nor has any functional effect been demonstrated. Although some synonymous variants can be pathogenic if they disrupt exonic splicing enhancers or silencers, no evidence from the literature or prediction tools suggests that p.Q241Q modifies the splicing of *GNAS*. On this basis, the variant is most appropriately regarded as a likely benign finding.

5.1.2 *USP8* mutations

USP8 activity is usually regulated through its interaction with 14-3-3 proteins at a specific binding site, which suppresses its deubiquitinase function and prevents uncontrolled signaling (75-77). When mutations such as p.S718F and p.P720R occur, this interaction is disrupted, thereby removing the inhibitory control (75). Functional studies have demonstrated that the loss of 14-3-3 binding renders *USP8* more susceptible to proteolytic cleavage, and this process generates a fragment with higher enzymatic activity than the full-length protein (75). As a consequence, EGFR is less efficiently degraded and instead becomes stabilized, which prolongs receptor signaling at the cell surface (75). The sustained activation of EGFR then drives persistent MAPK/ERK pathway signaling, ultimately leading to increased ACTH secretion in corticotroph tumor cells and providing a clear molecular link between *USP8* mutations and the development of Cushing's disease (75-77). In addition, a p.T739A substitution was identified. This variant lies adjacent to the regulatory motif, outside of the hotpsot (78). This variant (Thr739Ala) is classified as benign in ClinVar and is relatively common in the general population, with allele frequencies ranging from approximately 11% to 17% across databases like 1000 Genomes, gnomAD, ESP, TOPMed, and ExAC. Such high prevalence strongly supports its classification as a benign polymorphism, rather than a pathogenic mutation in the context of Cushing's disease.

5.2. Somatotroph PitNETs (acromegaly)

In somatotroph tumors, *GNAS* mutations were confirmed as a frequent recurrent alteration. In this cohort, mutations were identified in 24.8% of cases, a prevalence that falls a little below the range reported in the literature, where frequencies of up to 40% are consistently described (40) (42). The well-established p.R201C and p.R201H substitutions represented the majority of events, in agreement with their established role as oncogenic drivers of cAMP/PKA signaling (41) (42) (79). Less common and novel variants were also detected, including p.Q227R, p.G225D, p.R228Q, and p.(Q227HfsTer4).

Despite the relatively high prevalence of *GNAS* alterations, the clinical impact of these mutations remains uncertain. In the present series, no significant differences in age at diagnosis, number of surgeries, tumor subtype, sex, Ki-67 index, hormone secretion, or current insufficiencies were observed between WT and MUT cases. However, some studies have reported associations with smaller tumor size (40, 41). Other work has suggested sex-specific differences, with male patients carrying *GNAS* mutations showing higher baseline GH levels, and female patients displaying smaller tumor diameters and longer diagnostic delays (42). However, some studies show no significant difference between mutated and non-mutated groups in remission, size, GH-secretion, or age (42, 80-83).

In this cohort, remission at one year and the use of radiotherapy showed differences between wild-type and mutated cases, but only remission at one year reached statistical significance. Specifically, patients harboring *GNAS* mutations exhibited a lower remission rate compared with WT cases. Some studies also report lower percentages of remission in MUT cases compared to WT cases, however, these differences did not reach statistical significance (41, 42).

Radiotherapy displayed a difference between groups, with a higher proportion of mutated cases undergoing this treatment. However, the association did not reach statistical significance. Although this finding falls short of the conventional threshold for significance, it may reflect a trend worth noting, as it could suggest that patients with *GNAS*-mutated tumors are more likely to require adjuvant radiotherapy. No other clinicopathological variables demonstrated significant associations with *GNAS* mutation status. These findings suggest that GNAS mutations may influence the likelihood of achieving remission within the first year after surgery. However, given the limited number of mutated cases, these results should be interpreted with caution, and confirmation in larger cohorts will be necessary.

No *BRAF* mutations were detected in the somatotroph cohort. This is consistent with previous studies that reported either no mutations or very rare mutated cases in PitNETs (32, 53, 84). While activating *BRAF* mutations are well characterized in several other endocrine neoplasms, such as papillary thyroid carcinoma (52), current evidence suggests that they are not a recurrent feature of PitNETs (32, 53, 84).

5.3. Corticotroph PitNETs (Cushing´s disease)

In corticotroph tumors, *USP8* mutations were detected, including p.P720R, p.S718F, and p.S719F. In this cohort, *USP8* mutations accounted for 18.8.% of corticotroph adenomas, a prevalence that falls in the lower limit of the wide range previously reported, where values between 20% and 60% have been described depending on methodology and series (18, 32, 33, 75, 76, 85).

Comparison of clinical variables between *USP8* WT and MUT cases did not reveal significant differences in age at diagnosis, number of surgeries, sex distribution, Ki-67 index, radiotherapy, insufficiencies, hormone secretion, or remission at one year. Similar negative associations have been described in some studies (85), although other reports suggest that *USP8* mutations are linked to smaller tumors, and are more prevalent in younger and female patients (75, 76). The conflicting evidence across studies may reflect differences in patient cohorts, sample size, and statistical power, as well as variation in the clinical management of Cushing's disease.

No *BRAF* mutations were identified in this cohort, although isolated reports have described a low prevalence of such alterations in corticotroph tumors (55).

5.4. Prolactinomas

In prolactinomas, *SF3B1* and *BRAF* were analyzed, and no mutations were identified in both genes. *SF3B1* mutations are well established in other neoplasms, particularly uveal melanoma and myelodysplasia (86, 87). *SF3B1* mutations have recently been reported as recurrent events in prolactinomas, with a prevalence ranging from approximately 2% to 20% depending on the series (50, 51). These alterations are more common in male patients and have been associated with higher prolactin levels and shorter progression-free survival, suggesting a potential role in more aggressive disease behavior (51). *BRAF* mutations, in contrast, remain very rare and have not been consistently demonstrated in prolactinomas.

The absence of *SF3B1* and *BRAF* variants in this series may be explained by the relatively small number of lactotroph tumors analyzed, which limits the ability to capture low-frequency events.

Taken together, the findings from this cohort support the view that PitNETs are characterized by subtype-specific genetic alterations, but that the presence of these mutations does not translate into consistent clinical or pathological implications. The heterogeneity observed suggests that additional molecular mechanisms beyond single-gene mutations are likely to drive tumor behavior, highlighting the need for integrative studies that combine genomic, epigenomic, and clinical data.

5.5. Limitations and future directions

This study has several limitations that should be considered. The number of cases in some subgroups was small, particularly for prolactinomas, which reduces the statistical power for comparisons. The use of FFPE tissue also raises concerns about DNA quality, which can affect sequencing accuracy. In addition, only selected exons of a few candidate genes were analyzed, so other relevant alterations may have gone undetected. Information on tumor subtype was incomplete in some groups, especially in corticotroph adenomas, which restricted clinicopathological correlations. Another limitation is the absence of functional assays to clarify the biological effect of the novel variants detected.

5.6. Concluding Remarks

The genetics of PitNETs, is still poorly characterized, and there is still a lack of robust prognostic biomarkers. In this cohort, mutation status was shown to have an impact on clinical outcomes, with significant differences observed in remission at one year, which highlights the potential value of genetic profiling not only for understanding tumor biology, but also for anticipating treatment response. Nevertheless, the evidence remains limited by cohort size, and further studies are needed to determine whether these associations hold true across different populations and tumor subtypes, especially given that some studies have reported no clear differences in remission according to mutation status.

Future work should ideally involve larger, multi-center cohorts and broader sequencing approaches, such as whole-exome or whole-genome analysis, combined with transcriptomic and epigenetic data. These strategies could provide a more complete

picture of the molecular basis of PitNETs and help to determine the significance of rare or previously unreported variants. In the longer term, such efforts may support the development of improved molecular classifications and the identification of biomarkers with prognostic or therapeutic relevance.

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