Autonomous cortisol secretion – Mortality, morbidity and diagnostics

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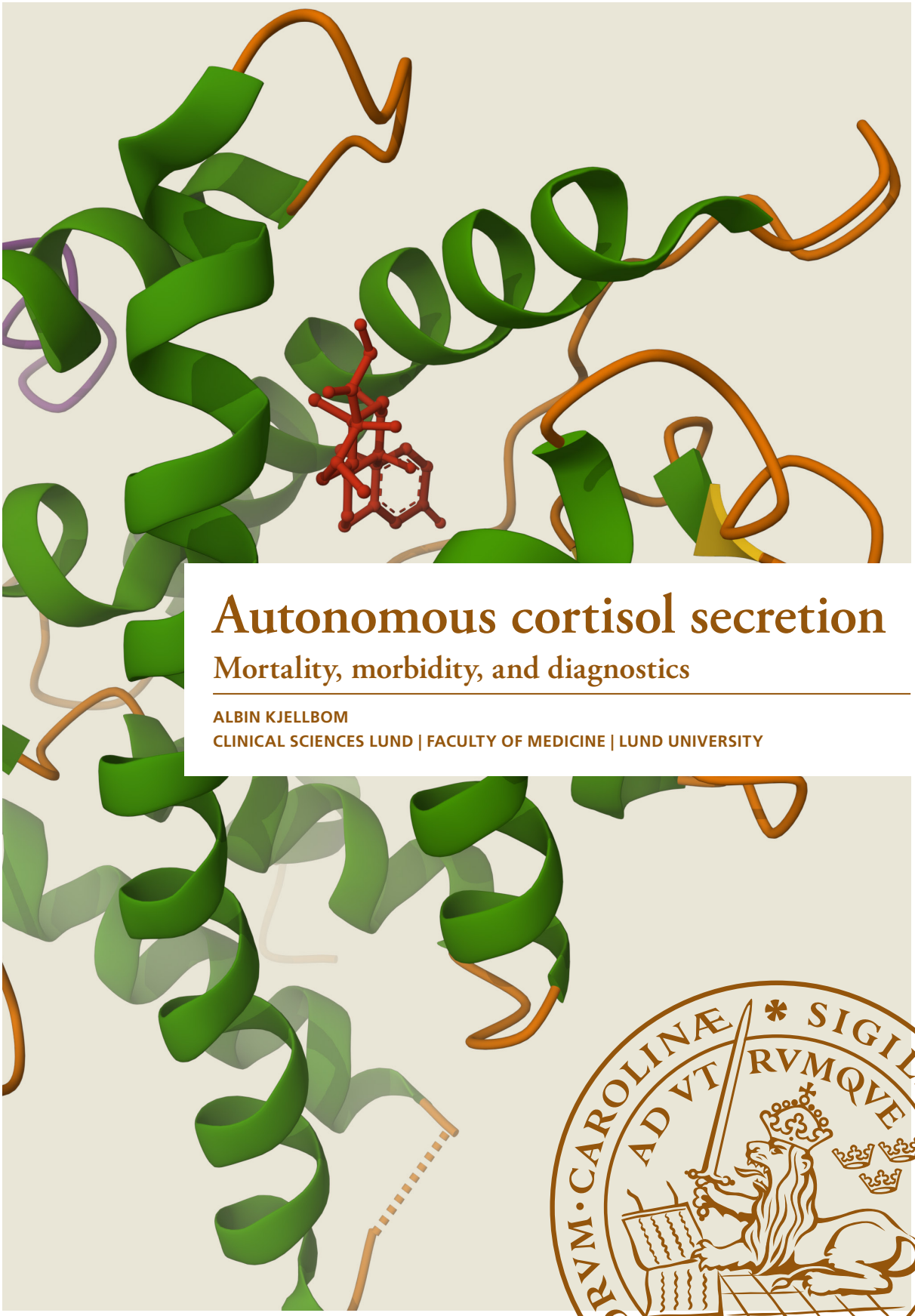

Autonomous cortisol secretion

Mortality, morbidity, and diagnostics

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[1] Kauppi B, et al. The three-dimensional structures of antagonistic and agonistic forms of the glucocorticoid receptor ligand-binding domain: RU-486 induces a transconformation that leads to active antagonism. J Biol Chem. 2003;278(25):22748-54.

[2] Berman HM, et al. The Protein Data Bank. Nucleic Acids Research. 2000;28(1):235-42.

[3] Sehnal D, et al. Mol* Viewer: modern web app for 3D visualization and analysis of large biomolecular structures. Nucleic Acids Research. 2021;49(W1):W431-W7.

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Everyone to whom much was given, of him much will be required, and from him to whom they entrusted much, they will demand the more.

Luke 12:48

Table of Contents

Abstract

Context
Up to half of patients with adrenal adenomas found as incidentalomas show biochemical signs of subtle cortisol hypersecretion without having clinical signs or symptoms of Cushing's syndrome. A condition called autonomous cortisol secretion (ACS). Previous studies have indicated that ACS might be associated with increased mortality.

Objectives
Explore if ACS is an independent risk factor for increased mortality. Evaluate low ACTH as a diagnostic marker of ACS. Investigate the prevalence of smoking in patients with adrenal adenomas.

Methods
Cohort and cross-sectional studies. Adult patients referred to two Swedish endocrine centres because of an adrenal adenoma, found as an incidentaloma, between 2005 and 2015 were enrolled. Mortality data were obtained from the Cause of Death Register. Patients were grouped according to predefined levels of cortisol after a 1-mg dexamethasone suppression test ($cortisol_{DST}$); non-functional adrenal adenoma (NFAA), defined as $cortisol_{DST}$ <50 nmol/L, and three levels of ACS ($cortisol_{DST}$ 50-82, 83-137 and ≥138 nmol/L)

Results
1048 patients were followed for 6.4 years. Compared with NFAA mortality was not increased in $cortisol_{DST}$ 50-82 nmol/L, hazard ratio (HR) 1.17 (95% CI, 0.79-1.73)), while $cortisol_{DST}$ 83-137 and ≥138 nmol/L were associated with a significant increase in mortality, HR 2.33 (1.53-3.53) and 2.87 (1.74-4.74). Mortality did not differ significantly between 632 patients with NFAA and matched controls (3:1) when followed for 6.6 years, HR 1.13 (0.87-1.46). Studying 198 patients with unilateral adrenal adenomas and 100 healthy controls, low ACTH (<2.0 pmol/L) was present in 53% of patients with ACS, 19% of patients with NFAA, and 4% of controls (NFAA vs. controls $p<0.001$). Smoking was associated with larger adrenal adenomas, bilateral adenomas, and ACS.

Conclusions
ACS is an independent risk factor for increased mortality, while NFAAs do not pose a relevant risk. The risk associated with ACS seems to become clinically relevant when the $cortisol_{DST}$ level is ≥83 nmol/L. Low ACTH is of limited value in diagnosing ACS, in part due to its high prevalence in patients with NFAA. Additionally, there appears to be a link between smoking, adrenal adenomas, and ACS.

Populärvetenskaplig sammanfattning

Avancerade röntgenundersökningar används allt oftare inom sjukvården. Dessa undersökningar ger oss möjlighet att tidigare diagnostisera olika sjukdomstillstånd men innebär också att vi hittar förändringar i kroppens organ som vi inte letar efter. Upp till var tionde vuxen person som genomgår en röntgenundersökning av magen får det oväntade beskedet att man funnit en tumör på ena binjuren. Tumörer som upptäcks på detta sätt kallas binjureincidentalom.

Binjurarna är två, för allmänheten ofta okända, körtlar som sitter ovanför njurarna och producerar en rad hormon som styr blodtrycket, energiomsättningen och saltbalansen i kroppen. Ett av dessa hormon är det livsnödvändiga kortisolet. Binjuretumörer som upptäcks som incidentalom är i majoriteten av fallen godartade men många av tumörerna uppvisar någon grad av mild kortisolöverproduktion.

Det är välkänt att sjukdomstillstånd med kraftigt förhöjda nivåer av kortisol är farligt, framför allt är det kopplat till en ökad risk för hjärtkärlsjukdom. Däremot har tidigare forskning på binjuretumörer med mild kortisolöverproduktion visat motsägelsefulla resultat gällande eventuella risker.

Målsättningen med forskningsprojektet var att undersöka om patienter med godartade binjureincidentalom, med och utan mild kortisolöverproduktion, har en ökad risk att drabbas av hjärtkärlsjukdom och att dö i förtid. Om så var fallet ville vi också undersöka om risken kunde kopplas till graden av kortisolöverproduktion.

Projektet är ett av de största i sitt slag, ca 4 000 patienter och kontrollpersoner från Skåne ingick i studierna. Resultaten visar att ungefär hälften av alla patienter med en godartad binjuretumör har en mild överproduktion av kortisol. Resultaten visar också att kortisolöverproduktion är kopplat till en ökad risk att dö i förtid, framför allt till följd av hjärtkärlsjukdom. Riskökning ses dock bara hos patienterna med de högsta kortisolnivåerna, vilka utgör en femtedel av patienterna. Riskökningen är jämförbar med den som till exempel rökning eller diabetes medför.

Resultaten ger vården möjlighet att identifiera och rikta sina insatser till de patienter som behöver den mest. Vi föreslår att patienterna med ökad risk skall erbjudas uppföljning, livsstilsrådgivning och behandling av kända riskfaktorer för hjärtkärlsjukdom som högt blodtryck, diabetes och höga kolesterolvärden. Resultaten innebär också att ett lugnande besked kan ges till majoriteten av patienterna med godartade binjuretumörer. Detta tror vi kommer minska onödiga undersökningar, uppföljning och inte minst oro hos denna patientgrupp.

Framtida forskning bör undersöka vilken specifik behandlingsstrategi som kan minska sjukdomsriskerna för patienter med godartade binjuretumörer och mild kortisolöverproduktion

List of Papers

Paper I

Olsen H, Kjellbom A, Löndahl M, Lindgren O. Suppressed ACTH Is Frequently Unrelated to Autonomous Cortisol Secretion in Patients with Adrenal Incidentalomas. J Clin Endocrinol Metab 2019;104(2):506-512.

Paper II

Olsen H, Kjellbom A, Löndahl M, Lindgren O. High prevalence of smoking in patients with adrenal incidentalomas: causality or case selection? Eur J Endocrinol 2020;183(3):335-341.

Paper III

Kjellbom A, Lindgren O, Puvaneswaralingam S, Löndahl M, Olsen H. Association Between Mortality and Levels of Autonomous Cortisol Secretion by Adrenal Incidentalomas: A Cohort Study. Ann Intern Med 2021;174(8):1041-1049.

Paper IV

Kjellbom A, Lindgren O, Danielsson M, Olsen H, Löndahl M. Mortality Not Increased in Patients with Nonfunctional Adrenal Adenomas: A Matched Cohort Study. J Clin Endocrinol Metab 2023.

Abbreviations

ACS	Autonomous cortisol secretion
ACTH	Adrenocorticotropic hormone
CI	Confidence interval
CRH	Corticotropin releasing hormone
$Cortisol_{DST}$	Plasma cortisol after DST
DST	1-mg dexamethasone suppression test
HPA	Hypothalamic-pituitary-adrenal
HR	Hazard ratio
IQR	Interquartile range
MACE	Major adverse cardiovascular event
NFAA	Non-functional adrenal adenoma
SCB	Statistics Sweden

Introduction

The adrenal glands are essential to human life. Since the Italian anatomist Eustachi first described the adrenals in the 16th century much has been learned about their function, but a lot still awaits to be elucidated. The adrenals are endocrine glands producing several hormones with numerous effects. The focus of this thesis revolves around the adrenal hormone cortisol.

Cortisol

Cortisol is a steroid hormone produced in the adrenal cortex, regulated by the pituitary through adrenocorticotropic hormone (ACTH). ACTH in turn is under the influence of corticotropin releasing hormone (CRH) produced by hypothalamic neurons. The interaction between these organs constitutes the hypothalamic-pituitary-adrenal (HPA) axis. Cortisol is essential for maintaining homeostasis in humans. Its release from the adrenal cortex is varying, though continuous, depending in part on different stimuli or "stressors". Fever and trauma are examples of such stimuli leading to an increase in cortisol. However, cortisol release is not only regulated by external stressors but is also under central regulation. Cortisol release has a pronounced circadian rhythm with the highest levels during the early morning hours.(1) In fact, the HPA-axis also has an ultradian rhythm with a pulsatile release of ACTH and cortisol, this oscillating activity seems to play a role in keeping the HPA-axis responsive.(2) An important part of the HPA-axis is its built-in downregulation that follows increased activity in response to stressors. The HPA-axis is a classic example of a negative feedback loop, with cortisol inhibiting the release of ACTH and CRH (Figure 1).(3) This negative feedback is essential for protecting the organism from the harmful effects of prolonged exposure to HPA-axis activation.(4)

Cortisol acts through binding to the intracellular glucocorticoid and mineralocorticoid receptors, which are present throughout the tissues of the body. The effects of receptor activation by cortisol are both rapid and slow. Rapid effects are mediated through nongenomic mechanisms, and slow effects by the activated receptor acting as a transcription factor. The latter resulting either in transcriptional activation and downstream protein synthesis, or transcriptional repression by for example inhibition of other transcription factors.(5, 6) Cortisol has a myriad of

effects in the human body, which is no surprise knowing that the glucocorticoid responsive genes comprise up to 20% of the genome. The most apparent effects of cortisol are easiest described by studying conditions with lack of cortisol and cortisol excess respectively. In the mid 19th century Thomas Addison described patients with a constellation of symptoms consisting of weight loss, fatigue, weakness, orthostatic hypotension, and salt craving.

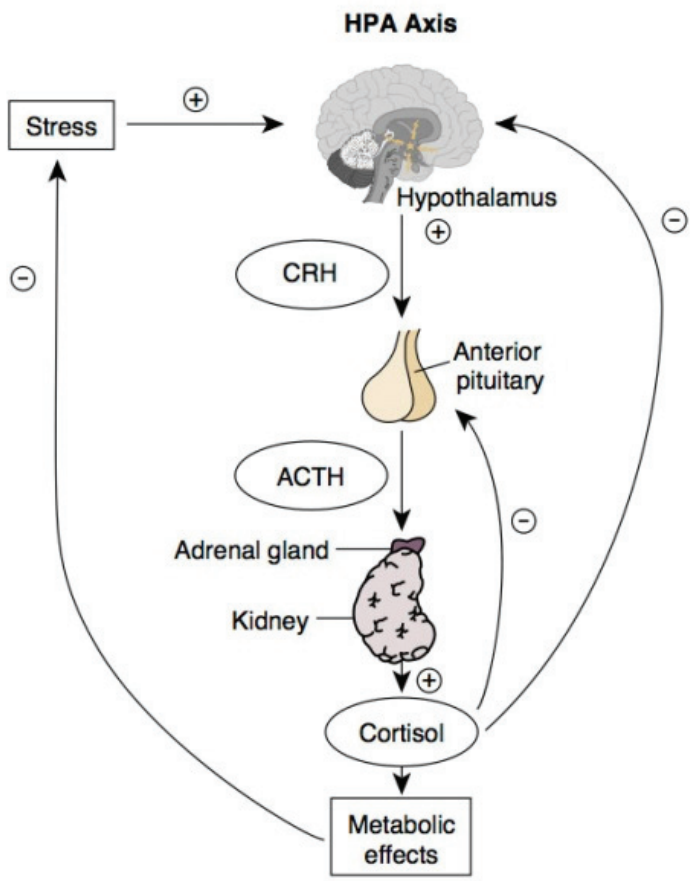

Figure 1 HPA-axis
Reprinted from: Hiller-Sturmhöfel and Bartke. Alcohol Research and Health 1998;22(3):153.(7)

Post-mortem revealed destruction of the adrenal cortex caused by tuberculosis. The described symptoms were in large caused by a lack of cortisol due to primary adrenal insufficiency. The condition was later named Addison's disease. Globally infections are still the most common cause but in the industrialised parts of the world autoimmune origin is dominating.(8) It is likely that many of the patients studied by Addison died due to what is now called an "Addisonian crisis", which is a situation

where the demand for cortisol is increased, typically due to a sudden stressor such as an infection. In a person with adrenal insufficiency this can result in a potentially lethal situation due to circulatory collapse. Cortisol has a key role in blood pressure control and in its absence the effect of vasopressors (catecholamines) on the smooth muscle in blood vessels is diminished, and the endothelial dilatation through nitric oxide is increased.(9, 10)

Harvey Cushing described the effects of cortisol excess in 1932. Cushing characterized a series of patients with cortisol excess caused by an ACTH-producing pituitary tumour, now termed Cushing's disease.(11) Endogenous cortisol excess can also be caused by a primary adrenal tumour or ectopic ACTH production as part of a paraneoplastic phenomenon. These conditions are often referred to as Cushing's syndrome and ectopic Cushing's syndrome. However, the most prevalent cause of Cushing's syndrome is exogenous in the form of glucocorticoid treatment. Symptoms and clinical findings in a person with cortisol excess do not differ depending on the cause. Classical findings and symptoms include central obesity, muscle wasting of arms and legs, easy bruising, purple striae, muscle weakness, hypertension, hyperglycaemia, depression, menstrual cycle disturbance, osteoporosis, and a decreased libido (Figure 2).(12)

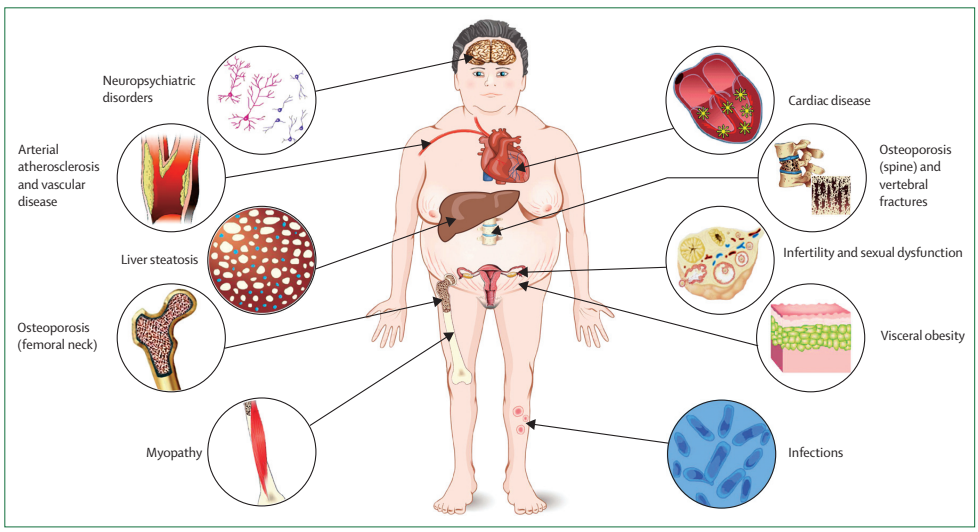

Figure 2 Main comorbidities and clinical complications associated with mortality in patients with Cushing's syndrome

Reprinted from: Pivonello, R., et al., Complications of Cushing's syndrome: state of the art. Lancet Diabetes Endocrinol, 2016. 4(7): p. 614, with permission from Elsevier.(12)

Cardiovascular disease is the leading cause of the increased mortality and morbidity in persons with Cushing's syndrome. Through which mechanisms cortisol excess causes the cardiovascular disease is not fully understood but key components are cortisol induced hypertension, endothelial dysfunction, atherosclerosis, and

myocardial hypertrophy.(13-15) Cushing's syndrome can lead to an especially severe form of metabolic syndrome, contributing to the observed morbidity and mortality.(16, 17) Removing the tumour responsible for the cortisol excess leads to normalized cortisol levels and a reduction in the morbidity/mortality risk. There is, however, a residual increase in risk even in patients who are in remission after being successfully treated for Cushing's syndrome.(16, 18-20) This indicates that some of the damage caused by the cortisol excess is long-standing or might be irreversible.

Adrenal incidentalomas

Adrenal tumours are common in the adult population, the prevalence increases with age, and they are more frequently found in women.(21) Adrenal tumours causing overt hormone excess, as for example the catecholamine-producing pheochromocytoma, a cortisol-producing adenoma causing Cushing's syndrome, or an adrenal carcinoma, often producing both cortisol and androgens, are rare. Most adrenal tumours do not give rise to clinical signs of hormone excess and are of benign origin in the form of an adenoma. Because of this, the exact prevalence is difficult to assess. From the 1950s and 60s, when most deceased in Sweden underwent post-mortem, studies based on autopsy results in persons without signs of adrenal disease while alive, reported a prevalence of adrenal tumours ranging between 2 and 9%. Studies from the time also found an association between hypertension and adrenal adenomas even after excluding aldosterone-producing adenomas.(22, 23) In more contemporary materials based on imaging studies, the prevalence is reported to be approximately 5%.(24-26) With the increasing use of imaging studies, a growing number of adrenal tumours are now being diagnosed while the patient is still alive. The most common situation is a patient undergoing a radiological study for other reasons than suspected adrenal disease and the adrenal tumour being a secondary finding. An adrenal tumour found this way is called adrenal incidentaloma.

Cortical adrenal tumours can be part of inherited conditions caused by germline mutations, such as multiple endocrine neoplasia type-1, primary pigmented nodular adrenocortical disease, and familial form of primary bilateral macronodular adrenal hyperplasia, but the vast majority of cortical adrenal tumours are sporadic.(27-29) The molecular pathogenesis of foremost tumours causing overt hormone excess have begun to be elucidated in recent years. As an example, a mutation in the PRKACA gene, encoding a catalytic subunit of protein kinase A, can be identified in up to 70% of adrenal adenomas causing Cushing's syndrome.(30, 31) These mutations are not seen in adrenal adenomas without overt cortisol excess, which might explain why a patient with an adrenal incidentaloma without Cushing's syndrome at presentation rarely progresses to overt disease.(27, 32) The gene most frequently reported to be associated with adrenal adenomas not causing overt

hormone excess involves the Wnt/β-Catenin pathway. A mutation involving this gene can be identified in approximately 50% of tumours.(33, 34) Our understanding of the pathogenesis of adrenal adenomas is still scarce. However, the development within the field of genetics will hopefully lead to enhanced knowledge surrounding tumour development, and steroidogenesis in patients with adrenal adenomas in the coming years.

Autonomous cortisol secretion

In the western world, computer tomography entered clinical medicine during the 1980s and 90s, and clinically silent adrenal tumours were being diagnosed in increasing amounts. As mentioned before, these tumours are called adrenal incidentalomas. Recommendations on the work-up of patients with adrenal incidentalomas were developed to identify the rare cases of malignancies and overt hormone secretion. During this period, case reports started to appear describing patients with adrenal incidentalomas, without clinical signs of cortisol excess but who showed biochemical signs of hypercortisolism or developed adrenal failure after unilateral adrenalectomy, indicating that the adenoma had been producing cortisol.(35-38) The condition was called subclinical Cushing's syndrome, and soon it became evident that it was associated with hypertension, diabetes mellitus, hyperlipidaemia, obesity, and osteoporosis.(39-42)

The reported prevalence of the condition has varied extensively, ranging between 20% and 45% in patients with adrenal incidentalomas, probably partly due to differing diagnostic criteria.(21, 43) Which diagnostic test and cut-off to use have been debated extensively. Most commonly one or more of the tests used in the work-up for suspected Cushing's syndrome have been proposed; 1-mg dexamethasone suppression test (DST), 24-h urine free cortisol, salivary cortisol at midnight, and basal ACTH.

As it became evident that patients with an adrenal incidentaloma and this subtle hypercortisolism very seldom progressed to a clinical Cushing's syndrome, a new name for the condition was introduced in the most recent guidelines on the topic, published by the European Society of Endocrinology in 2016. The term proposed was autonomous cortisol secretion (ACS). The guidelines state that the principal diagnostic test should be the DST. It tests the integrity of the HPA-axis. In an intact HPA-axis intake of the synthetic glucocorticoid dexamethasone leads to suppression of ACTH and CRH through negative feedback, with the effect that endogen cortisol secretion from the adrenal cortex is diminished. (Figure 1) Guidelines state that a plasma cortisol level <50 nmol/L after DST ($cortisol_{DST}$) excludes ACS, while a $cortisol_{DST}$ level between 50 and 137 nmol/L indicates "possible ACS", and a value

≥138 nmol/L indicates ACS. If, and in that case, which confirmatory test to be used is discussed in the guidelines but no recommendation is made.(21)

The DST was developed as a first-line screening test for Cushing's disease, and the cut-of value 50 nmol/L was selected to provide high sensitivity. Thus, the false positive rate can be significant.(44, 45) In the case of Cushing's disease/syndrome DST is supplemented with other tests such as salivary cortisol at midnight and 24-h urine free cortisol, which provide a solid arsenal in detecting true positive cases.(45, 46) However, this does not seem to be true in ACS. The circadian rhythm of cortisol secretion seems to be less affected in many patients with ACS than in the case of Cushing's syndrome, explaining the limited role of salivary cortisol at midnight.(47, 48) 24-h urine free cortisol evaluate total cortisol secretion during 24 hours. One might suspect that a tumour with autonomous cortisol secretion, even one not giving rise to clinical symptoms as in the case of ACS, would result in elevated urine cortisol levels. However, elevated levels of urine free cortisol are seldom seen in patients with ACS. This is due to several possible explanations, including the fact that the majority of cortisol in plasma is bound to corticosteroid-binding globulin and albumin. As a result, cortisol production must exceed the binding capacity of these proteins before it can result in elevated urine free cortisol levels.(49) In addition, the majority of cortisol is secreted in the urine as different metabolites, which means that increased excretion of these metabolites typically precedes that of free cortisol in states of hypercortisolism.(50, 51) A test often proposed, probably due to its simplicity, is a low basal ACTH level. The rationale is that a tumour producing cortisol would lead to a suppression of ACTH through negative feedback. While this often is the case in Cushing's syndrome, the diagnostic accuracy of basal ACTH for ACS is low.(51, 52) This could be due to the inability of some ACTH assays to detect low levels, the ultradian rhythm of ACTH secretion, and possibly increased cortisol response to ACTH.(53-55) As of now, DST is the cornerstones in the diagnostic work-up for ACS when it comes to biochemical tests.

The interest and debate regarding ACS grew stronger as three independent studies in the early 2000s showed an association between ACS and increased mortality risk in patients with adrenal incidentalomas. All three studies defined ACS as $\text{cortisol}_{\text{DST}}$ ≥50 nmol/L in a patient without clinical signs of Cushing's syndrome, and mortality was compared with patients with $\text{cortisol}_{\text{DST}}$ <50 nmol/L.(56-58) The studies were small and the number of events low, limiting inference regarding clinical implications. The discussion following these studies concerned whether ACS was an independent risk factor and if so, at what level ACS becomes clinically relevant.(59, 60)

ACS is not a binary condition. Cortisol hypersecretion in ACS ranges from mildly elevated to levels close to those seen in Cushing's syndrome, to which there is no clear-cut line. The available evidence suggests that the risk associated with ACS increases with more pronounced ACS, defined by the $\text{cortisol}_{\text{DST}}$ value.(21, 61) Some studies even indicate that non-functional adrenal adenomas (NFAA), defined

as $cortisol_{DST}$ <50 nmol/L, might be associated with increased cardiovascular risk.(62, 63)

The reason for this thesis

Patients with adrenal incidentalomas are common in clinical practice. We see several patients per week at our outpatient endocrine clinic at Skåne University Hospital, and ACS is the most common endocrine disruption in these patients. Against the backdrop of the growing evidence suggesting an association between ACS and increased morbidity, and even mortality, the project of which this thesis is a part, was initiated. The project´s overarching aim was to compile a database, containing data on patients with adrenal incidentalomas, large enough to study if ACS is an independent risk factor for morbidity and mortality, and if so, at what level of ACS this risk becomes clinically relevant to the affected patients.

Aims

Paper I: Low basal ACTH has been proposed as a marker for ACS in patients with adrenal incidentalomas. However, studies have indicated that ACTH is of limited value in the diagnostics. Our objective was to study low basal ACTH as a marker for ACS and investigate possible reasons behind its limited diagnostic value.

Paper II: Smoking has not previously been studied in patients with adrenal incidentalomas. Our aim was to study the prevalence of smoking, and its relation to ACS, in a large group of patients with adrenal incidentalomas.

Paper III: Studies have indicated that ACS is associated with increased mortality compared with NFAA. Our objective was to investigate if ACS is an independent risk factor for increased mortality, and if so, study mortality risk at different, predefined, levels of ACS.

Paper IV: Indirect evidence suggest that NFAA might be associated with increased cardiovascular risk. Since previous studies on mortality in patients with adrenal incidentalomas, with and without ACS, have used the NFAA group as reference, they provide no insight into whether NFAA is a clinically relevant risk factor. Our objective was to study mortality in patients with NFAA or ACS compared with matched controls.

Material and methods

Study setting and population

In Sweden's southern healthcare district regional guidelines recommending all patients with an adrenal incidentaloma to be referred to their regional hospital-based endocrine clinic for evaluation have been in place since Jan 1st, 2005. In this thesis the study populations of all included studies consist of consecutively included adult patients who were evaluated at the endocrine department at Helsingborg Hospital or Skåne University Hospital between January 1st, 2005, and September 15th, 2015, due to a previously unknown adrenal incidentaloma. Sweden has a universal healthcare system and the two study sites were the only available healthcare providers for patients living in the catchment areas of the hospitals who needed specialised endocrine care.

Patients had at least one visit to the outpatient endocrine clinic for clinical evaluation. They underwent DST, 1-mg of dexamethasone was taken at 11 PM, and fasting plasma cortisol was collected at 8 AM the following day. Patients were also screened for pheochromocytoma and primary aldosteronism. Imaging was done with computer tomography or magnetic resonance imaging and tumour size was defined as maximal axial diameter. If the adrenal incidentaloma could not be deemed a lipid-rich adenoma on initial imaging, at least one repeat imaging study with computer tomography was performed after six months.

All studies employed a shared set of exclusion criteria, which included incidentaloma <10 mm, metastatic malignancy, non-adenoma lesion (such as myelolipoma, haemorrhage or cyst), pheochromocytoma, primary aldosteronism, clinical Cushing's syndrome, oral corticoid treatment, medication affecting dexamethasone metabolism, and lack of DST.

Study design and endpoints

Paper I

We did a retrospective cohort study including a control group. Patients evaluated for unilateral adrenal incidentalomas at the endocrine department at Helsingborg Hospital were eligible for inclusion. In addition to shared exclusion criteria, patients

with body mass index <19.0 kg/m^2, age >75 years, treatment with inhalation steroids, and estimated glomerular filtration rate <60 ml/min/1.73 m^2 were excluded. Basal ACTH and cortisol$_{DST}$ were measured at baseline and at a 2-year follow-up. 100 healthy blood donors constituted the control group, in whom one basal ACTH was measured. ACTH <2.0 pmol/L was defined as low, and ACS was defined as cortisol$_{DST}$ ≥50 nmol/L. Endpoints were the relationship between low ACTH and ACS at baseline and 2-year follow-up, and prevalence of low ACTH in patients, with and without ACS, compared with controls.

Paper II

Paper II was a cross-sectional study. Patients evaluated for an adrenal incidentaloma at any of the two study sites during the inclusion period were eligible for inclusion. In addition to shared exclusion criteria, patients treated with inhalation steroids or systemic oestrogen were excluded. Current smokers were defined as daily smokers. Data regarding duration, dose, or former smoking were not available. Endpoints were the association between smoking and ACS, adenoma size, and bilateral adenomas.

Paper III

A retrospective cohort study was executed. Patients evaluated for an adrenal incidentaloma at any of the two study sites during the inclusion period were eligible for inclusion. In addition to shared exclusion criteria, patients treated with inhalation steroids or systemic oestrogen were excluded. Patients were grouped according to predefined levels of cortisol$_{DST}$, <50, 50-82, 83-137, and ≥138 nmol/L. Patients with cortisol$_{DST}$ <50 nmol/L were the reference group.

The primary endpoint was all-cause mortality. Secondary endpoints were cause-specific mortality (cardiovascular, cancer, infection, or other causes), and major cardiovascular adverse event ((MACE), a composite of cardiovascular mortality, non-fatal myocardial infarction, non-fatal stroke, hospital admission due to heart failure, and coronary intervention in the form of percutaneous coronary intervention or coronary by-pass graft). Low ACTH (<2.0 pmol/L) was analysed as a predictor for all-cause mortality. The study period for the mortality endpoints was from inclusion until December 31st, 2018. For MACE the study period ended December 31st, 2017.

Paper IV

A retrospective matched cohort study was implemented. Patients evaluated for an adrenal incidentaloma at any of the two study sites during the inclusion period were eligible for inclusion. In addition to shared exclusion criteria, patients with systemic oestrogen treatment were excluded. Patients were grouped according to the same levels of cortisol$_{DST}$ as in paper III. Cortisol$_{DST}$ <50 nmol/L was defined as NFAA. Control subjects from the general population were recruited with the help of

Statistics Sweden (SCB). Controls were individually matched to each patient for age, sex, and residency in a 3:1 ratio. Outcomes were compared between patient groups and their respective controls. The primary endpoint was all-cause mortality, and the secondary was cause-specific mortality (cardiovascular, cancer, infection, or other causes). The study period ended December 31st, 2018.

Data collection

Patient data were collected by the authors (A.K, H.O, and O.L) from the electronic medical records. Imaging studies were assessed by the clinical radiologist and confirmed by two authors (A.K and H.O). Out-of-range values were examined, and database lock was performed before outcome data from external sources were obtained.

Outcome data

Data on all-cause and cause-specific mortality were obtained from the National Board of Health and Welfare Cause of Death Register. Data on the other components of MACE were obtained from the National Board of Health and Welfare National Inpatient Register.

Assays

Plasma cortisol was analysed using the same assay during the inclusion period, a 1-step competitive immunoassay (Roche Diagnostics). Reference range 171-536 nmol/L, coefficient of variation 2.1% at 94.9 nmol/L, and detection limit 0.5 nmol/L.

ACTH was measured using two different 2-step immunometric sandwich assays: Cobas, Roche Diagnostics, reference range 1.6-13.9 pmol/L, coefficient of variation 5.4% at 1.1 pmol/L, and detection limit 0.23 pmol/ L, and Nichols Institute Diagnostics, reference range 2.0-10.0 pmol/L, coefficient of variation 9% at 8.0 pmol/L, and detection limit 2.0 pmol/L. The Cobas assay was used in all patients included in Paper I.

Statistical analysis

Paper I

Patients were divided into four groups based on ACTH </≥2 pmol/L, and $cortisol_{DST}$ </≥ 50 nmol/L. Measured hormone levels and anthropometrics were non-normally distributed. The Chi-squared and Mann-Whitney tests were used when analysing categorical and continuous data respectively. Hormone values were ln-transformed when used in regression models. The correlation between ACTH and cortisol, $cortisol_{DST}$, and adenoma size was analysed with simple linear regression. Multivariable linear regression, adjusted for sex, was used to analyse differences in ACTH between patient groups and controls. A two-tailed P-value <0.05 was considered statistically significant. Continuous data are presented with medians and ranges. We used IBM SPSS Statistics for Windows, version 22.0 (Armonk, NY: IBM Corp), for the statistical analysis.

Paper II

Patients were grouped according to smoking status (yes/no). In between group analyses of categorical data were done using the Chi-squared test, and for continuous data the Mann-Whitney test was used. The prevalence of $cortisol_{DST}$ ≥50 nmol/L in smokers vs. non-smokers and unilateral vs. bilateral adenomas was studied using multivariable logistic regression adjusted for age. Multivariable logistic regression was also used in analysing the prevalence of $cortisol_{DST}$ ≥50 nmol/L and ACTH <2.0 pmol/L in smokers vs. non-smokers with unliteral adrenal adenomas, adjusted for sex, age, BMI, adenoma size, and in the case of low ACTH also for ln-$cortisol_{DST}$. A two-tailed P-value <0.05 was considered statistically significant. Continuous data are presented with medians and ranges. IBM SPSS Statistics for Windows, version 25.0 (Armonk, NY: IBM Corp), was used for the statistical analysis.

Paper III

Multivariable Cox regression was used to calculate hazard ratios (HR) and 95% CI for the primary endpoint, all-cause mortality, between patient groups. Two models were used, one adjusted for sex, age, previous cardiovascular disease, smoking, and reduced kidney function (eGFR <60 ml/min). In the second model outcomes were additionally adjusted for diabetes mellitus and medical history of cancer. The reference category was patients with $cortisol_{DST}$ <50 nmol/L. The proportional hazards assumption was tested with Schoenfeld residuals. Interaction analysis was performed by adding a 2-way interaction term to the Cox model. Sensitivity analysis was performed for the primary endpoint using E-values.(64)

The secondary endpoints, cause-specific mortality and MACE, were analysed using the Fine and Grey multivariable regression, which accounted for competing risks.(65)

The functional form of the association between $cortisol_{DST}$ and relative risk of all-cause mortality was assessed using restricted cubic splines. A 2-tailed P-value <0.05 was considered statistically significant. Missing values for ACTH were addressed with multiple imputation. Continuous data are presented with medians and interquartile ranges (IQR).

SAS software, version 9.4 (SAS Institute, Cary NC) was used for the statistical analysis, excluding E-value calculation which was done using a web-based calculator.(66)

Paper IV

Unadjusted cumulative all-cause mortality between patients and controls was visualized with Kaplan-Meier plots, and differences were tested with the log rank test. Multivariable Cox regression, adjusted for sex and age, was used to calculate HR and 95% CI between patient groups and their controls. Interaction analysis was performed by expanding the model with a 3-way full factorial term. The proportional hazards assumption was tested with Schoenfeld residuals.

Secondary endpoints were analysed with the Fine and Gray multivariable regression.

Continuous data are presented with medians and IQR.

Statistical analysis was performed using Stata Statistical Software: Release 17 (StataCorp. 2021. College Station, TX)

Ethics

All studies were approved by the regional ethical review board in Lund, Sweden. Studies constituting Papers III and IV were also registered at ClinicalTrials.gov (NCT03919734).

Main results

Paper I

Of 375 eligible patients, 198 were included in the study, and 177 were excluded by the exclusion criteria. The median age was 62.8 years (range, 24.7-74.2), and 54% were women. The median age in controls, 100 healthy blood donors, was 45 years (19-70), and 52% were male.

53 (27%) patients had $cortisol_{DST} \geq 50$ nmol/L, and ACTH was <2.0 pmol/L in 56 (28%) patients.

ACTH was significantly lower in patients with $cortisol_{DST}$ <50 nmol/L (n=145) compared with controls ($p<0.001$). ACTH <2.0 pmol/L was present in 19% of patients with $cortisol_{DST}$ <50 nmol/L, and in 4% of controls ($p<0.001$). (Figure 3)

53% of patients with $cortisol_{DST} \geq 50$ nmol/L had ACTH <2.0 pmol/L. (Figure 4)

ACTH correlated negatively to adenoma size in patients with $cortisol_{DST}$ <50 nmol/L ($p=0.002$)

Patients were divided into four groups based on $cortisol_{DST}$ </≥50 nmol/L, and ACTH </≥ 2.0 pmol/L at inclusion. Follow-up data after two years were available in 106 patients. Differences between groups regarding ACTH and $cortisol_{DST}$ were still statistically significant at follow-up.

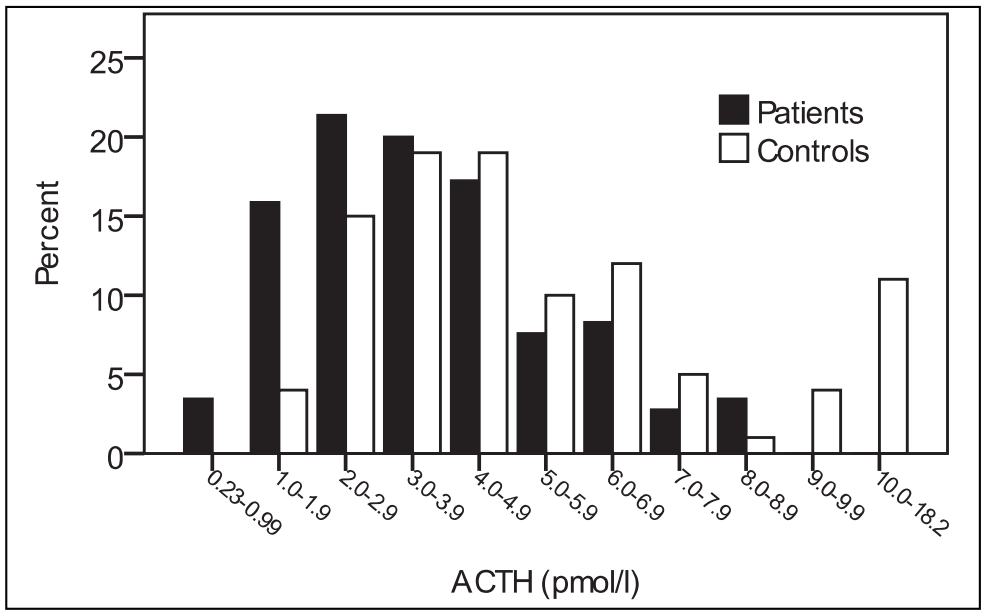

Figure 3 Distribution of ACTH in 145 patients with cortisol$_{DST}$ <50 nmol/L and 100 control subjects
ACTH was lower in patients than in controls (p<0.001).(67)

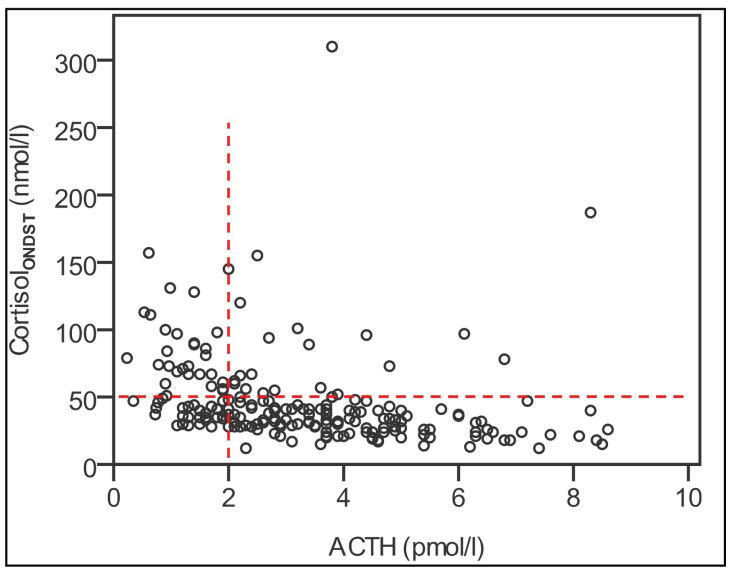

Figure 4 Cortisol$_{DST}$ in relation to ACTH in 198 patients with adrenal adenomas
ACTH was <2.0 pmol/L in 53% of patients with cortisol$_{DST}$ ≥50 nmol/L, and in 19% of patients with cortisol$_{DST}$ <50 nmol/L.Cortisol$_{ONDST}$ = cortisol$_{DST}$.(67)

Paper II

1594 patients were enrolled in the study, 550 were excluded based on the exclusion criteria, leaving 1044 patients in the final analysis. The median age was 64.8 years and 59% were women.

35% of patients were smokers. Smokers were younger, had lower BMI, larger unilateral adrenal adenomas, more often bilateral adrenal adenomas, lower ACTH, and higher $cortisol_{DST}$ than non-smokers. However, smokers had a lower prevalence of hypertension (treatment), diabetes mellitus, and treatment for dyslipidaemia than non-smokers. (Table 1)

Smokers had a higher prevalence of $cortisol_{DST}$ ≥50 nmol/L. In patients with unilateral adrenal adenomas, smoker´s adjusted odds ratio of having $cortisol_{DST}$ ≥50 nmol/L was 1.68 (CI, 1.21-2.33).

In the patient cohort, smoking was more prevalent in both men and women of all age groups when compared with the general population.(68)

Table 1 Patient characteristics and comparison between smokers and non-smokers
Continous data are presented with medians and range, and chategorical data as percentages. P-values are derived from comparison of non-smokers vs. smokers. BMI: body mass index.

[a] Available in 1021 patients.

[b] Available in 741 patients.

[c] Available in 761 patients.(69)

	All patients	Non-smokers	Smokers	p
Numbers	1044	674	370	
Women, %	59	56	64	0.009
Age, years	64.8(24.4-88.7)	66.3(24.4-88.7)	61.5(24.7-88.2)	<0.0001
BMI, kg/m^2 [a]	27.4(12.8-55.2)	28.1(12.8-50.4)	26.5(15.0-55.2)	<0.0001
Bilateral adrenal adenoma, %	16	12	22	0.0001
Unilaterall adenoma size, mm	20 (10-72)	19 (10-70)	22 (10-72)	0.0002
Cortisol, nmol/L [b]	473 (131-1299)	473 (131-1299)	472 (157-1244)	0.81
ACTH, pmol/L [c]	3.1(<0.23-26.0)	3.7(<0.23-26.0)	2.0(<0.23-11.0)	<0.0001
$Cortisol_{DST}$, nmol/L	46 (9-487)	43 (9-487)	53 (11-449)	<0.0001
$Cortisol_{DST}$ ≥50, %	45	40	54	<0.0001
Hypertension (treatment), %	52	57	44	<0.0001
Diabetes mellitus, %	19	21	15	0.017
Dyslipidaemia (treatment), %	31	35	23	<0.0001

Paper III

During the inclusion period, 1593 consecutive patients who met the inclusion criteria were enrolled, of which 545 were excluded according to the exclusion criteria, leaving 1048 patients in the study. 58.5% of patients were women, and the median age was 64.9 years (IQR, 54.4-69.6). 575 (54.9%) patients had $cortisol_{DST}$ <50 nmol/L, and 473 (45.1%) ≥50 nmol/L. Patients with $cortisol_{DST}$ ≥50 nmol/L were older, had a higher prevalence of hypertension (treatment), diabetes mellitus, and cardiovascular disease, and were more often smokers than patients with $cortisol_{DST}$ <50 nmol/L. (Table 2)

During a median follow-up period of 6.4 years, 170 patients died. Results for the primary endpoint all-cause mortality showed that when compared with $cortisol_{DST}$ <50 nmol/L, $cortisol_{DST}$ 50-82 was not associated with increased mortality (adjusted HR 1.17 (CI, 79-1.73)), while a $cortisol_{DST}$ 83-137, or ≥138 nmol/L was associated with significantly increased mortality, adjusted HR 2.33 (1.53-3.53), and 2.87 (1.74-4.74) respectively. (Table 2)

Analysis of the secondary endpoint, cause-specific mortality, showed a significantly increased risk of cardiovascular mortality in patients with $cortisol_{DST}$ ≥83 nmol/L compared with $cortisol_{DST}$ <50 nmol/L, adjusted HR 2.33 (1.27-4.28). There were no significant differences in mortality due to cancer, infections, or other causes.

Median follow-up for the composite endpoint MACE was 5.17 years, during which 151 patients had at least 1 MACE event. Patients with $cortisol_{DST}$ ≥138 nmol/L had a significantly increased risk of MACE (adjusted HR 2.41 (1.45-4.02)), while there were no significant differences in patients with $cortisol_{DST}$ 50-82, or 83-137 nmol/L compared with patients with $cortisol_{DST}$ <50 nmol/L.

The functional form of the association between $cortisol_{DST}$ and adjusted risk of all-cause mortality was analysed with restricted cubic splines. The result indicates that the association is linear, at least up to a $cortisol_{DST}$ level of 200 nmol/L. (Figure 5)

HR for all-cause mortality was 1.02 (0.65-1.59) in patients with ACTH <2.0 pmol/L compared with ACTH ≥2.0 pmol/L. HR adjusted for sex, age, previous cardiovascular disease, smoking, reduced kidney function, and ln-transformed $cortisol_{DST}$.

Table 2 Patient characteristics and mortality during follow-up

Continous data are presented with medians and IQR. Reference group (ref) in mortality analysis was $cortisol_{DST}$ <50 nmol/L. Hazard ratios (HR) adjusted for age, sex, smoking, previous cardiovascular disease, eGFR <60ml/min, medical history of cancer, and diabetes mellitus.

BMI: body mass index. eGFR: estimated glomerular filtration rate.

[a] Available in the 4 patient groups in 558, 268, 118, and 81 patients, respectively.

[b] Available in the 4 patient groups in 410, 206, 88, and 58 patients, respectively. (70)

	$Cortisol_{DST}$ <50 nmol/L	$Cortisol_{DST}$ 50–82 nmol/L	$Cortisol_{DST}$ 83–137 nmol/L	$Cortisol_{DST}$ ≥138 nmol/L
Numbers	575	272	119	82
Women, %	56.0	62.9	64.7	52.4
Age, years	62.8 (54.4-69.6)	65.8 (60.6–73.9)	68.0 (60.7–75.1)	69.1 (61.3–74.6)
BMI, kg/m^2 [a]	28.2 (25.3–32.0)	26.9 (23.7–30.8)	27.0 (23.6–30.7)	25.7 (21.4–29.4)
Current smoker, %	29.6	38.6	48.7	48.8
Hypertension (treatment), %	44.7	58.8	67.2	69.5
Diabetes mellitus, %	16.5	22.1	22.7	17.1
Cardiovascular disease, %	17.0	23.2	27.7	26.8
eGFR <60 ml/min/1.73 m^2, %	8.0	19.1	21.8	25.6
Medical history of cancer, %	16.0	17.6	22.7	25.6
ACTH <2.0 pmol/L, % [b]	15.9	33.0	48.9	53.4
Mortality, % (n)	10.8 (62)	17.3 (47)	31.1 (37)	29.3 (24)
HR (95% CI), all-cause mortality	ref	1.17 (0.79–1.73)	2.33 (1.53–3.53)	2.87 (1.74–4.74)

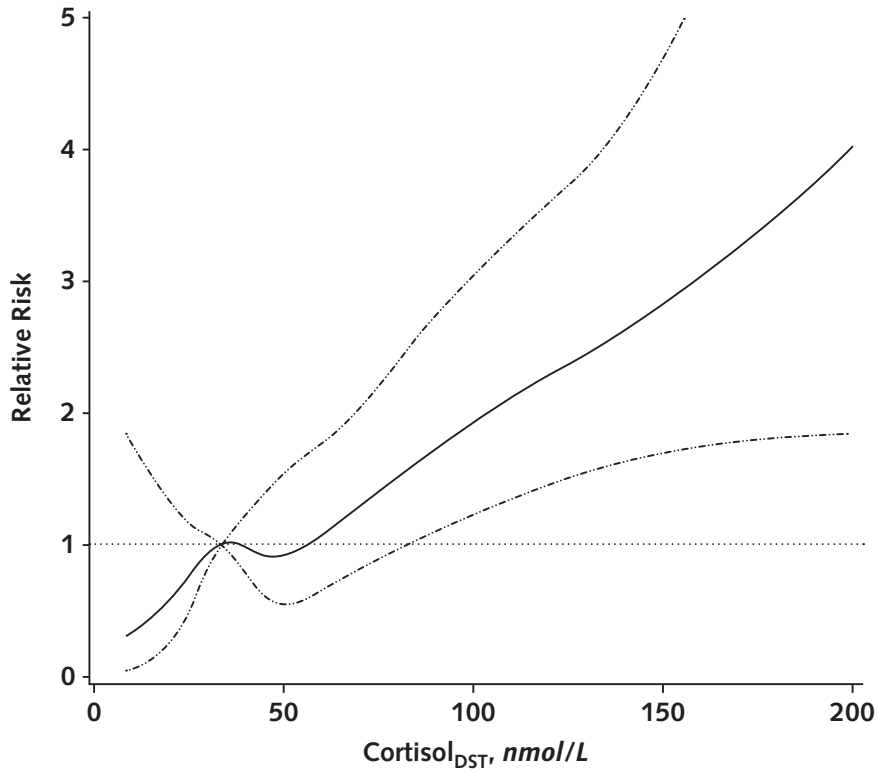

Figure 5 Functional form of the association between cortisol$_{DST}$ and relative risk of all-cause mortality

Population median of cortisol$_{DST}$ in the cortsiol$_{DST}$ <50 nmol/L group (34 nmol/L) was set as relative risk of one.(70)

Paper IV

In this study, 1154 patients and 3462 controls were included. (Figure 6) Controls were well-matched, median age was 65.1 years, and 59.5% were women in both the patient and the control group.

The median follow-up was 6.3 years (IQR, 4.3-8.7) in patients, and 6.7 years (4.7-9.1) in controls, during which 210 (18,2%) patients and 505 (14.6%) controls died.

There was no statistically significant difference in all-cause mortality between patients with NFAA or $cortisol_{DST}$ 50-82 nmol/L and their controls, adjusted HR 1.13 (0.87-1.46) and 1.13 (0.85-1.51), respectively. In patients with $cortisol_{DST}$ 83-137 or ≥138 nmol/L, mortality was increased compared with controls, HR 1.99 (1.38-2.88) and 4.09 (2.41-6.93) respectively. (Figure 7)

In the $cortisol_{DST}$ 50-82 nmol/L group, interaction analysis showed a significant interaction between the age and patient variables and all-cause mortality. Subgroup-analysis was performed, dividing patients with $cortisol_{DST}$ 50-82 nmol/L and their controls into two groups, </≥ 65 years of age. Results showed a significantly increased mortality risk in patients <65 years compared with controls, HR 2.33 (1.30-4.17), while no significant difference was seen in patients ≥ 65 years compared with controls (HR 0.92 (0.65-1.28).

Cardiovascular mortality was increased in patients with $cortisol_{DST}$ 83-137 and ≥138 nmol/L compared with controls, HR 1.96 (1.10-3.49) and 4.28 (1.81-10.15) respectively. In patients with $cortisol_{DST}$ ≥83 nmol/L cancer-associated mortality was also increased compared with controls, HR 2.01 (1.12-3.62). (Table 3)

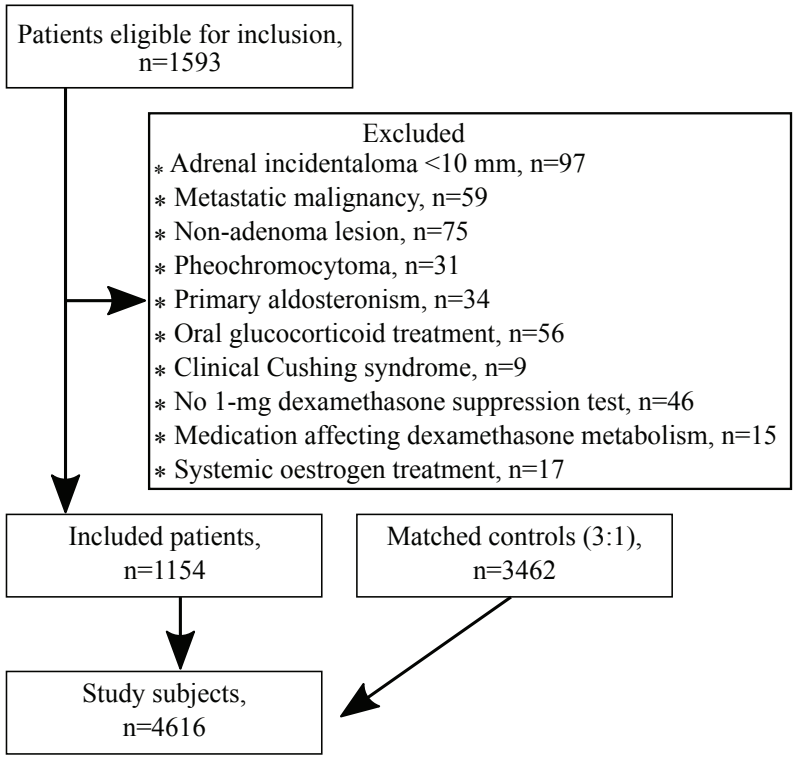

Figure 6 Inclusion flow diagram (71)

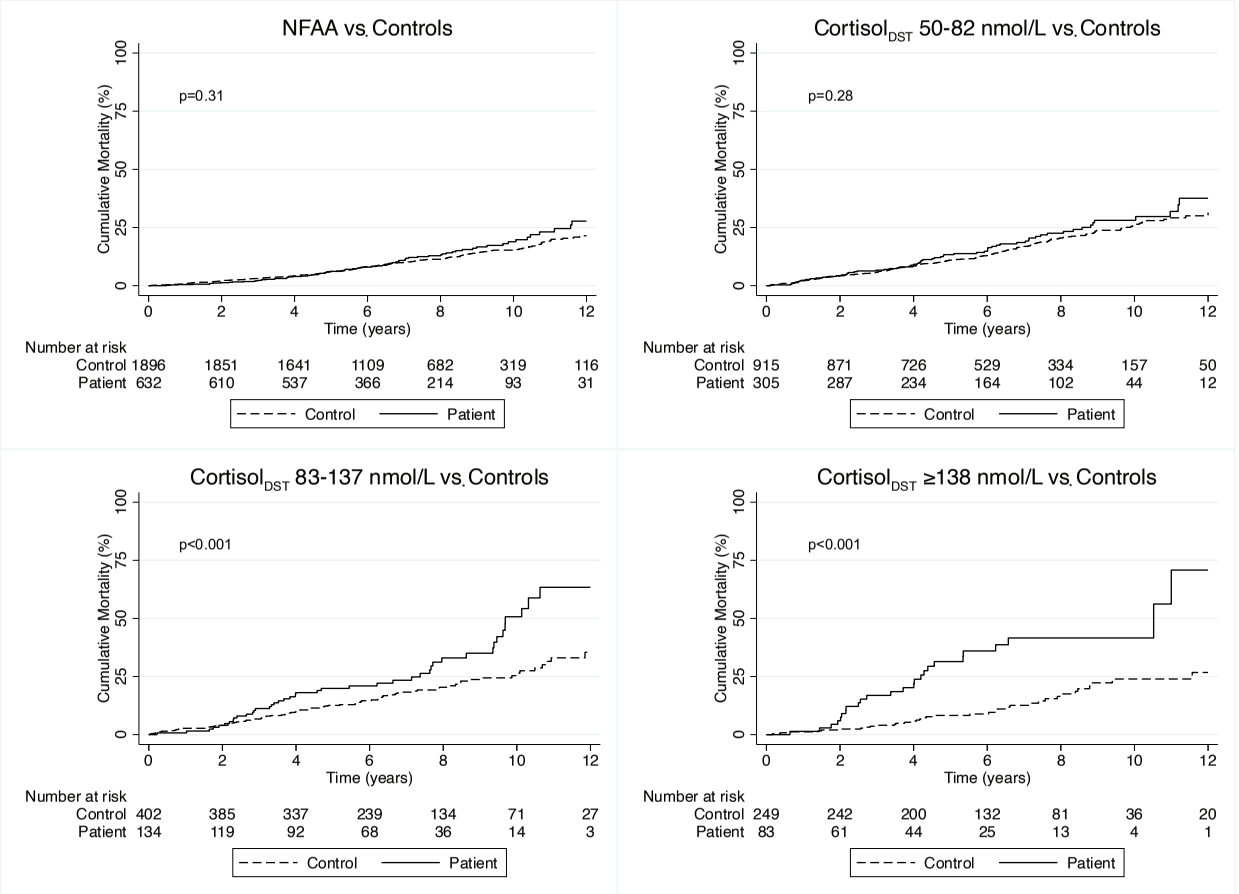

Figure 7 Kaplan-Meier plots of all-cause mortality between patient groups and controls
P-values derived from the log rank test.(71)

Table 3 Hazard ratios for all-cause mortality and cause-specific mortality

Hazard ratios (HR) and 95% CI for patient groups compared with controls. HR adjusted for sex and age.(71)

	All-cause mortality	Cardiovascular mortality	Cancer mortality	Infection mortality	Other mortality
	HR (95% CI)				
NFAA ($Cortisol_{DST}$ <50 nmol/L)	1.13 (0.87-1.46)				
$Cortisol_{DST}$ 50-82 nmol/L	1.13 (0.85-1.51)				
$Cortisol_{DST}$ 83-137 nmol/l	1.99 (1.38-2.88)	1.96 (1.10-3.49)	1.83 (0.93-3.61)	1.03 (0.21-5.09)	1.59 (0.78-3.27)
$Cortisol_{DST}$ ≥138 nmol/L	4.09 (2.41-6.93)	4.28 (1.81-10.15)	2.83 (0.88-9.09)	1.26 (0.12-13.53)	2.02 (0.87-4.68)
$Cortisol_{DST}$ <83 nmol/L	1.13 (0.93-1.37)				
$Cortisol_{DST}$ ≥83 nmol/L	2.48 (1.84-3.35)	2.51 (1.56-4.04)	2.01 (1.12-3.62)	1.08 (0.29-4.06)	1.73 (1.00-2.99)

Discussion

The principal findings of this thesis are that ACS is an independent risk factor for increased mortality, primarily caused by cardiovascular disease, and that the association between mortality risk and degree of ACS seems linear. It also provides evidence that NFAA does not seem to pose a clinically relevant risk to the affected patients. Smoking prevalence was higher in patients with adrenal incidentalomas than in the general population. Smoking was associated with larger adrenal adenomas, bilateral adenomas, and ACS, which gives rise to questions on the effect of smoking on adrenal physiology. The finding of the previously not described group of patients with low basal ACTH and normal DST provides a possible explanation for the limited value of ACTH in diagnosing ACS. It also raises questions on tumour physiology of adrenal adenomas, and the notion that cortisol secretion in ACS is solely autonomous.

Role of ACTH

Low ACTH has been suggested to indicate ACS and was often included in older diagnostic criteria.(72, 73) However, when using $cortisol_{DST}$ as the primary test for ACS, low ACTH has been found to have limited diagnostic value.(74, 75) Results from **Paper I** corroborate this, with only approximately half of patients with $cortisol_{DST}$ ≥50 nmol/L having low ACTH. DST is in no way a perfect test and one could argue that a high rate of false positives might cause these results. However, this risk was limited by the exclusion criteria containing common causes of false positive DST, such as old age, low body mass index, and reduced renal function.(76-79) Low ACTH showed no significant association with all-cause mortality in **Paper III**, further underlining its limitations.

The most interesting finding in **Paper I** was that an unexpectedly large group of patients with $cortisol_{DST}$ <50 nmol/L had low ACTH compared with controls. The difference between patients and controls was still significant when using a lower $cortisol_{DST}$ cut-off (<40 nmol/L), indicating that the high frequency of low ACTH was not caused by discrete cortisol hypersecretion. In patients with $cortisol_{DST}$ <50 nmol/L and low ACTH, the cortisol/ACTH ratio was increased, and a negative correlation was found between adenoma size and ACTH, which implies increased responsiveness to ACTH and that the factor behind the low ACTH is associated with the adrenal adenoma. The hypothesis of increased responsiveness to ACTH as

a possible explanation for the high rate of low ACTH is supported by previous findings. Adrenal adenomas have functional ACTH receptors in increasing numbers related to the degree of hypercortisolism. The cortisol response to ACTH stimulation test in patients with adrenal incidentalomas has been shown to have an inverse relationship to basal ACTH, and patients with NFAA have a stronger cortisol response to ACTH stimulation than controls.(63, 80-83) Results in **Paper I** cannot prove that increased ACTH responsiveness is the cause behind the large group of patients with low basal ACTH, and other unidentified factors are possible. However, regardless of cause, our findings have clinical implications.

Clinical implications

Low ACTH is frequent even in patients with adrenal incidentalomas and normal $cortisol_{DST}$. This finding provides yet another explanation of the limited diagnostic value of ACTH. If low ACTH is used as a diagnostic marker for ACS, it could lead to both false positives and negatives. False positive in the case of a patient with low ACTH and falsely elevated $cortisol_{DST}$ due to, for example, fast dexamethasone metabolism. False negative in the case of a patient with ACS not resulting in a suppression of the HPA-axis.

Repeat DST in some form is already recommended in patients with $cortisol_{DST}$ ≥50 nmol/L.(21) Based on findings in **Paper I,** patients with $cortisol_{DST}$ ≥50 nmol/L should undergo simultaneous measurement of dexamethasone during repeat DST, or if this assay is not available undergo a 48-h DST (0,5 mg dexamethasone four times daily for 48 h) to ensure adequate suppression of the HPA-axis during DST, reducing the rate of false positives.(84, 85) This is especially important in patients with low basal ACTH.

While ACTH has a limited role in diagnosing ACS it is still important in evaluating a patient with an adrenal incidentaloma, not least in the differential diagnostics of Cushing's disease. ACTH should be measured both before and after DST. In patients with basal ACTH in the normal range, ACTH after DST can be used to assess HPA-axis suppression during the test. A low ACTH after DST (<0,6 pmol/L) indicate adequate HPA-axis suppression and makes the DST result reliable, after considering other sources of error.(86)

Smoking

The prevalence of smoking was high in patients with adrenal incidentalomas. In **Paper II** we observed that patients with adrenal incidentalomas were more often smokers compared with the general population regardless of sex or age group. Smoking was associated with larger adrenal adenomas, bilateral adenomas, $cortisol_{DST}$ ≥50 nmol/L, and low ACTH. Inference regarding causality cannot be made from a cross-sectional study, but the findings raise several interesting

questions. The most obvious is if smoking is a risk factor for developing adrenal adenomas. While smoking is a well-established risk factor for developing malignant tumours there are no previously published data on a possible link to adrenal adenomas. A few studies indicate that smoking is associated with benign tumours in other organs, such as thyroid, colon, and parotid.(87-89) An effect of smoking on the HPA-axis, is on the other hand, reported in numerous papers. Cortisol levels are reported to be higher in smokers, often measured by repeated samples of saliva cortisol during the day.(90, 91) Smoking, and more precise nicotine, leads to HPA-axis activation through the hypothalamus, even if the exact mechanism is unclear.(92) Additionally, evidence suggests that nicotine might have a direct stimulatory effect on steroidogenesis in the adrenal cortex.(93) Some evidence also suggests that smoking leads to activation of the sympathetic nervous system.(92, 94) The latter could be of importance since increased activity in the splanchnic nerve, innervating the adrenal medulla, have been shown to increase ACTH sensitivity in adrenocortical cells in rats.(95) If this is the case in humans, it could explain the association between smoking and low ACTH observed in **Paper II**. It might also partially explain the large group of patients with low ACTH and cortisol$_{DST}$ <50 nmol/L identified in **Paper I**. However, it does not explain the association between smoking and cortisol$_{DST}$ ≥50 nmol/L. Studies on DST in smokers are few and results are conflicting.(96, 97) Neither is there evidence that smoking affects dexamethasone metabolism.(84, 98) Taken together this might signify that smoking is a risk factor for ACS by mechanisms yet to be revealed.

Mortality risk associated with ACS

Cortisol hypersecretion in ACS is continuous. Even though the association between mortality risk and ACS had been indicated in previous studies, (56-58) questions remained on at what magnitude ACS becomes clinically relevant.(59, 60, 99) In **Paper III** we aimed to explore this question. In contrast to previous studies, we analysed mortality at three different levels of ACS. Cortisol$_{DST}$ cut-off levels were selected based on prior studies indicating cardiometabolic risk at these levels.(21, 41, 100) Results showed that patients with discrete ACS (cortisol$_{DST}$ 50-82 nmol/L) did not have an increased mortality risk, while the risk was increased twofold in patients with cortisol$_{DST}$ 83-137 nmol/L, and threefold in patients with cortisol$_{DST}$ ≥138 nmol/L compared with patients with cortisol$_{DST}$ <50 nmol/L. Analysis of the functional form of the association between cortisol$_{DST}$ and mortality risk revealed a linear association, further strengthening the notion of a dose-dependent response. The increased mortality risk seems to be driven by an increased risk of cardiovascular disease. Patients with cortisol$_{DST}$ ≥83 nmol/L exhibited a higher risk of cardiovascular mortality, and those with cortisol$_{DST}$ ≥138 nmol/L showed an increased risk of major adverse cardiovascular events (MACE).

All studies on mortality risk in patients with ACS have used patients with NFAA ($cortisol_{DST}$ <50 nmol/L) as the reference group, thus providing limited information regarding the potential risk associated with NFAA.(56-58, 70, 101) The question of potential risks associated with NFAA has come into focus following studies giving indirect evidence of increased cardiometabolic risk in patients with NFAA.(62)

Paper IV presents results of a matched cohort study designed to investigate this question. Results showed no increase in mortality risk in patients with NFAA, nor $cortisol_{DST}$ 50-82 nmol/L, compared with matched controls. While this does not exclude that a difference exists, we deem the large number of included patients and controls to provide sufficient power to detect a clinically relevant difference between groups.(102-104) The many metabolic alterations reported in patients with NFAA might still be associated with discrete cortisol hypersecretion given the continuous nature of ACS. However, the results from **Paper IV** suggest that these alterations do not entail a clinically relevant increase in mortality.(63, 105-108)

All-cause mortality, as well as cardiovascular and cancer-associated mortality, were increased in patients with $cortisol_{DST}$ ≥83 nmol/L compared with controls. The association between ACS and cancer-related mortality has been shown in one previous study.(58) Several mechanisms could support this relationship. Systemic glucocorticoid treatment has been linked to an increased risk of several cancers in humans, possibly through an anti-apoptotic effect involving the glucocorticoid receptor.(109-115) Alterations in endogenous cortisol regulation have been associated with cancer-related mortality, tumour progression, and cancer relapse in humans. However, whether these observations signify a causative role of cortisol regulation in tumour progression in humans remains to be clarified.(116, 117)

Results of **Paper IV** also suggest that there might be an age-related disparity regarding the risk associated with ACS. In the $cortisol_{DST}$ 50-82 nmol/L group, patients <65 years of age had increased mortality risk, while no increase in risk was observed in patients ≥65 years compared with controls. This may infer that ACS cause more severe consequences in younger patients. The HPA-axis is subject to changes related to aging; cortisol levels increase, the diurnal variability is decreased, and the HPA-axis responsiveness is altered.(118) Hypothetically this could explain a more severe phenotype of ACS in younger individuals. The signal of age-related disparity partly supports previous findings of research in a large multicentre study, suggesting that especially women under the age of 65 years with ACS are at increased risk of excess mortality.(101) However, conclusions drawn from sub-group analyses should be made with caution.

The study populations in **Paper III** and **IV** consisted of consecutively included patients from two hospitals in southern Sweden. Sweden has a universal healthcare system, and the study sites covered all inhabitants in the catchment areas of each hospital. These factors minimize the risk of selection bias and render strong external validity. Patients were grouped according to prespecified levels of $cortisol_{DST}$, and

cortisol was analysed using the same assay in all patients during the entire study period. Due to the study design, there is a risk of unmeasured bias. However, sensitivity analysis of the observed association between ACS and all-cause mortality in **Paper III** showed that it is unlikely that an unmeasured confounder would explain the findings. The primary outcome, all-cause mortality, was based on data obtained from the Cause of Death Register, which is more than 99% complete.(119) In **Paper IV,** controls were matched for residency, reducing the potential influence of socioeconomic factors.(120) Taken together, we consider the main results from **Paper III** and **IV** to have high internal validity.

The results from **Paper III** add to previous evidence of an association between ACS and mortality risk and provide evidence that ACS is an independent linear risk factor for mortality, the risk becoming clinically relevant at $cortisol_{DST} \geq 83$ nmol/L. **Paper IV** shows that NFAA does not seem to pose a clinically relevant risk to the afflicted patients and confirms that $cortisol_{DST} \geq 83$ nmol/L seems to be a relevant threshold.

Clinical implications

Patients with ACS should be regarded as having high cardiovascular risk. Until specific treatment for ACS is available, patients should be offered lifestyle intervention and pharmacological treatment of classical cardiovascular risk factors. Intervention should be aimed at patients who benefit most, namely those with $cortisol_{DST} \geq 83$ nmol/L and younger patients with $cortisol_{DST}$ 50-82 nmol/L. Patients with NFAA should be given a reassuring message and do not need any follow-up.

However, data implies that patients with ACS probably have a residual increase in risk even after optimal treatment of risk factors such as hypertension and diabetes mellitus.(51) This residual risk can be suspected to be linked to specific cortisol effects. The effect of cortisol excess on, for example, myocardial cells has shown to be associated with more severe fibrosis and more pronounced hypertrophy in patients with cortisol excess compared with blood pressure matched controls.(12) This highlights the need for intervention studies specific to ACS. So far, studies on adrenalectomy are few and conflicting, and specific pharmacological treatment, while promising, is still in its infancy.(121-126) At present we are referred to treatment of known risk factors, and it is not possible to make a general recommendation on which patients might benefit from adrenalectomy. A multidisciplinary team still needs to make recommendations for surgery on an individual basis. However, the evidence of ACS being an independent linear risk factor for increased mortality should be considered in the decision-making.

Limitations

Paper I was a retrospective cohort study, a design accompanied by the risk of unmeasured bias. As discussed, smoking might be such a factor and to better elucidate this, controls matched for smoking habits would have been beneficial. The study provides indirect evidence that increased responsiveness to ACTH might explain the large group of patients with low ACTH and normal DST. This hypothesis could have been tested by performing an ACTH stimulation test.

In up to 10% of the population, adequate levels of dexamethasone are not achieved during the DST. This can be caused by, for example, rapid metabolism of dexamethasone due to a polymorphism in the cytochrome P450 system or variable gastrointestinal absorption, which can result in a false positive DST.(84, 85, 127, 128) This potential source of error was not checked for in **Paper I.** This can be done by simultaneous measurement of dexamethasone during DST**.** However, even after considering this, an ACTH in the normal range does not let us exclude the presence of ACS in a patient with $cortisol_{DST} \geq 50$ nmol/L.

The main limitation of **Paper II** is the lack of information on former smoking, duration, and dose of smoking, which hampered the analysis of the association between smoking and adrenal incidentalomas with and without ACS. As previously mentioned, the cross-sectional design does not allow inference on causality, and the observations in **Paper II** could be caused by case selection. Smoking is accompanied by co-morbidity, and as a group, smokers probably undergo radiological imaging more often than non-smokers, possibly leading to a higher detection rate of adrenal incidentalomas in smokers. If ACS and smoking have synergistic detrimental effects on co-morbid conditions their coexisting might also lead to an increased detection rate in affected patients.

The diagnosis of ACS was based on results from one DST with the risk of a proportion of patients with ACS being false positives. In **Papers III** and **IV** this would lead to underestimating the risk associated with ACS. Length of follow-up limits inference regarding risks associated with ACS beyond the scope of approximately ten years. When interpreting results of the secondary endpoints in **Papers III** and **IV**, one should be aware that the specific cause of death reported in the Cause of Death Register and diagnosis in the Inpatient Register is inaccurate in up to 20% of cases.(119, 129, 130)

Patients referred to the study sites were consecutively included but it is possible that the frailest patients with adrenal incidentalomas were not referred, which would lead to referral bias. Since controls were collected from the general population without respect to comorbidities this might result in patients having a healthy entrant effect in **Paper IV**. However, results from post hoc analysis, excluding patients and controls with observation time shorter than 12 and 24 months, did not differ significantly from the main results.

Conclusion

This thesis provides evidence that ACS is a linear independent risk factor for increased mortality, and the risk seems to become clinically relevant at $cortisol_{DST}$ ≥83 nmol/L. On the other hand, the presence of an NFAA does not pose a relevant risk. The DST is the only test to repeatedly demonstrate a significant correlation between results and mortality risk that is of clinical value. Smoking seems more prevalent in patients with adrenal adenomas and is associated with adenoma size, bilateral adenomas, and ACS.

The clinical implications are that patients with ACS should be viewed as having high cardiovascular risk and vigilance is recommended based on the signal of a potential link between ACS and cancer. Follow-up and treatment of classical cardiovascular risk factors are recommended, especially in patients with $cortisol_{DST}$ ≥83 nmol/L and younger patients with ACS. Knowledge of the risks associated with ACS should be considered when discussing surgical intervention. Patients with NFAA can be given a reassuring message and is not to be put through follow-up.

Future perspective

Findings in the thesis raise new questions, such as a possible link between smoking and both adrenal tumour genesis and ACTH responsiveness. An important question for future research is whether ACS is involved in cancer genesis and progression.

The substantial body of evidence of ACS being a dose-dependent risk factor for morbidity and mortality highlights the need for intervention studies. The most pressing question for clinicians seeing patients with ACS is what treatment to offer. As previously described, available studies on adrenalectomy offer little guidance. Current guidelines advocate that adrenalectomy could be an option in patients with ACS and co-morbidities that can be referred to hypercortisolism, such as diabetes mellitus.(21) However, it is not certain that patients with ACS without co-morbidities would not benefit from surgery. In Cushing's syndrome, disease duration is associated with overall mortality and morbidity risk, and early treatment is recommended to decrease residual risk after remission.(12) If this is true also in ACS is uncertain but further emphasises the pressing need for intervention studies. I believe that international collaboration is required to complete a study on adrenalectomy in patients with ACS, which would provide clinically relevant outcome data within a reasonable time frame.

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References

1. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature. 2002;418(6901):935-41.
2. Lightman SL, Conway-Campbell BL. The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. Nature Reviews Neuroscience. 2010;11(10):710-8.
3. Keller-Wood ME, Dallman MF. Corticosteroid inhibition of ACTH secretion. Endocr Rev. 1984;5(1):1-24.
4. Sheng JA, Bales NJ, Myers SA, Bautista AI, Roueinfar M, Hale TM, et al. The Hypothalamic-Pituitary-Adrenal Axis: Development, Programming Actions of Hormones, and Maternal-Fetal Interactions. Front Behav Neurosci. 2020;14:601939.
5. Rosen J, Miner JN. The search for safer glucocorticoid receptor ligands. Endocr Rev. 2005;26(3):452-64.
6. Gomez-Sanchez E, Gomez-Sanchez CE. The multifaceted mineralocorticoid receptor. Compr Physiol. 2014;4(3):965-94.
7. Hiller-Sturmhöfel S, Bartke A. The endocrine system: an overview. Alcohol Health Res World. 1998;22(3):153-64.
8. Neary N, Nieman L. Adrenal insufficiency: etiology, diagnosis and treatment. Curr Opin Endocrinol Diabetes Obes. 2010;17(3):217-23.
9. Melmed S, Auchus RJ, Goldfine AB, Koenig RJ, Rosen CJ. Williams textbook of endocrinology. 14th edition. ed. Philadelphia: Elsevier; 2020.
10. Téblick A, Gunst J, Van den Berghe G. Critical Illness-induced Corticosteroid Insufficiency: What It Is Not and What It Could Be. J Clin Endocrinol Metab. 2022;107(7):2057-64.
11. Cushing H. The basophil adenomas of the pituitary body. Ann R Coll Surg Engl. 1969;44(4):180-1.
12. Pivonello R, Isidori AM, De Martino MC, Newell-Price J, Biller BM, Colao A. Complications of Cushing's syndrome: state of the art. Lancet Diabetes Endocrinol. 2016;4(7):611-29.
13. Isidori AM, Graziadio C, Paragliola RM, Cozzolino A, Ambrogio AG, Colao A, et al. The hypertension of Cushing's syndrome: controversies in the pathophysiology and focus on cardiovascular complications. J Hypertens. 2015;33(1):44-60.
14. Pereira AM, Delgado V, Romijn JA, Smit JW, Bax JJ, Feelders RA. Cardiac dysfunction is reversed upon successful treatment of Cushing's syndrome. Eur J Endocrinol. 2010;162(2):331-40.

15. Albiger N, Testa RM, Almoto B, Ferrari M, Bilora F, Petrobelli F, et al. Patients with Cushing's syndrome have increased intimal media thickness at different vascular levels: comparison with a population matched for similar cardiovascular risk factors. Horm Metab Res. 2006;38(6):405-10.
16. Dekkers OM, Horváth-Puhó E, Jørgensen JO, Cannegieter SC, Ehrenstein V, Vandenbroucke JP, et al. Multisystem morbidity and mortality in Cushing's syndrome: a cohort study. J Clin Endocrinol Metab. 2013;98(6):2277-84.
17. Pivonello R, Faggiano A, Lombardi G, Colao A**.** The metabolic syndrome and cardiovascular risk in Cushing's syndrome. Endocrinol Metab Clin North Am. 2005;34(2):327-39, viii.
18. Colao A, Pivonello R, Spiezia S, Faggiano A, Ferone D, Filippella M, et al. Persistence of increased cardiovascular risk in patients with Cushing's disease after five years of successful cure. J Clin Endocrinol Metab. 1999;84(8):2664-72.
19. Papakokkinou E, Olsson DS, Chantzichristos D, Dahlqvist P, Segerstedt E, Olsson T, et al. Excess Morbidity Persists in Patients With Cushing's Disease During Long-term Remission: A Swedish Nationwide Study. J Clin Endocrinol Metab. 2020;105(8).
20. Clayton RN, Jones PW, Reulen RC, Stewart PM, Hassan-Smith ZK, Ntali G, et al. Mortality in patients with Cushing's disease more than 10 years after remission: a multicentre, multinational, retrospective cohort study. Lancet Diabetes Endocrinol. 2016;4(7):569-76.
21. Fassnacht M, Arlt W, Bancos I, Dralle H, Newell-Price J, Sahdev A, et al. Management of adrenal incidentalomas: European Society of Endocrinology Clinical Practice Guideline in collaboration with the European Network for the Study of Adrenal Tumors. Eur J Endocrinol. 2016;175(2):G1-G34.
22. Shamma AH, Goddard JW, Sommers SC**.** A study of the adrenal status in hypertension. J Chronic Dis. 1958;8(5):587-95.
23. Hedeland H, Ostberg G, Hokfelt B**.** On the prevalence of adrenocortical adenomas in an autopsy material in relation to hypertension and diabetes. Acta Med Scand. 1968;184(3):211-4.
24. Bovio S, Cataldi A, Reimondo G, Sperone P, Novello S, Berruti A, et al. Prevalence of adrenal incidentaloma in a contemporary computerized tomography series. J Endocrinol Invest. 2006;29(4):298-302.
25. Reimondo G, Castellano E, Grosso M, Priotto R, Puglisi S, Pia A, et al. Adrenal Incidentalomas are Tied to Increased Risk of Diabetes: Findings from a Prospective Study. J Clin Endocrinol Metab. 2020;105(4).
26. Hammarstedt L, Muth A, Wängberg B, Björneld L, Sigurjónsdóttir HA, Götherström G, et al. Adrenal lesion frequency: A prospective, cross-sectional CT study in a defined region, including systematic re-evaluation. Acta Radiol. 2010;51(10):1149-56.
27. Lacroix A, Feelders RA, Stratakis CA, Nieman LK**.** Cushing's syndrome. Lancet. 2015;386(9996):913-27.
28. Maillet M, Bourdeau I, Lacroix A**.** Update on primary micronodular bilateral adrenocortical diseases. Curr Opin Endocrinol Diabetes Obes. 2020;27(3):132-9.

29. Chevalier B, Vantyghem MC, Espiard S**.** Bilateral Adrenal Hyperplasia: Pathogenesis and Treatment. Biomedicines. 2021;9(10).
30. Beuschlein F, Fassnacht M, Assié G, Calebiro D, Stratakis CA, Osswald A, et al. Constitutive activation of PKA catalytic subunit in adrenal Cushing's syndrome. N Engl J Med. 2014;370(11):1019-28.
31. Cao Y, He M, Gao Z, Peng Y, Li Y, Li L, et al. Activating hotspot L205R mutation in PRKACA and adrenal Cushing's syndrome. Science. 2014;344(6186):913-7.
32. Rege J, Hoxie J, Liu CJ, Cash MN, Luther JM, Gellert L, et al. Targeted Mutational Analysis of Cortisol-Producing Adenomas. J Clin Endocrinol Metab. 2022;107(2):e594-e603.
33. Ronchi CL, Di Dalmazi G, Faillot S, Sbiera S, Assié G, Weigand I, et al. Genetic Landscape of Sporadic Unilateral Adrenocortical Adenomas Without PRKACA p.Leu206Arg Mutation. J Clin Endocrinol Metab. 2016;101(9):3526-38.
34. Wu L, Xie J, Qi Y, Su T, Jiang L, Zhou W, et al. Mutational landscape of non-functional adrenocortical adenomas. Endocr Relat Cancer. 2022;29(9):521-32.
35. Beyer HDR**.** Cortisol Secretion by an Incidentally Discovered Nonfunctional Adrenal Adenoma*. J Clin Endocrinol Metab. 1986;62(6):1317-21.
36. Bogner U, Eggens U, Hensen J, Oelkers W**.** Incidentally discovered ACTH-dependent adrenal adenoma presenting as 'Pre-Cushing's syndrome'. Acta Endocrinologica. 1986;111(1):89-92.
37. Huiras CM, Pehling GB, Caplan RH**.** Adrenal Insufficiency After Operative Removal of Apparently Nonfunctioning Adrenal Adenomas. JAMA. 1989;261(6):894-8.
38. Reincke M, Nieke J, Krestin GP, Saeger W, Allolio B, Winkelmann W**.** Preclinical Cushing's syndrome in adrenal "incidentalomas": comparison with adrenal Cushing's syndrome. J Clin Endocrinol Metab. 1992;75(3):826-32.
39. Newell-Price J, Grossman A**.** Adrenal incidentaloma: subclinical Cushing's syndrome. Postgraduate Medical Journal. 1996;72(846):207-10.
40. Rossi R, Tauchmanova L, Luciano A, Di Martino M, Battista C, Del Viscovo L, et al. Subclinical Cushing's syndrome in patients with adrenal incidentaloma: clinical and biochemical features. J Clin Endocrinol Metab. 2000;85(4):1440-8.
41. Morelli V, Masserini B, Salcuni AS, Eller-Vainicher C, Savoca C, Viti R, et al. Subclinical hypercortisolism: correlation between biochemical diagnostic criteria and clinical aspects. Clin Endocrinol (Oxf). 2010;73(2):161-6.
42. Terzolo M, Bovio S, Reimondo G, Pia A, Osella G, Borretta G, et al. Subclinical Cushing's syndrome in adrenal incidentalomas. Endocrinol Metab Clin North Am. 2005;34(2):423-39, x.
43. Elhassan YS, Alahdab F, Prete A, Delivanis DA, Khanna A, Prokop L, et al. Natural History of Adrenal Incidentalomas With and Without Mild Autonomous Cortisol Excess: A Systematic Review and Meta-analysis. Ann Intern Med. 2019;171(2):107-16.
44. Liddle GW**.** Tests of the pituitary-adrenal suppressibility in the diagnosis of Cushing’s syndrome*. J Clin Endocrinol Metab. 1960;20(12):1539-60.

45. Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM, et al. The diagnosis of Cushing's syndrome: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab. 2008;93(5):1526-40.
46. Bäcklund N, Brattsand G, Israelsson M, Ragnarsson O, Burman P, Edén Engström B, et al. Reference intervals of salivary cortisol and cortisone and their diagnostic accuracy in Cushing's syndrome. Eur J Endocrinol. 2020;182(6):569-82.
47. Masserini B, Morelli V, Bergamaschi S, Ermetici F, Eller-Vainicher C, Barbieri AM, et al. The limited role of midnight salivary cortisol levels in the diagnosis of subclinical hypercortisolism in patients with adrenal incidentaloma. Eur J Endocrinol. 2009;160(1):87-92.
48. Ceccato F, Barbot M, Albiger N, Antonelli G, Zilio M, Todeschini M, et al. Daily salivary cortisol and cortisone rhythm in patients with adrenal incidentaloma. Endocrine. 2018;59(3):510-9.
49. Raff H, Auchus RJ, Findling JW, Nieman LK**.** Urine free cortisol in the diagnosis of Cushing's syndrome: is it worth doing and, if so, how? J Clin Endocrinol Metab. 2015;100(2):395-7.
50. Kotłowska A, Sworczak K, Stepnowski P**.** Urine metabolomics analysis for adrenal incidentaloma activity detection and biomarker discovery. J Chromatogr B Analyt Technol Biomed Life Sci. 2011;879(5-6):359-63.
51. Prete A, Subramanian A, Bancos I, Chortis V, Tsagarakis S, Lang K, et al. Cardiometabolic Disease Burden and Steroid Excretion in Benign Adrenal Tumors : A Cross-Sectional Multicenter Study. Ann Intern Med. 2022;175(3):325-34.
52. Araujo-Castro M, García Cano A, Jiménez Mendiguchía L, Escobar-Morreale HF, Valderrábano P**.** Diagnostic accuracy of the different hormonal tests used for the diagnosis of autonomous cortisol secretion. Sci Rep. 2021;11(1):20539.
53. Russell GM, Henley DE, Leendertz J, Douthwaite JA, Wood SA, Stevens A, et al. Rapid glucocorticoid receptor-mediated inhibition of hypothalamic-pituitary-adrenal ultradian activity in healthy males. J Neurosci. 2010;30(17):6106-15.
54. Pecori Giraldi F, Saccani A, Cavagnini F**.** Assessment of ACTH assay variability: a multicenter study. Eur J Endocrinol. 2011;164(4):505-12.
55. Olsen H, Olofsson L, Mjoman M**.** Cortisol secretion from adrenal adenomas discovered as incidentalomas is responsive to ACTH. Clin Endocrinol (Oxf). 2014;80(3):335-41.
56. Debono M, Bradburn M, Bull M, Harrison B, Ross RJ, Newell-Price J**.** Cortisol as a marker for increased mortality in patients with incidental adrenocortical adenomas. J Clin Endocrinol Metab. 2014;99(12):4462-70.
57. Di Dalmazi G, Vicennati V, Garelli S, Casadio E, Rinaldi E, Giampalma E, et al. Cardiovascular events and mortality in patients with adrenal incidentalomas that are either non-secreting or associated with intermediate phenotype or subclinical Cushing's syndrome: a 15-year retrospective study. Lancet Diabetes Endocrinol. 2014;2(5):396-405.
58. Patrova J, Kjellman M, Wahrenberg H, Falhammar H**.** Increased mortality in patients with adrenal incidentalomas and autonomous cortisol secretion: a 13-year retrospective study from one center. Endocrine. 2017;58(2):267-75.

59. Park J, De Luca A, Dutton H, Malcolm JC, Doyle MA**.** Cardiovascular Outcomes in Autonomous Cortisol Secretion and Nonfunctioning Adrenal Adenoma: A Systematic Review. J Endocr Soc. 2019;3(5):996-1008.
60. Terzolo M, Reimondo G**.** Insights on the Natural History of Adrenal Incidentalomas. Ann Intern Med. 2019;171(2):135-6.
61. Terzolo M, Stigliano A, Chiodini I, Loli P, Furlani L, Arnaldi G, et al. AME position statement on adrenal incidentaloma. Eur J Endocrinol. 2011;164(6):851-70.
62. Araujo-Castro M**.** Cardiometabolic profile and urinary metabolomic alterations in non-functioning adrenal incidentalomas: A review. Clin Endocrinol (Oxf). 2022;97(6):693-701.
63. Androulakis, II, Kaltsas GA, Kollias GE, Markou AC, Gouli AK, Thomas DA, et al. Patients with apparently nonfunctioning adrenal incidentalomas may be at increased cardiovascular risk due to excessive cortisol secretion. J Clin Endocrinol Metab. 2014;99(8):2754-62.
64. VanderWeele TJ, Ding P**.** Sensitivity Analysis in Observational Research: Introducing the E-Value. Ann Intern Med. 2017;167(4):268-74.
65. Coemans M, Verbeke G, Döhler B, Süsal C, Naesens M**.** Bias by censoring for competing events in survival analysis. Bmj. 2022;378:e071349.
66. Mathur MB, Ding P, Riddell CA, VanderWeele TJ**.** Web Site and R Package for Computing E-values. Epidemiology. 2018;29(5):e45-e7.
67. Olsen H, Kjellbom A, Londahl M, Lindgren O**.** Suppressed ACTH Is Frequently Unrelated to Autonomous Cortisol Secretion in Patients With Adrenal Incidentalomas. J Clin Endocrinol Metab. 2019;104(2):506-12.
68. Ali SM, Chaix B, Merlo J, Rosvall M, Wamala S, Lindström M**.** Gender differences in daily smoking prevalence in different age strata: a population-based study in southern Sweden. Scand J Public Health. 2009;37(2):146-52.
69. Olsen H, Kjellbom A, Löndahl M, Lindgren O**.** High prevalence of smoking in patients with adrenal incidentalomas: causality or case selection? Eur J Endocrinol. 2020;183(3):335-41.
70. Kjellbom A, Lindgren O, Puvaneswaralingam S, Londahl M, Olsen H**.** Association Between Mortality and Levels of Autonomous Cortisol Secretion by Adrenal Incidentalomas : A Cohort Study. Ann Intern Med. 2021;174(8):1041-9.
71. Kjellbom A, Lindgren O, Danielsson M, Olsen H, Löndahl M**.** Mortality not increased in patients with non-functional adrenal adenomas: a matched cohort study. J Clin Endocrinol Metab. 2023.
72. Morelli V, Reimondo G, Giordano R, Della Casa S, Policola C, Palmieri S, et al. Long-term follow-up in adrenal incidentalomas: an Italian multicenter study. J Clin Endocrinol Metab. 2014;99(3):827-34.
73. Yener S, Ertilav S, Secil M, Akinci B, Demir T, Kebapcilar L, et al. Increased risk of unfavorable metabolic outcome during short-term follow-up in subjects with nonfunctioning adrenal adenomas. Med Princ Pract. 2012;21(5):429-34.

74. Di Dalmazi G, Vicennati V, Rinaldi E, Morselli-Labate AM, Giampalma E, Mosconi C, et al. Progressively increased patterns of subclinical cortisol hypersecretion in adrenal incidentalomas differently predict major metabolic and cardiovascular outcomes: a large cross-sectional study. Eur J Endocrinol. 2012;166(4):669-77.
75. Chiodini I**.** Clinical review: Diagnosis and treatment of subclinical hypercortisolism. J Clin Endocrinol Metab. 2011;96(5):1223-36.
76. Wilkinson CW, Petrie EC, Murray SR, Colasurdo EA, Raskind MA, Peskind ER**.** Human glucocorticoid feedback inhibition is reduced in older individuals: evening study. J Clin Endocrinol Metab. 2001;86(2):545-50.
77. Olsen H, Mjoman M**.** Moderately impaired renal function increases morning cortisol and cortisol levels at dexamethasone suppression test in patients with incidentally detected adrenal adenomas. Clin Endocrinol (Oxf). 2015;83(6):762-7.
78. Olsen H, Olsen M**.** Associations of age, BMI, and renal function to cortisol after dexamethasone suppression in patients with adrenal incidentalomas. Frontiers in Endocrinology. 2023;13.
79. Putignano P, Dubini A, Toja P, Invitti C, Bonfanti S, Redaelli G, et al. Salivary cortisol measurement in normal-weight, obese and anorexic women: comparison with plasma cortisol. Eur J Endocrinol. 2001;145(2):165-71.
80. Reincke M, Beuschlein F, Menig G, Hofmockel G, Arlt W, Lehmann R, et al. Localization and expression of adrenocorticotropic hormone receptor mRNA in normal and neoplastic human adrenal cortex. J Endocrinol. 1998;156(3):415-23.
81. Schubert B, Fassnacht M, Beuschlein F, Zenkert S, Allolio B, Reincke M**.** Angiotensin II type 1 receptor and ACTH receptor expression in human adrenocortical neoplasms. Clin Endocrinol (Oxf). 2001;54(5):627-32.
82. Beuschlein F, Fassnacht M, Klink A, Allolio B, Reincke M**.** ACTH-receptor expression, regulation and role in adrenocortial tumor formation. Eur J Endocrinol. 2001;144(3):199-206.
83. Di Dalmazi G, Fanelli F, Mezzullo M, Casadio E, Rinaldi E, Garelli S, et al. Steroid Profiling by LC-MS/MS in Nonsecreting and Subclinical Cortisol-Secreting Adrenocortical Adenomas. J Clin Endocrinol Metab. 2015;100(9):3529-38.
84. Ueland G, Methlie P, Kellmann R, Bjørgaas M, Åsvold BO, Thorstensen K, et al. Simultaneous assay of cortisol and dexamethasone improved diagnostic accuracy of the dexamethasone suppression test. Eur J Endocrinol. 2017;176(6):705-13.
85. Ceccato F, Artusi C, Barbot M, Lizzul L, Pinelli S, Costantini G, et al. Dexamethasone measurement during low-dose suppression test for suspected hypercortisolism: threshold development with and validation. J Endocrinol Invest. 2020;43(8):1105-13.
86. Puvaneswaralingam S, Kjellbom A, Lindgren O, Löndahl M, Olsen H**.** ACTH following overnight dexamethasone suppression can be used in the verification of autonomous cortisol secretion in patients with adrenal incidentalomas. Clin Endocrinol (Oxf). 2021;94(2):168-75.
87. Kim SY, Min C, Oh DJ, Choi HG**.** Tobacco Smoking and Alcohol Consumption Are Related to Benign Parotid Tumor: A Nested Case-Control Study Using a National Health Screening Cohort. Clin Exp Otorhinolaryngol. 2019;12(4):412-9.

88. He X, Wu K, Ogino S, Giovannucci EL, Chan AT, Song M**.** Association Between Risk Factors for Colorectal Cancer and Risk of Serrated Polyps and Conventional Adenomas. Gastroenterology. 2018;155(2):355-73.e18.
89. Galanti MR, Granath F, Cnattingius S, Ekbom-Schnell A, Ekbom A**.** Cigarette smoking and the risk of goitre and thyroid nodules amongst parous women. J Intern Med. 2005;258(3):257-64.
90. Steptoe A, Ussher M**.** Smoking, cortisol and nicotine. Int J Psychophysiol. 2006;59(3):228-35.
91. Badrick E, Kirschbaum C, Kumari M**.** The relationship between smoking status and cortisol secretion. J Clin Endocrinol Metab. 2007;92(3):819-24.
92. Rohleder N, Kirschbaum C**.** The hypothalamic-pituitary-adrenal (HPA) axis in habitual smokers. Int J Psychophysiol. 2006;59(3):236-43.
93. Rubin RP, Warner W**.** Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of the cat. Br J Pharmacol. 1975;53(3):357-62.
94. Miguel JP, Dias ADC, Bettini NR, Sousa S, Durão MPL, Oliveira LVC, et al. Cigarette smoke exposure causes systemic and autonomic cardiocirculatory changes in rats depending on the daily exposure dose. Life Sci. 2021;277:119498.
95. Ulrich-Lai YM, Arnhold MM, Engeland WC**.** Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. Am J Physiol Regul Integr Comp Physiol. 2006;290(4):R1128-35.
96. Vreeburg SA, Kruijtzer BP, van Pelt J, van Dyck R, DeRijk RH, Hoogendijk WJ, et al. Associations between sociodemographic, sampling and health factors and various salivary cortisol indicators in a large sample without psychopathology. Psychoneuroendocrinology. 2009;34(8):1109-20.
97. Direk N, Dekker MJ, Luik AI, Kirschbaum C, de Rijke YB, Hofman A, et al. The Very Low-Dose Dexamethasone Suppression Test in the General Population: A Cross-Sectional Study. PLoS One. 2016;11(10):e0164348.
98. Rose JQ, Yurchak AM, Meikle AW, Jusko WJ**.** Effect of smoking on prednisone, prednisolone, and dexamethasone pharmacokinetics. J Pharmacokinet Biopharm. 1981;9(1):1-14.
99. Cambos S, Tabarin A**.** Management of adrenal incidentalomas: Working through uncertainty. Best Practice & Research Clinical Endocrinology & Metabolism. 2020;34(3):101427.
100. Eller-Vainicher C, Morelli V, Salcuni AS, Battista C, Torlontano M, Coletti F, et al. Accuracy of several parameters of hypothalamic-pituitary-adrenal axis activity in predicting before surgery the metabolic effects of the removal of an adrenal incidentaloma. Eur J Endocrinol. 2010;163(6):925-35.
101. Deutschbein T, Reimondo G, Di Dalmazi G, Bancos I, Patrova J, Vassiliadi DA, et al. Age-dependent and sex-dependent disparity in mortality in patients with adrenal incidentalomas and autonomous cortisol secretion: an international, retrospective, cohort study. Lancet Diabetes Endocrinol. 2022;10(7):499-508.
102. Cohen J**.** Statistical power analysis for the behavioral sciences. 2nd ed. Hillsdale, N.J.: L. Erlbaum Associates; 1988.

103. Azuero A**.** A note on the magnitude of hazard ratios. Cancer. 2016;122(8):1298-9.
104. Taylor JM**.** Choosing the number of controls in a matched case-control study, some sample size, power and efficiency considerations. Stat Med. 1986;5(1):29-36.
105. Arruda M, Mello Ribeiro Cavalari E, Pessoa de Paula M, Fernandes Cordeiro de Morais F, Furtado Bilro G, Alves Coelho MC, et al. The presence of nonfunctioning adrenal incidentalomas increases arterial hypertension frequency and severity, and is associated with cortisol levels after dexamethasone suppression test. J Hum Hypertens. 2017;32(1):3-11.
106. Lopez D, Luque-Fernandez MA, Steele A, Adler GK, Turchin A, Vaidya A**.** "Nonfunctional" Adrenal Tumors and the Risk for Incident Diabetes and Cardiovascular Outcomes: A Cohort Study. Ann Intern Med. 2016;165(8):533-42.
107. de Paula MP, Moraes AB, de Souza M, Cavalari EMR, Campbell RC, Fernandes GDS, et al. Cortisol level after dexamethasone suppression test in patients with non-functioning adrenal incidentaloma is positively associated with the duration of reactive hyperemia response on microvascular bed. J Endocrinol Invest. 2021;44(3):609-19.
108. Athanasouli F, Georgiopoulos G, Asonitis N, Petychaki F, Savelli A, Panou E, et al. Nonfunctional adrenal adenomas and impaired glucose metabolism: a systematic review and meta-analysis. Endocrine. 2021;74(1):50-60.
109. Sørensen HT, Mellemkjaer L, Nielsen GL, Baron JA, Olsen JH, Karagas MR**.** Skin cancers and non-hodgkin lymphoma among users of systemic glucocorticoids: a population-based cohort study. J Natl Cancer Inst. 2004;96(9):709-11.
110. Dietrich K, Schned A, Fortuny J, Heaney J, Marsit C, Kelsey KT, et al. Glucocorticoid therapy and risk of bladder cancer. Br J Cancer. 2009;101(8):1316-20.
111. Jensen A, Thomsen HF, Engebjerg MC, Olesen AB, Friis S, Karagas MR, et al. Use of oral glucocorticoids and risk of skin cancer and non-Hodgkin's lymphoma: a population-based case-control study. Br J Cancer. 2009;100(1):200-5.
112. Herr I, Pfitzenmaier J**.** Glucocorticoid use in prostate cancer and other solid tumours: implications for effectiveness of cytotoxic treatment and metastases. Lancet Oncol. 2006;7(5):425-30.
113. Abukiwan A, Nwaeburu CC, Bauer N, Zhao Z, Liu L, Gladkich J, et al. Dexamethasone-induced inhibition of miR-132 via methylation promotes TGF-β-driven progression of pancreatic cancer. Int J Oncol. 2019;54(1):53-64.
114. Perez Kerkvliet C, Dwyer AR, Diep CH, Oakley RH, Liddle C, Cidlowski JA, et al. Glucocorticoid receptors are required effectors of TGFβ1-induced p38 MAPK signaling to advanced cancer phenotypes in triple-negative breast cancer. Breast Cancer Res. 2020;22(1):39.
115. Tian D, Tian M, Han G, Li JL**.** Increased glucocorticoid receptor activity and proliferation in metastatic colon cancer. Sci Rep. 2019;9(1):11257.
116. Andersen BL, Yang HC, Farrar WB, Golden-Kreutz DM, Emery CF, Thornton LM, et al. Psychologic intervention improves survival for breast cancer patients: a randomized clinical trial. Cancer. 2008;113(12):3450-8.

117. Volden PA, Conzen SD**.** The influence of glucocorticoid signaling on tumor progression. Brain Behav Immun. 2013;30 Suppl(0):S26-31.
118. Stamou MI, Colling C, Dichtel LE**.** Adrenal aging and its effects on the stress response and immunosenescence. Maturitas. 2023;168:13-9.
119. Brooke HL, Talbäck M, Hörnblad J, Johansson LA, Ludvigsson JF, Druid H, et al. The Swedish cause of death register. Eur J Epidemiol. 2017;32(9):765-73.
120. Marmot M**.** Social determinants of health inequalities. The Lancet. 2005;365(9464):1099-104.
121. Bancos I, Alahdab F, Crowley RK, Chortis V, Delivanis DA, Erickson D, et al. THERAPY OF ENDOCRINE DISEASE: Improvement of cardiovascular risk factors after adrenalectomy in patients with adrenal tumors and subclinical Cushing's syndrome: a systematic review and meta-analysis. Eur J Endocrinol. 2016;175(6):R283-R95.
122. Iacobone M, Citton M, Scarpa M, Viel G, Boscaro M, Nitti D**.** Systematic review of surgical treatment of subclinical Cushing's syndrome. Br J Surg. 2015;102(4):318-30.
123. Morelli V, Frigerio S, Aresta C, Passeri E, Pugliese F, Copetti M, et al. Adrenalectomy Improves Blood Pressure and Metabolic Control in Patients With Possible Autonomous Cortisol Secretion: Results of a RCT. Front Endocrinol (Lausanne). 2022;13:898084.
124. Debono M, Harrison RF, Chadarevian R, Gueroult C, Abitbol JL, Newell-Price J**.** Resetting the Abnormal Circadian Cortisol Rhythm in Adrenal Incidentaloma Patients With Mild Autonomous Cortisol Secretion. J Clin Endocrinol Metab. 2017;102(9):3461-9.
125. Belokovskaya R, Ravikumar A, Arumugam D, Izadmehr S, Goddard GM, Geer EB, et al. Mifepristone treatment for mild autonomous cortisol secretion due to adrenal adenomas: A pilot study. Endocr Pract. 2019;25(8):846-53.
126. Pivonello R, Bancos I, Feelders RA, Kargi AY, Kerr JM, Gordon MB, et al. Relacorilant, a Selective Glucocorticoid Receptor Modulator, Induces Clinical Improvements in Patients With Cushing Syndrome: Results From A Prospective, Open-Label Phase 2 Study. Front Endocrinol (Lausanne). 2021;12:662865.
127. Vogg N, Kurlbaum M, Deutschbein T, Gräsl B, Fassnacht M, Kroiss M**.** Method-Specific Cortisol and Dexamethasone Thresholds Increase Clinical Specificity of the Dexamethasone Suppression Test for Cushing Syndrome. Clin Chem. 2021;67(7):998-1007.
128. Genere N, Kaur RJ, Athimulam S, Thomas MA, Nippoldt T, Van Norman M, et al. Interpretation of Abnormal Dexamethasone Suppression Test is Enhanced With Use of Synchronous Free Cortisol Assessment. J Clin Endocrinol Metab. 2022;107(3):e1221-e30.
129. Eriksson A, Stenlund H, Ahlm K, Boman K, Bygren LO, Johansson LA, et al. Accuracy of death certificates of cardiovascular disease in a community intervention in Sweden. Scand J Public Health. 2013;41(8):883-9.
130. Ludvigsson JF, Andersson E, Ekbom A, Feychting M, Kim JL, Reuterwall C, et al. External review and validation of the Swedish national inpatient register. BMC Public Health. 2011;11:450.