Non-functioning pituitary adenomas: complications,
prognostic factors and tumor behavior

Thesis for the degree of Philosophiae Doctor

by

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2: Abbreviations

ACTH: Adrenocorticotropic hormone

AI: Adrenal insufficiency

DI: Diabetes insipidus

FPA: Functioning pituitary adenoma

FSH: Follicle stimulating hormone

GA: Gonadotroph adenoma

GH: Growth hormone

HPA: Hypothalamic-pituitary-adrenal

IHC: Immunohistochemistry / immunohistochemical

ITT: Insulin tolerance test

LC-MS/MS: Liquid chromatography tandem mass spectrometry

LH: Luteinizing hormone

MEN: Multiple endocrine neoplasia

NFPA: Non-functioning pituitary adenoma

OUS: Oslo University Hospital

PCR: Polymerase Chain reaction

RT-qPCR: Real-time quantitative reverse transcription polymerase chain reaction

SAI: Secondary adrenal insufficiency
TSH: Thyroid stimulating hormone
3: List of publications


4: Sammendrag av avhandling (Summary of thesis)


Stumme hypofyseadenomer kan utgå fra alle celle typene i den fremre delen av hypofysen, men oppstår aller oftest fra gonadotrope celler. Til tross for at gonadotrope svulster ikke gir symptomer på hypersekresjon av hormoner, produserer de fleste svulster FSHβ og LHβ, som kan synliggjøres med immunohistokjemisk farging av vev fra operasjonen. Det er usikkert om disse endringene i produksjon og utskillelse av hypofysehormoner kan
knyttes til vekst og modning av svulsten. Pasienter med stimme hypofyseadenom krever langvarig multidisiplinær oppfølgning fra endokrinolog, radiolog, oftalmolog og nevrokirurg. Forståelse av stimme hypofyseadenomers tumorbiologi og markører for vekst er viktig for å kunne tilpasse behandling og oppfølg av pasienter med slike svulster.

I artikkel 1 undersøkte vi forekomsten av hypofysevikt før og etter operasjon av pasienter med stimme hypofyseadenom. Målet med studien var å identifisere risikofaktorer for å utvikle sekundær binyrebarksvikt etter kirurgi, og å beskrive en normal respons på Synacthen-test 3 måneder etter operasjonen. Vi fant at pituitær apopleksi øker risiko for sekundær binyrebarksvikt etter operasjon. Videre viste vi at en kortisolstigning under Synacthen-test er betraktelig lavere enn den tradisjonelle grensen (>500nmol/l) når nye målemetoder for kortisol benyttes.

I artikkel 2 studerte vi sammenhengen mellom immunohistokjemisk farging for gonadotropiner (FSHβ og LHβ) i gonadotope adenomer og markører for aggressivitet, representert ved endringer i E-cadherin. Resultatene viste at svulster med høy gonadotropin farging hadde E-cadherin farging som taler for en aggressiv fenotyper (redusert membranøs E-cadherin, økt nukleær E-cadherin). Vi fant også en sammenheng mellom FSHβ farging og plasma-FSH hos pasienter.

I artikkel 3 ble 230 hypofyseadenomer (både fungerende og stimme) undersøkt for immunohistokjemisk farging av TGFBR3L, er et gen med økt uttrykk i hypofysen. Studier av mus og rotter har vist at TGFBR3L var spesifikt for gonadotrope hypofyseceller. Dette var den første studien som viste TGFBR3L på protein nivå hos mennesker, med farging med antistoff. Vår studie viste at TGFBR3L farging kun ses i gonadotrope celler hos mennesker, både normale og neoplastiske.
I artikkel 4 fortsatte vi å studere TGFBR3L i hypofyseadenomer, denne gangen i en ny gruppe pasienter der ytterligere kliniske data var tilgjengelig. Som i foregående studie var kun gonadotrope svulster positive for TGFBR3L. TGFBR3L var assosiert med høy LHβ farging, men med lavere verdier av plasma-LH og plasma-FSH. Høy TGFBR3L farging var også assosiert med større tumor volum. Funksjonen til proteinet TGFBR3L er ukjent, og dets effekt på gonadotropin produksjon/utskillelse, samt tumor vekst bør utdypes i videre studier.
5: Background

5.1: The pituitary gland and pituitary adenomas

The pituitary is a small gland located in the sella turcica at the base of the skull and is consists of two parts, the anterior (adenohypophysis) and posterior (neurohypophysis) (1). The anterior pituitary is made up of five different types of hormone-secreting cells: gonadotroph cells secrete FSH and LH, corticotroph cells secrete ACTH, thyreotroph cells secrete TSH, somatotroph cells secrete growth hormone, and lactotroph cells secrete prolactin (1). The cells of the anterior pituitary are regulated by the hypothalamus, from which they receives stimulatory or inhibitory signals (1). The posterior pituitary is made up of neurons that secrete the hormones ADH and oxytocin (1). The focus of this thesis is the function of the anterior pituitary gland, and tumors arising from it.

Pituitary adenomas (PA) are tumors of the anterior pituitary gland and can arise from any of the hormone-producing cell lines (2–5). Pituitary adenomas are classified as functioning or non-functioning based on clinical findings (3,6–8). Patients with functioning pituitary adenomas show clinical and biochemical signs of hormone hypersecretion, while patients with clinically non-functioning pituitary adenomas (NFPA) do not show evidence of hormone hypersecretion (9).

5.2: Epidemiology

Pituitary adenomas are the most common tumor in the sellar region (10,11) and among the most common intracranial neoplasms (12). The reported incidence and prevalence of pituitary adenomas vary widely. Epidemiological studies report a prevalence of 1/865 to 1/2688 (13–18). Some autopsy and MRI studies have found pituitary adenomas in 14-23% of the
population (19–23), with the majority being microadenomas (20,22). However, this high prevalence of pituitary adenomas is disputed and some studies report tumors in 1% (24), 4% (25), and 11% (11) of the population. Macro adenomas are less common, and has been reported in around 0.2% of the population (26,27), although other studies report a higher percentage of macro adenomas (28–30).

Overall the median age of diagnosis of pituitary adenoma is 40-52 years, and the tumors are slightly common in females than in males (13–15,17). The most frequent types of pituitary adenomas are prolactinomas and NFPAs (11,13–17). Whereas prolactinomas more are more often seen in younger females, NFPAs are more commonly diagnosed in males and their incidence increases with age (14–17,31). The median age of diagnosis of NFPAs is 53-60 years (15–17).

The etiology of pituitary adenomas is uncertain, and the majority of tumors are sporadic (32–34). However, a minority (<5%) develop in association with hereditary tumor syndromes such as multiple endocrine neoplasia (MEN) 1, familial isolated pituitary adenomas, and Carney complex (35–38).

5.3: Diagnosis and clinical findings

MRI is the radiological method of choice for diagnosis of pituitary adenomas (8,39). Based on tumor size, pituitary adenomas are classified as microadenomas (<10 mm) or macro adenomas (>10 mm) (4). Per definition, NFPAs are not associated with clinical findings of hormone hypersecretion (4). The diagnosis of NFPA is confirmed by histological and IHC examinations after surgery or biopsy (2–4). Other tumors in the sellar region are Rathke’s cleft cysts, cranio pharyngioma meningioma, granulomatous and inflammatory processes, and metastases (10,11,40,41).
NFPAs are frequently (21-49%) discovered incidentally on MRI or CT performed for causes unrelated to the pituitary gland (25,42). In the recent years the incidence of pituitary adenomas, and in particular NFPAs, have increased (14,15,17). This increase may be due to higher quality and frequency of radiologic investigations, leading to discovery of otherwise undetected pituitary adenomas (14,15,17). Clinically NFPAs constitute a majority of the incidentally discovered tumors, and they are usually microadenomas (11,15,17,25,28,41).

Clinical findings in patients with NFPAs ranges from asymptomatic to severe endocrinological and neurological disturbances (9,16,28,29,42–47). The most frequent findings are hypopituitarism, visual disturbances, ophthalmoplegia, hyperprolactinemia and headache (9,42,46,48,49), and are more common in patients with macroadenomas than microadenomas (28,42,48,50,51). Similarly, incidentally discovered adenomas are usually smaller and have less clinical symptoms (42,48,50,51).

Hypopituitarism is seen in in 25-33% patients with microadenomas (42,51), but as many as 40-80% of patients in surgical series (43,48,52–54). The rate of hypopituitarism in incidentally discovered tumors varies with the size of tumors in the studied population (50,51,55). Preoperatively, the most common pituitary insufficiencies are hypogonadotropic hypogonadism (63-77%), growth hormone deficiency (30-77%), secondary adrenal insufficiency (SAI) (15-36%), hypothyroidism (9-29%) (52–54,56–61). Diabetes insipidus is infrequently found preoperatively (53,54).

Visual disturbances develop due to compression of the optic chiasm, and is exclusively seen in macroadenomas (9,62,63). Overall, visual field defects are seen in in 14-22% of patients (29,43,48), and ophthalmoplegia in 4-16% (52,56,57,64–67). Pituitary apoplexy occurs in 4-14% of patients (17,28,30,43,44,58), and is commonly associated with
ophthalmoplegia (68–70). Hyperprolactinemia due to stalk compression is common in patients with NFPA, but serum-prolactin rarely exceeds 2000 mIU/L (19,44,53). Headache occurs in 10–70% of patients with NFPAs (52,53,71,72).

5.4: Hypopituitarism

Adrenal insufficiency is marked by low p-cortisol levels and symptoms of hypocortisolism, such as fatigue, anorexia, weight loss, nausea and abdominal pains and hyponatremia (73,74). The adrenal glands are part of the hypothalamic—pituitary-adrenal (HPA) axis. Primary adrenal insufficiency is caused by diseases of the adrenal gland (73–75), while SAI is caused by impaired pituitary ACTH signaling (73,76,77). Hypocortisolism is life threatening and patients must be substituted with glucocorticoids (73,78). The diagnosis of SAI will be discussed later.

Hypogonadism can cause fatigue, infertility, and decreased libido, and osteoporosis in adult patients (79,80). Secondary amenorrhea can also be observed in women (80). In adolescent, sex steroids are required for puberty (80). Biochemically, hypogonadotrophic hypogonadism is marked by low sex steroids (testosterone or estrogens) and low FSH and LH (81). For most patients treatment consists of substitution with testosterone or estradiol (80).

Symptoms of hypothyroidism include lethargy, cold intolerance, weight gain, among others. Patients with central hypothyroidism, low free-T4 in the presence of low TSH (82). Thyroid hormones show little variation during the day, and is assessed with routine blood sampling. Replacement with levothyroxine is the preferred treatment (82). Both the gonadal and thyroid axes can be evaluated based on routine blood sampling, and does not require dynamic testing (80,82).

Growth hormone deficiency causes changes in metabolism and body composition, and impairs physical performance (83–85). In children, growth hormone deficiency also causes
short stature (86). Growth hormone is secreted in a pulsatile manner and shows a diurnal variation (87), and IGF-1 is often used as an indirect marker of growth hormone production (88,89). Correct diagnosis of growth hormone deficiency often requires dynamic testing with the insulin tolerance test (ITT) or arginine-GHRH test (90). Replacement therapy with growth hormone injections in adults is indicated in selected patients, particularly younger and symptomatic patients (86).

Central diabetes insipidus is caused by insufficient ADH secretion by the posterior pituitary gland (91). It is characterized by polyuria, dehydration and polydipsia (91,92). Biochemically, blood samples show high osmolality and hypernatremia, while urine is hypotonic (91,92). Traditionally, the water-deprivation test has been used for diagnosis of DI, but recently the arginine-stimulated copeptin measurements has been suggested as an alternative (91,93). Central diabetes insipidus can be treated with desmopressin (94).

5.5: Natural course and management of NFPAs

NFPAs have a indolent course and the majority does not grow after diagnosis (19,28,30,41,43,48,95). Growth of the tumors within the first 5 years of diagnosis is seen 26-50% of macroadenomas, but is less common (10%) in microadenomas (9,28,30,41,43,47,48,95,96). Not all growing tumors become symptomatic and requires treatment, and studies have found that 20-30% of NFPAs grow and becomes symptomatic (30,42,43,48,95). A spontaneous decrease in tumor size can occur, but the frequency varies between studies and has been reported in 0-29% of NFPAs (30,41–43,47,48).

Due to the varying clinical course of NFPAs, it can be challenging to determine the frequency and duration of follow-up for the patients (8,30,43,48). This is particularly difficult with the increasing use of radiological investigations and discovery of pituitary incidentalomas.
Makers that predict tumor behavior would therefore be valuable to individualize patient treatment (6,7,97,98).

NFPAs can invade surrounding structures, but are usually benign and metastases are rare (6,99). Local invasion of surrounding structures is seen in approximately one third of NFPAs (50,52,67,100). Invasion of the cavernous sinus can be graded on MRI using the Knosp-score (101,102).

The management of NFPAs is based on tumor size and clinical findings (7–9,39,47,103,104). Observation alone is sufficient in patients with smaller tumors and no signs of local mass effect (43,47,48,104). Surgery is usually reserved for patients who develop local mass effects (visual field defects and ophthalmoplegia) (9,39,43,47,48,104,105). The frequency of radiological investigations and ophthalmological evaluation is individualized (9). Patients with NFPAs should also undergo endocrinological workup and receive substitution therapy when indicated (8,39,46,103).

The preferred treatment for NFPA is endoscopic transsphenoidal surgery (39,104,106–108,108,109). For larger tumors with suprasellar extension, transcranial approach might be required (110,111). Tumors with invasion of the cavernous sinus have increased risk of residual tumor postoperatively (101,102,112). Radiotherapy is also useful in the management of NFPAs, especially in cases where surgery is insufficient to control the growth of the tumor (7,9,39,100,113). Medical therapy of NFPAs with dopamine agonists and somatostatin analogs have been attempted, but their usefulness is limited (114–116). In some aggressive tumors and pituitary carcinomas chemotheraphy with temozolomide is an alternative (7,117).
5.6: Results of surgery

The results of surgery for NFPAs varies and residual tumors are reported in one third to two thirds of patients postoperatively (57,58,67,118–120). Residual tumor is more commonly present in tumors that invaded the cavernous sinus preoperatively (57,101,102,121). Tumor regrowth is observed in up to a third (10-36%) of patients, and is more frequently seen in patients with residual tumor after tumor where it occurs in up to a half of patients (46,58,118,119,122,123). Even in patients without visible residual tumor, regrowth is seen in approximately 12% (123). Regrowth usually occurs within the first 5-10 years of surgery, but can also occur several years later (119,121,123,124).

Overall, surgery offers tumor control without need for repeated surgery in most (80-90%) patients (52,121,125). Patients who receive radiotherapy in addition to surgery have lower rates of residual tumor and tumor regrowth (52,118). However, the majority of patients develop hypopituitarism following radiation therapy, and radiation is therefore usually reserved for patients where surgery in itself cannot control growth of tumors (100,113). Up to a third (12-34%) of patients with regrowth of tumor require reintervention (118,122). Postoperatively it is recommended that patients are followed clinically and radiologically for up to 15 years, as well with ophthalmological examination if indicated (126).

Transsphenoidal surgery for NFPA is generally safe with low mortality (<1%) mortality, while complications occur in 9-17% of operations (58,67,127,128). However, the rates of complications and mortality is higher in patients with larger tumors that require a transcranial approach (53,60,111,129–134). The most common surgical complications are CSF leak and transient diabetes insipidus or SIADH (58,104,127,128). Transient diabetes insipidus occurs in 6-28% (43,58,125), while permanent in only a few (1-5%) of patients (53,54,56,58,67).
Meningitis, intracranial hematoma and worsening of visual function can also occur but are seen in less than 2% of patients (58,67,127,128).

After undergoing surgery for NFPA, vision improves in the majority of patients (53,58,59,135). Most studies report improved pituitary function in 30-35% of patients postoperatively (53,57–59,125). Worsening of pituitary function is seen in 6-15% (53,58), while no change of pituitary function is seen in approximately half of patients (53,54,59). However, some studies report higher rates of hypopituitarism after surgery (52,107). Transcranial approach, larger tumors and invasive growth are associated with poorer surgical outcomes and risk of developing hypopituitarism (53,54,57–59,67,136).

NFPA and hypopituitarism are associated with increased morbidity and a small increase of mortality (137–142). In particular, SAI is life threatening and correct diagnosis is important (78,143). After surgery, new onset of SAI is seen in 1-8% of patients, while recovery from SAI is seen in 10-41% (53,54,58,67,144–146). The overall prevalence of SAI postoperatively varies between studies, with some reporting rates of 16-24% (53,57,59,61,144) while others report rates as high as 44-60% (43,61). Transcranial surgery and pituitary apoplexy increases the risk of developing SAI (53,69,70).

5.7: Hypothalamic-Pituitary-Adrenal axis

The HPA axis is responsible for regulating cortisol synthesis and secretion (147). The axis consists of corticotropin releasing hormone released by the hypothalamus which stimulates the pituitary to secrete ACTH, which in turn stimulates the adrenal glands to produce cortisol (147–149). Cortisol regulates many processes in the body such as metabolism, stress response, immune reaction and inflammation (150–152), and cortisol deficiency is life
threatening (76,78,141). In healthy humans, cortisol secretion has a circadian rhythm with highest levels in the morning, and a decrease throughout the day (147,153–155).

As a part of the normal physiological response, plasma-cortisol levels increase during stress such as acute disease, surgery, infection fasting and hypoglycemia (147,151,156–160). Abnormal sleep patterns and psychological disorders such as depression also affects cortisol levels (161–166). Increased cortisol levels is seen in women taking estrogen (oral contraceptive pills or hormone replacement therapy) due to up-regulation of cortisol-binding globulin (167–173).

5.8 Diagnosis of SAI

The diagnosis of SAI is based on morning p-cortisol levels or dynamic testing, either with the ITT or short Synacthen-test (SST) (75,174–180). Basal morning p-cortisol levels are predictive with p-cortisol response to ITT (180,181) and SST (175,177,177). Morning p-cortisol <100 nmol/l have by some been considered indicative of AI (174,177,180,182), while morning p-cortisol >226-350 nmol/l have been a considered normal (145,174,180,183,184), although dependent on the cortisol-assay used (174).

The ITT induces hypoglycemia, which stimulates the adrenal glands to produce cortisol via the hypothalamus and pituitary (75,178,180,185). The SST works by injecting a synthetic ACTH analog (Synacthen) to stimulate cortisol production in the adrenal gland (75,178,179). The ITT is considered the gold standard because it evaluates the entire HPA axis (hypothalamus, pituitary and adrenals), while the SST only tests the adrenal glands’ ability to produce cortisol. The results of ITT and SST tests correlate well and both tests are used to assess the HPA-axis (178,179,181,182,186–193). There is no consensus on when to use high-dose (250 µg) or low-dose (1 µg) SST (182,186,188,189,192,194). Traditionally, a normal
response to the ITT or SST have been peak cortisol >500-600 nmol/L (179,180), however the criteria for a normal test varies (195).

In patients with SAI, the adrenal glands atrophies due to lack of stimulation from the pituitary (182,186). However, it may take some time for the adrenals glands to become atrophic after the development of pituitary failure (182,186,196). Thus, when pituitary failure and SAI develops suddenly (e.g. damage during surgery or pituitary apoplexy), dynamic testing can produce a falsely reassuring result in the early phase after the injury (182,186,196). Testing of the HPA axis in the early postoperative phase is unreliable, and diagnosis of SAI should be delayed to 6-12 weeks after surgery (61,182,186,196,197). Some patients develop SAI in the months following operation for NFPAs, but it rarely develops later than 3 months postoperatively (61,197,198). Recovery of HPA-axis function can also be delayed, with most recovering within 1 year but can occur until 5 years postoperatively (61,145,198). In patients who undergo radiation therapy, hypopituitarism is common and may develop several years after the radiation (113).

P-cortisol is routinely measured using immunoassays (172,199,200). Older immunoassays are known to overestimate p-cortisol when compared to the precise methods such as Liquid Chromatography Mass Spectrometry / Mass Spectrometry (LC-MS/MS) (172). In the recent decade, more specific second-generation immunoassays have been introduced (172,199–203). These newer assays correlate better with LC-MS/MS, and gives up to 30% lower p-cortisol values than their older counterparts, especially at higher concentrations of p-cortisol (172,199–203). For this reason, several studies have suggested revising the reference limits for SST based on the laboratory method in use (199–206).
5.8: Histopathological classification of NFPAs

NFPAs are classified according to expression of pituitary hormones and cell-lineage specific transcription factors (2–4,6,207). This classification is based on IHC staining of the anterior pituitary hormones FSH, LH, GH, TSH and prolactin as well as the transcription factors SF-1, Tpit, Pit-1 (figure 1) (2–4,6). Most tumors express one or more pituitary hormones, however approximately 14-20% are hormone-negative (i.e. non-immunoreactive) (3,208,209). Transcription factors are recent additions to the classification of NFPAs and are particularly useful to determine the differentiation of hormone-negative adenomas (3,4,210). The transcription factor SF-1 is a marker of gonadotroph lineage, T-pit of corticotroph lineage, and Pit-1 of somato-thyro-lactotroph lineage (3,211,212).

Figure 1: Pituitary cell lines and their transcription factors

Hormone-negative (non-immunoreactive) tumors were previously designated null-cell adenomas, and their cell lineage could not be determined (3,4,210). However, recent investigations of transcription factors found that the majority of hormone-negative tumors showed positive staining for transcription factors and were re-classified accordingly (3,4,49).
Expression of SF-1 was seen in 66% of hormone-negative tumors, Tpit in 27%, and Pit-1 in 2%, while < 5% were true non-immunoreactive (null-cell) adenomas (3,4,49,49,213–215).

After this reclassification, the most common subtype of NFPAs overall are gonadotroph adenomas (73%), followed by corticotroph (10%) and somato-thyreo-lactotroph adenomas (9%) (3,4). True null-cell immune-nonreactive tumors now account for 1% of all NFPAs (3,4). The processes underlying the expression and secretion of pituitary hormones is unknown, It is unclear why adenomas of the gonadotroph lineage are mainly non-functioning and often hormone-negative, while corticotroph and Pit-1 positive tumors usually produce and secrete hormones (49,216–218).

Tumor behavior and clinical course differs between the pituitary cell lineages, and correct identification of differentiation is important (213,217,219–223). For instance, silent corticotroph adenomas show a more aggressive behavior (219,224–227), while gonadotroph adenomas (GA) tend to be less aggressive (6,214,228,229). However, it is worth noting that some of these studies did not include the staining of transcription factors.

Some markers that indicate aggressive behavior exists, including increased mitoses, elevated Ki-67 index and nuclear p53 staining (7,117,207,214,215,230,231). A Ki-57 index >3% have been associated with larger tumors, invasive growth and tumor recurrence after surgery (101,231–233). However, not all studies report similar correlation between Ki-67 index or p53 staining and tumor behavior or recurrence (234–237). Various other markers have also been proposed, including estrogen receptor (ER) α in GAs (207,215,228,230,238–241).

5.9: Gonadotroph pituitary adenomas (GAs)

Gonadotroph NFPAs are defined by the expression of the transcription factor SF-1 (3,4). In addition, most (79%) of the tumors also show positive staining for one or more of the
gonadotropin subunits FSHβ, LHβ or α subunit (3,4,209). GAs rarely produce clinical symptoms of hormone hypersecretion and the vast majority are clinically non-functioning (49,216,242,243). Still, some clinically non-functioning GAs secrete intact gonadotropins or their FSHβ, LHβ or α subunit, without causing clinical manifestations (114,209,242,244–260).

The gonadotropins FSH and LH are glycoprotein hormones formed by a dimerization of one α subunit and one FSHβ or LHβ subunit (261,262). Production of gonadotropin subunits are heterogeneous within GAs, and are seen in approximately 5-15% of tumor cells (245). IHC staining for α subunit is seen in 50-84% of GAs, FSHβ in 58-71%, LHβ in 45-71%, and the majority of tumors show staining for multiple subunits (114,209,256,263). Similarly, gonadotropin subunit mRNA is seen in most tumors (209,263), however FSHβ mRNA occurs more commonly and in higher concentration than LHβ mRNA (209,263).

Cell culture studies also show that most GAs secrete gonadotropins or their subunits in vitro, even tumors that are negative for pituitary hormones on IHC analyses (248–250,255,256,263–268). When cultured in vitro, α subunit was secreted by 85% of GAs, FSHβ by 82%, and LHβ by 66%, while intact FSH or LH was secreted by 75% tumors (263). A strong correlation between FSHβ mRNA in tumor and FSH secretion in culture medium was seen (263). Other studies report similar high rates of gonadotropin secretion from NFPAs cultured in vitro (249,250,255,267,269,270). In normal gonadotroph cells α subunit is normally produced in excess of the β subunits, both on mRNA and protein levels (271,272). However, in GAs this balance is disrupted and FSHβ (mRNA and protein) production is increased compared with α subunit and LHβ (263). In cultures FSHβ is also secreted in excess of α subunit (263). Correspondingly, in vivo secretion of FSH/FSHβ is most common, followed by α subunit and rarely LH/LHβ (245,249,250,254,255,257,269,270,273).
Signs of elevated gonadotropins may be subtle and not easily identifiable, which partly explain the rarity of clinically functioning GAs (216,242,244,245). In females, gonadotropin hypersecretion can cause elevated estradiol, menstrual irregularities, infertility, galactorrhea, ovarian hyperstimulation-syndrome (216,242,274–276). In males it can lead to testicular enlargement and hyper- or hypogonadism, but this is rarely seen (216,242,277–279).

5.10: Regulation of FSH and LH

The regulation of FSH and LH synthesis and secretion is complex and involves multiple pathways (262,280,281). As described above, FSH and LH are dimers formed from one common α subunit and one β subunit, which is distinct for each hormone (282). In normal gonadotroph cells, the production and secretion of gonadotropins are regulated by pulsatile release of GnRH from the hypothalamus (281–286). Activin, inhibin, follistatin and negative feedback from sex steroids, also play a part in the regulation of FSH and LH (figure 2) (262).

GnRH-receptor is expressed in most GAs (287), however its signaling differ from normal pituitary cells (252). In healthy subjects, chronic administration of GnRH leads to decreased circulating FSH and LH, while in patients with GAs chronic treatment with GnRH analog has been shown to increase serum α-subunit, without change in circulating FSH and LH (252). GnRH stimulation of cultured GAs, increases FSH and LH synthesis and secretion (270). LH secretion also increases in response to LH (270,273).

Activin is produced by the gonads and the pituitary glands, and has both endocrine and paracrine effects on gonadotroph cells (286,288–292). Activin stimulates transcription of FSHβ and GnRH-receptor mRNA, as well as FSH glycoprotein production and secretion (266,288,289,293–296). Activin belongs to the transforming growth factor β (TGFRβ) family, and signals through the TGFβ1- and TGFβ2-receptors (281,288,291,297,298). It has been
shown to work as a growth and differentiation factor, but in some tissues it has anti-proliferative effects (266,289,299–302). Both activin and activin receptors are present in GAs (266,290,291,303,304). In GAs, activin increases FSHβ mRNA levels as well as FSH synthesis and secretion (figure 2) (266,289,293,305), although one study found increased secretion of the FSHβ subunit only (266).

Follistatin is produced by gonadotroph cells in the pituitary and functions by binding to activin and inhibin (280,288,306–309). In particular, follistatin binds to activin and neutralizes its effect on FSH production and secretion (306–308). Studies have found lower levels of follistatin in GAs than normal gonadotroph cells (310). Further, cultures of GAs have found increased FSH secretion in tumors with elevated activin A (mRNA and protein) and decreased follistatin (305,311,312).

Inhibin is also produced by the gonads and the pituitary (286,289,293). It has the opposite effects to those of activin, and inhibits FSH synthesis and secretion (286,289,293,313). Inhibin belongs to the TGFβ family and exists in two forms, Inhibin A and B (286,289,293). Inhibin A binds to the receptor TGFB~R3, but the receptor for inhibin B is unknown (314–316).
Figure 2: Regulation of FSH in gonadotroph pituitary cells

Gonadal steroids also have negative feedback on the pituitary and regulates FSH and LH in males and females, both via the estrogen receptors (ER) α and β and androgen receptors (281,282,284,286,318–322). Pituitary ERs are necessary for normal reproduction (323,324), and is also present in most (70-83%) GAs (325–330). The expression of ERα and ERβ is lower in GAs than in normal pituitaries (325,326). Estradiol decreases FSH and LH mRNA and protein levels, both in vivo and in vitro (318–321,331–333). Estradiol also decreases FSH and LH (mRNA
and protein) in pituitary cultures (333). It also has inhibitory effects on the α subunit (331). Testosterone directly regulates LH secretion, but its effects on FSH requires aromatization to estradiol (321).

Different expression of ERs are also related to the behavior and aggressiveness in several tumor types, including pituitary tumors (327,334–340). This is in part due to estrogen-mediated downregulation of E-cadherin, which cell-cell adhesion molecule seen on the membrane of epithelial cells (336,341–343). However, this process is complex and dependent on the balance of ERα and ERβ receptors, as well as the presence of ligands (344).

5.11: Epithelial to Mesenchymal Transition

Loss of epithelial phenotype and development of a mesenchymal phenotype has been referred to as epithelial to mesenchymal transition (EMT) and is associated with aggressive behavior in many tumor types (345–347). One hallmark of EMT is loss of membranous E-cadherin (336,341,342,345,346,348–353). EMT is often associated with presence/accumulation of E-cadherin in the nucleus, where it is normally not present (354–358). In somatotroph and corticotroph pituitary adenomas, loss of membranous E-cadherin is associated with larger and invasive tumors, with poorer response to therapy (359–364). Decreased membranous E-cadherin and increased nuclear E-cadherin is also associated with larger NFPAs (334,365), however not all studies replicate these findings (366,367). Altered E-cadherin expression is also associated with changes in the secretory capacity of hormone-producing cells (368–371).

5.12: Transforming Growth Factor Beta Receptor 3 Like (TGFBR3L)

Previous studies had shown that the expression of TGFBR3L mRNA was higher in the pituitary than other organs, and TGFBR3L was considered pituitary enriched (i.e. 4x higher expression
in the pituitary than in any other tissue) (372). Other pituitary enriched genes include the pituitary hormones and transcription factors. Out of 26 pituitary enriched proteins, three were lacking evidence at the protein level, TGFBR3L being among them (373). Data from single-cell RNA sequencing indicated that TGFBR3L mRNA was expressed in gonadotroph pituitary cells (374,375). However, TGFBR3L had not been demonstrated at the protein level and its subcellular location was unknown. The amino acid sequence of TGFBR3L suggested that it was a single-pass membrane protein (376). The name of TGFBR3L originates from its sequence homology (34% of amino acids) with TGFBR3 (also known as betaglycan) (373).

TGFBR3 functions as a co-receptor for inhibin A (315,377). TGFBR3 also functions as receptor for other proteins in the transforming growth factor family (e.g. bone morphogenic proteins and growth differentiation factors) (378,379), and it is involved in embryological development, cell differentiation and in signaling in cancer cells (380,381). However, little is known about TGFBR3L and its downstream signaling. A single study reported that increased TGFBR3L expression was associated with the development of neuroblastomas (362). Beyond this, it is unknown if TGFBR3L shares some of these functions of TGFBR3, or if it only has a similar amino acid sequence.
6: Aims

6.1: Paper 1:

The primary aim of this study was to describe the prevalence of hypopituitarism and SAI in before and after surgery for NFPA. In addition, we wanted to identify risk factors for developing SAI after surgery. The secondary aims were to examine if evaluation of basal hormonal values could replace the short Synacthen-test endocrine work up 3 months after surgery, and to investigate what constitutes a sufficient p-cortisol response during SST on the new cortisol assays. We hypothesized that transsphenoidal surgery rescues pituitary function in patients with NFPAs, and that pituitary apoplexy is the main cause of post-operative SAI.

6.2: Paper 2

The primary aim was to investigate the relationship between EMT (marked by E-cadherin) and the IHC staining of FSH and LH in gonadotroph NFPAs. Further, E-cadherin and gonadotropin staining was compared to ER status of tumors. A secondary aim was to compare the staining of FSH and LH in the PA’s to the levels in the circulation. We hypothesized that FSH and LH accumulate intracellularly as tumor cells undergo EMT and lose their epithelial differentiation (and their ability to secrete hormones).

6.3: Paper 3

The primary aim of this study was to investigate the staining and expression of TGFBR3L in normal and neoplastic pituitary tissue from humans. We wanted to determine the subcellular location of TGFBR3L, which pituitary cell types that express TGFBR3L, and the relationship between TGFBR3L and pituitary hormones. Furthermore, we investigated the association
between TGFBR3L and EMT, ERs and SSTRs. Our hypothesis was that TGFBR3L was a membranous protein present on gonadotroph cells.

6.4: Paper 4

The primary aim of this study was to compare the TGFBR3L staining to the circulating levels of gonadotropins, and to tumor size and invasiveness. The secondary aim was to validate the results from paper 3 regarding TGFBR3L in a different cohort of patients with NFPAs, and this time in whole tissue sections. Furthermore, we wanted to investigate any association between TGFBR3 and TGFBR3L staining. Based on our findings in the previous study, we hypothesized that TGFBR3L was involved in gonadotropin regulation in NFPAs, and that TGFBR3L associated with larger invasive tumors.
7: Material and Methods

7.1: Population for study 1

Patients in studies 1 were included in a prospective study at the Section of Specialized Endocrinology at OUS that started in 2014. The inclusion criteria were (i) adult patients undergoing surgery for pituitary tumors, (ii) no clinical or biochemical signs of hypersecretion of hormones, and (iii) no previous surgery or radiotherapy to the pituitary gland. Recruitment will finish when 230 patients are included, which is expected by the end of 2021. Prior to surgery all patients were evaluated clinically, blood samples were analyses, and MRI/CT of the pituitary region performed. Ophthalmologist also examined the majority of patients preoperatively. The diagnosis of pituitary adenoma was confirmed by a neuropathologist. (Frozen tissue were also collected at the operation theater and stored at -80°C, but were not analyzed for the studies in this thesis). All operations were performed by four neurosurgeons. The endoscopic transsphenoidal approach has been used at OUS since 2005 (128), and was used in all patients operated with transsphenoidal approach. Informed consent was obtained from all patients to participate in the study. After surgery, patients receive routine follow-up, with visits at 3 months, 12 months, then yearly. The follow-up visits included clinical, biochemical and radiological examinations.

In study 1, patients that had been operated for NFPA and had undergone 3-months postoperative control by November 2018 were included. This included 117 patients, 65 (56%) males and 52 (44%) females. The mean age was 59 years, with a standard deviation (SD) of ± 14.9 years and a range 18-93 years. MRI was available from 116 patients, and CT from one patient. Routine descriptions from radiologists were consulted for information on tumor size.
The mean largest tumor diameter was 26.8 mm (SD ± 8.2 mm; range 13-61 mm). 114 patients were operated with endoscopic transsphenoidal approach and three patients with were operated transcranially. In the group that was operated transsphenoidally, the mean largest tumor diameter was 26.2 ± 6.8 mm, while in the transcranial group it was 53.7 ± 11.8 mm. The indication for surgery was visual impairment in 93 patients, tumor growth and elevated optic chiasm in 16 patients, pituitary apoplexy in 6 patients, and headache in 2. All the patients operated due to pituitary apoplexy had visual disturbances, and overall vision was affected in 85% of patients. Prior to surgery, any hypopituitarism was present in 71% of patients and SAI in 17% of patients. The demographic data and indication for surgery corresponded well with findings in other studies (15,42,52,53,67,128).

Upon discharge from hospital following surgery, the decision to substitute the patients with glucocorticoids was made by the attending neurosurgeon or endocrinologist. Postoperatively 76 (65%) patients were given cortisone until the 3 month postoperative visit. Of the 117 patients included, the low dose SST using the new p-cortisol assay was performed in 82 patients (figure 3). The need for further glucocorticoid therapy was evaluated by the physician who saw the patient, and was based on clinical findings and biochemical analyses. This decision was not affected by the study. Patient’s records were consulted for records of hospital admissions for hypocortisolism.

**Figure 3: Inclusion of patients and testing of HPA axis in study 1**

![Figure 3: Inclusion of patients and testing of HPA axis in study 1](Image)

Figure 3: SST: Short Synacthen test. ITT: Insulin tolerance test.
7.2: Population in studies 2 and 3

The subjects in the second and third studies were operated for pituitary adenoma at OUS between 1998 and 2009 and has been included in previous publications (240,367,382). Patients with confirmed pituitary adenoma and available paraffin embedded tissue were included in these studies. Many of the patients had been included in previous studies on NFPAs, Cushing’s disease and Acromegaly. For these studies, frozen tissue had been collected at surgery and stored at 80°C. Only patients without previous surgery or radiotherapy to the pituitary were included. Routine blood samples with hormonal analyses were available for most patients and MRI images were available after 2002. Patients’ records were consulted to determine whether the tumors were considered clinically functioning or non-functioning. Routine blood samples were consulted for circulating hormone levels. The majority (>90%) of the operations were performed by three surgeons. The diagnosis of pituitary adenoma was confirmed with hematoxylin and eosin staining. Informed consent were collected from all patients.

In study 2, only gonadotroph tumors with available paraffin embedded tissue were included. This comprised 105 patients with a mean age of 59.9 (SD ± 13.0; range 31-84) years. 72 (69%) patients were male and 33 (31%) were female. Frozen tissue for mRNA analysis were available from 74 patients and MRI were available from 47 patients. The MRI’s had been examined independently by two investigators in previous publication (383). The mean age of menopause in Norway is 51 years old (384) and women older than this were considered postmenopausal, and excluded from the analyses of circulating p-FSH and p-LH.

Study 3 included all types of pituitary adenomas (both functioning and non-functioning) operated between 1998 and 2009. Paraffin embedded tissue were available from
all 230 patients. Polymerase chain reaction (PCR) analyses for gene expression were available from gonadotroph tumors only. Clinical data, hormonal analyses and radiological examinations were not included in the study. The gonadotroph tumors were the same tumors used in study 2. In addition, whole tissue sections from 20 NFPAs from the Uppsala Biobank were included. Eight of the whole tissue sections contained parts of tissue from normal pituitary gland.

7.3 Population in study 4

This study included 145 patients operated for NFPA between December 2014 and September 2020, comprising all subtypes of NFPAs. This cohort was the same as that in study 1, and the patients received the same work-up and follow-up as described above. Only patients with available paraffin embedded tissue for further IHC investigations were included. 19 patients were not included due to missing pathology slides of for further analyses, necrotic tissue samples, pituitary adenoma not visible in slides, or diagnosis other than pituitary adenoma.

The population consisted of 42% females and 58% males, with a median age of 61 years (IQR 50-70). Radiological examination was performed on all patients (MRI in 144 patients, CT in one patient) prior to surgery. Preoperatively collected blood samples were available from 142 of 145 patients. The blood samples were analyzed using routine laboratory methods. The transsphenoidal approach was used in all patients except three who were operated by transcranial approach.

In this study, entire tissue sections were used for IHC analyses. Of the 145 tumors included in the study 80% were gonadotroph, 14% corticotroph, 4% somato-thyreolactotroph, 1% plurihormonal, and 2% were negative for both hormones and transcription
factors (null cell adenomas). Staining for pituitary hormones were performed on tissue from all patients. Prior to June 2019, IHC staining for transcription factors were only investigated in hormone negative tumor, but was investigated in all tumors thereafter.

7.4: Evaluation of HPA axis

Blood samples taken between 08.00 and 10.00 was used for analyses of p-cortisol and evaluation of HPA axis, both before and after surgery. Patients had abstained from glucocorticoids since 15.00 the previous day. Preoperatively, SAI was diagnosed in patients with symptoms of hypocortisolism in the presence of low morning p-cortisol. SAI was also diagnosed in patients with clinical symptoms of hypocortisolism who improved symptoms with glucocorticoid replacement, even if no morning p-cortisol was measured. After surgery, the attending endocrinologist or neurosurgeon evaluated the need for glucocorticoid substitution, based on preoperative pituitary function, morning p-cortisol and clinical evaluation. Upon discharge from the hospital, 76 patients (65%) received glucocorticoid replacement postoperatively.

In addition, most patients underwent dynamic testing of the HPA axis, on the routines of the hospital where they attended follow-up. The decision to continue/discontinue cortisone was made by the evaluating physician, and was not affected by the study. Records were collected to investigate hospital admissions for SAI.

SST was performed in 99 patients, where 1 µg Synacthen (Alfasigma, Milan, Italy) was used in 92 patients, while 250 µg was used in 7 patients. Synacthen was injected intravenously, and blood samples were collected before injection, then 30 and 60 minutes after the injection. The ITT was performed in four patients who were followed at their local hospital, and
consisted of infusion of insulin until hypoglycemia (p-glucose <2.2 mM) was reached, before measuring p-cortisol.

Evaluation of the other pituitary axes were based on clinical information and routine blood sampling, and dynamic testing were no performed. Blood hormone levels below the laboratory’s reference ranges were considered insufficient.

7.5: Measurement of plasma-cortisol:

In study 1, p-cortisol was measured using electrochemiluminescence assays on all patients. However, the methods of the analysis of p-cortisol changed during the study period, both the laboratory machines and the assay that was used. At the OUS, p-cortisol was measured by electrochemiluminescence assay on Roche Modular E170 (Roche Diagnostics, Rotkreuz, Switzerland) until 23 May 2016, and thereafter on Roche Cobas e602. The change in machinery did not affect the reference ranges of p-cortisol. (In addition 13 patients were followed in their local hospital that used Roche Cobas e801 for measuring p-cortisol, but these patients were not included in the analysis of the SST).

However, in September 2015 the laboratory at OUS changed to a new and more specific cortisol assay (changed from Roche Elecsys Cortisol I to Roche Elecsys Cortisol 2). The cortisol II assay was used on Roche Modular E170 and Roche Cobas e602 in the time periods specified above. The newer assay measured 10-25% lower levels of p-cortisol, with larger differences at increasing concentrations of p-cortisol. The reference range of p-cortisol on the old assay was 138-690 nmol/l, and on the new assay 112-502 nmol/l. The coefficient of variation was < 5.5% for both methods given by the laboratory at Department of Clinical Biochemistry, OUS.
7.6: Immunohistochemistry (IHC):

IHC uses two sets of antibodies to detect and visualize specific protein antigens in tissue. The primary antibodies bind to specific antigens in the tissue, while the secondary antibody binds to the primary antibody. The secondary antibody is conjugated to a label that allows its visualization by the microscope. The IHC staining for pituitary hormones in study 1 were based on routine laboratory reports, and included only pituitary hormones. More extensive IHC analyses were performed in the remaining studies. All tumors were stained for transcription factors and classified accordingly. IHC investigations for studies 2-4 were performed by the neuropathologist Olivera Casar-Borota at Uppsala University Hospital, blinded to the clinical data. IHC TGFBR3L staining and triple-labelling was performed and scored by Evelina Sjöstedt at Karolinska Insitutet and Uppsala University, who was also blinded to the clinical data. The anti-TGFBR3L antibody was developed by the Human Protein Atlas according to an established procedure (385).

In studies 2 and 3, IHC examinations were performed on tissue micro arrays (TMA) (386,387). The microarrays were created from two core biopsies of 1 mm taken from representative areas of each tumor sample. A neuropathologist had selected the areas of the tumor prior to sampling. The cores were then fixed in paraffin along with the samples from multiple other tumors. Samples from normal pituitary gland were also added to the microarrays and served as controls. The TMAs were then stained for pituitary hormones and transcription factors, and E-cadherin, N-cadherin, ERα, SSTRs and TGFBR3L. Study 3 also investigated TGFBR3L staining in 20 samples from the Human Protein Atlas’ biobank. For study 4, whole tissue sections were used for IHC, instead of the microarrays. This was done to validate the findings from study 3 in larger sections, and in a different cohort of patients.
The staining for the membranous E-cadherin, ERα, N-cadherin and SSTRs was scored using the immunoreactivity score (388). The immunoreactivity score is the product of the percentage of positively stained cells (0 = 0%; 1 = 1-10%; 2 = 10-50%; 3 = 50-80%; 4 ≥ 80%) multiplied with the staining intensity range (0: no staining; 1: weak staining; 2: moderate staining; 3: strong staining). This gives a scale from 1 to 12, but the scores 5, 7, 10 and 11 cannot be attained.

In study 2 and 3, the staining of FSHβ and LHβ was scored based on the percentage of positive cells (low: <10% positive cells, moderate: 10-50% positive cells, and high: >50% staining cells), while in study 4 they were scored using the abovementioned immunoreactivity score. TGFBR3L was also graded based on the percentage of positive cells (negative: 0% positive cells; low: ≤10% positive cells; moderate: 10-30% positive cells; high: ≥30% positive cells).

The triple staining was performed using the tyramide signal amplification (389). The samples were incubated with a single primary antibody (anti SF-1, anti-TGFBR3l, or anti-FSHβ. This was followed by incubation with secondary antibody conjugated with horseradish peroxidase, then addition of a tyramide signal amplification fluorophore. The samples were then heated to deactivate the first antibodies and the process was repeated for the next antigen, with a different fluorophore. All the antibodies originated from rabbits.

7.7 Radiological investigations:

In study number 1, the data regarding tumor size and invasiveness were collected from routine radiological descriptions. Thus, size and invasiveness were not assessed by the same observer using similar criteria. In study 4, based on the same population as study 1, all MRI and CT images was evaluated by the author under the guidance of Geir Ringstad, a
neuroradiologist. Tumor volume was calculated using the formula for an ellipsoid \((4/3 \times \pi \times (\text{height}/2) \times (\text{width}/2) \times (\text{depth}/2))\) and Knosp-score was used to determine invasiveness. Tumors with a Knosp score of 3 or higher were considered invasive. There was a strong correlation between largest tumor diameters from study 1 and the calculated volume \((r=0.829, p<0.0001)\).

The radiological results in study 2 was collected by Kristin Astrid Berland Øystese in collaboration with Geir Ringstad, and has been used in previous publications (240,367,383). All coronal MRI section from each tumor had been analyzed, and the measurements were used to calculate the tumor volume. Tumors with a Knosp score ≥3 was considered invasive. Radiological data were not included in study 3. This because MRI images were available from a minority of tumors investigated for TGFBR3L, and only a few \((n=13)\) of the tumors positive for TGFBR3L.

**7.8: Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)**

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed on pituitary tissue to investigate gene expression. Frozen tissue were collected at the operation theatre, frozen immediately after resection and stored at \(-80^\circ\text{C}\). mRNA was extracted and RT-qPCR was performed. The results of RT-qPCR was standardized against the geometric mean of the reference genes \(GADPH\) and \(ALAS1\) (390).

**7.9: Statistics**

The STATA statistical software was used for analyses in all studies. Student’s \(t\) test was used if the data was normally distributed. Mann-Whitney \(U\) test was used for comparison of the IHC
scores. Chi-square test was used for comparison between groups. Spearman’s rank correlation was used for correlation analyses. P<0.05 was considered significant.
8: Ethical considerations

The studies in this thesis were approved by the independent Regional Ethics Committee of South Eastern Norway (Study 1 and 4: REK number 2014/1680 approved 23.10.2014, extended 14.02.2020; study 2 and 3: REK number 2014/635 approved 08.05.2014, extended 09.05.2019) and the hospital administration of OUS. In study 3, additional material from the Uppsala Biobank, was used, which had been approved by Regional Ethics Review Board in Uppsala (Reference #2002-577, 2005-338 and 2007-159, approval dates 20.11.2002, 20.12.2005 and 31.07.2008). Informed consent was obtained from all patients, and patients had the right to withdraw from the studies at any time. The projects conformed to the ethical standards of the Helsinki Declaration of 1964 and its later amendments.

All patients in this study underwent normal medical work-up, treatment, and follow-up. The patients did not undergo additional tests or procedures for the purpose of this thesis, and participation in the studies did not pose any additional risk of harm to the patients.

Personal and clinical data was used in the study and patient’s confidentiality was an important issue. Clinical, biochemical and radiological data was collected and stored in an anonymized database, accessible only through OUS for selected users. The tumor tissue used in this study was anonymized and stored in a biobank at OUS. Investigators performing IHC grading and mRNA analyses were blinded to the clinical data.
9: Results/Summary of papers

9.1: Paper 1

The prevalence of hypopituitarism and SAI in patients with NFPAs varies greatly between studies. In this prospective study, we investigated the rate hypopituitarism in patients undergoing operation for NFPAs, with particular focus on the HPA axis. We also investigated what was considered a sufficient response to the SST 3 months after surgery for NFPA. This study included 117 patients operated for NFPA. Patients underwent clinical and biochemical evaluation preoperatively and 3 months postoperatively. Follow-up visits were scheduled yearly thereafter. The median follow-up time of the patients were 33 months (range 8-54 months).

Primary aim: We found that transsphenoidal surgery for NFPAs was safe and that the risk of developing hypopituitarism after surgery for NFPA was low. Hypopituitarism preoperatively was seen in 83 patients (71%), while after surgery it was seen in 65 (55%) patients postoperatively. Surgery rescued pituitary function in 22 (27%) patients with hypopituitarism, while new hypopituitarism developed in only 4 (12%) patients.

SAI was diagnosed in 20 (17%) patients before surgery and 17 (15%) after surgery. For patients undergoing transsphenoidal surgery without pituitary apoplexy, SAI was seen in 14 (13%) preoperatively and 10 (9%) postoperatively. Of the 20 patients with SAI preoperatively, eight (40%) recovered after surgery and were weaned off glucocorticoids at the 3-month postoperative visit. Twelve patients had SAI both before and after surgery. New SAI developed in five patients after surgery, three of which were operated with transsphenoidal approach, and two with open transcranial approach. Thus, the risk of developing SAI in patients undergoing routine transsphenoidal surgery for NFPA was <3%. SAI was seen in five of six
patients (83%) operated acutely for pituitary apoplexy, indicating that it was a risk factor for SAI postoperatively.

Secondary aim: In this study, the peak p-cortisol during SST was considerably lower than what has traditionally been considered a normal response (500-550 nmol/l). We found that cortisone could be safely tapered in patients with p-cortisol >320 mM during SST. Early morning p-cortisol >168 nmol/l were predictive of p-cortisol >320 during an SST in most patients. However, some patients with morning p-cortisol <168 nmol/l had a p-cortisol >320 nmol/l during SST, thus early morning p-cortisol was not a reliable predictor for the response to SST. None of the patients who discontinued cortisone were admitted to the hospital for hypocortisolism during the follow-up period.

The 1-year postoperative visit had been attended by 100 patients, of whom 13 had SAI at the 3-months visit. Three of these patients had recovered HPA axis function and cortisone was discontinued. No patients developed SAI between the 3-months and 1-year visits.

9.2: Paper 2

NFPAs most commonly arise from gonadotroph cells. Although clinically non-functioning, the majority of gonadotroph tumors produce FSH and/or LH, evident on IHC staining. In this study we compared gonadotropin staining to the degree of EMT (represented by changes in E-cadherin), and to the concentration of gonadotropins in the peripheral circulation. ER have been shown to regulate both E-cadherin and FSH, and we also investigated the relationship between ER-status and E-cadherin and gonadotropins.

Paraffin embedded tissue from 105 patients operated for GAs were available for inclusion in this study. Tumors with high FSH staining showed decreased staining for membranous E-cadherin and increased staining for nuclear E-cadherin. FSH staining
correlated positively with both ERα staining and mRNA. Further, the staining of FSH in tumors correlated with the circulating levels of p-FSH. There was also an inverse correlation between membranous E-cadherin and LH staining, although this correlation was weaker than for FSH. LH staining did not correlate with nuclear E-cadherin, ER staining or mRNA, or P-LH.

9.3: Paper 3

Earlier studies had found that the expression of TGFBR3L (mRNA) was higher in the pituitary than in other organs. Single-cell sequencing of mice and rat pituitary cells had shown that TGFBR3L was expressed in gonadotroph cells. However, TGFBR3L had not previously been detected on the protein level. In this study, we examined TGFBR3L IHC in pituitary adenomas and normal pituitary tissue. TMAs from 230 pituitary adenomas and whole tissue sections from 20 patients were included. Eight of the whole tissue sections contained parts of tissue from the normal pituitary gland.

IHC staining for TGFBR3L was seen in gonadotroph tumors only, not in tumors of the other pituitary lineages. However, only 37 (34%) of the 110 gonadotroph adenomas investigated showed staining for TGFBR3L, indicating that the TGFBR3L protein is produced by a subset of gonadotroph cells (figure 4). In normal pituitary tissue, TGFBR3L staining was solely seen in gonadotroph cells. This was visualized with both IHC and triple staining, which showed that TGFBR3L staining only in SF1 positive cells. In both normal and neoplastic pituitary tissue, the TGFBR3L staining showed a membranous pattern. TGFBR3L staining had positive correlation with FSH and LH staining, and an inverse correlation with E-cadherin staining. There was also a correlation between TGFBR3L staining and TGFBR3L gene expression (mRNA).
Figure 4: TGFR3L staining in gonadotroph NFPA

Figure 4: Immunohistochemical TGFR3L staining in gonadotroph tumors with score 1 (A) and 3 (B). 200x magnification.

9.4: Paper 4

The primary aim of this study was to investigate TGFR3L staining in NFPAs and to compare it to clinical, biochemical and radiological data unavailable in study 3. In particular, we wanted to study whether TGFR3L was associated with levels of circulating gonadotropins or with tumor size and invasiveness. Further, the aim was to validate the findings on TGFR3L by performing IHC staining on whole tissue sections from a different cohort of patients with NFPAs. We also wanted to compare TGFR3L staining to the staining of TGFR3.

This study included 145 patients operated for NFPAs between December 2014 and September 2020. Paraffin embedded tissue were available from all patients. Similar to study 3, TGFR3L was only seen in gonadotroph tumors, and in one plurihormonal tumor with gonadotroph elements (i.e. positive for FSH and SF-1 in addition to ACTH and T-pit). In gonadotroph tumors, high TGFR3L staining was closely associated with strong LHβ staining, while there was no association between TGFR3L and FSHβ staining. However, there was an
inverse relationship between TGFBR3L staining and p-LH and p-FSH in males with gonadotroph tumors.

Strong TGFBR3L staining was associated with larger tumor volume in patients with gonadotroph tumors, but there was no relationship between TGFBR3L and invasiveness. There was no association between TGFBR3L and the age or sex of patients. TGFBR3L did not correlate with TGFBR3. Strong TGFBR3 staining was seen in nearly all corticotroph tumors, but only weakly and in a minority of gonadotroph tumors.
10: Discussion of main findings

10.1: Hypopituitarism and the short Synacthen test

We found that surgery for NFPAs was safe, improved vision in the majority of patients, and that complications were infrequent, in accord with previous studies (52–54,58,67,127). New onset of SAI after surgery occurred in 2.6% of patients undergoing routine transsphenoidal surgery. Although surgery rescued HPA axis function in 40% of patients, the rate of SAI before and after (17% vs 15%) surgery was not significantly different. Other studies have found similar rates of SAI pre- and postoperatively, as well as a low rate of new onset of SAI (53,54,58,67). Patients with pituitary apoplexy were at increased risk of SAI, as has been shown earlier (69), but we did not find any other risk factors for developing SAI or hypopituitarism after surgery.

Hypopituitarism was more common before than after surgery for NFPA (71 vs 55% of patients), also corresponding to previous studies (53,54,58,67). One study reported worsening of pituitary function postoperatively (52). The same study (43) reported less tumor recurrence than studies where pituitary function improved after surgery (90% vs 63-65% tumor free recurrence) (53,58,67). Taken together, this suggest that the radicality of surgery affect the postoperative endocrine function. In this study, the postoperative MRIs were not systematically investigated by a radiologist, therefore prevalence of hypopituitarism/SAI could not be compared to presence of residual tumors. Other studies have found that larger tumors were associated with development of hypopituitarism after surgery, and that younger patients and absence of CSF leak were associated with recovery of pituitary function, but this was not the case in our study (53,54).

Our study showed that glucocorticoids could be safely tapered in patients with a peak p-cortisol during the SST that was substantially lower than the traditional cut-offs. A normal
p-cortisol response to the SST has traditionally been set at 500-600 nmol/l (179,180,189,192,193). In this study, p-cortisol >320 nmol/l during the SST three months after surgery for NFPA indicated a sufficient response. None of the patients were admitted to the hospital for hypocortisolism after tapering glucocorticoids, with the shortest follow-up being 8 months. Further, we found that all patients with morning cortisol >168 nmol/l had peak p-cortisol >320 nmol/l during the SST. However, 4 patients with a sufficient response to SST had morning p-cortisol below 168 nmol/l, indicating that morning p-cortisol alone is inadequate to determine HPA axis function.

It has been documented that older assays overestimates p-cortisol concentrations (172,200,205), in part because of cross-reactivity with other steroid hormones (200). The newer assays measure p-cortisol that is up to 30% lower than the older assays (172,203), and correlate better with LC-MS/MS, the gold standard for measuring steroid hormones (172,199,200). As a result of this, a re-evaluation of the normal response to SST has been suggested, both for the newer assays and for LC-MS/MS (199,201–203). Three studies that used the same assay as we did (Roche second generation assay) have suggested to revise a normal SST response to >350, >351, and >374 nmol/l, respectively (201–203), slightly higher than our results. One of these studies suggested that peak p-cortisol between 300 and 350 nmol/l indicates a grey area where diagnosis is uncertain (202).

These studies differed from ours in that they determined the cut-offs from ROC curves (202,203) or from the correlation between the first and second generation assays (201). Our study was purely observational, and in cases where the result of the SST was uncertain, slow tapering of cortisone was attempted. Cortisone was continued if signs of hypocortisolism
developed. This clinical approach may explain why we found a lower SST peak p-cortisol as sufficient.

10.2: FSH Staining and EMT in Gonadotroph Pituitary Adenomas

We found that tumors with high FSH staining showed decreased levels of membranous E-cadherin and increased levels of nuclear E-cadherin, both of which are considered markers of EMT (345,354,365). This suggests that the FSH content within gonadotroph tumors increase as they undergo EMT (figure 5). However, we also found that FSH IHC staining in tumors correlated with circulating p-FSH levels, indicating that some of the FSH entered the circulation. However, we could not determine whether this was due to increased production of FSH or change in secretory capabilities.

E-cadherin is known to affect hormone secretion in several cell types. A study of rat pituitary lactotroph cells found that prolactin content decreased after the forced expression of membranous E-cadherin (368). Prolactin mRNA levels were similar in the groups with/without E-cadherin, and the differences in prolactin protein content were presumably caused by changes in the secretory machinery (368). In pancreatic β-cells, lower levels of membranous E-cadherin is also associated with decreased insulin secretion (369). Further, incubation of β-cells with anti-E-cadherin antibodies inhibit secretion of insulin (371), while E-cadherin adhesion stimulated insulin secretion (370). Gonadotroph adenomas also show lower levels of membranous E-cadherin than other NFPA subtypes (240,359,360,367), but are rarely functional (216). Taken together, these results suggests that E-cadherin is important for the secretion of hormones and may explain the intracellular accumulation of E-cadherin in our study. However, it does not provide an explanation for the correlation between FSH IHC staining and p-FSH.
Figure 5: Loss of membranous E-cadherin and nuclear accumulation of the intracellular domain of E-cadherin are hallmarks of EMT. Our study found that changes in E-cadherin staining was associated with an increase in cellular content of FSH. EMT: epithelial to mesenchymal transition. This figure was created using images modified from Servier Medical Art (317), which is licensed under a Creative Commons Attribution 3.0 Unported License.

It is known that the production of FSHβ (mRNA and protein) is increased relative to LHβ and α-subunit in gonadotroph adenomas (263). Several studies have found that gonadotroph pituitary adenomas are associated with increased p-FSH in vivo and secretion of FSH in vitro (246,249,251,253,263,273), in accord with our results. A strong correlation between FSHβ mRNA and FSH secretion in vitro has also been reported (263), which indicates an intact secretory capacity of the tumors cells. However, it has also been reported that two thirds of gonadotroph tumors do not respond to GnRH stimulation, even though they contain FSH intracellularly (391). We found no correlation between LH staining and p-LH, although FSH and LH showed equal staining.
In our study there was a positive correlation between FSH staining and ERα (staining and mRNA). Although FSH synthesis and secretion is normally inhibited by estradiol (282,318,319,321,330,332), we found a positive correlation between FSH and ERα by IHC. Our data do not provide a good explanation for this. Possibly, this is due to the demography of the subjects as the majority of patients were either male (72 of 105 patients) or postmenopausal (27 of 105). Both groups have low levels of circulating p-estradiol which may obfuscate the negative feedback of estradiol.

EMT and the loss of membranous E-cadherin is associated with more aggressive tumor behavior in several tumor types throughout the body (349,392,393). This has also been reported for corticotroph and somatotroph pituitary adenomas, as well as NFPAs, where it has been associated with larger and more invasive tumors (334,359,360,365). However, we found no significant correlation between E-cadherin levels and tumor size or invasiveness.

Estrogen receptors (ER) are known to regulate E-cadherin expression (334,336,341). This relationship appears to be complex, where stimulation of ERα decreases membranous E-cadherin, while ERβ increases membranous E-cadherin (334,344,394). In accordance with these studies, we found that tumors with higher ERα IHC staining had lower levels of membranous E-cadherin.

10.3: TGFBR3L in pituitary adenomas

Study 3

In this study we show that TGFBR3L protein is present on the plasma membrane of gonadotroph pituitary cells, both neoplastic and non-neoplastic. TGFBR3L staining was not seen in the other anterior pituitary cell lineages. We found that TGFBR3L correlates with FSH
and LH staining in the gonadotroph cells. There was an inverse correlation between TGFBR3L and membranous E-cadherin.

Our results are supported by earlier studies that have found enriched TGFBR3L mRNA expression in pituitary gonadotroph cells of humans (395) and rodents (374,375). These studies also indicate that TGFBR3L is preserved across species. Our study was the first study to demonstrate TGFBR3L at the protein level and its subcellular location. Although the function of TGFBR3L is not known, its membranous location, and the correlation between TGFBR3L and FSH and LH staining suggests that TGFBR3L may be involved in the regulation of gonadotropins. It is of interest that TGFBR3 (which TGFBR3L is named after) functions as a receptor for Inhibin A (315). However, whether this is a coincidence or TGFBR3L also regulates gonadotropins requires further studies.

IHC staining for TGFBR3L was present in approximately a third of the gonadotroph tumors. This study was performed on TMAs which showed heterogeneity within the tumor. Thus, since only 2 areas of 1 mm each were investigated, it is possible that a larger number of tumors are positive for TGFBR3L.

We found an inverse relationship between TGFBR3L and membranous E-cadherin. In addition, TGFBR3L positive tumors showed increased staining for nuclear of E-cadherin. It is well established that the TGFβ-superfamily is involved in numerous signaling processes including EMT (396–399), but from our data we could not determine the mechanisms behind this correlation.

**Study 4**

In this study we continued the investigation of TGFBR3L staining in pituitary adenomas. Similar to the previous study, TGFBR3L was only seen in gonadotroph tumors, and was
strongly associated with LHβ staining. Unlike study 3, there was no association between TGFBR3L and FSHβ staining. In study 4 a different scoring system was used for FSHβ and LHβ staining than in study 3. Applying the older scoring system to study 4 did not change the results, and we could not explain this discrepancy between the two studies.

There was an inverse relationship between TGFBR3L staining and p-LH and -FSH in males with gonadotroph tumors. This was surprising, considering the close association between TGFBR3L and LHβ staining, and may suggest that TGFBR3L plays a role in the release of gonadotropins from the gonadotroph cells in the pituitary. It is well established that TGFBR3 is an important regulator of gonadotropins by functioning as a inhibin A co-receptor (315,400). Furthermore, the similarity between TGFBR3L and TGFBR3 lies in the zona pellucida domain, which appears to be the binding site for inhibin (373,400). Unfortunately, we could not investigate the function of TGFBR3L, its ligand(s), or downstream signaling cascade.

TGFBR3L correlated with size of gonadotroph tumors, but not with invasiveness. This finding is interesting in light of the inverse correlation between TGFBR3L and E-cadherin as demonstrated in study 3. Whether TGFBR3L is somehow involved in EMT and tumor progression also requires further investigations.
11: Methodological considerations

Evaluating the HPA axis is difficult and several different tests and cut-offs have been used for diagnosis of adrenal insufficiency. This is further complicated by change in laboratory methods and assays for measuring p-cortisol. While the ITT is often considered the gold standard, the SST is frequently used as an initial test of HPA axis. We have in recent years routinely used the low dose (1 µg) SST at the work up of our patients 3-months after surgery for a pituitary adenoma. In our study, patients did not routinely repeat the SST nor were the results confirmed with an ITT, which is a limitation. Thus, our definition of a sufficient SST was based partly on p-cortisol and partly on whether patients developed symptoms of hypocortisolism without glucocorticoids. Although this may seem an unsatisfactory definition, our study showed that patients with markedly lower p-cortisol response to SST than traditional cut-offs can safely taper glucocorticoids. Exactly where the cut-off value is, if a precise number exists, could not be determined. Since there are no agreed cut-off values for the SSTs on the newer p-cortisol assays we could not calculate sensitivity or specificity for the test.

The TMA method allowed rapid evaluation and characterization of many tumors. The limitation of this method was that only two small (1 mm) samples from each tumor were analyzed. We tried to overcome this by selecting two samples taken from different representative areas of each tumor, selected by an experienced neuropathologist. IHC staining of TMAs correlated well with mRNA expression in the frozen adenoma tissue (FSH stain vs mRNA R= 0.69, p<0.0001; TGFBR3L stain vs mRNA: R 0.38, p=0.006, ERα stain vs mRNA R=0.76, p<0.0001, E-cadherin stain vs mRNA R=0.43, p=0.0001). We took this as indirect evidence that the TMA samples were representative of the entire tumor. Furthermore, in the fourth study,
we tried to address this limitation by examining the same proteins in whole tissue sections and to verify our findings from the smaller TMAs.

We could not be certain that the frozen samples were adenomatous tissue either. However, the expression mRNA for pituitary hormones and transcription factors, and their close correlation with IHC, strongly suggests that the frozen samples were representative.

Different IHC scoring systems were used for the various proteins. E-cadherin, ERα, SSTRs and N-cadherin was scored using the immunoreactivity score (staining intensity x percentage of positive cells). In study 2 and 3, scoring of FSHβ and LHβ was based solely on percentage of positive cells. This method was chosen because intensity of FSHβ and LHβ staining was heterogeneous within each tumor. In study 4, the immunoreactivity score was used for grading staining for FSHβ and LHβ staining as well, in an attempt to account for the different staining intensity of tumors.

TGFBR3L staining was also scored based on percentage of positive cells. However, it was graded differently than FSHβ and LHβ. This was done in order to reflect that TGFBR3L staining was weak overall, and that only a minority of tumor cells were positive for TGFBR3L. The same score was used for TGFBR3L in study 3 and study 4.

In study 3 and 4, the blood samples used for analysis of p-FSH and p-LH were collected when patients attended routine preoperative visits and measured by standard laboratory methods. As consequence, the blood was not samples at the same time during the menstrual cycle in all females. Furthermore, the routine laboratory methods only measured intact p-FSH and p-LH, and not FSHβ, LHβ or α-subunits, which could have given additional insight into gonadotropin production and release from the NFPAs. This would have been interesting to
investigate, as other studies have found an imbalance in the production of gonadotropin subunits by gonadotroph adenomas (263).
12: Conclusions and implications

The diagnosis, treatment, and follow-up of patients with NFPAs have evolved in the recent decade with the advent of new radiological, laboratory, and surgical methods. Patients with NFPA require a multidisciplinary approach and long-term follow-up. Transsphenoidal surgery for NFPA is safe, and infrequently causes new SAI. Pituitary apoplexy and transcranial surgery increase the risk of developing SAI. SST is useful in the diagnosis of SAI after surgery for NFPA. Newer cortisol assays gives lower levels of p-cortisol than older assays, and the assay used during the SST should be taken into account when interpreting results.

As NFPAs undergo EMT, FSHβ appears to accumulate intracellularly. IHC staining of FSHβ correlates with circulating levels of p-FSH, suggesting that some of the FSH enters the circulation. TGFBR3L is a membrane protein specific to normal and neoplastic gonadotroph cells in the pituitary. In gonadotroph NFPAs, TGFBR3L is associated with markers of EMT, larger tumors and higher intracellular content of gonadotropins. TGFBR3L may be involved in gonadotropin regulation, since gonadotroph NFPAs with high TGFBR3L staining show high intracellular content of gonadotropins, but lower circulating gonadotropins. However, TGFBR3L staining is only present in up to one half of gonadotroph tumors, which may indicate different subtypes of gonadotroph cells. More studies are needed to elucidate to function and downstream signaling of TGFBR3L, and its ligand.
13: Future perspectives

Better understanding the biology and behavior of NFPAs will be necessary for optimizing treatment of patients with NFPAs. Identification of markers of aggressive tumors may guide clinicians in determining which patients require close follow-up and have risk of tumor regrowth postoperatively. In the future, a new definition of what constitutes a sufficient response to the Synacthen-test will also be helpful to endocrinologists.

The role of TGFBR3L in gonadotroph tumors and normal gonadotroph cells merits further investigations. In vitro studies of gonadotroph cells could be performed in order to determine the function of TGFBR3L. For instance, one could stimulate gonadotroph cell cultures with inhibin before and after silencing the TGFBR3L gene, in order to determine whether TGFBR3L is involved in inhibin signaling. The effect of silencing TGFBR3L on cell growth could thus also be evaluated. Furthermore, it would be interesting to discover TGFBR3L’s ligand(s) and its downstream signaling mechanism. Since the availability of normal pituitary tissue is limited, the study of NFPAs may also reveal details of the function of normal human pituitary cells.
13: References


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372. Tissue expression of TGFBR3L - The Human Protein AtlasAvailable from: https://www.proteinatlas.org/ENSG00000260001-TGFBR3L/tissue


Papers 1 to 4
Abstract

Objective  The aim was to study the prevalence of secondary adrenal insufficiency before and after surgery for non-functioning pituitary adenomas, as well as determine risk factors for developing secondary adrenal insufficiency. A secondary aim was to determine adequate p-cortisol response to a 1-μg Short Synacthen Test after surgery.

Design  Longitudinal cohort study.

Methods  One hundred seventeen patients (52/65 females/males, age 59 years) undergoing primary surgery for clinically non-functioning pituitary adenomas were included. P-cortisol was measured in morning blood samples. Three months after surgery, a Short Synacthen Test was performed.

Results  All tumours were macroadenomas (mean size 26.9 mm, range 13–61 mm). The surgical indications were visual impairment (93), tumour growth (16), pituitary apoplexy (6) and headache (2). Before surgery, 17% of the patients had secondary adrenal insufficiency (SAI), decreasing to 15% 3 months postoperatively. Risk of SAI was increased in patients operated for pituitary apoplexy (p < 0.001), while age, sex, tumour size and complication rate were not different from the remaining cohort. Three months after surgery, all patients with baseline p-cortisol ≥ 172 nmol/l (6.2 μg/dl) and peak p-cortisol during Short Synacthen Test ≥ 320 nmol/l (11.6 μg/dl) tapered cortisone unproblematically. In patients with intact hypothalamic-pituitary-adrenal axis, p-cortisol peaked < 500 nmol/l (18.1 μg/dl) during Short Synacthen Test in 48% of patient.

Conclusion  Pituitary surgery is safe and transsphenoidal surgery rarely causes new SAI. Relying solely on morning p-cortisol for diagnosing secondary adrenal insufficiency gives false positives and the Short Synacthen Test remains useful. A peak p-cortisol ≥ 320 during (11.6 μg/dl) Short Synacthen Test indicates a sufficient response, while < 309 nmol/l (11.2 μg/dl) indicates secondary adrenal insufficiency.

Keywords  Non-functioning pituitary adenoma · Short Synacthen Test · Secondary adrenal insufficiency · Pituitary surgery

Introduction

Pituitary adenomas (PA) account for 10–15% of all primary brain tumours, and non-functioning pituitary adenomas (NFPA) are the most common subtype accounting for 43–54% of the PAs [2, 28]. NFPAs without clinical or biochemical signs of hormone overproduction are usually benign and slow-growing tumours. As a consequence of suprasellar growth and compression of anterior visual pathways, visual impairment is a common symptom [12]. Some patients present with acute symptoms due to pituitary apoplexy, evolving symptoms of local mass effect or hypopituitarism [12]. When the tumour is incidentally found on imaging, surgery is usually postponed until the patients develop symptoms or the tumour approaches the optic chiasm. Beyond hormonal...
substitution, there is no well-established medical treatment for NFPA [16].

Patients with pituitary adenomas are at an increased risk for hypopituitarism, as well both pre- as postoperatively [12, 14, 27, 29]. Hypopituitarism is associated with increased mortality, morbidity and also quality of life [10, 14, 29]. The integrity of the hypothalamus-pituitary-adrenal (HPA) axis is particularly important, as is the correct diagnosis of secondary adrenal insufficiency (SAI) [7, 26]. However, as glucocorticoid use may have adverse effects, unnecessary replacement therapy should be avoided [15, 20, 31].

SAI is common in patients with NFPA, and the frequency preoperatively has been reported to be 22–53% [6, 11, 13, 18, 25, 30]. After surgery, recovery from SAI occurred in 16–62% of the patients [6, 13], while the development of new SAI ranged from 1 to 44%, making the prevalence of SAI after surgery to be 18–60% [6, 13, 18, 25, 30].

The insulin tolerance test (ITT) and the Short Synacthen Test (SST) can be used for evaluation of the HPA axis and have been shown to be well correlated [1, 17, 23]. When performing the SST, both a high (250 μg) and a low dose (1 μg) can be used, and they yield similar results [1, 17, 24]. Previous studies have shown that p-cortisol peaks around 30 min during the low dose SST, but some centres perform a 60-min test [1, 17, 24].

The aim of this study was to investigate surgery’s ability to rescue the HPA axis function in patients with NFPA, and potential risk factors for developing new SAI after surgery. Further, the laboratory methods for analyses of p-cortisol have changed to more specific assay in the recent years, while the normal cut-off values for SST was defined decades ago. We therefore also aimed to define new cut-off values for a normal cortisol response in this population.

We hypothesized that (i) endoscopic transsphenoidal surgery rescues the HPA axis in most patients with NFPA; (ii) pituitary apoplexy is the main cause for postoperative SAI; and (iii) medical history and basal hormone values render SST unnecessary to diagnose SAI.

Material and methods

A total of 117 patients operated for a clinical NFPA were prospectively included in this study between December 2014 and October 2018. Inclusion criteria were as follows: (1) patients above 18 years of age undergoing surgery for pituitary adenoma; (2) no clinical or biochemical signs of hormone overproduction; (3) no previous surgery or radiation to the pituitary gland. Prior to surgery, the patients underwent clinical and biochemical evaluation, either at the Department of Endocrinology, Oslo University Hospital (OUS) or in the referring hospital.

All patients had a sellar tumour on radiologic examination (116 by MRI and 1 by CT). Routine radiology reports were used when describing tumour size. Neuropathologists confirmed the diagnosis of pituitary adenoma in 113 patients using routine stains (H&E, reticulin, immunohistochemical stains). In four cases (3%), the specimen was necrotic, but diagnosis of pituitary adenoma was most likely. Immunohistochemical analyses showed expression of FSH/LH in 62%, ACTH in 9%, TSH/GH/prolactin in 6%, while 20% adenomas were hormone staining negative, respectively. We did not perform immunohistochemical analyses for transcription factors in this study. Fourteen patients operated on a suspicion of NFPA were not included in the study due to another histopathological diagnosis.

Routine follow-up visit was scheduled 3 months postoperatively, with clinical evaluation, hormonal analyses, and MRI of the pituitary region. One hundred four patients attended follow-up at the Department of Endocrinology, OUS, while 13 patients attended follow-up at their local hospital.

HPA axis was evaluated by morning p-cortisol in blood samples taken between 08.00 and 10.00 both pre- and postoperatively. Preoperative SAI was defined as low morning p-cortisol in the presence of symptoms of hypocortisolism and/or improvement of symptoms with glucocorticoid therapy. Patients that underwent pituitary apoplexy who were given glucocorticoids before p-cortisol was measured were classified as having SAI.

On discharge from the hospital after surgery, the decision to substitute patients with glucocorticoids was made by the attending neurosurgeon or endocrinologist. In the cases where substitution was given, cortisone acetate was used in doses of 12.5–37.5 mg daily.

At the 3-month follow-up, fasting morning samples were taken and a 1-μg SST was performed in 99 patients. Before p-cortisol measurements, patients had abstained from cortisone since 15.00 the previous afternoon. The decision to continue cortisone substitution was then made by the evaluating clinician, based on clinical signs and biochemical results. The study did not interfere with this decision. Patients in whom cortisone was continued following the visit were referred to as having SAI, and those where it was tapered as not having SAI.

For 92 patients, SST test was performed with 1 μg (low dose) Synacthen (Alfasigma, Milan, Italy), while 250 μg (high dose) was used in seven patients followed at their local hospital. The Synacthen was injected intravenously in all patients. Samples were collected immediately before injection, then 30 and 60 min thereafter. ITTs were performed in four patients with infusion of insulin until hypoglycaemia was reached (< 2.2 mmol/l) before p-cortisol was measured.

Plasma cortisol was analysed by electrochemiluminescence assay on Roche Modular E170 (Roche Diagnostics, Rotkreuz, Switzerland) until 23 May 2016, and after by Roche Cobas e602. A single hospital
used Roche Cobas e801 for measuring p-cortisol in 13 patients in the follow-up period. The reference range was not affected by the change of platform. From 01 September 2015, the cortisol assay changed from a polyclonal to a monoclonal antibody. The new assay is more specific for cortisol and has reference range 112–502 nmol/l (4.1–18.2 μg/dl), while the old assay had reference range 138–690 nmol/l (5.0–25.0 μg/dl). The coefficient of variation was < 5.5% for both methods.

The other pituitary axes were evaluated using standard laboratory methods and the laboratory’s reference values. Secondary hypothyroidism was diagnosed by free T4 below reference interval in the presence of low or normal TSH. TSH insufficiency could not be diagnosed in patients with known disease of the thyroid gland.

Hypogonadotropic hypogonadism was defined as testosterone below reference interval associated with low or normal gonadotropins in males. In postmenopausal females, gonadotropins inappropriately low for age (FSH < 15 IU/l, LH < 8 IU/l) was used. In premenopausal females, hypogonadism was defined as amenorrhea, and/or low gonadotropins associated with low oestrogen.

We used values of IGF-1 as an indication of growth hormone deficiency. Age- and gender-specific lower limit of normal IGF-1 levels for each of the patients was used as cut-off. Dynamic testing of growth hormone was not performed routinely at the 3-month follow-up.

Patients in need of vasopressin for polyuria and hypernatremia were categorized as having diabetes insipidus (DI). DI was considered transient if vasopressin was tapered and urine output and sodium concentration normalized. Persistent DI was defined as continued polyuria and use of vasopressin. None of the patients performed an overnight water deprivation test.

Statistics

Continuous data were analysed by Student’s t test if normally distributed, otherwise Mann-Whitney U test was performed. Chi-squared test was used for comparison of categorical variables. Values are given as mean ± standard deviation and p < 0.05 was considered significant. Statistical analyses were performed using STATA version 15.1. All patients gave signed informed consent and the study was approved by the regional Ethics Committee.

Results

Surgery

The operations were performed in a single tertiary referral centre, OUS, from December 2014 to October 2018. Two surgeons performed the majority of the operations (103), while other two surgeons performed the remaining 14. All tumours were macroadenomas (Table 1). An endoscopic transphenoidal approach was used in 114 patients, while three patients were operated by craniotomy. Indications for surgery were visual impairment in 93, tumour growth and elevated/compressed optic chiasm in 16, pituitary apoplexy in 6 and typical headache in 2 patients. All patients with pituitary apoplexy had affected vision.

Vision improved in 77 (87%) of 89 patients, remained unchanged in 11, and worsened in a single patient following surgery. Visual status after surgery was lacking in 10 patients.

Complications

No complications were described in 98 patients. In the early phase after surgery, 23 complications developed in 19 patients (Table 2). Cerebrospinal fluid leakage included patients with reoperation or lumbar drainage with positive β-trace. Three patients developed haemorrhage (two after transcranial surgery), all of whom were re-operated with evacuation of a postoperative hematoma. Meningitis was not seen in any of the patients.

Secondary adrenal insufficiency

Before surgery, SAI was diagnosed in 20 (17%) patients based on clinical evaluation and early morning plasma cortisol values. There was no significant difference in tumour size, age or sex among patients with and those without SAI, neither pre- nor postoperatively (Table 3). All patients with preoperative SAI were operated through the transphenoidal approach.

Upon discharge from the hospital, 76 patients (65%) were substituted with cortisone and remained on cortisone until the

Table 1  Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Male/female</th>
<th>Age (mean ± SD)</th>
<th>Largest tumour diameter (mean ± SD)</th>
<th>Patients with preoperative secondary adrenal insufficiency</th>
<th>Patients with any preoperative pituitary failure</th>
<th>Number of pituitary axes with preoperative failure</th>
<th>Visual impairment preoperatively</th>
<th>Transphenoidal surgery</th>
<th>Median duration of follow-up:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65/52</td>
<td>59 ± 14.7 years</td>
<td>26.8 ± 8.2 mm (range 13–61 mm)</td>
<td>20/117 (17%)</td>
<td>83/117 (71%)</td>
<td>131 (mean per patient: 1.1 ± 1.0, range 0–4)</td>
<td>99/117 (84.6%)</td>
<td>114/117 (97.4%)</td>
<td>33 months (range 8–54)</td>
</tr>
</tbody>
</table>

SD standard deviation
3-month evaluation. No patient in the cohort underwent secondary surgery or irradiation within 3 months and all (117) came to the follow-up visit.

At the 3-month postoperative visit, 17 patients (15%) were diagnosed with SAI, 12 of which had preoperatively SAI. Of the five patients who developed a new SAI, two were operated with open transcranial approach. There was no difference in age, tumour size, complications or sex among those with and without SAI, neither pre- nor postoperatively.

Eight of the 20 patients with preoperative SAI recovered function of the HPA axis by surgery. Age, sex, tumour size and complication rate were not different between patients who recovered from SAI versus those who did not (data not shown). Neither did analysis of the five patients who developed SAI after surgery shows any difference from the remaining cohort with respect to age, sex, tumour size or complications (data not shown).

Six patients underwent transsphenoidal surgery for pituitary apoplexy (headache, nausea, visual impairment and radiological findings). Pathologic examination confirmed the presence of an adenoma with bleeding in all specimens. At the 3-month follow-up, five of six had SAI, indicating a significantly increased risk of developing SAI after pituitary apoplexy ($p < 0.001$).

Of the 76 patients who used cortisone in the early postoperative period, 59 patients were weaned off cortisone substitution at 3-month visit. After tapering of cortisone, cortisone had not been re-instated in any patient, nor had any patient been hospitalized due to hypocortisolism. The shortest follow-up of any patients who tapered cortisone was < 11 months (i.e. 8 months after discontinuing cortisone at the 3-month visit).

No patients developed SAI after 3-month visit (shortest follow-up 8 months).

### Cut-offs for 1 μg SST

The new monoclonal cortisol assay was used for measuring early morning cortisol in 92 patients, and a low dose SST was performed in 82 of these. In addition, 13 patients underwent high dose test or were tested using the old assay. There was no significant difference in p-cortisol (at baseline or after 30 min) between low and high dose tests, nor between the new and old assay (data not shown).

Only results for the low dose (1 μg) SST and p-cortisol measured with the new assay are considered in the following sections ($n = 92$ for morning p-cortisol; $n = 82$ for SST). The results of the p-cortisol at baseline and during to SST is shown in Fig. 1 and Table 4. Of the patients without SAI, p-cortisol peaked at < 400 nmol (14.5 μg/dl) in 13% of patients and below 500 nmol/l (18.1 μg/dl) in 48% of patients.

Cortisone was tapered 3 months postoperatively, without adverse events, in all patients with early morning p-cortisol ≥ 172 nmol/l (6.2 μg/dl), or p-cortisol ≥ 320 nmol/l (11.6 μg/dl) at 30 min during SST. The highest baseline p-cortisol in a patient with SAI was 168 nmol/l (6.1 μg/dl) and the highest 30-min response was 309 nmol/l (11.2 μg/dl) (Fig. 1).

In patients without SAI, the lowest baseline p-cortisol was 64 nmol/l (2.3 μg/dl) and the lowest 30 min value was 320 nmol/l (11.6 μg/dl). Thus, the baseline p-cortisol of the two groups overlapped, whereas the peak p-cortisol response during SST did not. Four patients without SAI had baseline p-cortisol < 168 nmol/l (6.1 μg/dl) but considered to have normal HPA axis after ST, indicating that SST remained useful.

### Other pituitary axes

Hypopituitarism of one or more axes was diagnosed in 83 patients (71%) prior to surgery, of which 22 patients (27%) recovered (Table 5). Preoperatively, hypopituitarism was

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Preoperatively (n = 114)</th>
<th>3 months postoperatively (n = 114)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>63.3 ± 16.4</td>
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<td>Tumour size (mm)</td>
<td>26.8 ± 7.2</td>
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<td>Females</td>
<td>35.0%</td>
<td>41.2%</td>
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<tr>
<td>Pituitary axes with failure</td>
<td>2.1 ± 1.0</td>
<td>2.5 ± 1.2</td>
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<table>
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<tr>
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<th>SAI (n = 17)</th>
<th>No SAI (n = 100)</th>
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<tr>
<td>Age (years)</td>
<td>57.7 ± 14.3</td>
<td>58.2 ± 13.5</td>
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<tr>
<td>Tumour size (mm)</td>
<td>26.0 ± 6.7</td>
<td>25.9 ± 6.7</td>
<td>25.1 ± 6.7</td>
</tr>
<tr>
<td>Females</td>
<td>46.4%</td>
<td>45.0%</td>
<td>45.0%</td>
</tr>
<tr>
<td>Pituitary axes with failure</td>
<td>0.9 ± 0.8</td>
<td>0.7 ± 0.8</td>
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</tr>
</tbody>
</table>

Numbers given as mean ± SD. $p$ values indicate comparison between patients with and without secondary adrenal insufficiency (SAI). TSS transsphenoidal surgery.
diagnosed in one axis in 49 patients, while 21 had insufficiency of two axes, 12 of three axes and 1 patient had insufficiency of four axes. In the 34 patients with normal pituitary function before surgery, new hypopituitarism was seen in 4 patients (12%).

In total, hypopituitarism was seen in 65 patients (55%) after surgery. Postoperatively, 31 patients had insufficiency of one axis, 24 patients of two axes, 7 patients of three axes, 2 patients of four axes and a single patient had deficiency of all five pituitary axes. The average number of axes with insufficiency was 1.1 before surgery and 1.0 after surgery ($p = 0.48$) (Table 4). Patients with SAI had failure of significantly more pituitary axes compared to patients without SAI, both pre- (2.4 ± 0.8 vs 0.9 ± 0.8, $p < 0.001$) and postoperatively (1.8 ± 1.0 vs 0.8 ± 1.0, $p < 0.001$). The number of failing pituitary axes was not significantly associated with age, sex nor tumour size.

Three months after surgery, all patients who had SAI also developed failure of at least one additional pituitary axis. In the four patients with p-cortisol < 168 nmol/l (6.1 μg/dl) that did not have SAI, three patients had insufficiency of other axes (1, 2 and 3 axes, respectively), while one patient had entirely normal pituitary function.

**Discussion**

In the present prospective study of patients with NFPA, we demonstrated that a large proportion of patients with preoperative SAI recovered by surgery and that the majority of patients who developed SAI had undergone pituitary apoplexy or were operated by a transcranial procedure. Thereby, transphenoidal surgery of NFPA was an uncommon cause of new SAI. We demonstrate that an early morning p-cortisol > 168 nmol/l (6.1 μg/dl) or p-cortisol ≥ 320 nmol/l (11.6 μg/dl) following SST indicates a sufficient function of the HPA axis.

The prevalence of SAI pre - and postoperatively in our cohort was similar to the rate reported in other studies (13–53%) [3, 8, 9, 11, 13, 21, 22, 25]. Development of SAI after surgery has been reported to be from 1 to 7.5% [9, 13, 21, 25]. In accordance with the present study, the rate of HPA axis recovery following surgery seems to be high (21–41%) [9, 13, 25]. While some studies report an overall decrease in SAI after surgery [8, 13, 25], others however do report an increase [9, 11, 21, 22]. The reason for this is uncertain, but may be linked to the degree of radical surgery, and the intention to completely resect the tumour. The criteria for diagnosing SAI also vary between studies, potentially influencing the reported prevalence.

Pituitary apoplexy increased the risk for SAI postoperatively in our study, as reported elsewhere [5]. However, we found no correlation between tumour size, age, sex, the surgical approach or complications. A recent study found that postoperative SAI occurred more frequently in patients above 50 years, most common in males, in patients with CSF leak at surgery and in patients with visual impairment at presentation [3]. In the present series, we could not confirm these findings.

**Table 4  P-cortisol response to Synacthen test 3 months postoperatively**

<table>
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<th>30 min</th>
<th>60 min</th>
<th>Change 0 to 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>No SAI ($n = 74$)</td>
<td>317.7 ± 92.0 nmol/l</td>
<td>502.5 ± 98.9 nmol/l</td>
<td>418.1 ± 105.3 nmol/l</td>
<td>184.8 ± 90.5 nmol/l</td>
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<tr>
<td></td>
<td>11.5 ± 3.3 μg/dl</td>
<td>18.2 ± 3.6 μg/dl</td>
<td>15.2 ± 3.8 μg/dl</td>
<td>6.7 ± 3.3 μg/dl</td>
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<tr>
<td>SAI ($n = 8$)</td>
<td>102.0 ± 51.4 nmol/l</td>
<td>201.2 ± 73.3 nmol/l</td>
<td>156.0 ± 69.5 nmol/l</td>
<td>99.0 ± 38.9 nmol/l</td>
</tr>
<tr>
<td></td>
<td>3.7 ± 1.9 μg/dl</td>
<td>7.3 ± 2.7 μg/dl</td>
<td>5.7 ± 2.5 μg/dl</td>
<td>3.6 ± 1.4 μg/dl</td>
</tr>
</tbody>
</table>

Only patients given 1 μg Synacthen and measured using new assay is included. Values given as mean ± SD.
Neither could we identify factors predisposing for development of or recovery from hypopituitarism after surgery. In other studies, development of hypopituitarism has been associated with larger tumours, old age and transcranial approach [4, 13, 25, 30], while recovery of hypopituitarism after surgery has been associated with young age, small tumour, absence of CSF leak and transsphenoidal approach [13, 25].

A normal response to SST has traditionally been defined as a peak value of cortisol above 500 nmol/l (18.1 μg/dl) [23] based on older assays. In the present study, we found that many patients with peak values below this level were not in the need of cortisone substitution. This difference seems to be best explained by the change in assay, where the new and more sensitive assay gives 15–20% lower p-cortisol values, however non-linear and depending on the actual cortisol level (unpublished data from Department of Clinical Biochemistry, Oslo University Hospital).

The diagnosis of SAI was not routinely confirmed by repeat SST or ITT, but by observation only. Particularly, the patients who discontinued cortisone were of interest. The majority of these patients have been followed for more than 1 year without developing new signs of hypocortisolism, indicating that the values reported here indicate a sufficient response to ST.

We speculated that in patients with low morning p-cortisol, the presence of insufficiency of other pituitary axes could be predictive of SAI and render the SST unnecessary to perform. However, our data indicates that a postoperative SST is most useful to avoid unnecessary cortisol replacement therapy.

We found that hypopituitarism of one or more axes were common prior to surgery, with recovery of approximately one quarter of these by surgery. Other studies have reported rates of hypopituitarism of any axis in 80–86% prior to surgery [11, 13, 25], decreasing to 72% 3 months after surgery [25]. However, only few patients recovered or developed new hypopituitarism after the 3-month follow-up [25]. The timing of recovery or new failure of HPA axis was similar in another study, where no patients developed SAI later than 3 months after surgery [22]. In accordance, no patients have developed new SAI after 3 months, while three had recovered at 1-year visit in our study.

Of major clinical importance, the vision improved after surgery in most patients in our series. This corresponds to other series where visual status improved in 79–86% of patients after surgery, but deteriorated in 2–3% [11, 19, 22]. Our complication rate for DI and CSF leakage was also similar to other studies [13, 19, 25].

**Limitations**

While observation of the patients indicates that the proposed p-cortisol values are adequate, repeat SST or ITT could have been performed after the 3-month visit.

We cannot speculate on what values it is safe to leave the patients without substitutional therapy after surgery, since p-cortisol was not measured systematically upon discharge from hospital.

Although all patients attended radiological follow-up after surgery, the images were not systematically evaluated for residual tumour by a radiologist. Thus, SAI cannot be related to the degree of the residual pituitary adenoma in the present study.

**Conclusion**

Surgery for pituitary adenoma is safe and improves vision in the vast majority of patients. However, surgery does not rescue pituitary function in most patients, and solely pituitary failure should therefore not give the indication for surgery. Pituitary apoplexy is a strong predictor of pituitary failure and these patients should be given cortisone substitution on admission and on discharge after surgery. Transsphenoidal surgery for NFPAs in experienced hands infrequently creates new SAI, and patients without SAI before surgery can usually be discharged after surgery without cortisone substitutional therapy.

| Table 5 Failure of pituitary axes pre- and postoperatively |
|-----------------|-----------------|
| Before surgery  | After surgery    |
| ACTH            | 20/109 (18.0%)  | 17/117 (15%)  |
| TSH             | 27/117 (23%)    | 29/115 (25%)  |
| FSH/LH          | 74/111 (67%)    | 54/111 (49%)  |
| GH              | 9/81 (11%)      | 7/108 (7%)    |
| Diabetes insipidus | 1/117 (1%)   | 6/117 (5%)    |
| Total number of axes | 131/535 (missing 50) | 113/564 (missing 21) |
| Avg. number axes with insufficiency | 1.1 ± 1.0 | 1.0 ± 1.1 |

Number of axis with failure and number of biochemical analyses available. Of 535 axes measured preoperatively, 125 were insufficient, and 50 axes were missing. Postoperatively 113 out of 564 axes were insufficient and 21 were missing.
Early morning p-cortisol > 168 nmol/l (6.1 μg/dl) or p-cortisol ≥ 320 nmol/l (11.6 μg/dl) during SST indicates a sufficient function of the HPA axis. Some patients with an early morning cortisol < 168 nmol/l (6.1 μg/dl) still have a normal response to SST, wherefore SST remains useful.

Acknowledgements We thank Kari Abelsen and Ansgar Heck for assistance and support. This study was based on routine practice at the Section of Specialized Endocrinology, Rikshospitalet, Oslo University Hospital in Oslo, and did not receive additional funding.

Compliance with ethical standards

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the hospital authority, regional ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

References


Comments
This is a welcome update regarding the most recent and most logical method of post operative assessment of adrenal insufficiency and the need for and calibration of cortisol replacement therapy, assessed initially and over time.
E.R. Laws
Boston, MA, USA

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TGFBR3L—An Uncharacterised Pituitary Specific Membrane Protein Detected in the Gonadotroph Cells in Non-Neoplastic and Tumour Tissue

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Simple Summary: Pituitary neuroendocrine tumours originate from the endocrine cells of the anterior pituitary gland and may develop from any of the cell lineages responsible for producing the different pituitary hormones. The details related to tumour differentiation and hormone production in these tumours are not fully understood. The aim of our study was to investigate an uncharacterised pituitary enriched protein, transforming growth factor beta-receptor 3 like (TGFBR3L). The TGFBR3L protein is highly expressed in the pituitary compared to other organs. We found the protein to be gonadotroph-specific, i.e., detected in the cells that express follicle-stimulating and luteinizing hormones (FSH/LH). The gonadotroph-specific nature of TGFBR3L, a correlation to both FSH and LH as well as an inverse correlation to membranous E-cadherin and oestrogen receptor β suggests a role in gonadotroph cell development and function and, possibly, tumour progression.

Abstract: Here, we report the investigation of transforming growth factor beta-receptor 3 like (TGFBR3L), an uncharacterised pituitary specific membrane protein, in non-neoplastic anterior pituitary gland and pituitary neuroendocrine tumours. A polyclonal antibody produced within the Human Protein Atlas project (HPA074356) was used for TGFBR3L staining and combined with S1F and FSH for a 3-plex fluorescent protocol, providing more details about the cell lineage specificity of TGFBR3L expression. A cohort of 230 pituitary neuroendocrine tumours were analysed. In a subgroup of previously characterised gonadotroph tumours, correlation with expression of FSH/LH, E-cadherin, oestrogen (ER) and somatostatin receptors (SSTR) was explored. TGFBR3L showed membranous immunolabeling and was found to be gonadotroph cell lineage-specific, verified by co-expression with S1F and FSH/LH staining in both tumour and non-neoplastic anterior pituitary tissues. TGFBR3L immunoreactivity was observed in gonadotroph tumours only and demonstrated intra-tumour heterogeneity with a perivascular location. TGFBR3L immunostaining correlated positively to both FSH (R = 0.290) and LH (R = 0.390) immunostaining, and SSTR3 (R = 0.315). TGFBR3L correlated inversely to membranous E-cadherin staining (R = −0.351) and oestrogen receptor β mRNA (R = −0.274). In conclusion, TGFBR3L is a novel pituitary gland specific protein,
located in the membrane of gonadotroph cells in non-neoplastic anterior pituitary gland and in a subset of gonadotroph pituitary tumours.

**Keywords:** gonadotroph cells; pituitary gland; pituitary neuroendocrine tumours; membrane protein; immunohistochemistry; hormone secretion

1. Introduction

Cell lineage-specific transcription factors of the anterior pituitary gland are important for the development of pituitary cells [1]. The classification of pituitary neuroendocrine tumours (PitNET) [2] is based on their cell lineages and is an important part of diagnosis and treatment planning [3]. The transcription factor SF1 (NR5A1) is specific for gonadotroph cell lineage (FSH/LH), T-Pit (TBX19) for corticotroph cell lineage (ACTH), and Pit-1 (POU1F1) is seen in the somato-, lacto- and thyrotroph cells [1]. PitNETs are of epithelial origin and can develop from any of the pituitary cell lineages. They can produce and/or secrete hormones or be endocrine-inactive [3]. Epithelial-to-mesenchymal transition (EMT) is a process where the tumours lose their epithelial phenotype and develop mesenchymal characteristics [4,5]. A hallmark of EMT is the loss of membranous E-cadherin and the presence of nuclear E-cadherin [6], which is associated with larger and more invasive PitNETs [7–9]. Oestrogen receptors α and β (ERα, ERβ) regulate the expression of E-cadherin and seem to influence the clinical course of the gonadotroph tumours [10–12]. Somatostatin receptors (SSTR) have been shown to influence PitNETs response to treatment and clinical course in somatotroph and corticotroph tumours [8–13]. SSTR3 is the most abundant SSTR in gonadotroph tumours [14]. However, the relationship between tumour differentiation, receptor status and hormone production is not fully understood.

In the publicly available Human Protein Atlas (HPA) database [15,16], RNA expression and protein localisation data are available for protein-coding genes in human tissues and cells. RNA abundance is used for the classification of genes based on their expression in different types of tissues representing the whole human body. Tissue enriched expression is defined as a 4-fold higher RNA expression in one tissue type compared to the highest expression level in any other tissue. In the current version of the HPA, 26 genes are classified as pituitary gland enriched. Among these, most of the pituitary hormones of the different cell lineages are found, as well as several pituitary specific transcription factors, such as the corticotroph specific T-Pit and the somato-, lacto- and thyrotroph Pit-1.

Only 3 out of these 26 genes are lacking evidence on the protein level, according to Uniprot protein existence annotation [17], and are thus far only verified at the transcript level. Transforming growth factor beta-receptor 3 like (TGFBR3L) is one of them. The gene TGFBR3L is predicted to encode for a 316 amino acid long single-pass membrane protein [18]. The gene name is based on sequence identity (34% positive amino acids) to the C-terminal region of transforming growth factor beta-receptor 3 (TGFBR3), which contains a conserved zona pellucida domain with the potential to bind growth factors [17,19]. TGFBR3, also called betaglycan, has been shown to function as an inhibin co-receptor [20] and detected in gonadotroph cells in the rodent pituitary gland [21,22]. The RNA expression of TGFBR3 in humans was detected across all tissue types and showed no enrichment in the pituitary gland [16]. For TGFBR3L, however, the RNA expression level was found to be 9 times higher in the pituitary gland compared to tissues with the second-highest RNA expression (cerebral cortex and small intestine).

In a search for additional specific biomarkers for pituitary tumours, we describe here the distribution of TGFBR3L in non-neoplastic anterior pituitary tissue and in a well-characterised cohort of patients with PitNETs. We verified the location of TGFBR3L protein in gonadotroph cells as well as in gonadotroph tumours only. The expression profile of TGFBR3L was heterogeneous within the tumour cell population, and positive cells often displayed a perivascular location. Further, we characterised and correlated the distribution
of TGFBR3L to previously known markers of pituitary cell differentiation and EMT in the pituitary tumours.

2. Results

2.1. TGFBR3L Tissue Profiling in the Human Protein Atlas

The immunohistochemical (IHC) staining performed within the pipeline of the HPA Tissue Atlas (https://www.proteinatlas.org/ENSG00000260001-TGFBR3L/tissue) suggested that the TGFBR3L protein was present in the human pituitary gland in accordance with the RNA data. Moreover, positive TGFBR3L immunolabeling was restricted to a subset of cells in the pituitary gland (Figure 1). Since protein location was consistent with the RNA expression profile, the anti-TGFBR3L antibody (HPA074356) was marked with enhanced validation [23], thus, according to the HPA standard, it provided evidence on the protein level.

![Figure 1](image-url) TGFBR3L is only detected in the pituitary gland on the protein level. (A) The protein localisation profiled by immunohistochemistry (HPA074356) indicates membranous positivity in subsets of cells in the anterior pituitary gland, while the remaining tissues are negative. (B) The RNA level of the gene TGFBR3L in the pituitary gland is reported to be 57 NX (short for Normalised Expression, a normalised version of the transcripts per million [24]), which is 9 times higher than tissues with the second-highest RNA expression (cerebral cortex and small intestine, both 6 NX). The tissues shown in A are indicated in bold font. Images and expression data are from www.proteinatlas.org [16]. Scale bar 50 μm.

2.2. TGFBR3L is Selectively Detected in a Subset of Gonadotroph Cells in the Non-Neoplastic Anterior Pituitary Gland

In order to verify the localisation of TGFBR3L in the non-neoplastic anterior pituitary cells, cell lineage-specific transcription factors were used. The gonadotroph specific transcription factor SF1 overlapped with the TGFBR3L positive cells, verifying that TGFBR3L positive cells belonged to the gonadotroph lineage. Conversely, we found no overlap with corticotroph transcription factor T-Pit or the somato-, lacto- and thyrotroph transcription factor Pit-1. Figure 2 provides the 3-plex staining of TGFBR3L, together with SF1 and FSH-β in the morphologically normal anterior pituitary cells, without pathological change.
A similar pattern was observed when combining TGFBR3L, SF1 and LH-β. The TGFBR3L fluorescent staining shows a membranous location, similar to what was observed with the chromogenic detection protocol (Figure 1). All cases of the morphologically normal pituitary gland, which was the non-neoplastic area of surgically removed tumour tissue, consistently showed positive membrane staining in a subset of cells (Supplementary Materials). However, we noted that several SF1 positive cells were TGFBR3L negative (Figure 2), indicating heterogeneity regarding TGFBR3L expression within the gonadotroph cell population.

**Figure 2.** Immunofluorescence staining of TGFBR3L with SF1 and FSH-β in the non-neoplastic anterior human pituitary gland. A 3-plex immunostaining protocol using TSA amplification and heat elution of antibodies was used for the analysis of the three gonadotroph specific proteins: TGFBR3L (green), SF-1 (blue) and FSH-β (red). All TGFBR3L positive cells were also SF-1 positive. The individual markers within the dashed area are shown separately above the combined image. Scale bar 50 μm.

### 2.3. TGFBR3L is Only Detected in Gonadotroph Tumours

We then investigated TGFBR3L in different subtypes of PitNETs in our tissue microarray (TMA) cohort. The same chromogenic IHC protocol, as used for non-neoplastic anterior pituitary (Figure 1), was used for staining of the tumour TMA sections. In total, 230 different tumours were stained (Table 1), and TGFBR3L staining was exclusively seen in the gonadotroph tumours. Of the 110 gonadotroph tumours, 37 (34%) showed positive immunolabelling for TGFBR3L. Figure 3A,B shows the variable staining pattern seen in the gonadotroph PitNETs, ranging from single cells to almost all cells in the TMA core. In total, 29 cases were scored 1 (with less than 10% positive cells), while 3 cases were scored 2 (10–30% positive cells) and 5 cases scored 3 (more than 30% cells stained). The TGFBR3L staining correlated with the TGFBR3L mRNA levels in the 52 tumours available for the mRNA analysis (Table 2). Since many tumours exhibited a low number of positive cells, we also examined several whole tissue sections for intra-tumour heterogeneity. TGFBR3L positive cells tended to show a perivascular orientation (Figure 3C). Among
the 10 stained gonadotroph tumours, 2 were negative, 4 with sparse positivity and 4 with >30% cell positivity in whole tumour sections. In addition, 10 non-gonadotroph tumours were stained (4 corticotroph, 3 lactotroph and 3 somato-lactotroph) and showed no positive immunoreactivity in the whole tumour sections.

Figure 3. TGFBR3L detection in pituitary tumours. Standard IHC protocol, using chromogen and the TGFBR3L antibody (HPA074356), was applied to a TMA cohort of 230 pituitary tumours and several whole tumour sections. (A) Representative images of TGFBR3L staining in gonadotroph tumours scored 2 or 3, with more than 10% (score 2) or 30% (score 3) cells stained positive. (B) Examples of staining in gonadotroph tumours scored 1, with less than 10% cells stained positive, represented by only a few cells. (C) Example of a whole gonadotroph tumour section stained for TGFBR3L, showing the perivascular location. (D) Two examples of similar perivascular FSH-β positivity. Scale bar 50 μm.

Table 1. Characterisation of the PitNET TMA cohort, including TGFBR3L.

<table>
<thead>
<tr>
<th>Tumour Type</th>
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<th>IHC Subtype</th>
<th>Clinical Phenotype</th>
<th>TGFBR3L Positive</th>
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<td>Gonadotroph</td>
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<td>SF1; FSH/LH</td>
<td>NF-PitNET&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>25</td>
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<td>2</td>
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<td>Null cell</td>
<td>7</td>
<td>TFs&lt;sup&gt;2&lt;/sup&gt; neg; Hormone neg.</td>
<td>NF-PitNET&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>230</td>
<td></td>
<td></td>
<td>37</td>
</tr>
</tbody>
</table>

<sup>1</sup> NF-PitNet: Non-functioning pituitary neuroendocrine tumour. <sup>2</sup> TF: Transcription factor.
Table 2. Correlation analysis of TGFBR3L staining and mRNA compared to gonadotroph hormones, E-cadherin, oestrogen receptors and SSTR3.

<table>
<thead>
<tr>
<th>Staining/mRNA</th>
<th>TGFBR3L Staining</th>
<th>TGFBR3L mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFBR3L mRNA</td>
<td>$R = 0.378$</td>
<td>$R = 0.086$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.0058$</td>
<td>$P = 0.482$</td>
</tr>
<tr>
<td></td>
<td>$N = 52$</td>
<td>$N = 70$</td>
</tr>
<tr>
<td>FSH staining</td>
<td>$R = 0.290$</td>
<td>$R = 0.290$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.004$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td></td>
<td>$N = 95$</td>
<td>$N = 95$</td>
</tr>
<tr>
<td>LH staining</td>
<td>$R = 0.390$</td>
<td>$R = 0.390$</td>
</tr>
<tr>
<td></td>
<td>$P &lt; 0.0001$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td></td>
<td>$N = 95$</td>
<td>$N = 95$</td>
</tr>
<tr>
<td>E-cadherin IRS $^1$</td>
<td>$R = -0.351$</td>
<td>$R = -0.274$</td>
</tr>
<tr>
<td>(membranous)</td>
<td>$P = 0.0005$</td>
<td>$P = 0.022$</td>
</tr>
<tr>
<td></td>
<td>$N = 95$</td>
<td>$N = 70$</td>
</tr>
<tr>
<td>E-cadherin mRNA</td>
<td>$R = -0.086$</td>
<td>$R = -0.049$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.482$</td>
<td>$P = 0.638$</td>
</tr>
<tr>
<td></td>
<td>$N = 70$</td>
<td>$N = 95$</td>
</tr>
<tr>
<td>ERβ mRNA</td>
<td>$R = -0.274$</td>
<td>$R = 0.049$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.022$</td>
<td>$P = 0.638$</td>
</tr>
<tr>
<td></td>
<td>$N = 70$</td>
<td>$N = 95$</td>
</tr>
<tr>
<td>SSTR3 IRS $^1$</td>
<td>$R = 0.049$</td>
<td>$R = 0.022$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.638$</td>
<td>$P = 0.068$</td>
</tr>
<tr>
<td></td>
<td>$N = 95$</td>
<td>$N = 70$</td>
</tr>
<tr>
<td>SSTR3 mRNA</td>
<td>$R = 0.315$</td>
<td>$R = 0.315$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.008$</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td></td>
<td>$N = 70$</td>
<td>$N = 70$</td>
</tr>
</tbody>
</table>

$^1$ IRS: Immunoreactivity score.

2.4. TGFBR3L and FSH/LH Staining

Of the 110 gonadotroph tumours included, additional IHC analyses were available for 95 tumours. Of these, 30 gonadotroph tumours were TGFBR3L positive, while 65 were negative. TGFBR3L staining correlated with tumour FSH-β and LH-β staining (Table 2). Interestingly, when examining the intra-tumour heterogeneity for FSH/LH, an occasional perivascular location could also be observed, similar to the TGFBR3L location (Figure 3D).

2.5. TGFBR3L Expression Shows an Inverse Correlation with E-Cadherin and Oestrogen Receptor β

In order to explore a potential role of TGFBR3L in the process of EMT, the relationship between TGFBR3L and E- and N-cadherin was examined. We found an inverse correlation between membranous E-cadherin immunostaining and TGFBR3L staining and mRNA level (Table 2). Additionally, nuclear presence of E-cadherin was more frequently seen in tumours positive for TGFBR3L than in TGFBR3L negative tumours (90% vs. 71%, $p = 0.039$). Neither TGFBR3L immunolabeling nor mRNA correlated with N-cadherin (IHC and mRNA).

We also explored how TGFBR3L correlated with oestrogen receptor (ER) and somatostatin receptors (SSTR), which are additional markers that we have explored previously in gonadotroph tumours [12,13]. ERβ mRNA showed an inverse correlation with TGFBR3L staining, while ERα did not correlate with TGFBR3L on the protein or mRNA level. The measured mRNA levels of SSTR3 correlated strongly to both TGFBR3L mRNA levels and positive immunostaining, whereas SSTR 1, 2 and 5 (IHC and mRNA) did not correlate with TGFBR3L on either level. We did not find any correlation between TGFBR3L positivity and gender, age, tumour size and invasiveness.
3. Discussion

We performed an in-depth characterisation of the, thus far, uncharacterised TGFBR3L gene, which shows enriched expression in the pituitary gland, according to the Human Protein Atlas resource. We demonstrated that TGFBR3L is a membrane-bound protein detected in a subset of gonadotroph cells, both in the non-neoplastic anterior pituitary cells and in a subset of gonadotroph PitNETs. Furthermore, we found that TGFBR3L correlated with tumour FSH and LH staining, and 3-plex staining revealed an overlap of TGFBR3L positive cells, SF1, as well as FSH and LH positivity.

Our result showing that TGFBR3L is a selective marker of the gonadotroph cells is supported by previous findings in mouse [25], rat [26] and developmental human [27] pituitary gland, where single-cell expression analyses highlighted TGFBR3L as highly enriched in the gonadotroph cells. However, this is the first study based on adult human samples as well as the first evidence of existence of TGFBR3L at the protein level. The data in agreement with the analyses performed in rodents indicate a preserved expression across species. Additionally, the selective expression in the human pituitary gland compared to other regions of the brain can be observed in both mice and pigs [16,28]. The detection of heterogeneous protein expression in the non-neoplastic adenohypophysis and within gonadotroph tumours led us to hypothesise the existence of different sub-populations of gonadotroph cells [29]. Further studies are needed to better characterise the heterogeneity, which could also be due to environmental factors in the tumours, especially since we observed a perivascular location of TGFBR3L positive tumour cells.

PitNETs can cause metabolic disorders related to the hypersecretion of pituitary hormones or be clinically non-functioning, with no signs of hormone over-production. Clinically non-functioning PitNETs of gonadotroph lineage are the most common subtype of pituitary tumours, accounting for approximately 80% of all non-functioning PitNETs [30,31]. Functioning gonadotroph tumours are only rarely described clinically and might be under-diagnosed [30]. Gonadotroph tumours are defined by expression of SF1, and in the majority of cases, also present FSH and/or LH, as identified by IHC [32]. Additional transcription factors involved in the differentiation of gonadotroph cells include GATA-2 [1] and GATA-3 [33]. However, these two transcription factors are also involved in the differentiation of TSH producing cells and tumours [1,33]. The function of TGFBR3L is thus far unknown, but the correlation to FSH-β and LH-β, as well as their co-localisation, suggest a gonadotroph–related function, both in the non-neoplastic anterior pituitary gland and in tumour cells. In addition, the correlation observed between TGFBR3L and SSTR3, the gonadotroph associated SSTR [14], further supports the gonadotroph specific function of TGFBR3L. In our cohort of PitNETs, TGFBR3L was positive in slightly more than one-third of the gonadotroph tumours. As our study was based on TMAs, and TGFBR3L positivity shows intra-tumour heterogeneity, we cannot exclude that even a higher proportion of gonadotroph tumours may be TGFBR3L positive.

Pituitary tumours without IHC detection of pituitary-specific transcription factors or anterior pituitary hormones have been classified as “null-cell adenomas” in the current WHO classification of pituitary tumours [3]. As currently defined, true “null-cell adenomas” represent less than 1% of all pituitary tumours [34]. The existence of this tumour type has been questioned because of its rarity and lack of evidence of the pituitary cell origin [32,35]. A proportion of “null-cell adenomas” may represent other PitNETs, probably gonadotroph tumours that could not be correctly classified due to the methodological aspects, such as pre-analytical problems or suboptimal IHC protocols [32,35]. None of the limited null-cell tumours in the present cohort stained positive for TGFBR3L. Whether TGFBR3L is useful, as an additional marker for characterisation of “null-cell” tumours needs to be clarified in larger cohort of pituitary tumours that fulfils criteria for “null cell adenoma”.

PitNETs are tumours of epithelial origin and may transit to a more mesenchymal phenotype during tumour progression (EMT) [8,9,11]. EMT is marked by loss of membranous E-cadherin [4,5] and, in some cases, nuclear translocation of the protein [6,7]. We observed an inverse relationship between TGFBR3L and membranous E-cadherin staining. Tumours
positive for TGFBR3L also had a nuclear accumulation of E-cadherin more frequently. Taken together, this suggests that TGFBR3L is related to down-regulation of E-cadherin and might be involved in mechanisms associated with epithelial-mesenchymal plasticity.

Oestrogen receptors (ERs) are known to regulate FSH secretion directly on pituitary cells [36] and affect FSH expression in the pituitary gonadotroph cells [37]. In addition, ERs influence the expression of E-cadherin [10,38]. In our study, TGFBR3L correlated inversely with ERβ mRNA levels, but not with ERα at the protein or mRNA level. Since TGFBR3L is related to both ERβ mRNA and FSH-β/LH-β staining, we hypothesise that the protein plays a role in gonadotroph cell differentiation and gonadotropin regulation. Interestingly, TGFBR3, which shows high sequence homology with TGFBR3L [17], functions as a receptor for Inhibin A and suppresses FSH production in gonadotroph cells [20]. Additionally, it has been shown that inhibin subunits (mRNA and protein) are present selectively in gonadotroph adenomas and are linked to FSH expression [39,40]. We have recently demonstrated that FSH staining in gonadotroph tumours, similar to TGFBR3L, is associated with lower membranous E-cadherin, increased nuclear E-cadherin and increased ERα staining [41]. Along with the results presented here, this indicates a complex relationship between gonadotroph differentiation and hormone production, which merits further investigation. Lacking functional analysis, the discussion on the possible role of TGFBR3L in gonadotroph differentiation, regulation and tumourigenesis is hypothesis generating. Thus, further mechanistic studies are needed to elucidate the role of this protein in the gonadotrophs.

Although TGFBR3L shows sequence homology to TGFBR3, we did not investigate its potentially related functions in this study. Despite the lack of evidence, it has often been assumed that there is a related function between TGFBR3L and TGFBR3, solely based on the sequence homology nomenclature strategies [19]. To highlight the TGFBR3L relation to gonadotroph cell biology, we suggest adding ‘Gonadotroph enriched membrane protein’ (GEMP) as a synonym to TGFBR3L.

Single nucleotide polymorphism (SNP) in TGFBR3L has been associated with the risk of neuroblastoma, especially primary neuroblastoma in the adrenal gland [42]. In the same publication, the authors suggested TGFBR3L to be transcriptionally regulated by N-myc proto-oncogene protein (MYCN), based on the site of the SNP and the correlation between MYCN and TGFBR3L levels. MYCN is a transcription factor associated with oncogenesis [43]. Interestingly, SF-1 has also been mentioned in association with MYCN and neuroblastoma progression [44,45]. The potential role of TGFBR3L in tumorigenesis related to SF-1 needs to be explored in other neoplasms. The association with TGF-beta receptor (although only based on sequence homology), possible regulation by MYCN, a relation to neuroblastoma and the selective detection in gonadotroph non-neoplastic and tumour cells, make this protein relevant for further studies and characterisation.

4. Materials and Methods

4.1. Patient Cohort

The study included 230 PitNETs (Table 1 and Figure 4) operated from 1998 to 2009 at a tertiary referral centre at Oslo University Hospital, Oslo, Norway. None of the patients had previously received radiotherapy for pituitary or brain tumour. For patients with more than one pituitary surgery, only one of the tumour samples was included in the result; additionally, samples with the highest IHC score were selected for positive cases. Informed consent was obtained from all participants. Ethical approval was obtained from the Regional Committees for Medical Research Ethics - South East Norway (REC south-east) and the Oslo University Hospital (REK 2020/24582, approval date 27.06.2014 and 07.04.2020).
Figure 4. An overview of the number of patients included in the different steps. In total, 230 different cases were annotated in the PitNET cohort, out of which 110 were gonadotroph tumours (Table 1). Of these gonadotroph tumours, 95 cases included additional IHC and mRNA characterisation data from previous studies, used here for correlation analyses, Table 2. Fifty-two cases out of the 95 gonadotroph tumours were available for further TGFBR3L mRNA analysis (RT-qPCR), Table 2.

4.2. Immunohistochemical Characterisation of the PitNET Cohort

Formalin fixed paraffin embedded tumour tissue was available from all patients. The diagnosis of PitNET was confirmed through haematoxylin and eosin staining, and cell lineage was determined using pituitary hormones and cell lineage-specific transcription factors. Two 1 mm cores from each tumour were used to construct tissue microarrays (TMA), which were used for further analysis, as previously described [46].

Gonadotroph tumours, defined by positivity for the transcription factor SF1 and/or FSH/LH, accounted for 110 of the tumours (Table 1). For these tumours, the following IHC analyses were available: Membranous E-cadherin (intracellular domain), SSTR1, SSTR2, SSTR3, N-cadherin in 95 patients, ERα in 93 patients and SSTR5 in 91 patients. The IHC staining details have been described previously for SF-1 [46,47], FSH and LH [12,46], E-cadherin [47], ERα [12], SSTR 1-3 and 5 [13].

FSH and LH immunostaining demonstrated variable intensity from weak to strong in gonadotroph tumours and were scored on a scale from 0 to 4 based on the percentage of positive cells; 0 = no positive cells; 1 = 0–10% positive cells; 2 = 10–50% positive cells; 3 = 50–80% positive cells; and 4 for >80% positive cells. IHC positivity for membranous E-cadherin, ERα and SSTRs was quantified using an immunoreactivity score (IRS). The IRS was the product of the percentage of positively stained cells (0 = 0%; 1 = 1–10%; 2 = 10–50%; 3 = 50–80%; and 4 = >80%) and the predominant staining intensity (0: No staining; 1: Weak staining; 2: Moderate staining; 3: Strong staining). Nuclear E-cadherin was considered to be either positive or negative in a binary manner. All previous IHC analyses were performed by OC-B.

4.3. Whole Tumour Sections and Non-Neoplastic Anterior Pituitary Gland

In addition to the cohort of PitNETs, TGFBR3L IHC was performed on 20 whole tumour tissue sections (10 gonadotroph tumours and 10 non-gonadotroph tumours; 4 corticotroph, 3 lactotroph and 3 somato-lactotroph). Eight out of these whole tumour sections contained morphologically normal (non-neoplastic) anterior pituitary tissue removed during the pituitary tumour surgery (TGFBR3L IHC images shown in Supplementary Materials). The distribution of positive cells and the staining pattern observed for TGFBR3L IHC were similar in all the samples; only one sample was used for representative multiplex

4.4. TGFBR3L Antibody

The generation of the anti-TGFBR3L antibody (HPA074356) followed the standardised procedure used within the Human Protein Atlas [15]. The human genome sequence (TGFBR3L was encoded by the human gene ENSG00000260001) was used as a template to design a protein fragment antigen with low homology to other human proteins. A peptide corresponding to 51 amino acids (FPGGLKGSARFLSFGPPFPAPPPFFAACPFLWR-RPLFLKLSDTEDVFP) was designed as an antigen. His tag and ABP were included, and the recombinant protein was then produced in E.coli, purified and used for immunisation. After dual-column solid-phase system purification of the antiserum, the antibody was tested on a protein array for affinity control containing the actual antigen together with 384 other random peptides for specificity testing; one single peak was shown (more information can be found online [48].

4.5. TGFBR3L Chromogen Immunohistochemistry

Initial IHC staining was performed within the Tissue Atlas pipeline, with horseradish peroxidase (HRP) polymer conjugated secondary antibody and chromogenic 3,3′-diaminobenzidine (DAB) visualisation in line with a previously described protocol [15], which can be found online [16]. An identical protocol was used for the chromogen IHC on the TMA from the pituitary tumour cohort and the whole tissue sections. Coverslip mounting was performed using Pertex (Histolab, Västra Frölunda, Sweden) following dehydration in alcohol and Tissue clear (VWR). Counterstaining was done with HTXplus (Histolab, Västra Frölunda, Sweden); moreover, all remaining reagents, including antigen retrieval, wash and blocking buffers, HRP polymer, DAB chromogen and substrate were from LabVision (Fremont, CA, USA). Staining protocols were performed in an Autostainer 480 (LabVision) and Leica CV5030 (Leica Biosystems, Nussloch, Germany) and antigen retrieval in a pressure boiler (Decloaking chamber, Biocare Medical, Walnut Creek, CA, USA) using a pH6 citrate buffer (LabVision). The primary antibody (anti-TGFBR3L, HPA074356) was diluted 1:100 from the stock concentration (0.03 mg/mL) and incubated for 30 min at room temperature. Image digitalisation was performed using Scanscope AT2 (Aperio, Vista, CA, USA) using a 20× objective.

In the TMAs, TGFBR3L staining was scored from 0 to 3: Negative = 0, 1 = less than 10% positive cells, 10–30% cells positive = 2 and score 3 for >30% cells stained positive. TGFBR3L immunoreactivity was assessed by ES and OC-B, who were blinded to the clinical data.

4.6. TGFBR3L Multiplex Fluorescence Immunohistochemistry

To enable multiplex staining using antibodies raised in the same species (rabbit), we chose to use a 3-plex TSA strategy [49], where antibodies were added one at a time, and insoluble tyramide signal amplification (TSA) was used for visualisation followed by heating and inactivation of the first antibody. This was then followed by the second antibody and a different TSA fluorophore (TSA-Plus; PerkinElmer). The same protocol and reagents used for chromogen staining, with 30 min primary antibody incubation and 30 min secondary HRP polymer, were applied, except for 2 additional washing steps (one 5 min wash after primary antibody and one 10 min wash after the secondary antibody). Aqueous mounting media with DAPI (ab104139 Abcam) was used after the final round of staining. Anti-SF1 (ab217317 Abcam) was diluted 1:300 and anti-FSH-β (MCA1028 bio-rad) was diluted 1:25,000. The anti-TGFBR3L (HPA074356 atlas antibodies) was diluted 1:300, incubated overnight at 4 °C; and HRP conjugated secondary antibody (P0217 Dako) was used. The protocol for TGFB4L differed from the other antibodies, based on technical optimisation to achieve a clear membranous staining, similar to the chromogen images,
without cytoplasmic spillover. Digital fluorescent images were obtained using a “VSlide” slide scanning microscope (MetaSystems) equipped with a CoolCube 2 camera (12-bit grey scale), a 10× objective. The images (vsi-files) were additionally extracted to high quality jpeg files for further analysis using the software Metaviewer® (Metasystems). Additional fluorescent immunohistochemical staining was performed using anti-T-PIT (HPA072686, Atlas antibodies, diluted 1:300), anti-PIT-1 (HPA041646, Atlas antibodies, diluted 1:1500) as well as anti- LH-β (AB944, Merck, diluted 1:2000).

4.7. Real Time-qPCR

Frozen tissue was collected at the operating theatre and stored at –80°C. mRNA was extracted, and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed as described previously [50,51]. Only samples from gonadotroph tumours included in the TMA were examined using RT-qPCR. mRNA analyses of E-cadherin, SSTR2, SSTR3, ERα, ERβ were available from 70 patients, partly described in a previous study [52]. SSTR1 and SSTR5 mRNA data were available for 69 patients, FSH data for 58 patients and TGFBR3L data for 52 patients.

TGFBR3L mRNA was detected using Forward primer (FP) 5′-GCTGGTGTTGGCAGCCTTC-3′ and Reverse primer (RP) 5′-GCTGGGTGTATCTCCGGACC-3′. ERβ was detected using FP 5′-TCTAAAGAGGGATGCTCACTTC-3′ and RP 5′-CCTCACAGGAACCCACTCC-3′; and FSH-β with FP 5′-TGCTAAGCTACGATCAGACTCTC-3′ and RP 5′-GCGCTCCGACACCATCAAT-3′. The remaining primers used for PCR have been described previously for E-cadherin and N-cadherin [52]; ERα, SSTR1-3 and SSTR 5 [12]. Gene expression was quantified using the delta-delta Ct ($\Delta\Delta$Ct) method and normalised to GAPDH and ALAS1 Ct levels, as they have previously shown to be some of the most stable reference genes in NFPAs, and expressed as relative mRNA levels [51].

4.8. Statistics

Between-group comparisons were performed using the Mann–Whitney U-test and Chi²-test. Spearman’s rank correlation was used for correlation analyses. A p-value of <0.05 was considered significant. Stata 16.0 for Windows (StataCorp LLC, College Station, TX, USA) was used for all statistical analyses.

5. Conclusions

TGFBR3L is a recently described pituitary gland specific protein, previously not investigated in the human pituitary. Here, we were able to show the membranous protein location and verify the selective expression in gonadotroph cells in the non-neoplastic anterior pituitary gland and in a subset of gonadotroph pituitary tumours. The function of TGFBR3L is currently unknown, but the results suggest a role in gonadotroph cell development and function based on the correlation with FSH/LH and possibly tumour progression related to the epithelial-to-mesenchymal transition process.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/1/114/s1, Figure S1: TGFBR3L positivity in non-neoplastic anterior pituitary.

Author Contributions: E.S., A.J.K., J.B. and O.C.-B. planned the project. E.S. performed staining for TGFBR3L, wrote the first draft of the manuscript and prepared all the figures. E.S. and O.C.-B. did the immunohistochemical scoring. A.J.K. and N.C.O. performed the mRNA analysis, and A.J.K. did the statistical analyses. E.S., F.H. and N.M. did the fluorescent staining. Å.S. performed the bioinformatics investigation. A.J.K., N.C.O., K.A.B.O., A.P., J.B. and O.C.-B. collected and characterised the studied cohort. TGFBR3L antibody was provided by Human Protein Atlas (HPA), and the initial analysis within the HPA was done by E.S., F.H. and C.L. All authors have read and agreed to the published version of the manuscript.
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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board and Ethics Committee. The patient cohort was based on a retrospective study and included both patients previously included in a coincident study and patients signing informed consent in 2014. The permits were approved by Regional Committees for Medical Research Ethics - South East Norway (REC south-east) (code REK 2014/635, approved 23.05.2014 and code REK 2020/24582 for the extension approved 07.04.2020). The Human Protein Atlas project includes protein profiling and RNA expression data, related to several ethical permits; Medicinska fakultets forskningsetikkomité at Uppsala University, (code Ups 2002-577, approved 20.11.2002) and Regionala etikprövningsnämnden (code Dnr 2005:338, approved 20.12.2005) both related to collection of anonymised human biobank tissue for protein profiling, which was later complemented by Dnr 2007:159 (Regionala etikprövningsnämnden, approval 31.07.2008). The Human Protein Atlas also includes permit to process RNA data from fresh frozen human biobank material, by Regionala etikprövningsnämnden (code Dnr 2011-473, approved 25.01.2012).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** The RNA expression profile in human tissues (Figure 1) is based on publicly accessible and downloadable data, available in the HPA Tissue Atlas [16]. The IHC images in human tissues are also available online [16]. The data presented in this study related to the tumour samples is available on request from the corresponding author.

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**Conflicts of Interest:** The authors declare that they have no conflict of interests.

**References**


