From the Division of Endocrinology and Metabolism,
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Incidentially discovered adrenal non-producing adenomas do show signs of glucocorticoid excess

Inaugural Dissertation

for achieving the academic degree
***Doctor medicinae* (Dr.med.)**
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Content

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II. Declaration of Originality

Herewith, I declare under oath that the submitted thesis is my original and independent work and was written by me without further assistance. This work has not be submitted for any other degree or professional qualification. Appropriate credit has been given where reference has been made to the work of others. The thesis was not examined before, nor has it been published. The submitted electronic version of the thesis matches the printed version.

A part of the presented results have already been published in the journal *Hormone and Metabolic Research* (2021; 53: 512-519), as »DHEAS and differential blood counts as indirect signs of glucocorticoid excess in adrenal non-producing adenomas« by Winzinger, Eliza-Paula; Jandikova, Hana; Haase, Matthias; Knauerhase, Andreas; Winzinger, Tudor; Schott, Matthias; Willenberg, Holger Sven. All of the above mentioned authors contributed to this study.

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Winzinger T, Palamaru AL, **Winzinger EP**, Toader E. **Ethical Implications Regarding the Use of Biotechnologies in Terminal Patient Care.** J Intercult Manag Ethics. 2022; 5: 49:55. doi: 10.35478/jime.2022.1.07.

IV. Theses

1. basal ACTH and DHEAS concentrations along with the differential blood counts give good information on the extent of glucocorticoid excess;
2. patients with adrenal non-producing adenomas show signs of glucocorticoid excess, including relative lymphocytopenia, lowered DHEAS and ACTH concentrations, when compared to the results of normal individuals;
3. a step-wise decrease in basal ACTH or DHEAS secretion ranges from group A (control) over groups B (non-producing adenomas) and C (possible autonomous cortisol secretion) to group D (autonomous cortisol secretion); differences in basal DHEAS concentrations are significant between groups A and B;
4. there is a step-wise increase in the leukocytes and neutrophils count, as well as a step-wise decrease in the relative and absolute counts of lymphocytes, ranging from group A, towards group D;
5. there is a weak but significant correlation between DHEAS and the relative portions of eosinophils;
6. ACTH correlates significantly to the relative portions of the neutrophils and to the relative portions of lymphocytes, as well as with leukocytes and eosinophils.
7. the extent of lymphocytopenia correlates well and significantly with the concentrations of DHEAS and ACTH;
8. the concentrations of DHEAS correlate well and significantly with ACTH;
9. there is a weak but significant inverse correlation between age and DHEAS that is no more visible in groups C and D where ACTH concentration are lowest;
10. there is also a weak inverse correlation between age and lymphocyte count, the count of leucocytes, thrombocytes and neutrophils;
11. the negative feedback on ACTH secretion by cortisol is reflected by a weak but significant inverse correlation between tumor size and basal ACTH as well as DHEAS.

1. INTRODUCTION

1.1 Adrenal gland overview

Historically, the first proof of the existence of the suprarenal glands was given by Leonardo da Vinci in 1550 when he realized the first drawing of the adrenal glands but the first written description was made in 1563 by Bartolomeus Eustachius. The functional importance of the adrenal glands was described by Thomas Addison 1849 and their vital role was proved by Brown-Séquard in 1856.

The suprarenal glands are composed of 2 parts, each of them having endocrine activity. The adrenal cortex (originating from mesoderm) is formed of *glomerular*, *fascicular* and *reticular zones* (Neville *et al*. Clin Endocrinol Metab 1985), which are responsible for the production of mineralocorticoids, glucocorticoids and adrenal androgens, respectively (Miller *et al*. Endocrinol Metab 1995). The adrenal medulla (10% of the adrenal, originating from neural crest) is located in the center of the adrenal glands gives rise to catecholamines, including epinephrine (Xing *et al*. Endocrinol Metab Clin North Am 2015).

1.2. Steroid biosynthesis in the cortex of the adrenocortical gland

Steroid hormones are lipophilic molecules that are able to cross the cell membrane and to activate intracellular nuclear receptors that are multi-domain ligand-dependent transcriptional regulators in the nucleus (Cole *et al*. Semin Fetal Neonatal Med 2019).

The synthesis of the steroid hormones *de novo* in the adrenal glands starts with the conversion of cholesterol to pregnenolone by CYP11A (cholesterol side-chain cleavage) (Parker *et al*. Vitam Horm 1995). CYP11A is bound to the inner membrane of the mitochondrion and is found in all steroidogenic tissues (Miller WL. Endocr Rev 1988) but is not or poorly expressed in nonsteroidogenic tissues. The enzymes involved in the steroid biosynthesis are cytochrome P450 monooxygenases, hydroxysteroid dehydrogenases or steroid reductases (Miller Endocr Rev 1988). Following side chain cleavage, adrenal steroid hormone biosynthesis may enter three different pathways.

In the mineralocorticoids pathway, pregnenolone is converted to progesterone which has progestogenic, antiandrogenic and antimineralocorticoid activity. Progesterone is then hydroxylated to the mineralocorticoid active steroids 11-desoxycorticosterone, corticosterone (which is also a glucocorticoid) and then to 18-hydroxycorticosterone. After an oxidation step, the final product aldosterone is synthesized.

Substrates for the synthesis of glucocorticoids can be both pregnenolone and progesterone. The products of the first hydroxylation catalyzed by 17α-hydroxylase are 17α-hydroxyprogesterone or 17α-hydroxypregnenolone which is also converted in 17α-hydroxyprogesterone. After another hydroxylation

step by the 21α-hydroxylase, 11-desoxycortisol is formed and then hydroxylated by 11β-hydroxylase to cortisol. Cortisol, which has a high affinity to the mineralocorticoid receptor, can be inactivated to cortisone by oxidation. The reaction is catalyzed by 11β-hydroxysteroid dehydrogenase type 2. The reverse reaction, the conversion of cortisone into cortisol by reduction, is mediated by 11β-hydroxysteroid dehydrogenase type 1.

For the pathway of sex hormone precursors in the adrenal cortex, mainly dehydroepiandrosterone and androstenedione are produced and converted into the active sex hormones by gonadal tissue. The synthesis starts with pregnenolone or progesterone which are, as in the synthesis of glucocorticoids, first hydroxylated by the hydroxylase activity of 17α-hydroxylase / 17,20- lyase into 17α-hydroxypregnenolone or 17α-hydroxyprogesterone. The lysis activity of the 17α-hydroxylase / 17, 20-lyase on 17α-hydroxypregnenolone or 17α-hydroxyprogesterone lead to the synthesis of dehydroepiandrosterone (DHEA) and androstenedione, respectively. Cytochrome b5 (CYB5) can enhance the 17, 20-lyase activity of CYP17 enzyme which has a positive impact on dehydroepiandrosterone sulfate (DHEAS) biosynthesis (Rainey et al. J Steroid Biochem Mol Biol 2007). Of note, the expression of cytochrome b5 is also ACTH-dependent.

DHEA can be converted into DHEAS by a sulfotransferase or into androstenedione by action of 3β-hydroxysteroid dehydrogenase. DHEA, DHEAS and androstenedione are considered to be relatively weak effective androgens but serve as precursors to more potent sex steroids, including 11OH-androstenedione, testosterone, 11OH- testosterone, estrone and estradiol. They may also be converted peripherally into dihydrotestosterone, 11keto-testosterone or estrogens as androstenediol, estradiol and estriol (Ghayee HK *et al*. Rev Endocr Metab Disord 2007). In humans, DHEAS is the most abundant steroid hormone in the peripheral circulation (Berr *et al.* Proc Natl Acad Sci USA 1996) and concentrations of DHEA and DHEAS are even higher in the brain as compared to the circulation (Lacroix *et al.* J Steroid Biochem 1987; do Vale *et al*. Vitam Horm 2018).

1.3. ACTH and DHEAS as indices of glucocorticoid excess

The cicardian production of glucocorticoids and of adrenal androgens is regulated by the hypothalamic-pituitary-adrenal (HPA) axis and the final steps in the synthesis of the mineralocorticoides through the renin-angiotensin-aldosterone system (RAAS). When the hypophysiotropic neurons in the paraventricular nucleus of the hypothalamus are stimulated these neurosecretory cells release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Once CRH reaches the anterior pituitary, it binds to CRF type 1 receptors of pituitary corticotrophic cells, while AVP binds to V3 receptors. Corticotropin, or adrenocorticotropic hormone (ACTH), is released into circulation binding to type 2 melanocortin receptors (MC2-R) in the adrenal cortex and causing activation of adrenal steroid biosynthesis thereby release glucocorticoids (Burford *et al.* Int J Mol Sci 2017). Due to their lipophilic

nature, glucocorticoids cannot be pre-synthesized and stored within adrenal cells but have to be rapidly synthesized (using a number of enzymatic reactions) upon ACTH stimulation. If the glucocorticoid concentrations are chronically elevated because of different endocrine pathologies, such as cortisol-producing adrenal adenomas, ACTH concentrations will decrease due to a negative feedback loop on the HPA axis. Therefore, a decrease in the level of ACTH is correlated with a glucocorticoid excess (Ramamoorthy *et al.* Rheum Dis Clin North Am 2016).

Low age and gender-adjusted levels of DHEAS are associated with a glucocorticoid excess because of the fact that they are usually found in Cushing's syndrome. Due to the negative-feedback inhibition of the normal adrenal gland in patients with cortisol-secreting adrenal adenomas, the concentrations of DHEAS which is an ACTH-dependent androgen are also reduced. Of interest, DHEAS often remains suppressed for a long time after removal of the hypersecreting adrenal gland, occasionally up to 8 years after surgery. This is in contrast with the recovery of ACTH and cortisol secretion, which usually occurs 12 to 18 months after surgery and indicates a dissociation of adrenal DHEAS and cortisol regulation (Cavagnini *et al.* Endocrinol Adult Pediatr 2016).

1.4. Adrenal incidentalomas

The term "incidentaloma" was firstly introduced in 1982 by Geelhoed and Druy (Geelhoed Surg 1982), who recognized this pathology that appeared due to the technological advancement in the last three decades. The increase in the use of diagnostic medical imaging has led to a higher frequency of diagnosing unexpected pathology. Therefore, clinicians were faced with the dilemma of early diagnosis of an asymptomatic pathology caused by an undefined adrenal mass (Eldeiry *et al.* J Clin Translat Endocrinol 2018).

The prevalence of adrenal incidenatolmas (AI) varies depending on the source of data that may come from studies in the field of forensic medicine (autopsies), surgery or radiology. In autopsy series with a large number of patients (series with more than 1000 patients), the reported prevalence of AI varies from 1.05% to 8.7% (Sherlock *et al.* Endocr Rev 2020). In 2006, Bovio *et al*. a frequency of AI was reported to be 4,4% in a prospective study on 520 patients (Bovio *et al*. J Endocrinol Invest 2006). While this number may be explained by the use of an increasingly powerful scanning technology, the study included patients older than 55 years, thus capturing patients only at an age where there is a higher prevalence of adrenal masses. Similarly, Song *et al.* reported a 5% prevalence for AI in a retrospective study using a series of CT scans (Song *et al.* Am J Roentgenol 2008). However, the study protocol included a program dedicated to the radiological analysis of adrenal gland imaging in most cases, thus resulting in a possible diagnosis of a larger number of AI than would have been encountered in routine clinical radiological practice, when imaging investigations are not specifically done to identify abnormalities of

the adrenal gland, which are being accidentally observed (Davenport *et al.* Endocrine 2011; Sherlock *et al.* Endocr Rev 2020).

An adrenal incidentaloma is defined as an adrenal mass, observed on medical imaging by coincidence. In a majority of patients, adrenal incidentalomas are seemingly non-producing adrenocortical adenomas (NPA). Nevertheless, a proper diagnosis must be provided in order to exclude other associated pathologies and to properly exclude the malignancy of the adrenal mass and the hormonal hypersecretion of the lesion (Fassnacht *et al*. Eur J Endocrinol 2016; Sherlock *et al.* Endocr Rev 2020).

1.5. Evaluation of the adrenal mass

Adrenocortical tumors (ACTs), as the name implies, originate within the adrenal cortex. Once an adrenocortical nodule is identified, the diagnostic procedures that must be considered range from indolent and benign, such as adrenal cortical adenoma (ACA), to often aggressive and malignant, such as adrenal cortical carcinoma (ACC). In this thesis, it was evaluated to what extend incidentially discovered ACTs are truly non-hormone producing when overt Cushing's syndrome could be exluded.

1.5.1. Adrenocortical carcinoma *vs*. non-adenoma

Once an AI is discovered, the abdominal computed tomography (CT) is the most used imaging technique in order to properly evaluate adrenal cortical lesions (Francis Cancer Imag 2003). The features that should be assessed include tumor size, appearance (integrity and invasiveness), heterogeneity, lipid content, and the rate of washout after intravenous administration of contrast material (Hodgson *et al.* Surg Pathol Clin 2019). Some studies have suggested that chemical-shift magnetic resonance imaging (MRI) may be the preferred imaging modality in young patients or in cases when an indeterminate adrenal mass on unenhanced CT is discovered in a patient with an iodinated contrast allergy (Francis R. Cancer Imag 2003). Adrenal ultrasonography is useful in case of neonates with congenital adrenal hyperplasia (Daneman *et al.* Endocrinol Metab Clin North Am 2005). The most important radiological characteristics of ACTs determined on CTs are presented in **Table 1**.

Table 1: Imaging features on the CT of benign and malignant adrenocortical tumors		
characteristics on CT	**benign**	**malignant**
tumor size	often < 4-6 cm	often 4-6 cm
tumor appearance	well-defined; regular border; round	not delineated, irregular border; areas of necrosis
tumor heterogeneity	homogeneous enhancement	heterogeneous enhancement
lipid content and density, appearance on unenhanced CT	lipid rich, low density on unenhanced CT (< 10 HU)	lipid poor, high density on unenhanced CT (> 10 HU)
contrast enhancement and washout pattern 15 min after intravenous administration of contrast	enhanced with rapid washout (40%) after 15 min	enhanced with less washout (15-25%) after 15 min
The table was adapted after Hodgson *et al.* Surg Pathol Clin 2019 and presents a comparison between the features of benign or malignant tumors that can be observed on computed tomographies. Abbreviations: CT – computed tomography, HU – Hounsfield units.		

As far as the morphologic diagnosis of adrenocortical tumors is concerned, the usual principles of macroscopic examination apply, including specimen painting, margin identification, specimen measurement, and specimen weighing. The external examination of the tumor as well as the non-tumoral adrenal parenchyma must be carefully evaluated (Hodgson *et al.* Surg Pathol Clin 2019). The normal adrenal cortex has an average thickness of at least 2 mm. In patients with a cortisol-secreting lesion, due to the negative feedback inhibition by the autonomous cortisol secretion on the HPA axis, the cortex becomes thinner and atrophied (Duan *et al.* J Clin Pathol 2015; Mete O *et al.* Histopathol 2012; Hodgson *et al.* Surg Pathol Clin 2019). Most commonly, ACAs are well delineated and homogeneous. In contrast, ACCs are more often heterogeneous and may show fibrous bands. In addition, hemorrhage, necrosis, and calcification are commonly observed. They may show an irregular border and be infiltrated into the adjacent structures such as the regional venous structures, adipose tissue surrounding the adrenal gland, and adjacent organs such as the kidney (Lloyd WHO Press 2017).

The morphological microscopic examination is based on various criteria and scores. The first and most used histological diagnostic tool is the Weiss scoring system, which is based on 9 histological parameters including the high nuclear grade, mitotic rate (more than 5 mitoses per 50 high-power fields (HPF), atypical mitotic figures, less than 25% clear cells, patternless sheets (diffuse architecture exceeding 30% of the tumor), non-diffuse growth (alveolar, columnar, trabecular or cordlike areas), tumor necrosis, venous invasion, sinusoidal invasion, and capsular invasion (if a parameter is absent it is scored with 0 and if it is present it is scored with 1) (Weiss *et al.* Am J Surg Pathol 1989; Papotti *et al.* Endocr Pathol 2014). A Weiss score above 3 was associated with an increased risk of malignancy. In

the initial study by Weiss, the characteristics with the strongest association with the result were the mitotic rate > 5/50 HPF, atypical mitosis and venous invasion (Weiss LM *et al*. Am J Surg Pathol1989).

More recently, The Lin-Weiss-Bisceglia criteria were described with an increased focus on invasiveness and mitotic count and less focus on some other morphologic features defined in the Weiss criteria (Bisceglia *et al.* Int J Surg Pathol 2004). The Lin-Weiss-Bisceglia criteria use a major and minor criteria framework to classify oncocytic ACTs for which the presence of 1 major criteria (mitotic rate >5 mitoses per 50 high-power fields, atypical mitotic figures, venous invasion) indicates malignancy and the presence of 1 to 4 minor criteria (tumor size > 10 cm and/ or weight > 200 g, necrosis, sinusoidal invasion, capsular invasion) indicates a tumor of uncertain malignant potential (Bisceglia *et al.* Int J Surg Pathol 2004; Hodgson *et al.* Surg Pathol Clin 2019).

Another key predictor of malignant behaviour is the Ki67 proliferative index (Iino *et al*. Mod Pathol 1997). The extent to which the Ki67 index confirms the diagnosis of ACC and predicts an aggressive form of ACC is debated and ranges from > 2.5-7.0% (Morimoto *et al*. Endocr J 2008; Nakamura *et al.* Endocrinol Metab Clin North Am 2015). However, due to the enormous intratumoral heterogeneity of ACC, depending on how the test is performed after which the Ki67 labeling index is obtained, the results are significantly influenced. In particular, the question of whether the Ki67 index should be calculated for the mass of the entire tumor specimen or for the sum of tumoral-transformed areas into specimens has not been resolved (Nakamura *et al.* Endocrinol Metab Clin North Am 2015). This limitation should be considered when using the Ki67 marking index when classifying adrenal lesions and as a prognostic marker for ACC (Nakamura *et al.* Endocrinol Metab Clin North Am 2015). The Helsinki score used in some centers incorporates the Ki67 index and is considered to be a better predictor of diagnosis (Duregon *et al.* Hum Pathol 2017).

1.5.2. Assessment of the functional status of adrenocortical tumors

Clarification of the endocrine status of patients with AI has a major importance when establishing the diagnosis, along with the exclusion of malignancy. Between 10% and 15% of AI present an excessive hormonal secretion (Barzon *et al*. J Clin Endocrinol Metab 1998). Clinical signs and symptoms of hormonal excess and associated comorbidities, along with a hormonal evaluation, underlie personalized management.

Every patient with AI should be examined in order to exclude the excess secretion of adrenal catecholamines (although recent data have suggested that it may not be necessary in lesions with low Hounsfield units) (Canu *et al.* J Clin Endocrinol Metab 2019) and to determine the excess glucocorticoids. Mineralocorticoid hypersecretion should be excluded in patients with hypertension and/ or hypokalemia. In the presence of hirsutism or virilization or if an ACC is suspected, it is recommended

to determine measurements of androgen and estrogen concentrations that could produce gynecomastia.

Several hormones can be produced by functional ACTs, including mineralocorticoids (e.g. aldosterone), glucocorticoids (e.g. cortisol), and sex steroids (androgens). The most important clinical, biochemical, and clinico-pathologic features of functioning ACTs are summarized in **Table 2** (Duan *et al.* Endocr pathol 2016; Funder *et al.* J Clin Endocrinol Metab 2008; Stowasser J Clin Endocrinol Metab 2015; Duan *et al*. Arch Pathol Lab Med 2015; Duan *et al.* J Clin Pathol 2015; El-Maouche *et al.* Lancet 2017; Hodgson *et al.* Surg Pathol Clin 2019).

Table 2: Overview of biochemical and clinical characteristics of functional adrenocortical tumors

	glucocorticoid excess (adrenal Cushing's syndrome)	**mineralocorticoid excess (primary aldosteronism)**	**sex steroid excess (feminization or virilization syndromes)**
biochemical aspects	- increased cortisol concentrations in at least 2 endocrine assays (24 hour urinary free cortisol, serum free cortisol following a 1 mg dexamethasone challange, or late-night salivary free cortisol), followed by a second test	- inadequate high plasma aldosterone for renin, followed by a confirmation test	- increased concentrations of DHEA, DHEAS, androstenedione, dihydrotestosterone, testosterone, estrone, hydroxyprogesterone, or estradiol
clinical features	- any age; women affected more commonly than men - central obesity, facial rounding, hirsutism, easy bruising, skin striae, poor wound healing, muscle weakness, hypertension, hyperglycemia, osteoporosis in adulthood; weight gain and growth failure in childhood; subclinical Cushing syndrome	- third-sixth decade; equal male/female ratio - hypertension with or without hypokalemia; nonspecific symptoms including muscle weakness, easy fatigability, headache, palpitations, nocturia, polyuria, or polydipsia	- variable clinical findings based on the patient's age, gender, and extent of hormone excess; virilization in women (increased muscle mass and facial hair, deep voice and amenorrhea); in prepuberty age (pubic hair growth); feminization in men (gynecomastia or impotence)
The table was adapted after Hodgson *et al.* Surg Pathol Clin 2019 and presents a comparison between the biochemical and clinical aspects of the adrenocortical tumors which are functional.			

In this thesis, we will mainly refer to the diagnostic characteristics of glucocorticoids hypersecretion in case of adrenal cortical adenomas, emphasizing endocrine characteristics of non-producing adrenal adenomas.

Screening for autonomous cortisol secretion in adrenal adenomas

Diagnosing cortisol excess in patients with AI may often be challenging due to the nonspecific and various signs and symptoms. For patients with overt Cushing's syndrome (with typical clinical features such as moon *facies*, *striae*, and proximal muscle weakness), the European Society of Endocrinology (ESE/ ENSAT) recommends the use of 2 of 3 screening tests: 24-hour urinary free cortisol (UFC) excretion, late-night salivary cortisol (LNSC) concentrations or plasma cortisol concentrations following an overnight dexamethasone suppression test (1-mg dexamethasone suppression test (DST); or low-dose DST) (Nieman LK *et al*. J of Clin Endocrinol and Metab. 2008). Nevertheless, there are a wide range of confounding factors that can interfere with the proper assessment of cortisol excess in patients with AI. Some of this confusing factors include different assays (antibody development, radioimmunoassay (RIA), enzyme-linked immunosorbent assay, automated chemiluminescence, high-performance liquid chromatography or mass spectrometry), patient comorbidities causing physiological hypercortisolism, and pseudo-Cushing's syndrome (Nieman *et al.* J of Clin Endocrinol and Metab 2008; Boscaro *et al. J* Clin Endocrinol Metab 2009; El-Farhan *et al.* Ann Clin Biochem 2017; Sherlock *et al.* Endocr Rev 2020).

Autonomous cortisol secretion (ACS) is characterized by excess cortisol that is independent from ACTH stimulation and often without clinical signs and symptoms of overt Cushing's syndrome. Multiple names were given to this phenomenon, e.g. "subclinical Cushing's syndrome," and "subclinical hypercortisolism" and "preclinical Cushing's syndrome". However, the universal use of the term "autonomous cortisol secretion" is proposed by ESE/ENSAT (Fassnacht *et al.* Eur J Endocrinol 2016; Sherlock *et al.* Endocr Rev 2020).

No gold standard has yet been established for diagnosing ACS. A summary of published guidelines is presented in **Table 3**, including a wide variety of recommended first screening, secondary screening and confirmation tests that may slightly vary depending on the guideline (Sherlock *et al.* Endocr Rev 2020).

Summarizing the recommendations presented below, the use of the 1-mg DST is considered to have the highest sensitivity for screening for ACS. A post-dexamethasone cortisol concentration ≤1.8 µg/dL (≤50 nmol/L) is considered "normal" and excludes cortisol excess in most cases. F concentrations between 1.9 and 5.0 µg/dL (50-140 nmol/L) may indicate "possible autonomous cortisol secretion," and F concentrations above 5.0 µg/dL (140 nmol/L) are suggested to confirm ACS (Di Dalmazi *et al.* Lancet Diabetes Endocrinol 2014; Debono *et al.* J Clin Endocrinol Metab 2014; Sherlock *et al.* Endocr Rev 2020).

Table 3: Summary of published guidelines for the diagnosis of autonomous cortisol secretion in patients with adrenal incidentalomas									
guideline (year)	**NIH (2003)**	**ES (2008)**	**AACE/AAES (2009)**	**FSE (2008)**	**IACE (2011)**	**ESE/ENSAT (2016)**	**KES (2017)**	**JES (2018)**	**SEEN (2020)**
country	USA	USA	USA	France	Italy	Europe	Korea	Japan	Spain
first screening test (cut-off)									
1-mg DST cut-off concentration [µg/dL]	yes > 5.0: possible ACS	yes > 1.8	yes > 5.0	yes > 1.8	yes < 1.8: exclude, >5.0: consider, 1.8–5.0: indeterminate	yes <1.8: exclude >5.0 ACS 1.8–5.0: possible ACS	yes <1.8: normal >5.0: ACS 1.8–5.0: addit. tests	yes >5.0 ≥ 3 with (any of A-D) or E ≥1.8 with (A and B) or E	yes <1.8: exclude >5.0: ACS If DST >3 or 1.8- 3: addit. tests, DHEAS
Late night salivary F	NM	yes	NR	NR	NR	NR	NM	NR	yes
second screening test									
24-hour UFC	NM	NR	NR	yes	yes	Yes if DST > 5 µg/dL	NR		yes if DST >3 µg/dL
late-night serum F	NM	NM	NM	yes	yes	NM	NM	≥5 µg/dL	yes if DST >3 µg/dL
ACTH	NM	NM	NM	NM	NM	NM	yes	NM	yes if DST >3 µg/dL
late-night salivary F	NM	NM	NR	NM	NR	yes if DST > 5 µg/dL	NM	NM	yes if DST >3 µg/dL
4-mg DST	NM	NM	NM	NM	NR	yes if DST > 5 µg/dL	NM	NR	NM

Table 3 (Continuation): Summary of published guidelines for the diagnosis of autonomous cortisol secretion in patients with adrenal incidentalomas									
guideline (year)	**NIH (2003)**	**ES (2008)**	**AACE/AAES (2009)**	**FSE (2008)**	**IACE (2011)**	**ESE/ENSAT (2016)**	**KES (2017)**	**JES (2018)**	**SEEN (2020)**
further confirmatory tests									
4-mg DST	NM	NR	yes	NM	NR	NM	NM	NR	yes
8-mg DST	NM	NR	NM	NM	NR	NM	NM	NR	NM
localization (adrenal in origin)									
ACTH	NM	yes	yes	yes	yes	yes	yes	<10 pg/mL	<10 pg/mL
DHEA-S	NM	Yes	yes	NR	NR	NM	NM	yes	yes

The table was adapted from Yanase *et al*. Endocr J 2018; Sherlock *et al*. Endocr Rev 2020 and completed with Araujo-Castro *et al*. Endocrinol Diabetes Nutr 2020 and presents the recommendations regarding endocrine function tests used for the diagnosis of autonomous cortisol secretion in patients with adrenal incidentalomas.

Abbreviations: DST – dexamethasone suppression test; F – cortisol; NM – not mentioned; NR – not recommended; UFC – urinary free cortisol.

NIH – National Institutes of Health (Grumbach *et al*. Ann Intern Med 2003), ES – Endocrine Society (Nieman *et al*. J of Clin Endocrinol and Metab 2008), AACE/AAES – American Association of Clinical Endocrinologists / American Association of Endocrine Surgeons (Zeiger *et al*. Endocr Pract 2009), FSE – French Society of Endocrinology (Tabarin *et al*. Ann Endocrinol 2008), IACE – Italian Association of Clinical Endocrinologists (Terzolo *et al*. Eur J Endocrinol 2011), ESE/ENSAT – European Society of Endocrinology / European Network for the Study of Adrenal Tumors (Fassnacht *et al*. Eur J Endocrinol 2016), KES – Korean Endocrine Society (Lee *et al*. Endocrinol Metab (Seoul) 2017), JES – Japan Society of Endocrinology (Yanase *et al*. Endocr J 2018), SEEN – Spanish Society of Endocrinology (Araujo-Castro *et al*. Endocrinol Diabetes Nutr 2020).

JES abbreviations: A – low plasma levels of ACTH in the early morning, B – no diurnal changes in serum cortisol concentrations; C – unilateral uptake on adrenal scintigraphy; D – low serum concentrations of DHEAS; E – transient adrenal insufficiency or atrophy of the attached normal adrenal cortex after removal of the adrenal tumor).

The biochemical diagnostic and follow-up of non-producing adrenal adenomas

An adrenocortical adenoma is a benign neoplasm of adrenocortical cells. Although the majority of these lesions are initially diagnosed as non-secretory some may later produce glucocorticoids independent of ACTH and mineralocorticoids independent of stimulation from the RAAS. Rarely, they may also produce androgens or estrogens which may result in virilization or feminization (Sherlock *et al.* Endocr Rev 2020).

An important role in the regulation of adrenocortical cell development in played by the 3′, 5′-cyclic adenosine 5′-monohosphate–protein kinase A (cAMP–PKA) pathway. ACTH binds to the ACTH receptor (a G-protein coupled receptor) in the adrenocortical cell, thereby activating adenylyl cyclase, cAMP synthesis and activation of PKA. Abnormally increased cAMP–PKA signaling is thought to be the key mechanism in the development of most benign adrenocortical tumors (Kamilaris *et al.* Hormones (Athens) 2018; Sherlock *et al.* Endocr Rev 2020).

Based on the results of the hormone and radiological study, the patients diagnosed with non-functioning adrenal adenoma are expected to present the following biochemical characteristics: normal hormonal findings and the following radiological features: AI showing < 10 HU on CT scan without contrast, presenting signal loss in opposed-phase in MRI and/or contrast washout in CT > 60% absolute and > 40% relative washout (Fassnacht *et al.* Eur J Endocrinol 2016; Terzolo *et al.* Eur J Endocrinol 2011; Tabarin *et al.* Ann Endocrinol (Paris) 2008; Araujo-Castro *et al*. Endocrinol Diabetes Nutr 2020).

Considering the fact that the majority of AIs are benign and non-functioning, surgical treatment is therefore not required (Grumbach *et al.* Ann Intern Med 2003, Mansmann *et al.* Endocr Rev 2004). The main problem lies in uncertainty when deciding whether follow-up is necessary or not and how often it is needed. The recommendations regarding the follow-up and its required duration in case of benign and non-functioning AIs present some discrepancies between different guides (**Table 4**; Terzolo *et al.* Eur J Endocrinol 2011; Fassnacht *et al.* Eur J Endocrinol 2016, Tabarin *et al.* Ann Endocrinol (Paris) 2008; Grumbach *et al.* Ann Intern Med 2003; Zeiger *et al.* Endocr Pract 2009). A reason for the lack of consensus is mainly explained by the lack of solid scientific evidence, since most existing publications correspond to retrospective studies with limited case series, or to prospective studies with a short follow-up (Araujo-Castro *et al.* Endocrinol Diabetes Nutr 2020).

Table 4: Proposed follow-up for benign and non-functioning AIs, according to different guidelines		
guideline and country	**biochemical monitoring**	**radiological monitoring**
NIH (USA)	repeat in 6 months, 2 and 5 years, no changes → discharge	repeat in 6−12 months, no changes → discharge
AACE/ AASE (USA)	catecholamines and cortisol annually, for 5 years; no changes → discharge	repeat in 3−6 months and annually, 2 years no changes → discharge
FSE (France)	catecholamines and cortisol in 6 months, 2 and 5 years, no changes → discharge	repeat in 6 months, 2 and 5 years, no changes → discharge
IACE (Italy)	repeat study only if clinical changes	repeat in 3−6 months if > 2 cm, no changes → discharge, do not repeat if < 2 cm
ESE/ ENSAT (Europe)	non-functioning: do not repeat study, ACS or possible ACS: clinical monitoring, repeat study only if changes occur	adenoma < 4 cm: no follow-up, adenoma > 4 cm: repeat in 6−12 months, indeterminate: repeat in 6−12 months, no changes → discharge
The table presents information adapted after Araujo-Castro *et al.* Endocrinol Diabetes Nutr 2020 which shows the actual recommendations regarding the follow-up investigations, once a benign and non-functioning adrenal incidentalom is diagnosed. Abbreviations: NIH – National Institutes of Health (Grumbach *et al.* Ann Intern Med 2003); AACE/AAES – American Association of Clinical Endocrinologists/American Association of Endocrine Surgeons (Zeiger *et al.* Endocr Pract 2009); FSE – French Society of Endocrinology (Tabarin *et al.* Ann Endocrinol (Paris) 2008); IACE – Italian Association of Clinical Endocrinologists (Terzolo *et al.* Eur J Endocrinol 2011); ESE/ENSAT – European Society of Endocrinology/European Network for the Study of Adrenal Tumors (Fassnacht *et al*. Eur J Endocrinol 2016).		

Weaknesses of current diagnostic recommendations on the assessment of subtle cortisol hypersecretion

The actual biochemical diagnostic process, as described above, does not pay attention to concurrent secretion of glucocorticoids other than cortisol (F), if not accidentally picked up by cross-reacting immunoassays, and does not take into account either the free or bound portions of hormones or the clinically important sensitivity to glucocorticoids (Vassilatou *et al.* Clin Endocrinol 2009). Although cortisol and other glucocorticoids are bound to cortisol-binding protein, which is not respected during the diagnostic workup, but may be regulated by multiple factors, the diagnosis is based only on hormone measurements (Hammond GL. Endocrilol 2016; Lewis *et al.* Clin Chim Acta 2005; Bae *et al.* Best Pract Res Clin Endocrinol Metab 2015) that significantly interfere with diagnostic procedures (Nieman *et al.* J Clin Endocrinol Metab 2008; Dhillo *et al.* Eur J Endocrinol 2002; Winzinger *et al.* Horm Metab Res 2021). Net glucocorticoid action can only be recognized by the clinical evaluation which may differ depending

on the experience and shows otherwise lack of specificity and sensitivity (Popp *et al.* Exp Clin Endocrinol Diabetes 2019; Wei *et al.* Neuroendocrinol 2020; Winzinger *et al.* Horm Metab Res 2021).

It has already been suggested that glucocorticoid secretion from NPA is subtle and below the detection limit of current laboratory investigations, characterized by qualitative alterations of glucocorticoid production rather than explicit quantitative changes and therefore not sufficient to cause overt signs, symptoms or diagnostic hormonal findings, but might adversely affect multiple pathways associated with metabolic and cardiovascular outcomes (Peppa *et al*. Metabolism 2010; Terzolo *et al.* J Clin Endocrinol Metab 2002; Sippel *et al.* Surg Clin North Am 2004; Rossi *et al.* J Clin Endocrinol Metab 2000; Maser-Gluth *et al.* Eur J Clin Invest 2000; Yener S. Eur J Intern Med 2013). Therefore, instead of relying on the classical determination of hormone concentrations it is increasingly discussed to take into account aspects of the case histories and the results of the clinical examination and other specific symptoms of glucocorticoid excess (Nieman *et al.* J Clin Endocrinol Metab 2008; Carroll *et al.* Rev Endocr Metab Disord 2010).
As previously shown in other studies, patients who were classified as having a non-producing adrenal adenoma showed later signs of glucocorticoid excess as compared to normal individuals (Leibowitz *et al*. Clin Endocrinol 1996; Angeli *et al.* Front Horm Res 2002; Catargi *et al*. J Clin Endocrinol Metab 2003; Sippel *et al*. Surg Clin North Am 2004). Interestingly, serum concentrations of DHEAS have repeatedly been reported as being low in patients treated with exogenous glucocorticoids, for example the treatment of asthma (Kannisto *et al.* Eur J Endocrinol 2004).
However, it is still unclear whether endocrine function testing, e.g. suppression with dexamethasone, aldosterone to renin ratio, determinations of basal hormone levels, are sensitive enough to detect subtle/ subclinical hypersecretion of the adrenal gland. In addition, blood concentrations of hormones may not necessarily accurately reflect the extent of hormone action (Winzinger *et al.* Horm Metab Res 2021).

1.6. Hypothesis and purpose of the work

Currently, there is debate whether the current concepts of diagnosing hypersecretion of glucocorticoids are sufficient to detect subtle signs of excess hormone secretion in formally non-producing adrenal adenomas. In this paper, we studied whether the adrenal adenomas classified as non-functional do indeed show signs of excess glucocorticoid secretion.

To pick up glucocorticoid action rather than concentrations of total cortisol, we studied a number of parameters regulated by glucocorticoids, including negative feedback on ACTH or DHEAS concentrations and asked whether basal ACTH correlates with cortisol concentrations after DST and whether basal DHEAS concentrations correlate with ACTH. Since glucocorticoids are potent immunosuppressants and interfere with differential blood counts (Forsham *et al.* J Clin Endocrinol Metab 1948; Hills *el al.* Blood 1948; Parillo *et al.* Annu Rev Pharmacol Toxicol 1979), we asked whether

changes in differential blood counts indicate glucocorticoid excess when measurements of F after DST do not.

In summary, the aim of our study was to test whether there are signs of excess hormone secretion in formally non-producing adrenal adenomas because such indicators may serve as additional predictive factors that could possibly improve current diagnostic recommendations.

2. PATIENTS, MATERIALS AND METHODS

2.1. Patients and endocrine assessment

The ENSAT (European Network for the Study of Adrenal Tumors) initiative (Ethical committee number A 2016-0020) and the SHIP-PAGE study (steroid hormones in patients with pituitary, adrenal or gonadal endocrinopathies), Ethical committee number A 2016-0088 enabled us to retrospectively analyse data of patients who were diagnosed between 2005 and 2019.

In a retrospective design, we searched two databases of patients. The first database was comprised of 192 patients from University Hospital Düsseldorf, Division for Specific Endocrinology, who were diagnosed between 2005 and 2009 in the context of incidentally diagnosed adrenal masses or due to suspicion of underlying hormonal excess. The results obtained from this database have been published (Winzinger *et al.* Horm Metab Res 2021).

In order to confirm our previously found results, we decided to employ a second database. This database was comprised of initially 148 patients from the Rostock University Medical Center, Division of Endocrinology and Metabolism, who were diagnosed between 2010 and 2019.

For control, patients have been included that did not have an adrenal tumor but underwent a dexamethasone suppression test for other reasons, e.g. hypertension or weight gain. Cushings's disease was excluded in these patients and their response to dexamethasone normal.

All of the patients included in our study had been examined clinically. As selection criteria for patients, the aldosterone-to-renin ratio as well as plasma metanephrines and normetanephines were required to be normal in order to exclude the excessive secretion of aldosterone or catecholamines. In addition to that, women had their estrogens paused for more than a month before hormonal analysis.

Computed tomography or magnetic resonance imaging techniques were used for the radiological evaluation of adrenal masses. Endocrine function tests were performed in order to assess hormone secretion. Moreover, blood counts and differential blood counts were analysed to assess a possible glucocorticoid effect.

The folowing guidelines for the management of patients with adrenal incidentalomas were employed for interpretation of results obtained from the first database: Grumbach *et al.* Ann Intern Med 2003, Mansmann *et al.* Endocr Rev 2004**.** The recommendations from the most current guideline from ESE/ENSAT were also taken into consideration for this this paper (Fassnacht *et al.* Eur J Endocrinol 2016). Based on the results of imaging studies and cortisol suppression in the DST, patients were classified in one of the following groups:

- **Group A**: control subjects, no tumour on imaging and serum F after DST below 1.8 µg/dL (50 nmol/l);
- **Group B**: patients with an adrenocortical tumour on imaging and exclusion of endogenous hypercortisolism (NPA, non-producing adenomas) with an F concentration after DST below 1.8 µg/dL (< 50 nmol/l);

- **Group C**: patients with an adrenocortical adenoma on imaging and possible autonomous cortisol secretion (SAGH), indicated by an F concentration after DST between 1.8 and 5.0 µg/dL (50-138 nmol/l);
- **Group D**: patients with an adrenocortical adenoma on imaging and autonomous cortisol secretion (ACS) with an F concentrations after DST over 5.0 µg/dL (138 nmol/l).

The reference group (A) included subjects who were tested on all the above mentioned parameters for other reasons, including weight gain or arterial hypertension and who did not have an adrenal mass on imaging. Patients from Rostock University Medical Center were only egligible to form the groups B, C or D.

2.2. Hormone determinations

Baseline serum F, ACTH and DHEAS were measured between 8 and 10 AM. In addition, the overnight dexamethasone suppression test (DST) was performed with oral administration of 1 mg of dexamethasone between 11 and 12 PM and a consecutive measurement of cortisol the following day from a blood sample taken between 8 and 10 AM.Cortisol concentrations were determined by ECLIA, an electrochemiluminescence-immunoassay (Elecsys 1010/2010 or ModularAnalytics E170, Roche, Switzerland). The analytical detection limit is 0.018 to 63 µg/dL (0.5-1750 nmol/L). The intra- and interassay coefficients of variation were 1.1 and 1.3 % at a concentration of 7.53 µg/dL (208 nmol/L) in patient sera, and 1.1 and 1.6 % at a concentration of 46 µg/dL (1268 nmol/L). ACTH concentrations were determined by ECLIA, an electrochemiluminescence-immunoassay (Roche, Switzerland). Sensitivity (detection limit) is 1.0 pg/mL (0.22 pmol/L). The intra- and interassay coefficients of variation were 2.9 and 5.4 % at a concentration of 4.9 pg/mL (1.08 pmol/L) in human plasma, and 2.1 and 2.6 % at a concentration of 1390 pg/mL (306 pmol/L). The plasma samples were obtained in the morning (8-10 a.m.). DHEAS concentrations were determined by a competitive immunoassay (Immulite 2000, Siemens Healthcare Diagnostics GmbH, Germany). The detection limit was 3 µg/dL (0.08 µmol/l). The intra- and interassay coefficients of variation were 8 and 9.8 % at a concentration of 163 µg/dl (442 mmol/l), and 4.9 and 7.9 % at a concentration of 659 µg/dL (1789 mmol/L).

2.3. Radiological studies

CT scans were performed as 4 mm thick continuous slices with a Somatotom 2, Somatotom DR, or Somatotom DRH scanner (Siemens, Erlangen, Germany) before and after i.v. administration of contrast material.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism for Windows (version 4.1, GraphPad Software, San Diego, CA, USA), MedCalc or SPSS (IBM, Version 22). For comparisons of continuous variables between the groups, the non-parametric Mann–Whitney test or the two-tailed Student's *t*-test were used, depending on normal distributions of parameters. For the analysis of categorical data contingency tables and Fisher's exact test were employed. *P*-values below 0.05 were considered statistically significant (confidence interval, CI, 95%). Results are given as mean ± standard deviation (SD) for normally distributed variables and as median and 25/75 percentile for variables with a non-Gaussian distribution. It was corrected for alpha errors that have been, however, negligible small.

3. RESULTS

We retrospectively searched two the databases of patient visits from 2005 to 2009 in University Hospital Düsseldorf (D), and between 2010 and 2019 in University Hospital Rostock (HRO). We included 192 (from D), respectively 148 patients (from HRO) who had been seen in our outpatient service in the context of incidentally diagnosed adrenal masses or due to suspicion of underlying hormonal excess. Due to missing data along the parameters required for our study and after applying the selection criteria, we could only keep 64 patients from HRO. Basic characteristics of the study population are given in **Table 5**, the mean age being 59.2± 13.1 years (in D) and 58.4± 14.8 years (R). In D, the age was significantly lowest in group A, followed by group D and the portion of woman was notably higher in group D. In R the age was the lowest in group B, also followed by group D (**Table 5**).

Table 5: Basic characteristics of patients (Düsseldorf and Rostock)

F after DST	**Group A** <1.8 µg/dL	**group B** <1.8 µg/dL		**group C** 1.8–5.0 µg/dL		**group D** >5.0 µg/dL	
City	**D**	**D**	**HRO**	**D**	**HRO**	**D**	**HRO**
patients, n	34	68	26	56	22	34	16
women	88.2%	64.7%	50.0%	67.9%	52.9%	91.2%	60.0%
age [years]	52.2 ± 11.6	59.6 ± 11.5	50.5 (51; 64.5)	63.9 ± 13.5	62 (56.2; 67.7)	57.4 ± 14.3	58.5 (45.0; 66.7)
average tumor size [cm]	–	2.0 (1.6-3.2)	2.4 (1.5; 3.0)	3.2 (2.3-4.2)	3.0 (2.5; 3.5)	3.4 (3.0-4.0)	2.1 (1.2; 3.8)
basal F [µg/dL]	11.9 (9.3-15.8)	11.4 (8.5-15.1)	10.4 (9.3; 13.0)	14.5 (10.6-18.4)	16.5 (10.8; 19.3)	21.2 (15.3-26.7)	15.0 (10.2; 18.8)
F after DST [µg/dL]	1.0 (0.6-1.4)	1.1 (0.8-1.4)	1.0 (0.7; 1.3)	2.9 (2.2-3.7)	2.8 (2.3; 3.1)	8.0 (6.1-18.8)	10.2 (7.9; 16.3)
DHEAS [µg/dL]	123 (78-170)	67 (35-103)	56.1 (37.4; 90.2)	35 (20-87)	27.8 (11.5; 43.8)	18.0 (15-52)	25.8 (10.3; 49.7)
ACTH [pg/ml]	17.8 (13.1-22.0)	16.5 (8.9-20.9)	8.2 (2.76; 12.7)	7.0 (5.0-16.7)	7.5 (2.9; 10.8)	5.0 (1.0-9.8)	4.96 (1.4; 8.1)

Patients' characteristics in the groups A-D. Group A – control subjects, Group B – non-producing adenomas, Group C – possible autonomous cortisol secretion, Group D – autonomous cortisol secretion (ACS), ACTH – corticotropin; DHEAS – dehydroepiandrostenedione sulphate, DST – 1 mg dexamethasone suppression test, F – cortisol. HRO – Rostock, D– Düsseldorf.

The data obtained from Düsseldorf is given as means ± standard deviation and was published (Winzinger *et al.* Horm Metab Res 2021). The data obtained from Rostock is given as median and 27^{th} / 75^{th} percentiles.

The different degrees in F concentrations after DST were set as criterion to define the groups A, B, C, and D and to separate groups also with statistical proof (**Table 5**). As also shown in Table 5, in Düsseldorf, basal F concentrations did not differ between the groups except for group D which had

significantly higher F values. Also in Düsseldorf, patients in group B had the smallest adrenal tumor diameters in comparison to groups C ($p<0.01$) and D ($p<0.01$) (**Table 5**).

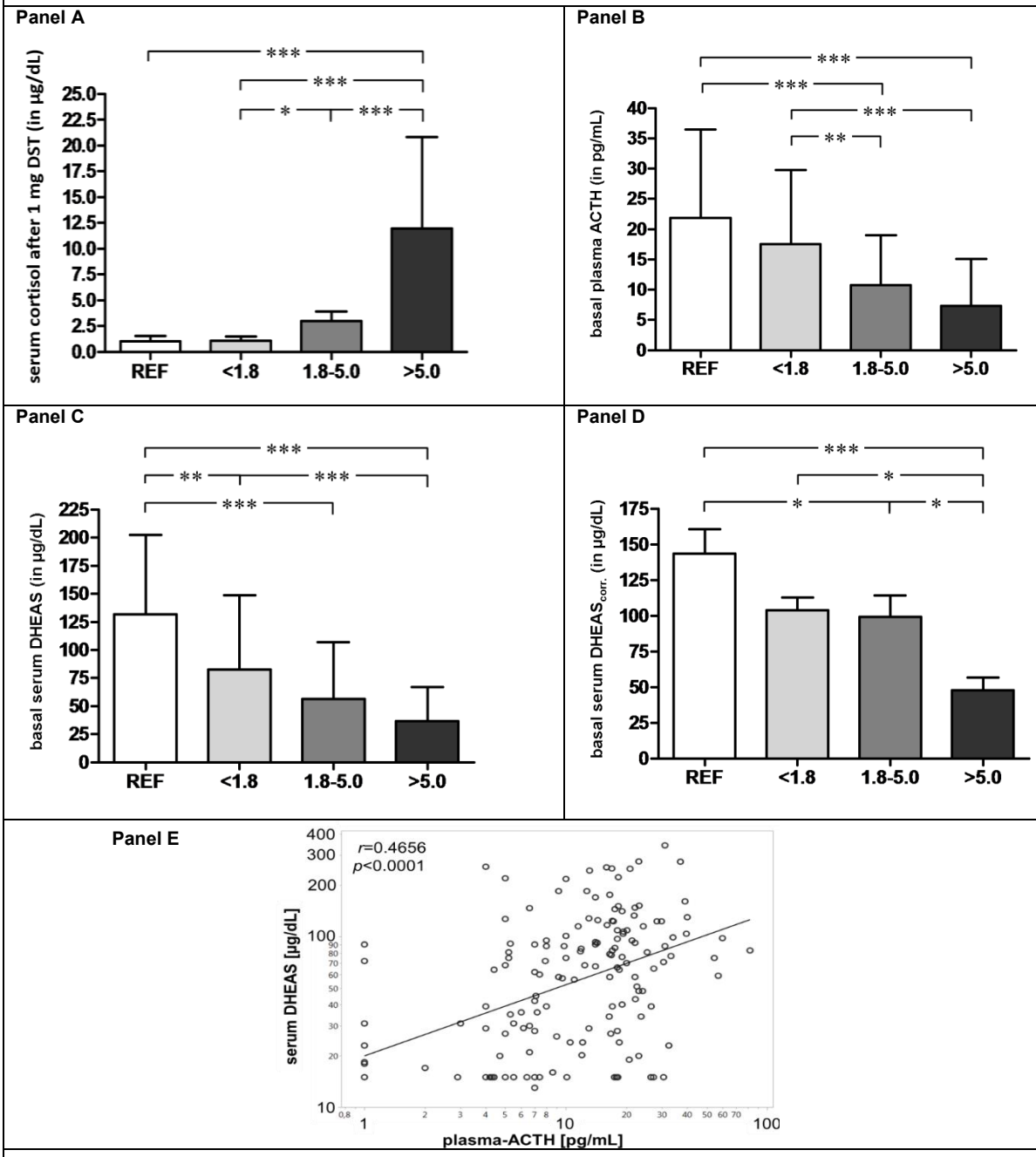
In Düsseldorf, as well as in Rostock, a step-wise decrease in basal ACTH or DHEAS secretion was observed ranging from group A (normal) over groups B and C (lower in comparison to group A) to group D (lowest). The data obtained from Düsseldorf is shown in **Figure 1**. These significant changes were observed in parallel for both ACTH and DHEAS whereby the difference in basal DHEAS was already significant between groups A and B. ACTH and DHEAS were related to each other (Spearman-r=0.466, p<0.0001) (**Figure 1**).

The different degrees in F concentrations after DST were not only reflected by step-wise changes in basal ACTH and DHEAS concentrations but also by the differences in the different blood counts (**Figure 2**) which correlated more or less well with hormone concentrations (**Table 6**). In Düsseldorf, a weak correlation between DHEAS and the relative portions of eosinophils (Spearman-r=0.263, p<0.01) can be observed, as well as between DHEAS and the relative portions of lymphocytes (Spearman-r=0.176, p<0.05). The ACTH was also related to the relative portions of the neutrophils (Spearman-r=0.277, p<0.001) and to the relative portions of lymphocytes (Spearman-r=0.284, p<0.001) (**Table 6**). Of interest, also in Rostock, DHEAS correlated again well with ACTH (Spearman-r=0.332, p<0.01). ACTH concentrations also correlated well with leukocytes (Spearman-r=0.243, p<0.01), the relative portions of lymphocytes (Spearman-r=0.227, p<0.01), neutrophils (Spearmanr=0.272, p<0.01) and eosinophils (Spearman-r=0,222, p<0.01) (**Table 6**).

We also found a weak but significant inverse correlation (in D) between age and DHEAS (Spearman-r= –0.24, p<0.01) that was stable in a subgroup analysis of groups A and B but no more visible in groups C and D where ACTH concentration were lowest. DHEAS concentrations were slightly higher in women than in men over the whole study population (p<0.01) even though age and ACTH were comparably high. There was also a weak inverse correlation between age and lymphocyte count (Spearman-r= –0.19, p<0.05). Moreover, age correlated weakly with the count of leucocytes (Spearman-r= –0.17, p<0.05), thrombocytes (Spearman-r= –0.22, p<0.01) and neutrophils (Spearman-r= –0.15, p<0.05).

There was an association between adrenal adenoma size and basal F concentrations (Spearman-r=0.22, p<0.01) as well as F after DST (Spearman-r=0.382, p<0.0001) and between basal F and F after DST (Spearman-r=0.536, p<0.0001). Of note, the negative feedback on ACTH secretion by cortisol was reflected by a weak but significant inverse correlation between tumor size and basal ACTH (Spearman-r= –0.208, p<0.05) as well as DHEAS (Spearman-r= –0.298, p<0.01).

Figure 1: Means and standard deviations of hormonal determinations in the patients groups from Düsseldorf



The figure comprises column bar graphs generated using the means and standard deviations of hormonal determinations in the patients groups as follows: serum cortisol after 1 mg DST (Panel A); basal concentrations of corticotropin (ACTH, Panel B) and dehydroepiandrostenedione-sulfate (DHEAS, Panel C) are given along with the more or less good correlation between basal ACTH and DHEAS values (Panel E). The stepwise increase in F concentrations after DST was a classifier for patients groups. However, there is also a stepwise decrease in both ACTH and DHEAS concentrations ranging from normal individuals over patients with non-producing adrenal adenomas (<1.8) and patients with adrenal adenomas causing mild cortisol excess (1.8-5.0) to patients with biochemically defined hypercortisolism (>5.0).

ACTH – corticotropin, DHEAS – dehydroepiandrostenedione sulfate, DST – 1 mg dexamethasone-suppression test

Asteriks indicate significance in the ANOVA analysis, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. Parts of the results presented by Figure 2 have already been published in Winzinger *et al.* Horm Metab Res 2021.

Table 6: Correlations of blood count indices with results of hormonal determinations (results obtained from Düsseldorf and Rostock)

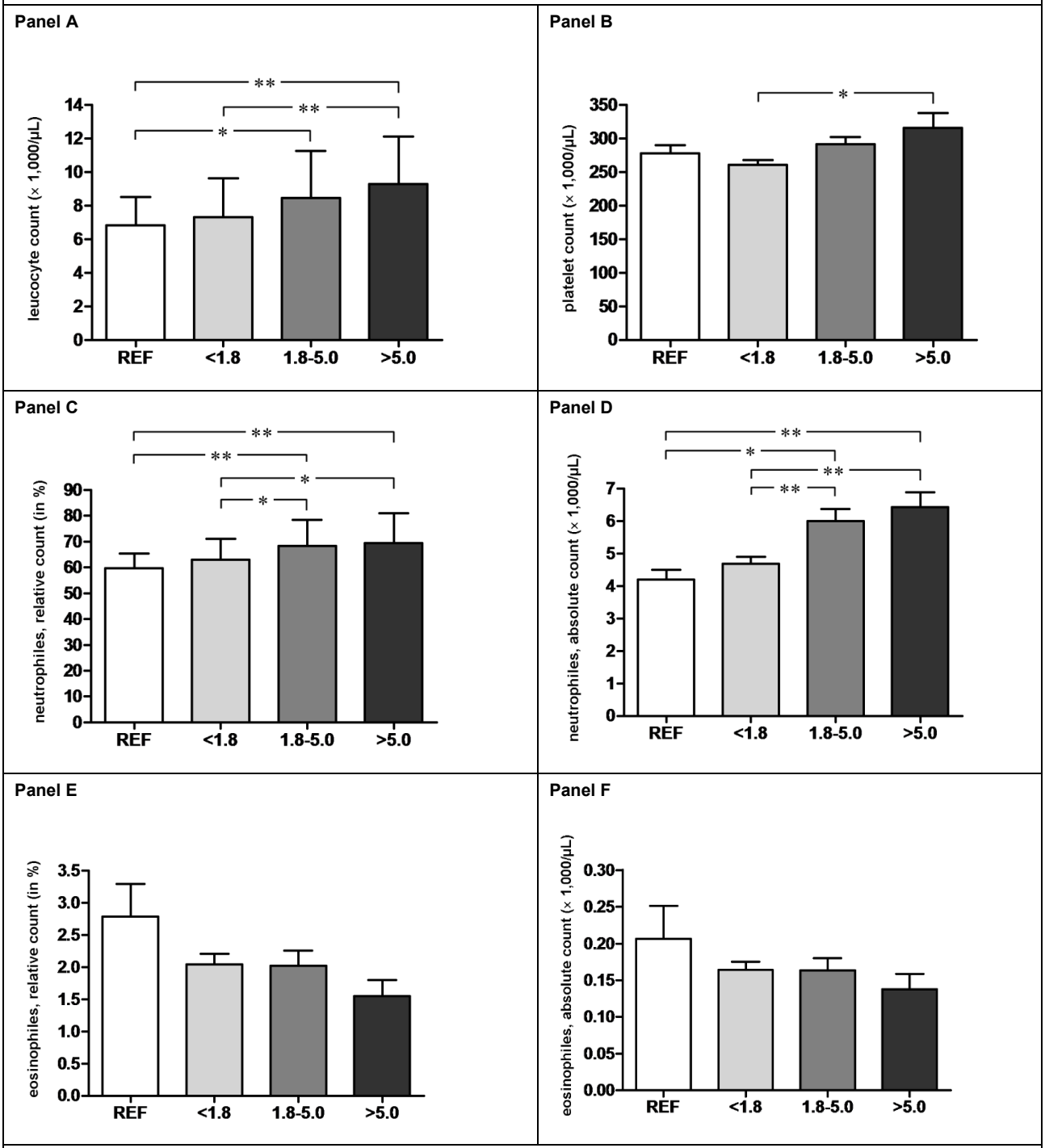
para-meter	TU size		basal F		DST		ACTH		DHEAS	
	D	HRO	D	HRO	D	HRO	D	HRO	D	HRO
leu	*r*=0.040 n.s.	*r*=0.042 n.s.	***r*=0.222 *p*<0.01**	n.t	***r*=0.356 *p*<0.0001**	n.t	***r*=–0.235 *p*<0.01**	***r*=–0.243 *p*<0.001**	*r*=–0.139 n.s.	*r*=0.116 n.s.
thr	*r*=0.055 n.s.	r=0.052 n.s.	***r*=0.156 *p*<0.05**	n.t	***r*=0.257 *p*<0.001**	n.t	*r*=–0.099 n.s.	*r*=–0.031 n.s.	*r*=–0.090 n.s.	*r*=0.014 n.s.
nph, rel	*r*=0.119 n.s.	r=0.141 n.s.	***r*=0.325 *p*<0.0001**	n.t	***r*=0.342 *p*<0.0001**	n.t	***r*=0.277 *p*<0.001**	**r=0.272 *p*<0.001**	*r*=–0.154 n.s.	*r*=–0.061 n.s.
nph, abs	*r*=0.057 n.s.	r=0.080 n.s.	***r*=0.323 *p*<0.0001**	n.t	***r*=0.391 *p*<0.0001**	n.t	***r*=–0.237 *p*<0.01**	**r=0.238 *p*<0.001**	*r*=–0.137 n.s.	*r*=–0.048 n.s.
ly, rel	*r*=–0.147 n.s.	**r=–0.165 *p*<0.05**	***r*=–0.371 *p*<0.0001**	n.t	***r*=–0.409 *p*<0.0001**	n.t	***r*=0.284 *p*<0.001**	**r=0.227 *p*<0.01**	***r*=0.176 *p*<0.05**	*r*=0.065 n.s.
ly, abs	*r*=–0.063 n.s.	r=–0.087 n.s.	***r*=–0.194 *p*<0.05**	n.t	*r*=–0.130 n.s.	n.t	*r*=0.140 n.s.	r=0.071 n.s.	*r*=0.110 n.s.	*r*=0.027 n.s.
eos, rel	*r*=–0.133 n.s.	r=0.145 n.s.	***r*=–0.168 *p*<0.05**	n.t	***r*=–0.204 *p*<0.05**	n.t	***r*=0,251 *p*<0,01**	**r=0.222 *p*<0.01**	***r*=0.2636 *p*<0.01**	*r*=0.092 n.s.
eos, abs	*r*=–0.121 n.s.	r=–0.093 n.s.	*r*=–0.057 n.s.	n.t	*r*=–0.099 n.s.	n.t	***r*=0.245 *p*<0.01**	**r=0.222 *p*<0.01**	***r*=0.206 *p*<0.05**	*r*=–0.097 n.s.

The table shows the results of the correlations between different blood count parameters and hormonal determinations. To the already published results from Düsseldorf (Winzinger *et al.* Horm Metab Res 2021), we added the results obtained from the Rostock analysis. Calculations of Pearson's or Spearman's-*r* (*r*) are given along with probability values (*p*) that show the relation of parameters to each other and their significance, respectively.

Abbreviations: TU – tumor, F – cortisol, DST – 1 mg dexamethasone-suppression test, ACTH – corticotropin, DHEAS – dehydroepiandrostenedione sulfate; D – Düsseldorf, HRO – Rostock; leu – leukocytes, thr – platelets, nph – neutrophils,ly – lymphocytes, eos – eosinophils; abs – absolute counts, rel – relative portions; n.s. – not significant, n.t. – not tested

Of importance, there was a step-wise increase in the leukocytes and neutrophils count, as well as a step-wise decrease in the relative and absolute counts of lymphocytes, ranging from group A, towards group D. The lymphocytes correlated well with the plasma ACTH concentrations (**Figure 2**).

Figure 2 – part 1: Means and standard deviations of indices of blood counts in the patients groups from Düsseldorf



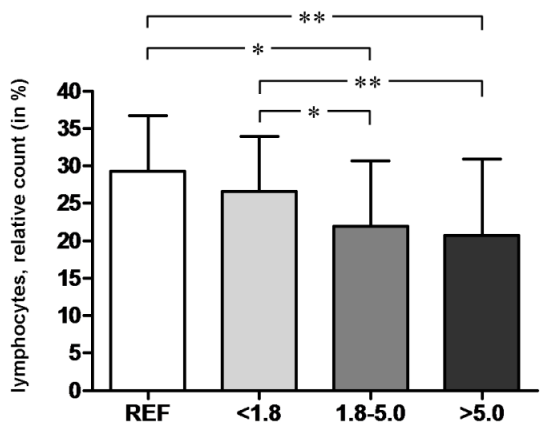
The figure comprises column bar graphs generated using the means and standard deviations of of indices of blood counts in the patients groups from Düsseldorf as follows:

leucocyte count (Panel A); platelet count (Panel B); neutrophils, relative (Panel C) and absolute (Panel D); eosinophiles, relative (Panel E) and absolute (Panel F) counts. There is a stepwise change to be observed in indices of whole and differential blood counts ranging from normal individuals over patients with cortisol producing adrenal adenomas (>5.0). Some results are not significant due to the small number of patients included in the study but the differences between the groups can still be observed.

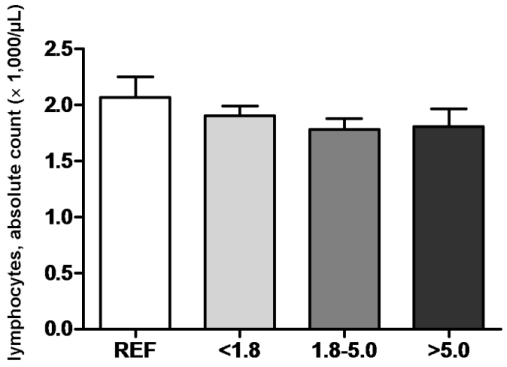
Asteriks indicate significance in the ANOVA analysis, * - p<0.05, ** - p<0.01, *** - p<0.001 (Some results presented by Figure 2 have already been published: Winzinger *et al.* Horm Metab Res 2021).

Figure 2 – part 2: Means and standard deviations of lymphocytes counts in the patients groups from Düsseldorf and correlation between lymphocytes and ACTH

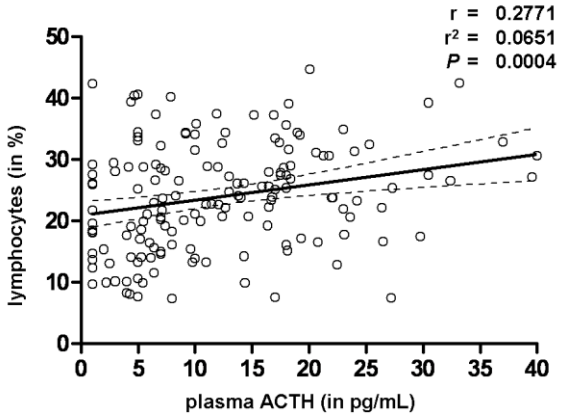
Panel G



Panel H



Panel



The figure comprises column bar graphs generated using the means and standard deviations of indices of blood counts in the patients groups as follows: lymphocytes, relative (panel G) and absolute (panel H) counts and a graphical representation of the correlation between lymphocytes and plasma ACTH (Panel I); ACTH – corticotropin.

There is a stepwise change to be observed in indices of whole and differential blood counts ranging from »normal individuals« over patients with cortisol-producing adrenal adenomas (>5.0). Some results are not significant due to the small number of patients included in the study but the differences between the groups are apperent.

Asteriks indicate significance in the ANOVA analysis, * - p<0.05, ** - p<0.01, *** - p<0.001 (some results of Figure 2 have already been published in Winzinger *et al.* Horm Metab Res 2021).

In Rostock, the graphical representation of the concentrations of studied parameters allowed us to observe again the step-wise differences between the patients groups. In spite of that, the statistical relevance was not so strong as in the previous study due to the smaller number of included participants.

4. Discussion

With the widespread application of ultrasound, CT and MRI, incidentally discovered adrenal tumors have been detected with increasing frequency in so described “clinically inapparent” subjects (Ichijo *et al.* Endocr J 2019). Although the majority of the adrenal incidentalomas are biochemically described as NPAs, subtil signs of glucocorticoid excess may be present or become clinically apparent only later (Leibowitz *et al*. Clin Endocrinol 1996; Angeli *et al*. Front Horm Res 2002; Catargi *et al*. Clin Endocrinol Metab 2003; Sippel *et al*. Surg Clin North Am 2004; Yener *et al.* Eur J Intern Med 2012). The relative risk of developing ACS in NPA and with possible ACS varies among the different studies from 6.6 to 31%, depending on the criteria used to define ACS, and the follow-up period involved (Araujo-Castro *et al.* Endocrine 2019; Araujo-Castro *et al.* Endocrinol Diabetes Nutr 2020).

An increased risk of developing ACS in NPA has been documented in lesions with a diameter measuring over 2.5-3 cm (Barzon *et al.* Eur J Endocrinol 2002; Morelli *et al.* J Endocrinol Investig 2017). The patients included in our study classified as having an NPA (group B) presented tumors with an average diameter of 2.0 cm (1.6- 3.2 cm) and the patients classified as having a possible ACS were found to have average diameters of 3.2 cm (2.3- 4.2 cm). However, a recent meta-analysis found that only 2.5% of the patients with NPA or ACS experienced significant changes in lesion size (≥ 10 mm) or function (4.3% of the NPA developed ACS), and there were no cases of malignant transformation. The study was based on a cohort of 4121 patients with a follow-up of 50.2 months (Elhassan *et al.* Ann Intern Med 2019). However, this observation interval may be too short to draw definite conclusions and the means to define hormone excess somewhat crude as shown by our data.

An oversecretion of glucocorticoids of NPA could also be demonstrated by their association with the metabolic syndrome (Ribeiro Cavalari *et al.* Clin Endo crinol Oxf 2018, Midorikawa *et al.* Clin Endocrinol Oxf 2001, Reimondo *et al.* J Clin Endocrinol Metab 2020). The first large scale data regarding prevalence of metabolic syndrome components were discussed in a multicenter survey which included 1004 patients (Mantero *et al.* J Clin Endocrinol Metab 2000). 85% of the participants had NPA and 9% had ACS. The frequency of hypertension, diabetes and obesity were 41%, 10% and 28%, respectively. In another study, which included 231 participants with NPA (Comlekci *et al.* Endocrine 2010), hypertension, metabolic syndrome, glucose intolerance and cardiovascular disease were found to have a prevalence of 52%, 46%, 42%, and 7%, respectively. These percentages, in case of adrenal adenomas which were considered to be non-functional, were higher than the prevalence rates obtained from health examination surveys of a general population (Erem *et al.* J Public Health (Oxf) 2009; Yener S. Eur J Intern Med 2013). Prevalence of hypertension was also found to be significantly higher in NPA subjects than the average population. Moreover, the frequency of the clinical complications increased in parallel to the degree of hypercortisolism (Yener S. Eur J Intern Med 2013). An increased cardiometabolic risk and the exacerbation of such risk during follow-up in AI with ACS versus NPA was also observed (Elhassan *et al.* Ann Int Med 2019). Moreover, an impaired cardiovascular profile is frequently found in patients with NPAs (Di Dalmazi *et al.* Curr Opin Endocrinol Diabetes Obes 2015, Kim

et al. Endocrinol Metab Seoul 2014), which also present a higher frequency of hypertension (Arruda *et al.* J Hum Hypertens 2017). They are also associated with the development of atherosclerotic risk factors (Yener *et al.* Med Princ Pract 2012), with elevated D-dimer levels (Yener *et al.* J Endocrinol Invest 2009) and with endothelial alterations (Yener *et al.* J Endocrinol Invest 2011). As a matter of fact, there are other reports in favor of the hypothesis that formally NPAs do indeed cause mild glucocorticoid excess (Arruda *et al.* J Hum Hypertens 2017).

Taking into consideration the fact that no gold standard has yet been established for diagnosing ACS, the current recommendations for the diagnosis of endogenous hypercortisolism are only based on the determination of F above 1.8 µg/dL (50 nmol/l) after DST (Nieman *et al. J Clin Endocrinol Metab* 2008, Fassnacht *et al.* Eur J Endocrinol 2016, Farrugia *et al.* Rom J Intern Med 2017). The biochemical definition does not pay attention to concurrent secretion of glucocorticoids other than F if not accidentally picked up by cross-reacting immunoassays and does not take into account either the free or bound portions of hormones or the clinically important sensitivity of glucocorticoids (Hammond GL. J Endocrinol 2016). As such, the diagnosis is based on hormone measurements although cortisol and other glucocorticoids are bound to cortisol-binding protein which is not respected during the diagnostic workup but may be regulated by multiple factors (Hammond GL. J Endocrinol 2016; Lewis *et al.* Clin Chim Acta 2005; Bae *et al.* Best Pract Res Clin Endocrinol Metab 2015) that significantly interfere with diagnostic procedures (Nieman *et al.* J Clin Endocrinol Metab 2008; Dhillo *et al.* Eur J Endocrinol 2002; Winzinger *et al.* Horm Metab Res 2021).

Various important guidelines (**Table 3**) from different countries include a wide variety of recommendations regarding the first screening, secondary screening and confirmation tests that may slightly vary depending on the guideline. The use of the 1-mg DST is considered to have the highest sensitivity for screening for ACS. A post-dexamethasone cortisol concentration ≤1.8 µg/dL (≤50 nmol/L) is considered “normal” and "excludes" cortisol excess in most cases (Di Dalmazi *et al.* Lancet Diabetes Endocrinol 2014; Debono *et al.* J Clin Endocrinol Metab 2014; Sherlock *et al.* Endocr Rev 2020). Additional markers such as ACTH and DHEAS are recommended to be tested only in order to determine the adrenal origin of the glucocorticoid hypersecretion (**Table 3**). As metioned above, NPAs are often associated with other comorbidities and may develop later signs of glucocorticoid excess. Following the guidelines, net glucocorticoid action is only recognized by the clinical evaluation which is dependent on experience and shows otherwise lack of specificity and sensitivity (Popp *et al.* Exp Clin Endocrinol Diabetes 2019; Wei *et al.* Neuroendocrinol 2020). It is not clear if the current diagnostic concepts that are mainly based on F measurements are accurate enough to determine and to predict a possible subclinical hyperfunction of NPAs.

Therefore, we have studied further markers of endocrine activity in order to identify adrenal masses at risk for autonomous hypercortisolism. In this study, we have evaluated the utility of ACTH, DHEAS and differential blood counts, as indicators of glucocorticoid action. As already mentioned into the study

protocol, we decided to use the data obtained from patients from Düsseldorf and to repeat the initial study using information achieved from patients from Rostock in order to verify the already obtained results. The results were similar but the statistical relevance was not so strong in the second study due to the smaller number of participants included and due to the missing data from some studied parameters. The outliers were not excluded.
Our results showed that F concentrations after the low dose DST correlate well with basal ACTH and DHEAS concentrations. Moreover, ACTH and DHEAS correlate well. Of interest, when we added the data obtained from our patients from Rostock, DHEAS correlated again well with ACTH.

The mean values of ACTH and DHEAS decrease step-wise ranging from group A (control), over groups B (NPAs) and C (SAGH), towards group D (CSA). Of interest are lower concentrations of ACTH and DHEAS in group B (NPAs) in comparison with group A (control), the difference in basal DHEAS being already significant (Figure 1). Decreased values of ACTH and DHEAS may indicate subtle cortisol excess in group B (NPAs) which demonstrates the utility of such markers (Winzinger *et al*. Horm Metab Res 2021). The graphical representation of the concentrations of studied parameters allowed us to observe again the step-wise differences between the patients groups from Rostock (data not shown because of mere confirmation).

Dehydroepiandrosterone and its sulfate are produced by the adrenal cortex. Their secretion is, at least partially, controlled by ACTH (Mantero *et al.* J Clin Endocrinol Metab 2000). In addition, in patients with cortisol-secreting adrenal adenomas (CSA), the concentrations of DHEAS and ACTH are known to be reduced. In case of the removal of the hypersecreting tumour pre-interventionally supressed ACTH and cortisol concentrations are reported to recover after 12 to 18 months while DHEAS concentrations often remain low for a longer period after surgery, occasionally up to 8 years. This may indicate a dissociation of adrenal DHEAS and cortisol regulation (Cavagnini *et al.* Endocrinol Adult Pediatr 2016), which may be of advantage to differentiate Cushing's disease from adrenal hypercortisolism along with basal ACTH (Masjkur *et al*. J Clin Endocrinol Metab 2019; Winzinger *et al*. Horm Metab Res 2021). The use of age-adjusted DHEAS ratio has already been shown to be a sensitive and specific tool for screening the ACS in patients with incidentally detected AIs (Dennedy *et al.* J Clin Endocrinol Metab 2017).

Current guidelines recommend to support the clinical diagnosis of Cushing's syndrome through biochemical testing which centers serum F concentrations after DST (Nieman *et al.* J Clin Endocrinol Metab 2008). However, while a blood concentration of a hormone may serve as a tumor marker, it may be limited in its concentration as a marker of hormone action that emphasizes the value of taking the case history and the physical examination in patients with Cushing's syndrome (Carroll *et al.* Rev Endocr Metab Disord 2010). Moreover, other parameters of hormone action may become helpful surrogates for answering the question whether correction of an elevated marker such as F after DST is useful to help a patient in the prevention of developing complications from an adrenal tumor with a more or less

autonomous glucocorticoid secretion. As a limitation, the power of this study is too low to obtain meaningful results from analysis of parameters such as osteoporotic fractures, osteoporosis defined by dual X-ray absorptiometry and even diabetes or hypertension. Thus, our study does not provide a cut-off value that was determined against the background of endpoints but rather shows that basal corticotropin and DHEAS concentrations may be distributed over a continuum reflecting the status of the negative feedback by net glucocorticoid action. Further evaluation of these parameters may however help in understaning the extent of excess cortisol secretion by diseased adrenal tissue (Winzinger *et al*. Horm Metab Res 2021).

In order to observe an alternative point of view of the actual diagnostic method we investigated differential blood counts and we observed changes that already indicate subtle forms of glucocorticoid (GC) excess (Winzinger *et al*. Horm Metab Res 2021). One of the glucocorticoid effects is immunosuppression with induced apoptosis of lymphocytes via the induction of G1 cell cycle arrest (Harmon *et al.* J Cell Physiol 1979) and programmed cell death of immature thymocytes (Wyllie Nature 1980) and mature peripheral T lymphocytes (Herold *et al.* Cell Mol Life Sci 2006). The mechanisms of GC-induced apoptosis seem to involve the loss of mitochondrial potential (Marchetti *et al.* J Exp Med 1996; Petit *et al.* J Cell Biol 1995) and the subsequent activation of caspases (Robertson *et al*. Cancer Res 1997; Chandra *et al*. J Blood 1997). The signal transduction pathway is initiated by binding of the glucocorticoid to the glucocorticoid receptor (GR) (Baxter *et al.* Science 1971). Furthermore c-myc down-regulation may be directly involved in the inhibition of cellular proliferation and in the apoptosis of lymphocytes (Yuh *et al.* J Biol Chem 1989). The most significant mechanism of action of GCs lies in their capacity to block cytokine production (Almawi *et al.* Cell Transplant 1998; Mori *et al.* Blood 1997). In addition to the above-mentioned mechanisms, recent studies suggested that GCs exerted their effects, at least in part, through induction of specific GC-regulated genes, GILZ (GC-induced leucine zipper) and GITR (GC-induced TNFR family-related) (Riccardi *et al.* Adv Exp Med Biol 2001; Cannarile *et al.* Cell Death Differ 2001; Winzinger *et al*. Horm Metab Res 2021).

As expected, we found that ACTH and DHEAS concentrations correlated well with the extent of relative portions of lymphocytes (**Table 6**). Furthermore, the concentrations of relative count of lymphocytes differed statistically significantly between the patient groups, decreasing gradually from group A (control), towards group D (CSA). The difference between group A (control) and group B (NPAs) can again be observed, the patients with NPAs already showing lower lymphocytes concentrations compared to normal subjects (**Figure 2**). This may also suggest a subtle glucocorticoid hypersecretion present by the formal NPAs (Winzinger *et al*. Horm Metab Res 2021). Coming along with these data, leucocytosis was direct proportional with the F after DST values, increasing step-wisely, being the most prominent in group D (CSA) (**Figure 2**). The results obtained from Rostock, also confirmed the above mentioned aspects: ACTH concentrations also correlated well with leukocytes, the relative portions of lymphocytes, neutrophils and eosinophils.

One of the strengths of our study is that the patients were assessed in a single center and laboratory, only the subjects with adrenal cortical adenomas were selected, those with carcinoma or with excessive secretion of aldosterone or catecholamines being rejected. Another strength could be the confirmation and verification of the already obtained results using data from another center from a different city and from a different time interval.
A weakness of our study could be the fact that we did not used the lowered calculated age- and sex-specific DHEAS ratios that could lead to more statistical significant results. However, age differences between the patient groups with adrenal tumors were almost negligible and DHEAS was found to be lower in group D as compared to groups B and C although individuals in group D were younger than in B or C. We conclude from our data that age is only a confounder in patients in whom glucocorticoid secretion is normally regulated or close to normal. In our opinion, this indicates that DHEAS concentrations are indeed rather driven by excess glucocorticoid secretion than by age in patients with adrenal adenomas (Winzinger *et al*. Horm Metab Res 2021).

DHEAS is secreted in a large plasma pool and therefore exhibits a longer half-life (10–20 h) and fewer circadian variations compared with cortisol (Bornstein *et al.* Ann Int Med 1999). Several studies reported low DHEAS levels in patients with documented hypopituitarism and HPA-insufficiency (Kasperlik-Zaluska *et al.* Clin Endocrinol (Oxf) 1997; Arnaldi *et al.* Braz J Med Biol Res 2000). Therefore, reduced DHEAS concentrations were thought to indicate subclinical autonomous glucocorticoid hypersecretion by adrenal incidentalomas as well (Bülow *et al.* Eur J Endocrinol 2006) but the sensitivity and specificity of this parameter were poor (51 and 65%, respectively, with PPV 10%), due to influencing factors, most importantly age and gender (Mantero *et al.* J Clin Endocrinol Metab 2000). Recently, it has also been shown that lowered calculated age- and sex-specific DHEAS ratios (derived by dividing the DHEAS by the lower limit of the respective reference range) is a sensitive and specific marker of subclical hypercortisolism (F concentrations after DST over 5.0 µg/dL (138 nmol/l) in adrenal incidentalomas (sensitivity 100%, specificity 91.9%) (Dennedy *et al.* J Clin Endocrinol Metab 2017; Winzinger *et al*. Horm Metab Res 2021).

Conflicting results may also be the consequence of methodological shortcomings, e.g. determination of DHEAS concentrations using different approaches, e.g. immunoassays that show more cross-reactivity in comparison to mass spectronomy-based techniques which may become a problem in patients with lower DHEAS concentrations. Though cortisol assays seem to render more or less similar results (Huayllas *et al.* Lab Med 2018) determination of other adrenal steroids may even render opposite information as shown for aldosterone (Constantinescu *et al.* Clin Chim Acta 2020; Winzinger *et al*. Horm Metab Res 2021).
Likewise, ACTH is known to be a fragile analyte that is prone to variations that are reported to be less significant with the method we have employed (Nandakumar *et al.* Clin Biochem 2020). Another

limitation is that dexamethasone concentrations have not been determined to ensure effective supression of HPA axis during the test and proper characterization of patients. However, it is also to mention that correlations may be less sensitive because ACTH and DHEAS concentrations sometimes hit the lower limit of detection what may have led to false high entries in our statistical analysis – especially in the group of patients with overt Cusing's syndrome (Winzinger *et al*. Horm Metab Res 2021).

Considerable discrepancy between recommendations offered by the different guidelines raise many issues when it comes to the follow-up suggestions regarding NPAs (**Table 4;** Terzolo *et al.* Eur J Endocrinol 2011; Fassnacht *et al.* Eur J Endocrinol 2016, Tabarin *et al.* Ann Endocrinol (Paris) 2008; Grumbach *et al.* Ann Intern Med 2003; Zeiger *et al.* Endocr Pract 2009). One main problem lies in the uncertainty when deciding whether follow-up is necessary or not and how often it is needed.
One of the most important points in planning a follow-up is the information obtained from the initial radiological assessment, since the subsequent follow-up depends on the initial radiological characteristics of the lesion. As mentioned above, there is considerable discrepancy between the radiological monitoring recommendations (**Table 4**)**.** Following the suggestions based on the results obtained from the meta-analysis conducted by Elhassan (Elhassan *et al.* Ann Int Med 2019) and from the European guidelines (Fassnacht *et al.* Eur J Endocrinol 2016), in case of an AI initially diagnosed as non-functional and measuring < 4 cm in size and with unequivocally benign radiological features, no further imaging studies are strictly recommended (Fassnacht *et al.* Eur J Endocrinol 2016). According to different studies that have analysed the risk of malignancy transformations of lesions initially diagnosed as NPAs is less than 1% in patients subjected to surgery due to an increase in lesion size during follow-up (Elhassan *et al.* Ann Int Med 2019; Lamas *et al.* Endocrinol Nutr 2009; Libe *et al.* Eur J Endocrinol 2002; Barzon *et al.* J Clin Endocrinol Metab 1999; Araujo-Castro *et al.* Endocrinol Diabetes Nutr (Engl Ed) 2020). Surgical treatment is therefore for benign and non-functioning adrenal adenomas not required (Grumbach *et al.* Ann Intern Med 2003, Mansmann *et al.* Endocr Rev 2004).

When referring to the biochemical work-up of NPAs, based on already published data (**Table 4**), and until prospective studies with longer follow-up periods become available, the re-examination using the DST suppression test is annually recommended, for at least 5 years in patients with AI in general. Other examinations including UFC, nocturnal cortisol, ACTH, DHEAS are suggested to be taken into consideration for patients with possible ACS and with ACS (Araujo-Castro Endocrinol Diabetes Nutr (Engl Ed) 2020). In these latter two groups, assessment moreover should also include screening and the control of comorbidities potentially related to hypercortisolism (type 2 diabetes, arterial hypertension, obesity, osteoporosis and dyslipidemia). In fact, this aspect should receive priority even over hormone testing, since it will condition the treatment decision in most patients with ACS and possible ACS. Repeated hormone testing to screen for PHA or pheochromocytoma is not recommended unless there

are new clinical-biochemical data giving reason to suspect such disorders (Araujo-Castro Endocrinol Diabetes Nutr 2020).

A reason for the lack of consensus between various guidelines is mainly explained by the lack of solid scientific evidence, since most existing publications correspond to retrospective studies with limited case series, or to prospective studies with a short follow-up (Araujo-Castro *et al.* Endocrinol Diabetes Nutr 2020). The clinical question remains whether patients should benefit from adrenalectomy when they have a supposedly benign adrenocortical tumour and show no clear clinical or laboratory signs of hormone excess (Ye *et al.* BMC Surg 2016, Nishikawa *et al.* Biomed Pharmacother 2002). In recent studies, the surgery is not required in unilateral, clearly benign, non-functioning, small adrenal tumors (<4 cm) (Papierska *et al.* Pol J Radiol 2013, Zeiger *et al.* J Clin Endocrinol Metab 2011). In our data, the biggest average size of the adrenal adenoma is 3.4 cm for group D (CSA) **(Table 5)**. In absence of randomised prospective studies, surgical or medication-based treatment of subclinical Cushing's syndrome remains a single-case decision, a multidisciplinary approach being also required (Vassiliadi *et al.* Nat Rev Endocrinol 2011, Di Dalmazi *et al*. Eur J Endocrinol 2015, Thomas *et al.* Eur Urol Focus 2016; Winzinger *et al*. Horm Metab Res 2021).

Our results showed that cortisol concentrations after DST correlate well with the extent of ACTH suppression, decreased DHEAS synthesis and the extent of relative lymphocytopenia as a parameter of GC action. Therefore, we were able to demonstrate that patients that were classified as having a non-producing adrenal adenoma show signs of GC excess when compared to "normal patients". As a novelty of our study it may be informative to determine such parameters as ACTH, DHEAS, relative count of lymphocytes and other blood counts to assess patients for subtle adrenal glucocorticoid excess (Winzinger *et al*. Horm Metab Res 2021). These additional examinations could represent a useful tool for an early determination of subtle glucocorticoid hypersecretion and for deciding the best follow-up recommendations for patients with NPAs.

5. Summary

The majority of incidentally discovered adrenal tumors are later characterized as non-producing adrenocortical adenomas (NPA). We asked whether subtle laboratory abnormalities in parameters that reflect glucocorticoid action can be found in patients with NPA despite their definition of being "silent".
Since glucocorticoids are potent immunosuppressants, we studied blood counts and differential blood counts along with corticotropin and dehydroepiandrostendione (DHEAS) blood concentrations, as well as cortisol values before and after an overnight 1 mg dexamethasone suppression test. We compared the results of normal individuals, of patients with adrenal adenomas and normal hormone profiles and with subclinical autonomous glucocorticoid hypersecretion, as well as overt cortisol excess.
We found that almost all indices of the blood counts were significantly different between the patient groups. In particular, patients with adrenal non-producing adenomas already showed signs of glucocorticoid excess, including relative lymphocytopenia, lowered DHEAS and ACTH concentrations.
We also found that the extent of lymphocytopenia correlated well with the concentrations of DHEAS and ACTH, and DHEAS correlated well with ACTH concentrations. In order to confirm the already obtained results from Düsseldorf, we repeated the same study protocol in a different centre in Rostock and in a different period of time.
We conclude that the basal ACTH and DHEAS concentrations along with the differential blood counts give good information on the extent of glucocorticoid excess and that silent adrenal adenomas seem to oversecrete glucocorticoids at concentrations that already alter these parameters.

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7. Abbreviations

AACE/AAES	American Association of Clinical Endocrinologists/American Association of Endocrine Surgeons
ACA	adrenal cortical adenoma
ACC	adrenal cortical carcinoma
ACT	adrenocortical tumor
ACTH	adrenocorticotropic hormone
ACS	autonomous cortisol secretion (subclinical Cushing's syndrome/ subclinical hypercortisolism/ preclinical Cushing's syndrome)
Addit.	additional
AI	adrenal incidenatolma
a.m.	*ante meridiem*
AVP	arginine vasopressin
cAMP–PKA	3′, 5′-cyclic adenosine 5′-monohosphate–protein kinase
CI	confidence Interval
CRF	corticotropin-releasing factor
CT	computed tomography
D	University Hospital Düsseldorf
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
DST	dexamethasone suppression test
ECLIA	electrochemiluminescence immunoassay analyzer
e.g.	*exempli gratia*, for example, example given
eos abs	eosinophils, absolute counts
eos rel	eosinophils, relative portions
ES	Endocrine Society
ESE/ENSAT	European Society of Endocrinology/ European Network for the Study of Adrenal Tumors
et al.	*et alii*, and others
F	cortisol
FSE	French Society of Endocrinology
GC	glucocorticoids
GR	glucocorticoid Receptor
Group A	control subjects
Group B	patients with non-producing adrenal adenomas
Group C	patients with adrenocortical adenoma on imaging and possible autonomous cortisol secretion
Group D	patients with adrenocortical adenoma on imaging and autonomous cortisol secretion
h	hour
HPA	hypothalamic-pituitary-adrenal axis
HPF	high-power fields
HRO	University Hospital Rostock
HU	Hounsfield unit
IACE	Italian Association of Clinical Endocrinologists
i.v.	intravenous
JES	Japan Society of Endocrinology

KES	Korean Endocrine Society
Leu	leukocytes
LNSC	late-night salivary cortisol
Ly abs	lymphocytes,absolute counts
Ly rel	lymphocytes,relative counts
MC2-R	melanocortin type 2 receptors
n.t.	not tested
µg/dL	micrograms per deciliter
min	minutes
mm	millimeter
MRI	magnetic resonance imaging
NIH	National Institutes of Health
Nph abs	neutrophils,absolute counts
Nph rel	neutrophils,relative portions
nmol/L	nanomoles per litre
Param	parameter
pmol/L	picomoles per litre
NPA	non-producing adrenal cortical adenoma
NM	not mentioned
NR	not recommended
n.s.	not significant
p	probaility values
pg/mL	picogramms per milliliter
r	Pearson's or Spearman's r
RAAS	renin-angiotensin-aldosterone system
RIA	radioimmunoassay
SAGH	possible autonomous cortisol secretion
SEEN	spanish Society of Endocrinology
SD	standar deviation
SHIP-PAGE	steroid hormones in patients with pituitary, adrenal or gonadal endocrinopathies
Thr	thrombocytes (platelets)
TU	tumor
UFC	urinary free cortisol
USA	united States of America
vs.	*versus*

8. Tables

9. Figures