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Growth and Aggressiveness in Clinically Non-Functioning Pituitary Adenomas

Dissertation Submitted for the Degree of Doctor of Philosophy

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Selected abbreviations

Adrenocorticotropic hormone	ACTH
Akaike Information Criteria	AIC
Coefficient of Variance	CV
Complementary DNA	cDNA
Cycle threshold	Ct
Dopamin Receptor	DR
Epithelial to Mesenchymal Transition	EMT
Follicle stimulating hormone	FSH
Formalin Fixed Paraffin Embedded	FFPE
Growth Hormone	GH
Immunohistochemistry	IHC
ImmunoReactive Score	IRS
Inter Quartile Range	IQR
Luteinising hormone	LH
Mesenchymal to Epithelial Transition	MET
Messenger Ribonucleic Acid	mRNA
Non-functioning Pituitary Adenoma	NFPA
Oestrogen Receptor	ER

Pituitary Adenoma	PA
Pituitary-specific transcription factor	Pit-1
Polymerase Chain Reaction	PCR
Progesterone Receptor	PR
Prolactin	PRL
Reverse transcription quantitative PCR	RT-qPCR
Somatostatin Receptor	SSTR
Somatostatin Receptor Ligand	SRL
Standard Deviation	SD
Steroidogenic factor 1	SF-1
T-box transcription factor	T-Pit
Thyroid-stimulating hormone	TSH
Tissue MicroArray	TMA
Tumour Volume Doubling Time	TVDT

List of publications

Paper 1

Normann KR, Øystese KA, Berg JP, Lekva T, Berg-Johnsen J, Bollerslev J, Olarescu NC 2016 Selection and validation of reliable reference genes for RT-qPCR analysis in a large cohort of pituitary adenomas. Molecular and Cellular Endocrinology 437 (2016) 183-189

Paper 2

Øystese KA, Zucknick M, Casar-Borota O, Ringstad G and Bollerslev J 2017 Early postoperative growth in non-functioning pituitary adenomas; A tool to tailor safe follow-up. Endocrine methods and techniques DOI:10.1007/s12020-017-1314-5

Paper 3

Øystese KA, Casar-Borota O, Normann KR, Zucknick M, Berg JP, Bollerslev J 2017 Estrogen Receptor α, a Sex-Dependent Predictor of Aggressiveness in Nonfunctioning Pituitary Adenomas: SSTR and Sex Hormone Receptor Distribution in NFPA. J Clin Endocrinol Metab 2017 102 (9):3581-3590

Paper 4

Øystese KA, Berg JP, Normann KR, Zucknick M, Casar-Borota O and Bollerslev J 2017 The Role of E- and N-cadherin in the postoperative course of Gonadotroph Pituitary Adenomas (Submitted)

Background

The pituitary gland

The pituitary gland is an endocrine organ, situated in the sella turcica at the base of the brain. It is surrounded laterally by the cavernous sinuses, and superiorly by the optic chiasm (1). The carotid arteries runs through the cavernous sinuses at both sides, giving of branches for blood supply for the pituitary gland (Figure 1) (2). The pituitary gland consist of the adenohypophysis and the neurohypophysis. The adenohypophysis originates from oral ectoderm, hence its epithelial phenotype, while the neurohypophysis develops from neuroectoderm early in the embryogenesis (3). The pituitary, together with the hypothalamus, is crucial in growth, development and reproduction and in sustaining vital homeostasis through secreting hormones that regulate peripheral endocrine glands (ACTH, TSH, FSH, LH), or through direct effect on target cells (GH, ADH, prolactin and oxytocin).

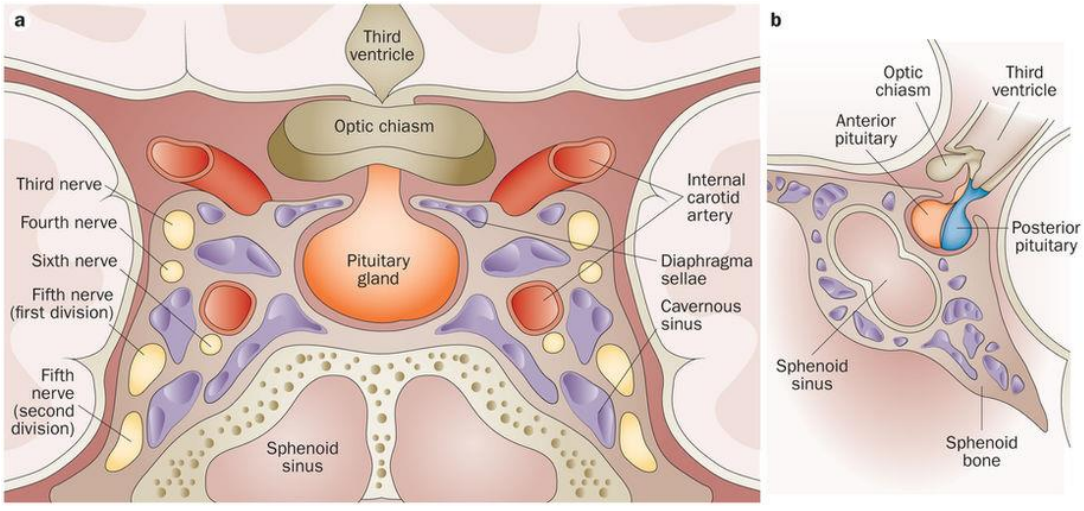


Figure 1: View of the normal sellar and parasellar region in coronal (a) and lateral (b) view. From Di Ieva et al 2014 (4); copyright obtained.

Pituitary adenomas

Adenomas arising from the anterior pituitary, pituitary adenomas (PAs), are the third most common intracranial neoplasm, according to cancer and tumour registries (5). A systematic review of PA showed a prevalence of 14.4% in post mortem studies, and 22.5% in imaging studies (6). A substantial proportion of the adenomas are discovered by incidence, hence the name pituitary incidentalomas (7). Tumours originating in the posterior pituitary are rare (8).

Clinically non-functioning pituitary adenomas (NFPAs), or silent pituitary adenomas, are adenomas without excess hormone secretion leading to a clinical syndrome of hormone overproduction (9). They comprise approximately half of the PAs (10). Both hormone secreting and non-secreting PAs can originate from any of the cell-types of the anterior pituitary, gonadotroph cells being the most common origin of the clinically NFPAs (11), while prolactin producing adenomas are the most frequently occurring functioning PA, followed by somatotroph adenomas (7, 10). Corticotroph cells can give rise to both functioning and non-functioning tumours, though both are relatively rare (7, 10, 12). Thyrotroph adenomas are rarely occurring, both as hormone secreting and non-secreting adenomas (10, 11). There is a slight male predominance in the clinically NFPAs, though not found in all studies (7, 10, 13, 14). Men also tend to be older and present larger tumours than women (7, 15).

The mainstay of treatment for the clinically NFPAs is transphenoidal surgery with removal or debulking of the adenoma. In some complicated cases, a transcranial approach is necessary (16). Radiation is primarily used as adjuvant therapy for patients with residual tumours, and have been

shown to be effective in reducing recurrence of tumour (17). However, the treatment modality is associated with several side effects in particular pituitary deficiency (18). The data on the long term complication risks is sparse, as summarized by Ntali et al (18). No medical therapy has been shown to be effective in the treatment of these tumours, though randomized controlled trials are lacking (19, 20).

PAs are mainly characterized as benign tumours and rarely metastasize, however a substantial portion of the tumours show invasive behaviour (12, 21). The risk of relapse is greater for tumours where residual tumour tissue is present after surgery, than after radical tumour resection (22-24). The relapse is greatest during the first five to ten years, though a delayed relapse may occur several years after primary surgery (22, 25). To date there are no reliable marker that can accurately prognosticate the postoperative course of the tumours (4). Hence, the postoperative follow-up of this patient group remains a challenge.

Classification of clinically NFPAs

NFPAs lack a clinical syndrome due to unregulated hormone secretion, but the cytoplasm of the adenoma cells may contain pituitary hormones, in which case they are named silent adenomas (9). NFPAs are a heterogeneous group of tumours, classified into subgroups clinically, radiologically and/or by immunohistochemical analyses. A correct classification has direct clinical impact on the follow-up of the patients, and may give information on the prognosis of the adenoma. In addition,

detailed characterization of subgroups in scientific studies is crucial to unveil the "truth" about the adenomas.

Clinical classification

The adenomas are traditionally categorised into a clinically aggressive or non-aggressive PAs. According to the recently published guidelines on aggressive pituitary tumours, the diagnosis of an aggressive pituitary tumour should be considered when there is invasion into surrounding structures on radiologic investigation together with a rapid tumour growth rate, or clinically tumour growth despite optimal standard therapies. However; tumour recurrence or invasiveness alone are not necessarily indicative of an aggressive tumour (26). Young age has been associated with a more aggressive tumour behaviour (27-29), though studies have not shown a consistent association between age and the risk of tumour recurrence (23, 30, 31). Pituitary carcinomas are defined as neoplasms originating from the anterior pituitary with craniospinal or systematic metastasis (32). These tumours are rare among pituitary tumours (12), and even more rare in NFPAs (33).

Radiological classification

Adenomas of the pituitary are classified by size and invasiveness from radiologic investigations. According to the largest diameter measured, the tumours are divided into micro (<10 mm) and macro adenomas (>10 mm), and further into pico (<3 mm) and giant adenomas (>40 mm) (34). Macroadenomas (>10 mm) have been associated with a higher tendency of growth progression, than microadenomas (35).

The level of invasiveness into the surrounding structures is frequently reported in everyday practice and in clinical studies. In the guideline concerning aggressive PAs, invasiveness together with rapid growth or growth despite optimal standard therapies were considered diagnostic for the term aggressive pituitary adenoma. Invasiveness alone was not considered a single determinant of tumour aggressiveness (26). Invasiveness gives information on the operability of the tumour and the possibility for radical removal, and hence affects the long-term prognosis of the adenomas (23, 30, 34). A systematic review of recurrence after total resection of tumour showed that preoperative invasion was not a convincing predictor of tumour recurrence in NFPAs. However the studies reviewed varied in their results, and there is an obvious relation between invasion and radical removal of tumour (25). Some studies indicate that the different immunohistochemical subtypes of clinically NFPAs show different levels of invasiveness (36). There are different approaches of addressing invasiveness. The above-mentioned guideline does not recommend or refer to one specific approach (26). The most common measures of invasiveness used in the literature are Knosp`s and Hardy`s classifications. Knosp et al based their definition of invasiveness in relation to the carotid arteries that runs through the cavernous sinuses laterally to the sella on both sides (37, 38). The Hardy classification relates to the invasion of the sphenoid bone structures (39). Recent reports have suggested to combine radiologically determined invasiveness and immunohistochemical properties in the prognostic characterization of pituitary adenomas (40).

Immunopathological classification

The five distinct hormone-secreting cells of the anterior pituitary can all give rise to both hormone secreting and non-secreting tumours. The differentiation between these tumour types is made by staining for the pituitary hormones, and may be supplemented by the associated cell lineage specific transcription factors (41). The immunohistochemical subtypes of NFPAs tend to show different clinical characteristics (11). Silent corticotroph, plurihormonal and null-cell adenomas are known to show a more aggressive clinical course than the gonadotroph adenomas (30, 42). Silent somatotroph, thyrotroph and lactotroph adenomas are rare (12) and studies comparing these subtypes are therefore a challenge. The gonadotroph subtype is the most prevalent determined by IHC, in series investigating clinically NFPAs, in particular after the introduction of transcription factors as part of the immunohistochemical classification (11, 43). However, detailed epidemiological studies including both clinical data concerning hormone production and subclassification by IHC of NFPAs are sparse (10, 12, 15).

Transcription factors

Adenomas not staining for any pituitary hormones (null-cell adenomas) have traditionally been the second largest immunohistochemical subtype of clinically NFPAs (30, 43). During the last years, staining for lineage specific transcription factors has been suggested as a supplement to the characterization of NFPAs together with staining for the pituitary hormones (41, 44). This classifies a substantial proportion of the hormone negative adenomas into gonadotroph and corticotroph adenomas based on a positive staining for the transcription factor SF-1 and T-Pit, respectively (45, 46); while the transcription factor Pit-1 is characteristic for the

combined group of somatotroph, thyrotroph and/or lactotroph adenomas (47). Accordingly, the remaining portion of "true" null-cell adenomas, negative for both the transcription factors and all the adenohypophysial hormones, is small (11).

Proliferation markers and malignancy

The world health organization (WHO) suggested in 2004 to classify pituitary tumours into three categories (typical and atypical PAs and pituitary carcinoma), based on the level and/or presence of the cell proliferation markers Ki-67 (> 3%), nuclear p53 and mitotic activity and for carcinomas; the presence of metastasis (32). However, the criterias for the distinction between typical and atypical were vague and has not been shown to correlate consistently with clinical variables of aggressiveness (48). WHO published a new *Classification of Tumors of Endocrine organs* in the fall of 2017, where the classification into typical and atypical no longer was withheld, though some of the markers are still kept as prognostic markers (41). Size, clinical presentation and invasiveness in addition to immunohistochemical markers was mentioned as parameters to consider in relation to aggressiveness. The definition of pituitary carcinoma was kept in the latest classification (41).

Growth and aggressiveness of clinically NFPAs

The follow-up of patients with clinically NFPAs is resource and time consuming for both the health care system and for the patients. To date there are no reliable marker that predicts tumour growth in these

adenomas, and no parameter that mirrors the disease activity as we have for the functioning pituitary adenomas.

Growth dynamics

Population based studies have found a very high prevalence of pituitary lesions, while the prevalence of clinically relevant lesions are much lower, indicating that there is a variable growth potential in these tumours (6, 13). Most studies report stable or slowly growing tumour remnants after surgery for clinically NFPAs (22). Tumours totally removed at surgery have a lesser risk of recurrence; however, a delayed recurrence five and ten years after primary surgery has been reported in several studies (22, 25).

Tumour growth dynamics have been of interest for scientist for decades. Knowing the course of a tumour`s growth pattern and pace would enable the clinician to tailor the timing and modality of treatment and the follow-up of the individual patient better. In their review from 2013 Rodriguez-Brenes et al (49) described five different growth patterns with concomitant biological models. The exponential growth pattern is associated with the biological model of simple and constant cell division. This model has been widely used in clinical studies, and is the background assumption for calculation of tumour volume doubling time (TVDT). In the linear growth curve, a fixed volume is added to the existent tumour volume, associated with a superficial cell division (49). The logistic growth model was originally based on empirical data. However, physiologic explanations have been suggested such as insufficient diffusion of nutrients. The logistic curve follows three phases; initial exponential growth, a linear phase and the plateau phase (Figure 2). The review also described atypical growth and

multistep growth. The first pointing to subexponential or sublinear (subcubic) growth but without retardation of growth leading to a sigmoid curve. The latter model with alternating growth and dormant periods (49). These models were not investigated in the present study.

To date only a single study has explored the growth kinetics of NFPAs in detail. Honegger et al studied 15 patients with a clinically NFPAs (12 of them after surgery), and found the logistic and exponential growth models to best describe the growth in their group of tumours (29). The TVDT of NFPAs has been estimated in a few studies, most assuming that the tumours exhibit exponential growth, with TVDT measured from a minimum of two measurement points (27, 28, 50, 51).

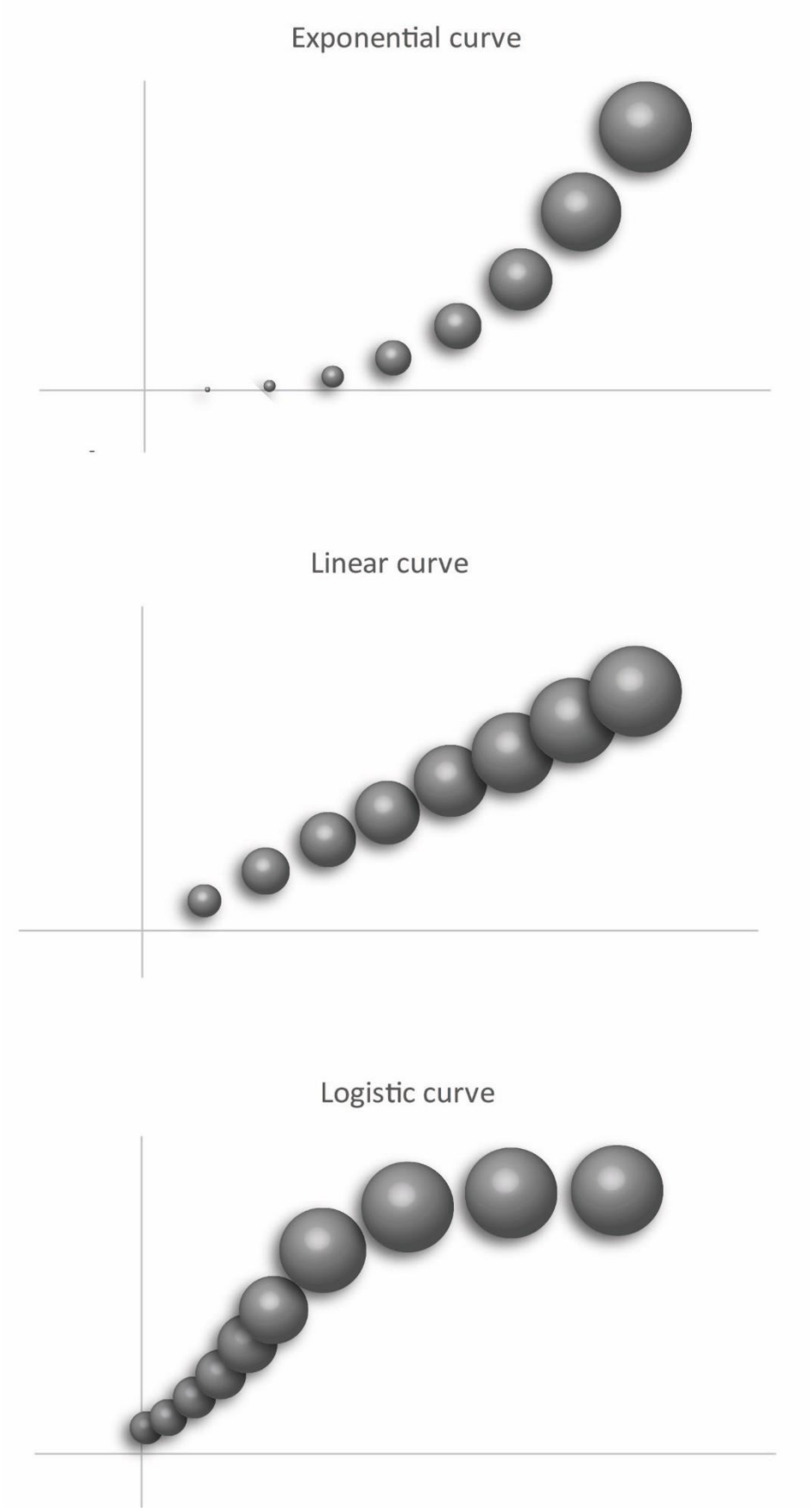


Figure 2: Examples of exponential, linear and logistic growth curves from top to bottom.

Somatostatin, Oestrogen and Progesterone Receptors

The different immunohistochemical subtypes of clinically NFPAs are known to have different clinical characteristics, and some of them equivalent to their functioning counterparts (11). The receptor distribution differs between the normal pituitary cells, though not all have been thoroughly studied (52, 53). Medical therapies directed towards the somatostatin receptors (SSTR) are available for GH, ACTH and TSH secreting adenomas (54-56), and towards the dopamine receptor (DR) for prolactin secreting adenomas (57). To date there are no effective medical therapy available for clinically NFPAs, and knowing the receptor pattern would give potential targets of medical therapy.

SSTRs are G-protein coupled membrane receptors (58). Five main subtypes (SSTR1-5) have been described and four of them are normally expressed in the pituitary gland (59). SSTR3 has been shown to be highly expressed in some clinically NFPAs, while the expression of the remaining SSTR-types has varied (60-62). Some studies have shown SSTR3 to be involved in apoptotic and antiproliferative cell functions, though this has not been studied in pituitary cells (63, 64). Studies in mice have shown a ligand-independent function of SSTR3, inhibiting ACTH-secretion (65). If SSTR3 holds a role in the pathogenesis and silencing of NFPAs is yet to be elucidated.

SSTR2 and SSTR5 are the two main targets for already existing somatostatin receptor ligands (SRL) used in the treatment of some of the functioning PAs (66). The SRL have shown to have effect in controlling the hormone secretion and reducing tumour volume (67-69). SSTR2 and SSTR5 are known to be highly expressed in functioning somatotroph

adenomas, while SSTR5 dominates in functioning corticotroph tumours (69, 70). Studies reporting the distribution of SSTR2 and SSTR5 in NFPAs have shown conflicting results (60, 62, 71). Only a few studies have reported the effect of SRL on growth in clinically NFPAs (72, 73), and randomized controlled trials have so far not been published.

Oestrogen and progesterone receptors (ERs and PRs) are nuclear steroid receptors. They function as transcription factors, and are known to participate in the growth and differentiation of several tissues (74, 75). Both are found in normal pituitary tissue (76, 77). ER is a known transcription factor for the gonadotroph and lactotroph lineage of pituitary cell development, and has been demonstrated in normal gonadotroph and lactotroph cells (53). There are two classes of Oestrogen receptor, ERα and ERβ, with several isoforms identified for each class (78). The level of ERα has been linked to aggressiveness in several tumour types, including prolactinomas (79-81). Only a few studies have investigated ERs and it`s association to aggressiveness in clinically NFPAs (61, 82-84).

The PR has been associated with proliferation in breast cancer (75). PR has two main isoforms, PRA and PRB, where PRA is the dominant isoform. PRB is an activator of genes in cells, while PRA inhibits the effect of PRB (85).The information of the role of PR in the development and progression of PAs are lacking.

Epithelial to Mesenchymal Transition (EMT)

Loss of epithelial differentiation is thought to be involved in the development of more aggressive tumours, and has been linked to the

process of Epithelial to Mesenchymal Transition (EMT), with a loss of the adhesion protein E-cadherin (86-88).

Both EMT and the counterpart Mesenchymal to Epithelial Transition (MET) are changes in a cell`s phenotype taking place in both normal and pathological conditions (88). Epithelial tissues are highly specialized with diverse functions. They are classically characterized by tight cell to cell contacts, with their main adhesion system being maintained by the adherence proteins (88). Cadherins are a family of glycoproteins with varied functions in different cells (89). The downregulation of E-cadherin has been viewed as the hallmark of EMT and several mechanisms for the downregulation of E-cadherin have been described in the EMT process addressing both transcriptional and posttranscriptional modifications (88, 90-92). E-cadherin has been found in the nucleus both in pituitary tumours as well as in other tumour types. The nuclear presence is associated with a lower E-cadherin level by the membrane (91, 93, 94). Some investigators have suggested that nuclear E-cadherin binds to DNA and is involved in signaling pathways (91). A lower level of E-cadherin has been associated with a more aggressive phenotype in some functioning PAs (86, 87, 95, 96), while the few studies on E-cadherin and aggressiveness in clinically NFPAs have given variable results (43, 84).

N-cadherin, also an adherence protein, is associated with a mesenchymal phenotype. It has been shown to be involved in a tumour's ability to invade and metastasize (97). Increased N-cadherin expression has shown effects on cell motility independent of the presence of E-cadherin (97). N-cadherin is present in the normal pituitary, though the distribution in the different pituitary cells may vary (95, 98). Chauvet et al studied N-cadherin in

somatotroph and lactotroph tumours and found de novo expression of N-cadherin to be linked to a more mesenchymal phenotype (95). Some studies have also suggested that both E-and N-cadherin might be linked to regulation of hormone secretion for some functioning adenomas (86, 99).

Knowledge gap

The clinically NFPAs have had less attention than their functioning counterparts, and there is a need for information in several areas concerning these adenomas. The tumours are heterogeneous, and some of the immunohistochemical subtypes are rare, blurring the results in different studies. There is a lack of reliable markers to prognosticate the clinical course of the adenomas. Likewise, the knowledge on adenoma growth is sparse, making a safe planning and termination of follow-up difficult. Finally, there is no effective medical treatment for the NFPAs, and invasive and irreversible treatment is the only alternative for cure or symptom control.

Aims of the study

- The overall aim of the study was to better characterize the morphology and behaviour of clinically NFPAs.
- We aimed to establish a platform for future analyses with qPCR through mapping the most reliable reference genes for different immunohistochemical subtypes of functional and non-functional pituitary adenomas.
- We also aimed to investigate the growth patterns and pace of clinically NFPAs, and at the same time create solid end-points of tumour growth, through obtaining growth curves and calculating TVDT.
- We aimed to pattern biological characteristics between the different immunohistochemical subtypes of NFPA, with details on receptor distribution which may be potential targets of medical therapy.
- Finally, the study aimed to describe the distribution of the E- and N-cadherin molecules, which are linked to an epithelial phenotype, and correlate them to clinical variables of aggressiveness in silent gonadotroph tumours.

Summary of results

Paper 1: Selection and validation of reliable reference genes for RT-qPCR analysis in a large cohort of pituitary adenomas

Background: A major challenge in quantification of gene expression by RT-qPCR is the lack of data on stable reference genes in the specific tissues investigated.

Aim: To characterize the most optimal reference gene or reference gene combination in different classes of pituitary adenomas.

Main results:

- Selection cohort: Twenty-one out of 27 genes investigated showed cycle threshold (Ct)-values with a SD < 1 indicating good stability as reference genes.
    - GeNorm: The optimal number of reference genes combined to obtain a stable normalization index was two. The recommended combination of reference genes for the whole cohort was ALAS1 and PSMC4.
    - NormFinder: The best candidate gene in all subclasses investigated was ALAS1. The best combination of genes were PSMC4 and GAPDH for the whole cohort.
    - BestKeeper: Of the 10 genes with the lowest SD; RPL30, RPS17, GAPDH and ACTB were the most stable.
- Validation cohort: Based on the results from the selection cohort PSMC4, ALAS1 and GAPDH were selected for the validation analyses. The combination of ALAS1 and GAPDH showed the most optimal stability in NFPAs, also when dividing into subtypes based

on IHC. The combination of PSMC4 and GAPDH was best for both GH and ACTH producing adenomas.

Conclusion: The combination of ALAS1 and GAPDH was the most stable combination of reference genes for NFPAs, while PSMC4 and GAPDH was optimal for both GH and ACTH- producing adenomas. We found that several reference genes showed good stability in PAs, and might serve as additional reference genes for further validation in PAs.

Paper 2: Early postoperative growth in non-functioning pituitary adenomas. A tool to tailor safe follow-up

Background: The postoperative growth of clinically NFPAs determines the follow-up and the time to potential reintervention. However, information on the growth patterns of these tumours is sparse.

Aim: To characterize the growth rate and growth pattern in clinically NFPAs.

Main results: Of 52 patients with sufficient follow-up, 39 patients (tumours) were available for growth curve modelling.

- Growth curves: 13 tumours showed exponential growth, 16 tumours logistic growth and 10 tumours evidenced linear growth. Thirteen tumours did not show regrowth.
- There was a higher rate of reintervention among tumours with exponential growth.
- Logistic growing tumours presented a lower initial TVDT than both linear and exponential growing tumours.
- Men showed a slightly lower initial TVDT than women.
- No tumours showed obvious signs of accelerated growth.

Conclusion: Postoperative tumour growth showed a good fit to models of exponential, linear and logistic growth. A substantial portion of the tumours showed a deceleration of TVDT with time, and there was no obvious signs of accelerated tumour growth in the cohort. These findings suggest calculation of initial TVDT as a tool to tailor safe follow-up in the care of clinically NFPAs.

Paper 3: Estrogen Receptor α; a Sex-Dependent Predictor of Aggressiveness in Nonfunctioning Pituitary Adenomas: SSTR and Sex Hormone Receptor Distribution in NFPA

Background: The treatment and follow-up of clinically NFPAs is multidisciplinary and resource demanding. No medical treatment is available and there is no reliable marker of tumour aggressiveness for these tumours.

Aim: We aimed to pattern the distribution of SSTRs, ERα and PR in different immunohistochemical subclasses of clinically NFPAs, and relate them to variables of aggressiveness.

Main results: IHC analysis revealed that SSTR3 was abundantly expressed in all immunohistochemical subtypes investigated, while the other SSTRs presented low IRS that differed between the subclasses. ERα showed a gender specific correlation with SSTR2. In addition; there was a gender specific association between the IRS of ERα and rate of and time to reintervention. ERα combined with age showed to be a reliable predictor for reintervention in male patients with gonadotroph adenomas.

Conclusion: SSTR3 was abundantly expressed in clinically NFPAs. ERα showed a gender specific association to the rate of and time to reintervention. The combination of ERα and age served as a predictive marker for reintervention in male patients with gonadotroph adenomas.

Paper 4: The Role of E- and N-cadherin in the postoperative course of Gonadotroph Pituitary Adenomas

Background: EMT is a process linked to invasive and metastatic properties of epithelial tumours. Gonadotroph pituitary adenomas show a wide range of aggressiveness. To date there is no reliable marker predicting the clinical course of these tumours and the mechanisms behind their aggressiveness is yet to be elucidated.

Aims: To pattern the distribution of E- and N-cadherin in gonadotroph pituitary adenomas, and investigate their link to variables of aggressiveness.

Main results: We found the IRS of N-cadherin to be high, and the IRS of the extracellular domain of E-cadherin to be low in the gonadotroph tumours, while the IRS of the intracellular domain of E-cadherin varied. A substantial portion of the tumours showed nuclear presence of the intracellular domain of E-cadherin. These tumours also presented significantly lower membranous staining for the intracellular domain of E-cadherin than tumours without nuclear staining. Absence of nuclear staining for the intracellular domain of E-cadherin was associated with a higher rate and shorter time to reintervention.

Conclusion: N-cadherin was abundantly expressed in gonadotroph pituitary adenomas. The extracellular domain of E-cadherin seemed to be missing in these adenomas, and a substantial part of the tumours presented nuclear staining for the intracellular domain of E-cadherin. Absence of nuclear staining for E-cadherin was associated with a higher rate of reintervention.

Discussion

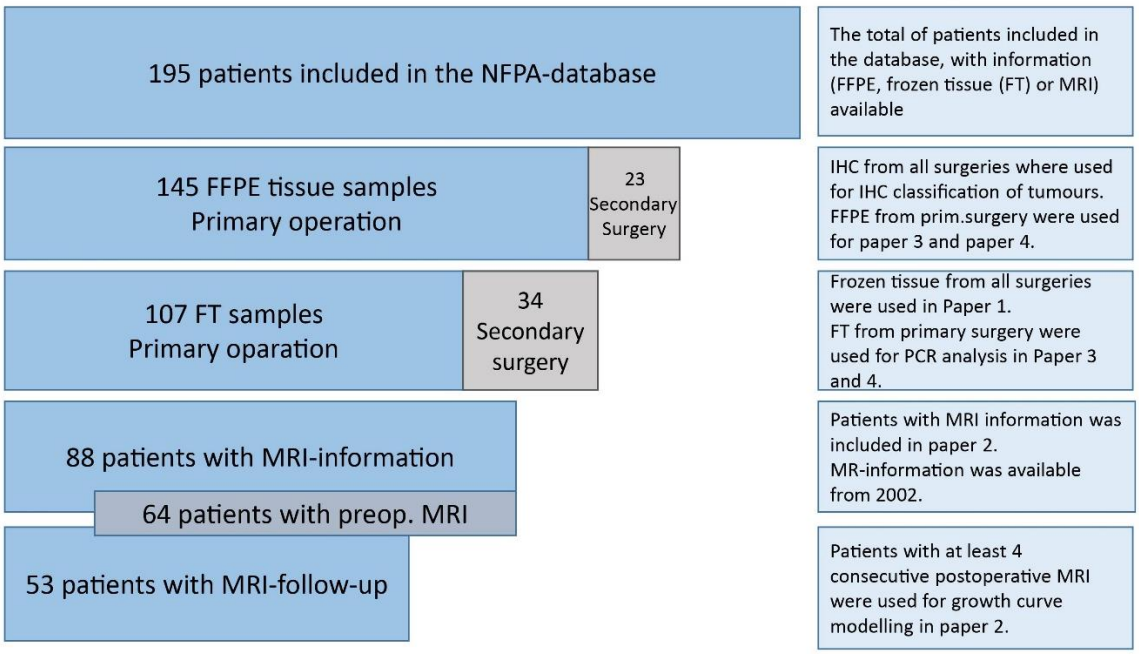
Methodological Considerations

Design and patient population

Adenoma tissue from surgery of PAs has been collected for systematic studies concerning Mb. Cushing, Acromegaly and clinically NFPAs at the Section of Specialized Endocrinology at OUS during the last decades. For the NFPAs, this ended in 2009 when there was no longer ongoing research on these specific tumours. Most of the patients included in the present study, were included in the previous investigation by IHC of KIT protein expression in different types of PAs (100). Patients from whom Formalin Fixed Paraffin Embedded (FFPE) tissue or frozen tissue were available, were retrospectively included in the present study of clinically NFPAs (Figure 3). Not all samples have been used in scientific analyses previously. Informed consent was retrieved from patients where this was missing. The associated patient records were studied to affirm that all tumours were evaluated as clinically non-functioning at the time of the primary operation either through their electronic patient record, or in the original paper version in the archives.

Patients with frozen adenoma tissue available for rt-qPCR analyses were included in Paper 1. Patients from other cohorts of acromegaly and Mb. Cushing were included in Paper 1, only. Patients where digital MRI-scans were available were included in the study of growth curves and TVDT (Paper 2), while patients with either FFPE or frozen tissue of sufficient quality from primary operation were included in the receptor study (Paper 3). Patients with gonadotroph tumours and FFPE or frozen tissue were included in the study of markers of EMT (Paper 4).

Digital MRI-scans were available from 2002. We needed at least four consecutive good quality scans to pattern growth curves for the postoperative growth of tumours. This might have given us a selected group of patients with less aggressive tumours than those going through reintervention before four MRI-scans were obtained, and less aggressive than tumours where the follow-up ended early. An attempt to describe this potential selection bias has been made in Paper 2.



Figur 3: Patient material available for analyses in the database of clinically NFPAs.

RT-qPCR

We used Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) for the quantification of mRNA levels in tissue samples from PAs. The PCR method is a method used for amplification of a target gene segment (101). RNA is fragile for denaturation and is therefore reverse transcribed from mRNA to cDNA (102). In the PCR procedure, a forward and reverse primer depicting the target gene is added to a solution containing the complementary DNA (cDNA) from the tissue of interest. The solution is run though a thermal cycler where denaturation, annealing of primers and transcription takes place at different temperatures. This reaction is repeated through a known number of cycles (101). A fluorescent dye added to the standard solution makes it possible to detect the amount of double stranded DNA throughout the cycling process, hence the name real-time PCR or quantitative PCR (qPCR) (103).

Before the RT-qPCR can be performed, tissue samples must be homogenized and mRNA extracted. The mRNA molecule is easily degraded by naturally occurring ribonucleases (RNAse). In our experiments strict precautions were made to prevent this during the homogenization and extraction of RNA.

The samples included in the study were collected over a long period of time. Some of the frozen tissue samples were dating as far back as 1995. However, all samples were scored with RNA integrity number (RIN), which is a measure of the amount of degraded RNA in a sample (104). Only samples with a RIN > 4 were included. Only 8 samples showed a RIN value between 4-5, seven of these from the primary operations. For these samples electropherograms accompanying the RIN analysis, were controlled to

approve of the sample quality. A RIN value >7 was present for the 12 hormone producing tumours and 12 clinically NFPAs included in the selection study in Paper 1. RNA quantity and purity was measured by OD readings on a Nanodrop ND1000 Spectophotometre, as described in Paper 1.

The amplification of the target cDNA is relying on the specificity of the primers used. The primers were designed and quality checked using primer-blast function in PubMed (105). The detection of the fluorescent dye is also depending on the specificity of the primers used, while the dye binds to all double stranded DNA (106). To control for this the fluorescence signal is plotted as a function, not just against time, but against temperature to visualize the melting curves of the amplicon (107). If the primers are not specific and several genes are amplified in the sample, there will be several or a low and broad melting curve.

The RT-qPCR method is prone to errors and variations during each step in the procedure. The exponential nature of the amplification makes insignificant initial errors and variations great at the end of the reaction. Therefore, all mRNA results for all genes in each sample (analysed in parallels) were checked according to melting point and standard curves, by the same two investigators (Kjersti Normann and the author of this thesis KAØ). Sample results of low quality, with a blurred melting point or with diverging parallels were not included in the statistical analyses.

We measured the relative quantity of the target gene against a standard curve of four samples and normalized to the combination of two reference genes, yielding a relative quantification of all the target genes investigated. Relative quantification in relation to at least two reference genes is

preferred when knowledge on the precise quantity of amplicons is not needed, as it does not depend on standards with known concentrations in the tumour tissue (108). One of the major limitations of this method is the lack of appropriate selection of reference genes and the validation of these. The Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE)-guidelines emphasized that validation of reference genes for each specific tissue is crucial for assessing reliable gene expression data (109). The data on validation of reference genes in NFPAs is however sparse (110). Therefore, validation of reference genes in Paper 1, laid the base for the relative quantification of the target mRNA analyses in Paper 3 and 4.

Immunohistochemistry

Immunohistochemical analyses are widely used both in everyday clinical practice and as a research tool. The IHC principle is based on the detection of specific antigens in tumour tissue by antibodies (111). The antibodies are immunoglobulin molecules with a specific binding site to a target molecule. Antibodies can themselves also function as an antigen, which is important in IHC for the visual detection of the antibody-antigen constellation (112). The antibodies used in IHC may be monoclonal or polyclonal, the latter being an antiserum from an immunized animal containing several antibodies (113). The monoclonal antibodies are produced from a single clone of B-cells activated by a specific antigen. Though produced specifically for one target protein, the monoclonal antibodies may cross react with other molecules presenting similar epitopes as the target (112).

The introduction of Tissue MicroArray (TMA) enabled large panels of tissue specimens to be investigated in one session making it a practical and effective high throughput analysis (114). For the construction of the TMA blocks in our study, small cylindrical biopsies (diameter of 1 mm) were punched from several single adenoma tissue samples, beforehand marked as suitable for investigation by the pathologist. The biopsies were transferred to a recipient paraffin array block with multiple cavities corresponding to the biopsies taken. The array block was then cut into thin sections which were placed on microscope slides for further antibody staining as presented in the papers.

One of the challenges with TMAs is variable depth in the tissue specimens, and hence a lack of tissue specimen for some of the samples from the deeper levels of the different biopsy cylinders in TMA block. The tumours may also show heterogeneity in the distribution of the target proteins, though studies have shown a good correlation between TMAs with biopsies as small as 0.6 mm and whole-tissue sections (115). The pathologist marks representative areas where the punches are taken from, while the deeper layers are not visualized, hence the heterogeneity along the depths of the biopsy are more challenging to account for (116). In our study, two biopsies (1 mm) from each of the original tumour blocks were taken to control for tumour heterogeneity.

The target proteins in our study were all scored manually using the Immuno Reactivity Score (IRS), as described previously (117). This scoring system takes both the intensity of the staining and the proportion of the staining cells into account. It was originally used for scoring of the oestrogen receptor in breast cancer (117). The IRS has previously been used

for immunohistochemical analyses in both PAs and other tumour types (60, 118, 119). For some of the analyses in our study the IRS was divided into negative (IRS 0-1), weakly positive (IRS 2-3), moderately positive (IRS 4-8) and strongly positive (9-12), as previously done (60). In Paper 3, the IRS of ERα was divided into negative and positive in accordance with the same grading system, as mentioned above (60). The division between these IRS grades is artificial, since there is a continuum and little difference between the adjacent scores. The IRS is a product of two variables, and the same score may present different morphology. E.g. a tumour with an intense colour reaction in a small proportion of the cells might appear very different from a tumour with a weak colour reaction in 51-80% of the cells, though the IRS will be the same in both cases (IRS 3). The IRS does not take the location of the target protein into account, and additional information on the location of the cadherins was added, independently of the IRS in Paper 4. Because of the nature of the IRS scale, only non-parametric analyses was performed on the data set.

The IRS is a manual scoring system which is dependent on the experience and the judgement of the pathologist, and hence liable to inter- and intra-rater (observer) variability. One advantage of the present studies was that an experienced pathologist (Olivera Casar-Borota), blinded to clinical data, performed all the immunohistochemical analyses.

Sub classification of tumours

The tumours included in the present study were sub classified according to immunohistochemical staining for the pituitary hormones and cell lineage specific transcription factors for all tumours where FFPE tissue samples were available. Staining for the pituitary hormones is normally performed

as a routine investigation of PAs in clinical practice. The recently published WHO classification of PAs includes complimentary staining for the cell lineage specific transcription factors (SF-1, T-Pit and Pit-1) (41). Up until recently there has been a lack of a commercially available reliable T-Pit antibody (120), hence this was not investigated in the TMAs in this study. This may have had implications for the size of the null-cell and corticotroph subgroups in our analyses. Some of the null-cell adenomas might have been T-Pit positive and thus belonging to the corticotroph subgroup. The results concerning these two subgroups must therefore be interpreted with caution in Paper 2 and 3.

For some tumours FFPE was not available from the primary operation, but from secondary surgery. In these cases, the classification of the tumour type was used, if frozen tissue for mRNA analyses was available. Only a few tumours in the cohort lacked immunohistochemical subtyping.

Detection of target proteins

Immunohistochemical staining was used as the main method of detecting target proteins in our study, accompanied and supported by analyses of mRNA levels. All antibodies used in the analyses were monoclonal.

Receptors

Hormone receptors are the main target for medical treatment of functioning PAs, with SSTRs being the most important in somatotroph, corticotroph and thyrotroph tumours, and DR the target in lactotroph tumours (54-57). Only a few series have previously described the distribution of SSTRs, ER and PR in some of the immunohistochemical subtypes of NFPAs, and the need for more information concerning the presentation of receptors in the different immunohistochemical subtypes of

NFPAs was needed. The antibodies used for detection of receptors have all been used in human pituitary studies previously, except for the antibody targeting the PR (60, 79, 121, 122). The DR and ERβ were not investigated in the present study, due to lack of satisfactory experience with the antibodies available.

Markers of EMT

E-cadherin and N-cadherin were chosen as markers of EMT in our gonadotroph cohort. Antibodies directed towards both the intracellular and extracellular domain of E-cadherin were used to detect the presence and localization of E-cadherin, as performed previously in somatotroph and corticotroph adenomas (86, 87). The specificity of the antibody towards the intracellular domain of E-cadherin used in Paper 4 has been debated (123), and the distributor warn that it might cross react with P-cadherin (124). P-cadherin, or placental cadherin, is an adherence protein normally located to the proliferating areas of epithelial tissues. It has also been associated with markers of aggressiveness in some epithelial cancer types (125). We found only a single study measuring the relative mRNA level of P-cadherin (CDH3) in pituitary tissue, where it was present at low levels. E-cadherin (CDH1) was the most abundantly expressed cadherin (95). The specificity of this antibody has been discussed as a part of the limitation section in Paper 4. The antibody directed towards the extracellular domain of E-cadherin (HECD-1) has previously been used to determine the level of the extracellular domain of E-cadherin both by immunohistochemistry and by Western Blot picking up fragments of both approximately 84 and 120 kDA corresponding to the extracellular domain of E-cadherin and the full length E-cadherin respectively (126-128). According to the distributor (Abcam) by

direct correspondence, the antibody targeting N-cadherin is directed against the intracellular domain of the molecule.

Growth curve modelling

Volume Measurements

The largest tumour diameter is a frequently used measure of size in every day clinical practise. However, this measure has been shown to have a high retest error (129). Tumour volume measures may be calculated from diameter in three perpendicular planes, or by slice-by-slice method, also known as the Cavalieri method.

The Cavalieri principle is a method of calculating volume in irregular structures, based on the work of Bonaventura F. Cavalieri (1598 – 1647). The volume is calculated by dividing a figure in to slices (*indivisibles*), summarizing the surface area of each slice and multiplying it with the thickness of the slices (130). This method can be transferred to MRI-scans where the surface area on each MRI-slice depicting tumour tissue is summarized and multiplied by the slice-thickness and distance between each slice (131). The slice-by-slice volume measurement has been used as the reference method when testing other methods of calculating size and volume (129, 132).

Figure 4: The Cavalieri principle exemplified with domino pieces. Each figure is made out of six identical domino pieces, which exemplifies the indivisibles. According to the Cavalieri principle, the volumes of the three different structures are the same.

We used the slice-by-slice method to calculate volumes for the establishment of growth curves and TVDT in Paper 2. The method is time consuming and subject to retest error, however it allows for irregular structures of the adenomas to be incorporated in the tumour volume. In particular, the tumour remains after surgery might show an irregular outline. In addition, the slice-by-slice method has shown to have less retest error than other frequently used tumour size measures (129). Based on clinical experience; the accuracy of the volume measurement is depending on the time after surgery, where tumour contours on early postoperative scans might be more difficult to outline due to scar tissue than for example well circumfered preoperative tumours. The reliability of the slice-by-slice method at different post-operative time points is however, not known. Tumour size may also influence the accuracy of the volume measurements, while small tumours might be harder to detect properly than large

tumours. The volume calculation of larger tumours may involve numerous slices and are therefore more prone to errors, which again will be multiplied by the slice thickness.

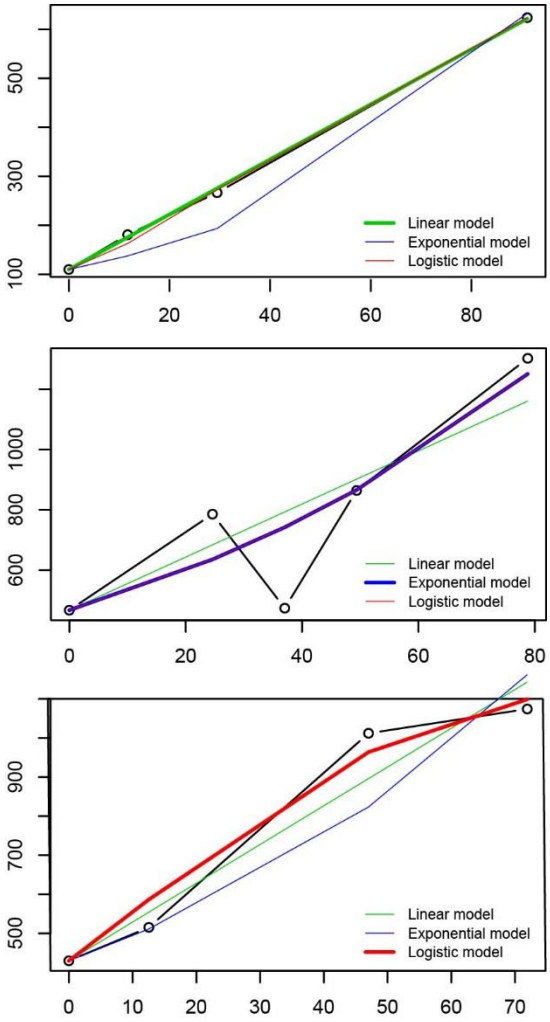
To overcome some of challenges concerning retest error all volume measures were performed by the same investigator (the author), and controlled by the same experienced neuroradiologist Geir Ringstad). Agreement on tumour outline was made for all tumours.

Growth curves

We plotted the volume measurements against time to make growth curves for all tumours where four or more consecutive MRI-scans not interrupted by secondary treatment were available (Paper 2). Standardized growth models, exponential, linear and logistic models, was then fitted to each curve (Figure 5). More time-points, or volume registrations, would have allowed for several and better fitted growth models. We were limited by the number of consecutive MRI-scans available in the retrospective cohort, and therefore the measured growth curves were fitted to the three mentioned standardized growth models. However, these models showed a good fit to the measured growth curves.

The best fitting model was determined by the lowest Akaike Information Criteria value (AIC). The AIC principle is used to select between different models that has been considered plausible before the analysis. For our analyses this was the linear model (formula 1), the exponential model (formula 2) and the logistic model (formula 3) as given in Figure 5. The AIC principle adjusts for the number of measurement points, in addition to calculating the maximal likelihood estimation when selecting the best fitting model (133). For some volume curves more than one model showed

a similar fit (as exemplified in curve 1 and 2 in Figure 5), the AIC will then prefer the simplest model.



(1). $f(t) = V_0 + rt$

Best model fit: linear.

(2). $f(t) = V_0 exp^{rt}$

Best model fit: exponential.

(3). $f(t) = \frac{V_{inf} + V_0 exp^{rt}}{V_{inf} + V_0 exp^{(rt-1)}}$

Best model fit: logistic.

Figure 5: Volume in mm³ (y-axis) plotted against time in months (x-axis). The standardized growth models; linear (green line), exponential (blue line) and logistic (red line), was fitted to the measured volume curves (black line with circles at each measurement point). The formulas for each growth model are given to the right of the growth curves. The best fitted model is marked with a bold line. The formulas for the growth models are given as a function of time f(t), and requires the following parameters: lowest measured volume (V_0), the slope gradient (r), the logistic model also requires the upper asymptote volume (V_{inf}).

Accelerated tumour growth, a shortening of TVDT with time, has previously not been described in PAs (29). This might be because rapid and increased growth excludes the possibility of following these tumours through the necessary amount of time points for growth curve modelling. As said, we needed more than four measurement points to formally asses an accelerated growth curve, and this was not available for all tumours. The growth curve of each tumour was therefore inspected to look for signs of accelerated growth. In a systematically planned prospective study, with sufficient number of volume measurements formal tests can be made to depict accelerated growth in addition to the other standardized growth patterns.

Tumour volume doubling time

TVDT is the time it takes a tumour to double the volume. At least two volume measures at two different time points, with a known interval, are needed to calculate TVDT (134). The TVDT formula assumes that the growth between the time-points is exponential. This, however, is not the case for most tumours. The finding of a logistic or linear growth pattern allows for a change in TVDT with time, and hence the measurements of TVDT based on two measures will be less accurate not taking the growth pattern of the tumour into account.

Since we did not find signs of accelerated growth in the tumours investigated, the initial phase of the growth curves showed the fastest growth rate for all tumours. To make the TVDT values comparable across the different models, a logistic model was fitted to all growth curves for the TVDT calculations. This model is the most general, and contains an

exponential and linear phase in addition to the decaying growth phase (Figure 2). The TVDT was then calculated at the very beginning of growth, based on the estimated growth rates from the models, as described in Paper 2. In this way, we were able to investigate the maximal growth potential for each tumour, and compare it across the different growth models.

Discussion of main findings

Reference genes in Pituitary Adenomas; Paper 1

In the study of reference genes in PAs, Paper 1, we first performed an investigation of 30 reference genes by three different program algorithms (Table 1) to find the best reference genes, the optimal number of genes to combine and the best combination of genes. Secondly, we performed a validation study of three reference genes based on the findings in the selection study. The MIQE guidelines calls for normalization of target genes against a combination of stably expressed reference genes. In addition, the optimal reference genes should be investigated for the particular tissues and specific experimental design (109).

ALAS1 and PSMC4 were found to be the best reference genes by NormFinder and GeNorm in the selection study. They were not included in the analyses by BestKeeper, while they were not among the ten reference genes with the lowest SD. However, of the reference genes studied, 21 had a Ct-values with a SD less than 1 pointing to a good stability. ALAS1 and PSMC4 has not been considered as reference genes in PAs previously. GAPDH has frequently been used as a reference gene in pituitary tumours (61, 87, 135, 136).

The best number of combination was found to be two by the GeNorm program. A single reference gene has frequently been used for normalization in PAs, this often being GAPDH, however the MIQE-guidelines recommends a combination of reference genes for optimal stability GAPDH was found to be stable in PAs in our cohort for both functioning and non-functioning tumours. GAPDH has also been found

stable in previous investigations of PAs and hence seems to be a good choice in combination with other reference genes (110, 137).

ALAS1, PSMC4 and GAPDH were chosen as reference genes for the validations study, based on the results from the selection study. The large number of tumour samples included in the validations study enabled us to differ between subtypes of PAs by the NormFinder algorithm (Table 1).

Table 1: Overview of the program algorithms used in determining the best reference genes in Paper 1.

Program	GeNorm	NormFinder	BestKeeper
Features	Calculates the best ref. genes. Calculates best combination of ref. genes. Calculates the optimal number for combination of ref. genes.	Calculates the best combination of two ref. genes Differentiates between inter- and intra-rater variations	Provides SD, CV and a correlation coefficient (r) for each gene to the BI.
Weaknesses	Does not correct for co-regulation (several transcripts regulated via the same mechanism).	Requires a samples set of at least 8 samples.	Includes only 10 genes. Does not provide a ranking order.
Outcome	Stability Value (M)	NormFinder Stability Value (M)	SD, CV and r
Use in the study	Selection cohort: Calculated and ranged the best reference genes. Found the optimal number for combination of ref. genes.	Selection cohort: Calculated and ranged best ref. genes and combination of ref. genes Validation cohort: Identified the optimal ref. genes in the immunohistochemical subtypes of PA.	Selection cohort: Provided SD and CV for the 10 selected ref. genes.

We found that the most stable reference gene combination in clinically NFPAs were ALAS1 and GAPDH, and PSMC4 and GAPDH in functioning adenomas. Considering that PAs arise from different cell lineages, they were expected to show some differences in their phenotype, and hence in their reference genes, which was in accordance with our results in Paper 1. When dividing between silent gonadotroph, null-cell and corticotroph adenomas, the combination of ALAS1 and GAPDH remained steady in our cohort.

The results from Paper 1 demonstrated that there are several good candidates that can be used and validated as reference genes in PAs. However, our results states that a combination of these should be used.

Growth rate and growth curves in clinically NFPAs, Paper 2

In Paper 2, we studied the postoperative growth of clinically NFPAs by growth curve patterning and calculation of initial TVDT.

TVDT is calculated from two measurement points, assuming exponential growth between the time points (138). A substantial portion of the tumours we investigated seemed to halt their tumour growth during the follow-up time, suggesting a logistic growth pattern (Figure 6). Honegger et al also recognized the logistic growth pattern in 4 out of 12 patients with MRI follow-up after surgery (29). Another large retrospective study focussing on the second and third regrowth in clinically NFPAs, showed that a considerable proportion of tumours monitored with MRI continued to grow, though not all (24). A third of the tumours we investigated showed a

growth curve fitting an exponential growth model. Accordingly, exponential growth is not applicable for all clinically NFPAs.

The growth curves and TVDT do not take the growth direction into account, which for these tumours is essential for the indication for surgery. The main indication for surgery is compression of the optic apparatus, which presupposes superior tumour growth (19). A model which takes growth direction into account would be helpful to identify the patients who are at risk for reintervention. Monsalves et al (28) studied TVDT and growth direction and compared with clinical data and some proliferation markers. They did not find growth direction to be related to TVDT, however TVDT was calculated from a minimum of two time-points and the growth curve of tumours was not reported in this study (28).

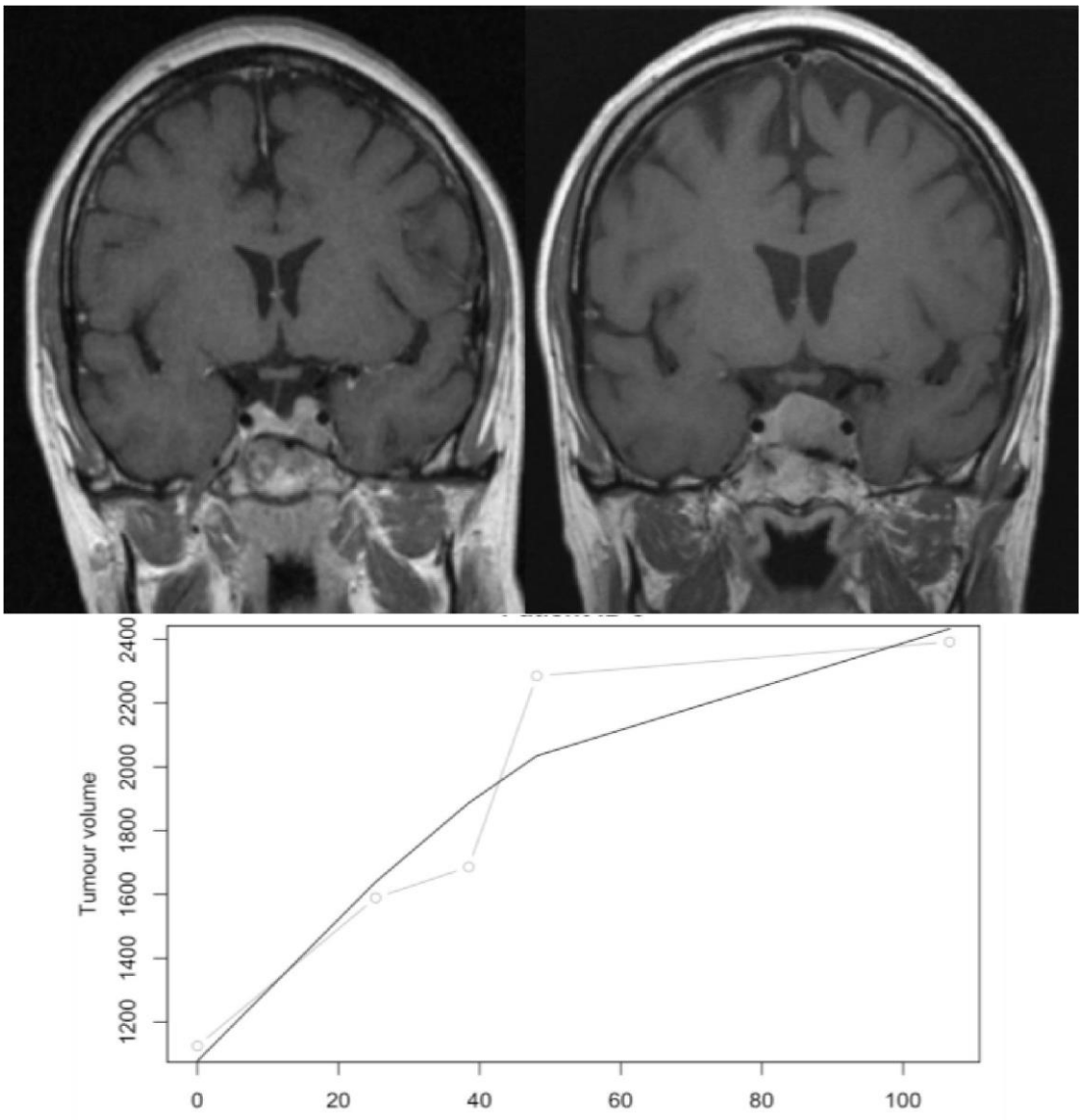


Figure 6: Example of tumour regrowth after surgery. The MRIs are taken one year (left) and nine years (right) after primary surgery. The measured growth curve is presented in light grey, with circles at each time point. The volumes in mm³ (y-axis) are plotted against months after primary surgery (x-axis), and fits a logistic growth model (dark grey line). The MRIs and growth curve are presented with the patients concent. Parts of the figure is retrieved from Øystese et al (2016) (139). Copyright Obtained.

We found the tumours with logistic growth patterns to have the lowest initial TVDT, while the exponentially growing tumours presented the slowest initial growth, but a slightly higher rate of reintervention.

Whether the rapid growth by itself causes insufficient nutrition and thereby slows down, or whether there are other factors causing deceleration of the growth remains speculative. Stensjøen et al (140) studied growth patterns in glioblastoma, and found that growth models with an increase in TVDT with time (Gompertz and linear radial) were better fitted to their volume registrations than an exponential growth model, and moreover a slower growth in large compared to small tumours (140). Their median doubling time was, however, 29.8 days and the theory of nutrition depending decay of growth might seem more plausible for these fast growing tumours than for the slow growing NFPAs.

We did not find a correlation between age and TVDT in our cohort. It has previously been shown by several studies that age is positively correlated to TVDT in NFPAs (27-29). However, most of these studies have been performed with TVDT calculated from only two time points. The patients not included for growth modelling in Paper 2 due to insufficient follow-up not caused by reintervention was significantly older than the patients included. This might obscure the association between age and TVDT, and prospective studies with a standardized follow-up are needed to explore this possible correlation in a standardized way.

Men had faster initial TVDT than women in our cohort, though the rate of reintervention did not differ between genders. Tampourlou et al found the male patients to have a lower risk of second regrowth than females, but there was no gender difference in the risk of later regrowth (24). Honegger et al. did not find gender differences in their study, however only four female patients were included (29). There are some suggested gender differences when it comes to aggressiveness in the subtypes of silent PAs

(36, 141), however; we found an even gender distribution in the different immunohistochemical subtypes presented in Paper 2.

If we can assume that tumour growth decelerates, rather than accelerates, with time, the early growth rate gives us valuable information in planning a safe postoperative follow-up algorithm. We did not find obvious signs of accelerated growth in our cohort of clinically NFPAs. To our knowledge, it has not been described in NFPAs previously. However, cases of cancerous transformation of silent PAs several years after the diagnosis or primary surgery have been reported in the literature, which also indicates a change in growth rate (142-144). Tampourlou et al, reported two patients with a malignant transformation 20 and 35 years after primary surgery (24). Though very rare, this complicates the discussion on when to terminate the follow-up of this patient group. In addition, the most aggressive tumours are not available for growth curve patterning due to their need of early reintervention, and hence their growth pattern is a challenge to study. Recently published guidelines have recommended life-long follow-up for all patients identified with an aggressive pituitary adenoma, defined by radiologically invasive tumours with unusually rapid tumour growth rate or clinically relevant tumour growth despite optimal standard therapies (26). The guidelines do not define what rapid tumour growth is, or how this should be calculated.

As previously mentioned, some studies report delayed regrowth in NFPAs after surgery, both in patients with and without residual tumour (22, 24). These findings might have several explanations. Small tumour remnants, not visible on MRI, might grow steadily until they reach a detectable size. Slowly growing tumours might reach a predetermined level defining

growth, often a volume increase of 20% or diameter an increase of 2 mm in any direction. In addition, new growth might be initiated after a period of no growth. Rodriguez-Brenes et al report of a multistep growth as one of the five growth patterns in their review, linking growth to genetic alterations in the tumour which leads to alternately tumour growth and dormancy (49).

Our results demonstrate that postoperative growth of clinically NFPA might be fitted to different types of growth models, and shows a deceleration of TVDT with time in a substantial proportion of the adenomas. The clinical usage of the models implies that the exponential model, with constant TVDT, is the worst way the early growth rate can evolve. Hence, early TVDT may be used to estimate a safe follow-up regime. However, as previously mentioned, the risk of transformation of tumours should be kept in mind.

Somatostatin, Oestrogen and Progesterone receptor distribution in clinically NFPAs, Paper 3

In Paper 3, we described the immunohistochemical distribution of SSTR1, 2, 3 and 5, ERα and PR in clinically NFPAs of different immunohistochemical subclassifications and compared it to variables of aggressiveness.

SSTR3 was abundantly expressed by IHC throughout the whole cohort and in the immunohistochemical subtypes investigated. Previous IHC studies have shown SSTR3 to be the most abundant SSTR in gonadotroph tumours (60), though this has not been found by all (62). However, different methods for immunohistochemical scoring have been used. Of the SSTRs, we found the SSTR3 to have the highest relative mRNA level, followed by SSTR2 when examining the whole cohort. Gonadotroph adenomas showed a higher mRNA expression of SSTR3 than corticotroph and null-cell adenomas. However, the groups of corticotroph and null-cell adenomas were small for the mRNA analyses. A previous report has also shown the mRNA levels of SSTR3 to be higher in the gonadotroph subgroup than in silent corticotroph adenomas (145). Drastikova et al studied a mixed cohort of PA, including a large cohort of clinically NFPAs where they found the mRNA of DR to be most abundant receptor, followed by ERα. SSTR3 and SSTR2 were the most abundant of the SSTRs (146). In our cohort, ERα showed a similar mRNA level as SSTR3. Nishioka et al compared functioning and non-functioning pituitary adenomas, and found the mRNA levels of SSTR3 to be higher in clinically NFPAs than in some functioning adenomas (somatotroph and lactotroph), while the levels of SSTR2 and 5 were lower (61).

SSTR2 is known to be the most abundant somatostatin receptor in functioning somatotroph adenomas, while SSTR5 dominates in corticotroph tumours (69, 118). In our cohort, SSTR5 was significantly higher in the corticotroph subgroup than in the gonadotroph subgroup, though the IRS was generally low. The difference between the corticotroph subgroup and the null-cell adenomas was not significant, possibly due to lack of power, or lack of staining for the corticotroph transcription factor T-Pit. The silent subtypes of PAs have been shown to have some similar clinical features as their functioning counterparts (11). However, the SSTR3 receptor is known to be absent or expressed at low levels in the functioning corticotroph adenomas (69, 147), while we found the IRS of SSTR3 to be high in the silent corticotroph subgroup. In vitro studies based on cell cultures have shown an association between SSTR3 function and regulation of hormone production and secretion in somatotroph and corticotroph cells; and that the presence of SSTR3 was related to a reduced hormone expression (65, 148).

Due to the presence of SSTRs in NFPAs, SRL are considered as potential agents for medical treatment. Octreotide, a specific SSTR2 ligand, has been tested in some small clinical trials, with limited follow-up time (72, 73). The lack of obvious effect of Octreotide is in concert with our findings where SSTR2 was present at relatively low levels and not in all immunohistochemical subtypes of the clinically NFPAs. Pasireotide, is a less selective SRL with a varied affinity for SSTR1, 2, 3 and 5 (66). Ibanez-Costa et al performed an in vitro study on the effect of SRL on cell cultures collected from different types of PAs and found cells collected from NFPAs to be poorly responsive to both octreotide and pasireotide. In addition, they observed a paradoxical increase in cell-viability. This study also found

SSTR3 to be the most abundant receptor in NFPAs at the mRNA level (149). The results of ongoing clinical trials studying the effect of pasireotide in clinically NFPAs are awaited during the next years (ClinicalTrials.gov ID: NCT01283542 and NCT02749227).

The literature concerning SSTR distribution in clinically NFPAs is characterized by a lack of consistent methods and well-defined cohorts. However, there seems to be a difference between the receptor distribution between functioning and non-functioning adenomas, and also an internal difference among the immunohistochemical subtypes of clinically NFPAs. Our results add to these previous findings in stating the abundance of SSTR3 in NFPAs, with weak and subgroup dependent staining for the latter SSTRs. A further exploration of the difference in receptor distribution between functioning and non-functioning adenomas would be of great interest; though it should be performed comparing silent and functioning tumours of the same cell-lineage.

ERα is known to be a transcription factor in gonadotroph and lactotroph cell differentiation (53). Hence, the IRS for ERα was significantly higher in the gonadotroph subgroup than in the corticotroph and null-cell subgroup. The IRS was however, low in the gonadotroph subgroup. Previous studies that have investigated ERα by IHC in clinically NFPAs have also found the staining scores to be low (82, 84). We did the analyses of ERα and variables of aggressiveness both with and without dividing between pre- and postmenopausal women, and for the whole cohort and the gonadotroph tumours separately, to control for potential variations in the ERα.

We found a gender dependent association between the presence of ERα and reintervention, time to reintervention and correlation to SSTR2,

examined both with and without premenopausal women and only in gonadotroph adenomas. These gender differences seemed not to be depending on lack of statistical power in the female group.

Nishioka et al found a significant correlation between ERα and SSTR2 at the mRNA level in clinically NFPAs for patients aged over and under 50 years. They did not investigate the genders separately, though men were overrepresented (61). Drastikova et al did not find a correlation between these two receptors (146). In breast cancer cell-line studies, the ERα has been suggested to participate in the regulation of the transcription of SSTR2 (150, 151). The gender dependency of the correlation between the receptors are yet to be elucidated.

Delgrange et al have studied the gender differences in relation to ERα in lactotroph adenomas. Lactotroph adenomas are common in women, but relatively rare and more aggressive in men (152). The IRS of ERα in lactotroph adenomas was found to be inversely correlated to tumour size and markers of proliferation using the same antibody and IRS, as in our study (79). This association was present in both genders. The women in this study were younger than the women with clinically NFPAs in our study, and showed a high IRS of ERα. The men showed a low IRS similar to the IRS found in both genders in our cohort (79). We found that the absence of ERα in men aged below 60 years predicted reintervention with high precision. A low ERα was also suggested as a marker indicative of poor prognosis in the study on prolactinomas (79). Gao et al studied the ERα in GH3 cell-lines and different types of PA. They found the distribution of ERα in non-invasive and invasive PA to differ between the PA-types. In the NFPAs the proportion of tumours with ERα staining in < 50% of the

cells were higher in the invasive NFPAs, than in the non-invasive (153). These findings resemble the results of our study, where absence of ERα was related to a more aggressive clinical course. However, the staining score and end-points were different, and this study did not investigate men and women, or the different immunohistochemical subtypes of NFPAs separately (153).

Gender differerences in clinically NFPAs are not well elucidated, though some differences are found in relation to age at diagnosis and proportion of macroadenomas in epidemiological studies (7, 10). We have found differences between men and women in both Paper 2, where the initial TVDT was lower in men, and in Paper 3 as presented above.

The presence and function of PR has hardly been studied in NFPAs, though it is known to be present in both normal and adenomatous pituitary tissue (76, 154). A cell study performed on a mixed set of pituitary adenoma cells (two non-functioning) showed progesterone to inhibit cell proliferation, while estradiol stimulated cell proliferation. The effect of progesterone was related to the level of PR (155). In the present study, we did not find PR to be associated with rate of reintervention, and not correlated to the other receptors investigated.

SSTR3 seems to be the most abundant SSTR across all clinically NFPAs, and not just in gonadotrophs, making this an interesting target for comparing functioning and non-functioning adenomas and for exploring a potential target of medical treatment. In addition, we present data on ERα as a gender dependent prognostic marker in gonadotroph NFPAs. ERα has not been tested as a marker of aggressiveness in gonadotroph adenomas previously.

E-cadherin and N-cadherin in Gonadotroph adenomas, Paper 4

In Paper 4, E-cadherin and N-cadherin in gonadotroph pituitary adenomas was characterised by IHC and mRNA expression and related to factors of aggressiveness.

We found that the immunohistochemical staining for the extracellular domain of E-cadherin was nearly absent and the IRS for the intracellular domain of E-cadherin varied with most tumours presenting a medium IRS (4-8). The IRS for N-cadherin was high throughout most of the cohort. Epithelial tissue is known to have a high level of E-cadherin (97). However, other adherence proteins are also present in epithelial tissues. Both E-cadherin and N-cadherin have previously been found in normal pituitary tissue (98). Two small studies with double staining for hormones and cadherins in normal pituitary material have shown variable cadherin distribution between the different types of adenohypophysial cells. The gonadotroph cells were found to present N-cadherin in both studies. In the same studies the somatotroph and lactotrophs cells presented with high membranous E-cadherin, while N-cadherin was found in some somatotroph and lactotroph cells in one of the studies (95, 98). Another study found N-cadherin to be nearly absent in normal somatotroph and lactotroph cells (156).

Information on the regulation and role of N-cadherin is sparse in PAs and with deviant results. In a previous study, reduced membranous staining for N-cadherin was associated with invasion in gonadotroph and null-cell adenomas. The same study showed a strong membranous staining for N-cadherin in most of the gonadotroph adenomas, and in none of the somatotroph and lactotroph adenomas investigated (156). Jia et al investigated transcripts from CDH2 (gene coding for N-cadherin) in a

mixed cohort of PAs. They found a higher CDH2 transcript to be associated with larger tumours, when investigating all tumours together. They also found the CDH2 transcript to be higher in the non-functioning tumours than in PRL, GH and mixed-hormone secreting tumours (157). The studies points to a variable expression of N-cadherin in different subtypes of PAs.

The adherence proteins are dynamic structures that are regulated by different transcriptional or posttranscriptional processes (91, 92, 136, 158). There is a plethora of possible factors influencing their production and degradation (92). A switch in cadherin expression is described as part of EMT, most commonly a downregulation of E-cadherin and a corresponding upregulation of N-cadherin. For some tumours, however, a de novo expression of N-cadherin without down regulation of E-cadherin occur (97). We did not find a negative correlation between N-cadherin and the intracellular domain of E-cadherin, indicative of a cadherin switch. On the contrary, there was a slight positive correlation between the two cadherins. Coexistence of E- and N-cadherin has been found in other tumour types and in normal tissues (98, 159, 160). In vitro studies on breast cancer cells have shown that invasive and motile features dominates when both E- and N-cadherins are present, in spite of a normal E-cadherin level (161, 162).

We did not find a difference in the IRS of N-cadherin in tumours between patients needing reintervention compared to patients operated only once. However, we found a slightly higher mRNA expression of N-cadherin in tumours from patients going though reintervention, than those who did not. The majority of tumours investigated showed a high IRS (>9) for N-cadherin (N= 94) while only ten patients showed a moderate staining (IRS 4-8). With this little variation in the IRS for N-cadherin in the cohort,

differences between groups were hard to detect. Zhou et al found the levels of E-cadherin to be lower in invasive than non-invasive, while the levels of N-cadherin did not differ, in a mixed cohort of clinically NFPAs. However, only a few of the included adenomas in this study stained for gonadotropins and the majority were plurihormonal (84).

There was a discrepancy between the IRS for the extracellular domain of E-cadherin and the intracellular domain of E-cadherin in our study. This discrepancy might be due to biological or methodological causes. A study on cancer cell lines showed that the extracellular domain of E-cadherin was cleaved off by proteases, while the intracellular domain was degraded (163). Another in vitro study of the intracellular cleavage of E-cadherin reported on nuclear translocation of the protein (91). Hence, this suggests that cleavage of E-cadherin may take place on both the inside and outside of the membrane. Previous studies in colonic adenomas have also shown low levels of the extracellular domain of E-cadherin, while the intracellular domain was intact (128). The specificity of the antibodies used may also affect the results presented. This has been discussed previously in the section on methodological considerations.

As for N-cadherin, we did not find an association between the IRS of E-cadherin scored by the cell membrane and reintervention. More than two thirds of the cohort presented nuclear staining for the intracellular domain of E-cadherin. There was a clear association between the presence of nuclear E-cadherin and low levels of membranous staining for the intracellular domain of E-cadherin. This association has also been shown in both functioning and non-funtioning pituitary adenomas previously (86, 87, 93). The ten tumours with moderate IRS (IRS 4-8) for N-cadherin, were

all tumours presenting nuclear staining for the intracellular domain of E-cadherin, mirroring the positive correlation between the IRS of the intracellular domain of E-cadherin and the IRS of N-cadherin. The pathogenesis behind the presence of nuclear E-cadherin in pituitary adenomas is not known, though a translocation of the intracellular domain of E-cadherin after cleavage of the extracellular domain, has been suggested (93). In vitro studies inducing EMT may elucidate the fate and function of the cadherins in the gonadotroph tumours.

Previous studies in subgroups of pituitary adenomas have found the presence of nuclear E-cadherin to be linked to a more aggressive tumour type, more resistant to treatment (86, 87, 93). On the contrary, nuclear E-cadherin has also been found in normal tissues (164). We found the rate of reintervention to be lower in tumours presenting nuclear E-cadherin than tumours not presenting nuclear E-cadherin. In light of this result, it was expected that the IRS of the intracellular domain of E-cadherin was higher in tumours needing reintervention. This was however, not the case most likely caused by lack of statistical power (Type 2 error). Elston et al studied a mixed cohort of pituitary adenomas with over half being NFPAs, though not further subclassified by IHC (93). They found nuclear staining for E-cadherin to be associated with a higher level of invasion. Reintervention and time to reintervention, which was used as an end-point in Paper 4 is not necessarily depending on rate of tumour invasion. The results are therefore not directly comparable. In addition, their immunohistochemical scoring system was different from the IRS used in our paper, and hence the staining levels of E-cadherin not corresponding (93).

The parameters of aggressiveness between studies of pituitary adenomas differ, making it hard to compare the effect of nuclear E-cadherin on the tumour course in different subtypes of PAs. However, all studies taken together suggests that there is a link between presence of nuclear E-cadherin and a lower membranous level of E-cadherin in both functioning and clinically non-functioning adenomas, and that the presence of nuclear E-cadherin is more frequently occurring in NFPAs (93). The association between the presence of nuclear E-cadherin and aggressiveness have shown variable results in different subtypes of PAs.

In Paper 3 we found a gender specific association between the absence of ERα and rate of reintervention, while the absence of nuclear E-cadherin in the Paper 4 gave a higher rate of reintervention. The correlation between nuclear E-cadherin and reintervention was still significant when adjusting for ERα and age. However, the correlation between ERα and reintervention became weaker in the same regression model ($p=0.06$, OR = 0.79). The correlation remained significant for the location of E-cadherin, and not significant for ERα when investigating women and men separately. Zhou et al presented a negative correlation between the immunohistochemical staining of E-cadherin and ERα (84). This resembles our findings where nuclear absence of E-cadherin, and hence a high membranous E-cadherin score, and absence of ERα was both related to reintervention. A study on rat somatolactotroph GH3 cells, showed that Estradiol 17β(E_2) promoted non-adherence and lowered the levels of N-cadherin. They found N-cadherin to be high and E-cadherin to be low in the same cells in preliminary studies (165). The above-mentioned findings points to a possible link between ER and EMT in the gonadotroph adenomas, though this could be cell-specific mechanisms.

N-cadherin showed to be an abundant cadherin in gonadotroph adenomas, possibly reflecting the normal distribution of cadherins in gonadotroph pituitary cells. The cadherins have not been thoroughly studied separately in gonadotroph adenomas previously. Our findings, together with previous studies on clinically NFPAs and PAs, points to a cell-specific distribution of E-and N-cadherin in pituitary tumours, that may be associated with the aggressiveness of the adenomas.

Limitations

The main challenge of the studies is the lack of systematic inclusion. We used the material available from previous studies where tissue samples were collected. Information on how many patients and which patients who were operated in the same time period and not included in the cohort was not available to us. This might bias the selection of patients included, and systematic prospective studies are needed to validate the hypotheses generated. However, we assume that the tissue available was random.

Originally, the study aimed to compare gonadotroph and null-cell adenomas in relation to growth and markers of aggressiveness. With the introduction of transcription factors to the immunohistochemical subclassification, the number of null-cell adenomas was substantially reduced. This made comparison between subclassification subject to lack of power. However, staining for transcription factors improves the distinction between the different immunohistochemical subtypes of clinically NFPAs, in accordance with their clinical features (11), and could possibly have improved the characterization of the subtypes. We did not stain for the transcription factor T-pit due to lack of a good available antibody when

performing the IHC analyses (120). This might have obscured the difference between the corticotroph and null-cell adenomas.

There was a preponderance of male patients in our cohort. It is known from some, but not all, register-based studies that NFPAs are more common in men than in women, and that more men present with macroadenomas than women (7, 10, 15). However, the proportion of men in our cohort was larger than what is the case in previous studies. The reason for this gender difference in our cohort is not known to us.

According to the recent guidelines on management of aggressive PAs; the diagnosis of aggressiveness is based on information from series of radiologic investigations (invasiveness and growth) and/or clinical data (resistance to treatment). In our retrospective cohort, this information was only available for a proportion of the cohort. Sufficient MRI follow-up was only available for approximately a fourth of the included patients, and therefore not applicable as an end-point in most of the analyses. Preoperative tumour volume, invasiveness and information on residual tumour would improve the data presented in the studies substantially. Composite end-points like reintervention (both radiation and surgery) and time to reintervention, were used as variables of aggressiveness. These are clinically relevant end-points, but they are depending on several factors not necessarily related to the growth potential of the tumours. In an attempt to adjust for this, reintervention taking place less than 12 months after primary surgery was not included as part of the primary intervention.

In Paper 2, the same investigator, in agreement with an experienced neuroradiologist, performed all volume estimates. However, the variability of the measures was not formally investigated. The intra- and inter-rater

variability of postoperative investigations have not been previously investigated in clinically NFPAs. The manual summary of slices method is to date the best available method of investigating volumes.

We dichotomized between women over and under 51 years, according to the mean age for menopause in Paper 3, due to lack of exact clinical information concerning this (166). The exact time for menopause is difficult to obtain in this group of patients due to potential pituitary deficiency.

Lack of normal material is in general a challenge in the study of PAs, due to the size of the organ, location of the pituitary and the heterogeneity of the pituitary tissue. The study of transformation from normal to tumourous tissue demands knowledge on the normal phenotype.

Main Conclusions

Reference genes should be determined specifically for the experiments performed and tissue investigated. We found the combination of ALAS1 and GAPDH to serve as the most stable reference genes for clinically NFPAs, while PSMC4 together with GAPDH served as the best reference gene combination for functioning corticotroph and somatotroph adenomas.

Growth rate seem to stay constant or decelerate with time in clinically NFPAs, hence TVDT may serve as a tool to predict a conservative and safe follow-up for most NFPAs. The tumours with the shortest initial TVDT are more likely to decelerate their growth rate during the follow-up, and hence less likely to need reintervention.

Somatostatin Receptor 3 is highly expressed across the different immunohistochemical subtypes of clinically NFPAs. The other SSTRs investigated showed a low or moderate expression, which also was subtype specific. The presence of ERα is associated with reintervention in male patients with gonadotroph adenomas. The combination of young age and absence of ERα may serve as a prognostic marker for reintervention in male patients with gonadotroph adenomas.

The expression of N-cadherin is high, while the expression of the extracellular domain of E-cadherin is low in gonadotroph adenomas. The presence of nuclear E-cadherin is strongly associated with a low membranous staining for the intracellular domain of E-cadherin. Absence of nuclear staining for E-cadherin is related to a higher rate of reintervention in gonadotroph adenomas.

Future perspectives

The studies of clinically NFPAs are characterized by a lack of well-defined study groups and end-points. The diagnostic tools have improved and changed during the last decades, and consequently there is a need for updated information. With the mapping of reference genes and development of methods for investigating tumour growth, the survey basis for upcoming studies is more easily accessible. We have already initiated a comprehensive systematic prospective study, planned to validate the hypotheses made in the work presented here.

In the presented studies, we have tested out robust end-points of aggressiveness like TVDT and growth pattern. Held together with preoperative information on invasiveness and clinical information this will enable us to better distinguish between aggressive and non-aggressive tumours, in accordance with the guidelines on aggressive PAs (26).

A large cohort of clinically NFPAs with systematic inclusion of patients followed postoperatively with MRI at fixed time points will give us valid information on postoperative growth patterns and TVDT. The growth patterns and growth rate held together with data from analyses made on preoperative MRI, blood samples and tumour tissue from primary surgery, will give us the opportunity to search for biomarkers prognosticating a clinical aggressive course. Reliable prognostic markers of aggressiveness are long awaited in caring for this patient group.

The tumour receptors may potentially serve as targets for medical treatment. Medication that potentially can reduce tumour volume or inhibit further tumour growth will aid the neurosurgeons in curing the

patients and/or potentially reduce the need for surgery. SSTR3 points out as one of the interesting receptors to stimulate and silence in further in vitro studies. In addition, the stimulation and knockdown of ERα in gonadotroph tumours will be of great interest for future cell studies.

We found a gender specific link between ERα and rate of reintervention in clinically non-functioning gonadotroph adenomas in Paper 3 and presence of nuclear staining for the intracellular domain of E-cadherin and reintervention in clinically non-functioning gonadotroph adenomas in Paper 4. Previous studies in different types of epithelial tumours have associated the process of EMT with ERα-function (167-170). Whether there is a link between ERα and EMT in gonadotroph PAs needs exploration through in vitro studies.

The results presented on E- and N-cadherins and on SSTRs in Paper 3 and 4 differ from some previous studies of functioning pituitary adenomas. Possible mechanisms for silencing of hormone producing tumour cells will be of great interest to explore in vitro. The results of such studies might give valuable information applicable to both functioning and non-functioning pituitary adenomas, and possibly other endocrine tumours.

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