UNIVERSIDADE FEDERAL DE MINAS GERAIS

Graduate Program in Molecular Medicine

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# WHOLE-EXOME SEQUENCING IDENTIFIES *RXRG* AND *TH* GERMLINE VARIANTS IN FAMILIAL ISOLATED PROLACTINOMA

Belo Horizonte

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Doctoral thesis submitted to the Graduate Program in Molecular Medicine of Universidade Federal de Minas Gerais as partial requirement for obtaining the title of PhD in Molecular Medicine.

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To all my family, those of my blood and those I have conquered in life, specially to Guilherme, Ana, Fernanda, Gustavo, Aristides, Alice, Arthur and Pedro, source of support, comfort and strength, without whom this work would not be possible or so pleasurable.

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“Start by doing what's necessary; then do what's possible; and suddenly you are doing the impossible.”

Francis of Assisi

## ABSTRACT

Pituitary adenomas are common intracranial tumors that occur sporadically. In some rare cases this condition is identified in familial clusters and has no involvement with other endocrine tumors, a disorder identified as Familial Isolated Pituitary Adenoma (FIPA). FIPA development has been associated with genetic abnormalities, especially in *AIP* gene, where germline mutations have been reported in approximately 20% of cases. Mutations in the *MEN1* gene have been described in a subset of pituitary adenoma families, but with *bona fide* multiple endocrine neoplasia type 1 feature. Mutations in prolactin receptor (*PRLR*) have also been associated to pituitary adenoma in animal models. Thus, in most FIPA cases the exact genetic defect that lead to disease development remains unknown. Therefore, the aim of this work is to determine the genetic basis of FIPA in a Brazilian family. The studied family is composed of three siblings presented with isolated prolactin-secreting pituitary adenoma diagnosed through clinical, biochemical and imaging testing. Sanger sequencing was used to genotype candidate genes *AIP*, *MEN1* and *PRLR*. Further mutation screening was performed using whole-exome sequencing. *In silico* analysis and additional predictive algorithms were applied to prioritize likely pathogenic variants. No mutations in the coding and flanking intronic regions in the *MEN1*, *AIP* and *PRLR* genes were detected. Whole-exome sequencing revealed novel, predicted damaging, heterozygous variants in three different genes: *RXRG*, *REXO4* and *TH*. The *RXRG* and *TH* possibly pathogenic variants may be associated with isolated prolactinoma in the studied family and the possible contribution of these genes to additional FIPA families should be explored.


**Keywords:** *RXRG*, *TH*, *FIPA*, prolactinoma, *AIP*, *MEN1*, *PRLR*

## RESUMO

Adenomas hipofisários são tumores intracranianos comuns que ocorrem de forma esporádica. Em alguns casos raros, esta doença é identificada em grupos familiares e não tem envolvimento com outros tumores endócrinos, uma condição identificada como adenomas de hipófise familiais isolados (FIPA). O desenvolvimento de FIPA tem sido associado a anormalidades genéticas, especialmente no gene *AIP*, no qual mutações germinativas foram relatadas em aproximadamente 30% dos casos. Mutações no gene *MEN1* foram descritas em famílias com adenoma de hipófise, mas com fenótipo específico para neoplasia endócrina múltipla tipo 1. Mutações no receptor de prolactina (*PRLR*) também têm sido associadas ao desenvolvimento de adenoma hipofisário em modelos animais. Assim, na maioria dos casos de FIPA o defeito genético exato que leva ao desenvolvimento da doença permanece desconhecido. Portanto, o objetivo deste trabalho é determinar a base genética de FIPA em uma família brasileira. A família estudada é composta por três irmãos diagnosticados com adenoma hipofisário secretor de prolactina através de testes clínicos, bioquímicos e de imagem. O sequenciamento Sanger foi utilizado para genotipagem dos genes candidatos *AIP*, *MEN1* e *PRLR*. Além disso, uma pesquisa por novas mutações foi realizada utilizando-se a técnica de sequenciamento de todo exoma. Uma análise *in silico* e algoritmos de previsão foram aplicados para priorizar variantes provavelmente patogênicas. Nenhuma mutação foi identificada na região codificadora e reguladora de *MEN1*, *AIP* e *PRLR*. O exoma revelou variantes novas e provavelmente patogênicas em três genes diferentes: *RXRG*, *REXO4* e *TH*. As variantes em *RXRG* e *TH* podem estar associadas ao prolactinoma isolado na família estudada e a possível contribuição desses genes para outras famílias FIPA devem ser exploradas.

**Palavras-chave:** *RXRG*, *TH*, *FIPA*, prolactinoma, *AIP*, *MEN1*, *PRLR*

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# LIST OF ABREVIATIONS

| | |
|---|---|
| *a*-MpT | *a-methyl-p-tyrosine* |
| ACTH | Adrenocorticotrophic Hormone |
| AHR | Receptor aril hidrocarboneto |
| AIP | Aryl-hydrocarbon receptor-interacting protein |
| *AIP* | Aryl-hydrocarbon receptor-interacting protein gene |
| *CDKN1B* | Cyclin-dependent kinase inhibitor 1B gene |
| CRH | Corticotrophic Releasing Hormone |
| D2R | Dopaminergic receptor |
| FIPA | Familial isolated pituitary adenoma |
| FSH | Follicle-Stimulating Hormone |
| GH | Growth Hormone |
| GHRH | GH-Releasing Hormone |
| GnRH | Hypothalamic-derived Gonadotropin-Releasing Hormone |
| GPCRs | G Protein Coupled Receptors |
| IGF1 | Hepatic Insulin-like Growth Hormone |
| Kb | Kilobase |
| kDa | Kilodalton |
| LH | Luteinizing Hormone |
| MEN1 | Multiple endocrine neoplasia type 1 |
| *MEN1* | Multiple endocrine neoplasia type 1 gene |
| MEN4 | Multiple Endocrine Neoplasia Type 4 |
| NGS | Next Generation Sequencing |
| PIF | Prolactin inhibitory factors |

| | |
|---|---|
| PRF | Prolactin releasing factors |
| *PRKAR1A* | Protein kinase cAMP-dependent type I regulatory subunit alpha gene |
| PRL | Prolactin |
| PRLR | Prolactin receptor |
| *PTTG* | Pituitary tumor-transforming gene |
| REXO4 | REX4 Homolog, 3'-5' Exonuclease protein |
| *REXO4* | REX4 Homolog, 3'-5' Exonuclease gene |
| RXRG | Retinoid X Receptor, Gamma protein |
| *RXRG* | Retinoid X Receptor, Gamma gene |
| T3 | Tri-iodo-thyronine |
| TH | Tyrosine hydroxylase protein |
| *TH* | Tyrosine hydroxylase gene |
| TRH | Thyrotropin-Releasing Hormone |
| TSH | Thyrotrophin |
| *USP8* | Ubiquitin-specific protease 8 |

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# 1. INTRODUCTION

## 1.1. Anatomy and physiology of the anterior pituitary gland

The pituitary is a small endocrine organ located within the sphenoid bone depression, called *sella turcica*. Together with the hypothalamus the pituitary gland orchestrates diverse body functions, including growth, reproduction and metabolic homeostasis. Scientists and artists have explored the anatomy and function of the hypothalamus and pituitary gland since the 2nd century AD. The importance of the hypothalamic-pituitary region has even influenced the work of the Renaissance artist Michelangelo Buonarroti in the Sistine Chapel ceiling at the Vatican, Italy (Figure 1). In this painting the creation of man sets in an arrangement that represents the brain outline, including the hypothalamic-pituitary region, suggesting the main role of this structure to life maintenance (Lechan and Toni, 2013).

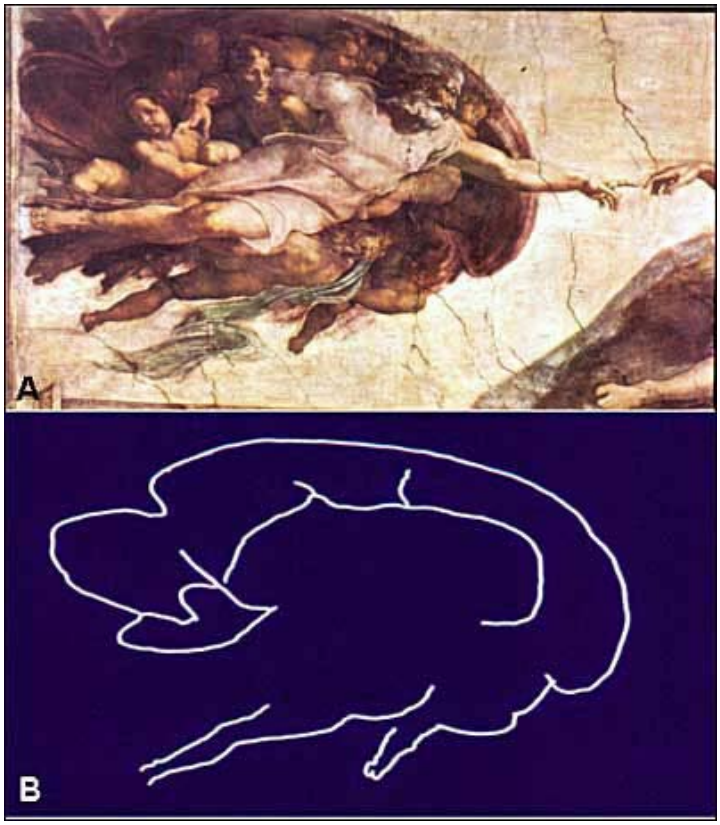


**Figure 1.** ***Creation of Adam*** **(Michelangelo Buonarroti, 1508-1512).** (a) Photograph of the ceiling of the Sistine Chapel at the Vatican, Italy; (b) The outline of the painting represents the midline sagittal section of the brain (From Toni *et al*., 2004. In: Lechan and Toni, 2013).

The adult pituitary weights about 600 mg and measures about 13 mm in the longest transverse diameter, 6 to 9 mm vertically, and around 9 mm anteroposteriorly (Melmed and Kleinberg, 2004). Dura mater surrounds the pituitary gland and form a roof over the *sella turcica* superiorly, such that the arachnoid membrane cannot enter the *sella*, thus pituitary gland maintains its anatomical and functional connections with the brain yet sits outside the blood-brain barrier (Nussey and Whitehead, 2001). The pituitary (Figure 2) is 5 mm beneath the optic chiasm, and positioned between the cavernous sinuses, that contain the internal carotid artery, oculomotor, trochlear and abducens cranial nerves, and also the first and second branches of the trigeminal nerve (Hong *et al*., 2016).

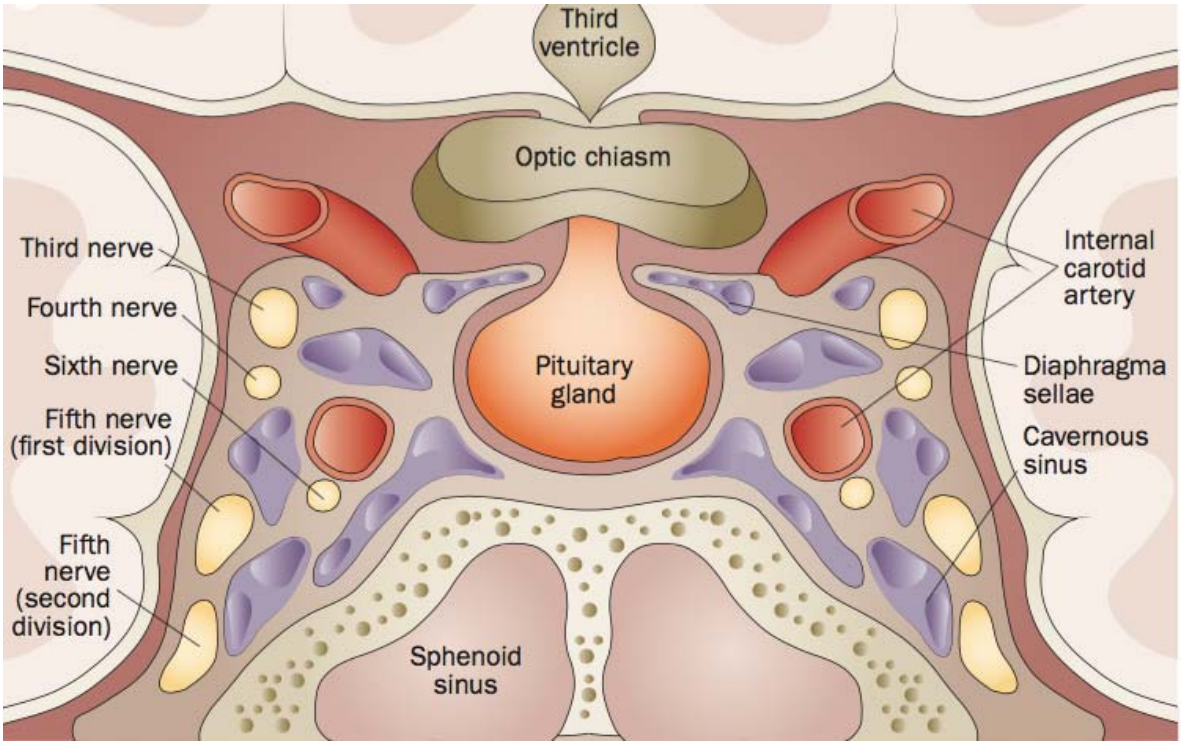


**Figure 2.** Normal anatomy of the sellar and parasellar regions surrounding the pituitary gland in a coronal view (Di Ieva *et al*., 2014).

The pituitary is attached directly to the median eminence of the hypothalamus and is composed of two morphologic and functional different components: the anterior lobe (adenohypophysis) and the posterior lobe (neurohypophysis) (Melmed and Kleinberg, 2004). The anterior pituitary gland derives from the invagination of the Rathke's pouch, a primitive ectodermal tissue (Treier and Rosenfeld, 1996). The anterior pituitary is divided in three parts: *pars intermedia*, *pars tuberalis* and *pars distalis* (Figure 3a). The *pars intermedia* is composed of epithelial cells from the posterior limb of Rathke's pouch, being rudimentary in humans. The *pars tuberalis* is a small rim of the adenohypophysis that involves the pituitary stalk. The bulk of the gland is the *pars distalis*, which represents 80% of the total pituitary volume (Asa and Ezzat, 2002; Drummond *et al*., 2003).

The adenohypophysis consists of five distinct types of differentially distributed hormone producing and secreting cells (Figure 3b). The functional development of these cell types involves complex spatiotemporal regulation of cell lineage-specific transcription factors expressed in pluripotential pituitary stem cells. The most frequent anterior pituitary cell line is the somatotroph, which comprises 45 to 50% of cells and produce growth hormone (GH). The lactotroph comprises between 9% of hormone-secreting anterior pituitary cells in males and nulliparous woman and up to 25% in multiparous females. These cells are specialized in prolactin (PRL) production. The corticotroph constitute 10 to 20% of anterior pituitary cells and produce adrenocorticotrophic hormone (ACTH). The gonadotrophic cells, 10 to 15% of cells, produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The thyrotrophic cells account for 5% of hormone-secreting anterior pituitary cells and produce thyrotrophin (TSH) (Asa and Ezzat, 2002; Drummond *et al*., 2003; Heaney and Melmed, 2004; Hong *et al*., 2016).

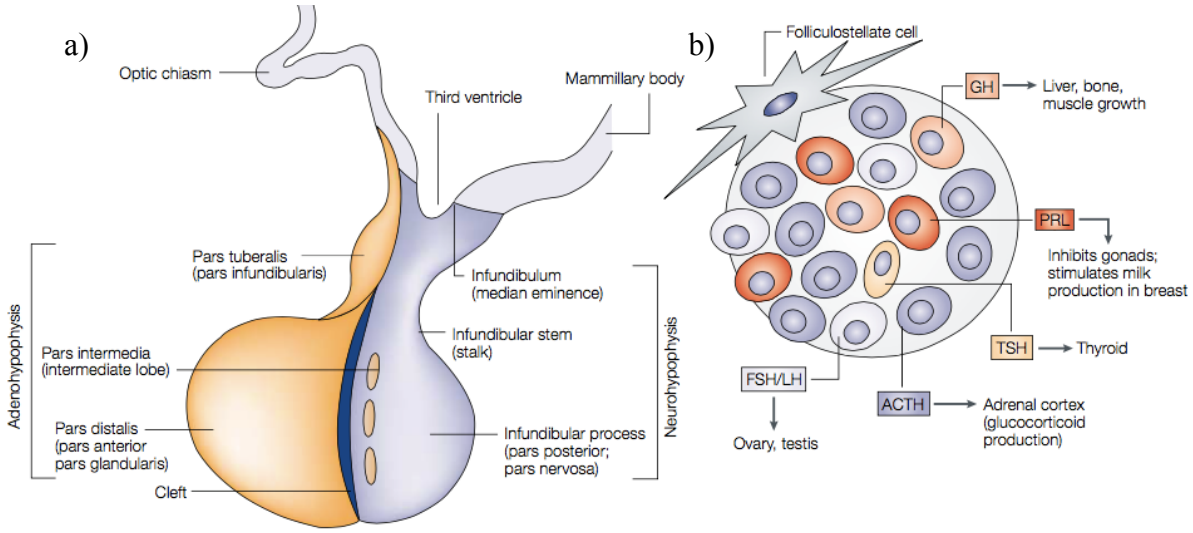


**Figure 3. Pituitary anatomy and cell types.** (a) Distinct parts of anterior pituitary; (b) Discrimination of pituitary cell types according to their hormone production (Asa and Ezzat, 2002).

Different levels of control regulate the anterior pituitary hormone secretion. Hypothalamic control is mediated by adenohypophysiotropic hormones ,which are secreted into the portal system and bind directly to the anterior pituitary cell surface G-protein coupled receptors. A second control system is based on peripheral hormones, which act through negative feedback regulation of trophic hormones and their respective target hormones. The third regulation occurs inside the pituitary, where paracrine and autocrine soluble growth factors and cytokines locally regulate neighboring cell development and function. In consequence, a controlled pulsatile secretion of the six trophic pituitary hormones, ACTH, GH, PRL, TSH, FSH and LH, is achieved through these different regulatory mechanisms (Asa and Ezzat, 2002; Hong *et al*., 2016).

The regulation of ACTH secretion through corticotrophic cells is taken by hypothalamic-derived corticotrophic releasing hormone (CRH) and inhibited by cortisol. ACTH target organ is the adrenal gland where it regulates steroid secretion, leading to glucose, sodium and water homeostasis (Figure 4a). GH-releasing hormone (GHRH) and ghrelin, both hypothalamic hormones, induce GH secretion. Somatostatin (hypothalamus),

hepatic insulin-like growth hormone (IGF1), thyroid hormone and glucocorticoids inhibit GH secretion. GH regulates bone and muscle growth and maintains lean growth in adults (Figure 4b). Thyrotropin-releasing hormone (TRH), from the hypothalamus, and estrogen stimulate PLR secretion. PRL is negatively regulated by dopamine, released by cells in the median eminence. PRL-receptor signaling prepares and maintain the breast for postpartum and lactation (Figure 4c). TSH is positively regulated by TRH. TSH regulates thyroidal iodine metabolism, thyroid-hormone synthesis and thyroid growth, leading to thermogenesis and protein synthesis control. Tri-iodo-thyronine (T3), regulate TSH and TRH synthesis, providing control of TSH-directed thyroid hormone action (Figure 4d). Hypothalamic-derived gonadotropin-releasing hormone (GnRH) stimulates FSH and LH (Figure 4e). These hormones regulate sex-steroid synthesis and secretion, also participating in germ-cell development (Heaney and Melmed, 2004).

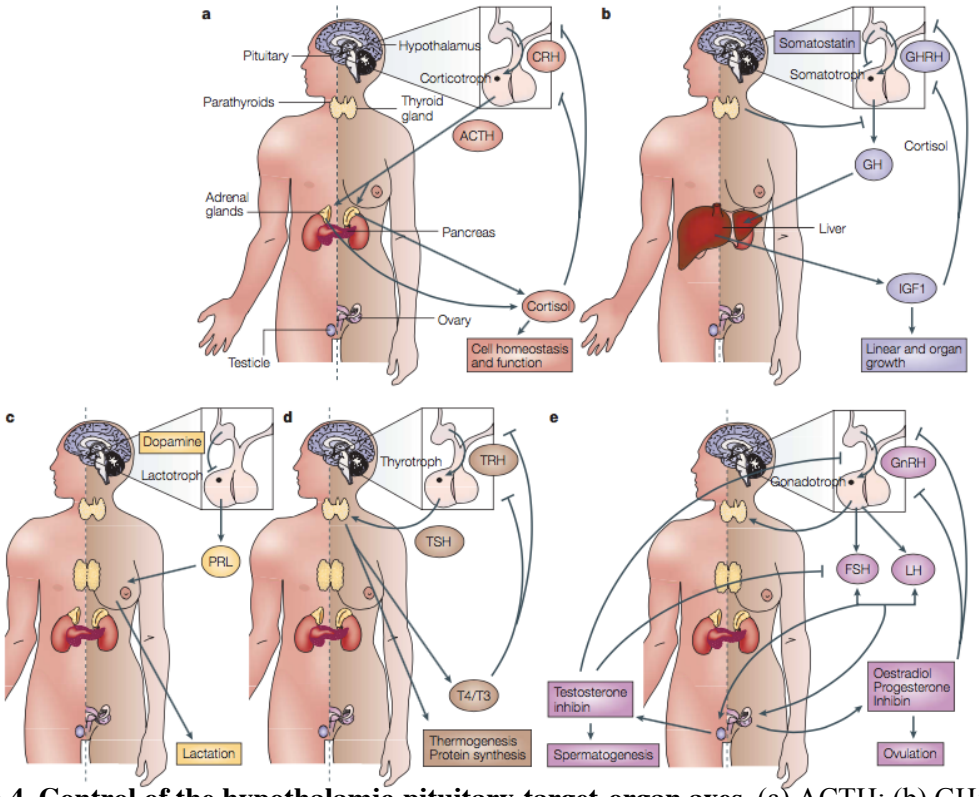


**Figure 4. Control of the hypothalamic-pituitary-target-organ axes.** (a) ACTH; (b) GH; (c) PRL; (d) TSH; (e) FSH and LH (Heaney and Melmed, 2004).

## 1.2. Prolactin regulation

Prolactin is a 23 kDa polypeptide hormone that plays multiple homeostatic roles in the organism and is vital to mammogenesis, lactogenesis and galactopoiesis. The main cells that synthetize and secrete PRL are the lactotrophs, located at the anterior pituitary. Other organs and tissue are as well capable of PRL production and secretion, but little is known about the function of these PRL-secreting tissues (Freeman *et al*., 2000; Bernard *et al*., 2015b).

Synthesis and secretion of PRL by the lactotrophs are under the control of multiple prolactin releasing factors (PRF) and prolactin inhibitory factors (PIF). Dopamine secreted by tuberoinfundibular hypothalamic neurons (TIDA) is the primary inhibitory regulator of prolactin (Freeman *et al*., 2000; Mancini *et al*., 2008). Dopamine suppresses PRL though activation of $D_2$ receptors. In fact, this is the physiologic basis for the therapeutic approach for hyperprolactinemia treatment, in which dopamine agonist is prescribed to in order to reduce PRL uncontrolled secretion (Neill, 1980).

Other PIF are somatostatin and gamma-aminobutyric acid. The major PRFs are TRH, oxytocin and neurotensin. These molecules are released into the long or short portal veins, as well across paracrine and autocrine mechanisms and target receptors in lactotrophic cells (Figure 5). Another regulating system is supported by PRL itself, which is capable of promoting feedback. PRL serum elevation activates PRL-receptors located at dopaminergic neurons and promote hypothalamic dopamine synthesis and increase the concentration of dopamine in the portal veins (Freeman *et al*., 2000; Mancini *et al*., 2008).

The causes of serum PRL elevation can be divided in physiologic, pharmacologic, or pathologic causes. Pregnancy and lactation are common cause of hyperprolactinemia. PRL levels increase after exercise, meals, stimulation of the chest wall, physical and

psychologic stress, treatment with dopamine receptor antagonist drugs and pituitary adenomas (Mancini *et al*., 2008; Vilar *et al*., 2008; Bernard *et al*., 2015b).

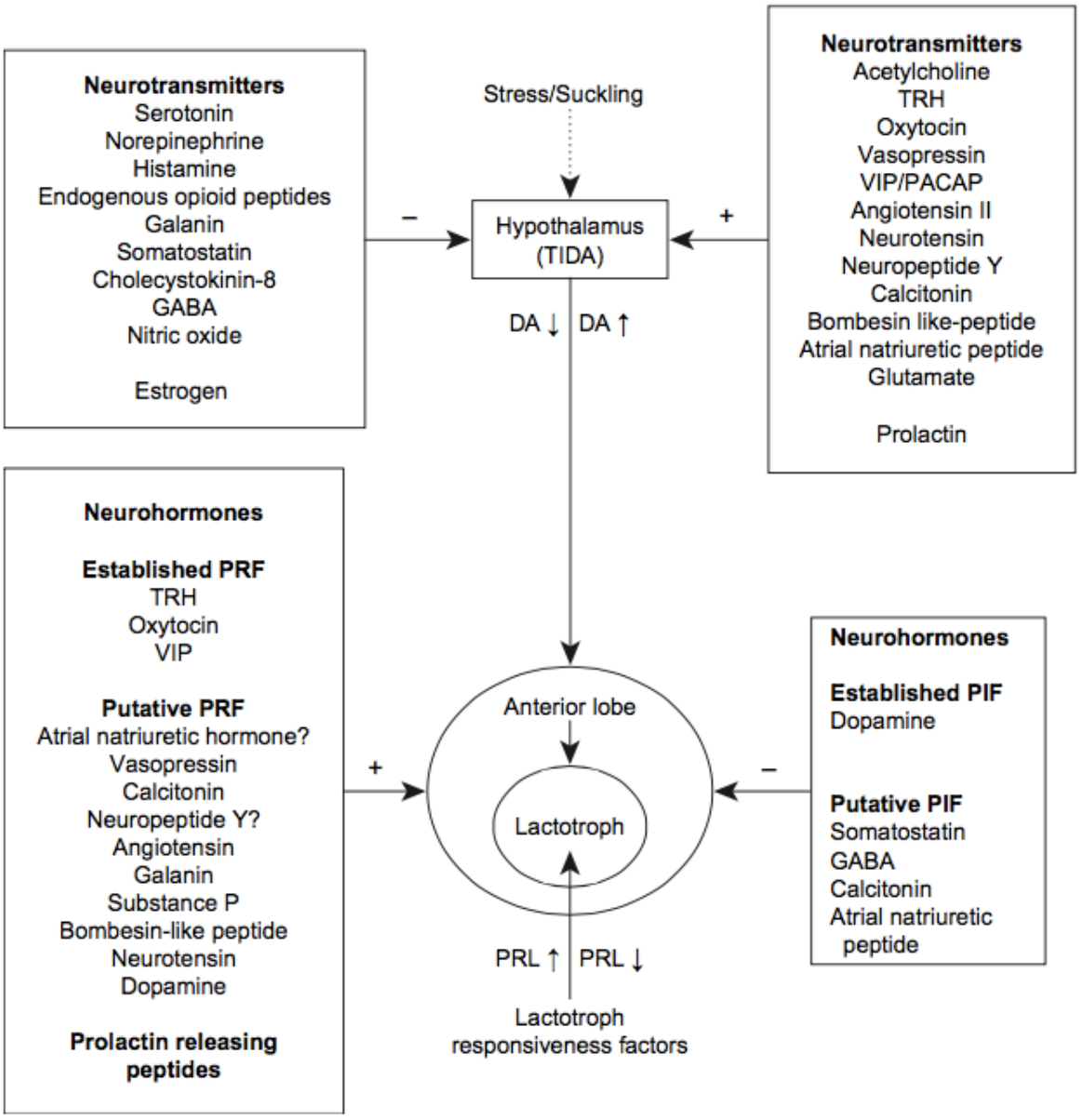


**Figure 5. Overview of PRL regulation.** Tuberoinfundibular dopaminergic system (TIDA) activity controlling PRL in response to diverse stimulus. PIF and PRF are secreted by neuroendocrine neurons and also regulate PRL secretion (Mancini *et al*., 2008).

## 1.3. Epidemiology of prolactinomas

Pituitary adenomas are monoclonal tumors, that is, the tumor arises from a single cell that has been transformed by genetic events that converted it into a neoplastic tissue. The transformations consist in acquisition of unique proliferative advantage and excessive proliferation of anterior pituitary hormone-producing cell lines (Asa and Ezzat, 2002; Melmed, 2011). Pituitary tumors rarely progress to become true metastatic carcinomas (Di Ieva *et al*., 2014). Despite exhibiting important growth, they present low mitotic activity when compared to other tumor types (Melmed, 2011).

Although not metastatic, these adenomas are associated to significant morbidity due to over-production of specific anterior pituitary hormones, leading to endocrine syndromes. Moreover, pituitary adenomas may promote a local space occupying effect. Prolactinomas arise from lactotrophic cells and secrete prolactin, causing symptoms such as hypogonadism, galactorrhea and bi-temporal hemianopsia (Asa and Ezzat, 2002; Heaney and Melmed, 2004; Hong *et al*., 2016).

European population-based studies report a pituitary adenoma prevalence of one per 1,277 individuals. These tumors account for 15% of all intracranial neoplasms, being the third most frequent tumor type after meningiomas and gliomas. The main pituitary adenomas are prolactinomas, which represent 50% of all cases on average (Aflorei and Korbonits, 2014).

Prolactinomas can be classified by their size as micro and macroprolactinomas. Microadenomas are less than 10 mm in diameter, basically restrict to the *sella turcica* and for the most part are not associated to mass effects. Nevertheless, macroadenomas are associated to the invasion of surrounding areas as well as optic chiasm compression

(Ciccarelli, *et al*., 2005). Over 90% of prolactinomas are small, intrasellar tumors that rarely increase in size (Casanueva *et al*., 2006).

Prolactinomas have been reported in patients from two to 80 years, and its prevalence varies widely among different age groups, being the most prevalent pituitary adenoma type between the second and fourth decades of life (Mindermann and Wilson, 1994; Casanueva *et al*., 2006). There is also a difference of prolactinoma prevalence according to sex. In adults, prolactinomas arise more frequently in women than in men and become more diagnosed in men than in woman during the sixth decade of life (Ciccarelli *et al*., 2005; Aflorei and Korbonits, 2014).

### 1.4. Inherited prolactinomas

Although the vast majority of prolactinomas arise sporadically, some cases have been reported in family clusters, and can be defined as inherited prolactinomas. The classical familial syndromes that predispose patients to prolactinoma are Multiple Endocrine Neoplasia Type 1 (MEN1), Multiple Endocrine Neoplasia Type 4 (MEN4), Carney complex (CNC), and Familial Isolated Pituitary Adenomas (FIPA) (Ciccarelli *et al*., 2005; Lee and Pellegata, 2013).

MEN1 is an autosomal dominant syndrome that is caused by an inactivating mutation in the *MEN1* gene (AgarwaL *et al*., 2009). *MEN1* is located at 11q13, consists of 10 exons that encode a 610 amino acid protein referred to as menin (Chandrasekharappa *et al*., 1997). Menin is predominantly a nuclear protein that has roles in transcriptional regulation, genome stability, cell division, and proliferation (Lemos and Thaker, 2008). This syndrome was first described in a familial cluster as *Wermer syndrome*, characterized by the combined occurrence of tumors of the parathyroid glands, pancreatic islet cells, and

anterior pituitary gland (Wermer, 1954). The most frequent tumors in MEN1 syndrome are those of the parathyroid glands (95% of cases), endocrine gastroenteropancreatic tract (30-80% of cases), and anterior pituitary (15-90% of cases) (Gribil *et al*., 2004). This disorder affects all age groups and has high penetration, with clinical manifestations developing in more than 80% of affected individuals by the fifth decade of life. Approximately 60% of pituitary adenomas occurring in MEN1 are PRL-secreting (Agarwal *et al*., 2009).

A variation of MEN1 syndrome, called MEN1-Burin, was described in four large kindred from the Burin peninsula, Canada. These patients have prominent features of prolactinomas in addition to carcinoids, and parathyroid tumors. These patients also show disruption in 11q13. A nonsense mutation in the MEN1 gene has been found to be responsible for the disease in all four MEN1-Burin families, suggesting that a common ancestral mutation in the MEN1-Burin phenotype is responsible for this prolactinoma variant of MEN1 (Olufemi *et al*., 1998).

Nevertheless, in a subset of MEN1-phenotype familial cases, around 10 to 30%, test negative for mutations in the *MEN1* coding region (Georgitsi, 2010). To understand these cases animal studies were established and led to the identification of *CDKN1B*, which encodes the 196 amino acid protein cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$, and predispose rats to a MEN-like phenotype, including parathyroid adenomas, pancreatic islet-cell hyperplasia, tyroid C-cell hyperplasia, bilateral phaeochromocytoma, paragangliomas and cataracts (Pellegata *et al*., 2006; Lee and Pellegata, 2013). This disease was assigned as MEN4 in human and classified as a novel and rare familial syndrome seen in patients with MEN1-like features, but no *MEN1* gene mutations (Thakker, 2014). Mutations in *CDKN1B* have been shown in a group of patients with PRL-secreting pituitary adenoma (Lee and Pellegata, 2013). Full description of the phenotype associated with *CDKN1B* mutations needs to be better clarified. Study of 426 MEN4

suspected cases identified mutation in *CDKN1B* in only 11 patients (2.4%), of which four were asymptomatic (Georgitsi, 2010). Thus, this gene and disease related to it need further investigation.

Carney Complex is an autosomal dominant multiple endocrine neoplasia syndrome characterized by the complex of "myxomas, spotty pigmentation, endocrine overactivity, and schwannomas". This disease was first described in by Carney in 1986 in a family in which symptoms occurred in three successive generations (Carney *et al*., 1986). Studies to unveil the genetic landscape of CNC identified mutations in protein kinase cAMP-dependent type I regulatory subunit alpha gene (*PRKAR1A*) in several families and also in patients with the sporadic form of the disease. Three unrelated families and one sporadic case shared the same 2bp deletion in exon 4B of *PRKAR1A*, suggesting it could be a hot spot for mutation (Kirschner *et al*., 2000). Actually, mutations in *PRKAR1A* have been reported in approximately 60% of patients with CNC. The hyperprolactinemia detected in such patients is for the most part asymptomatic and almost exclusively associated with clinical or subclinical acromegaly (Ciccarelli *et al*., 2005).

Familial Isolated Pituitary Adenoma classification was first mentioned in 2005 and has been a widely used concept since than (Ciccarelli *et al*., 2005; Daly and Beckers, 2014). Long before this classification, familial isolated pituitary adenomas were described. In 1967 Linquette and coworkers reported a family with isolated prolactinoma (Linquette *et al*., 1967). In 2005, Berezin and Karasik described four families with more than one family member holding prolactinoma diagnosis. Three of the families contained two members with a prolactinoma and a fourth one contained one man with prolactinoma and his only son with idiopathic hyperprolactinaemia. The authors discus that a transmissible genetic defect must be responsible for the familial occurrence of prolactinomas, however since families studied were small and no consistent pattern of transmission was observed,

no further conclusions were taken (Berezin and Karasik, 1995).

FIPA is defined when two or more related individual reported with pituitary adenomas and no other syndromic features are diagnosed. In FIPA may occur pituitary tumors of the same type in all affected members of the same family (homogeneous presentation), or tumors of different cell types (heterogeneous presentation) (Daly and Beckers, 2014). Prolactinomas are the most commonly observed tumor (39.9%) in FIPA families, followed by GH-secreting or mixed GH-secreting and prolactin-secreting adenomas (30% and 7%, respectively) (Daly *et al*., 2006).

In about 20% of FIPA families, a mutation in Aryl hydrocarbon receptor-interacting protein (*AIP*) gene has been described and part of them have been associated to PRL-secreting pituitary adenomas (Daly and Beckers, 2014). Germline mutations in the *AIP* were first reported after whole-genome, single-nucleotide polymorphism genotyping of a family from northern Finland (Vierimaa *et al*., 2006). After years of studies, it is now known that the most occurring type of pituitary adenoma in *AIP*-related FIPA are GH-secreting adenomas (40 to 50%), followed by prolactinomas (10 to 15%), somatomammotropinoma and non-functioning pituitary adenoma (4 to 7%). The median age of diagnosis of *AIP* mutated FIPA families is 23 years, which is younger than other pituitary adenoma-related syndromes. These tumors are less responsive to treatment and are more aggressive (Korbonits *et al*., 2012).

## 1.5. Genetics of prolactinomas

Although prolactinomas are common pituitary adenomas, the mechanisms that control the abnormal proliferation of this tumor type remains unclear for the most part. Studying the molecular profile of pituitary adenomas might be a challenging and technically difficult effort, since treatment might not include tissue resection and biopsy materials are rarely available; thus samples to provide information of when and how the lactotrophs acquire molecular modifications are scarce (Melmed, 2011). Based on the monoclonal nature of these neoplasms, evidence supports the hypothesis that pituitary tumors are caused by intrinsic pituitary-cell defects (Asa and Ezzat, 2002).

Common cancer-associated genes are rarely mutated in pituitary tumors (Melmed, 2011). This fact might explain the benign profile of these tumors. Besides, premature pituitary tumor senescence appears to bypass pro-proliferative signals and maintain cell viability. However, proto-oncogenes have been found to be mutated or overexpressed in prolactinoma, such as pituitary tumor-transforming gene (*PTTG*), which is expressed at high levels in most pituitary tissue. Experiments showed that besides overexpressed in pituitary tissue, this gene induces cellular transformation and is tumorigenic in nude mice (Melmed, 1997). However, *PTTG* role in pituitary tumorigenesis remains unclear (Asa and Ezzat, 2002)

As stated earlier, MEN1, MEN4, CNC and FIPA are familial syndromes that predispose patients to prolactinomas. Carney complex and MEN4 are rare causes of inherited forms of pituitary adenomas, especially prolactinomas. Thus mutations in *MEN1* and *AIP*, although rare, are the most frequent type in such tumors (Agarwal *et al*., 2009; Daly *et al*., 2006). *MEN1* and *AIP* are both located at the chromosome 11q13 locus (Lecoq *et al*., 2014). While *MEN1*-associated mutations have been described in pedigrees with

bona fide MEN1 features (Agarwal *et al*., 2009), *AIP* mutations have been reported in ~20% of FIPA cases (Daly and Beckers, 2014).

In addition, it has been shown that prolactin receptor (*PRLR*) knockout mice develop prolactinoma (Schuff *et al*., 2002) and that mutation in this same gene is associated to familial idiopathic hyperprolactinemia. The p.His188Arg variant of PRLR was found in three sisters with hyperprolactinemia, two of whom were presented with oligomenorrhea and the third with infertility (Newey *et al*., 2013). However, the role of *PRLR* mutation in clinical manifestations has been discussed in the literature (Bernard *et al*., 2015a). Recently, Bernard *et al*. (2015a) investigated 88 patients with sporadic prolactinoma and found four *PRLR* mutations (p.Ile76Val, p.Ile146Leu, p.Glu108Lys and p.Glu554Gln) in 16 patients. However, the four variants were tested in vitro and had no effect on PRLR expression, localization and signaling after prolactin stimulation. Thus no phenotypically similar patients were reported to harbor inactivating germline mutations in this gene so far (Bernard *et al*., 2015b).

Experiments have been conducted in order to characterize other genes likely to be associated to prolactinoma tumorigenesis. The majority of studies is being held in the sporadic form of prolactinomas and has highlights new genetic targets. Large-scale expression profile analysis has pointed genes pertaining to prolactinoma formation, but further investigation is needed to access their role in lactotrophic proliferation (Evans *et al*., 2001; Evans *et al*., 2008; Jiang *et al*., 2010; Tong *et al*., 2012; Zhao *et al*., 2014; Seltzer *et al*., 2015; Zhou *et al*., 2015).

The latest approach to access genes associated prolactinoma formation and phenotype is whole exome-sequencing. Recently this technique was used to evaluate the genetic profile of dopamine-agonist resistant sporadic prolactinoma. Prolactinomas evaluated in this study lack *AIP*, *BMP-4*, *CDKN1B*, C*DKN2A*, *CDKN2C*, *Cyclin D1*, *D2R*,

*GADD45G*, *Gsp*, *MEG3a*, *MEN1*, *p53*, *Pdt-FGFR4*, *PKC*, *PRKAR1A*, *PTTG*, *RAS*, *SSTR2*/*SSTR5*, *WIF* and *ZAC1* mutations. However PRDM2 emerged in this study as a drug-resistance and tumor recurrence driver (Gao *et al*., 2015).

Although current effort, it is still unclear how these new expression and genetic findings correlated to tumor development, and if any of these are also associated to familial settings.

## 1.6. Clinical features and diagnosis of prolactinomas

Prolactinomas are the most common causes of hyperprolactinemia (Ciccarelli, *et al*., 2005) and hyperprolactinemia is a well-established cause of hypogonadrotropic hypogonadism and anovulatory infertility. Scientific evidence suggests that PRL inhibits GnRH secretion and this leads to low circulating levels of LH and FSH and loss of ovarian stimulation, which can result in infertility (Bernard *et al*., 2015b).

In women, hyperprolactinemia is associated with oligo/amenorrhea in 90% of cases, 80% of patients also exhibit galactorrhea and may also manifest anovulatory infertility. Moreover, a chronic elevated PRL serum level is associated with reduced spinal bone mineral density. Hyperprolactinemia may also be identified in men and usually causes impotence, infertility and decreased libido (Figure 6). Due to the general aspects of symptoms and delayed recognition of them, men commonly present larger tumors than woman and are more susceptible to neurological symptoms (Casanueva *et al*., 2006).

Symptoms related to *sellar* mass effect, such as visual impairment and headaches, may also be detected in macroprolactinoma patients. Visual impairment is caused by suprasellar extension, leading to compression of the optic chiasm and bi-temporal hemianopsia. Enlargement gland symptoms include extraocular muscle dysfunction (from

palsies of cranial nerves 3, 4 or 6), or ipsilateral facial pain (from involvement of the V1 and V2 branches of the 5th nerve) (Melmed and Kleinberg, 2004; Hong, *et al*., 2016).

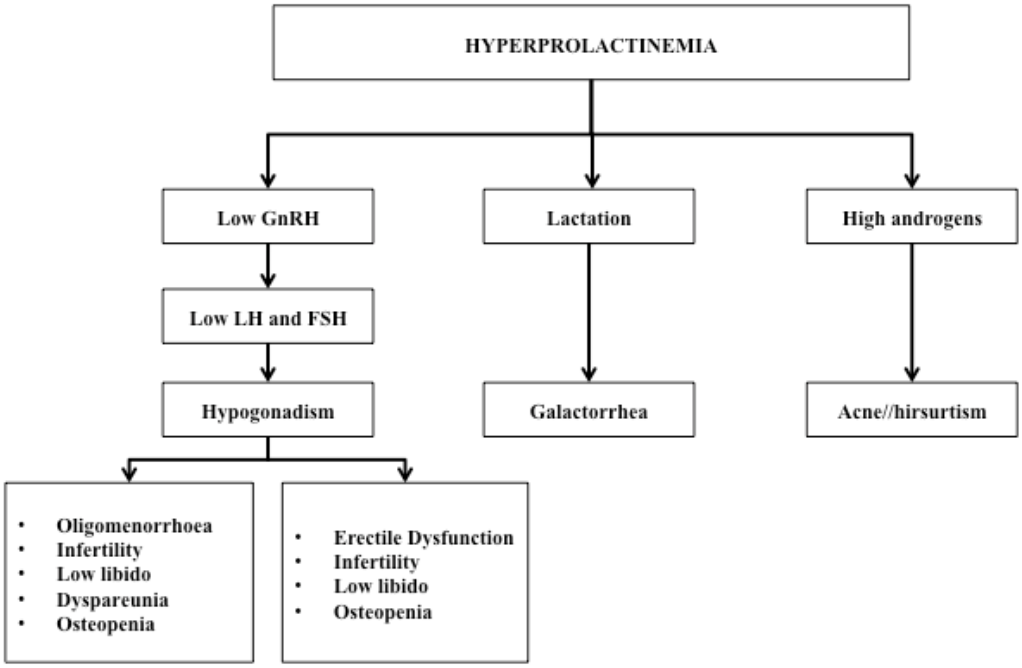


**Figure 6. Clinical manifestations of hyperprolactinemia** (modified from Vilar and Naves, 2012).

Prolactinoma clinical assessment starts with questioning the history of the patient. It is important to query about pregnancy, medications, headaches, and visual symptoms. However, the final diagnosis of prolactinoma requires both image exam of the pituitary adenoma, and laboratory analyses documenting the presence of sustained hyperprolactinemia (Casanueva *et al*., 2006). Figure 7 shows a diagnostic algorism for prolactinomas.

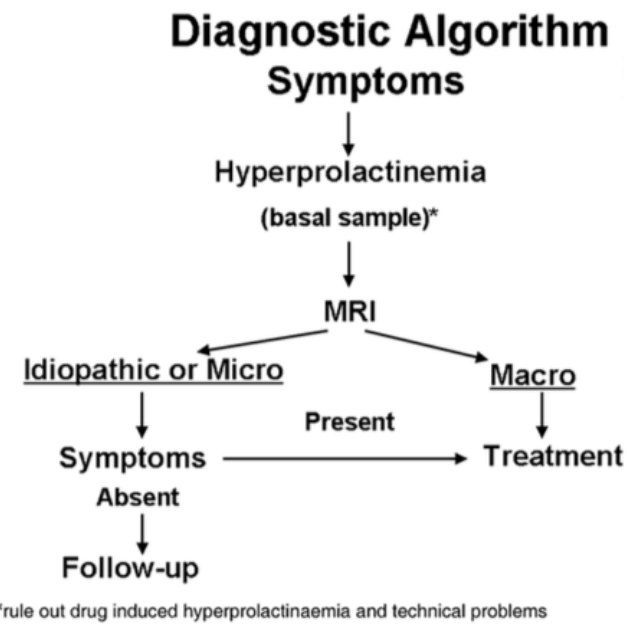


**Figure 7. Recommended diagnostic algorism for prolactinomas** (Casanueva *et al*., 2006).

## 1.7. Treatment of prolactinomas

Therapeutic goals for hyperprolactinemia include control of excessive hormone secretion and infertility, sexual dysfunctions, and osteoporosis, removal and relief of any disturbance in vision and cranial nerve function, and prevention of recurrence or progression (Auriemma *et al*., 2016).

Dopaminergic agonists such as bromocriptine and cabergoline are the primary therapy for patients with prolactinoma. Treatment restores menses in 90% of patients and shrinks tumor mass by more than 50% in about 80% of patients (Heaney and Melmed, 2004). Large comparative studies of cabergoline and bromocriptine have convincingly demonstrated the superiority of cabergoline in terms of patient tolerability and convenience, reduction in prolactin secretion, restoration of gonadal function, and decrease in tumor volume. Cabergoline is effective in most patients, including those not responsive to bromocriptine (Bloomgarden and Molitch, 2014).

Experimental therapies with somatostatin analogues, nerve growth factor, interferon-a and dopastatins are being developed to be used when first line therapy fails. These studies are in various phases of development, but none of these approaches has received approval or a demonstration to be advantageous. Thus, for medication non-responsive prolactinoma, surgical and radiation treatment are both available options, although are not frequently required (Capozzi *et al*., 2015).

## 1.8. Whole-exome sequencing

The genetic study of tumors uses the DNA sequencing, which is one of the main tools for medical research (Rabbani *et al*., 2014). The union of two techniques: the chain termination sequencing by Sanger *et al*. (1977), and the polymerase chain reaction (PCR) by Mullis and collaborators (1986), established many marked events such as the completion of the Human Genome Project (HGP). This approach provided a reference genome so that latter on genetic alterations could be associated to disease phenotypes (Sachidanandam *et al*., 2001; Venter, 2003; Rabbani *et al*., 2014).

Eventually, newly developed technologies are replacing the traditional methods for whole-genome sequencing (WGS) and whole-exome sequencing (WES) (Rabbani *et al*., 2014). Whole-exome sequencing is a high-throughput sequencing technology that is capable to determine the arrangement of DNA base pairs specifying the protein coding regions of an individual's genome, called exome. The exome represents only 1-2% of the human genome, however, studies have shown that genetic diseases are more likely to be caused by mutation in this region, than mutations in noncoding regions (Isakov, 2013a). These revolutionizing sequencing technologies, are promising to be used in clinic to improve human health, although their costs, ethical issues related to the produced genetic

data and the need for user-friendly software in the analysis of the raw sequence have to been addressed (Rabbani *et al*., 2014). The recent advances in these techniques are accelerating the pace of discovery in genetic disorders and cancer. As a result, they have entered the clinical practice and have been used to evaluate genes associated with phenotype for which no genetic abnormalities has been described (Bick and Dimmock, 2011).

Thus, next-generation sequencing technologies are useful tools to decipher the genetic events driving multiple diseases that lack known causal genetic mutation. Over the past few years, whole-exome sequencing has been used to detect causative mutations in endocrine related traits such as nonfunctioning pituitary adenomas. Genomic DNA from seven pituitary non-functioning pituitary adenoma were investigated and revealed 24 somatic variants identified and confirmed. However, DNA sequence analysis of these variants in a set of 24 pituitary non-functioning adenomas did not reveal any mutations, indicating that these genes are unlikely to contribute significantly in the etiology of sporadic pituitary (NEWEY *et al*., 2013).

Whole-exome was used to search for the causal mutation in an isolated hyperparathyroidism family. At this study DNA extracted from two sibs and one offspring from this family, all affected with multiglandular hyperparathyroidism revealed a very rare previously described missense mutation (p.W341R) in *MEN1* (Isakov *et a*l., 2013b). Parathyroid carcinomas were also recently investigated through whole-exome sequencing. Seven patients were investigated and *PRUNE2* variant emerged as the probably causal mutation in such patients (Yu *et al*., 2015). Moreover whole-exome sequencing has been used to access the causal mutation in 12 ACTH-secreting pituitary adenomas. This study revealed different somatic mutations in a single candidate gene, *USP8* (encoding ubiquitin-specific protease 8) in Cushing´s disease patients (Ma *et al*., 2015). Recently in sporadic

prolactinomas Gao has used whole-exome sequencing to establish genetic difference between six dopamine-responsive and six dopamine-resistant prolactinomas. Multiple genes emerged at this study, however PRDM2 was pointed out as an important gene for prolactinoma tumorigenesis (Gao *et al*., 2015).

Thus, the present study applied whole exome sequencing in an attempt to identify the causative mutation in *AIP*, *MEN1* and *PRLR* mutation-negative Brazilian family presenting with familial isolated prolactinoma.

# 2. PURPOSE

This work aims to study a rare case o familial isolated prolactinoma and unveil the genetic characteristics of this family, and how they might be associated to tumor development. For that, the following specific purpose were considered:

**2.1. Determine the presence of *MEN1* mutation in this kindred.**

**2.2. Determine the presence of *AIP* mutation in this kindred.**

**2.3. Determine the presence of *PRLR* mutation in this kindred.**

**2.4. Perform whole exome in DNA samples from this kindred if genes referred to above had wild type alleles.**

**2.5. Analyze whole-exome sequencing results and select candidate single nucleotide variants.**

**2.6. Validate the found single nucleotide variants.**

# 3. MATERIALS AND METHODS

### 3.1. Clinical Case study (summary)

The index case (II.5 figure 8), a Brazilian Caucasian male born in 1956, was referred to our endocrine clinic at Hospital Felicio Rocho (Belo Horizonte, Brazil) after a head CT scan revealed pituitary macroadenoma with sellar expansion. Upon questioning, the patient reported low libido and erectile dysfunction that started 5 years prior to admission. Hormonal analysis showed hyperprolactinemia (serum prolactin (PRL), 362 ng/mL; NR = 3.0-18.6 ng/mL). Following dopamine agonist treatment, there was a rapid and sustained normalization of plasma PRL levels and tumor shrinkage. Patient family history revealed two siblings who were subsequently diagnosed with prolactinoma. An older sibling (born 1951) (II.3 Figure 8) was diagnosed with hyperprolactinemia (PRL 62.9ng/mL) associated with a sellar mass of 0.6 x 0.6 cm in a reach out study to assess the familial nature of the disorder in the proband. Dopamine agonist therapy was initiated and this patient (II.3 Figure 8) was lost to follow up. The proband's younger female sibling (II.13 Figure 8), born in 1969, was diagnosed with hyperprolactinemia (PRL 92.7ng/mL) and macroadenoma with sellar expansion (size 0.9 x 0.6 cm) at age 36 years. Dopamine agonist was initiated followed by a spontaneous pregnancy with subsequent tumor remission. Eight other asymptomatic family members (seven siblings and the mother) had normal prolactin serum levels on several occasions from 2010 to 2015.

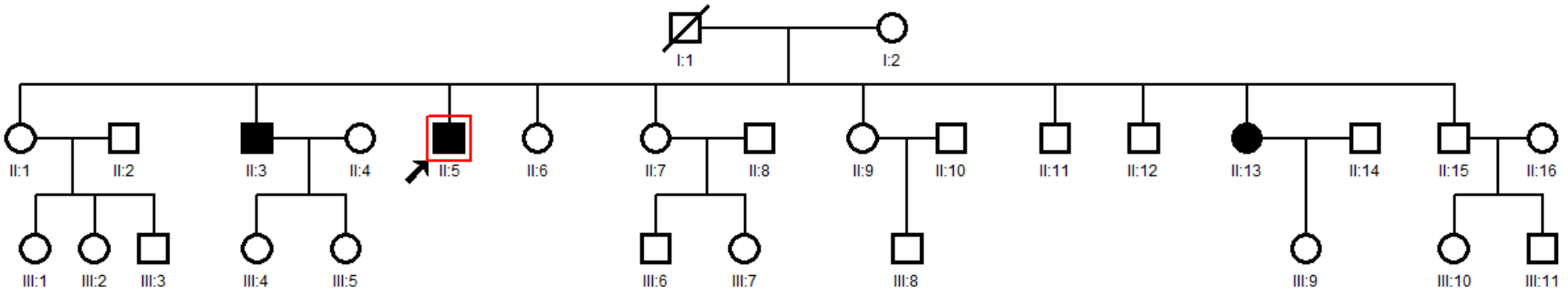


**Figure 8. Familial isolated prolactinoma pedigree.** Black filled shapes represented affected siblings. White shapes are asymptomatic subjects. Proband is pointed by the arrow.

### 3.2. Sanger sequencing

About 5ml of peripheral blood of all patients and controls were collected in vacuum tubes with EDTA after obtaining written informed consent of the patients. Genomic DNA was isolated from all study participants using saline concentration method of Lahiri and Nurnberger (Lahiri and Nurnberger, 1991). All participants gave informed consent which was approved by the Ethics Committee of the Universidade Federal de Minas Gerais.

Genotyping for germline mutations in the *MEN1*, *AIP*, and *PRLR* genes was carried out in the three clearly affected family members (II.3, II.5, II.13 figure 8). Exon-specific flanking primers (Table 1) were either designed according to previously published studies (Vierimaa *et al*., 2006; Newey *et al*., 2013) or using PrimerBlast software, available at *http://www.ncbi.nlm.nih.gov/tools/primer-blast/*. The DNA extracted from the blood patients was quantified and polymerase chain reactions (PCR) were performed using 50ng/ml DNA, 2.5ml of 10x Buffer IIB (40mM NaCl, 10mM TrisHCl, pH8.4, TritonX -100 0.1 %; 1.5MM MgCl2), dNTPs 2.5ml (0.2mm), 0.5ml of each primer pair (10 pmol/ml) and 0.25ml of Taq polymerase (0.625U). The products were amplified in a thermocycler Eppendorf Mastercycler Gradient, using the following steps: denaturation at 94° C for 3 minutes, and 35 cycles of denaturation at 94$^{o}$C for 30 seconds. Annealing at 55-60$^{o}$C for 30 seconds and extension at 72° C for 30 seconds. At the end of the cycles, the reactions undergone a final extension at 72° C for 5 minutes. PCR reaction products were gel-verified and purified using the PCRLink$^{TM}$ Quick PCR Purification Kit (Life technologies, Carlsbad, California), then submitted to sequencing reaction with the ABI BigDye Terminator Cycle Sequencing Kit v3.1 on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA).

**Table 1. Primer list and PCR conditions.**

| GENE | EXON | SEQUENCE (5´- 3´) | SIZE (bp) |
|---|---|---|---|
| *AIP* | 1 | **F** AACCAATCACCATCCGTTTC<br>**R** GTCGAGTTGTGCATGTGAGC | 397 |
| *AIP* | 2 | **F** GGACTGGACTTCTCCTTGGG<br>**R** GTCTAGCAGAGGGTGGAGGG | 246 |
| *AIP* | 3 | **F** GATGGTGGTGGGGAAGG<br>**R** ACCCCTGGGTGGACAGG | 359 |
| *AIP* | 4 and 5 | **F** CTCTGCTGCTGGTGTGTGAT<br>**R** GAAAGGCCACTCTCTGACCA | 597 |
| *AIP* | 4 and 5 | **F** GAGCCCGCTGTGATATG<br>**R** TGCACTGGCAGTAGTTGAGC | 387 |
| *AIP* | 6 | **F** ATGGTGCCAGGAGACATGA<br>**R** AACAGCCACCCAAGTACCAG | 477 |
| *MEN1* | 2.1 | **F** GGAACCTTAGCGGACCCTGGGAG<br>**R** GGAAGGTGAGCTCGGGAACGTTGG | 288 |
| *MEN1* | 2.2 | **F** GTTTTCCCAGTCACGACGTGGAGCAT<br>**R** ACCCCCTTCTCGAGGATAGA | 204 |
| *MEN1* | 2.3 | **F** CGACCTGTCCCTCTATCCTC<br>**R** GAGGGTTTTGAAGAAGTGGG | 197 |
| *MEN1* | 3 | **F** AGGGTGTAAACAGGGAGAGAGAGTC<br>**R** GGCTTGGGCTACTACAGTATGAA | 453 |
| *MEN1* | 4 | **F** GAGACATAATGATCTCATCC<br>**R** AAGTCTGGCCTAGCCCAGTC | 202 |
| *MEN1* | 6 | **F** GGGTGGCAGCCTGAATTATG<br>**R** CCCCCAACACACAAAGTTCT | 220 |
| *MEN1* | 8 | **F** CGACCCTACAGAGACCCCAC<br>**R** CCATCCCTAATCCCGTAC | 273 |
| *MEN1* | 9 | **F** ATCGTGCCCTCCCTTCCCC<br>**R** CTGTCACCACCTGTAGTGCC | 240 |
| *PRLR* | 2 | **F** CAATGTTCAGCCATGCTACG<br>**R** CAGCAGAATGTGCCAGTGTAG | 300 |
| *PRLE* | 3 | **F** CCCAGAATAAAGTGGTGGATG<br>TCCACCCTGTTGACAAACAC | 245 |
| *PRLR* | 4 | **F** AAGGGTCAAATGGTTAAATGGA<br>**R** GGCCTGGAGAATGGGAGTA | 250 |
| *PRLR* | 5 | **F** CCAAAGGCCAGTGGTATTGA<br>**R** TCCA TCCAAAACCCAAGAAG | 353 |
| *PRLR* | 6 | **F** AAGCCAAAGAAAAGGTGCAA<br>**R** TATCCTTGCCAAAGGCCATA | 277 |
| *PRLR* | 7 | **F** AGGGGAAAACTCTCTTTCTTCA | 257 |

| GENE | EXON | SEQUENCE (5´- 3´) | SIZE (bp) |
|---|---|---|---|
| *PRLR* | 8 | **R** ACCATTTAAAACATATTTAGGGACA<br>**F** GAA TGGAGGAAAACACTCTTGG<br>**R** TGACTATCATGATTGGGAGGAA | 248 |
| *PRLR* | 9 | **F** AGCTGCCAAACCAAGTCCTA<br>**R** AAGGCTGGCTGAAACTACCA | 293 |
| *PRLR* | 10.1 | **F** GGGA TGCTGA TTTGGAA TGT<br>**R** GGTAAGAGGATCTGGGGTTG | 500 |
| *PRLR* | 10.2 | **F** CCCTTTTGTCTGAAAAGTGTGA<br>**R** GGCGTATCCTGGTCAGTCTC | 400 |

### 3.3. Whole-exome sequencing (WES)

There are different deep-sequencing platforms to choose from when performing whole exome sequencing. Hybridization is the most optimal and commonly used method for targeted exome. For this purpose, peripheral blood from the three clinically affected sibs (II.3, II.5, II.13 Figure 8) was collected and DNA extracted. Subsequently, DNA was subjected to whole-exome capturing and sequencing using the Roche NimbleGen V2 chip (Madison, Wisconsin) or Nextera (San Diego, California) with the Illumina HiSeq2000 sequencing platform (San Diego, California).

The Illumina sequencing workflow begins with Nextera sample preparation, followed by cluster generation on a system flow cell, sequencing with Illumina's proprietary sequencing by synthesis technology and culminating with data analysis.

Samples consisting of longer fragments are first sheared into a random library of 100-300 base-pair long fragments. After fragmentation the ends of the obtained DNA-fragments are repaired and an A-overhang is added at the 3'-end of each strand. Afterwards, adaptors which are necessary for amplification and sequencing are ligated to both ends of the DNA-fragments. These fragments are then size selected and purified. The Cluster Generation is performed on the Illumina cBot. Single DNA-fragments are attached

to the flow cell by hybridizing to oligos on its surface that are complementary to the ligated adaptors. The DNA-molecules are then amplified by a so called *bridge amplification* which results in a hundred of millions of unique clusters. Finally, the reverse strands are cleaved and washed away and the sequencing primer is hybridized to the DNA-templates. During sequencing the huge amount of generated clusters are sequenced simultaneously. The DNA-templates are copied base by base using the four nucleotides (ACGT) which are fluorescently-labeled and reversibly terminated. After each synthesis step, the clusters are excited by a laser which causes fluorescence of the last incorporated base. After that, the fluorescence label and the blocking group are removed allowing the addition of the next base. The fluorescence signal after each incorporation step is captured by a built-in camera, producing images of the flow cell.

### *3.3.1. Variant Calling and annotation*

Variant calling is the part of the process that perform the initial mapping of the reads, improvement of alignments and quality scores, variant identification, and recalibration of the variants quality scores. In general, a coverage of 20X to 50X at each nucleotide is considered acceptable when identifying variations.

Raw sequence files were prepared using the Genome Analysis Tool Kit (GATK) for each of the sequenced samples. Each fastq file was aligned against the human hg19/GRCh37 reference genome. PCR duplicates were removed using Picard (*http://picard.sourceforge.net/)*, reads around known and detected indels were realigned, and base quality was recalibrated using GATK. In order to call variants from the processed BAM files, a variant calling pipeline from GATK was applied.

All generated VCF files were analyzed as a familial group using three different tools. The first software used was *Mendel, MD,* developed by the Clinical Genomic Laboratory of Universidade Federal de Minas Gerais and available at *http://mendel.medicina.ufmg.br* (Cardenas *et al*., 2015). VCF files were also clustered together and analyzed by *Ingenuity® Variant Analysis*$^{TM}$ software, available at *www.ingenuity.com/variants*. The third analysis was performed using the pipeline developed by Noam Shomron, Ofer Isakov and Marie Perrone at the Tel-Aviv University Medical School as previously detailed (Isakov, 2013a). For these analyses only variants with call quality of at least 40.0 and read depth of at least 20.0 were considered. Additionally, variants with allele frequency greater than or equal to 1.0% of the genomes reported in the 1000 genomes project (*www.1000genomes.org)*, the public Complete Genomics (*http://www.completegenomics.com/public-data/*) or NHLBI ESP exomes (*http://evs.gs.washington.edu/EVS/)* were also excluded from further analyses. The selected genes carried identical homozygous or heterozygous sequence variants that co-occurred in all genotyped cases.

### *3.3.2. Ingenuity® Variant AnalysisTM*

In addition to the above listed confidence and frequency criteria, variants associated with gain or loss of function, compound heterozygote, heterozygous ambiguous, haploinsufficiency, homozygous, or hemizygous that occurred in all WES genotyped samples at the variant level were chosen to be studied.

Filtering criteria also selected genes that were either pathogenic, possibly pathogenic, established gain of function in the literature, gene fusions, inferred activating mutations, predicted gain of function by BSIFT, within a microRNA binding site, a

frameshift, in-frame indel, stop codon change, a missense unless predicted to be innocuous by SIFT or Polyphen-2, predicted to disrupt splice site up to 2.0 bases into intron, deleterious to a microRNA or a structural variant.

Considering the biological context, the following key words were selected from *Ingenuity® Variant Analysis™* (Ingenuity biological analysis): hyperprolactinaemic disorder, prolactinoma, pituitary adenoma predisposition, prolactin excess, amount of prolactin-secreting pituitary gland adenoma (quantity of prolactinoma), amount of prolactin-producing pituitary adenoma (quantity of prolactinoma), autosomal dominant prolactin-producing pituitary adenoma (autosomal dominant prolactinoma), formation of prolactin-secreting pituitary gland adenoma (formation of prolactinoma), prolactin-producing pituitary adenoma (prolactinoma), familial isolated pituitary adenoma or diseases consistent with phenotypes. Analysis that considered differentially expressed published prolactinoma genes was also applied. In this analysis, a list of differentially expressed genes in human prolactinomas reported from 1993 until 2015 was created. Gene list was extracted from six published papers (Evans *et al*., 2001; Evans *et al*., 2008; Jiang *et al*., 2010; Tong *et al*., 2012; Zhao *et al*., 2014; Zhou *et al*., 2015). This list was used to filter if any of the mutations detected was noted within a gene that was previously found to be differentially expressed in prolactinomas.

### *3.3.3. Mendel, MD*

The pathogenicity prediction of the variants using this algorithm was performed by selecting variants that were present in all three patients and were assigned as pathogenic by all of the below listed prediction models PROVEAN (Choi *et al*., 2012), SIFT, PolyPhen and CADD (Dong *et al*., 2015).

*Mendel, MD* was also used to analyze indels. In this analysis, confidence, frequency and pathogenicity criteria were maintained and a filter for the pathogenicity impact of the alteration was added and only variants with high or moderate impact were selected.

### *3.3.4. Mutation Analysis*

The results available from the *Ingenuity®* platform that considered a list of previously published differentially expressed proteins in prolactinomas was added to the list of genes selected through the *Ingenuity®* analysis of prolactinoma biological context and resulted in one mutated genes single list. This list was than combined to the analyses performed using *Mendel, MD* and only those genes that were present in both analyses were kept. Than, this single gene list was compared to the independent analysis performed using the Isakov-Shomron pipeline and the final single-nucleotide variant list that was detected by all three schemes was subsequently analyzed (Figure 9).

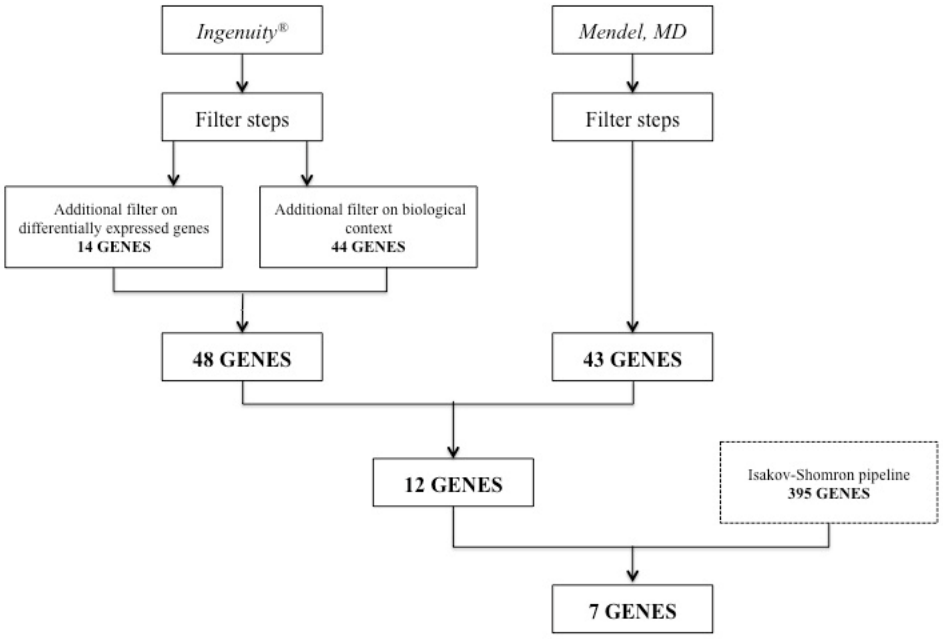


**Figure 9. Flowchart of variant analyses method.** Three methods of whole-exome variant analyses were gathered together to produce a final list of seven genes selected for subsequent study.

### *3.3.5. Indel analyses*

Using an annotation approach, genes that harbored indels were analyzed for their potential relevance to prolactinoma tumorigenesis. The following criteria were taken under consideration: (i) Pathway annotation, which includes all pathways in which a given gene product has reportedly been involved in. Pathway information was gathered from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (*http://www.genome.jp/kegg/);* (ii) Interaction annotations from the STRING protein functional interactions database (*http://string-db.org*) (iii) Publications relating each gene to pituitary adenomas were accessed in the Medline database.

### *3.3.6. Structural damage prediction and pathway analysis*

In order to obtain a homology model of mutated genes, a psiBLAST (ProteinDataBank, *http://blast.ncbi.nlm.nih.gov/)* was performed. The crystallographic coordinates were obtained from a PDB template with high homology to the translated protein sequence from the affected patients.

Pathway analysis was performed using *Ingenuity® Variant Analysis*$^{TM}$ tool of pathway to phenotype, where a possible connection between protein and disease is established according to its protein to proteins interaction profile.

### *3.3.7. WES sequence data validation*

Peripheral blood from all consenting family members as well as 95 ethnicity-matched elderlies (65 years of age or older) controls without any discernible personal or

family history of benign or malignant tumors (recruited under an Ethics Committee approved protocol from amongst individuals who currently attend the Geriatric Clinic of the Universidade Federal de Minas Gerais) were tested for selected sequence variants that were seemingly pathogenic and causative. After DNA isolation from the cases and controls, all relevant genes were amplified by PCR with primers specific for each region (Table 2). PCR products were purified using PCRLink$^{TM}$ Quick PCR Purification Kit (Life technologies, Carlsbad, California) following manufacturer's protocol and visualized on a silver-stained 6.5% polyacrylamide gel. Sequences were obtained on ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Bidirectional sequence data were analyzed by using Sequencer 4.9 software.

**Table 2. Primer list for whole-exome sequencing validation.**

| GENE | EXON | SEQUENCE (5′-3′) | SIZE (bp) |
|---|---|---|---|
| *RXRG* | 1 | **F** GGGGGGATGTGCAGAGCCATAAGTCAGG<br>**R** GCACTACCCAGAGGTTCATGCCCACGTG | 464 |
| *REXO4* | 9 | **F** CTCCACTCACCTGCACAGTC<br>**R** TGCTCTTTCACGAGGCTGAG | 223 |
| *TH* | 11 | **F** ACCAAGACCAGACGTACCAG<br>**R** TTCTCATCTGTGACCTGGGC | 183 |
| *MAP2K3* | 17 | **F** TGTGAAGCCCTCCAATGTCC<br>**R** ATCCTCTCCTGAGCCTGGG | 165 |
| *PABPC1* | 18 | **F** GTTATGATGGAGGGTGGTCG<br>**R** CTTCTAAAACCTACCTGTGGGA | 287 |

# 4. RESULTS

### 4.1. AIP, MEN1 and PRLR mutation analyses

Mutations in the coding and flanking intronic regions of the *AIP*, *MEN1* and *PRLR* genes were not detected in any affected family members genotyped (data not shown).

### 4.2. Whole exome sequencing analysis

Variant calling from WES of the three genotyped patients resulted in 57,509 common variants in 8,498 genes, the mean base call quality was 1,547 and average read depth was X83. A average of 1,2337 missense single nucleotide variations were found in the three sequenced patients, and the mean number of nonsense variants were 125, mean silent SNV total of 12,702 and a mean total of 1,947 indels (Table 3).

**Table 3. Description of sequence variants detected by whole exome sequencing.**

| Variants | Patients | | | Mean |
|---|---|---|---|---|
| | II.3 | II.5 | II.13 | |
| Missense SNV | 11231 | 11465 | 14317 | 12337 |
| Nonsense SNV | 89 | 97 | 189 | 125 |
| Silent SNV | 12116 | 12606 | 13384 | 12702 |
| Total number of indels | 2516 | 2578 | 747 | 1947 |

The *Ingenuity®* analysis, considering the biological context of prolactinoma, yielded a list of 44 mutated genes. The second analysis performed using the *Ingenuity®* platform that considered a list of published differentially expressed proteins in prolactinomas resulted in a list of 14 mutated genes. Both combined analyses resulted in a list of 48 genes. The analysis performed using *Mendel, MD* resulted in a list of 43 selected genes that fulfilled the selection criteria. These two gene lists (48 genes and 43 genes) were

combined and only 12 genes emerged to be shared by both lists. The independent analysis performed using the Isakov-Shomron pipeline combined with the 12 genes list mentioned above resulted in seven selected genes (Figure 10, Table 4).

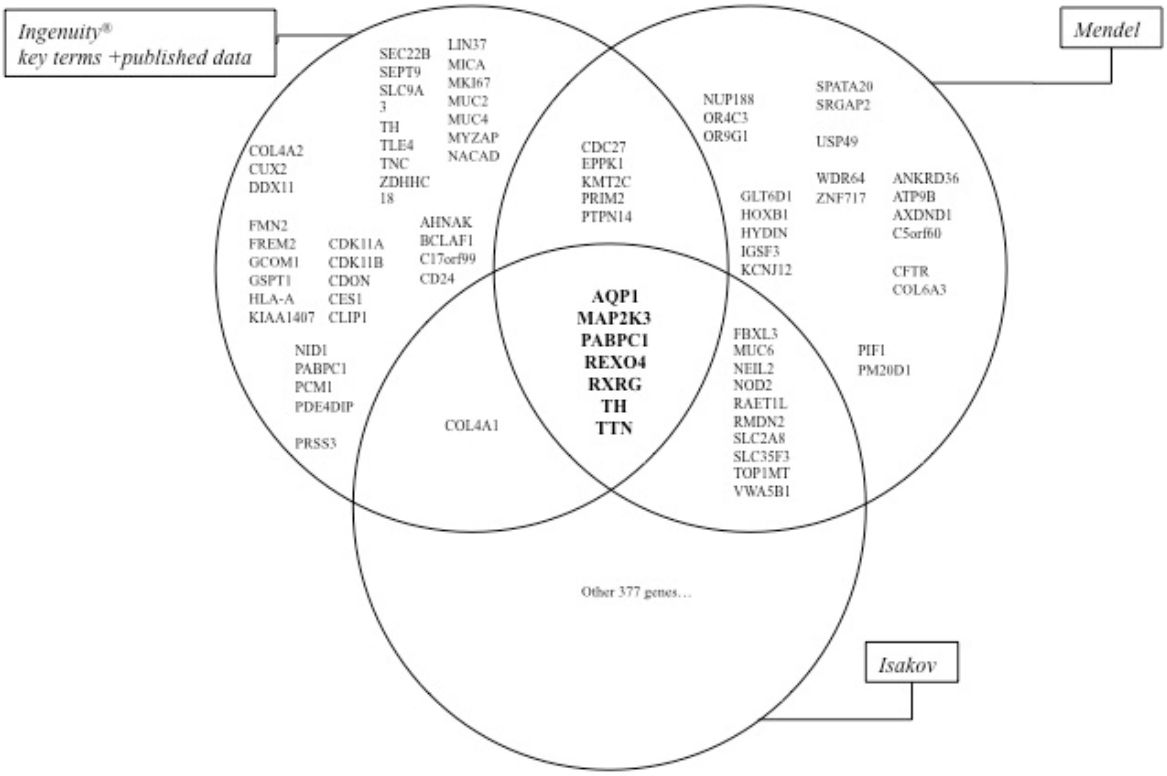


**Figure 10. Diagram representing genes selected through different analyses.**

**Table 4. Genes selected through different analyses.** Shaded squares indicate the analysis where the gene emerged. Bold genes were those that emerged in *Ingenuity®*, *Mendel* and Isakov analyses.

| | *Ingenuity®* key terms | *Ingenuity®* published data | *Mendel, MD* | Isakov-Shomron pipeline |
|---|---|---|---|---|
| AHNAK | ■ | ■ | | |
| ANKRD36 | | | ■ | |
| **AQP1** | ■ | | ■ | ■ |
| ATP9B | | | ■ | |
| AXDND1 | | | ■ | |
| BCLAF1 | ■ | | | |
| C17orf99 | ■ | | | |
| C5orf60 | | | ■ | |
| CD24 | ■ | ■ | | |
| CDC27 | ■ | | ■ | |
| CDK11A | ■ | | | |
| CDK11B | ■ | | | |
| CDON | ■ | | | |
| CES1 | ■ | | | |
| CFTR | | | ■ | |
| CLIP1 | ■ | | | |
| COL4A1 | ■ | | | |
| COL4A2 | ■ | ■ | | ■ |
| COL6A3 | | | ■ | |
| CUX2 | | ■ | | |
| DDX11 | | ■ | | |
| EPPK1 | ■ | | ■ | |
| FBXL3 | | | ■ | ■ |
| FMN2 | ■ | | | |
| FREM2 | ■ | | | |
| GCOM1 | ■ | | | |
| GLT6D1 | | | ■ | |
| GSPT1 | ■ | | | |
| HLA-A | ■ | ■ | | |
| HOXB1 | | | ■ | |
| HYDIN | | | ■ | |
| IGSF3 | | | ■ | |
| KCNJ12 | | | ■ | |
| KIAA1407 | ■ | | | |
| KMT2C | ■ | | ■ | |
| LIN37 | ■ | | | |
| **MAP2K3** | ■ | | ■ | ■ |
| MICA | ■ | | | |
| MKI67 | ■ | | | |
| MUC2 | ■ | | | |
| MUC4 | ■ | | | |
| MUC6 | | | ■ | ■ |
| MYZAP | ■ | | | |
| NACAD | ■ | ■ | | |
| NEIL2 | | | ■ | ■ |
| NID1 | ■ | | | |
| NOD2 | | | ■ | ■ |
| NUP188 | | | ■ | |
| OR4C3 | | | ■ | |
| OR9G1 | | | ■ | |
| **PABPC1** | ■ | | ■ | ■ |
| PCM1 | ■ | ■ | | |
| PDE4DIP | ■ | | | |
| PIF1 | | | ■ | |
| PM20D1 | | | ■ | |
| PRIM2 | ■ | ■ | ■ | |
| PRSS3 | ■ | | | |
| PTPN14 | ■ | | ■ | |
| RAET1L | | | ■ | ■ |
| **REXO4** | ■ | | ■ | ■ |
| RMDN2 | | | ■ | ■ |
| **RXRG** | ■ | | ■ | ■ |
| SEC22B | ■ | | | |
| SEPT9 | ■ | ■ | | |
| SLC2A8 | | | ■ | ■ |
| SLC35F3 | | | ■ | ■ |
| SLC9A3 | ■ | ■ | | |
| SPATA20 | | | ■ | |
| SRGAP2 | | | ■ | |
| **TH** | ■ | | ■ | ■ |
| TLE4 | | ■ | | |
| TNC | ■ | ■ | | |
| TOP1MT | | | ■ | ■ |
| TTN | ■ | | ■ | ■ |
| USP49 | | | ■ | |
| VWA5B1 | | | ■ | ■ |
| WDR64 | | | ■ | |
| ZDHHC18 | | ■ | | |
| ZNF717 | | | ■ | |
| | | | | |

Variants list were then filtered by PROVEAN, SIFT, Polyphen and CADD algorithms, resulted in six SNV in five genes predicted to be deleterious: *RXRG*, *REXO4*, *TH*, *PABPC1* and *MAP2K3* (Table 5).

**Table 5. Combined exome results and analyses of single nucleotide variants (SNV) effect on protein structure and function by Polyphen, Sift, Provean and CADD.**

| GENE | CHR | CODON CHANGE | AA CHANGE | PROVEAN | SIFT | PPH2 | CADD |
|---|---|---|---|---|---|---|---|
| RXRG | 1 | cGc/cAc | p.R317H | -4.58 | 0.022 | 1.000 | 34 |
| TTN | 2 | cAa/cGa | p.Q9198R | -2.11 | 0.370 | ND | 7.214 |
| AQP1 | 7 | gTc/gGc | p.V284G | -1.99 | 0.072 | ND | 20.4 |
| PABPC1 | 8 | gAa/gGa | p.E372G | -6.14 | 0.000 | 0.999 | 27.6 |
| PABPC1 | 8 | Cgc/Tgc | p.R374C | -7.17 | 0.000 | 0.988 | 32 |
| REXO4 | 9 | tGg/tAg | p.W195* | ND | ND | ND | 38 |
| TH | 11 | Aag/Tag | p.K474* | ND | ND | ND | 36 |
| MAP2K3 | 17 | tTg/tGg | p.L215W | -5.84 | 0.000 | 1.000 | 25.4 |

Shaded lines represent SNV that are highly expected to be deleterious according to the *in silico* analysis.
ND – No data; Cutoff: PROVEAN (-2.5), SIFT (0.05), PPH2 (0.95) CADD (15).

Indel analysis was also performed using data generated from all three samples from the affected patients. After filtration steps (see methods) a total of eight genes were selected (Table 6). Following the aforementioned steps of gene annotation, none of the indel listed genes were selected for further investigation.

**Table 6. Small insertions and deletions analyses.**

| GENE | CHR | CODON CHANGE | MUTATION | FUNCTIONAL CLASS |
|---|---|---|---|---|
| *HRNR* | 1 | atg/ | HOMO | FRAME_SHIFT+START_LOST |
| *ZNF717* | 3 | ttt/ | HETERO | FRAME_SHIFT |
| *MAP3K1* | 5 | tcaaca/tca | HOMO | CODON_DELETION |
| *PHPT1* | 9 | tgtctg/ | HETERO | FRAME_SHIFT |
| *ATRNL1* | 10 | ccttct/cct | HETERO | CODON_DELETION |
| *HYDIN* | 16 | att/ | HETERO | FRAME_SHIFT |
| *PKD1L2* | 16 | aac/ | HETERO | FRAME_SHIFT |
| *CNDP1* | 18 | gtg/gTGCtg | HETERO | CODON_INSERTION |

### *4.3. Exome detected pathogenic variants validation via Sanger sequencing*

The missense variants found in *PABPC1* and *MAP2K3* genes were not validated or confirmed in the three affected siblings. Variants detected in the *RXRG*, *REXO4* and *TH* genes that were validated in all three affected family members and were subsequently sequenced in all available clinically unaffected family members (Figure 11, table 7) as well as in the 95 healthy ethnically matched controls. None of population controls carried any of the genotyped variants (data not shown). Family sequencing revealed that some clinically and serologically asymptomatic siblings (p.R317H RXRG: n=3, p.w195* REXO4: n=5; p.K474* TH: n=4) also harbored these variants.

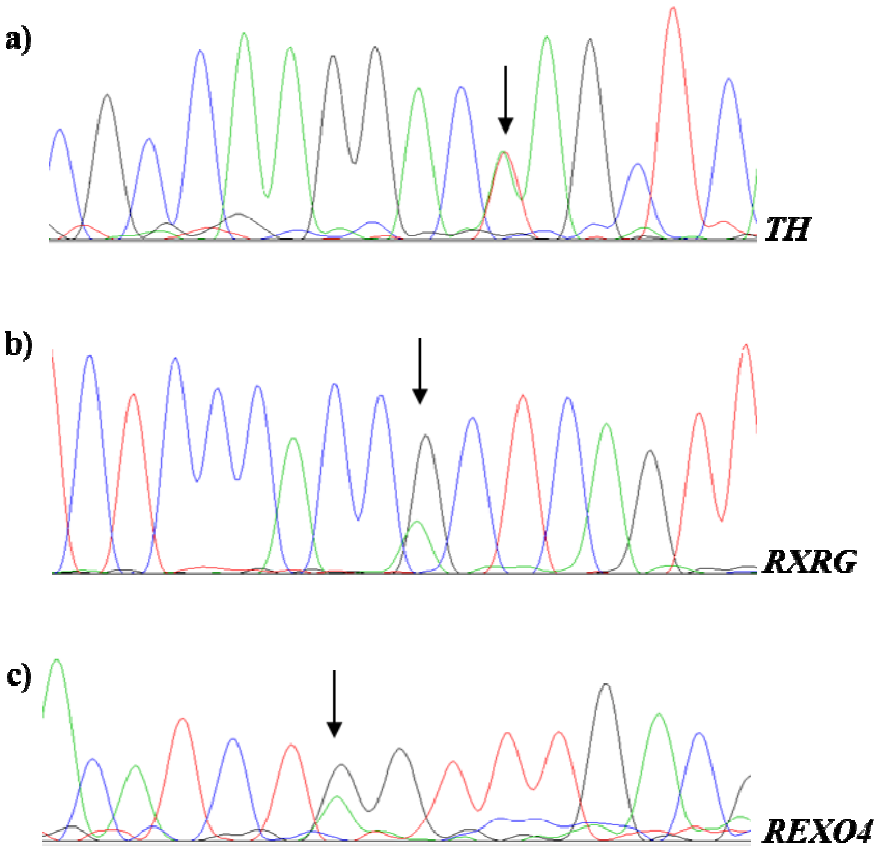


**Figure 11. Representative Electropherograms of validated genes for affected individuals through the family.** (a) *TH* sequencing results; (b) *RXRG* sequencing results; (c) *REXO4* sequencing results. Arrows point to the mutation.

**Table 7. Summary of validation sequencing results.** Shaded squares indicate affected subjects.

| SUBJECTS | *RXRG* p.R317H cGc/cAc | *REXO4* p.W195* tGg/tAg | *TH* p.K474* Aag/Tag | *MAP2K3* p.L215W tTg/tGg | *PABPC1* p.E372G gAa/gGa | *PABPC1* p.R374C Cgc/Tgc |
|---|---|---|---|---|---|---|
| **I.2** | G/G | **G/A** | A/A | T/T | A/A | C/C |
| **II.1** | **G/A** | **G/A** | **A/T** | T/T | A/A | C/C |
| **II.3** | **G/A** | **G/A** | **A/T** | T/T | A/A | C/C |
| **II.5** | **G/A** | **G/A** | **A/T** | T/T | A/A | C/C |
| **II.6** | G/G | G/G | A/A | T/T | A/A | C/C |
| **II.7** | G/G | **G/A** | A/A | T/T | A/A | C/C |
| **II.9** | G/G | G/G | A/A | T/T | A/A | C/C |
| **II.11** | **G/A** | G/G | **A/T** | T/T | A/A | C/C |
| **II.12** | G/G | **G/A** | **A/T** | T/T | A/A | C/C |
| **II.13** | **G/A** | **G/A** | **A/T** | T/T | A/A | C/C |
| **II.15** | **G/A** | **G/A** | **A/T** | T/T | A/A | C/C |

### *4.4. Protein pathogenicity Prediction and pathway analysis*

The RXRG p.R317H mutation leads to major structural abnormality and predictably deleteriously affects protein function is located in RXRG-Retinoic acid binding site, a region evolutionarily highly conserved, as shown in Figure 12.

Protein interaction pathways that might lead to disease were constructed for TH and RXRG proteins (Figure 13 and 14). These pathway maps are not conclusive to show the exact function of TH and RXRG in prolactinoma development, but theses figures demonstrate how close both proteins are to hyperprolactinemia control and indicate further investigation in such pathways might be interesting.

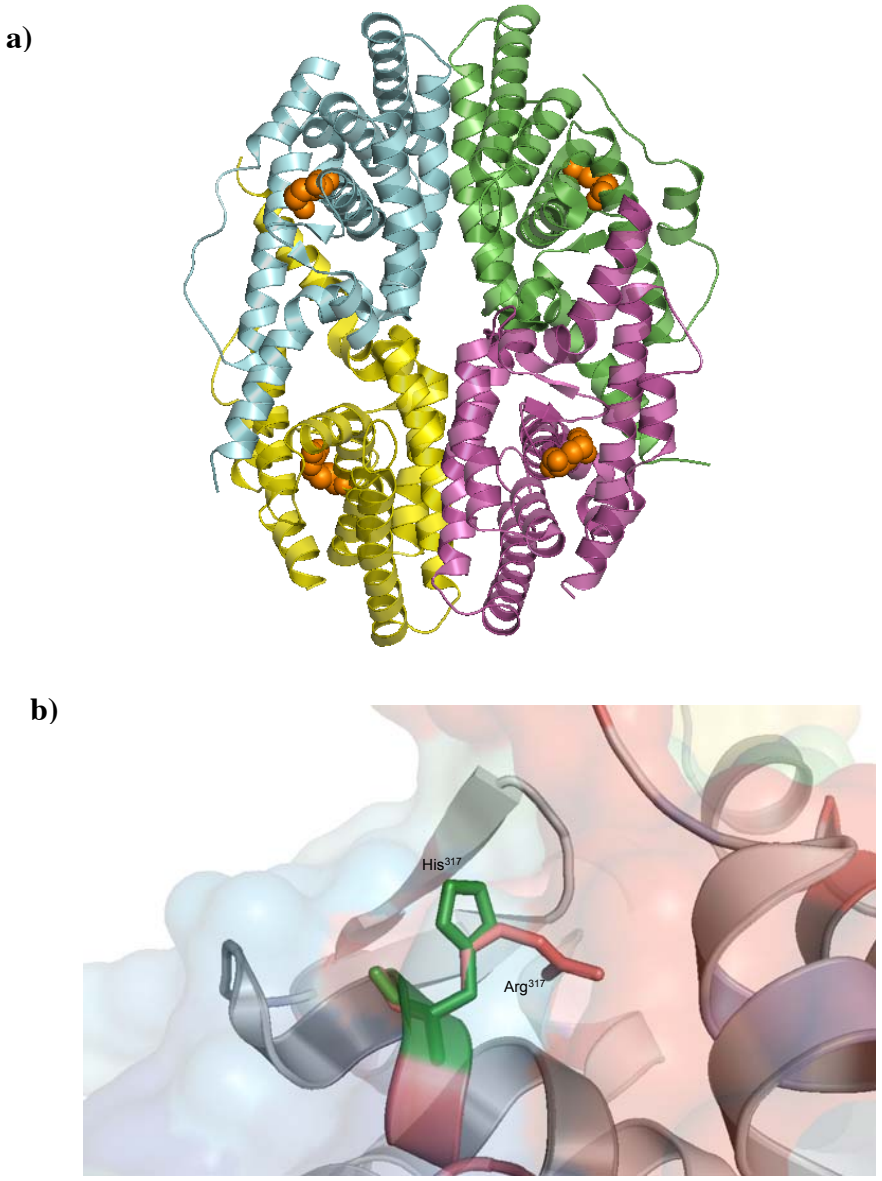


**Figure 12. RXRG protein structure.** (a) Overall structure of the tetrameric RXRG (PDB code 1G1U) shown in a cartoon diagram as A1 (light blue), B1 (green), B2 (yellow) and A2 (magenta). Side-view showing the location of the mutation p.R317H in orange spheres on each chain; (b) A close-up showing the side chain of amino acid 317 of RXR (Histidine, in green and Arginine in pink) at an α helix.


**Figure 13. Pathway demonstrating TH probable interaction with hyperprolactinemia phenotype.**

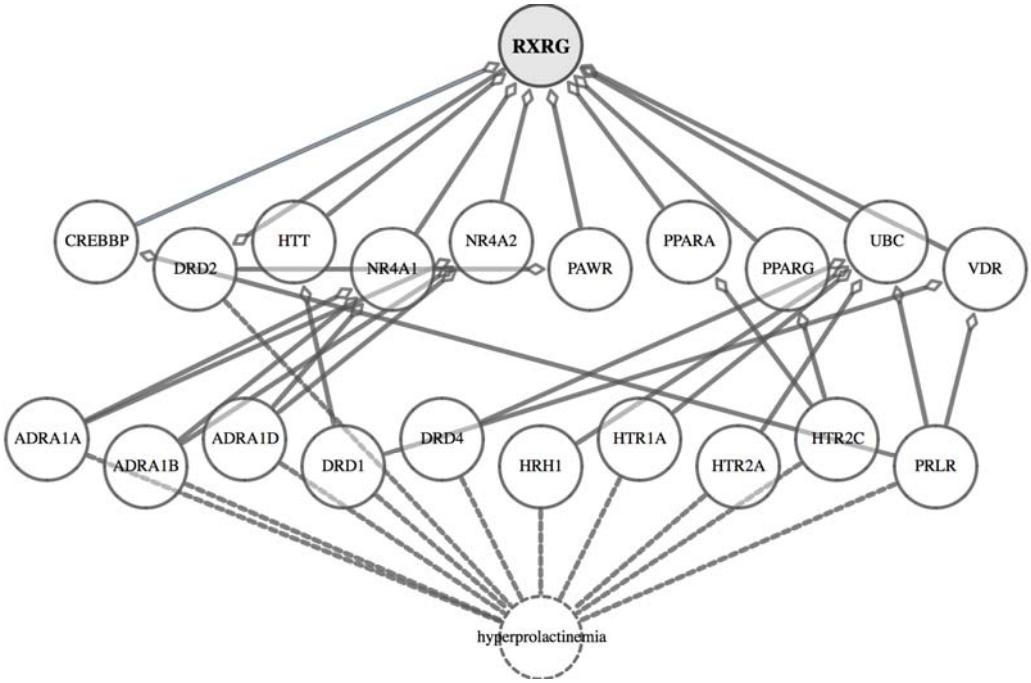


**Figure 14. Pathway demonstrating RXRG probable interaction with hyperprolactinemia phenotype.**

# 5. DISCUSSION

The pituitary tumorigenesis is a complex process and the identification of genes that are critical for the characterization of the disease is a demanding task due to the infinitely possibilities. Thus familial cases, although rare, are an interesting starting point to seek for novel mutations that could occur throughout a family setting and promote pituitary adenoma tumorigenesis.

This work describes three affected siblings in a familial set. All affected individuals manifest typical hyperprolactinemia phenotypes and were responsive to dopamine-agonist treatment. In fact, dopaminergic agonists are the primary therapy for patients with hyperprolactinemia and prolactinoma (Casanueva *et al*., 2006), and as reported in the present study, these drugs normalize PRL levels and significantly reduce tumor volume (Heaney and Melmed, 2004). It is also interesting to notice that one of the reported patients presented spontaneous tumor remission after pregnancy. This event has actually been reported in two-thirds of pregnant patients (Almalki *et al*., 2015). This mechanism of remission could be related to the activity of lysosome enzymes in degradation of PRL granules pituitary cells, as previously shown in the lactotroph involution after cessation of lactation (De Marco *et al*., 1982).

Classically, PRL levels >250 ng/ml have been considered to be highly suggestive of the presence of a macroprolactinomas (Vilar *et al*., 2014), as shown in patient II.5. However, macroprolactinomas are associated to Circulating PRL levels of 100-200 ng/mL, but not infrequently they may be <100 ng/mL (Vilar *et al*., 2014), as observed in the two other patients described in this study, II.3 and II.13. Thus lower concentration of serum PRL might be present in prolactinoma patients since overlap in PRL values regardless of the etiology of hyperprolactinemia has been shown (Vilar *et al*., 2014).

Traditional Sanger sequencing was the first protocol adopted by this study to identify common variants in family setting. *AIP*, *MEN1* and *PRLR* were investigated but

affected family members were negative for mutations in these genes. Thus, a Whole-exome sequencing protocol was selected to continue the genetic studies of this family.

In this single Brazilian family with an isolated prolactinoma phenotype, three novel, seemingly pathogenic heterozygous variants in the *RXRG*, *REXO4,* and *TH* genes were identified. Of these, both *REXO4* and *TH* variants are likely to be pathogenic as these are clearly inactivating (stop codon and premature termination of protein translation).

RNA exonuclease 4 (*REXO4*) is a nuclear expressed exonuclease, also known as *XPMC2H* and *REX4*, located to the long arm of chromosome 9 (Kwiatkowska *et al*., 1997). Although the variation in this gene emerged as possibly pathogenic, its allele frequency in the ExAC Browser Exome Aggregation Consortium (*http://exac.broadinstitute.orgI*) is 6:1,000, which was considered high for a variant causing this disease. Also, based on the paucity of published data on the function of this gene product, no direct involvement of mutations in this gene can be inferred to contribute to prolactinoma formation and predisposition.

The second gene that emerged as a strong prolactinoma susceptibility gene was the Tyrosine Hydroxylase (*TH*) gene, located to the short arm of chromosome 11 (Craig *et al*., 1986) that carries the *AIP* and *MEN1* genes (Lecoq *et al*., 2015). Not only the type of variant found in this gene and its location made it an attractive candidate prolactinoma formation gene, but also its function. TH enzyme converts L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), the essential and rate-limiting step to formation of dopamine and other catecholamines. Since dopamine released from the hypothalamus negatively regulates the secretion of prolactin (PRL) from the anterior pituitary gland, TH is intimately involved in the prolactin signaling pathway (Bernard *et al*., 2015b).

This result is consistent with as once speculated nature of the inherited mutation in prolactinoma families. It was thought that mutations do not reside in classic oncogenes,

since patients respond well to dopamine-agonist therapy, but might reside in genes that confer increased sensitivity to stimulatory neurohormones (Berezin and Karasik, 1995). It had been also speculated if the functional dopamine uncoupling from $D_2$ receptor could contribute to the development of prolactin (PRL)-secreting pituitary tumors. However, mutations in the coding exons of the $D_2$ could not be demonstrated (Friedman *et al*., 1994), Thus other elements of dopamine pathway might be disrupted in prolactinoma development and TH enters these criteria.

Experimental studies examined the role of TH expression in pituitary cell proliferation and have shown that hyper expression of tyrosine hydroxylase in human lactotroph adenomas enhances dopamine synthesis and diminishes prolactin secretion (Freese *et al*., 1996), as well as suppression of tumor growth (Williams *et al*., 2002). Studies using genetically modified organisms as model systems yielded inconclusive results in terms of teasing out the possible involvement of TH in prolactinoma development. Mice homozygotes for targeted null mutations are catecholamine deficient and usually die of cardiac failure (Kobayashi *et al*., 1995).

Although 62 TH mutations are recorded in *The Human Gene Mutation Database HGMD®* (*http://www.hgmd.cf.ac.uk/ac/index.php*), the p.K474* does not appear in this database. Thus, we suggest that the variant herein described, p.K474*, truncates the protein, leading in turn to reduced dopamine production, with consequent prolactin overexpression. Homozygous or compound heterozygous mutations in this gene have been associated with autosomal recessive Segawa syndrome, a disorder characterized by perinatal severe complex encephalopathy or a progressive hypokinetic-rigid syndrome and dystonia (Ludecke *et al*., 1995; Wijemanne *et al*., 2015), phenotypes clearly not presented by the current family.

The missense variant found in the *RXRG* gene (R317H) was assigned a likely damaging score by several prediction algorithms. Although these are predictions and estimates, it has been shown that protein damage prediction algorithms have ~70% sensibility and ~15% specificity (Flanagan *et al*., 2010).

The *RXRG* gene is located to the long arm of chromosome 1 and encodes a protein member of the steroid/thyroid hormone superfamily of nuclear receptors, called retinoid X receptor gamma (Almasan *et al*., 1994). This gene is highly conserved across species and is expressed at low levels throughout the body, with higher levels in skeletal muscle, pituitary gland and certain areas of the brain. RXRG is involved in diverse cellular processes, from proliferation to metabolism. This receptor forms dimers with the retinoic acid, thyroid hormone, and vitamin D receptors, increasing both DNA binding and transcriptional function on their respective response elements. Noteworthy, the ligand-binding domain of the protein is where p.R317H variant found in this study locates to (Lefebvre *et al*., 2010). This missense heterozygous mutant was previously reported as a somatic mutation in endometrial cancer, but no association to pituitary adenomas was reported (*COSMIC*, *http://cancer.sanger.ac.uk/cosmic*).

It has been shown that RXRG is expressed in pituitary adenomas and may co-localize to Pit-1 (Sanno *et al*., 1999), a pituitary specific transcription factor that binds to and transactivates pituitary hormone genes such as PRL (INGRAHAM *et al*., 1997). Moreover, treatment with retinoic acid has been proved beneficial and well tolerated for Cushing's disease patients, further supporting the possible importance of this pathway to pituitary tumor development (Giraldi *et al*., 2012).

Studies using mice have shown that *RXRG* mutation may lead to premature death and altered responses to the administration of dopamine antagonists (Saga *et al*., 1999). Thus, it is unclear how mechanistically the *RXRG* p.R317H mutation may be involved in

pituitary tumor development but one could speculate that protein expression is needed to negatively regulate pituitary cells growth, and that an inactivating mutation could lead to accelerated proliferation and PRL protein overexpression.

Furthermore, it is interesting to notice that two of these variants (*RXRG and TH*) are not present in the ExAC Browser Exome Aggregation Consortium (*http://exac.broadinstitute.orgI*) and present signaling pathways that could lead to hyperprolactinemia, although these mechanisms should be further investigated. In addition, these possibly pathogenic variants in the *RXRG* and *TH* genes were found concomitantly in the three affected and also in two asymptomatic, normal prolactin levels siblings. The lack of hyperprolactinemia and mass effect symptoms in these two family members at the time of study cannot exclude that they have pituitary microadenoma or that they could manifest the disease later in life. This lack of symptoms could also be explained by incomplete penetrance of found mutations as previously shown for *AIP* in FIPA families (Korbonits *et al*., 2010). Thus, further studies with FIPA families are needed to ascertain the role of these variants alone or in combination in prolactinoma susceptibility and development.

# 6. CONCLUSION

The present study suggests that the two genes previously unrelated to pituitary tumorigenesis *RXRG* and *TH*, may underlie prolactinoma in a Brazilian family. The mechanism by which these genes are associated with pituitary tumor development remains unclear and requires further investigations. The possible contribution of these genes in other unrelated ethnically diverse FIPA families also need to be addressed.

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**UNIVERSIDADE FEDERAL DE MINAS GERAIS**

**PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR**

# ATA DA DEFESA DE TESE DA ALUNA
# FLAVIA MARQUES DE MELO

Realizou-se, no dia 03 de maio de 2016, às 14:00 horas, Faculdade de Medicina da UFMG sala 029, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *Whole-exome identifies germline variants in familial isolated prolactinom*, apresentada por FLAVIA MARQUES DE MELO, número de registro 2012786078, graduada no curso de CIENCIAS BIOLOGICAS/DIURNO, como requisito parcial para a obtenção do grau de Doutor em MEDICINA MOLECULAR, à seguinte Comissão Examinadora: Prof(a). Luiz Armando Cunha de Marco - Orientador (UFMG), Prof(a). Luciana Bastos Rodrigues (UFJF), Prof(a). Bárbara Campolina Carvalho Silva (UniBH), Prof(a). Eduardo Pimentel Dias (UFMG), Prof(a). Eitan Friedman (Tel Aviv University), Prof(a). Maria Marta Sarquis Soares (UFMG).

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.
Belo Horizonte, 03 de maio de 2016.

Prof(a). Luiz Armando Cunha de Marco ( Doutor )

Prof(a). Luciana Bastos Rodrigues ( Doutora )

Prof(a). Eduardo Pimentel Dias ( Doutor )

Prof(a). Barbara Campolina Carvalho Silva ( Doutora )

Prof(a). Eitan Friedman ( Doutor )

Prof(a). Maria Marta Sarquis Soares ( Doutora )

05/05/16

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Faculdade de Medicina - UFMG

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**UNIVERSIDADE FEDERAL DE MINAS GERAIS**

**PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR**

# FOLHA DE APROVAÇÃO

**WHOLE-EXOME IDENTIFIES GERMLINE VARIANTS IN FAMILIAL ISOLATED PROLACTINOM**

## FLAVIA MARQUES DE MELO

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em MEDICINA MOLECULAR, como requisito para obtenção do grau de Doutor em MEDICINA MOLECULAR, área de concentração MEDICINA MOLECULAR.

Aprovada em 03 de maio de 2016, pela banca constituída pelos membros:

Prof(a). Luiz Armando Cunha de Marco - Orientador
UFMG

Prof(a). Luciana Bastos Rodrigues
UFJF

Prof(a). Bárbara Campolina Carvalho Silva
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Prof(a). Eduardo Pimentel Dias
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Belo Horizonte, 3 de maio de 2016.