Daniel Bengtsson

Cushing's Disease and Aggressive Pituitary Tumours

- Aspects on Epidemiology, Treatment and
Long-term Follow-up

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Linköping 2021

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Cover illustration: *Plumuitary* by Ana Norlén

Printed in Sweden by LiU-tryck, Linköping, 2021

ISSN: 0345-0082

ISBN: 978-91-7929-652-0

A physician is obligated to consider more than a diseased organ, more even than the whole man - he must view the man in his world.

Harvey W. Cushing

ABSTRACT

This thesis focuses on clinical and epidemiological aspects of aggressive pituitary tumours/carcinomas and Cushing's disease. Pituitary carcinomas account for only 0.1-0.2% of the tumours originating from the anterior pituitary gland and are defined solely by the event of distant metastases, whereas aggressive pituitary tumours are defined by their clinical behaviour of rapid/progressive growth despite optimal treatment with surgery, radiotherapy and medical agents. The prognosis for individuals with aggressive tumours/carcinomas has been poor with few treatment options. However, case reports indicated better outcomes after treatment with the alkylating agent temozolomide. In **study I and III**, we investigated 24 patients (16 aggressive tumours and 8 carcinomas) given treatment with temozolomide. We found an initial response rate (tumour regression $\geq 30\%$) in 10/21 evaluable patients, with complete regression in two carcinomas. Favourable response was associated with low tumour expression of the DNA repair protein MGMT; in responders median 9% (range 5-20%) *vs* non-responders median 93% (50-100%). Our results also indicated a longer survival in patients with low MGMT. Out of 11 patients with MGMT $>10\%$, nine died with an estimated median survival of 26 months (95% CI 14-38), whereas only 1/6 patients with lower MGMT died from tumour progression during a follow-up of median 83 months (range 12-161).

One of the patients in study I and III had a corticotroph pituitary carcinoma and in addition, Lynch syndrome (LS), a hereditary cancer-predisposing syndrome caused by germline mutations in DNA mismatch repair (MMR) genes and primarily associated with colon and endometrial carcinomas. In **study II**, we investigated the characteristics of the pituitary carcinoma and found loss of MSH2 and MSH6 protein expression, consistent with the patient's germline mutation in *MSH2*. This was the first published case of a pituitary tumour associated with LS. In addition, we identified all known Swedish patients with LS ($n=910$) and searched for diagnostic codes consistent with a pituitary tumour in the Swedish national patient register. We found in total three patients with clinically relevant pituitary tumours, the reported prevalence in the background population is around 1:1000.

The last two studies in the thesis focused on Cushing's disease (CD), i.e. an ACTH-secreting pituitary tumour resulting in excess levels of cortisol. CD is associated with multiple comorbidities and increased mortality. The reversibility of comorbidities and mortality risk after remission of cortisol levels have been under debate. **Study IV** examined psychiatric consequences of CD, measured by the use of psychotropic drugs. 179 patients with CD and a quadrupled matched control group were followed from diagnosis and at 5- and 10-year follow-up. We found that use of antidepressants remained at around 25% of patients with CD, regardless of remission status, at diagnosis and follow-up, whereas drugs for somatic comorbidities decreased. Use of antidepressants, sleeping pills and anxiolytics was higher in patients with CD

compared to controls at diagnosis and 5-year follow-up. A cross-sectional analysis of 76 patients in sustained biochemical remission for median 9.3 years showed that 25% were taking antidepressants, a significantly higher use than controls, OR 2.0 (95% CI 1.1-3.8). In addition, patients with CD had a higher use of psychotropic drugs, already in the 5-year period before diagnosis.

Study V investigated mortality and causes of death in 371 patients with CD, compared to a quadrupled matched control group. Follow-up was median 10.6 years (IQR 5.7-18.2) after time of diagnosis. Overall mortality was increased in patients with CD, HR 2.1 (95% CI 1.5-2.8) and remained elevated for patients in remission at last follow-up (n=303), HR 1.5 (1.02-2.2). For patients not in remission (n=31), HR was 5.6 (2.7-11.6). Cardiovascular diseases (32/66) and infections (12/66) were overrepresented causes of death in patients with CD.

Main conclusions of the thesis:

- Temozolomide improves outcome in patients with aggressive pituitary tumours/carcinomas and a low MGMT expression in the tumour predicts a favourable outcome. As additional therapies evolve, MGMT may help to tailor the treatment.
- Germline mutations in MMR genes may contribute to the development and clinical course of pituitary tumours and may be a novel cause of hereditary pituitary tumours.
- Patients with Cushing's disease have a high use of psychotropic drugs that remains elevated despite achievement of biochemical remission, suggesting persisting negative effects on mental health and highlighting the need for long-term monitoring of psychiatric symptoms. In addition, psychiatric symptoms may be early and important signs of CD.
- Efforts to achieve biochemical remission are crucial to reduce mortality in CD. However, patients in remission still have an increased mortality compared to controls. This underscores the need for life-long monitoring and treatment of associated comorbidities in patients with CD.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals.

- I. **Bengtsson D**, Schröder HD, Andersen M, Maiter D, Berinder K, Feldt Rasmussen U, Rasmussen ÅK, Johannsson G, Hoybye C, van der Lely AJ, Petersson M, Ragnarsson O, Burman P. Long-term outcome and MGMT as a predictive marker in 24 patients with atypical pituitary adenomas and pituitary carcinomas given treatment with temozolomide. *J Clin Endocrinol Metab*. 2015 Apr;100(4):1689-98. doi: 10.1210/jc.2014-4350.
- II. **Bengtsson D**, Joost P, Aravidis C, Askmalm Stenmark M, Backman AS, Melin B, von Salomé J, Zagoras T, Gebre-Medhin S, Burman P. Corticotroph pituitary carcinoma in a patient with Lynch Syndrome (LS) and pituitary tumors in a nationwide LS cohort. *J Clin Endocrinol Metab*. 2017 Nov 1;102(11):3928-3932. doi: 10.1210/jc.2017-01401.
- III. **Bengtsson D**, Schröder HD, Berinder K, Maiter D, Hoybye C, Ragnarsson O, Feldt-Rasmussen U, Krogh Rasmussen Å, van der Lely A, Petersson M, Johannsson G, Andersen M, Burman P. Tumoral MGMT content predicts survival in patients with aggressive pituitary tumors and pituitary carcinomas given treatment with temozolomide. *Endocrine*. 2018 Dec;62(3):737-739. doi: 10.1007/s12020-018-1751-9.
- IV. **Bengtsson D**, Ragnarsson O, Berinder K, Dahlquist P, Edén-Engström B, Ekman B, Höybye C, Burman P, Wahlberg J. Psychotropic drugs in patients with Cushing's disease before diagnosis and at long-term follow-up - a nationwide study. *J Clin Endocrinol Metab*, accepted 2021 Feb 4, doi: 10.1210/clinem/dgabo79
- V. **Bengtsson D**, Ragnarsson O, Berinder K, Dahlquist P, Edén-Engström B, Ekman B, Höybye C, Järås J, Valdemarsson S, Burman P and Wahlberg J. Increased mortality persists after treatment of Cushing's disease: A nationwide matched study of 371 patients. *Unsubmitted manuscript*.

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ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
APT	Aggressive pituitary tumour
ATC	Anatomical therapeutic classification system
CD	Cushing's disease
CI	Confidence interval
CS	Cushing's syndrome
ESE	European Society of Endocrinology
FSH	Follicle stimulating hormone
GH	Growth hormone
HR	Hazard ratio
ICD	International statistical classification of diseases
IQR	Interquartile range
LH	Luteinising hormone
LS	Lynch syndrome
MGMT	O6-methyl-guanine-DNA methyltransferase
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSH	MutS homologue
MSI	Microsatellite instability
NFPA	Non-functioning pituitary adenoma
NPR	Swedish national patient register
OR	Odds ratio
PC	Pituitary carcinoma
PDR	Swedish prescribed drug register
PRL	Prolactin
SPR	Swedish pituitary register
TSH	Thyroid stimulating hormone
TSS	Transsphenoidal pituitary surgery

INTRODUCTION

Although pituitary tumours in general are benign, a small part of the tumours has a progressive growth pattern and recurs despite treatment with surgery, radiotherapy and medical agents. Such tumours are referred to as “aggressive” based on their clinical behaviour; the prognosis is poor and treatment options are sparse. Being a very rare event and defined solely by the presence of metastasis, pituitary carcinomas account for less than 0.2% of pituitary tumours. Out of the apparently benign adenomas, Cushing’s disease, i.e. excess levels of cortisol due to an ACTH-secreting pituitary tumour, stands out as the gravest, and is associated with significant comorbidities. Although often smaller than other pituitary tumours, patients with Cushing’s disease have the highest mortality rates and the effects of previous hypercortisolism may persist despite achievement of biochemical remission.

This thesis is mainly focused on clinical and epidemiological aspects of aggressive pituitary tumours/carcinomas and Cushing’s disease. The first and third study investigated the effect of temozolomide in aggressive pituitary tumours/carcinomas and the potential of the DNA repair protein MGMT as a predictor of outcome. In the second study, we explored the role of germline mutations in DNA mismatch repair genes in a patient with a pituitary carcinoma. Finally, the last two studies examined neuropsychiatric complications and mortality in Cushing’s disease.

BACKGROUND

In general, the term “pituitary tumour” in the thesis refers to neuroendocrine tumours originating from the anterior pituitary gland.

Tumours of the anterior pituitary gland

In autopsy series, approximately 10% of unselected patients have a pituitary adenoma (1). However, most pituitary tumours will remain unrecognized during life and the prevalence of clinically significant pituitary tumours, meaning those who come to medical attention, is around one per 1000 (2-4).

Pituitary adenomas are often divided into clinically functioning tumours according to their hormone secretion or defined as clinically non-functioning tumours. Immunohistochemistry of hormone subgroups and transcription factors arrange the tumours according to cell origin (5), **Table 1**. In rare cases, the tumours may be plurihormonal. Most clinically non-functioning adenomas stain positive for gonadotropins, and on rare occasions for prolactin, ACTH or growth hormone, and are referred to as “silent” gonadotrophs, lactotrophs, corticotrophs and somatotrophs respectively. Less than 3% are immunonegative for all hormones/transcription factors and are termed null cell adenomas (5,6). Evaluation of proliferation markers, somatostatin receptors and granulation patterns may have prognostic and/or predictive implications.

Table 1. Simplified classification of pituitary adenomas. Proportion of each clinical subtype derived from population-based data, reviewed in ref (7).

Clinical phenotype	Hormone secreted	Approximate proportion(7)	Cell origin	Transcription factor
Hyperprolactinemia	PRL	32-66%	Lactotroph	PIT-1
Gigantism/acromegaly	GH	8-16%	Somatotroph	PIT-1
Cushing's disease	ACTH	2-6%	Corticotroph	T-PIT
Hyperthyroidism	TSH	<1%	Thyrotroph	PIT-1
Hypergonadism	LH/FSH	Very rare	Gonadotroph	SF-1
Clinically nonfunctioning	-	15-54%	Gonadotroph (most tumours) Lactotroph, somatotroph, corticotroph (uncommon) True null cell (<3%)	SF-1/ PIT-1/ T-PIT or absent

Magnetic resonance imaging (MRI) is the preferred imaging method and the extension of the tumours into the cavernous sinus can be graded according to the modified Knosp classification (8). Approximately 35-45% invade into adjacent structures (5,9). In

addition, the tumours are often classified according to their largest diameter, as microadenomas (<10 mm), macroadenomas (≥ 10 mm) and giant adenomas (≥ 40 mm) (5).

Figure 1. Normal anatomy of the sellar and parasellar regions surrounding the pituitary gland. **a**, Coronal view. **b**, Lateral view. Reprinted from Di Ieva, A. et al. *Nat. Rev. Endocrinol.* doi:10.1038/nrendo.2014.64 (10), with permission from the publisher.

Pituitary tumours may cause headache or visual disturbances due to the location close to the optic chiasm and cranial nerves III-VI, **Figure 1**. In addition, the tumour might compromise pituitary hormone secretion with associated symptoms such as menstrual disturbances, hypothyroidism, growth hormone deficiency, hypocortisolism and in rare cases diabetes insipidus (7,11). Hypersecreting tumours cause symptoms related to the hormones secreted, Table 1. Pituitary surgery with transsphenoidal approach is the primary treatment of hypersecreting tumours and enlarging clinically non-functioning tumours with local mass effects, with the exception of lactotroph tumours that most often are controlled with medical agents (7,12,13). In addition, radiotherapy and/or medical treatment are used as adjuvant therapy to control remaining hormone secretion and/or tumour growth or when surgery is not possible. Pituitary hormone insufficiencies are treated with hormone replacement therapy (11).

Aggressive pituitary tumours and pituitary carcinomas

Definition and epidemiology

The current World Health Organization (WHO) classification, published in 2017 (5), divides tumours of the anterior pituitary into adenomas and carcinomas. Because histopathological signs of malignancy are lacking, carcinomas are only considered when metastases are present. In the previous WHO classification from 2004, tumours exhibiting a Ki67 labelling index > 3%, a high mitotic count and extensive p 53-staining

were called “atypical adenomas” (14). With that definition, atypical adenomas were found in 2.7 to 15% of patients that underwent surgery (14,15). However, the term “atypical adenoma” was abandoned in the last WHO classification since the prognostic value was uncertain and no agreement on methods and cut-offs were reached (5). Low Ki67 labelling index and negative p-53 are seen in approximately 20% of clinically aggressive adenomas and carcinomas (16). Thus, the definition of aggressive pituitary tumours cannot be based on proliferation markers only. Instead, the definition recommended by the European Society of Endocrinology (ESE), relies on the clinical behaviour of the tumour, i.e. a radiologically invasive tumour with unusually rapid growth, or continued tumour growth despite optimal standard therapies including surgery, radiotherapy and medical treatments (17). This definition leaves room for arbitrary judgement and hampers accurate estimations of incidence and prevalence (18). It should be noted that invasiveness is not equal to aggressiveness, although indicating a higher risk of recurrence (19). Invasion into the cavernous sinus, sphenoid sinus or skull base have been reported in 40-50 % of pituitary adenomas in surgical series (20). Thus, an aggressive tumour is invasive but locally invasive tumours that respond to standard treatment are not per se aggressive.

Carcinomas have been found in 0.1-0.2 % of surgically treated pituitary tumours (14,15). The true rate of aggressive tumours is unknown. Dekkers et al recently reviewed the epidemiology of aggressive pituitary adenomas/carcinomas and suggested that of all detected pituitary tumours it is likely that less than 0.1% will turn malignant and less than 0.5% will exhibit true aggressive behaviour, although emphasizing the difficulties in estimating the true rates (18). Functioning tumours are overrepresented in carcinomas and aggressive adenomas. In contrast to benign pituitary adenomas, in which corticotroph adenomas are a minority, corticotroph tumours are the most common subtype in aggressive pituitary adenomas and carcinomas, representing about 30-50 % (16,21).

Markers of aggressiveness

As previously mentioned, the vast part of pituitary tumours remains unrecognized during life and most of the detected tumours will respond to standard therapies. To date, no tumour markers exist that can reliably predict an aggressive behaviour at an early stage and the presentation might be identical to an “ordinary” adenoma. In fact, pituitary carcinomas have a median lag time from diagnosis to appearance of metastases of around 5-7 years (22,23). The latest WHO classification does not provide clear cut-off values but recommends that elevated proliferative activity (as measured by mitotic count and Ki-67) and some tumour subtypes are indicative of a higher probability of recurrence, **Table 2** (5,24). Attempts to predict risk of recurrence have been conducted in French studies proposing a grading scale that include both pathological (proliferative) and radiological features (9,25). A finding of invasiveness on MRI together with markers of increased proliferation was shown to predict recurrence (9,25). In addition, young age at diagnosis, especially in the setting of an inherited tumour syndrome has been proposed to indicate a higher likelihood of

aggressiveness (26). Recently, pathogenic variants of *TP53* and *ATRX* have been linked to larger and more aggressive tumours, foremost of the corticotroph subtype (27,28).

Table 2. Likelihood of recurrence of pituitary neuroendocrine tumours. Adopted from Lopes M.B.S, Acta Neuropathol 134, 521-535, 2017, ref (24). With permission from the publisher.

Low probability of recurrence	High probability of recurrence	Malignant (metastatic disease)
Pituitary adenoma	Adenomas with elevated proliferative activity	Pituitary carcinoma
	<u>Special subtypes (variants) of adenomas:</u>	
	Sparsely granulated somatotroph adenoma	
	Lactotroph adenoma in men	
	Silent corticotroph adenoma	
	Crooke cell adenoma	
	Plurihormonal PIT-1 positive adenoma	

It is noteworthy that aggressive adenomas and carcinomas share many clinical and pathological features and have comparable survival rates after treatment with temozolomide (16). This has raised the question whether these tumours represent “two sides of the same coin”, both harbouring a “malignant potential” (20). The term pituitary neuroendocrine tumour (PitNET) rather than adenoma has been proposed to better reflect the heterogeneity and clinical spectrum of tumours of the anterior pituitary (29).

Treatment and outcome

Patients with aggressive pituitary adenomas/carcinomas should be offered optimal standard therapies based on decisions in multidisciplinary teams (17). Given the rarity of the condition and that therapies often are considered as “rescue treatment” when standard treatments with surgery, radiotherapy or medical agents fail, no randomised trials of chemotherapies have been conducted. Most attempts with conventional cytotoxic drugs including cisplatin, carboplatin, etoposide, CCNU and 5FU, showed discouraging results (22,30). Historically, outcome in pituitary carcinomas was poor, two thirds being deceased within 1 year after occurrence of metastasis (23). The first case reports with successful treatment of aggressive pituitary adenomas and carcinomas with temozolomide (TMZ) were published in 2006 (31-33). In study I and III, we investigated initial response rates and long-term outcome after TMZ treatment.

Temozolomide

Temozolomide (TMZ) is an orally administered alkylating agent that has a very high bioavailability and crosses the blood-brain barrier. It is most widely used in the treatment of glioblastoma multiforme. The side effects include fatigue, nausea and myelosuppression.

TMZ acts by inserting a methyl group to DNA bases (preferably guanine) and subsequently causes DNA strand breaks and cell death (34). The modified bases formed are O⁶-MeG (5-10%), N⁷-MeG (60-80%) and N³-MeA (10-20%) (35). Cytotoxicity is primarily mediated through O⁶-MeG (36). The DNA repair protein O⁶-methyl-guanine-DNA methyltransferase (MGMT) can remove the methyl group in O⁶-MeG (direct repair) and hence counteract the effect of TMZ. One MGMT molecule is required for each repair and the enzyme is inactivated and degraded during the process, (37). Intact mismatch repair (MMR) proteins are required for the effect of TMZ in the O⁶-guanine pathway. If MGMT is absent and normal guanine not restored, the mismatched base pair is recognized by the MMR system, resulting in futile cell cycles and eventually apoptosis, **Figure 2**. The two other methylated DNA bases are repaired via the base excision repair (BER) system and methylpyrine-N-glycosylase (MPG) (34). Theoretically, the best effect of TMZ would occur if both MGMT and MPG are depleted together with an intact MMR system. In patients with glioblastomas, promoter methylation of MGMT (epigenetic silencing) is associated with a better outcome after TMZ treatment (38). The use of MGMT as a predictive marker of TMZ response in pituitary tumours/carcinomas was explored in study I and III in this thesis.

Figure 2. Temozolomide mechanisms of action and drug resistance. (Reprinted from Liu L, Gerson SL. Targeted modulation of MGMT: clinical implications. Clin Cancer Res. 2006;12(2):328–31. With permission from AACR). TMZ, temozolomide; MGMT, O⁶-methyl-guanine-DNA methyltransferase.

Lynch syndrome

In the second work of this thesis, we propose that germline mutations in genes encoding the DNA mismatch repair (MMR) proteins, i.e. Lynch syndrome (LS), a hereditary tumour complex syndrome, contribute to the development of aggressive pituitary tumours. LS is an autosomal dominant cancer-predisposing syndrome caused by germline mutations in one of the MMR genes (39). Numerous mutations in four different key genes have been described as disease causing; are MutS homologue 2 (*MSH2*), mutS homologue 6 (*MSH6*), MutL homologue 1 (*MLH1*) and postmeiotic segregation increased 2 (*PMS2*). In addition, mutations within the epithelial adhesion molecule (*EPCAM*) gene adjacent to the *MSH2* gene may cause silencing of *MSH2* (40). With few exceptions, an additional somatic mutation or loss of function in the second (wild-type) allele is necessary for the development of tumours (41). Protein products of *MSH2/MSH6* and *MHL1/PMS2* respectively form heterodimeric complexes engaged in the identification and repair of DNA damages (42). Defective MMR proteins result in an inability to recognize and repair DNA mismatches effectively, leading to a phenotype called microsatellite instability (MSI). This is characterised by insertion of small repetitive DNA sequences resulting in mutations in cancer-related genes (42).

The penetrance of LS is high, 52-82% of mutation carriers develop colon cancer before the age of 70 years (43). The second most common manifestation is endometrial cancer (25-60%), followed by gastric cancer (6-13%) and ovarian cancer (4-12%) (44,45). Malignant brain tumours (glioblastoma, astrocytoma), small bowel, pancreatic, urothelial, and hepatobiliary cancers are also thought to be associated with Lynch syndrome (43). In the recent years, a neuroendocrine tumour, adrenocortical carcinoma (ACC), was shown to be a manifestation of LS (46-48). Case reports of pheochromocytoma (49), pancreatic neuroendocrine neoplasm (50) and neuroendocrine neoplasm in the liver (51) have also been reported. However, pituitary tumours had never been addressed as a part of LS prior to study II in this thesis.

Cushing's disease

Cushing's disease (CD) refers to a pituitary neuroendocrine tumour that secretes excess amounts of adrenocorticotropin (ACTH) that in turn lead to uncontrolled production of cortisol from the adrenals. CD is the leading cause of endogenous hypercortisolism (Cushing's *syndrome*, CS), accounting for around 50-80% depending on population studied and period of data collection (52-54). Endogenous CS might also arise due to ectopic ACTH- or CRH-secreting tumours or in ACTH-independent causes such as adrenal adenomas/carcinomas or in rare hereditary syndromes such as Carney complex (54). The most common cause of CS is however the use of exogenous glucocorticoids in therapeutic situations. This thesis is about pituitary diseases and other causes of hypercortisolism than CD are not covered. However, many clinical features are shared regardless of aetiology and much research refer to cohorts with CS of both pituitary and adrenal origin.

Epidemiology and pathogenesis

CD represents about 2-5.7% of pituitary adenomas in population-based cohorts (4) with a 75 vs 25 % female predominance and peak incidences around the age of 40 years (3,4,55,56). In a large surgical series, corticotroph tumours (including both silent and active tumours) represented 15% of pituitary adenomas (14). Reported population-based prevalence is between 1.2 and 6.2 per 100 000 (2-4,57) and the annual incidence is estimated to 1.2-2.4 per million and year (Spain, Denmark, Finland and Sweden) (55,56,58,59). The prevalence may be underestimated due to mild and undetected cases (54). The most recent study, (Swedish, nationwide) found a slightly higher annual incidence between 2005 and 2013 compared to 1987-1995 (2.0 vs 1.5, $p < 0.05$), possibly reflecting improved awareness and diagnostic procedures during the last decades (55).

Most cases of CD occur sporadically and only a few cases of CD have been described in a hereditary setting with germline mutations. These rare syndromes include familial isolated pituitary adenoma (FIPA), multiple endocrine neoplasia (MEN) type 1 and 4, Carney complex and DICER 1-syndrome (pituitary blastoma in children) (60).

A major advance in the understanding of Cushing's disease was achieved during the last decade, when somatic mutations in the gene encoding for ubiquitin-specific protease 8 (*USP8*) was shown in around half of sporadic CD tumours (61,62). Mutations in *USP8* lead to increased expression of epidermal growth factor (EGF) receptors, which mediates ACTH secretion (63). Recently, mutations in another gene encoding for deubiquitinase, *USP48*, were frequently found in wild-type *USP8* tumours (27).

Clinical features and comorbidities

Approximately 80-90% of adenomas causing CD are < 10 mm or invisible at MRI and symptoms due to local mass effects are uncommon (14,62). On the other hand, hypercortisolism might cause severe symptoms and significant comorbidities (64,65). The symptoms may be vague in early stages of the disease and overlap with common disorders such as obesity, diabetes, polycystic ovarian syndrome and depression. Hence, many patients have a delay of several years from first symptoms to confirmation of diagnosis (66,67). Nevertheless, hypercortisolism is associated with life-threatening complications including infections, hypokalaemia, thromboembolism, hypertensive crisis and arrhythmia (68).

The typical signs of centripetal redistribution of adipose tissue, skin bruising, purple striae, weight gain, a flushed ruddy face and hirsutism are often recognized as features of CD (64,69). Less obvious symptoms such as back pain, fatigue, muscle weakness, decreased libido, menstrual irregularities, nephrolithiasis, depressed mood, insomnia, decreased cognitive ability and increased number of infections may be harder to relate to undiagnosed CD in the clinical situation (70). **Table 3** depicts the most common signs and symptoms at presentation of CD in the European Registry on Cushing's syndrome (ERCUSYN).

Table 3. Signs and symptoms at presentation of Cushing's disease reported in the ERCUSYN study. Data are expressed as number of patients with a sign or symptom/total number of patients with records available for that sign or symptom. (Extracted from Valassi et al, Eur J Endocrinol. 2011 Sep;165(3):383-92. doi: 10.1530/EJE-11-0272.)

Weight gain	240/294 (82%)
Hypertension	233/306 (76%)
Skin alterations	227/292 (78%)
Myopathy	181/272 (67%)
Hirsutism (female patients)	145/232 (63%)
Menstrual irregularities (female patients)	123/195 (63%)
Reduced libido	61/123 (50%)
Depression	93/243 (38%)
Diabetes mellitus	96/294 (33%)
Hair loss	76/224 (34%)
Fractures	55/263 (21%)

Several complications and comorbidities may arise as a consequence of chronic hypercortisolism and are linked to the increased mortality in CD. Hypertension, induced by mechanisms including activation of the mineralocorticoid receptor (71), is seen in 55-85% of CD patients in active phase (72). Increased gluconeogenesis and impaired beta-cell function result in diabetes in 20-47% and impaired glucose tolerance in 21-64% (72). Dyslipidaemia in 38-71% reinforces the picture resembling the metabolic syndrome. Structural and functional changes in the cardiovascular system, such as premature atherosclerosis and left ventricular hypertrophy have been described in 27-62% (73) of patients with Cushing's *syndrome*. Hypercortisolism induces changes in the haemostatic and fibrinolytic systems and a more than 10-fold increase risk of thromboembolic events are seen compared to the background population, (74). Hypercortisolism causes immunosuppression (75) and increases the risk of infections (68,76). Cortisol excess alters bone metabolism and osteoporosis is seen in 38-50% (72). Vertebral and rib fractures were observed in 25 and 28% respectively in 57 CD patients at diagnosis (64). Psychiatric and cognitive alterations are discussed below.

Neuropsychiatric comorbidities

Excess cortisol levels have major negative impact on the brain, with corresponding neuropsychiatric symptoms (77). In 1932, in his original description of 12 patients with Cushing's syndrome, dr Harvey Cushing highlighted emotional disturbances as a feature of the disease (78). Subsequently, studies have confirmed mood disturbances as a common complication in hypercortisolemic patients and major depression is estimated to be present in 55-80% of patients with CD in active phase (72). Depression may be periodical and can include suicide thoughts or attempts emphasizing the need for awareness and monitoring (79). Other neuropsychiatric symptoms linked to hypercortisolism include anxiety, sleep disturbances, fatigue, cognitive impairment, and less commonly manic behaviour (79-82). Patients with glucocorticoid excess

performed poorer compared to control subjects on visual and spatial information, reasoning and concept formation as well as in verbal and language performance (83). Impairment of quality of life (QoL) is significant in patients with CD, scoring worse than both controls (84) and patients with other pituitary tumours (85). Psychiatric symptoms may relieve or totally regress after successful treatment of hypercortisolism (86-89). In a study by Dorn et al (89), including 33 patients with Cushing's syndrome, significant psychopathology was present in two thirds at diagnosis, and decreased to 24% 12 months after remission. However, compared to controls, cross-sectional studies have shown increased psychopathology and cognitive impairment after long-term remission for 11 (range 1-32), and 13 (5-19) years respectively (90,91). Longitudinal studies with longer follow-up are lacking which was one of the reasons for conducting study IV in this thesis. We investigated psychotropic drug use in patients with CD, before diagnosis and at long-term follow-up. The impact of biochemical remission status was evaluated.

Diagnosis of CD

After excluding exogenous glucocorticoid use (systemic and topical), three main ways to establish a diagnosis of Cushing's syndrome are employed in clinical practice. 1) 24-hour urinary free cortisol; 2) dexamethasone suppression test and 3) late night salivary cortisol (69). All three tests have acceptable accuracy (92) but are accompanied by methodological caveats including high fluid intake and renal impairment (in test 1), elevated cortisol due to oestrogen and/or slow metabolism of dexamethasone (test 2) and fluctuations of cortisol in normal subjects as well as altered diurnal rhythm in shift-workers (test 3) (70). Some conditions other than CD, termed "pseudo-cushing" states, may result in abnormal screening tests. These include alcoholism, anorexia nervosa, extreme obesity, uncontrolled diabetes and psychiatric disorders (70,93). Finally, a small proportion of patients with CD have a cyclic secretion of cortisol and repeated testing may then be needed.

Once a diagnosis of CS is established, the next task is to determine the cause. Suppression of ACTH is indicative of ACTH-independent causes (most common adrenal adenomas or carcinomas). An elevated or normal ACTH indicates an ACTH-dependent cause (CD most common, followed by ectopic ACTH production) Additional testing include pituitary MRI, however a tumour is identified in only around 50-80% of CD patients depending on MRI technique and incidental small pituitary tumours are present in up to 10 % of healthy individuals (54). For that reason inferior petrosal sinus sampling (IPSS) is used in cases with small or absent tumours to confirm pituitary origin (69). IPSS has high sensitivity and specificity (around 95%) but has the disadvantage of being invasive and requires highly specialised centres.

Treatment and outcome

The goals of treatment in patients with CD are to normalise cortisol excess, maintain normal pituitary function and achieve tumour control. The mainstay of treatment is removal of the tumour by transsphenoidal surgical (TSS) resection (12), which results in initial biochemical remission in about 80 percent of patients (94). A dedicated and

experienced surgeon, a visible microadenoma at preoperative MRI, and a low postoperative morning cortisol are factors that predict higher rates of remission (94). After initial remission, around 18% recur with a wide heterogeneity and variability between studies (reported recurrence rates between 1 and 38%). Macroadenomas are more likely to recur than microadenomas, 30 vs 17% (94).

For patients with persistent and/or recurrent disease repeat surgery and/or radiotherapy are treatment options (12). Radiotherapy leads to tumour control in >80% while biochemical remission rates vary widely between 28-84% (95). The major drawbacks of radiotherapy are the delayed effect and the increased risk of subsequent hypopituitarism. Rapid control of hypercortisolism can effectively be achieved by bilateral adrenalectomy, however associated with permanent adrenal insufficiency and risk of subsequent development of corticotroph tumour progression (96).

A number of medical agents are available to control severe hypercortisolism in the acute phase prior to surgery or to bridge the period until control is achieved by radiotherapy. The drugs target either the steroid genesis in the adrenals (including ketoconazole, metyrapone, osilidrostat and levoketoconazole), the excess ACTH-production in the pituitary (including pasireotide) or the glucocorticoid receptor (mifepristone) (97,98). All pharmacological treatment options have considerable side effects that may limit the chronic use of these agents.

Comorbidities in CD may not fully resolve after remission, although improvement generally can be expected (72,99). Accordingly, incidence rates compared to the background population (standardised incidence rates, SIRs) were found increased for stroke, thromboembolism and sepsis in a recent study of patients with CD in long-term remission (76). Emphasis is put on the management of acute and chronic complications, such as infections, thromboembolic events, diabetes mellitus, hyperlipidaemia, psychiatric symptoms, osteoporosis and hypertension (12). Finally, proper hormone replacement therapy of hypopituitarism after treatment of hypercortisolism is mandatory (100).

CD has the highest mortality rates of pituitary adenomas with standardised mortality rates (SMRs) ranging from 1.9 to 9.3 in the most recent publications (101-107), **Table 4**. Compared to non-functioning tumours treated with TSS, patients with CD had an age-adjusted hazard ratio of 2.35 (95% CI 1.13-4.09) (108). SMR in CD have remained high during the last decades (106) while the mortality in acromegaly decreased (109,110).

Table 4. Standardised mortality rates (SMR) in Cushing's disease, studies published after 2010. CI, confidence interval.

First author	Publication year	Period covered	No of CD patients	SMR (95% CI)
Clayton	2011	1958-2010	60	4.80 (2.79-8.27)
Bolland	2011	1960-2005	188	3.25 (2.18-4.85)
Hassan-Smith	2012	1988-2009	80	3.17 (1.70-5.43)
Yaneva	2013	1965-2010	240	1.88 (0.69-4.08)
Ntali	2013	1967-2009	182	9.3 (6.2-13.4)
Ragnarsson	2019	1987-2014	502	2.5 (2.1-2.9)
Roldán-Sarmiento	2021	1979-2018	172	3.1 (1.9-4.8)

The majority of deaths in CD are related to cardiovascular diseases (59,101-106,108). Fatal infections also represents a common cause of death (111,112) and recently, suicide was shown to contribute to increased mortality in patients with CD (106).

Failure to achieve biochemical remission is closely associated with increased mortality (101,106). However, data are inconsistent in regards of mortality risk for patients that achieved remission. Some studies found that SMRs for patients in remission were not significantly elevated (59,103,104,108,113), raising a hope that a “cure” of CD normalised survival, while other studies reported an elevated SMR despite remission (101,102,105). The most recent meta-analysis concluded that “cured” patients still had an elevated SMR (pooled SMR from eight studies 2.5 [95% CI 1.4-4.2]) (114). Following that meta-analysis, one study on 502 patients with CD (in remission n=419) demonstrated a significantly higher SMR (1.9, 95% CI 1.5-2.3) for patients in remission (106) while another smaller study did not (107). In a publication from 2016, Clayton et al presented survival data in patients with CD that had already been in remission for 10 years. This cohort had an overall SMR of 1.61 (95% CI 1.23-2.12) (115). Interestingly, patients in remission after one TSS only, had a SMR comparable to the general population. However, survival bias must be considered when interpreting these results, since deaths prior to ten years after diagnosis were not evaluated. Thus, mortality after remission of CD has been debated and formed a rational for study V in this thesis.

AIMS OF THE THESIS

The overall aim of this thesis was to improve the knowledge and management of aggressive pituitary tumours/carcinomas and Cushing's disease.

Specific aims in each study

- Study I** To study the outcome after treatment with temozolomide in patients with aggressive pituitary adenomas and carcinomas, and to study the predictive value of MGMT.
- Study II** To study if germline mutations in DNA mismatch repair genes (Lynch syndrome) contribute to the development of pituitary tumours and if pituitary tumours are a manifestation of Lynch syndrome.
- Study III** To study if tumour content of MGMT influences survival after temozolomide treatment in patients with aggressive pituitary adenomas and carcinomas and to study if a second treatment period with temozolomide is effective.
- Study IV** To study psychotropic drug use in patients with Cushing's disease compared to a matched control group, before diagnosis and at long-term follow-up, and to investigate in what way the drug use is influenced by biochemical remission status.
- Study V** To study the rates of mortality in an unselected cohort of Swedish patients with Cushing's disease compared to a matched control group and to investigate if mortality is influenced by biochemical remission status.

METHODS

Study populations and collection of data

Study I and III

In study I, collection of baseline and treatment data was performed retrospectively by using detailed clinical research forms completed by each treating physician. Patients were identified via a collaboration through the Northern European NeuroEndocrine Group (NENEG); seven university hospitals participated (Lund/Malmö, Gothenburg, Stockholm, Brussels, Rotterdam, Copenhagen and Odense). All patients with an aggressive pituitary tumour or carcinoma that had earlier received or had ongoing treatment with TMZ were included in the study. In total 24 patients were included; eight with pituitary carcinomas, 16 with aggressive pituitary tumours.

In study III, we constructed a new clinical research form to collect data on long-term outcome and survival in the same patient cohort.

Study II

The study population in study II consisted of all known carriers of germline MMR mutations in Sweden, identified from existing registers in six Swedish university hospitals (Lund/Malmö, Gothenburg, Linköping, Stockholm, Uppsala and Umeå). Pituitary tumours in the cohort was sought for via linkage to the Swedish National Patient Register (NPR), followed by review of the medical history records.

Study IV-V

In study IV-IV, we used the Swedish Pituitary Register (SPR) to recruit patients with confirmed CD. Patients diagnosed from 1991 to 2018 were included, in total 374 subjects. Age and gender distribution are shown in **Figure 3**. Four controls per CD patient, matched by age, sex and residential area at diagnosis, were recruited from the Swedish total population register. We excluded two patients with CD due to invalid personal identification numbers that made matching to controls impossible. We analysed the remaining 372 patients with CD in study IV. In the workup of Study V, one patient was found to have no clinical data at all in the SPR and was removed from the analysis. We analysed all the remaining 371 patients in study V.

In study IV, the major part of the analyses were dependent on data from the prescribed drug register, initiated in July 2005 and the population studied was adapted accordingly. Hence, 179 patients with CD, diagnosed from 2006 to 2018, were followed longitudinally from diagnosis. In addition, a cross-sectional subgroup analysis of patients in sustained long-term remission with a valid follow-up after 2006 was carried out. This selected group of patients (n=76) were diagnosed from 1995 to 2010.

National registers used in the thesis

Swedish pituitary register (Study IV-V)

The Swedish pituitary register (SPR), initiated in 1991, is based on the national INCA (Information Network for Cancer treatment) IT-platform, and located at the Regional Cancer Centre (RCC), Stockholm-Gotland, Sweden. Data is added to the register at diagnosis, at regular follow-ups and when surgery or radiotherapy are performed. The register contains date of diagnosis, type of pituitary tumour, pituitary hormone levels, MRI findings of the tumour, ophthalmological findings, and information on performed treatments (surgery, radiotherapy, medical). At follow-ups, evaluation of biochemical remission is performed and noted in the SPR. The coverage of patients is varying dependent on type of tumour. In this thesis only patients with CD are evaluated and the coverage of CD patients is around 95% based on a comparison of annual incidences, 1.54 per million inhabitants in the SPR 1992-2017 compared to a recent nationwide Swedish investigation that used national patient registers and showed an annual incidence of 1.6 during 1987-2013 (55). Also, in our neighbour countries annual incidences per million inhabitants have been reported to be 1.2-1.7 in Denmark (59) and 1.7 in Finland (56). In regards of internal validity in the SPR specific efforts have been made during 2017-2018 to improve the register data for Cushing's disease, in particular the evaluation of biochemical remission status.

Figure 3. Age and gender distribution of Cushing's disease in the Swedish Pituitary Register. N=374 (females 283, 76%). Median age 44 years.

Total population register (Study IV-V)

The total population register (TPR) is held by Statistics' Sweden and was started in 1968. It contains data on date of birth and death, residential area and migration data.

Near 100% of births and deaths and 91-95% of migrations are reported within 30 days to the TPR (116). We used the TPR to recruit a control population and to identify migrations in study IV-V.

National patient register (Study II and IV)

The Swedish national patient register (NPR) is held by the National Board of Health and Welfare. It was initiated in 1964 and became nationwide 1987. It includes dates of hospital admission/discharge and diagnostic codes according to the International Statistical Classification of Diseases (ICD). The register has near full coverage (99%) since 1987 (117). Accuracy of diagnostic codes in the register varies but is generally good for diagnoses with high incidences (118). Since 2001 hospital out-patient visits (but not primary care visits) are also included in the register. Coverage in the out-patient register was low in the first years but then improved to > 95% (117). The accuracy of diagnostic codes related to pituitary tumours have not been validated systematically in the NPR. However, a recent nationwide study found that only 534 of 1317 subjects with a diagnosis of Cushing's disease in the NPR had a confirmed CD diagnosis (55). Therefore, review of medical records is essential when investigating pituitary diseases. We used the NPR in study II to identify patients with ICD codes consistent with pituitary tumours, followed by medical chart reviews. In study IV, we looked for inward care and outpatient visits to the specialized psychiatry. The NPR was not used to identify patients with Cushing's disease (they were identified in the SPR, see above).

Swedish prescribed drug register (Study IV)

The Swedish prescribed drug register (PDR) is held by the National Board of Health and Welfare and contains complete national data on drug prescriptions and dispenses (119). It includes information on type of drug according to the Anatomical Therapeutic Chemical (ATC) classification system, date of prescription and dispense, dosage and category of care-provider prescribing the drug. Over-the-counter drugs and drugs used in in-patient care are not included but in contrast to the NPR, it includes the important data from the primary care. The PDR was initiated in July 2005 and has since then near 100% coverage (120).

Cause-of-death register (Study V)

The cause-of-death register reports the underlying cause of death and alternative multiple causes of death, coded by ICD. The accuracy is acceptable for most diagnoses and is especially good for malignant neoplasms (90%) and ischemic heart disease (87%) (121). The number of deaths are virtually complete and less than 1% are missing an underlying cause of death (122).

Definition of response to temozolomide (Study I and III)

In contrast to common malignant diseases, there is no general agreement on the definition of response to TMZ in pituitary tumours. In study I and III we used the following criteria; for all patients a complete response was defined as complete regression of tumour mass and normalization of hormonal excess in functioning tumours. Partial response in aggressive tumours was defined as $\geq 30\%$ tumour volume regression on MRI and/or $\geq 50\%$ decrease in hormonal excess. In carcinomas, a partial response was defined as decrease of tumour burden $\geq 30\%$. These criteria are uniform with those used in a large compilation of 166 cases, published in 2018 (16). However, in that report they added a category of patients with “stable disease”, defined as tumour regression $< 30\%$ and $> 10\%$.

CD diagnosis and evaluation of remission status in the SPR (Study IV-V)

The diagnoses were made according to local routines at each hospital and included typical symptoms of CD, elevated levels of 24-hour urinary free cortisol levels (UFC), pathological overnight dexamethasone tests, abnormal late-night salivary or blood cortisol, petrosal sinus sampling and histopathological confirmation of the diagnosis. At regular clinical follow-ups, the remission status was evaluated by the treating physician and noted in the SPR. Biochemical remission was defined as normal midnight salivary cortisol levels, and/or s-cortisol < 50 nmol/L at an overnight dexamethasone suppression test, and/or 24-hour UFC below the upper reference limit, or hypocortisolism after pituitary surgery or radiotherapy, or bilateral adrenalectomy.

Immunohistochemistry and DNA extraction (Study I-III)

Immunohistochemistry (IHC) is widely used in the detection of proteins in tissue. Major advances in diagnostics of pituitary tumours are owing to IHC. Main advantages include the possibility to identify the hormonal type of pituitary tumour cells, proliferation markers (Ki67, mitotic count, p53), identification of transcription factors unique to different cell lineages and other proteins such as somatostatin receptors (5). Possible drawbacks of IHC involve methodological difficulties as well as biological diversification in tumour cells and interindividual differences between pathologists interpreting the results. IHC typically uses a primary antibody that identifies the antigen of interest in the tissue, together with a secondary antibody (binding to the primary antibody) that is stained. The tissue will appear stained where the antigen of interest is present and the results are most often presented as percentage of positive cells in a view field or according to a pre-defined cut-off as positive or negative. The method includes several steps, each of which risks to compromise the analysis. Type of fixation, time in fixation, improper deparaffinisation, uneven application of the primary or secondary antibodies and imperfect blocking of endogenous enzymes allowing for unspecific binding with resulting background staining are examples of pitfalls in IHC (123). Proper antigen retrieval protocols, use of a positive control in each

staining and if not possible the use of an internal control, e.g. vascular epithelial cells is of great importance.

In study I and III immunohistochemistry was used to examine the expression of Ki67, p53, MGMT and MMRs. The system used was Ventana BenchMark Ultra. Staining for MGMT, MLH 1, MSH2 and MSH6 were performed with tyramide signal amplification. Positive stainings of endothelial cells were used as internal controls. As primary antibodies Ki67 (Ventana clone G291-1129), p53 (Ventana clone Do7, RTU), MGMT (Invitrogen clone MT23.2, 1:100), MLH1 (Novocastra clone ES05, 1:50), MSH2 (Ventana clone G219-1129, RTU) and MSH6 (Epitomics 1:50) were used.

In study II the EnVision (DAKO) detection kit was used. MMR protein expressions in pituitary tumour cells were classified as retained (presence of staining in nuclear cells), lost (loss of staining in nuclear cells with retained staining in non-neoplastic cells) or reduced (weaker than in the surrounding cells). The applied antibodies were MLH1 (clone G168-15, 1:50, BD Pharmingen), PMS2 (clone A16-4, 1:300, BD Pharmingen), MSH2 (clone FE-11, 1:100, Calbiochem), and MSH6 (clone EPR3945, 1:100; Epitomics, Burlingame).

In addition to IHC in study II, we also analysed microsatellite instability (MSI) as part of the determination of MMR status. MSI is characterized by insertion of small repetitive DNA sequences resulting in mutations in cancer-related genes (42) and can arise as a consequence of MMR deficiency. For analysis of MSI, we used a system primarily developed for the established tumour forms in Lynch syndrome that included five mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27). Instability for one marker was classified as MSI-low, ≥ 2 markers as MSI-high and stability for all markers as microsatellite stable. Due to its novel use in pituitary tumours it had not been validated for those tumours.

Statistical methods

Descriptive statistics

Descriptive data are presented as total numbers (percentage) and median (range or interquartile range) for categorical and continuous variables respectively.

Comparing continuous variables

When comparing continuous variables between two groups you have to consider the distribution of the variables. If not normally distributed, you are referred to use non-parametric tests, i.e. tests that compare the rank of the values and not its absolute value. In this thesis, only non-parametric tests were used (the Mann-Whitney U-test) to compare continuous variables between groups. To compare a change of values within a group (change of Ki67 and MGMT over time in study I) the Wilcoxon signed rank test was employed.

Comparing proportions

The comparison of proportions between groups were made by 2-sided Fisher exact test. Unlike a one-sided test, it does not assume a change in a pre-specified direction and

the p-value generated is hence higher. The test compares the observed proportions in each group with the expected proportion (based on the whole cohort). The advantage of this test is that it is suitable for comparisons of proportions with few events. In study IV the change of proportions within a group (CD patients with psychotropic drugs at diagnosis and 5-year follow-up) was also analysed with the paired samples McNemar change test.

Logistic regression

Binary logistic regression is used when the dependent variable of interest is categorical and has two possible outcomes. It can be used to predict which of two categories (e.g. using a drug or not) a subject is likely to belong to, dependent on explaining variables that can be both categorical (e.g. Cushing's disease or matched control) or continuous (e.g. age). In its simplest form, only one explaining variable is used and the analysis is then called univariable. Unlike continuous variables, categorical cannot directly be put in a linear equation (linear line). Instead the probability of an event occurring is calculated from logit transformed values of the explaining variable. Via repeated calculations, called maximum likelihood estimation, an equation that best represents the observed observations is produced. To extend the model one can put in multiple variables and control for different explaining variables in the same model. The use of a multivariable design requires at least 10 events of the dependent variable per each explaining variable in the model and that the explaining variables are independent from each other. Logistic regression produces an odds ratio that illustrates the impact of the explaining variables. In this thesis, logistic regression was used in study IV to examine use of drugs in CD patients in comparison to controls and in study V to investigate predictors of biochemical remission status.

Survival analysis

Unlike the comparison of proportions, described above, survival analysis takes into account the time elapsed to an event. It compares differences between groups at multiple time-points and presents an estimate of the difference, often expressed as a hazard ratio. In Kaplan-Meier survival analysis only one variable (such as Cushing's disease *vs* control group) are compared. In Cox regression, multiple variables, such as sex and age can be fitted in the model. Cox regression assumes that observations are independent from each other and that the hazards are proportional over time. In study V, survival in Cushing's disease was evaluated in comparison to controls and predictors of outcome was looked for. The proportional hazard assumptions were assessed graphically.

P-values and confidence intervals

Statistical methods often compare a null hypothesis (e.g. there is no difference in MGMT levels between responders to TMZ and non-responders) with an alternative hypothesis (there is a difference). The p-value describes the probability of finding the observed results when the null hypothesis is actually true. A lower p-value indicates stronger evidence in favour of the alternative hypothesis. In all statistical analyses used

in this thesis, p-values < 0.05 were considered significant. The confidence interval (CI) is related to the p-value but offers a description of the statistical precision and compliments the p-value in the interpretation of the results and judgement of clinical usefulness. Confidence intervals for ORs and HRs were calculated as for a 95 % probability that the true value lies within the interval.

Ethical considerations

None of the five studies included any additional blood sampling or other efforts from the studied subjects. Data was collected from existing registers or medical records and, in the case of study I and II, IHC and DNA extraction were performed on existing tumour material from previous surgeries. However, reviewing medical records always mean a potential harm to the subjects' integrity. All data analyses were therefore handled with unidentified records. Only age and sex was available at the analysis phase of the studies and statistical results were presented on a group level. In study I-III patients were presented with age and sex in tables but we considered the risk of identifying any individual patient as small.

Study I and III were approved by The Regional Ethics committee of Lund, approval No. 2014/347 and No. 2015 /365. Study II was approved by The Regional Ethics committee of Lund, approval No. 2015/365. Study IV-V were approved by The Regional Ethics committee of Linköping, No. 2017/60-31 and No. 2018/130-32. The Swedish Pituitary Registry was approved by the Ethics Committee at Karolinska Institute (Stockholm, Sweden), 2003 (No. 2003/515/03) and 2012 (No. 2012/915-32).

RESULTS

Study I

Long-term outcome and MGMT as a predictive marker in 24 patients with atypical pituitary adenomas and pituitary carcinomas given treatment with temozolomide.

Descriptive data

In total 24 patients were included in the study, of those 8 were pituitary carcinomas (PC) and 16 locally aggressive adenomas (LAPT). Median age at diagnosis was 48 years (range 13-71), 16/24 were males. Nineteen of the tumours were hormone secreting (PRL 9, ACTH 4, GH 4, GH/PRL 2). Ki67 was 2-50% in LAPTs and 5-80% in PCs, $p=0.038$. In all patients, tumour growth was not controlled despite standard treatment with pituitary surgery ($n=24$), radiotherapy ($n=20$) and medical treatment ($n=19$) including dopamine agonists, somatostatin receptor analogues, steroid synthesis inhibitors and cytotoxic agents other than temozolomide. In PCs, median time from diagnosis to metastasis was 85 months (range 14-266). Out of the 16 LAPTs, 7 presented with an aggressive behaviour at diagnosis. The other nine had a lag-time to aggressive behaviour for median 110 months (5-300).

Response to temozolomide

All patients had been given treatment with temozolomide (TMZ) in doses of 150-200 mg/m² per day for 5 days every 28 days. Median time on TMZ treatment was 6 months (range 1-23). Treatment response data are based on 21/24 patients. Three patients were excluded of which two patients were given only 1 cycle of TMZ due to side effects and in a third, there was no available data on hormone levels at start of TMZ. Complete response to TMZ was defined as a complete regression of tumour mass and normalization of hormone levels. A partial response was defined as a decrease in pituitary tumour volume $\geq 30\%$ at MRI and/or a hormonal decrease $\geq 50\%$ in LAPTs and a decrease of tumour burden (including metastases) $\geq 30\%$ in PCs. At the end of first treatment period with TMZ (median 6, range 2-23 months after start of TMZ) 10/21 had a decreased tumour volume by $\geq 30\%$ and the effect of TMZ was seen after a median of 3 months (range 1-6). One additional patient (PRL-secreting LAPT) had a stable tumour but decreased hormone secretion by 71%.

Two (both carcinomas) displayed a complete initial response but relapsed after 60 and 138 months respectively. Five patients with partial response had a sustained effect of TMZ for median 40 months (range 16-69) after stop of TMZ during the observation time in study I. However, two relapsed during the extended observation time in study III. Two patients with initial response to TMZ progressed in their pituitary tumours shortly after the last cycle of TMZ and another two after 3 and 15 months respectively. Patient status at last observation after extended observation time is outlined in **Table 6** (in results, Study III).

Impact of MGMT and other predictors of response to TMZ

The IHC staining pattern of MGMT in the pituitary tumours was expressed as the percentage of cells with positive staining. Material was available in 20/21 patients with evaluable effect of TMZ treatment. Six patients had MGMT expression in $\leq 10\%$ of tumour cells, 11 had MGMT $>10\%$ and in three patients the staining was heterogeneous. Examples of MGMT staining in three patients are shown in **Figure 4**.

Figure 4. MGMT immunohistochemistry staining patterns in pituitary tumours. (A) Densely stained tumour with MGMT expression in most nuclei; (B) sparsely stained tumour; note positive endothelial cell nuclei; (C) heterogeneous staining. Left column: x 10 magnification; right column: x 40 magnification. (Reprint from Study I, with permission from the publisher.)

In patients with homogeneous MGMT staining, the absolute values of MGMT expression correlated to TMZ treatment response. MGMT in seven responders was median 9% (range 5-20%) compared to 93% (50-100%) in 10 non-responders, $p < 0.001$. **Figure 5** summarizes the response to TMZ in relation to MGMT staining. See results from study III for the impact of MGMT status on survival.

Study II

Corticotroph Pituitary Carcinoma in a Patient With Lynch Syndrome (LS) and Pituitary Tumors in a Nationwide LS Cohort.

All known carriers of germline mutations in mismatch repair proteins, consistent with Lynch syndrome (LS), were recruited from LS registers in Swedish university hospitals. In total 910 (426 men and 484 women) were included. The study identified five cases with a pituitary disease of which three had a clinically significant pituitary tumour. One (the index case identified in study I) had a corticotroph carcinoma, another had an invasive non-functioning macroadenoma and the last one had a microprolactinoma. In the last two cases, the pituitary tumour preceded the presentation of other tumours classically associated to Lynch syndrome, **Table 5**.

Table 5. Patients with pituitary tumors in the Swedish LS Cohort (n=910).

Age*/Sex	Germline mutation	MMR (IHC)	MSI	Pituitary tumour	Pituitary treatment	Clinical presentation and course	Other tumours
51/M	MSH2	loss of MSH2 and MSH6	low (1 of 5 markers)	corticotroph carcinoma, liver and skeletal metastases	surgery*3, radiation, bilat adrenalectomy, temozolomide, capecitabine	Severe Cushing's disease. Progression, died two yrs after diagnosis	colon ca 39 yrs old
39/F	MSH6	NA	NA	Micro-prolactinoma	dopamine agonist (DA)	Infertility, galactorrhea. PRL normalised on DA, no shrinkage of tumor	endometrial ca 49 yrs old
48/M	PMS2	NA	NA	invasive non-functioning macro-adenoma	transsphenoidal surgery	Vision defect. Normalised after surgery, no hormonal deficits, no relapses	colon ca 52 yrs old

*age at diagnosis of pituitary tumour; MMR DNA mismatch repair proteins; IHC immunohistochemistry of pituitary tumour; MSI microsatellite instability of pituitary tumour; NA not available.

Tumour tissue was only available in the corticotroph carcinoma. Immunohistochemistry showed loss of MSH2 and MSH 6 consistent with the known germline mutation in *MSH2*, **Figure 6**. Microsatellite instability (MSI) was shown in one of five markers, thus the tumour was classified as MSI-low.

Figure 6. Immunohistochemistry staining of mismatch repair proteins in the corticotroph carcinoma of the patient with a germline mutation in *MSH2*. Loss of MSH2 and MSH6. Preserved MLH1 and PMS2.

Study III

Tumoral MGMT content predicts survival in patients with aggressive pituitary tumors and pituitary carcinomas given treatment with temozolomide.

MGMT as a predictor of survival after temozolomide therapy and effects of a second treatment course.

This study was an extension of Study I and included the patients with a homogeneous staining pattern of MGMT, $n=17$ (11 aggressive adenomas and 6 carcinomas). The median (range) time from diagnosis to start of TMZ was 100 months (3-285) and median follow-up time since start of TMZ was 30 months (6-161). Eleven patients died during the observation time and the estimated median survival time after start of TMZ was 36 months (95% CI 13-59) in the whole cohort. When categorized by MGMT tumour content into low (0-10%) or high (>10%) the group of patients with low MGMT ($n=6$) survived longer, $p=0.032$, **Figure 7**. Few events make the statistical conclusion unreliable. The estimated median survival in the group with high MGMT ($n=11$) was 26 months (95% CI 14-38), in the group with low MGMT, only one patient died from pituitary progression during a median follow-up of 83 months (range 12-161). Age, sex, tumour type (aggressive adenoma or carcinoma), number of TMZ cycles and duration from diagnosis to start of TMZ did not differ between the two MGMT groups. Age, sex, tumour type, time from diagnosis to start of TMZ, initial response to TMZ and Ki67 (in categories of 0-10% and >10%) did not correlate to survival.

Figure 7. Kaplan-Meier estimates showing survival in patients with low (0-10%) vs high (>10%) MGMT tumour content. The low MGMT group survived longer, $p=0.032$, Log Rank (Mantel-Cox). In the low MGMT group, one patient died from unrelated cause and was censored at 29 months. All other deceased patients died from progressive disease.

In study III, only patients with homogeneous MGMT staining (n=17) were evaluated and presented in the original paper. **Table 6** outlines the outcome after TMZ treatment with extended observation time in all 21 patients (including the three patients with heterogeneous MGMT staining and the one with no data on MGMT). Thirteen of 21 (62%) patients progressed in their pituitary tumours and died median 24 months (range 6-56) after start of TMZ. Eight (38%) had prolonged disease stabilisation and were still alive after an observation time of median 77.5 months (range 29-161). Three patients remained stable with no other treatment than the first course of TMZ. One of these was a patient with an aggressive prolactinoma and low MGMT that died from an unrelated cause 29 months after start of TMZ. The other two remained stable for 120 and 68 months respectively, both were non-functioning aggressive tumours with a low Ki-67 index of 2%, one had low MGMT and one high. One patient with an aggressive prolactinoma and heterogeneous MGMT remained stable on cabergoline only, at 80 months after start of TMZ. In addition, one patient with a PRL/GH-secreting carcinoma and low MGMT received a second course of TMZ and remained stable 161 months after start of the first TMZ course. Three patients were alive at 62, 75 and 91 months respectively with slow progression of their tumours. All three had received additional treatment with radiotherapy and one of them a second course of TMZ.

In total, five patients with progressive/recurrent disease had received a second course of TMZ, median 19 months (range 2-117) after completing the first one. Two of five patients had high MGMT and the tumours progressed in both. Out of the three with low MGMT, tumour regression of > 30% was observed in one, borderline regression (27%) in one and there was no effect in the last one.

Table 6. Summary of characteristics and outcome after TMZ treatment in 21 patients with aggressive pituitary tumours/carcinomas (study I + study III).

*Age at start of TMZ. # Initial response defined as $\geq 30\%$ tumour regression on MRI and/or $\geq 50\%$ hormonal decrease. TMZ, temozolomide. APT, aggressive pituitary tumour; PC, pituitary carcinoma; GH, growth hormone; PRL, prolactin; NF, non-functioning; ACTH, adrenocorticotroph hormone; met, metastasis.

Sex/ Age*	Tumour	Ki6 7 IHC (%)	MGM T IHC (%)	TMZ cycles (=mon ths)	Initial respon se to TMZ#	Time to relaps from start 1st TMZ	Treatmen t after 1st TMZ	Final outcome
F/36	APT (GH)	7	9-100	6	yes	regrowth 14 mo	no	progression, † in pit tumour 29 mo after start TMZ
M/57	APT (PRL/G H)	23	10	3	yes	slow progressio n 60 mo after start TMZ	cabergoli ne, lanreotide radiothera py	slow progresion, alive 75 mo after start TMZ

Sex/ Age*	Tumour	Ki67 IHC (%)	MGMT IHC (%)	TMZ cycles (=months)	Initial response to TMZ [#]	Time to relapse from start 1st TMZ	Treatment after 1st TMZ	Final outcome
M/32	APT (PRL)	8	90	12	no (25% tumour regression)	progression 17 mo after start TMZ	3 cycles TMZ, 5 mo after stop 1st TMZ	no effect of 2nd TMZ, † in pit tumour 45 mo after start 1st TMZ
M/36	APT (PRL)	6	9-100	4	yes (stable tumour, PRL decrease by 71%)		cabergoline	stable, alive 80 mo after start TMZ
M/49	APT (PRL)	2	100	5	no	PRL↑ after 53 mo, frontal mets 60 mo after start TMZ	proton beam on multiple tumour rests, cabergoline, surgery on frontal met	alive 62 mo after start TMZ, slow progression
M/60	APT (PRL)	10	20	11	yes	rapid progress after 8 cycle TMZ, poor compliance	no	† in pit tumour 24 mo after start TMZ
M/82	APT (PRL)	2	9	21	yes		no	† due to unrelated cause 29 mo after start TMZ
M/29	APT (PRL)	41	100	4	no		68- Gallium dotatate	progressive growth, † in pit tumour 11 mo after start TMZ
M/37	APT (NF)	2	9	12	yes		no	alive 120 mo after start TMZ, stable
M/46	APT (NF)	2	100	18	no (28% tumour regression)		no	alive 68 mo after start TMZ, stable
F/73	APT (NF)	10	90	5	no		no	progressive growth, † in pit tumour 26 mo after start TMZ

Sex/ Age*	Tumour	Ki67 IHC (%)	MGMT IHC (%)	TMZ cycles (=months)	Initial response to TMZ [#]	Time to relapse from start 1st TMZ	Treatment after 1st TMZ	Final outcome
M/62	APT (NF)	10	90	6	no		177- Lutetium octreotate	progressive disease, † in pit tumour 10 mo after start TMZ
M/65	APT (NF)	3	95	3	no		9 cycles TMZ 19 mo after stop 1st TMZ, + velipside last 4 cycles	progressive disease, † in pit tumour 36 mo after start TMZ
M/52	PC (ACTH)	80	0-60	8	yes (50% tumor regression, progress 6 mo after start TMZ)		+ capecitabine last 2 TMZ cycles	progressive disease, † in pit tumour 8 mo after start TMZ
M/67	PC (ACTH)	10	95	12	no		+ capecitabine last 4 TMZ cycles	progressive disease, † in pit tumour 19 mo after start TMZ
M/70	PC (ACTH)	70	9	2	yes	quick relapse after stop TMZ	4 cycles TMZ 2 mo after stop 1st TMZ	no effect of 2nd TMZ, progressive mets 3 mo, † in pit tumour 12 mo after start 1st TMZ
M/49	PC (GH)	60	90	2	no		palliative radiotherapy, 90- yttrium dotatate	progressive disease, † in pit tumour 6 mo after start TMZ
F/52	PC (GH)	20	9	6	yes	stable for 60 mo, then new occipital met, 2nd relapse 74 mo, 3rd relapse 91 mo	radiosurgery on occipital nodule, 12 cycles TMZ 69 mo after stop 1st TMZ	progression of pit tumour, new occipital met, started 3rd course of TMZ, alive 91 mo after start 1st TMZ

Sex/ Age*	Tumour	Ki67 IHC (%)	MGMT IHC (%)	TMZ cycles (=months)	Initial response to TMZ [#]	Time to relapse from start 1st TMZ	Treatment after 1st TMZ	Final outcome
F/61	PC (PRL/GH)	5	9	23	yes	stable for 138 mo, then met os sphenoidal e	12 cycles TMZ 117 months after stop 1st TMZ, cabergoline and sandostatin continuously	stable, in good shape and normal hormones, alive 161 mo after start TMZ
F/52	PC (PRL/GH)	20	50	14	no		Radiotherapy	progressive disease, † in pit tumour 30 mo after start TMZ
F/82	PC (PRL)	10	not done	10	yes	Progression of mets 16 mo after start TMZ	9 cycles TMZ 10 months after stop 1st TMZ, radiotherapy, cabergoline	progressive disease, † in pit tumour 56 mo after start TMZ

Study IV

Psychotropic drugs in patients with Cushing's disease before diagnosis and at long-term follow-up - a nationwide study.

In total, we recruited 372 patients with CD from the Swedish pituitary register. A quadrupled control group (n=1488), matched for age, sex and residential area at diagnosis was recruited from Statistics Sweden's total population register. Since the analyses were dependent on the Swedish prescribed drug register, established in July 2005, only patients diagnosed from 1 year after that were eligible for a longitudinal follow-up (n=179, females 78%). Median age at diagnosis was 45 years (IQR 35-59) and median follow-up was 7.0 years (IQR 4.0-9.8). At 5- and 10-year, 129 (females 81%) and 56 (females 82%), patients respectively had a follow-up in the SPR.

Use of psychotropic drugs in patients with CD at diagnosis and at long-term follow-up

We analysed drug dispenses from 1 year before to 1 year after each time point. Two or more dispenses of a drug was regarded as evidence of taking the drug. The proportions of CD patients with dispenses of antidepressants were nearly unchanged at diagnosis (26%), 5-year (26%) and 10-year (23%) follow-up. For sleeping pills the rates were 22 % at both diagnosis and 5-year follow-up and then decreased to 16 % at 10-year follow-up. Anxiolytics were dispensed to 17 %, 9 % and 5 % at the 3 respective time-points. Use of drugs for diabetes and hypertension decreased significantly from diagnosis to 5-year follow-up, **Figure 8**. At 5-year follow-up, but not at diagnosis, female sex and older age positively correlated to use of antidepressants in CD patients.

Figure 8. Proportion of CD patients with CD drug dispenses at diagnosis, 5-year and 10-year follow-up. Difference of proportions were tested with Fisher exact 2-sided test at two levels: diagnosis vs 5-year and diagnosis vs 10-year respectively. Only significant changes are indicated: * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001. (Reprint from Study IV. With permission from the publisher)

When compared to controls, CD patients had a higher use of antidepressants (OR 2.3, 95% CI 1.6-3.5), anxiolytics (3.9, 2.3-6.6), and sleeping pills (3.8, 2.4-5.9) at diagnosis. At 5-year follow-up, the use of antidepressants (2.4, 1.5-3.9) and sleeping pills (3.1, 1.9-5.3) remained higher among patients, whereas at 10-year follow-up, the use of these drugs was not different from the control group, **Table 7**.

Table 7. Odds ratios (OR) with corresponding 95% confidence intervals (CI) in patients with CD compared to a quadrupled control group matched by age, gender and residential area. At diagnosis CD patients n=179, 5-year follow-up n=129, 10-year follow-up n=56. Psychotropic drugs refer to antidepressants, anxiolytics and sleeping pills in combination. Drug dispense defined as ≥ 2 dispenses ± 1 year in relation to date of follow-up.

	OR (95% CI)		
	At diagnosis	5-year follow-up	10-year follow-up
Antidepressants	2.3 (1.6-3.5)	2.4 (1.5-3.9)	1.7 (0.8-3.6)
Anxiolytics	3.9 (2.3-6.6)	1.6 (0.8-3.1)	1.0 (0.3-3.5)
Sleeping pills	3.8 (2.4-5.9)	3.1 (1.9-5.3)	1.7 (0.7-3.9)
≥ 1 psychotropic drug	3.2 (2.2-4.5)	2.3 (1.5-3.6)	1.7 (0.9-3.3)
≥ 2 psychotropic drug	3.3 (2.0-5.4)	2.8 (1.6-5.0)	1.5 (0.6-3.7)
Opioids	6.4 (4.1-9.8)	3.6 (2.1-6.0)	1.6 (0.7-3.9)
Ca-inhibitors	8.5 (5.5-13.3)	1.5 (0.8-2.9)	2.4 (0.9-6.4)
RAS-blockade	10.6 (7.2-15.5)	2.0 (1.3-3.1)	1.6 (0.7-3.3)
Both Ca-inhib and RAS-blockade	11.2 (6.6-19.3)	1.3 (0.5-3.2)	1.6 (0.5-5.2)
Antidiabetics including insulin	8.1 (5.0-13.4)	3.1 (1.6-6.1)	10.1 (3.0-34.0)

Influence of biochemical remission status

At 5- and 10-year follow-up biochemical remission was achieved by 110/129 (85 %) and 52/56 (93 %) respectively. We found no differences in use of psychotropic drugs according to remission status in the longitudinal study. With the aim to investigate the influence of long-term remission, we also performed a cross-sectional subgroup analysis of patients in sustained remission. Only patients in biochemical remission at all consecutive follow-ups in the SPR and only those who had not undergone pituitary surgery or radiotherapy in the 5 years before last follow-up were included. In addition, the patients had to have a completed follow-up in the SPR after July 2006 (to enable analysis of drug dispenses from 1 year before). In total, 76 patients with CD (females 82%, median age at diagnosis 38.5) were eligible for this analysis. Time in remission, calculated as the time elapsed from last surgery or radiotherapy to date of follow-up was median 9.3 years (IQR 8.1-10.4). In comparison to controls, CD patients in long-

term remission had a higher use of antidepressants (25% vs 14 %) and sleeping pills (22% vs 11%), **Table 8**.

Table 8. Odds ratios (OR) for drug dispenses in 76 CD patients in sustained long-term remission for median 9.3 years compared to a quadrupled control group (n=292) matched by gender, age and residential area. Psychotropic drugs refer to antidepressants, anxiolytics and sleeping pills in combination. Drug dispense defined as ≥ 2 dispenses ± 1 year in relation to date of follow-up.

	CD n (%)	Controls n (%)	OR (95% CI)	p-value
Antidepressants	19 (25)	41 (14)	2.0 (1.1-3.8)	0.023
Anxiolytics	9 (12)	18 (6)	2.1 (0.9-4.8)	0.097
Sleeping pills	17 (22)	31 (11)	2.4 (1.3-4.7)	0.008
≥ 1 psychotropic drug	27 (36)	60 (21)	2.1 (1.2-3.7)	0.007
≥ 2 psychotropic drugs	15 (20)	23 (8)	2.9 (1.4-5.8)	0.003
Opioids	9 (12)	29 (10)	1.2 (0.6-2.7)	0.626
Ca-inhibitors	7 (9)	24 (8)	1.1 (0.5-2.7)	0.782
RAS-blockade	15 (20)	40 (14)	1.6 (0.8-3.0)	0.191
Both Ca-inhibitors and RAS-blockade	7 (9)	14 (5)	2.0 (0.8-5.2)	0.146
Antidiabetics including insulin	11 (15)	11 (4)	4.3 (1.8-10.4)	0.001

Use of psychotropic drugs before diagnosis of CD

We analysed dispenses of drugs in the period 5 years *before* to date of diagnosis. We compared the proportion of patients and controls with two or more dispenses during that period. Twenty-nine of 103 (28%) CD patients were dispensed antidepressants at least two times compared to 62/412 (15%) in the control group (OR 2.2, 95% CI 1.3-3.7, $p=0.002$). As well, patients with CD had a higher use of ataractics (21 vs 8.5 %; OR 2.9, 1.6-5.3, $p<0.001$) and sleeping pills (20 vs 11 %; OR 2.1, 1.2-3.7, $p=0.011$). The difference between the two groups was apparent from several years before diagnosis. **Figure 9** shows the cumulative proportion of the subjects' first drug dispense from start of the observation time (5 years before diagnosis).

Figure 9. Cumulative proportion of subjects (CD n=103, controls n=412) with first drug dispense from start of observation (5 years before) to date of diagnosis. x-axis; years before diagnosis. y-axis; proportion of patients. P-values refer to Log Rank (Mantel-Cox). (Reprint from Study IV, with permission from the publisher)

Study V

Increased mortality persists after treatment of Cushing's disease: A nationwide matched study of 371 patients.

We investigated 371 patients with CD (females n= 281, 76%) diagnosed from 1991 to 2018. Median age at diagnosis was 44 (IQR 32-56) years and median follow-up after diagnosis was 10.6 (5.7-18.2) years. We compared the patients with a quadrupled control group (n=1484), matched by age, gender and residential area at diagnosis, in survival analyses.

At 1, 5, 10, 15 and 20-year follow-ups, remission rates were 80, 92, 96, 91 and 97 % respectively. Age, gender and tumour size at diagnosis did not predict remission status at any of the specified follow-ups. In a comparison of remission status at last available follow-up, older age (OR 1.03, 95% CI 1.01-1.06, $p=0.01$) but not gender was associated to not being in remission.

Mortality

Older age at diagnosis (HR 1.09, 95% CI 1.07-1.12) and not being in remission at last follow-up (HR 5.1, 2.6-10.1) were associated to increased mortality within the patients with CD. Compared to matched controls, overall mortality was increased in CD patients (HR 2.1, 95%CI 1.5-2.8). Men and women had comparable HRs. The estimated 5- and 10-year survival in patients with CD was 0.92 (95% CI 0.89-0.95) and 0.86 (0.81-0.89) respectively, compared to 0.98 (0.97-0.99) and 0.94 (0.93-0.96) in controls. Patients in remission at last follow-up had an increased HR, whereas HR for patients in remission after one pituitary surgery only was not significantly elevated, **Table 9** and **Figure 10**.

Table 9. Hazard ratios (HR) for CD patients compared to matched controls. Cox regression analyses with corresponding 95% confidence intervals (CI).

	CD, deaths/total no (%)	Controls, deaths/total no (%)	HR (95% CI)	p- value
Overall mortality	66/371 (18)	139/1484 (9)	2.1 (1.5-2.8)	<0.01
Males	20/90 (22)	40/360 (11)	2.2 (1.3-3.8)	<0.01
Females	46/281 (16)	99/1124 (9)	2.0 (1.4-2.9)	<0.01
Age at diagnosis ≤44 years	9/191 (5)	10/766 (1)	3.6 (1.5-8.9)	0.01
Age at diagnosis >44 years	57/180 (32)	129/718 (18)	2.2 (1.6-2.9)	<0.01
In remission at last follow-up	35/303 (12)	97/1212 (8)	1.5 (1.02-2.2)	0.04
Not in remission last follow-up	15/31 (48)	15/124 (12)	5.6 (2.7-11.6)	<0.01
Remission status unknown at last follow-up	7/16 (44)	11/64 (17)	3.3 (1.3-8.5)	0.01
No follow-up	9/21 (43)	16/84 (19)	4.1 (1.6-10.3)	<0.01
In remission after 1 pit surg only	21/188 (11)	55/752 (7)	1.6 (0.98-2.7)	0.06
Disease-specific causes of death				
Cardiovascular	32/371 (9)	48/1484 (3)	2.9 (1.9-4.5)	<0.01
Infections	12/371 (3)	12/1484 (1)	4.3 (1.9-9.5)	<0.01
Cancer	9/371 (2)	44/1484 (3)	0.9 (0.4-1.9)	0.79

Figure 10. Kaplan-Meier survival estimates in patients with CD vs a quadrupled matched control group. Upper left, all CD patients (n=371); upper right CD patients in remission at last follow-up (n=303); lower left, CD patients in remission after 1 pituitary surgery only (n=188); lower right, CD patients not in remission at last follow-up (n=31)

Causes of death

Cardiovascular diseases (32/66) and infections (12/66) were the most common causes of death in patients with CD, **Figure 11**. Ten out of 12 fatal infections occurred during the first six years after diagnosis. Compared to controls, death due to cardiovascular diseases (HR 2.9, 95% CI 1.9-4.5) and infections (4.3, 1.9-9.5) was more common whereas death due to cancer was not, **Table 9**.

Figure 11. Sixty-six causes of death in 371 patients with CD, plotted in relation to time of diagnosis.

Infection; pneumonia n=6, staphylococcus sepsis n=1, diverticulitis and sepsis n=1, unspecified sepsis/infection n=4.

Cardiovascular; ischemic heart disease n=18, cerebrovascular disease n=9, ruptured aortic aneurysm n=2, myocarditis n=1.

Other causes; neurodegenerative disease n=1, unspecified chock n=1, perforation of colon n=1.

Cause of death unknown; cause of death not reported at time of study end.

DISCUSSION

Study I and III

Is temozolomide effective in aggressive pituitary tumours and carcinomas?

Before the publication of study I, the experience of temozolomide treatment in aggressive pituitary adenomas/carcinomas was limited. The first case reports appeared in 2006 (31-33) and were followed by small case series of 6-13 patients (124-127).

In study, I we collected information from seven European centres on all patients that had been treated with TMZ. We showed that 10/21 (47%) patients responded initially to TMZ with $\geq 30\%$ tumour volume regression. In responding patients, the effect was seen within 3 months of treatment. Two carcinomas responded with complete tumour regression that persisted for 60 and 138 months respectively. Partial tumour regression was demonstrated in another two carcinomas and in five patients with aggressive pituitary tumours. In addition, one patient decreased prolactin by 71 % with a remaining stable tumour volume. Following our study, one Italian (128) and one French (129) study found slightly lower response rates in their cohorts of 31 (35.5% with initial tumour regression) and 43 (42% with initial tumour regression) cases respectively. Subsequently the European Society of Endocrinology (ESE) performed an electronic multinational survey, including 157 patients treated with TMZ and found complete response in 6%, partial response in 31% and in addition, 31% were considered as stable disease (16). Altogether, it seemed like around 40% of cases resistant to conventional treatment and other cytotoxic agents, responded initially to TMZ with tumour regression and another 30% would have an initial disease stabilisation. A recent German study, including 42 cases, similarly found an initial tumour regression in 33% and stable disease in 37% (130). The ESE guidelines, issued in 2018, recommend TMZ as the first-line cytotoxic drug in therapy of aggressive pituitary tumours that progress despite standard therapies with surgery, radiotherapy and medical agents (17).

Is MGMT associated with the response to temozolomide?

Lower MGMT was associated with a better initial response to TMZ in study I. Out of the 17 patients with homogeneous MGMT staining, the 7 responders had MGMT staining percentages between 5 and 20 (median 9) and the 10 non-responders between 50 and 100 (median 93). Thus, we concluded that a low MGMT predicts a high likelihood of response. Moreover, study III indicated a more favourable response to a second course of TMZ and longer survival in patients with MGMT $\leq 10\%$. In addition to study I, some other studies reported a relationship between MGMT expression and response (16,131-133), while others did not (125-129). In the large ESE cohort, a complete response was observed only in patients with low MGMT and partial response was more likely in patients with low MGMT (16). On the other hand, tumours with high

MGMT expression may occasionally respond to TMZ and in parallel, not all with low MGMT respond (16). Thus, the predictive value of MGMT has been debated. The ESE guidelines concluded that a low MGMT is associated with higher likelihood of response to TMZ; however, a high MGMT should not exclude a patient from a three months trial of TMZ (17).

The lack of standardized IHC methods might explain some of the discordances between studies. Fixation methods, time in fixation, antigen retrieval protocols, sensitivity of antibodies/detection systems and lack of internal positive controls may influence the results (134-136). Kontogeorgos et al showed the impact of MGMT methodology in a recent study (136). Three out of 25 cases that initially stained immunonegative for MGMT turned positive after pre-treatment with antigen retrieval protocols (136). This implies that some protocols may yield falsely negative MGMT staining and account for cases that do not respond to TMZ despite a low MGMT. In study I, we tried to minimize methodological pitfalls in immunohistochemistry. A highly experienced pathologist performed all the analyses to avoid arbitrary judgement (the drawback being that a second pathologist did not confirm the results). All specimens were pre-treated with heated buffer and we used a tyramide signal amplification (TSA) technique that allows for lower concentrations of primary antibody, which in turn reduces the risk of nonspecific binding. Positive internal controls (epithelial cells) were used and we analysed MGMT on pituitary tumour tissue from the surgery closest to TMZ treatment. In the statistical analysis, we excluded tumours with heterogeneous staining. In many of the larger cohorts, evaluation of MGMT was not centralised and standardised; the methods have not been reported in detail (128-130). Thus, development of a validated IHC method should have a high priority to aid future research and clinical use of MGMT in the treatment decisions of aggressive pituitary tumours.

It should be noted that even with the robust method used in study I, one patient (aggressive non-functioning tumour), with 100% MGMT staining, had a near partial response (28% tumour shrinkage) and remained stable 50 months after discontinuation of TMZ. The patient received radiotherapy 6 months prior to TMZ. A marked effect of TMZ was also noted in the other three patients treated with radiotherapy within 6 months prior to TMZ. This implies a synergistic effect of radiotherapy as described in glioblastomas (137) and was also observed in the large ESE survey of pituitary tumours (16). A recent Italian study (138) investigated the outcome of re-irradiation and concomitant TMZ in 21 patients with recurrent aggressive adenomas and carcinomas (14 with previous TMZ treatment). A complete response was seen in two and partial response in 11. With a median follow-up of 27 months, the estimated 2-year local control rate was 73%. Low MGMT protein expression was associated with local control and overall survival, further supporting the role of MGMT as a predictive factor.

Epigenetic silencing of MGMT by promoter methylation is associated to a better response to TMZ in patients with glioblastomas (38) but in contrast to glioblastomas, promoter methylation status does not have a close association to protein staining

determined by IHC, in pituitary tumours (125,132). Silencing through other mechanisms or genetic variants of MGMT may explain some of the discordances.(139,140). In pituitary tumours, the preferred method to measure MGMT should be by IHC (17,140).

Although MGMT seem to be a main determinant of TMZ effect, other mechanisms of resistance have been considered. Acquired resistance during treatment might occur due to drug- or radiotherapy-induced epigenetic changes or by selection of pre-existing resistant cell clones (139). Heterogeneous MGMT distribution in the tumour might result in an initial or partial response, i.e. cells with low MGMT will respond to TMZ, while the cells with high MGMT will eventually continue to grow. Loss of MSH6 was observed in one patient progressing from a prolactinoma to a carcinoma with concomitant escape from TMZ effect (141) and loss of MSH6 expression in four of 13 patients with aggressive pituitary tumours was associated with unfavourable response to TMZ (127). In our study, one patient had a germline mutation in the MMR genes. This patient rapidly developed resistance to TMZ, supporting that an intact MMR pathway is required for the effect of TMZ (34). In glioblastomas, expression of MPG (that mediates base excision repair) has been correlated with a worse outcome (142); in the case of pituitary tumours, this has not yet been investigated (35). Theoretically, this could explain why some tumours with low MGMT do not respond to TMZ.

Other predictive factors of outcome

In our study, the material was too small for conclusions of outcome in relation to hormonal status of the tumours. However, only one out of five patients with non-functioning tumours (the only one with low MGMT) showed an initial response to TMZ. A lesser response to TMZ in non-functioning tumours, independent of MGMT status, was later shown in a larger compilation of patients (16). In contrast to benign adenomas, in which corticotroph tumours are a minority (14), they are the most common subtype in aggressive adenomas and carcinomas, accounting for >40% (16). In our cohort, the three patients with corticotroph tumours all had a poor final outcome and were deceased 8-19 months after start of TMZ, despite an initial response to TMZ in two of the cases. No difference in response was seen according to subtype of functioning tumours in the large ESE survey (16).

Interestingly, we found no differences in initial response or survival between aggressive tumours and carcinomas, nor did the Ki67 index predict outcome. Several authors have reached the same conclusion (16,128-130,143). The tumours may present as “ordinary” pituitary tumours with years of benign behaviour ahead of transformation to a carcinoma or an aggressive tumour (Study I and (22,23,130). Markers to identify patients who might benefit from TMZ treatment at an early stage of the disease are lacking.

What is the long-term outcome after TMZ treatment and what to do if TMZ fails?

The introduction of TMZ has improved outcome in patients with aggressive pituitary tumours/carcinomas. Historical data were discouraging, only one third of patients with a pituitary carcinoma survived one year after appearance of metastases (23). Heterogeneity between studies and different methods of reporting survival hampers clear survival estimates. In a review of 54 case reports published in 2016, the estimated 5-year survival after start of TMZ was > 50% (143). However, it is likely that this review was influenced by publication bias of early cases with favourable outcome; responders to TMZ have a significantly longer survival than non-responders (129). Lasolle et al estimated an overall median survival of 40 months after *stop* of TMZ with a significantly longer survival in patients that responded to TMZ (129). Losa et al reported an estimated 4-year survival of 60% after *start* of TMZ (128). In the German survey by Elbelt et al, an estimated median progression-free survival of 29 months was reported in 30 patients receiving ≥ 6 cycles of TMZ (23 months for the total cohort of 46 patients) (130). In study III, we found an estimated median survival of 36 months after *start* of TMZ in our total cohort. Notably, the survival seemed longer for patients with low MGMT of whom only one out of six patient died during a follow-up of median 83 months.

There is no agreement on the duration of TMZ therapy in responding patients but a minimum of 6 months is recommended (17). In our study 2/24 patients stopped TMZ after the first cycle due to side effects (hearing loss and urinary tract infection plus extreme fatigue respectively), which is in line with the ESE survey in which 11% of patients stopped TMZ due to side-effects (most frequently bone marrow suppression). Side-effects are otherwise mostly mild (fatigue and nausea/vomiting most common) and in responders tolerating TMZ a prolonged duration of > 12 months might be favourable in terms of survival (129,139,143,144). Discontinuation of TMZ followed by a second attempt is generally accompanied by a lesser response, (16,128-130). Notably though, in study III we observed that a second treatment with TMZ might be worthwhile in patients with low MGMT. Out of five patients with repeated TMZ therapy, two patients (both with low MGMT) had a regression of their tumour volume by 30 and 27 % after a pause of TMZ for 69 and 117 months respectively.

Despite the improvement compared to the era before TMZ, overall prognosis is still poor. During the extended observation time in study III, 13/21 (62%) died due to progression of the pituitary tumours. Only four patients remained stable with no other treatment than the first course of TMZ (except for cabergoline in one) and additionally one was stable after a second course of TMZ. Three patients were alive with slow progression of their tumours after the addition of radiotherapy and/or a second course of TMZ. Progression after TMZ cessation was also demonstrated in 42 % after a median of 12 months in the ESE survey (16). In the German survey, the median estimated progression-free survival was 23 months (95% CI 18-28). Thus, there is a need of alternative treatment options in case of failure or progression after initial response to TMZ. Inactivation of MGMT by administration of O6-benzylguanine increases the effect of TMZ but concomitant increased hematopoietic toxicity reduces its clinical

value (139,145). Dose-dense regimens of TMZ, have a theoretical potential to deplete MGMT storages, but also increases the risk of side effects (146) and the experience in pituitary tumours is limited to few patients (139). Concomitant radiotherapy and TMZ was associated with a higher response rate compared to TMZ alone in the ESE survey (16) and re-irradiation with concomitant TMZ has shown relevant initial response in patients with recurring tumours (138), see above. The use of peptide receptor radionuclide therapy (PRRT) might be an option in selected cases. PRRT exhibits radiation internally by linking a radiopharmaceutical (lutetium mostly used in pituitary tumours) to a somatostatin receptor ligand (147). In our cohort, three of the patients that did not respond to TMZ received PRRT (lutetium, yttrium and gallium respectively) after stop of TMZ, in all three cases with a poor outcome. However, in a recent review of published case series, 6/12 patients were considered as responders to PRRT, warranting further evaluation of this treatment option (147). Immune checkpoint inhibitors has gained rapid interest due to its improvement of outcome in other cancers. Infiltrating lymphocytes and expression of PD-L1 have been demonstrated in pituitary tumours, forming a theoretical base of immunotherapy (148,149). Case reports (150-153) have shown varying results and a clinical trial is ongoing (153). Finally, anti-VEGF therapy, mTOR-inhibition and tyrosine kinase inhibitors have resulted in disease stabilisation in a few case reports and need further evaluation (154).

Conclusions of study I and III

In summary, Study I and III provided evidence of TMZ as an important treatment option in aggressive pituitary adenomas and carcinomas. Around 50% responded initially and MGMT, measured by a centralised method, predicted both the initial response and survival. With growing number of reported cases in the literature, the response rate to TMZ is now around 40%. A low protein expression of MGMT indicates a higher likelihood of response. However, despite high MGMT, some patients may respond to TMZ, warranting a three months trial of TMZ in these patients as well. A validated and standardised MGMT IHC method is needed to aid further research and the clinical use of MGMT. Regardless of the improvement in outcome after the initiation of TMZ, overall prognosis is still poor with frequent recurrences, underscoring the need of continued search and evaluation of other treatment options and predictive factors to tailor the treatment.

Study II

Do germline mutations in MMR genes contribute to the development and course of pituitary tumours?

The fact that one of our patients with a corticotroph carcinoma belonged to a family with Lynch syndrome (LS) prompted us to investigate the nature of his pituitary tumour. We found loss of function of *MSH2* and *MSH6* consistent with the known germline mutation in the family (*MSH2*). Thus, the germline mutation in the MMR genes most likely contributed to this patient's pituitary carcinoma. At time of submission, this was to our knowledge the first case that described a pituitary tumour as a manifestation of LS. However, in the same year (2017) another case study reported an invasive corticotroph adenoma in a patient with LS (155). The authors suggested that this patient's germline mutation in *MLH1* contributed to tumour development by inducing somatic mutations in *MSH6* and *MEN1* found in the pituitary tumour.

While somatic mutations in *USP8* have been identified in around half of corticotroph tumours, they often present as small functioning adenomas (5,61). Only rare cases of CD have been associated to inherited syndromes including *MEN1*, *MEN4*, Carney complex, *MEN2*, and *DICER1* syndrome (pituitary blastoma) (5,26). Our results imply that germline mutations in the MMR genes might be a relevant driver of the pathogenesis of corticotroph pituitary tumours.

In aggressive pituitary tumours, MMR deficiency have implications in the choice of treatment since a response to TMZ is unlikely (127,139). This was reflected by the rapid tumour progression after six months of TMZ treatment in our index case. We proposed therapy with immune checkpoint inhibitors (ICI) as an option since other types of MMR-deficient tumours had responded to treatment with pembrolizumab (156). Moreover, microsatellite instability (MSI), which is a consequence of MMR deficiency, has been linked to the efficacy of ICI (157). Increased expression of programmed death ligand 1 (PD-L1) and presence of tumour-infiltrating lymphocytes are thought to be more common in functioning and aggressive tumours, supporting the use of PD-L1 blockade in selected cases (148). However, a recently published case report described rapid progression of an MMR-deficient invasive corticotroph adenoma during pembrolizumab treatment (152) and more experience is needed. The role of combination therapy with ICI, temozolomide and/or radiation also needs further investigation.

Are pituitary tumours a manifestation of Lynch syndrome?

A nationwide search in the Swedish Patient Register yielded in total three cases of pituitary tumours in the cohort of 910 individuals with LS. The reported prevalence of pituitary tumours in the general population is around 1/1000 (3,4), which could indicate that pituitary tumours are possible manifestations in LS. With respect to the small numbers, this might however be a chance finding. Unfortunately, it was only possible to investigate the tumour tissue in one of the cases (the index case described above). One patient with a prolactinoma never underwent surgery and the tumour

specimen of the invasive non-functioning tumour was by mistake not sent for histopathology.

The tumour spectrum of Lynch syndrome is expanding and another neuroendocrine tumour, adrenocortical cancer (AAC), is now considered as part of the syndrome (46,47). Interestingly, in contrast to colon carcinomas, MSI was stable in most LS-associated AACs. Our case was defined as MSI-low (unstable in one of five markers). Raymond et al suggested that DNA MMR gene deficiency in AAC might be a secondary event that occurs in later stages of carcinogenesis, thus preventing the accumulation of detectable MSI (46). Also, the MSI method used in our study was primarily developed for colon carcinomas and has not been validated in neuroendocrine tumours. To date, IHC should be the first line diagnostic tool in neuroendocrine tumours to investigate MMR deficiencies.

Conclusions of study II

In summary, study II suggests that germline mutations in MMR genes may contribute to the development and clinical course of pituitary tumours. Especially in aggressive pituitary tumours, analysis of MMR proteins by IHC might facilitate targeted treatment since response to TMZ is unlikely in MMR deficient tumours. Pituitary tumours are possible manifestations of LS and conversely, the management of pituitary tumours should include a proper family history including tumours associated with LS.

Study IV

How common is the use of psychotropic drugs in patients with CD and are the psychiatric symptoms reversible after treatment of hypercortisolism?

Study IV illustrates the high burden of psychopathology in CD. The study showed a higher use of antidepressants in patients with CD compared to controls from several years before diagnosis and during long-term follow-up. The proportion of CD patients with antidepressants remained at a rate of around 25% at diagnosis, and at 5- and 10-year follow-up. Further, the cross-sectional analysis of patients in sustained remission for median 9.3 years showed the same rate of antidepressant dispenses (25%), a significantly higher use than controls (OR 2.0, 95% CI 1.1-3.8). Increased use of sleeping pills was also observed before diagnosis as well as after long-term remission. In contrast, there was a significant decrease in the use of RAS-blockers, Ca-inhibitors and antidiabetics after treatment of hypercortisolism. Previous prospective studies of Cushing's *syndrome* with shorter follow-up (86-89), have shown regression or improvement of depressive symptoms in a major part of patients after correction of hypercortisolism. We found that 66% of the patients who were taking antidepressants at diagnosis had continued the therapy at 5-year follow-up. In addition, 16/129 (12%) had started treatment between diagnosis and 5-year follow-up. Although grading of symptoms was not possible in our study, the results imply persisting mental health issues despite achieved biochemical remission. The findings in study IV support two previous cross-sectional studies in which increased prevalence of psychopathology and cognitive impairment was found after remission for 11 (range 1-32), and 13 (5-19) years, respectively (90,91).

The underlying mechanisms of incomplete recovery after normalisation of cortisol levels are not fully understood. Brain imaging studies in patients with hypercortisolism suggest persisting effects following treatment of hypercortisolism. Cerebral atrophy found at active CS was only partly reversible following treatment (158), structural abnormalities in the anterior cingulate cortex have been demonstrated after long-term remission of CD (159) and in one study of CD patients in long-term remission, reductions in grey matter integrity in the left uncinate fasciculus were associated to severity of depressive symptoms (160). The brain is rich in glucocorticoid and mineralocorticoid receptors, both activated by cortisol (161), and neurotoxic effects of chronic hypercortisolism has been demonstrated in animal models (162).

One secondary finding in study IV was the high use of opioids in patients with CD. Thirty-two percent used opioids at diagnosis, the use remained high (24%) at 5-year follow-up and then decreased to 14% at 10-year follow-up. A higher incidence of fractures in CD (76) may account for some of the increased use of opioids, yet the sustained use during the first 5 years after diagnosis indicates that pain is a major issue in CD. Bodily pain contributes to decreased quality of life in patients with hypercortisolism (163,164). Another feature of cortisol excess, muscle weakness, may persist despite biochemical remission and further decrease quality of life (165). A

structured follow-up of pain and myopathy, including patient education of illness perception and coping strategies (85,166) as well as evaluation/treatment by physiotherapists and encouragement of physical exercise might be helpful to improve overall quality of life in CD (12).

Sleeping disorders improved after treatment of Cushing's *syndrome* in studies by Starkman et al (86,167). However, our results showed sustained and elevated use of sleeping pills compared to controls, even after long-term biochemical remission. Although sleeping pills may be hard to discontinue, this indicates remaining sleep quality issues in patients with CD.

Higher urinary cortisol levels at diagnosis, older age and female sex have been associated to depression in CD patients (168). In our findings, older age and female sex were associated to dispense of antidepressants at 5-year follow-up but not at diagnosis. However, the small number of men might impair statistical comparisons. Delay of diagnosis has also been correlated to depression in patients with Cushing's syndrome of both pituitary and adrenal origin (169,170). In a recent meta-analysis, the median time from first symptom to CD diagnosis was 38 months (67). In our study, duration of hypercortisolism was impossible to assess. Considering our results of increased use of psychotropic drugs and visits to a psychiatrist from 5 years prior to diagnosis it is likely that a substantial part of patients have a longer delay. Our findings also indicate that psychiatric symptoms are important to recognize as early signs of CD.

Conclusions of study IV

Psychiatric symptoms are early manifestations of CD in a considerable part of the patients, reflected by the increased use of psychotropic drugs from several years before diagnosis. Regardless of biochemical remission status, psychotropic drug use remains elevated, suggesting persisting negative effects on mental health. The findings of study IV highlights the importance of early diagnosis of CD and the need for life-long monitoring of mental health issues in patients with CD.

Study V

Is mortality increased in CD despite biochemical remission?

Study V demonstrated an overall doubled mortality in CD compared to matched controls. Although achievement of biochemical remission was a major predictor of survival, patients in remission still had an increased mortality compared to controls, HR 1.5 (95%CI 1.02-2.2). This finding highlights the importance of careful monitoring and follow-up of CD and its associated co-morbidities, regardless of remission status. Previous studies have been inconsistent in regards of mortality after remission with a wide variability between studies (101,103,104,106,108,113,114). Methodology may have contributed to this since sample sizes, time of follow-up, study period, geographical area and different criteria for inclusion and remission have varied. The majority of studies have used unmatched national data as the reference and calculated standardized mortality rates (SMR). In that context, the major strength of study V was the properly matched controls together with the large and unselected cohort of patients with CD. The most important finding was the increased mortality regardless of remission status. This supports the most recent meta-analysis of eight studies that found a pooled SMR of 2.5 (95% CI 1.4-4.2) for patients with CD in remission (114).

Why is the mortality increased in CD?

Cardiovascular diseases were the leading cause of death in study V, as expected from previous studies (101,105,106,108), representing nearly half of all deaths and a HR of 2.9 (1.9-4.5) compared to controls. This supports previous observations of persistent alterations in the cardiovascular system despite biochemical remission (73,171) and underscores the importance of aggressive treatment of these comorbidities. Hypercortisolism also induces changes in the haemostatic and fibrinolytic systems (172) and a more than 10-fold increase in thromboembolic events have been demonstrated, in particular in patients undergoing pituitary surgery (74,76). We found two cases of fatal pulmonary embolism, one of them in close relation to surgery. Both occurred before 1996, possibly reflecting a more aggressive approach to treatment and prophylaxis of thromboembolism in the modern management of patients with CD.

Infections were the second most common cause of death in our study (12/66, HR 4.3, 1.9-9.5). The major part, 10/12, occurred during the first 6 years after diagnosis. This finding supports the recently published data from the ERCUSYN register, encompassing 1045 CD patients, in which infections was the most common cause of death during the early years after diagnosis (111). It also in line with the findings by Clayton et al (115), investigating mortality in CD after 10 years of remission, in which infections were uncommon causes of death, 2/51 (4%). Severe hypercortisolism alters the immune system and increases the risk of viral, bacterial and fungal infections (75,173). The current Endocrine Society guidelines (12) states as an “ungraded best practice recommendation” that vaccinations against pneumonia, influenza and herpes zoster should be discussed and offered to patients with CD. In addition, hypocortisolism after treatment of CD might contribute to fatal infections (174,175) emphasizing the importance of adequate dose adjustments of cortisol replacement

therapy during intercurrent disease. The present study design did not allow for review of medical charts and details surrounding the infections but it could be questioned whether some of the fatal infections in our study were preventable. Increased attention to these issues including education of patients, relatives and health professionals might improve outcome. Some authors have also pointed out the increased risk of opportunistic infections such as pneumocystis pneumonia and suggested prophylactic treatment in selected cases with severe hypercortisolism (75).

One major limitation in study V was the lack of reliable data on comorbidities and hormone replacement therapy. Although data are not uniform, previous studies have associated increased mortality with co-existing hypertension (58,101), depression at diagnosis (176) and glucocorticoid replacement therapy (106). Diabetes mellitus that possibly increases the risk of both cardiovascular and infectious diseases was a risk factor in some previous studies (101,102,107) but not in others (106,176). In our study, age at diagnosis was associated with mortality in patients with CD, which is in line with most previous studies (3,11,19,21,28,29). Men and women had a comparable increased HR in comparison to matched controls, indicating no significant gender differences.

It is tempting to suggest that previous exposure to cortisol excess increases the mortality since our patients in remission failed to achieve a normal survival compared to the matched controls. However, the duration of glucocorticoid excess was not possible to evaluate in our study. The total duration of hypercortisolism is very hard to assess since symptoms might be vague in the beginning of the disease and time from first recalled symptoms to diagnosis most often exceeds several years, as demonstrated in study IV and other reports (67). In addition, the level of endogenous hypercortisolism and/or glucocorticoid replacement doses in patients with hypocortisolism are seldom known. Lambert et al found higher mortality in patients with longer duration of hypercortisolism, defined as time from first symptom of CD to time of established remission (176), however no association was found in patients in remission. Ragnarsson et al reported that time from diagnosis to remission was not associated with increased mortality; however duration of hypercortisolism prior to diagnosis was not evaluated (106). Clayton et al found that a subgroup of CD patients who required only one single TSS to achieve remission and survived for the first ten years reached a life expectancy comparable to the background population, possibly reflecting a shorter duration of hypercortisolism (115). This is encouraging but may reflect a survival bias phenomenon since deaths prior to ten years after diagnosis were not evaluated. Patients in remission after one TSS only in our study (n=188) did not significantly differ from the matched controls (HR 1.6, $p=0.06$). However, the HR was not lower than for all patients in remission (n=303, HR 1.5, $p=0.04$) and the significance level might be related to the smaller sample size. Still, the results point in the direction that prompt normalisation of hypercortisolism is crucial. In support of this is Clayton et al's finding of an association between numbers of treatments to achieve remission and mortality (115).

It might be so, that a subset of patients with mild disease, short delay of diagnosis and rapid normalization of cortisol excess has a normal life expectancy. Nevertheless,

taken together our results and previous findings, CD patients must always be considered to have an increased mortality risk no matter the initial outcome of treatment. This is logical since comorbidities, including neuropsychiatric symptoms and vascular alterations are not fully reversible despite achievement of biochemical remission (99).

Conclusions of study V

The most important contribution of study V was the study design that compared a large and unselected cohort of patients with verified CD, with a properly matched control group. Mortality remained increased in patients with CD despite achievement of biochemical remission. This may reflect effects of previous hypercortisolism and highlights the importance of early diagnosis and treatment of persisting comorbidities.

Methodological discussion

Immunohistochemistry

We used immunohistochemistry in study I-III. IHC might be compromised in several steps of the procedure (see method and discussion sections). Interpretation of IHC is arbitrary and dependent on the performing pathologist. Ideally, the analyses should be blinded and performed by two or more pathologists. Highly specialised and experienced pathologists in study I (Henrik Daa Schroeder) and study II (Patrick Joost) performed the IHC. The clinical characteristics and outcomes were blinded to the pathologists during examination. However, a second pathologist did not confirm the results. Still, we consider the centralised IHC analyses of MGMT as a particular strength compared to other studies.

Study design, using registers, internal and external validity

In study I and III, selection bias must be considered since the studied patients all were given TMZ based on clinical decisions before the study was conducted. On the other hand, this could be regarded as a strength. All patients had progressive tumours and were treated with TMZ as a “rescue” option. The chance of spontaneous recovery regardless of TMZ treatment was nearly non-existent in all of these patients. All participating centres included every patient that had been given TMZ to avoid falsely high response rates (publication bias). Expectedly, most previous case reports had reported a positive outcome of TMZ. A randomized trial comparing TMZ *vs* placebo is not advisable based on the “rescue treatment” character of TMZ.

Study I-III included small number of patients, which was unavoidable due to the rarity of the diseases. Comparison of response rates and mortality with other studies might be compromised due to varying selection criteria, response criteria and different starting points. In study III, the censored cases in the survival analysis were included in the Kaplan-Meier graph to allow for the readers own cautious interpretations.

In study IV-V, we used the Swedish pituitary register (SPR) to recruit patients with CD and collect clinical data, such as age at diagnosis, tumour size, treatment modalities and biochemical remission status. The confirmation of diagnosis and evaluation of remission status in the SPR are dependent on the best efforts from each treating clinician and misclassification cannot be completely ruled out. However, before data outtake, we reviewed the remission status variable and completed missing data when possible (in a few cases, medical charts from smaller hospitals were not available). We therefore regarded the remission status variable as accurate.

The SPR contains the very major part of Swedish patients with CD from all health care regions in Sweden (see background section) and should represent an unselected nationwide cohort of patients with CD. Tumour size was not reported in 78/371 (21%) cases. Likewise, we are not fully confident that all treatments (pituitary surgery, radiotherapy, adrenalectomy and medical treatment) were reported; hence, we have been careful with conclusions associated to treatment regimens or tumour size. For hormone deficiencies, the way to report these variables in the SPR has changed during

the years, and these variables are currently under review. At this time, we have chosen not to include these variables in our studies. Instead, we used the Swedish Prescribed Drug Register (PDR) in study IV to look for hormone replacement therapy. The coverage in the PDR is complete (see background section) and is very suitable for research (119).

The unique personal identification number allows for linkage to national registers in Sweden and easy access to large volumes of data. However, the researcher has a great responsibility to interpret the data prudently. Validation studies of the Swedish National Patient Register (NPR) have shown that overall 85-95% of all diagnoses in the NPR are valid (118). However, this was not true for CD as a recent study found that only 534/1317 (41%) with a CD diagnosis in the NPR had confirmed CD (55). In that context, the use of the SPR instead of the NPR to identify patients with CD was a strength in study IV-V. Equally important was the review of medical charts in study II, in which we found five Lynch patients with a diagnosis consistent with pituitary disease; only three had a pituitary tumour.

The Swedish Cause of Death Register, used in study V, has a near complete coverage (122), and reports both the main and alternative underlying causes of death. The accuracy is acceptable for most diagnoses and is especially good for malignant neoplasms (90%) and ischemic heart disease (87%) (121). Still, details surrounding each death can only be attained by review of the medical charts, which was impossible in study V. In the one case with an “external” cause of death, no specified cause could be determined. In addition, four deaths occurred in close relation to study end and causes of death were not yet reported at time of data output. In all other cases, the combinations of the main cause of death and the alternative causes of deaths were logical and considered accurate.

Immortal time bias should always be considered in observational studies. In study IV-V, the matching of controls was performed at date of diagnosis (i.e. the start of follow-up), minimizing the risk of such bias. However, the true incidence or death rate in CD can never be calculated in any study as patients with undiagnosed CD obviously are not included (i.e. left-censoring). Our results in study IV showed that many patients with CD live years with mental health issues prior to diagnosis. Other studies have shown an increased rate of infections and cardiovascular diseases ahead of diagnosis (76,173). Thus, an unknown number of CD patients may possibly never be diagnosed ahead of death.

Surveillance bias, i.e. the increased attention to symptoms in patients with repeated visits to health care professionals might have influenced the rate of drug dispenses in CD patients in study IV. This may partly explain the increased rate of antidiabetics and psychotropic drugs in CD patients in long-term remission. However, the major part of psychotropic drugs was prescribed in the primary care indicating that CD patients seek care outside of their regular follow-ups at endocrine departments.

It should be mentioned that dispenses of psychotropic drugs is a surrogate variable for psychiatric illness and might not be equivalent to specified psychiatric disorders. Still, we considered the drug use as good indicator of mental health, especially when

compared to matched controls. One specific strength in study IV was that recall bias, i.e. the increased attention to previous symptoms in ill patients, was diminished by the study design. In that aspect, study IV is a good indicator of the problem with delayed diagnosis in CD.

In study IV-V we used controls matched by gender, age and residential area at date of diagnosis. Other variables, including socioeconomic factors, comorbidities/treatments or unidentified confounders, might have influenced the results. However, adjusting for comorbidities such as diabetes and hypertension could as well have compromised the results since they also can be regarded as direct effects of hypercortisolism.

We considered the external validity in study IV-V as good based on the nationwide design including the vast majority of CD patients from all health care regions in Sweden. In addition, the data represents outcome in the modern era of treatment and can be generalised in many countries with similar treatment patterns.

Risk and odds ratios

In study IV we presented both proportions of patients with dispense of a drug, i.e. the risk using the drug being you have Cushing's disease or not, and odds ratios (OR), i.e. the odds of having Cushing's disease given you are using the drug divided by the odds of being a control if you used the drug. An alternative would have been to present risk ratios (relative risk) instead of ORs. In many ways, risk and odds resemble each other but in the circumstance of more than 10% taking the drug, the odds tend to overestimate the risk and ORs and risk ratios might then not be equivalent. However, after calculating both types of ratios, differences were very small and did not change any conclusions. The calculation of ORs require at least 10 events per dependent variable included in the model and the dependent variables should be independent from each other. Adjustment of a large number of variables should be avoided. We used controls matched by age, sex and residential area, thereby avoiding the need for adjustment when comparing CD and controls. However, in some of the analyses, the number of events were small, e.g. the drug use in men, and we chose not to draw any major conclusions in such circumstances.

Survival analysis and hazard ratios

We used Kaplan-Meier survival analysis and Cox regression to estimate survival in study V. These methods account for the time elapsed to the event (death) and assume that the hazard is proportional during the whole study time. For example, if a new treatment option is introduced during the study and improves outcome, this assumption does not hold. In our cohort of patients with CD, spanning from 1991 to 2018, we believe that no major improvement in treatment has occurred. The assumption of proportionality can be assessed by statistical methods or graphically. For patients in remission, the death rates were low in the first years, then increased and eventually decreased again. Thus, the proportional hazard assumption was violated. Under such circumstances, the HRs could be looked upon as an average during the study time and estimates of survival at different time-points can complement the analysis (177). Survival tables are included in the manuscript. It

should also be noted that the number of remaining patients decreased at the longest follow-ups and interpretations at these time-points should be cautious.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

At the time of publication of study I, it represented the largest cohort of patients with aggressive pituitary tumours/carcinomas that had been treated with TMZ. In the troublesome clinical situation when all standard treatment options have failed, 10/21 patients showed an initial response to TMZ. Subsequently, other publications have added experience and number of cases in the literature now exceeds 300 (139). Combining all studies, the response rate to TMZ is around 40%. Study I contributed to the European Society of Endocrinology clinical practice guidelines for management of aggressive pituitary tumours and carcinomas, published in 2018 (17), in which TMZ is recommended as the first-line chemotherapeutical treatment. We demonstrated that a low expression of MGMT, as measured by a robust and standardised IHC method, was a predictive biomarker of TMZ effect. In study III, which was a follow-up of study I, we found that low MGMT tumour content was associated with longer survival and better response to a second course of TMZ. The predictive value of MGMT is still debated since patients with high MGMT occasionally respond to TMZ treatment. Therefore, current guidelines recommend a three-month trial with TMZ regardless of MGMT status (17). However, the likelihood of response to TMZ is far higher in patients with low MGMT (17,139).

More than half of the patients with aggressive tumours/carcinomas are unresponsive to TMZ and a considerable part recurs after initial response. Efforts to understand and overcome TMZ resistance in pituitary tumours such as evaluation of the base excision repair pathway and mechanisms of acquired resistance during treatment have not been fully addressed. Likewise, treatment combinations that might potentiate TMZ need further investigation. The ongoing evaluation of alternative treatment options such as PRRT, immunotherapy or combined TMZ with radiotherapy is important. Since it is unlikely that we will find a “one-fits-all” treatment, development of predictive factors to tailor treatment is crucial. In that context, a validation study to determine the best MGMT IHC protocol could aid further research and clinical use of MGMT. Another major challenge is to develop markers that identify aggressive potential of a tumour at an early stage. To date, TMZ has mostly been given in late stages of the disease and it is unclear whether earlier intervention improves outcome.

One of the patients in study I, suffering from a corticotroph carcinoma also had Lynch Syndrome (LS), a hereditary cancer-predisposing syndrome caused by germline mutations in DNA mismatch repair (MMR) genes. In study II, we demonstrated that the pituitary carcinoma showed loss of MMR function consistent with the patient’s germline mutation (*MSH2*). This finding might help in the ongoing research of pituitary tumour pathogenesis and is now gaining growing interest (60,178). Our

results also indicated a higher prevalence of pituitary tumours among LS patients compared to the expected prevalence. Thus, LS might be a rare hereditary cause of pituitary tumours and in the work-up of pituitary tumours, patients should be asked for a relevant family history including tumours in the LS spectra (colon and endometrial cancer being the most common). In parallel, clinicians that treat LS patients should be aware of pituitary tumours as a possible manifestation of LS. Our findings also have clinical implications in regards of treatment options since MMR-deficient aggressive pituitary tumours are likely resistant to TMZ. Instead, they might be susceptible to immune checkpoint inhibitors based on their high level of infiltrating lymphocytes and expression of PD-L1 (148). Further research is needed to confirm our findings and to elucidate best treatment options for this rare group of patients.

The clinical implications of study IV-V are also relevant. The studies investigated a large group of unselected patients with Cushing's disease compared to matched controls. Patients with CD had a 2-fold increased mortality even in the modern era of treatment. The main ways to reduce mortality are to minimize delay of diagnosis and achieve biochemical remission as quick as possible once the diagnosis is established. Importantly though, we also showed that the mortality was elevated despite achievement of biochemical remission. The leading causes of death were cardiovascular diseases and infections. This underlines the need of monitoring and treating of comorbidities during life-long follow-up.

Apart from reducing mortality, improving quality of life and every-day functions should be a main goal in the management of CD. Study IV showed that patients with CD had an increased use of psychotropic drugs from many years before diagnosis illustrating the problem of delayed diagnosis and highlighting psychiatric symptoms as important early signs of CD. Moreover, our results also showed remaining mental health issues despite long-term remission of CD, indicating persisting effects of previous hypercortisolism and emphasizing the need for life-long monitoring of mental health.

What can we do to improve survival and psychiatric health in CD, apart from our best efforts to decrease duration of hypercortisolism and treat/prevent comorbidities? After treatment of CD, hypopituitarism and dosages of replacement therapies may affect both psychopathology/quality of life (81,179) and mortality (106,174). The impact of educational level and other socioeconomic factors might also be clues to identify patients at higher risk, which we now aim to investigate.

Finally, it should be of importance to keep and improve the Swedish pituitary register. In addition to the clinical data, biobanks can provide access to a large number of pituitary tumour tissues for retrospective analyses as new potential tumour markers evolve. Combining register data with pathological investigations could gain much knowledge.

SAMMANFATTNING PÅ SVENSKA

Behandling och uppföljning av aggressiva hypofystumörer och Cushings sjukdom

Den här avhandlingen handlar om ovanliga men allvarliga tumörer i hypofysen. Hypofysen är ett livsnödvändigt hormonproducerande organ som är beläget under hjärnan och nära synnervskorsningen. Ungefär var tusende person har en hypofystumör som i de allra flesta fall är godartad och växer mycket långsamt. Tumörerna ger symtom om de trycker på synnerven och andra viktiga strukturer eller genom hämning av den viktiga hormonproduktionen. Dessutom tillverkar en del av tumörerna hormoner, till exempel prolaktin, tillväxthormon eller kortisol, som i sin tur ger upphov till särskilda symtom. I vissa fall kan tumörerna behandlas med hormondämpande läkemedel som också kan krympa tumören. Om tumörerna är stora eller överproducerar hormoner som är skadliga för kroppen krävs oftast operation och ibland även strålbehandling. I de fall det uppstår hormonbrister kan dessa ersättas effektivt med läkemedel.

En liten andel av tumörerna växer mer aggressivt och kan vara mycket svårbehandlade. I ytterst sällsynta fall kan det uppstå dottertumörer i andra delar av kroppen. Det har tidigare saknats bra behandlingsalternativ för aggressiva hypofystumörer och prognosen har varit dålig. I två av delarbetena i den här avhandlingen (studie I och III) undersöktes effekten av cellgiftet temozolomid på aggressiva hypofystumörer som fortsatt växa trots behandling med upprepade operationer, strålning och andra cellgifter. Projektet inkluderade 24 individer och var ett internationellt samarbete. Studie I visade att temozolomid hade bra effekt på ca hälften av dessa svårbehandlade tumörer. Dessutom kunde vi visa att effekten var beroende av ett särskilt protein, MGMT. Tumörer med ett lågt innehåll av MGMT var mycket mer känsliga och nivån av proteinet kunde användas för att förutsäga behandlingseffekten. I studie III visade vi att överlevnaden efter behandling med temozolomid var betydligt bättre för patienter vars tumörer hade lågt innehåll av MGMT. Behandling med temozolomid är nu förstahandsvalet om standardbehandling misslyckats och överlevnaden för dessa patienter har förbättrats.

I studie II kunde vi visa att en nedärvd mutation var ansvarig för uppkomsten av en aggressiv hypofystumör. Hos alla människor finns olika mekanismer för att reparera skador som uppstår i arvsmassan, så kallade DNA-reparationsproteiner. Vid den ärftliga sjukdomen Lynch syndrom är dessa proteiners funktion påverkad vilket ökar risken för ett flertal olika tumörer. Vi redovisade det hittills första fallet av en hypofystumör hos en patient med Lynch syndrom och kunde visa att hypofystumören uppkommit pga den nedärvda mutationen. Fyndet är viktigt eftersom det ökar förståelsen för uppkomsten av hypofystumörer och möjliggör en mer precis och riktad

behandling mot den speciella tumörtypen. Studien visar också att hypofystumörer bör övervägas som en möjlig del av Lynch syndrom och belyser vikten av att noggrant gå igenom släkthistorien vid uppföljning av hypofystumörer.

De sista två delarbetena, studie IV-V, fokuserade på en särskild form av hypofystumör, Cushings sjukdom. Dessa tumörer drabbar bara runt 15 personer per år i Sverige men kan ge mycket svåra symtom genom överproduktion av hormonet kortisol. Flera organsystem i kroppen blir påverkade och det är vanligt med följsjukdomar såsom diabetes och högt blodtryck. Kortisol påverkar också hjärnans funktioner. De allra flesta patienterna blir opererade och cirka 80% återfår då en normal kortisol-nivå i blodet. Tyvärr har det visat sig att negativa effekter av kortisol kan kvarstå även efter behandling. Studierna har använt det unika svenska kvalitetsregistret för hypofystumörer och vi kunde undersöka 371 individer med Cushings sjukdom, många med en uppföljning över 10 år. I studie IV undersökte vi förskrivning av läkemedel kopplade till mental ohälsa och visade att patienter med Cushings sjukdom använde mer läkemedel mot depression, ångest och sömnstörningar jämfört med en kontrollgrupp. Det viktigaste fyndet var att patienter som haft normala kortisolvärden i över 9 år fortfarande använde mer antidepressiva läkemedel än kontrollerna. Detta talar för kvarstående negativa effekter på hjärnans funktioner trots många års normala kortisolvärden. Ett annat viktigt fynd var att den ökade förskrivningen var tydlig redan flera år före diagnostillfället vilket belyser problemet med försenad diagnos hos många av dessa patienter.

Studie V visade att patienter med Cushings sjukdom har en dubbelt så hög dödlighet som en matchad kontrollgrupp och den viktigaste faktorn för att förbättra överlevnaden är att uppnå normala kortisolvärden. Även hos patienter med normaliserade kortisolvärden fanns dock en överdödlighet vilket talar för kvarstående negativa effekter av tidigare kortisolöverproduktion. Den största delen av överdödligheten kunde förklaras av hjärt-/kärlsjukdomar respektive infektioner. För att minska dödligheten hos patienter med Cushings sjukdom är det därför viktigt att vara uppmärksam på infektioner och behandla följsjukdomar såsom högt blodtryck, diabetes och blodfetterubbnings.

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to my supervisors **Jeanette Wahlberg Hughes and Pia Burman** who not only have supported and inspired me, but also have become valuable friends during my PhD studies. **Pia** – You made it all happen from the beginning. Thank you for including me in exciting research projects, for sharing your outstanding skills in endocrinology and research, for your enduring 24-hour availability and for never settling with second best. **Jeanette** – You kept me going until the end with positive energy and enthusiasm. Thank you for your encouragement, for your help in limiting my sometimes-overambitious efforts and for your patience with my unfinished manuscripts. My sincere thanks for all the time and energy you both have put in my PhD studies.

I also want to express my sincere thankfulness to **Oliver Gimm**, my co-supervisor and professor of the institution, for help and support during my time as a PhD student.

My gratitude to all **co-authors** for inspiring discussions and important contributions. A special thanks to **Oskar Ragnarsson** for extensive and invaluable input in paper IV-V, for sharing your great knowledge in the field of Cushing's disease and for pointing out what is important and what is not. Thank you **Jacob Järås**, for numerous demanding, yet joyful and interesting zoom-meetings digging deep into the statistical mine. Thank you also **Henrik Daa Schroeder** and **Patrick Joost** for performing the immunohistochemistry in study I and III.

Thank you **Olivera Casar-Borota** for comprehensive guiding in the world of pituitary immunohistochemistry and for happy days in Uppsala.

Lars Brudin – Thank you for statistical advice and for your unrestrained interest in statistics and medical questions, no matter the issue. Special thanks for the graph in paper I.

Pär Wanby, my first mentor in clinical endocrinology and now my inspiring colleague and co-author. The best thing with my working room is that it is next to yours.

My humble thanks for support and patience to all colleagues, nurses, secretaries and friends at the **Endocrine Department in Kalmar**. Special thanks to **Marianne Fagerberg, Jesper Johnsson, Åsa Bratt, Panagiota Chatzimichail, Etsko Engel Blaaauwweikel** and **Herbert Krol** for taking care of my patients whenever I was buried in research.

Region Kalmar County, head and former head of the **Department of Internal Medicine**, for encouragement and for providing the time I needed to carry out my research. Thank you to the **Research section at Region Kalmar** for financial support as well as great encouragement. I am also very grateful for the financial support from the **Medical Research Council of Southeast Sweden**.

Martin Carlsson for enabling and supporting my first attempts in conducting science.

Bertil Ekman, for your interest in my research and for your tendency to always come up with unexpected aspects on clinical or research-related questions.

Thank you to the holder of the **Swedish pituitary register**, **Britt Edén Engström**, all colleagues in the Swedish pituitary society and people working with the register. A special thanks to **all participating patients** that made study IV-V possible.

Many friends have, knowingly or unknowingly, supported me during my PhD studies, I cannot mention all. Thank you all for keeping in touch when I have not. Special thanks to long-term friends, **Jacob Skov** and **Erik Melén** with whom I discuss everything (including medical science). To **Fredrik Ellin**, **Mats Ranebo**, **Jonas Bonnedahl** for instant help and debriefing when needed (including medical science). A special thanks also to **Adam Hjort Ström** – without knowledge in medical science but with master knowledge in friendship.

My amazing parents, **Inger and Kenneth**, my older brother **Jonas** and my uncle **Krister**. Thank you for your peculiar and unlimited belief in my abilities from birth and ongoing, for all you that have done and keep doing.

My beloved wife **Ana**. Even though you painted the plumuitary on the cover page, that is probably the least important of your contributions. Thank you for keeping me on track and for always standing strong when the wind blows the hardest. In the end, the most important things in life will always be our family. **Olle** and **Malte**, you are the true inspiration!

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FACULTY OF MEDICINE AND HEALTH SCIENCES

Linköping University Medical Dissertation No. 1782, 2021
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