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Copy Number Alterations of Aggressive Pituitary Neuroendocrine Tumors

A Thesis Presented

by

Grace Zhao Zhang

To the Keck Science Department

of

Claremont McKenna, Scripps, and Pitzer Colleges

In Partial Fulfillment of

The Degree of Bachelor of Arts

Senior Thesis in Biology

December 12th, 2022

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Abstract:

Pituitary neuroendocrine tumors (PitNETs) are tumors of the pituitary gland. Although most are benign, they can cause severe morbidity if compression of surrounding tissue and/or endocrinopathies occur.

Aggressive PitNETs are notably detrimental and difficult to predict, and their effects are further exacerbated by challenges in treatment. Although histological studies can detect certain markers of tumor aggressiveness, they are insufficient at wholly predicting PitNET aggressiveness, making the clinical behavior of PitNETs challenging to determine. Since treatment of aggressive tumors also remains suboptimal, this further results in negative impacts on health and quality of life.

Genetic markers, such as copy number variations (CNVs), could be particularly powerful in detecting PitNET aggressiveness prior to the manifestation of clinical signs of tumor progression. If CNVs are a biomarker for aggressive PitNETs, this would greatly improve their diagnosis and treatment process.

To identify whether CNVs predict PitNET aggressiveness, this retrospective study examined clinical and genetic features of non-functional (NF) PitNETs, prolactinomas, corticotropinomas, and somatotropinomas. DNA extraction and quantification of PitNETs was performed. Pituitary DNA that had undergone whole-genome sequencing (WGS) was previously analyzed for CNVs and data extraction of the respective PitNET patients' clinical charts was performed. Features of clinical aggressiveness were compared to the CNV data. It was found that PitNETs with copy number gains were the most clinically aggressive, and that the subtypes of PitNETs arose from different combinations of copy number gains and losses on different chromosomes. These findings likely reflect results from recently collected pituitary tumor and

blood DNA sent for whole-genome sequencing. This research supports that aggressive PitNETs can be identified by CNVs and suggests that subtypes of aggressive PitNETs arise from different tumor suppressor genes and oncogenes, which supports the notion that PitNETs are heterogeneous. Knowledge of aggressive PitNET heterogeneity would ultimately allow for more effective diagnosis and treatment for aggressive PitNETs.

Introduction:

Pituitary Gland:

The pituitary gland (hypophysis) is a small organ at the base of the brain. By working with the hypothalamus, the pituitary gland regulates many bodily functions through the production of hormones which control many parts of the endocrine system. By regulating endocrine systems, the pituitary gland ensures homeostasis within the body.

Pituitary Gland General Anatomy and Physiology—Posterior Lobe:

The hypothalamus's nerve cells, supraoptic and paraventricular nuclei, produce the hormones released from the posterior pituitary lobe (neurohypophysis), vasopressin/antidiuretic hormone (ADH), and oxytocin (OT), which are transported directly to the posterior lobe through the pituitary stalk and released to their target organs (Daniel, 1976; Ginnard & Nella, 2019).

Pituitary Gland General Anatomy and Physiology—Anterior Lobe:

While the posterior pituitary lobe stores hormones produced by the hypothalamus, the anterior pituitary lobe (adenohypophysis) synthesizes its hormones, regulating its levels through a negative feedback loop. The pituitary stalk connects the pituitary gland to the hypothalamus, which releases regulatory hormones directly to the anterior pituitary through the hypophyseal-portal system, a network of capillaries that connects the hypothalamus to the anterior pituitary lobe, to stimulate production of six main hormones: growth hormone (GH), thyroid-stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL). The acidophils and basophils are the two types of cells that comprise the anterior pituitary that secrete these hormones. TSH, ACTH, FSH, and LH are tropic hormones that stimulate the function of other endocrine glands by entering the bloodstream and flowing to their respective target organs, binding to membrane or nuclear

receptors of these organs, and stimulating them to produce their hormones. These hormones are then sent to their respective target organs. When sufficient hormone has been produced, the hormones secreted by the target glands then communicate back to the hypothalamus and pituitary gland, stopping releasing hormone and tropic hormone production in a negative feedback loop to control hormone production and maintain homeostasis (Figure 1).

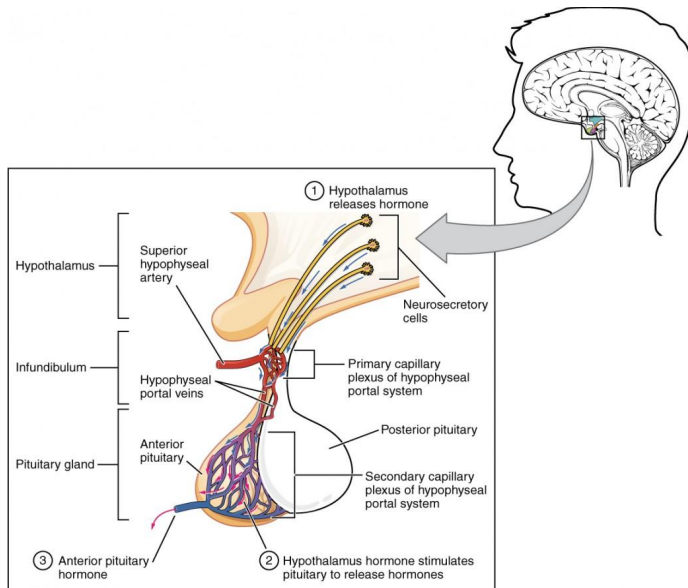


Figure 1. Hypothalamus and Pituitary Gland Feedback Loop; <https://courses.lumenlearning.com/suny-ap2/chapter/the-pituitary-gland-and-hypothalamus/>

While the anterior pituitary also secretes gonadotrophs (follicle-stimulating hormone and luteinizing hormone), which is integral for sexual development, and thyroid-stimulating hormone, which stimulates production of the thyroid hormones, the hormones essential to this study are growth hormone, prolactin, and adrenocorticotropic hormone. These hormones are explained below.

Anterior Pituitary Physiology of Hormones—Growth Hormone (GH):

About 50% of the acidophil is responsible for producing somatotroph cells, which possess a characteristic round shape (Osamura et al., 2009) and secrete growth hormone (GH). The majority of somatotroph cells are located in the lateral wings of the anterior pituitary lobe,

but are also located in the median wedge (Figure 2). The release of GH (somatotropin) is imperative for muscle mass and bone and muscle growth. GH stimulates the rate of protein synthesis and thus body growth in bones and skeletal muscles (Endocrine System, n.d.; Lim et al., 2020; Lumen Learning & OpenStax; Paxton & Knibbs, 1970). The negative feedback loop of GH secretion is regulated through GHRH: GHRH is excreted from the hypothalamus and travels to the anterior pituitary lobe to stimulate the production of GH, which travels to its target organs, including the liver, bone cells, muscle cells, and nervous system cells, to stimulate them to release insulin-like growth factor (IGF-1), which stimulates cartilage, bone and skeletal muscle growth through cellular reproduction, cell death inhibition and amino acid utilization for protein synthesis. High IGF-1 levels inhibit GHRH production and stimulate the GH inhibitor, somatostatin (SRIF), to be released from the hypothalamus. This stops growth hormone production in the pituitary gland to regulate IGF-1 levels and thus body growth (Figure 3).

Anterior Pituitary Physiology of Hormones—Prolactin (PRL):

20% of the acidophil also produces mammatroph (lactotroph) cells, which secrete PRL. The need to develop breast tissue and produce milk during pregnancy increases the amount of lactotroph cells. These cells are primarily localized at the lateral wings of the gland (Figure 2). The primary purpose of PRL produced by lactotroph cells is for normal breast milk production (lactation) by the mammary glands in women, but it also regulates the immune system, reproductive systems, metabolism, behavior and bodily fluids (Endocrine System, n.d.; Lim et al., 2020; Lumen Learning & OpenStax; Paxton & Knibbs, 1970). Immunohistochemistry, the identification of the antigens present in cells through studying antibody-binding to antigens, has detected that both PRL and GH can be co-expressed in certain types of cells (Osamura et al., 2009). Unlike the other tropic hormones of the anterior pituitary, PRL levels are controlled by

inhibition from the hypothalamus (Majumdar & Mangal, 2013). Dopamine from the hypothalamus inhibits PRL production, and high levels of estrogen (in pregnant or lactating females, which promotes the growth of mammary tissue) increase it. PRL secretion stimulates the release of dopamine from the hypothalamus to inhibit prolactin secretion when sufficient PRL has been released (Figure 3).

Anterior Pituitary Physiology of Hormones—Adrenocorticotrophic Hormone (ACTH):

20% of the basophil produces corticotroph cells, which are located in the median mucoid wedge and produce ACTH (Figure 2). Levels of ACTH follow a diurnal rhythm—they are high in the morning and lower as the day progresses. Corticotropin-releasing hormone (CRH) from the hypothalamus is released to stimulate ACTH to be secreted from the anterior pituitary lobe. ACTH flows through the blood to the adrenal glands to signal its outer layer, the adrenal cortex, to produce hormones called corticosteroids, which include glucocorticoids, mineralocorticoids, and androgens. Glucocorticoids are steroid hormones that impact the body's ability to cope with stress, convert food into energy, and modulate the immune system's inflammatory response. This includes cortisol, the main type of glucocorticoid. Mineralocorticoids influence the balance of sodium and potassium, and therefore electrolyte and fluid balance, to maintain normal blood pressure levels. Lastly, androgens are sex hormones that affect muscle mass, sexual desire, sense of wellbeing in men and women, and sexual development in men (Endocrine System, n.d.; Lim et al., 2020; Lumen Learning & OpenStax; Paxton & Knibbs, 1970). Glucocorticoids released from the adrenal glands complete a negative feedback loop by stopping the hypothalamus from producing CRH, which stops the pituitary gland from producing ACTH and thus the adrenal glands from producing corticosteroids (Figure 3).

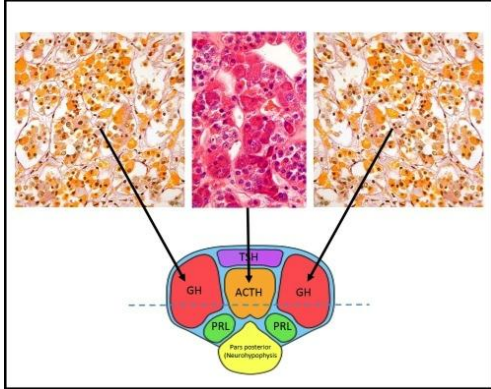


Figure 2. Axial Section of the Anterior Pituitary Lobe (Adenohypophysis) Demonstrating the Anatomy of Neuroendocrine Cells through Histochemical Staining; <https://www.ncbi.nlm.nih.gov/books/NBK425703/>

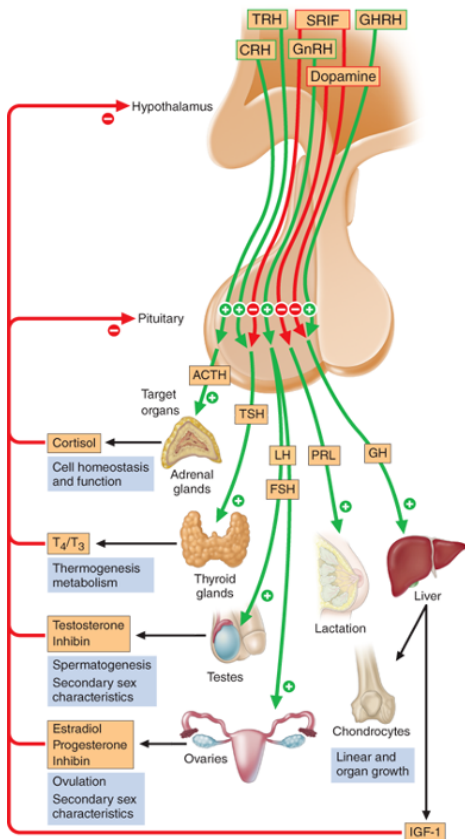


Figure 3. Hypothalamic Hormones and Anterior Pituitary Hormone Negative Feedback Loop; <https://accessmedicine.mhmedical.com/content.aspx?bookid=1130§ionid=79751425#1139425051>

Anterior Pituitary Physiology of Hormones—Summary

The hypothalamic peptide hormones GHRH and SRIF, CRH, influence the release of the anterior pituitary hormones GH, ACTH, PRL, which then promote the release of hormones from

their respective target glands and other metabolites (Asa & Ezzat, 2002; Sadow & Rubin, 1992). The pituitary gland is necessary for many functions in the body, including growth, metabolism, response to stress, and water/electrolyte balance, and is therefore an essential structure of the brain.

Pituitary Neuroendocrine Tumors (PitNETs)—Overview:

Pituitary neuroendocrine tumors (PitNETs) are neuroendocrine neoplasms which grow in the base of the brain in the sella turcica that holds the pituitary gland, and they can have detrimental effects by affecting the regulation of hormones and other systems in the body.

PitNETs are one of the most common types of intracranial tumors, accounting for 10-15% of all intracranial tumors (Asa et al., 2022; Brue and Castinetti, 2016; Vankelecom and Roose, 2017).

Pituitary neuroendocrine tumors form from neuroendocrine epithelial cells (Larkin & Ansorge, 2017). Most PitNETs form in the adenohypophysis, which contains the anterior and intermediate lobes of the pituitary gland (Asa & Ezzat, 2002). They likely arise from early progenitor or fully differentiated hormone-expressing cells, and disrupt signaling pathways, including those related to mitochondrial function, oxidative stress, mitogen-activated protein kinase signaling and cell cycle regulation (Melmed, 2011). Depending on the subtype of pituitary tumor and thus the tumor's cells of origin, functional/secretory PitNETs surround the pituitary gland and cause hypersecretion of hormones, while non-functional PitNETs do not.

Pituitary Neuroendocrine Tumor (PitNET)—General Phenotype:

The clinical phenotype of pituitary tumors is determined by their differentiated hormone-expressing cell type (Figure 4). The four types of pituitary neuroendocrine tumors analyzed in this study are corticotropinomas, somatotropinomas, prolactinomas, and non-secretory/non-functioning (NF) pituitary neuroendocrine tumors. While non-functioning pituitary

neuroendocrine tumors do not secrete excess hormone, functional (secretory) pituitary neuroendocrine tumors arise from their respective endogenous cell types and over-secrete hormones, impacting normal pituitary function (Larkin & Ansorge, 2017).

Pituitary Neuroendocrine Tumor (PitNET)—Phenotypes by Subtype:

Depending on the type of pituitary tumor, different hormones will be secreted and severe clinical complications could be observed. Their slow growth and non-specific clinical symptoms often worsen patient outcomes through inefficient diagnosis (Brue and Castinetti, 2016; Russ, Anastasopoulou, & Shafiq, 2022).

Corticotropinomas are ACTH-secreting pituitary neuroendocrine tumors that lead to Cushing's syndrome, the condition of prolonged exposure to excess cortisol. Although glucocorticoids from the adrenal glands will inhibit CRH production in the hypothalamus in a negative feedback loop to control ACTH and glucocorticoid production, if a corticotropinoma is present, its cells continuously secrete ACTH, causing excess ACTH to be made, leading to hypercortisolism (HCM), defined as excess exposure of tissue to cortisol and/or other related glucocorticoids. This eventually results in Cushing's disease, and is often, but not always, associated with high serum cortisol (hypercortisolemia), and can lead to the development of hypertension, diabetes mellitus, and bone loss (Uwaifo & Hura, 2022). Common symptoms of hypercortisolism include weight gain, muscle weakness, and mood disorders (Asa et al., 2022; American Cancer Society, 2022; Russ, Anastasopoulou, & Shafiq, 2022).

In contrast, somatotropinomas are GH-secreting pituitary neuroendocrine tumors, which therefore cause acromegaly, the condition of exposure to high levels of growth hormone secreted from the pituitary gland. Although high IGF-1 levels regulate the amount of GH in the body by suppressing its production in the pituitary gland and stimulating somatostatin production in the

hypothalamus, GH-secreting pituitary neuroendocrine tumors will continuously secrete GH. This causes acromegaly, defined as abnormally high levels of GH. Because both PRL and GH can be coexpressed in the same cells for certain types of cells (Osamura et al., 2009), these tumors may also variably secrete PRL, causing hyperprolactinemia, high levels of prolactin (Osamura et al., 2009). Excessive sweating, joint pain, enlarged hands and feet, and coarse facial features are common symptoms of acromegaly (Asa et al., 2022; American Cancer Society, 2022; Russ, Anastasopoulou, & Shafiq, 2022). Abnormally high levels of GH and IGF-I also increase the likelihood of development of diabetes mellitus, hypertension, obesity, obstructive sleep apnea syndrome, and heart disease, and acromegaly is therefore associated with high mortality and a low quality of life (Biermasz et al., 2005).

The most common type of PitNET, prolactinomas, are PRL-secreting pituitary neuroendocrine tumors that cause hyperprolactinemia: high levels of prolactin. This can cause galactorrhea, abnormal milky discharge from the nipples, and suppresses gonadotropin (LH and FSH), causing low estrogen and progesterone levels, resulting in infertility and oligomenorrhea/amenorrhea, irregular menstrual cycles. A major consequence of low estrogen is bone loss, causing increased risk of osteoporosis (Majumdar & Mangal, 2013). Both sexes may also encounter decreased libido, and women may also experience vaginal dryness, while men may encounter decreased sexual desire and breast enlargement (Brue and Castinetti, 2016; Russ, Anastasopoulou, & Shafiq, 2022). Such effects significantly decrease prolactinoma patients' quality of life.

Although NF tumors are pituitary neuroendocrine tumors that comprise various subtypes (identified through immunostaining of adenohypophyseal hormones), unlike secretory PitNETs, NF PitNETs are not associated with production of biochemical levels of hormones and are

therefore called “silent” PitNETs. The most common subtype of NF pituitary neuroendocrine tumor is the silent gonadotroph PitNET (which stains for follicle-stimulating hormone/luteinizing hormone), followed by silent corticotroph tumors, PIT1 (POU1F1) gene lineage, and null cell tumors, which are tumors made of adenohypophyseal cells in which immunohistochemistry is unable to detect any cell type specific differentiation nor transcription factors (Drummond et al., 2022). Although NF PitNETs do not secrete excess hormone, non-secretory tumors, along with secretory tumors, can grow into large macrotumors, causing mass effect (increased pressure on adjacent structures in the brain) from tumor growth, impacting other structures in the brain. This can cause headaches from increased pressure on nerves in the brain, visual impairment from compression of the optic apparatus, and/or loss of normal pituitary function, which is called hypopituitarism (Figure 5; Asa et al., 2022; Brue and Castinetti, 2016; Dai et al., 2021; Elkington, 1968; Freda et al., 2011; Russ, Anastasopoulou, & Shafiq, 2022; Tatsi & Stratakis, 2019).

Pituitary neuroendocrine tumors significantly decrease patients’ quality of life, and such nonspecific clinical symptoms cause challenges in providing correct diagnoses of pituitary neuroendocrine tumor patients’ conditions. This further delays their treatment, worsening their condition due to progression of disease. Although pituitary neuroendocrine tumors generally remain benign and rarely develop into their malignant form, carcinomas, and metastasize (Pernicone et al., 1997), they can have drastic effects if invasion of nearby anatomical structures and/or abnormal endocrine levels manifest.

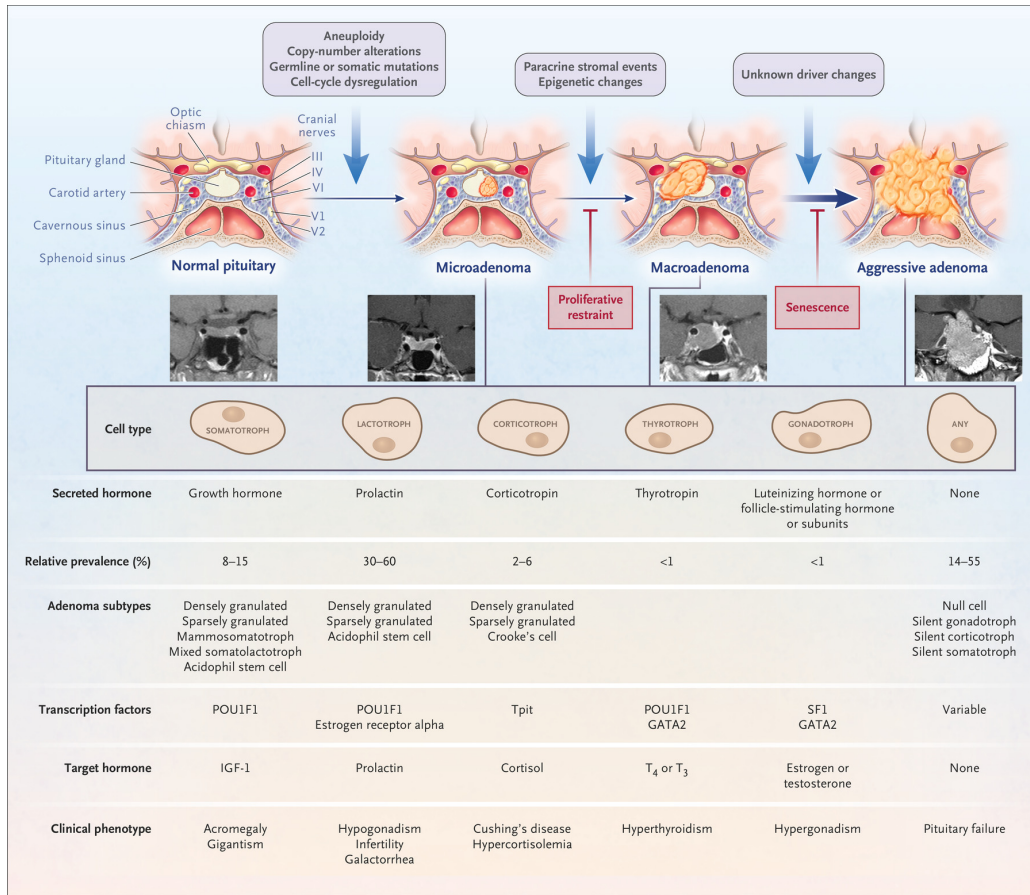


Figure 4. Pituitary Neuroendocrine Tumor Pathogenesis; <https://www.nejm.org/doi/10.1056/NEJMra1810772>

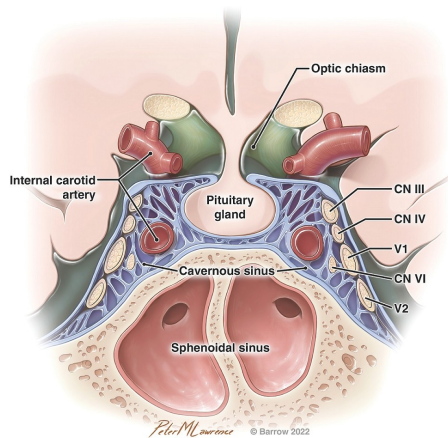


Figure 5. Anatomy of Pituitary Gland Location; <https://www.barrowneuro.org/resource/about-the-pituitary-gland/>

Aggressive Pituitary Neuroendocrine Tumors (PitNETs)—Overview:

Aggressive pituitary neuroendocrine tumors comprise 10% of all pituitary neuroendocrine tumors and have detrimental effects (Kasuki & Raverot, 2020).

Functional/secretory pituitary tumors that are aggressive are characterized by invasive expansion to surrounding structures, recurrent disease due to rapid growth and resistance to conventional therapies (including surgery, radiotherapy, and medication), failure to biochemically cure disease, and persistent hormone production (thus elevated hormone levels). Because they do not secrete excess hormone, aggressive nonfunctional pituitary neuroendocrine tumors are characterized by tumor growth, which can cause recurrent disease (Chatzellis et al., 2015; Dai et al., 2021; Kasuki & Raverot, 2020; Raverot et al., 2018). This can be visualized through invasion into nearby structures, including cavernous sinus invasion, bony invasion, mucosal invasion, and dural invasion.

Aggressive Pituitary Neuroendocrine Tumors (PitNETs)—Difficulties in Treatment:

Aggressive pituitary tumor treatment is inadequate, exacerbating their effects on the body. Because resistance to therapies and tumor recurrence are characteristic of aggressive pituitary neuroendocrine tumors, their treatment is increasingly challenging (Molitch, 2017; Chang et al., 2021). Repeated targeted therapies are often used, such as repeat surgery in conjunction with subsequent radiotherapy or chemotherapy. Such repeated treatments are often detrimental to quality of life. After 3-4 surgeries, potential assistance in tumor resection diminishes (Forster et al., 2018). Additionally, although repeat radiotherapy can be used when medical therapy is ineffective or intolerable, 25%-90% of patients who undergo radiotherapy develop anterior hypopituitarism, and repeat radiotherapy greatly increases the risk of radiation necrosis (death of tissue) or the destruction of the nearby structures, including the carotid artery wall. Medication therapy is also a common modality of treatment for PitNETs and is the first line of treatment for prolactinomas. Dopamine agonist therapies, such as bromocriptine and cabergoline, decrease levels of prolactin and often decrease the size of prolactinomas.

Pegvisomant and somatostatin analogs, such as octreotide, are often used for GH-secreting neuroendocrine tumors. Pegvisomant inhibits the effects of over-secretion of growth hormone, while somatostatin analogs, including lanreotide and octreotide, reduce production of growth hormone and may decrease somatotropinoma size. Lastly, ketoconazole, including nizoral, is used to treat corticotropinomas by reducing levels of cortisol. However, ketoconazole does not affect the growth of corticotropinomas, nor does it decrease ACTH production. Instead, somatostatin analogues or steroidogenesis inhibitors are used. If patients are unresponsive to such conventional therapies, temozolomide (TMZ) therapy is often used (Lee, 2022). It has been the most widely-used option for aggressive PitNETs, as patients who respond to treatment have significant improvement in their five-year survival rate. However, only one third of recipients demonstrate partial or complete response to the first course of TMZ and repeat TMZ treatment is often less effective than the first. TMZ also causes severe side effects that occur in 10% of users, including increased risk of infection, nausea, vomiting, constipation/diarrhea, weakness, seizures (Syro et al., 2018; *Temozlomide (Temodal)*, 2019). Although other therapies have been developed to treat aggressive PitNETs, such as immune checkpoint inhibitors, peptide receptor radionuclide therapy (PRRT), and vascular endothelial growth factor receptor-targeted therapy (VEGF-targeted therapy), the efficacy of these treatments remains uncertain (Cooper, Bonert, & Mamelak, 2021; Ilie, Lasolle, & Raverot, 2019). Similarly, response to SRL therapy and EGFR- and MTOR-targeting therapy is also inconsistent (Cooper, Bonert, & Mamelak, 2021). Patients with aggressive pituitary neuroendocrine tumors through the manifestation of clinical symptoms are advised to receive care from a multidisciplinary team of endocrinologists, neurosurgeons, radiation oncologists, neuro-radiologists and neuro-ophthalmologists so that they can partner together to provide the most optimal care possible for aggressive pituitary tumor patients

(Casanueva et al., 2017; Freda et al., 2011; Melmed, 2020). Despite persistent treatments, aggressive pituitary neuroendocrine tumors tend to progress over time, with the 10-year recurrence rate of pituitary neuroendocrine tumors being 7-12% (Chang et al., 2021; Salomon et al., 2018; Reddy et al., 2011). Therefore, the variability in aggressive pituitary tumor response to different treatments is not always promising and consequently could worsen patients' overall health.

Aggressive Pituitary Neuroendocrine Tumors (PitNETs)—Difficulties in Diagnosis:

Aggressive pituitary neuroendocrine tumors are identified through clinical markers (Kasuki & Raverot, 2020), making aggressiveness impossible to absolutely predict and difficult to manage. After PitNETs are surgically resected, there is not an assured methodology to identify whether or not the PitNET is aggressive and will therefore present with the aforementioned complications. Pituitary tumor patients are reexamined for progression of disease through both histological studies that stain tumor tissue for microscopic study and continuous assessment of pituitary hormone levels to analyze if oversecretion of hormone is present from a secretory pituitary tumor. Additionally, histological invasion of surrounding structures, including cavernous sinus invasion, bony invasion, mucosal invasion, and dural invasion, can also be detected through histological studies. However, neither histological studies nor examination of hormone levels accurately depict the aggressiveness of pituitary neuroendocrine tumors (Chatzellis et al., 2015; Dai et al., 2021). For example, high Ki-67 is a measure of active proliferation of tumor cells (Zhang et al., 2021), while abnormal expression of the p53 protein is indicative of TP53 missense mutations, a marker of cancer (Köbel et al., 2019). However, approximately 20% of aggressive PitNETs and pituitary carcinomas demonstrate low Ki-67 index and negative p53 immunohistochemical staining (McCormack et al., 2018), demonstrating

that although such markers from immunohistochemical staining are related to risk of recurrence (Rutkowski et al., 2018), which is a marker of tumor aggressiveness, they do not perfectly predict aggressive PitNET behavior. Therefore, unlike other neuroendocrine tumors, pituitary neuroendocrine tumors cannot be classified into grades by their Ki-67 proliferation index due to more effective clinical biomarkers of aggressiveness explained prior (Asa et al., 2022, Dekkers et al., 2020). Instead, aggressive pituitary neuroendocrine tumors persist despite conventional therapies. Therefore, although patients' histological studies and hormonal evaluations may appear normal, their lesions may continue to grow. Examination of patients' clinical and radiographic state are the only viable ways to analyze aggressiveness of the pituitary tumor. Hence, analysis of clinical and radiographic features are analyses conducted during follow-up of patients' health, which does not allow the ability to effectively determine pituitary neuroendocrine tumor progression prior to its clinical manifestations.

Copy Number Variations (CNVs) as a Potential Genetic Biomarker of Aggressive PitNETs:

PitNETs cause significant clinical issues, and aggressive PitNETs cannot be identified prior to manifestation of clinical symptoms and are often resistant to conventional treatment. It is therefore crucial to diagnose pituitary tumors as aggressive or non-aggressive prior to the manifestation of clinical/radiographic symptoms to optimize the efficacy of treatment. However, current technologies are insufficient for identification of aggressive pituitary tumors prior to the development of aggressive clinical symptoms. Copy number variations (CNVs)/copy number alterations (CNAs) may be an identifying factor of aggressiveness in pituitary neuroendocrine tumors. Copy number variations are changes in the structures of chromosomes wherein alterations in the number of copies of DNA sequences (segments of genes) occur in the genome. As compared to single nucleotide variants (SNVs) wherein individual nucleotides are altered and

insertions/deletions (InDels), in which small (less than one kilobase pair long) insertions or deletions of regions of chromosomes occur, copy number variations (CNVs) are stretches of DNA that are one kilobase or longer that have changed in number. Typically, CNVs are either genetic amplifications in which there are greater than two copies of the gene in the genome, or deletions, in which there are less than two copies of the gene in the genome (Hastings et al., 2009; Pfaffl, n.d). In addition to being associated with cancer through differential gene expression (Shao et al., 2019), CNVs have been found in large parts of the genome of pituitary neuroendocrine tumors. Additionally, tumors with CNVs have been found to have higher gene expression levels and are associated with poor prognosis (Bi et al., 2017). This supports the notion that CNVs may be a biomarker for pituitary neuroendocrine tumor aggressiveness.

The Present Study–Purpose and Predictions:

This study analyzed pituitary neuroendocrine tumors at the genome level to determine if chromosomal copy number variations predict aggressiveness in pituitary neuroendocrine tumors. I hypothesized that copy number variations of pituitary neuroendocrine tumors predict aggressiveness of pituitary neuroendocrine tumors.

It has been found that prolactinomas with CNVs are more likely to recur (Chen et al., 2020; Lasolle et al., 2020), losses of chromosome arms 1p and 11p (losses in the copies of genetic DNA segments in the short arms (p) of chromosomes 1 and 11) are significant CNVs of secretory and atypical PitNETs (Bi et al., 2017; Pack et al., 2005; Szymas et al., 2002), and that allelic loss of chromosome 11p is mostly seen in prolactinomas (Chatzellis et al., 2015). Therefore, I predicted that genetic deletions in chromosomes 1 and 11 predict aggressiveness in prolactinomas, somatotropinomas and corticotropinomas. I also predicted that CNVs in

chromosome 11 are the driving mutations at the chromosomal level that caused most aggressive prolactinomas.

It has also been found that recurrent neuroendocrine tumors have more alterations than primary tumors, especially DNA gains (Szymas et al., 2002). Also, invasive neuroendocrine tumors were found to carry more overrepresentations at chromosome 1p34 than non-invasive neuroendocrine tumors (Szymas et al., 2002). From our study's previous whole-genome sequencing (WGS) analysis, common CNV amplifications were found in chromosomes 3, 5, 7, 8, 9, 12, and 14. Thus, I also predicted that copy number amplifications in chromosomes 1, 3, 5, 7, 8, 9, 12, and 14 predict aggressiveness in somatotropinomas, prolactinomas, corticotropinomas, and NF pituitary neuroendocrine tumors.

Methods:

Summary:

In The Ferreira Lab, we focus on connecting genetic alterations to clinical pathologies with the goal of discovering novel treatments and biological targets for treatments of disease. Following surgical resection, tumor tissue and blood samples of patients who underwent surgery for pituitary neuroendocrine tumors were collected. The DNA of 16 pituitary tumor tissue and 20 blood samples was extracted and 200 pituitary DNA samples were quantified to be prepared for future sequencing. 129 samples of previously-extracted pituitary DNA were used for multiple experiments, including whole-exome sequencing (WES), whole-genome sequencing (WGS), and other experiments. Whole-genome sequencing data was analyzed for copy number variations. The medical records of 72 pituitary neuroendocrine tumor patient surgical cases (11 non-functioning neuroendocrine tumors, 20 prolactinomas, 19 corticotropinomas, and 22 somatotropinomas) between 2001 and 2021 at the University of Washington Medical Center in Seattle, Washington and affiliated hospitals whose tumors underwent whole-genome sequencing were retrospectively reviewed. Patient demographics and clinical outcomes were collected. Copy number variation amplifications and deletions were examined in relation to pituitary neuroendocrine tumor clinical characteristics to identify genetic biomarkers of aggressive pituitary neuroendocrine tumors by subtype.

Tumor Tissue and Blood Collection and Preservation:

Pituitary tumors were resected by the neurosurgical team at The University of Washington affiliated hospitals. With subjects' consent to donate tumor tissue and/or blood, and tumor-related information for medical research purposes, the tumor and blood specimens were collected from the operating room to be frozen in liquid nitrogen for research related to somatic

and germline DNA extractions. All specimens collected for research were obtained with appropriate consent and protocols were reviewed and approved by the University of Washington Institutional Human Subjects Division Review Board.

DNA Extraction:

DNA extraction was performed on frozen pituitary tumor tissue and blood utilizing the QS GeneRead DNA FFPE (Qiagen) protocol. Manual extraction of the DNA of 20 pituitary blood samples and 16 pituitary tissue samples was performed. Modifications of tissue extraction included use of a tube rotator to disperse the sample during overnight incubation and an optimized volume of 80 μ L of nuclease-free (distilled) water for elutions, which were repeated twice. DNA was then quantified using the Qubit Fluorometric Quantification protocol. Quantification of 200 pituitary DNA samples was performed for future whole-exome sequencing and other research techniques. If the measured volume was lower than the desired volume, identical DNA samples from the same source (i.e., blood or tissue) were pooled together by pipetting duplicated or triplicated DNA samples into a single sample tube. If the measured volume was higher than the desired volume, DNA samples were placed into a preheated Savant SpeedVac Vacuum Concentrator to reach the optimal volume.

Copy Number Alterations (CNAs) in Cases of Pituitary Neuroendocrine Tumors (PitNETs)

Using Whole-Genome Sequencing (WGS) Analysis:

To find if there were common copy number alterations that can explain the pathogenesis of all these tumors, the DNA of pituitary blood and tumor tissue was sent for whole-genome sequencing. Whole-genome sequencing was conducted on 129 PitNETs at 4x depth and 90% coverage, utilizing the standard Genome Analysis Tool Kit pipeline to align the sequencing reads to the reference genome. BAM files for all 129 cases were generated. Control-FREEC software

program was utilized to call the copy number variations from the BAM files. Genomic Identification of Significant Targets in Cancer (GISTIC) software program was utilized to identify the driver copy number alterations, using the notion that they would occur more frequently in tumors than the passenger copy number alterations.

These analyses were conducted separately on the four subtypes of pituitary neuroendocrine tumors utilized for the present study: somatotropinomas, prolactinomas, corticotropinomas, and non-functional PitNETs. Each heatmap depicted the significant copy number alterations and their genomic coordinates. The map was thoroughly analyzed to extract significant partial and whole chromosome copy number gains and losses.

Patient Clinical Data Extraction:

Clinical endpoints of patients whose pituitary tumors underwent WGS were extracted from patient charts in the UW Medicine's medical database, Epic, and recorded in a database dedicated for research purposes. Demographic endpoints, including sex and race, were found from the main patient chart. The neuropathology report number, age at surgery, date of surgery, tumor pathology, pituitary pathology notes, immunohistochemical staining of pancytokeratin, notes on pancytokeratin staining, synaptophysin, p53, ki67, GH, ACTH, PRL/GH, TSH, LH/FSH immunohistochemistry, and mucosal, dural, bony, histological cavernous sinus invasion were found from neuropathological reports. The patient's type of surgery, gross total cavernous sinus invasion, and pituitary gland extraction were found through examination of patients' operative reports. Prior treatments, including prior surgery, prior surgery date, prior radiation therapy and type, prior medications and type; pre-op endocrine labs, post-op endocrinopathies, MRI-based knosp scores, assessment for biochemical cure, additional therapy after surgery, and pre-op symptoms, including headaches, visual field fullness, syndromes, apoplexy, and other

endocrinopathies/symptoms were found from patients' progress notes. Genetic tests that had been performed on samples, including WGS, low-pass WGS, whole-exome sequencing (WES), validation panel sequencing, RNA sequencing, single-cell RNA sequencing, and cell-free DNA sequencing, were also listed. The clinical markers of biochemical remission, persistent disease despite surgical/medical/radiation therapy, and invasion into surrounding structures (gross cavernous sinus invasion, mucosal invasion, dural invasion, and bony invasion) were used to assess tumor aggressiveness. The number of clinical markers of aggressiveness that each case reflected was used to classify the tumor's level of aggressiveness.

Results:

The results of the CNV heatmap from the WGS of 72 pituitary samples (Figure 6) and the corresponding clinical data of the patient cases were thoroughly analyzed to discern whether CNVs are important markers which reflect the pathogenesis and aggressiveness of PitNETs.

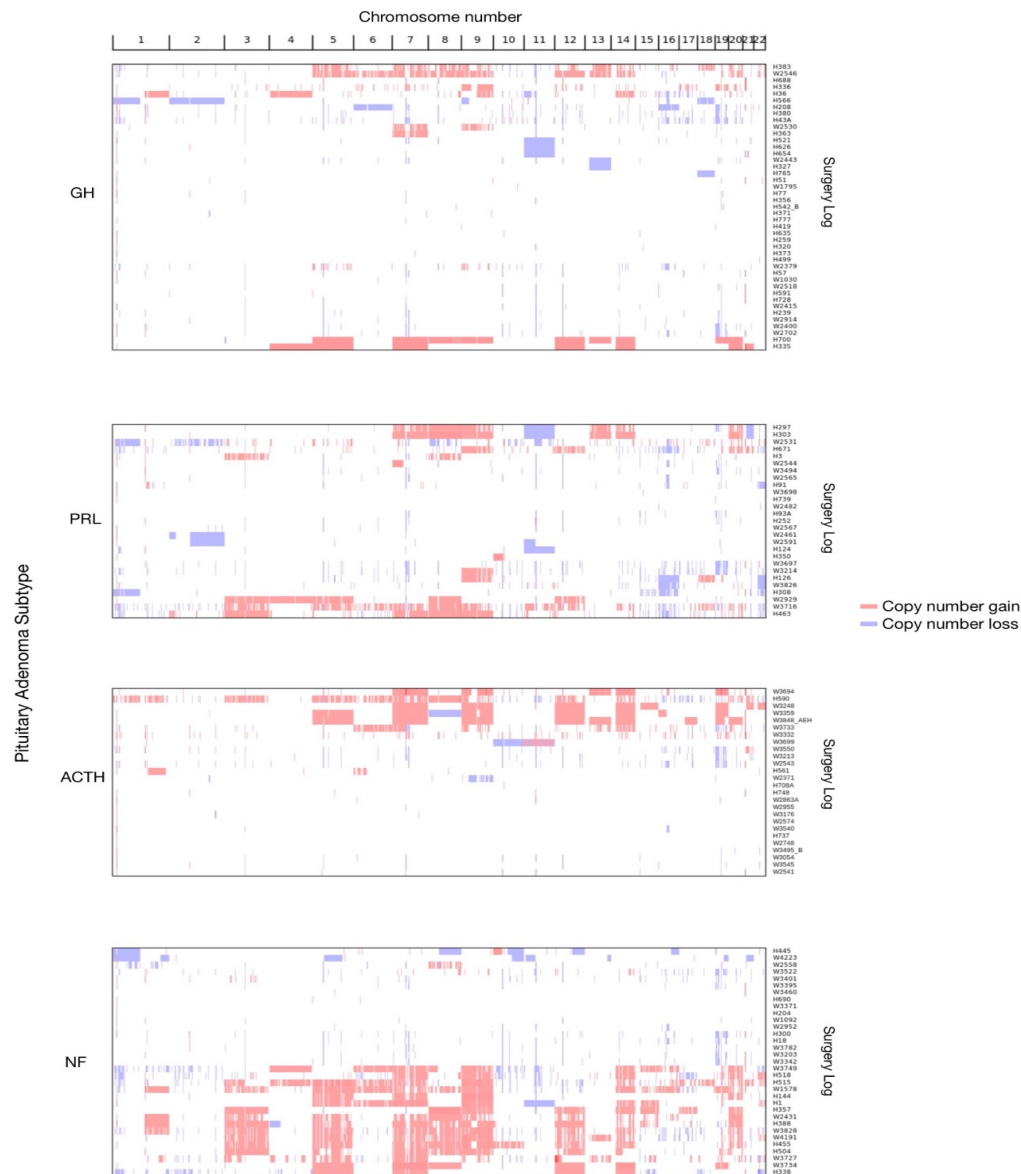


Figure 6. Copy Number Alteration Analysis of PitNETs; Retrieved from The Ferreira Lab

Copy number gains and losses that were detected on all pairs of autosomes (chromosomes 1-22) were correlated with patient outcomes to identify if and where CNVs were associated with aggressive features of PitNETs. It was found that pituitary neuroendocrine

tumors with genomic copy number variations were associated with aggressive features.

Conversely, tumors without copy number alterations were found to be non-aggressive. A total of 34 out of 72 total pituitary neuroendocrine tumor cases were classified as aggressive/very aggressive. This consisted of 4 out of 11 NF PitNETs (Table 1), 10 out of 20 prolactinomas (Table 2), 11 out of 19 corticotropinomas (Table 3), and 9 out of 22 somatotropinomas (Table 4).

Surgery Log	CNV Type	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22	Features of Aggressiveness	Aggressive? 0 = No; 1 = Mildly Aggressive; 2 = Aggressive; 3 = Very Aggressive	
W1030																								Macroadenoma; Additional therapy after surgery (Radiation); No cure	2	
W1795																								No cure? (lost to follow up)	0	
W2379	Losses																partial chr.								Macroadenoma; Gross cavernous sinus invasion	1
W2400	Losses																								Prior med (resistant to cabergoline); Macroadenoma	1
W2415																									Macroadenoma; Discordant cure; (has permanent DI and AI)	1
W2443	Losses														whole chr.										Giant macroadenoma; Discordant cure; Gross cavernous sinus invasion	2
W2518																									Macroadenoma	0
W2530	Gains							whole chr.	whole chr.	whole chr.	whole chr.														Giant NF macroadenoma (3.5 cm)	1
W2546	Gains					whole chr.	whole chr.	whole chr.	whole chr.				whole chr.	whole chr.	whole chr.										Hemorrhagic 2.5 cm macroadenoma	1
W2702	Losses																								2 Prior Surgeries (this surgery is another recurrence and needed an ETSS and craniotomy); Gross total cavernous sinus invasion	3
W2914																									Additional therapy after surgery (TSS on 4/19/21 for residual tumor)	2

Table 1. CNVs of All NF PitNETs

Surgery Log	CNV Type	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22	Features of Aggressiveness	Aggressive? 0 = No; 1 = Mildly Aggressive; 2 = Aggressive; 3 = Very Aggressive		
W1092																									Prior surgery; Hemorrhagic macroadenoma; No cure, Additional therapy after surgery (radiation)	3	
W1578	Gains	partial chr.		whole chr.		whole chr.	whole chr.	whole chr.	whole chr.	whole chr.					whole chr.	partial chr.	whole chr.				whole chr.	partial chr.			2.9 x 2.4 x 1.7 cm Macroadenoma; Additional therapy after surgery (Prolactinoma on Cabergoline, which makes residual tumor stable/no growth); Bony invasion; Gross cavernous sinus invasion	2	
H1	Gains and Losses					whole chr.	whole chr.	whole chr.	whole chr.			whole chr.			whole chr.	whole chr.								partial chr.	Prior meds (was resistant to cabergoline and bromocriptine); Macroadenoma; Gross cavernous sinus invasion	2	
W2431	Gains			whole chr.		whole chr.		whole chr.	whole chr.	whole chr.				whole chr.	whole chr.		partial chr.								Prior med (resistant to cabergoline); Aggressive giant macroadenoma; Additional therapy after surgery (TSS and XRT and cabergoline); No cure	3	
W2558	Gains and Losses	partial chr.							whole chr.																	Hemorrhagic lesion	1
W2952																									Prior med (resistant to cabergoline); Gross cavernous sinus invasion	1	
W3203	Losses																								Prior med (intolerant to cabergoline; resistant to bromocriptine); Gross cavernous sinus invasion	1	
W3342	Losses																								Prior med (intolerant to cabergoline); Macroadenoma	1	
W3371																									Prior med (resistant to bromocriptine, and then also cabergoline after); No cure; Additional therapy after surgery (bromocriptine, maybe NF (as of 1/29/2019))	3	
W3395	Losses																									Prior med (intolerant of cabergoline)	1
W3401	Losses																								Giant macroadenoma invading cavernous sinus and skull base erosion; Prior med (octreotide; cabergoline (was placed on cabergoline with very good response, but presented with a CSF leak from rapid tumor shrinkage; he has continue with CSF leak.) BUT stabilizing levels suggest medication resistance); No cure; Additional therapy after surgery (cabergoline); and possible histological bony and mucosal invasion	3	
W3460																									Prior med (cabergoline (The prolactin and testosterone normalized, but serial imaging demonstrated that the adenoma continued to grow.); Macroadenoma (1.5 cm); No cure; Maybe Histological bony invasion; Gross cavernous sinus invasion	2	
W3522	Losses																								Growth of adenoma from 0.9 x 0.6 x 0.9cm on 4/17/2017 to 1.4 x 1.3 x 1.4cm on 3/18/2019 (thus 1/2 cm in 2 years); Gross cavernous sinus invasion	1	
W3727	Gains			partial chr.	partial chr.	partial chr.		whole chr.		partial chr.			partial chr.	partial chr.		partial chr.									Prior med (resistant to cabergoline); Macroadenoma (4.9 x 3.1 x 2.4 cm); Additional therapy after surgery (temozolomide concurrently with radiation; Histological bony invasion; Gross cavernous sinus invasion	3	
W3734	Gains					whole chr.		whole chr.	whole chr.				whole chr.		whole chr.											Prior med (resistant to cabergoline); Gross cavernous sinus invasion	1
W3749	Gains and Losses	partial chr.	partial chr.		whole chr.		whole chr.	whole chr.	partial chr.	whole chr.	partial chr.	partial chr.			whole chr.	whole chr.	partial chr.									Large macroadenoma	0
W3782	Losses																								Large hemorrhagic and apoplectic macroadenoma (2 x 3 cm)	1	
W3828	Gains and Losses	partial chr.		whole chr.		whole chr.		whole chr.	whole chr.	whole chr.				whole chr.	whole chr.		partial chr.	partial chr.								Prior med (resistant to cabergoline and bromocriptine); No cure (was on DA, fibrous tumor, needed GK) and then Additional therapy after surgery (9/22/2020: SRS (stereotactic radiation) to residual pituitary adenoma, (19 Gy to 50% IDL) and Bromocriptine); Histological bony invasion; Gross cavernous sinus invasion	3
W4191	Gains			whole chr.		whole chr.		whole chr.	whole chr.	whole chr.				whole chr.	whole chr.	whole chr.	partial chr.	partial chr.								Hemorrhagic macroadenoma (2.8 cm); No cure (on DA); thus Additional therapy after surgery (cabergoline since April 2021)	2
W4223	Losses	partial chr.				partial chr.						partial chr.	partial chr.													Macroadenoma; Gross cavernous sinus invasion	1

Table 2. CNVs of All Prolactinomas

Surgery Log	CNV Type	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22	Features of Aggressiveness	Aggressive? 0 = No; 1 = Mildly Aggressive; 2 = Aggressive; 3 = Very Aggressive	
W2371	Losses									whole chr.															Prior surgery (2 adrenalectomies: 1991, 1993); Gross cavernous sinus invasion	1
W2541																	partial chr.			partial chr.					No cure; Additional therapy after surgery (recurrence, thus had repeat TSS, then no cure, thus did bilateral adrenalectomy); Gross cavernous sinus invasion	2
W2543	Losses																								Prior surgery (2011) (this current case is a recurrence); No cure; Additional therapy after surgery (2 TSS that were unsuccessful, thus had bilateral adrenalectomy for persistent and aggressive Cushing's); Gross cavernous sinus invasion	3
W2574																									Macroadenoma; Histological dural invasion; Gross cavernous sinus invasion	2
W2748																									Macroadenoma	1
W2955																									Prior surgery (TSS in 2011); Prior med (cabergoline-NOT resistant); No cure; Additional therapy after surgery (radiation); Gross cavernous sinus invasion	2
W3054																									Prior Surgery (TSS, without cure); this surgery was a craniotomy and ETSS; Gross cavernous sinus invasion	2
W3176																									Prior surgery (TSS); this surgery is a recurrence; Gross cavernous sinus invasion	2
W3213	Losses																				partial chr.				Gross cavernous sinus invasion	1
W3248	Gains								whole chr.	whole chr.			whole chr.	whole chr.	whole chr.						whole chr.	whole chr.	partial chr.		Gross cavernous sinus invasion	1
W3332																									0	0
W3359	Gains and Losses					whole chr.	whole chr.	whole chr.	whole chr.			whole chr.	whole chr.	whole chr.	whole chr.	partial chr.					whole chr.				2 Prior surgeries (not resectable because of cavernous sinus invasion). Prior XRT (Cyberknife radiosurgery); Prior med (resistant to temozolomide); Macroadenoma 3.1 x 2.0 x 1.7 cm; No cure; Additional therapy after surgery (surgery, proton radiation, gamma knife, temozolomide, adjuvant temozolomide); Histological bony invasion	3
W3540																									Macroadenoma; No cure; Additional therapy after surgery (ETSS x 2 + Crani, s/p GK); Gross cavernous sinus invasion	3
W3545																									Prior surgery (ETSS); this surgery was a recurrence (thus persistent disease); No cure; Additional therapy after surgery (spironolactone medical therapy, likely gamma knife treatment); Gross cavernous sinus invasion	3
W3550	Gains and Losses																				partial chr.	partial chr.			Macroadenoma; Additional therapy after surgery (ETSS and Gamma knife); Gross cavernous sinus invasion	2
W3694	Gains							whole chr.	partial chr.				whole chr.	whole chr.	partial chr.						whole chr.				Macroadenoma; Histological bony invasion; Gross cavernous sinus invasion	2
W3699	Gains and Losses										whole chr.	partial chr.													Macroadenoma; Gross cavernous sinus invasion	1
W3733	Gains and Losses							whole chr.	partial chr.	partial chr.				partial chr.	whole chr.						partial chr.	partial chr.			0	0
W3848_A EH	Gains					whole chr.	whole chr.	whole chr.				whole chr.	whole chr.	whole chr.				partial chr.		partial chr.	whole chr.				Prior surgery (2020); Macroadenoma (1.2 x 1.1); No cure; Gross cavernous sinus invasion	1

Table 3. CNVs of All Corticotropinomas

Surgery Log	CNV Type	chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22	Features of Aggressiveness	Aggressive? 0 = No; 1 = Mildly Aggressive; 2 = Aggressive; 3 = Very Aggressive	
H3	Gains			whole chr.					whole chr.																0	0
H91	Losses																partial chr.						partial chr.		Macroadenoma; Histological dural invasion; Histological bony invasion	1
H93																										0
H93A																						partial chr.				0
H126	Gains and Losses																whole chr.		whole chr.				partial chr.		Gross cavernous sinus invasion	0
H297	Gains and Losses							whole chr.	whole chr.	whole chr.		whole chr.	whole chr.	whole chr.							partial chr.	partial chr.			Giant, invasive, hemorrhagic macroadenoma (5cm); Additional therapy after surgery (craniotomy, XRT); Gross cavernous sinus invasion	3
H303	Gains							whole chr.	whole chr.	whole chr.		whole chr.	whole chr.	whole chr.								whole chr.	partial chr.		Prior surgery (2013); this case was further resection fo macroadenoma; Additional therapy after surgery (XRT); Gross cavernous sinus invasion	3
H308	Gains and Losses	partial chr.															partial chr.	whole chr.					partial chr.		2cm Macroadenoma; maybe Histological bony invasion	1
W2461	Losses		partial chr.																						Macroadenoma; Histological dural invasion; Gross cavernous sinus invasion	2
W2482																									Prior surgery; Giant macroadenoma that needed 2 surgeries to resect; Discordant cure; Gross cavernous sinus invasion	3
W2531	Gains and Losses	partial chr.	partial chr.			partial chr.			partial chr.		partial chr.	partial chr.					partial chr.		partial chr.		partial chr.				Giant macroadenoma; No cure; Additional therapy after surgery (somatoline injections); Histological mucosal invasion; Histological bony invasion; Gross cavernous sinus invasion	3
W2544	Gains and Losses								partial chr.													partial chr.			Prior surgery (2003); Prior XRT; Prior med (resistant to bromocriptine, octreotide, and GH antagonists) (Thus is a severe medically resistant and radiation resistant GH-secreting adenoma causing severe acromegaly); No cure; Additional therapy after surgery (GK); Gross cavernous sinus invasion	3
W2565																						partial chr.			Macroadenoma; Gross cavernous sinus invasion	1
W2567																									Macroadenoma; Gross cavernous sinus invasion	1
W2591	Losses		partial chr.									partial chr.													2cm Macroadenoma; Discordant cure; Gross cavernous sinus invasion	1
W2929	Gains			whole chr.	whole chr.	whole chr.			whole chr.				whole chr.												2cm Macroadenoma (1.6 x 1.4cm); Gross cavernous sinus invasion	1
W3214	Gains and Losses											whole chr.									partial chr.	partial chr.	partial chr.		2 Prior surgeries (2014 and 2015); Prior med (cabergoline-- didn't reduce sleep apnea and hypertension); thus he underwent surgery for residual/resistant tumor; Histological dural invasion; Gross cavernous sinus invasion	2
W3494	Losses																						partial chr.		2 Prior surgeries (L and 2018); Prior med (resistant to octreotide); Additional therapy after surgery (TSS with cure, then recurrence and thus taking octreotide, may start radiotherapy); Gross cavernous sinus invasion	3
W3697	Losses								partial chr.			partial chr.	partial chr.								partial chr.	partial chr.	partial chr.		1.5 cm Macroadenoma; Gross cavernous sinus invasion	1
W3698																									Large (1.8 x 2.5 x 2.7cm -> nearly 3.5cm) Macroadenoma; Additional therapy after surgery (another ETSS); Maybe Histological bony invasion; Gross cavernous sinus invasion	2
W3716	Gains and Losses			whole chr.	whole chr.	whole chr.	partial chr.	whole chr.	whole chr.	whole chr.	partial chr.	partial chr.	partial chr.		partial chr.	partial chr.	partial chr.	whole chr.		partial chr.	partial chr.	partial chr.	partial chr.	Gross cavernous sinus invasion	0	
W3826	Losses																						partial chr.		Macroadenoma; Histological bony invasion; Gross cavernous sinus invasion	1

Table 4. CNVs of All Somatotropinomas

Of the 34 PitNET cases classified as aggressive and very aggressive, 21 cases (61.76%) were identified to have CNVs, whereas 13 aggressive/very aggressive cases (38.23%) did not (Table 5). Therefore, CNVs were found to correlate with PitNET aggressiveness. The most significant CNVs for aggressive behavior were different based on PitNET subtype (Table 5).

Significant CNVs of Aggressive/Very Aggressive NF PitNETs		
Type	Copy Number Losses	
Number of Samples	1 out of 4	1 out of 4
Location	Chromosome 13	Chromosome 19
Total Number of Samples with CNVs	2 out of 4	

Significant CNVs of Aggressive/Very Aggressive PRL PitNETs					
Type	Copy Number Gains				
Number of Samples	5 out of 10	6 out of 10	6 out of 10	6 out of 10	5 out of 10
Location	Chromosome 3	Chromosome 5	Chromosome 7	Chromosome 9	Chromosome 13
Total Number of Samples with CNVs	7 out of 10				

Significant CNVs of Aggressive/Very Aggressive ACTH PitNETs		
Type	Copy Number Losses	Copy Number Gains
Number of Samples	3 out of 11	2 out of 11
Location	Chromosome 19	Chromosome 19
Total Number of Samples with CNVs	5 out of 11	

Significant CNVs of Aggressive/Very Aggressive GH PitNETs			
Type	Copy Number Losses		
Number of Samples	3 out of 9	3 out of 9	3 out of 9
Location	Chromosome 11	Chromosome 16	Chromosome 19
Total Number of Samples with CNVs	7 out of 9		

Table 5. Summary of Most Prevalent CNVs of Aggressive PitNETs by Subtype

Copy number losses in chromosomes 13 and 19 were found to predict aggressive NF PitNETs. Of the four aggressive/very aggressive NF PitNETs, one sample had a whole copy number loss in chromosome 13, while the other sample had a partial copy number loss in chromosome 19 (Table 6). Copy number losses in chromosome 19 were also found to be a predictor of aggressive somatotropinomas and corticotropinomas, and copy number gains in

chromosome 19 were also found to be a predictor of aggressive corticotropinomas, but CNVs in chromosome 19 were not found to be a predictor of aggressive prolactinomas.

Legend																								
Copy Number Gain:																								
Copy Number Loss:																								
CNV Analysis of Aggressive/Very Aggressive NF PitNETs																								
Surgery Log	Number of Copy Number Gains	Number of Copy Number Losses	Total CNVs	Location of CNV and CNV Type																				
				chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21
W2702	0	1	1																		partial chr.			
W2443	0	1	1										whole chr.											
W1030	0	0	0																					
W2914	0	0	0																		1 loss			
Total CNVs				0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 loss	0	0

Table 6. CNVs of Aggressive NF Pituitary Neuroendocrine Tumors

Copy number gains in chromosomes 3, 5, 7, 9 and 14 were found to predict prolactinoma aggressiveness, but not in NF PitNETs, somatotropinomas, or corticotropinomas. Out of ten aggressive/very aggressive prolactinoma cases, five had copy number gains in chromosome 3, six had copy number gains in chromosome 5, six had copy number gains in chromosome 7, six had copy number gains in chromosome 9, and five had copy number gains in chromosome 14 (Table 7). Additionally, seven out of ten aggressive/very aggressive prolactinoma cases (70%) were of male patients, four of which had over eight genetic amplifications, while the other three had none. Among these four cases, genetic amplifications on chromosomes 3, 5, 7, and 9 were a shared trait (Table 8). Patterns of CNVs by sex were not seen among other PitNET subtypes.

Legend																									
Copy Number Gain:																									
Copy Number Loss:																									
CNV Analysis of Aggressive/Very Aggressive Prolactinomas																									
Surgery Log	Number of Copy Number Gains	Number of Copy Number Losses	Total CNVs	Location of CNV and CNV Type																					
				chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22
W1092	0	0	0																						
W3371	0	0	0																						
W3460	0	0	0																						
W3401	0	1	1																		partial chr.				
W3727	8	0	8		partial chr.	partial chr.	partial chr.		whole chr.		partial chr.		partial chr.	partial chr.		partial chr.									
H1	7	1	8				whole chr.	whole chr.	whole chr.		whole chr.		whole chr.		whole chr.								partial chr.		
W2431	9	0	9			whole chr.		whole chr.		whole chr.		whole chr.		whole chr.		partial chr.					whole chr.				
W3828	9	2	11	partial chr.		whole chr.		whole chr.		whole chr.		whole chr.		whole chr.		partial chr.	partial chr.				partial chr.				
W1578	12	0	12	partial chr.		whole chr.		whole chr.		whole chr.		whole chr.				whole chr.	partial chr.	whole chr.			whole chr.	partial chr.			
W4191	10	2	12			whole chr.		whole chr.		whole chr.		whole chr.			whole chr.	whole chr.	whole chr.		partial chr.	partial chr.		partial chr.	partial chr.		
Total CNVs				2 gains	0	5 gains	1 gain	6 gains	2 gains	6 gains	4 gains	6 gains	0	1 loss	4 gains	2 gains	5 gains	3 gains	2 gains, 2 losses	2 losses	0	1 loss, 1 gain	4 gains	0	2 gains

Table 7. CNVs of Aggressive Prolactinomas

Legend	
Copy Number Gain:	
Copy Number Loss:	

CNV Analysis of Aggressive/Very Aggressive Prolactinomas--By Sex																										
Surgery Log	Sex	Number of Copy Number Gains	Number of Copy Number Losses	Total CNVs	Location of CNV and CNV Type																					
					chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22
W1092	M	0	0	0																						
W3371	F	0	0	0																						
W3460	M	0	0	0																						
W3401	M	0	1	1																	partial chr.					
W3727	M	8	0	8			partial chr.	partial chr.	partial chr.		whole chr.		partial chr.		partial chr.	partial chr.		partial chr.								
H1	F	7	1	8				whole chr.	whole chr.	whole chr.		whole chr.		whole chr.		whole chr.	whole chr.					partial chr.				
W2431	F	9	0	9			whole chr.	whole chr.	whole chr.	whole chr.	whole chr.		whole chr.		whole chr.	whole chr.		partial chr.			whole chr.					
W3828	M	9	2	11	partial chr.		whole chr.	whole chr.	whole chr.	whole chr.	whole chr.				whole chr.	whole chr.		partial chr.	partial chr.		partial chr.					
W1578	M	12	0	12	partial chr.		whole chr.	whole chr.	whole chr.	whole chr.	whole chr.				whole chr.	partial chr.	whole chr.			whole chr.	partial chr.					
W4191	M	10	2	12			whole chr.	whole chr.		whole chr.	whole chr.				whole chr.	whole chr.	whole chr.		partial chr.	partial chr.		partial chr.				
Total CNVs					2 gains	0	5 gains	1 gain	6 gains	2 gains	6 gains	4 gains	6 gains	0	1 loss	4 gains	2 gains	5 gains	3 gains	2 gains, 2 losses	2 losses	0	1 loss, 1 gain	4 gains	0	2 gains

Table 8. CNVs of Aggressive Prolactinomas by Sex

For corticotropinomas, copy number gains and losses in chromosome 19 were found to be predictive of aggressive cases. Out of eleven aggressive/very aggressive corticotropinomas, two cases were found to have copy number losses in chromosome 19, and two cases were found to have copy number gains in chromosome 19 (Table 9). Copy number losses in chromosome 19 were also found to be potentially predictive of aggressive somatotropinomas and NF PitNETs, but CNVs in chromosome 19 were not found to be predictive of aggressive prolactinomas.

Legend	
Copy Number Gain:	
Copy Number Loss:	

CNV Analysis of Aggressive/Very Aggressive Corticotropinoma																									
Surgery Log	Number of Copy Number Gains	Number of Copy Number Losses	Total CNVs	Location of CNV and CNV Type																					
				chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22
W3359	7	1	8					whole chr.		whole chr.	whole chr.	whole chr.			whole chr.		whole chr.		partial chr.			whole chr.			
W3540	0	0	0																						
W3545	0	0	0																						
W2543	0	2	2																partial chr.			partial chr.			
W3550	1	1	2																		partial chr.		partial chr.		partial chr.
W2541	0	2	2																		partial chr.		partial chr.		
W3694	7	1	8						whole chr.			partial chr.			whole chr.	whole chr.		partial chr.			whole chr.				
W2574	0	0	0																						
W2955	0	0	0																						
W3054	0	0	0																						
W3176	0	0	0																						
Total CNVs				0	0	0	0	1 gain	0	2 gains	1 loss	2 gains	0	0	1 gain	1 gain	2 gains	0	2 gains, 1 loss	0	0	2 gains, 3 losses	0	1 gain	0

Table 9. CNVs of Aggressive Corticotropinomas

For somatotropinomas, copy number losses in chromosomes 11, 16, and 19 may be predictive of aggressiveness. Out of nine aggressive/very aggressive somatotropinomas, three

cases were found to have copy number losses in chromosome 11, three cases were found to have copy number losses in chromosome 16, and three cases were found to have copy number losses in chromosome 19 (Table 10). Copy number losses in chromosome 19 were also found to be a predictive factor for aggressive NF and ACTH-secreting neuroendocrine tumors, and copy number gains in chromosome 19 were also found to predict aggressive ACTH-secreting neuroendocrine tumors. However, CNVs in chromosome 19 were not found to be predictive of aggressive prolactinomas.

Legend				CNV Analysis of Aggressive Somatotropinomas																					
Copy Number Gain:				Location of CNV and CNV Type																					
Copy Number Loss:				chr1	chr2	chr3	chr4	chr5	chr6	chr7	chr8	chr9	chr10	chr11	chr12	chr13	chr14	chr15	chr16	chr17	chr18	chr19	chr20	chr21	chr22
H297	5	3	8							whole chr.	whole chr.	whole chr.		whole chr.		whole chr.	whole chr.					partial chr.		partial chr.	
W3494	0	1	1																			partial chr.			
W2531	3	6	9	partial chr.	partial chr.			partial chr.			partial chr.		partial chr.	partial chr.					partial chr.		partial chr.		partial chr.		
H303	6	2	8							whole chr.	whole chr.	whole chr.		whole chr.		whole chr.	whole chr.						whole chr.	partial chr.	
W2544	1	1	2							partial chr.									partial chr.						
W2482	0	0	0																	partial chr.	partial chr.				
W3214	1	3	4									whole chr.							partial chr.	partial chr.		partial chr.			
W2461	0	1	1		partial chr.																				
W3698	0	0	0																						
Total CNVs				1 loss	2 losses	0	0	1 gain	0	3 gains	2 gains, 1 loss	3 gains	1 loss	3 losses	0	2 gains	2 gains	0	3 losses	1 loss	1 gain	3 losses	2 gains	2 losses	0

Table 10. CNVs of Aggressive Somatotropinomas

Discussion:

Through these findings, this retrospective analysis supports the idea that the study of chromosomal copy number alterations predicts aggressiveness of pituitary neuroendocrine tumors. Aggressive PitNETs yielded greater frequency of genetic amplifications, demonstrating that my hypothesis that CNAs can be used to predict aggressive PitNETs was supported through this analysis. Pituitary neuroendocrine tumors with copy number alterations were found to behave more aggressively, which also supports findings from previous literature that pituitary neuroendocrine tumors with CNVs have poorer prognosis (Chen et al., 2020). This supports that PitNET tumor tissue and blood that had undergone DNA extraction to undergo future WGS would also carry many CNVs and have similar types of CNVs by subtype as compared to the present study.

My predictions were partially supported by this study. Copy number losses in chromosome 1 were not found to predict aggressiveness in PitNETs. However, copy number losses in chromosome 11 were found to be a marker of aggressiveness in somatotropinomas from this study. This was found to be a distinctive feature of aggressive somatotropinomas and was not present in the other PitNET subtypes. Additionally, although copy number gains in chromosomes 1 and 12 were not found to be predictive of aggressive PitNETs, copy number gains in chromosomes 3, 5, 7, 9, and 14 were predictive of aggressive prolactinomas. This was also found to be a predictor specific to prolactinomas but was not present in other subtypes.

Because this study contained a small sample size of 11 NF PitNETs, 20 prolactinomas, 19 corticotropinomas, and 22 somatotropinomas, and also, it has been found that allelic loss of the short arm of chromosome 11 (11p) in prolactinomas is an identifying factor of aggressiveness (Chatzellis et al., 2015) which was not found in the current study, there may need to be further

research using a larger cohort of PitNET patients on what genes on which chromosomes are significant identifiers of aggressive pituitary neuroendocrine tumors.

Previous literature has emphasized the importance of GNAS (Guanine Nucleotide Activating Subunit) as a proto-oncogene in chromosome 20 that disrupts the cAMP signaling pathway in somatotropinomas (Chang et al., 2021; Chen et al., 2020; De Sousa & McCormack, 2022; Thapar et al., 1996). Of aggressive somatotropinomas, it was found that two samples had copy number gains on chromosome 20 in the present study. Therefore, GNAS mutations were not found to be an identifier of aggressive somatotropinomas, supporting that somatotropinomas with GNAS mutations are smaller, less invasive, are less refractory to somatostatin analog medication therapies, and typically respond to dopamine agonist therapies (Buchfelder et al., 1999; Gadelha et al., 2013; Neou et al., 2020; Zhou et al., 2014) and are thus less aggressive.

Research has also found that USP8 (Ubiquitin Specific Peptidase 8) is a proto-oncogene that affects the EGFR pathway in chromosome 15 for corticotropinomas (Chang et al., 2021; Chen et al., 2020; De Sousa & McCormack, 2022; Thapar et al., 1996). However, in the present study, CNVs on chromosome 15 were not found to be a substantive predictor of aggressive corticotropinomas, supporting the notion discussed in previous literature that USP8 mutations and EGFR overexpression are not associated with aggressive corticotropinomas (Albani et al., 2018; De Sousa, 2018; Faucz et al., 2017; Hayashi et al., 2016; Ma et al., 2015; Perez-Rivas et al., 2015).

Past research has also found that p53 is a tumor suppressor gene in chromosome 17 for PitNETs (Chang et al., 2021; Chen et al., 2020; De Sousa & McCormack, 2022; Thapar et al., 1996). This present study found that only two cases of aggressive prolactinomas had copy number losses on chromosome 17 and that only one case of aggressive somatotropinoma had

copy number losses on chromosome 17. It was shown from the present study that mutations in chromosome 17, including mutations of p53, were not a crucial predictor of aggressiveness in PitNETs. This supports the notion that p53 mutations are not a sufficient marker for PitNET aggressiveness (Oliveira et al., 2002).

It was found in this retrospective study that CNVs are correlated with aggressive PitNETs. Additionally, it was found that genetic amplifications were correlated with aggressive prolactinomas and aggressive corticotropinomas. Therefore, although the proto-oncogenes of GNAS and USP8 were not specifically found to be predictors of PitNETs, this study supports the notion that proto-oncogenes could be markers of certain subtypes of aggressive PitNETs.

Each individual subtype of PitNET demonstrated different CNVs that were associated with clinical aggressiveness. Since copy number losses in chromosomes 13 and 19 may be a predictive factor in aggressive NF neuroendocrine tumors, these results support that the BRCA2 gene on chromosome 13q, an important tumor suppressor that could be driving fallopian tube and ovarian cancer, could be an important tumor suppressor causing NF neuroendocrine tumors (Jongsma et al., 2002). Deletions of DNMT2, SLC44A2 and CDKN2D on chromosome 19 have been found to lead to poor prognosis in neuroblastoma patients and could therefore be tumor suppressor genes that lead to aggressive NF neuroendocrine tumors (Lasorsa et al., 2020).

For prolactinomas, copy number gains in chromosomes 3, 5, 7, 9 and 14 predict prolactinoma aggressiveness, and the GDNF gene could thus be an important oncogene on chromosome 5 that is causing aggressiveness in prolactinomas, since GDNF has been found to be overexpressed in squamous non-small cell lung carcinoma and that the GDNF protein exists in early-stage lesions and that it is thus involved in early tumorigenesis in lung cancer (Garnis et al., 2005). Additionally, overexpression of genes including EGFR, FTSJ2, NUDT1, TAF6, and

POLR2J on chromosome 7 have been proposed to be associated with non-small cell lung cancer (NSCLC) and may also be a predictor of aggressive prolactinomas (Campbell et al., 2008; Tsiambas et al., 2017). Increased expression of PLD1 on chromosome 9 could also be a driving factor for aggressive prolactinomas, as increased PLD1 mRNA and protein were found in human breast cancer tissue and human renal cancer, as well as NSCLC (Ahn et al., 2012; Menter & Tzankov, 2019). Also, it was found that seven out of ten aggressive/very aggressive prolactinomas cases were of male patients, indicating that prolactinomas in males may oftentimes be aggressive. Additionally, of these seven cases, four of them had over eight genetic amplifications, while the other three had none. Thus, over half of the aggressive/very aggressive prolactinomas from men had copy number gains in over eight chromosomes. Furthermore, the common genetic amplifications among the four cases that had over-expression in eight chromosomes were in chromosomes 3, 5, 7, and 9. Since the other prolactinomas of both sexes also harbored common gene amplifications in chromosomes 3, 5, 7, 9 and 14, although men appear to have more aggressive prolactinoma cases than females, which are marked by copy number amplifications, the sites of the genetic amplifications remain the same for males with aggressive/very aggressive prolactinomas compared to females with genomic amplifications and aggressive/very aggressive prolactinomas. This study therefore supports that the aggressiveness of tumors among male prolactinoma patients may be caused by a different biological mechanism other than genetic amplifications (Larkin & Ansorge, 2017; Yoo et al., 2018).

For corticotropinomas, copy number gains and losses in chromosome 19 are predictive of aggressive ACTH-secreting pituitary neuroendocrine tumors, which support the notion that the amplification of ERCC1 gene, which is a gene on chromosome 19p that could be a contributor to lung cancer (Wang et al., 2015), could be an important oncogene on chromosome 19 that could

cause aggressive corticotropinomas. However, the deletion of ERCC1 gene has also been proposed to be involved in glial tumorigenesis, and the ERCC1 tumor suppressor gene may also cause aggressive corticotropinomas (Deimling et al., 1992).

Genetic deletions in chromosomes 11, 16, and 19 may be predictive of aggressive somatotropinomas, suggesting that the MEN1 gene, a tumor suppressor gene on chromosome 11q13 that could contribute to pancreatic endocrine tumors and multiple endocrine neoplasia type I syndrome (MEN I), could also cause aggressive somatotropinomas (Wang, 1998; Eubanks et al., 1994). Like NF neuroendocrine tumors, it could be possible that downregulation of the genes DNM2, SLC44A2 and CDKN2D on chromosome 19 could also cause aggressive somatotropinomas (Lasorsa et al., 2020). However, it has also been found that the mutated gene STK11(LKB1) on chromosome 19p13.3 is a tumor suppressor gene that might be associated with hamartomas and adenocarcinomas in Peutz-Jeghers syndrome (PJS), wherein polyps form in the intestines, and it is the third most mutated gene in lung adenocarcinomas (Larsen et al., 2014). Thus, STK11 may also be an important tumor suppressor on chromosome 19 that could cause somatotropinoma tumorigenesis.

This research suggests that aggressive pituitary neuroendocrine tumors can be identified through genetic analysis of their genetic mutations. With knowledge of the presence of copy number alterations through genetic testing of tumor tissue and blood, this study supports that aggressive pituitary neuroendocrine tumors can be distinguished from non-aggressive pituitary neuroendocrine tumors through the presence of CNVs, which will be greatly beneficial in diagnosing aggressive from non-aggressive PitNETs, and subsequently deciding the most effective course of treatment for these cases (Cooper et al., 2021).

Conclusion:

Not only might it be possible to predict aggressive PitNETs through the presence of CNVs, but through the correlation of specific types of CNVs on the different subtypes of aggressive PitNETs, they can be further classified by subtype: the type of genetic copy number alteration distinguishes the aggressive PitNET phenotype.

Impact of the Use of CNVs in the Diagnosis and Treatment of Aggressive PitNETs:

In diagnosing PitNETs, it is essential to recognize aggressive PitNETs from non-aggressive PitNETs to treat patients based on their tumor's behavior (Kasuki & Raverot, 2020). If CNVs can predict PitNET clinical aggressiveness, the diagnosis and treatment of aggressive PitNETs could be drastically improved. By achieving knowledge of PitNET aggressiveness, clinicians would be able to immediately recognize aggressive cases that require more vigilant observation and extensive treatment. If the presence of CNVs is distinct for specific aggressive PitNET subtypes, clinicians could greatly improve aggressive PitNET treatment by developing therapies that target the specific genetic mutations that give rise to aggressive PitNETs. Further study using single-cell RNA sequencing can detect the specific cell populations of tumors (Anaparthi et al., 2019). Therefore, further research using single-cell RNA sequencing could be beneficial for further study on the genetic makeup of aggressive PitNETs and how they diverge from non-aggressive PitNETs' clinical behavior. This way, the identification of aggressive and non-aggressive PitNETs through the presence of specific CNVs could drastically improve the diagnostic and treatment process for aggressive PitNET patients.

Results In Relation to PitNET Heterogeneity:

This CNV analysis also supports the notion of pituitary tumor heterogeneity, which would revolutionize the management of patients' treatment. Tumor heterogeneity (polyclonality)

is defined as tumor cell populations that undergo advantageous mutations, which increases its chance of survival and division, allow it to make a new clonal population (Abécassis et al., 2019; Dentre et al., 2017). Pituitary neuroendocrine tumors are generally viewed as monoclonal (made of one cell type) in origin (Alexander et al., 1990; Aflorei and Korbonits, 2014). However, additional research has demonstrated that pituitary neuroendocrine tumors have various clonal origins prior to and following recurrence, suggesting that pituitary neuroendocrine tumors are polyclonal (Carreno et al., 2017; Clayton and Farrell, 2006; Kumar and Prusty, 2013; Moreno et al., 2005; Vankelecom and Roose, 2017; Zhan et al., 2014; Zhan et al., 2016).

Since the significant copy number alterations that were present in each subgroup of aggressive PitNET was distinct to each subtype, this study suggests that PitNETs are heterogeneous, since different genetic alterations were found to exist on different chromosomes to give rise to the different subtypes of aggressive PitNETs. In addition to microprolactinomas forming into macroprolactinomas, with sporadic mutations as the suggested mechanism of growth (Kumar & Prusty, 2013) and that various proteome pathway systems occur in NF PitNETs (Zhan et al., 2014), our study acts as further support for the heterogeneous makeup of PitNETs.

The present study also supports recent research that has revealed the presence of clonal variations based on the different types of copy number alterations of PitNETs (Jain et al., 2020; Jain et al., 2021). Thus, CNVs in PitNETs have been found to be related to PitNET heterogeneity. Research has also found that recurrences could arise from the different cell populations that the pituitary neuroendocrine tumor developed from (Carreno et al., 2017; Clayton and Farrell, 2006). This suggests that recurrence, a characteristic of PitNET aggressiveness, could be related to the various subpopulations of cells that create the PitNET,

potentially causing its aggressive behavior. Thus, CNVs in PitNETs could be a genetic marker of PitNET heterogeneity, which could be linked with tumor aggressiveness. Therefore, developments in treatment for aggressive PitNETs could target specific genetic mutations that the cell populations arise from in order to more effectively treat patients' aggressive PitNETs by targeting the pathways that genetic CNVs disrupt in their heterogeneous cell populations.

Gaining the ability to detect which pituitary neuroendocrine tumors are aggressive and non-aggressive through the presence of unique CNVs on each subtype of PitNET would allow for proper treatment of aggressive PitNET patients. Since this study signifies that PitNETs are heterogeneous and that the types of CNVs present on aggressive PitNETs are specific to their PitNET subtype, this also supports that, through targeted therapies that can detect the specific pathways of aggressive PitNETs' genetic mutations, treatments of aggressive PitNETs could also be improved to target specific CNVs within the heterogeneous cell population of PitNETs. This study acts as evidence for possible novel diagnosis and treatment options that could revolutionize care for aggressive PitNET patients.

Acknowledgements:

I would like to thank my thesis advisors, Dr. Manuel Ferreira Jr, MD, PhD; and Professor Pete Chandrangsou. I would like to thank Dr. Ferreira for his continuous guidance and mentorship for me. You have always been by my side, and your constructive feedback and advice for me has been absolutely invaluable. No words can describe how thankful I am for your tremendous leadership and how much I have learned from you. Professor Chandrangsou, I am so very grateful for how receptive, kind, and supportive you have been in helping me along this journey. From all of your advice, support, and resources you have offered me, I'm so thankful for all of your help and kindness. I'm so grateful to have had you both as my readers—I couldn't have hoped for a better learning experience from you both.

Thank you so much to my wonderful colleagues at the Ferreira Lab for your continued support for me throughout this process. I'd like to thank Mallory Tucker, Oscar Parrish, Jessica Eaton, MD; Lucia Paredes, ARNP; Maheswara Duvvari, PhD; Jacob Ruzevick, MD; Carolina Parada, PhD; and Jennifer Delegard, BS. I'm so incredibly grateful for all of the support and mentorship you have given me.

Lastly, but certainly not least, thank you to all of my wonderful friends and family who have been so unconditionally loving and supportive of me throughout this process.

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