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The role of E47 in patients with endogenous glucocorticoid excess

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Zusammenfassung (Deutsch):

E47 ist ein Transkriptionsfaktor und wurde kürzlich als Modulator von Glukokortikoidrezeptor-Zielgenen identifiziert. Sein Verlust schützt Mäuse vor den nachteiligen metabolischen Auswirkungen von Glukokortikoiden. Patienten mit Cushing-Syndrom (CS) sind aufgrund eines Überschusses an endogenen Glukokortikoiden stark von verschiedenen metabolischen Komorbiditäten betroffen. In der vorliegenden Arbeit wurde die Rolle von E47 bei Patienten mit endogenem Glukokortikoid-Überschuss analysiert.

Wir führten eine retrospektive Kohortenstudie mit 120 Patientinnen mit CS (ACTH-abhängig = 79; ACTH-unabhängig = 41) und 26 gesunden weiblichen Kontrollen durch. Die E47 mRNA-Expression wurde zwischen verschiedenen CS-Untergruppen, bei floridem Cuhsing-Sydrom und in Remission nach der Operation sowie nach ACTH-Stimulation und Dexamethason-Suppressionstest bei den Kontrollen gemessen. Ebenso wurde die E47-Genexpression mit metabolischen Komorbiditäten bei CS korreliert.

Wir fanden heraus, dass die E47-Genexpression bei Patienten mit floridem CS (n = 29) im Vergleich zu Patienten in Remission (n = 91; $p = 0.0474$) signifikant niedriger war. Die E47-Genexpression war in der prä-chirurgischen Untergruppe der CS-Patienten im Vergleich zu den Patienten nach erfolgreicher Operation (n = 14; $p = 0.0353$) signifikant niedriger. Die Verabreichung von 1 mg Dexamethason bei gesunden Kontrollen zeigte keine Veränderungen in der E47 mRNA-Expression. Die Stimulation mit Synacthen© von Gesunden hingegen führte zu einer positiv signifikanten Abnahme der E47 mRNA-Expression nach 30 Minuten intravenöser Verabreichung im Vergleich zur Ausgangsmessung ($p = 0.0015$). Die E47-Genexpression korrelierte außerdem positiv mit den Messungen des Serum-Gesamtcholesterins ($p = 0.0036$), des LDL-Cholesterins ($p = 0.0157$) und des Verhältnisses von Taille zu Arm ($p = 0.0138$) in der Untergruppe der CS-Patienten in Remission.

Zusammenfassend beschreibt diese Arbeit, dass die E47 mRNA-Expression sowohl

bei CS-Patienten als auch bei Kontrollen eine hohe Streuung aufwiesen. E47 scheint ein GC-abhängiges Gen zu sein, das bei einem endogenen GC-Überschuss herunterreguliert wird und möglicherweise darauf abzielt, die Nebenwirkungen von metabolischen Glukokortikoiden zu reduzieren.

Abstract (English):

E47 is a transcription factor and has recently been identified as a modulator of glucocorticoid receptor target genes. Its loss protects mice from the adverse metabolic effects of glucocorticoids. Patients with Cushing's syndrome (CS) are severely affected by various metabolic comorbidities due to an excess of endogenous glucocorticoids associated with tumour formation.

The aim of this study was to analyse the role of E47 in patients with endogenous glucocorticoid excess. We performed a retrospective cohort study with 120 female patients with CS (ACTH-dependent = 79; ACTH-independent = 41) and 26 healthy female controls. E47 mRNA expression was measured in different CS subgroups, before and after successful surgery, and after ACTH stimulation and dexamethasone suppression test in controls. We also investigated the correlation between E47 gene expression and metabolic comorbidities in CS .

We found that E47 gene expression was significantly lower in patients with overt CS (n = 29) compared to patients in remission (n = 91; $p = 0.0474$). E47 gene expression significantly decreased in the pre-surgical subgroup of CS patients compared to their corresponding post-surgical samples (n= 14; $p = 0.0353$). Administration of 1 mg dexamethasone in healthy controls showed no changes in E47 mRNA expression. Stimulation with Synacthen© in healthy controls, however, resulted in a positive significant decrease in E47 mRNA expression after 30 minutes of intravenous administration compared to baseline measurements ($p = 0.0015$). E47 gene expression was positively correlated with serum total cholesterol ($p = 0.0036$), LDL cholesterol ($p = 0.0157$) measurements and waist-to-arm ratio ($p = 0.0138$) in the subgroup of CS patients in remission.

Taken together, this thesis describes that E47 mRNA expression showed a high dispersion in both CS patients and controls. E47 appears to be a GC-dependent gene that is downregulated in the presence of endogenous GC excess and may aim to reduce the side effects of metabolic glucocorticoids.

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CS	Cushing’s syndrome
GCs	Glucocorticoids
ACTH	Adrenocorticotropic hormone
CD	Cushing’s disease
ECS	Ectopic Cushing's syndrome
EAT	Ectopic ACTH secreting tumors
CRH	Corticotropin-releasing hormone
BMAH	Bilateral macronodular adrenal hyperplasia
PPNAD	Primary pigmented nodular adrenocortical disease
HPA	Hypothalamic-pituitary-adrenal
CT	Computed tomography
MRI	Magnetic resonance imaging
SCN	Suprachiasmatic nucleus
AVP	Arginine vasopressin
PVN	Hypothalamic paraventricular nucleus
11β-HSDs	11β-hydroxysteroid dehydrogenases
PTMs	Post-translational modifications
MRs	Mineralocorticoid receptors
UFC	Urinary free cortisol
DST	Dexamethasone suppression test
HDDST	High dose dexamethasone suppression test
oCRH	ovarian corticotropin-releasing hormone
Gd-DPTA	Gadopentetate dimeglumine
HU	Hounsfield units
TSS	Transsphenoidal selective pituitary adenomectomy
CYP17A1	17 alfa-hydroxylase
CYP11β	11 beta-hydroxylase
EMA	European Medicines Agency

SS	Somatostatin
DA	Dopamine
NET	Neuroendocrine tumors
SSTRs	Somatostatin receptors
SSTR5	SSTR subtype 5
DR2	DR subtype 2
FDA	The US Food and Drug Administration
GRE	Glucocorticoid-responsive elements
TF	Transcription factors
bHLH	Basic helix-loop-helix protein
TCF3	Transcription factor 3
AS	Alternative splicing
RR-sys	Blood pressure systolic
RR-dia	Blood pressure diastolic
HbA1c	Glycated hemoglobin
TG	Triglyceride
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
24h- UFC	24- hour urinaryfree cortisol
HOMA - IR	Homeostatic model assessment - insulin resistance
Id protein	Inhibitors of DNA binding and cell differentiation protein
Mdn	Median
IQR	Interquartile range
n	Number
SEM	Standard error mean

1. Introduction

1.1 Cushing's syndrome

1.1.1 Definition, clinical presentation and pathophysiology

Cushing’s syndrome (CS) can be defined as a disease which contains a series of symptoms that is caused by exposure to a high level of glucocorticoids (GCs) for a long time. These symptoms encompass moon-shaped face, plethora, weight gain with limb thinning but central body fatty deposits, buffalo hump, purple stretch marks on the abdomen, acne, menstrual irregularities, and fatigue (1, 2). Most cases of CS are caused due to pharmacologic dosis of GC in medical therapy (3), as GCs are common, effective medicines that are used to treat for example inflammatory, oncologic or autoimmune diseases. Besides iatrogenic CS due to pharmacotherapy, CS can be caused by endogenous glucocorticoid excess. Endogenous CS is a rare condition. Although, endogenous CS has been known for several decades, the identification of hypercortisolism, the determination of its etiology and the achievement of optimal treatment outcomes remain challenging.

Endogenous CS can be divided into adrenocorticotropic hormone (ACTH)-dependent (about 80%) and ACTH-independent (about 20%) causes (4). The most common cause of endogenous CS is ACTH overproduction from a pituitary adenoma, this ACTH-dependent CS is also called Cushing’s disease (CD), which accounts for 70% of endogenous CS (5). Among ACTH-dependent forms, the ectopic Cushing's syndrome (ECS) which is caused by ACTH producing from non-pituitary neuroendocrine tumors is a unique type. It is also known as paraneoplastic CS. ECS accounts for 9% to 18% of cases of ACTH-dependent Cushing's syndrome (6). Ectopic ACTH secreting tumors (EAT) can lead to glucocorticoid excess through unregulated ACTH expression and secretion, secondary cortisol secretion by the adrenal glands. EAT have different histological forms, locations, and prognoses, they include small cell carcinoma of the lungs; carcinoid tumors in the bronchus, pancreas, or thymus; medullary carcinomas of the thyroid; pheochromocytomas, and other lesions, mostly of neuroendocrine origin (7). Cases with unknown source of ACTH

production are designated as occult ectopic CS.

The ACTH-independent form of CS is caused by adrenal oversecretion of cortisol. This mostly is due to an unilateral adrenal adenoma, but can rarely also be due to an adrenal carcinoma; or bilateral macronodular adrenal hyperplasia (BMAH) or primary pigmented nodular adrenocortical disease (PPNAD) and its non-pigmented variant, isolated micronodular adrenocortical disease (9). Excessive secretion of cortisol inhibits the release of ACTH.

The overall incidence of endogenous CS is approximately 0.2 to 5 new cases per million people per year and a prevalence of 39-79 per million in various populations (10). A nationwide Swedish study reported the incidence of CD in Sweden to be 1.6 cases per million per year (11). The median age of diagnosis is 41.4 years with a female: male ratio of 3:1 (8, 12). Recently, more research suggested that patients with diabetes or hypertension may include undiagnosed cases of CS (10, 11). Furthermore, the widespread use of computed tomography (CT) and magnetic resonance imaging (MRI) scans has led to an increasing number of incidental adrenal masses being discovered (13). Often these incidental findings of adrenal adenomas may co-secrete smaller amounts of glucocorticoids known as autonomous cortisol secretion (previously subclinical CS). It is a common disorder occurring in up to 20% of patients with incidentally detected adrenal adenomas, which are found in approximately 4% of middle-age population and in more than 10% of elderly persons (14).

Normal cortisol secretion depends on the feedback of the hypothalamic-pituitary-adrenal (HPA) axis, CS always occurs when the HPA system's physiological balance is destroyed towards endogenous GC excess. Under stress, input from the suprachiasmatic nucleus (SCN) stimulates the hypothalamic paraventricular nucleus (PVN) to release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH enters the anterior pituitary gland through hypophysial portal vessels. CRH activates corticotroph cells by binding to its receptor and induces the release of ACTH into the genetal circulation. The main target of circulating ACTH is the adrenal cortex, where it stimulates the synthesis and secretion

of GCs from the zona fasciculata (15).

GCs are downstream effectors of the HPA axis, regulating physiological changes through ubiquitous intracellular receptors. The feed-forward mechanism within the HPA axis is balanced by negative feedback from GCs, which act on the anterior pituitary and hypothalamus respectively to inhibit further release of ACTH and CRH (Figure. 1) (16).

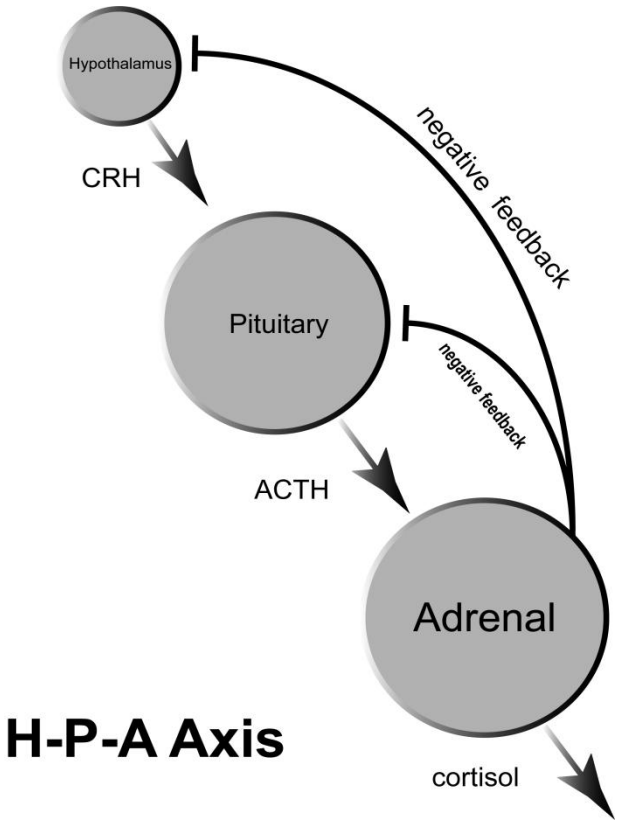

Figure 1. Hypothalamus pituitary adrenal axis. The HPA axis is balanced by negative feedback from cortisol, which act on the anterior pituitary and hypothalamus respectively to inhibit further release of ACTH and CRH. CRH, corticotropin releasing hormone; ACTH, adrenocorticotropic hormone.

The supply of GCs at the cellular level is maintained by the tissue-specific metabolic enzymes 11β-hydroxysteroid dehydrogenases (11β-HSDs) (17). 11β-HSD2 is a potent dehydrogenase which function is to rapidly inactivate GCs (convert cortisol to cortisone) so that aldosterone selectively enters and binds mineralocorticoid receptors (MRs) in the kidneys and pancreas. On the contrary, 11βHSD1 acts as a major

11β-reductase in GC target tissues such as liver, adipose tissue, and brain. It facilitates the conversion of the inactive precursor cortisone to biologically active cortisol, thereby utilizing high concentrations of inert cortisone in the circulation to regenerate active GCs in tissues (18). At the cellular level, the functional balance of these two functionally distinct isoenzymes 11β-HSD1 and 11β-HSD2 play crucial roles in maintaining GC availability and activity. If an abnormality occurs at any point in the above process this may lead to an excessive secretion of GCs which will result in CS.

Complications of Cushing's syndrome CS is associated with a variety of complications involving multiple systems, including the endocrine system, musculoskeletal system, gastrointestinal system, infections and even neuropsychiatric disorders. These side effects caused by excess glucocorticoid concentrations can range from mild side effects to life-threatening ones that may require immediate interventions (19, 20).

GCs can induce the development of diabetes. Hyperglycemia also may increase the risk of infection (21), leading to longer hospital stays As it exerts a catabolic effect on skeletal muscle and skin, increased protein consumption and type II muscle fiber atrophy with marked muscle weakness are the consequence. In addition, patients with CS can develop osteoporosis accompanied by pathological fractures due to the inhibition of bone formation and excessive bone resorption by high cortisol concentrations, especially in elderly patients or post-menopausal women (22). Further, patients with endogenous CS may present with hypertension, hypokalemia, dyslipidemia, and androgen excess. These features of the metabolic syndrome, combined with a hypercoagulable state, result in an increased cardiovascular risk that may not completely return to baseline levels after treatment (12). Hepatic steatosis and increased visceral adipose tissue are common as well. CS patients have an increased risk of suffering from gastritis and peptic ulcer (23). Psychiatric and cognitive disorders, including memory disturbances, depression, mania, anxiety disorders and psychosis, have also been reported in patients with CS (24).

Addressing hypercortisolism is essential to improve the metabolic profile and halt the progression of these complications. However, even after CS remission, the risk of

some complications remains high. In a prospective longitudinal study investigating CS-associated myopathy showed that patients with CS in remission continued to have varying degrees of impairment in muscle strength, even after several years in remission (25). Therefore, long-term follow-up data on various complications after surgical treatment of CS can help determine the optimal follow-up strategy for CS (26).

1.1.2 Diagnosis and treatment of Cushing's syndrome

An early diagnosis and correct classification is extremely meaningful for CS patients to get appropriate interventions. The detailed medical history, clinical evaluation, laboratory and imaging confirmations are needed. The diagnosis involves several phases: the confirmation of hypercortisolism, the differentiation between pseudo-Cushing states and the true Cushing's syndrome (27), the differentiation between ACTH-independent and ACTH-dependent causes of the CS, and the differentiation between pituitary and ectopic sources of the ACTH-dependent CS (28). The first step is to confirm that hypercortisolism exists. Because of the rhythmic nature of GCs secretion, random circulating cortisol and ACTH measurements are of little significance (29). A good recommended screening test for hypercortisolism is the 24-hour urinary free cortisol (UFC) excretion corrected by body surface area. The UFC results are also influenced by stress, depression, excessive water intake (>5 L/d), malnutrition and pregnancy. These situations may lead to a falsely high UFC (30). On the contrary, a falsely low UFC can be obtained in the condition of inadequate urine collection. Some studies have reported that baseline urinary free cortisol values in patients with CS vary over a wide range (31). So the reliability and reproducibility of the UFC test is very important. The Endocrine Society recommendations state that the diagnosis of CS should be made with at least twice measuring 24-hour UFC (32). Although UFC is a practical measurement to determinecortisol concentrations, it's still difficult for some patients to implement. The gold standard to diagnose CS is the low-dose dexamethasone suppression test (DST). Dexamethasone is about 30 to 40 times more potent than cortisol and has no significant mineralocorticoid activity (33).

Dexamethasone binds to glucocorticoid receptors in the paraventricular nucleus of the hypothalamus and pituitary cortical cells and inhibits the secretion of CRH and ACTH. In humans, dexamethasone does not directly inhibit the production of adrenal steroids (34). If the HPA axis is functioning normally, any supraphysiologic dose of dexamethasone is sufficient to suppress pituitary ACTH secretion. The production of cortisol will be reduced accordingly. The DSTs assesses the hypothalamic and pituitary corticotroph cell responses to glucocorticoid negative feedback inhibition of CRH and ACTH secretion (35).

In addition, night time salivary cortisol is also used as test for the assessment or management of hypercortisolemic states. Its samples are easy to collect avoiding the stress of venipuncture and unaffected by salivary flux (38).

After confirming hypercortisolism, patients will be suspected of having a diagnosis of CS. Identifying the cause of hypercortisolism is the key to diagnosis. Several tests can be used to differentiate ACTH-dependent CS from the ACTH-independent ones. A high dose dexamethasone suppression test (HDDST) can be used to identify CD. The HDDST takes advantage of the physiological features of CD that neoplastic ACTH secretion may retain part of its responsiveness to negative feedback inhibition by GCs, so that cortisol is not inhibited in either the overnight 1mg or the two-day low dose test. ACTH secretion can be partially suppressed in majority of patients with CD by increasing the dose of dexamethasone by 4-8 times. However, many ectopic ACTH-producing non-pituitary tumours are usually unresponsive to negative feedback from GCs.

Most of CD patient's plasma ACTH and cortisol level go up in response to CRH. For patients with ectopic ACTH, they always don't respond to CRH (43-46).

Once hypercortisolism has been confirmed biochemically, the tumour needs to be identified and localized by imaging tests. Pituitary MRI is always used when CD was suspected. MRI with high resolution and with contrast is useful to localize and characterize both macroadenomas and microadenoma (47). Corticotroph adenomas always show characteristically hypodense signal on MRI and do not show enhancement after administration of gadopentetate dimeglumine (Gd-DPTA) contrast

(48). Although MRI sensitivity is high, it is important to note that the small size of the tumour or the similarity of signal characteristics of ACTH-secreting tissue as normal pituitary tissue may result in false-negative results (49). Adrenal imaging will be performed in patients who were suspected having ACTH-independent causes of CS (50). Adrenal adenomas are generally small and uniformly dense with well-defined borders on CT imaging (51). The attenuation value of adenomas on unenhanced CT is usually less than 18 Hounsfield units (HU) (52). Another feature is the rapid washout of iv contrast media (53). The imaging features of adrenocortical carcinomas are very different from adrenal adenomas, as they are usually large, irregular, and easily invade surrounding structures (54). In short, combined with functional hormone tests, blood test related results, experienced imaging physicians are critical in the localization and diagnosis of CS, as well.

Surgical resection of the source of glucocorticoid excess (pituitary adenoma, nonpituitary tumor-secreting ACTH or adrenal tumors) remains the first-line treatment for all forms of CS (12). The treatment goal of overt CS is to normalize cortisol levels and to treat comorbidities associated with hypercortisolism. Laparoscopic unilateral or bilateral adrenalectomy is the treatment of choice for CS due to adrenal causes (55). For all benign unilateral disease, unilateral resection is the treatment of choice. When surgery and medical therapy are not successful in ACTH-dependent CS or for the bilateral adrenal disorders, bilateral adrenalectomy can be used. Generally, after bilateral adrenalectomy, patients need lifelong glucocorticoid and mineralocorticoid replacement treatment.

For Cushing's disease, trans-sphenoidal selective pituitary adenomectomy (TSS), selectively removing the corticotroph adenoma is generally the first-choice treatment of CD's patients (56). Long- term remission rates after TSS is 50% – 80% (57). During the first 2 weeks after TSS, measuring serum sodium and assessing free T4 and prolactin are critical to evaluate surgical outcome. But if the surgery is not effective, further treatment needs to be taken, for example repeat surgery, pituitary radiotherapy, adrenalectomy, or pharmaceutical. Bilateral adrenalectomy will be performed for occult ACTH secretion or EAS or as a life-preserving emergency

treatment in patients with very severe ACTH-dependent disease that cannot be promptly controlled by medical therapy (55).

Medical treatment of hypercortisolemia may be used before surgery, after surgery and radiotherapy if no complete remission can be achieved by these methods

There are two approaches to achieve the medical control of hypercortisolism: firstly, blocking the secretion of cortisol to bring it to normal levels; secondly, completely blocking the secretion of cortisol with concomitant glucocorticoid replacement (blocking and replacement). Medical treatments for hypercortisolemia comprise steroidogenesis inhibitors (ketoconazole, metyrapone, mitotane, and etomidate), ACTH release modulator (somatostatin and dopamine agonists) or glucocorticoid receptor blocker (mifepristone) (58, 59).

1.2 The role of glucocorticoids and glucocorticoid receptors

Glucocorticoids (GCs) are steroid hormones which are synthesized and released by the adrenal zona fasciculata. Biologically, GCs are synthesized from cholesterol. Because of GCs' lipophilic nature, they cannot be pre-synthesized and stored in the adrenal glands, but must be rapidly synthesized under the feed-forward mechanism of the HPA axis (71). GCs enter target tissues and regulate numerous physiological processes, including metabolism, immune function, cardiovascular function, reproduction and cognition (72). The basic role is energy mobilization, especially carbohydrate metabolism at basal GC level. GCs thus influence the expression of thousands of genes. GCs function mainly through two kinds of receptors, including glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). Of note GCs also bind to membrane-bound receptors. On the molecular level, GCs have a higher affinity to MRs than GRs. At low to middle concentrations of circulating GCs, GCs bind to the MRs primarily (73). The binding of GRs mostly occurs after MRs have been saturated (74). GCs act both in a circadian and stress-directed manner to help to adapt to changing environments (75). At low to moderate levels, GCs influence feeding behavior and act on the liver, adipose tissue and muscle to maintain adequate

circulating concentrations of glucose and free fatty acids (76). When faced with an unexpected challenge, individuals often go into a state of stress or overload. High levels of GCs will be secreted to bind to low-affinity GRs and initiate physiological and behavioral changes in response to the challenge. High GCs can suppress the immune system, growth, and metamorphosis. They also promote protein loss, deposition of fat and atherosclerotic plaques, and hypertension; disrupt second messenger systems; and cause neuronal cell death (74). MRs regulate basal HPA tone, while GRs mediate negative GCs feedback after stress. During a stress response, elevated GCs facilitate a shift in energy balance to facilitate coping with a stressor and to enable individual survival (77-79).

As mentioned above, the function of GCs will be mediated by the GRs. The GR is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (80). It is expressed in almost all cell types of our body and its gene activity is regulated by both developmental and tissue-specific factors, so actions of GCs are particular to specific cell types. GR up- and downregulation is complex and a common process in GC modulated GR expression. The GR will be downregulated when exposured to high GCs for a long time (81). For decades, a large number of cell line and animal tissue-related experiments have revealed that treatments with GCs lead to the reduction in the expression of GR mRNA and protein (82). Of note, tissue-specific activation of GR mRNA abundance also can be observed. An artificially mimic hyperglucocorticoid situation in fish showed that GR mRNA was upregulated in liver, heart, muscle， however there was a downregulation in intestine (83). In humans, GCs diminish the GR mRNA level in peripheral lymphocytes, and individual basal GC mRNA expression varies widely (84).

The GR is composed of three main domains: the N-terminal transactivation, the C-terminal ligand binding domains and the central DNA-binding, with the central domain containing two zinc fingers (85, 86).Without GCs, GR is mainly located within a large multi-protein complex in the cytoplasm. Once bound to GCs, GRs form dimers before acting as transcription factors and can form either homodimers or

heterodimers (87-89). Afterwards, these dimers bind to glucocorticoid-responsive elements (GRE) and regulate the expression of target genes.

The GR gene can be spliced into a large group of receptor isoforms, each with a different expression and function, expanding the diversity of GCs which GR acts. The activated GR acts as a transcription factor (TF) and controls the expression of thousands of genes, whether promoted or repressed (90, 91). Three components determine the role that GR plays in the human body. Firstly, the activity of GR is directly related to the amount of GC molecules ready in cell. Secondly, the expression of active GR in the nucleus determines the molecular response to GCs. This is regulated by GR synthesis and catabolism, the expression of different GR isoforms, post-translational modifications (PTMs) and nuclear translocation. In the end, the genomic role of GR is constrained by the accessibility of cell-type-specific GREs. They act in concert with cell-specific TFs, coregulators and regulatory RNAs in the genome (92). With binding to GR, functions in different levels of GCs were transferred to changes of downstream gene expressions. Importantly, GR related interactions with DNA are regulated by a large number of transcription factors (TF). Some of these interactions lead to significant changes in DNA binding of specific TFs. For example, NF-κB can be repressed by GR (93). Additionally, GR plays a role in boosting the expression of STAT5-controlled genes (94).

1.3 Transcription factors and E47 function

Transcription factors are nuclear proteins that regulate the expression of downstream target genes. They serve to guide genomic expression by recognizing specific DNA sequences to control chromatin and transcription. TFs have been shown to drive cell differentiation, dedifferentiation, and trans-differentiation. Mutations in TFs and TF binding sites cause a variety of human diseases (95, 96). Human TFs are divided into 23 TF families (97). The transcription factor E47 is a kind of class I family of basic helix-loop-helix protein (bHLH) which is also known as E protein. Other three E proteins identified in vertebrates contain E12, E2-2 (Tcf4), and HEB (Tcf12) (98). E

proteins regulate growth and development in many tissues, including cell differentiation, development of skeletal muscle, and neurogenesis (99). Most importantly, they play a role in different aspects of B-cell development processes (100). They can dramatically suppress the differentiation of group2 innate lymphoid cells from bone marrow and thymic progenitors, and also serve as a critical checkpoint to promote B and T cell development (101). E proteins are also widely expressed in non-lymphoid cell types and hetero-dimerize with the tissue-specific bHLH proteins (also called class II bHLH proteins) (102). In general, the conserved bHLH domain is involved in homo- or hetero-dimerization to form a functional transcription unit. This transcription unit binds to the canonical E-box response element (CANNTG), then gives its function (103, 104).

E47 is encoded by*E2A* or *TCF3*, E47 (Tcf proteins). Basically, E47 is always known as acting crucial roles in lymphocyte survival, expansion, and developmental progression (105). It plays a pivotal role in the activation of B cell-specific genes. In fact, E47 has crucial roles in many situations such as vertebrate embryogenesis, organ and tissue homeostasis, and cancer formation (106-109). Restricted E2A expression can be observed in the human pituitary, kidney, adrenal and liver (110). In summary, molecular function of E47 includes DNA-binding transcription factor activity, cis-regulatory region sequence-specific DNA binding, bHLH transcription factor binding, protein hetero-dimerization activity, repressing transcription factor binding.

E2A gene and E47 protein

The transcription factor 3 gene (TCF3 or E2A) is located to chromosomal band 19p13.3 encoding several transcription factors. The E47 protein encoded by TCF3 gene is one of the transcription factors, E12 is its splicing format (111, 112). The difference between them is generated by alternative splicing of pre-mRNA. Alternative splicing (AS) forms two mutually exclusive exons, 18a (E12) or 18b (E47)(111). Notably, 18a and 18b mean the exons in the mRNA, while E12 and E47 refer to the resulting protein isoforms. This variation contributed to their unique affinity: E47 binds strongly to DNA, while E12 binds weakly to DNA (113).

E47 includes a HLH domain and DNA binding domain. The HLH domain is a parallel,

four-helix bundle with two protruding basic region helices, each 15-20 residues long. Two highly conserved amino acids mediated DNA-binding specificity - a glutamate and an arginine residue in the DNA-binding region (114). Both homodimers and heterodimers have been shown to bind to the consensus E-box sequence (CANNTG), and activate gene transcription (115).

E47 has mostly been recognized in its function in lymphocyte development so far. By committing lymphoid progenitors to the B cell lineage, it promotes the developmental process, expansion and survival of lymphocytes (116). It also functions as a tumour suppressor and has been associated with multiple chromosomal translocations associated with childhood leukaemia, impared E47 activity is associated with the development of cancer, for instance, prostate cancer (117, 118). E47's DNA-binding activities regulated by the inhibitors of DNA binding and cell differentiation (Id) proteins. Since Id proteins lack the DNA-binding region, they inactivate DNA binding of E proteins upon heterodimerization. Besides, post-translational phosphorylation has also been reported to affect the stability and activity of E47 proteins (119).

In 2004, E47 was identified as a participant in the GRα cross-talk for the first time. A system for the simultaneous profiling of multiple transcription factor activities using protein-DNA arrays was applied to investigate the potential cross-talk between GRα and TFs. It was finally determined that the DNA binding activity of E47 was regulated by GRα. The results were then confirmed by both in vitro electrophoretic mobility shift assays and in vivo assays (120). Recently, Professor Uhlenhaut's research group described that the crosstalk of E47 and GR plays a role in hepatic lipid and glucose metabolism. Their team carried out a series of mice experiments which confirmed that E47 mutant mice were protected from steroid-induced hyperglycemia, dyslipidemia and hepatic steatosis (121). Establishing links between E47 and glucocorticoid metabolism therefore may offer new insights into the metabolic mechanism in conditions with chronic hypercortisolism such as CS. The aim of this dissertation was to declare the function of E47 in patients with endogenous glucocorticoid excess.

2. Hypothesis and aim

Patients with Cushing's syndrome (CS) suffer from an endogenous glucocorticoid excess due to tumour formation associated with a variety of metabolic comorbidities seriously affecting patients' health status.

E47 has been identified as a modulating transcription factor of glucocorticoid receptor target genes, its loss protecting mice from metabolic adverse effects of glucocorticoids.

We hypothesized that increased expression of E47 is associated with existence of comorbidities in patients with CS.

We aimed to analyze the role of E47 in patients with endogenous glucocorticoid excess and its association with disorders of the lipid and glucose metabolism.

3. Subjects and methods

3.1 Subjects

Subjects of this study were recruited from the Department of Endocrinology of the University Hospital Munich, Germany. All patients provided written informed consent to participate in the German Cushing's Registry (NeoExNET, ethical approval no. 152-10). A total of 120 female patients with CS were included in our analysis. The majority of subjects were patients with ACTH-dependent CS, 68 patients diagnosed with Cushing's disease due to excessive ACTH secretion from benign pituitary adenomas and 11 patients diagnosed with excess cortisol due to ectopic ACTH secretion. The remaining 41 patients had ACTH-independent CS.

In addition, 26 healthy female subjects were recruited as controls. For the purpose of data analysis, CS patients were further subdivided into overt CS with persistent cortisol excess and CS in remission. Diagnostic criteria were applied as outlined in the 2008 Endocrine Society evidence-based clinical guidelines (32). Exclusion criteria included intake of glucocorticoid or ACTH-related drugs or with psychiatric disorders.

In addition, data on clinical and biochemistry were collected, including weight, body

fat mass, waist-arm-ration, blood pressure, glucose, HbA1c, insulin, cholesterol, TG, HDL, LDL, and 24h-UFC.

3.2 Material

Item	Model	Manufacturer
Analytics software	SPSS 25	IBM
Drawing software	GraphPad Prism 8	GraphPad Software
Real time PCR system	Quantistudio5	Thermo Fisher
Autoclave	VX-75	Systec
centrifuge	5702 R	Eppendorf
Mini Centrifuge	Galaxy mini C1213	VWR
Vortex Mixer	Vortex-Genie 2	IKA
Thermal mixer	Thermomixer™	Eppendorf
Spectrophotometer NanoDrop	ND-1000	PeqLab
Thermal cycler	T100™	Bio-Rad
Thermal mixer	5355 000.011	Eppendorf
Ultra-Low freezer (-86°C)	HFU 586 Basic	Thermo Fisher
Pipettes	Research® plus	Eppendorf

Kit name	Product Number	Company
SsoFast™ EvaGreen® Supermix with Low ROX	1725211	Bio-Rad
NucleoSpin RNA Blood *Midi* kit,	740210.2	MACHEREY-NAGEL
SupersScript™ II Reverse Transcriptase	18064022	Thermo scientific

Item	Product Number	Company
Ethanol, absolute (100%)	No. 9065.3	Roth
Ethanol, technical		Central Warehouse
RNase/DNase-free water	10977023	Invitrogen
H_2O (sterile)		Central Warehouse
96-well reaction plate	MicroAmp (0.1 ml)	Thermo Fisher
Q-Stick^{TM} qPCR Seal	4ti-0565	4titude
EDTA Monovettes	K3E / 2.7 ml	Sarstedt

Name	Company	Country
Dexamethasone (1mg)	Mibe	Germany
Synacthen (250 µg)	Alfasigma,	Italy

3.3 Sample collection

Of each enrolled participant 2.7 ml venous blood were drawn using EDTA Monovettes. The blood samples were directly placed on ice and transferred to the lab. Bloodwasmixed with DL buffer (NucleoSpin RNA Blood Midi kit) containing guanidinthiocyanate in a 1:1 ratio and immediately frozen at -80 °C avoiding RNA degradation. It is important to note that some blood specimens were directly frozen at -80 °C, as these blood samples were collected before the start of this project. Frozen samples were then used for RNA extraction.

3.4 Dexamethasone suppression test

The aim of the dexamethasone suppression test is to measure the response of the adrenal glands to ACTH. For the low dose dexamethasone suppression test, patients were administered 1 mg of dexamethasone at 10 p.m. and serum cortisol was measured at 8 a.m. the following morning. In people with a normal HPA axis function,

cortisol levels should decrease with the use of dexamethasone beyond a defined threshold (If cortisol levels remain above 50 nmol/L, the patient has hypercortisolism).

3.5 ACTH stimulation test

Via ACTH stimulation test, the adrenal and pituitary gland function can be determined. In our project, patients were asked to remain in a lying position. A peripheral line was placed, blood was drawn for cortisol at baseline and 30 minutes after intravenous administration of Synacthen (250 µg) introvenously. An increase in cortisol above 20 mcg/dL or 552 nmol/L after stimulation by ACTH is expected as normal and excludes adrenal insufficiency.

3.6 RNA isolation

RNA isolation from whole blood samples using NucleoSpin blood RNA Midi protocols

1. Thaw the frozen 2 mL mixture blood (1 mL whole blood+ 1 mL DL buffer)
2. Add 30 µL liquid proeinase K and close the lid. Shaking 10-15 min at room temperature (18 – 25 ℃) on shaker. Centrifuge briefly to clean the lid (1 s at 2000 g).
3. Provide the mixture to 15 mL tube (provided)
4. Add 1 mL 70% ethanol to the tube and mix vigorously.
5. Transfer the complete lysate (maximum volume 4000 µL) into a NucleoSpin RNA Blood Midi Column placed in a 15 mL Collection Tube (Note: Avoid foam and aerosol formation).
6. Cengrifuge 3 min at a speed of 4500 g and leave the column in the tube with the flow-through
7. Add 1 mL MDB (Membrane Desalting Buffer) onto the column, then centrifuge 3 min at a speed of 4500 g
8. Discard flow-through and Collection Tube and place the column in a fresh

Collection Tube (15 mL, provided)

9. Add 200 µL rDNase onto the column. Incubate at room temperature, 15 min.
10. First time wash - add 1 mL Buffer RB2 to the NucleoSpin RNA Blood Midi Column. Centrifuge for 3 min at a speed of 4500 g. Leave the NucleoSpin RNA Blood Midi Column in the tube with the flow-through
11. Second time wash - add 3 mL Buffer RB3 to the NucleoSpin RNA Blood Midi Colmn. Centrifuge for 3 min at a speed of 4500 g.
12. Place the column into a nuclease-free Collection Tube (15 mL, provided) and discard the collection Tube with flow-through from the previous step
13. Discard wash buffer flow-through from the tube, insert the column into the tube and perform a further centrifugation (3 min, 4500 g) to dry the column
14. Reinsert the column into a fresh tube
15. Add 100 µL Rnase-free H_2O onto the column. Centrifuge for 3 min at a speed of 4500 g.
16. An additional elution step as step 15.

Total RNA concentration measurement

Total RNA concentrations were measured by NanoDrop Spectrophotometer with an optical density of 260 nm (OD260) and to verify RNA integrity and absence of RNA degradation, indicated by an OD260 / OD280 ratio of approximately 2.0.

Our protocol set the qualified threshold at OD260 / OD280 > 1.8 which can be used for cDNA synthesis. Among all of RNA samples, an average (± SD) ratio of 1.98 (± 0.17) was obtained. The RNA samples were stored at -80°C immediately before the cDNA synthesis step.

cDNA synthesis

The SuperScript II cDNA synthesis kit was used for reverse-transcription of 100 ng RNA following the company's instruction. In this project a 20 µL reaction volume is used for 100 ng of total RNA for each probe.

1. Add the following components of each probe to a nuclease-free microcentrifuge tube:

- 1 µL random primers (50 ng/µL)
- 100 ng total RNA
- 1 µL dNTP Mix (10 mM each)
- 1 µL Nuclease-free water to 13 µL

2. Heat mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation, then add:

- 4 µL 5 X First-Strand Buffer
- 2 µL MDTT

3. Mix contents of the tube gently, incubate at 25 °C for 2 min.
4. Add 1 µL (200 units) of SuperScript™ II RT and mix by pipetting gently up and down, then incubate tube with the thermal cycler (mode set: 25°C for 10 min, 42°C for 50 min, 70°C for 15 min, 4°C forever).

After these steps, already cDNA samples were stored at -20°C until down-flow use.

3.7 Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed in the Quantstudio 5 real-time PCR systems (Applied Biosystems) according to the manufacturer's protocols. PCR reactions were carried out in a 10 µL reaction mixture. For each PCR reaction, 5 µL (5 ng) of template cDNA , 4 µL SsoFast EvaGreen Supermix with Low ROX (Bio-Rad Laboratories Inc, USA), 0.5 µL (5 pmol) of each primer were used. Triplicate were used for each sample. Gene expression levels were normalized to β-Actin to account for possible differences in the amount of starting RNA. Relative fold changes were determined from the threshold cycle (Ct) values(122).And ΔCt = (ct of E47) - (ct of β-Actin). E47-mRNA expression was expressed as arbitrary units calculated by the formula 10^4 x ($2^{-\Delta Ct}$). The relative expression was analyzed using the $2^{-\Delta\Delta Ct}$ method (ΔΔCt = (ΔCt of CS) - (ΔCt of control)).

The primer pairs used were as follows (Eurofins Genomincs, Germany):

- E47 fwd 5’-CCTGAACTTGGAGCAGCAAG-3’

- E47 rev 5’-TACCTTTCACATGTGCCCGG-3’
- β-Actin fwd 5’-CATGTACGTTGCTATCCAGGC-3’
- β-Actin rev 5’-TGAGGATCTTCATGAGGTAG-3’

The PCR cycling conditions:

- Initial denaturation step (95 °C, 10 min),
- Denaturation followed (95 °C, 15 sec) ×40 cycles,
- Annealing/elongation (60 °C, 60 sec).

3.8 Statistical analysis

Results are expressed as median with 95% CI, *p*- value less than 0.05 was considered statistically significant. For PCR analysis, data was tested for statistical significance using normalized E47. To test for normality, Shapiro-Wilk’s test was used. As values of E47 mRNA expression were non-normally distributed Kruskal-Wallice test and Dunn's multiple comparisons test was used for comparison of E47 expression between overt CS, remission CS and the control group. E47 expression pre- and post-surgery as well as the two described endocrine function tests were analyzed using Wilcoxon matched-pairs signed rank test. Comparisons between different CS entities were analyzed by Mann-Whitney test. Bivariate correlations between variables were performed using nonparametric Spearman's correlation. SPSS software version 25.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) for graphical presentation of obtained data sets.

4. Results

4.1 Characteristics of study participants

Of the 120 female patients with CS included in our analysis, 14 patients with both pre- and post-operative peripheral blood specimens. Characteristics of participants, including numbers, age, related 24-h-urine cortisol levels and other metabolic parameters were collected and displayed (Table 1, table 2). Average age of control group was younger than CS group. Median age was 54 years (IQR41 - 63 years) in the overt group and 52.0 years (IQR42 - 63 years) for patients in remission. Median age of the control cohort was 31.5 years (IQR26 - 36 years). The median age of the control group is significantly lower than in the two subgroups of patients with CS ($p < 0.0001$) using one-way ANOVA. Positive significant differences were also observed for glucose, 24-h-urine cortisol, insulin, HOMA and systolic blood pressure among overt CS, remission CS and the control group. Glucose concentrations in the overt CS subgroup are higher than those of patients with CS in remission ($p = 0.0037$) (Figure 2A). Regarding 24-h-urine cortisol measurements, the overt CS group presents with increased level than patients in remission and healthy controls ($p< 0.0001$) (Figure 2B). Systolic blood pressure of patients with overt CS is higher than in patients in remission and healthy controls ($p = 0.0326$) (Figure 2C). Insulin and homeostatic model assessment- insulin resistance (HOMA- IR) were both higher in patients with overt CS ($p = 0.0086$, $p = 0.0050$) (Figure 2D, 2E).These positive statistics results were all consistent with the clinical features of CS.

Figure 2.

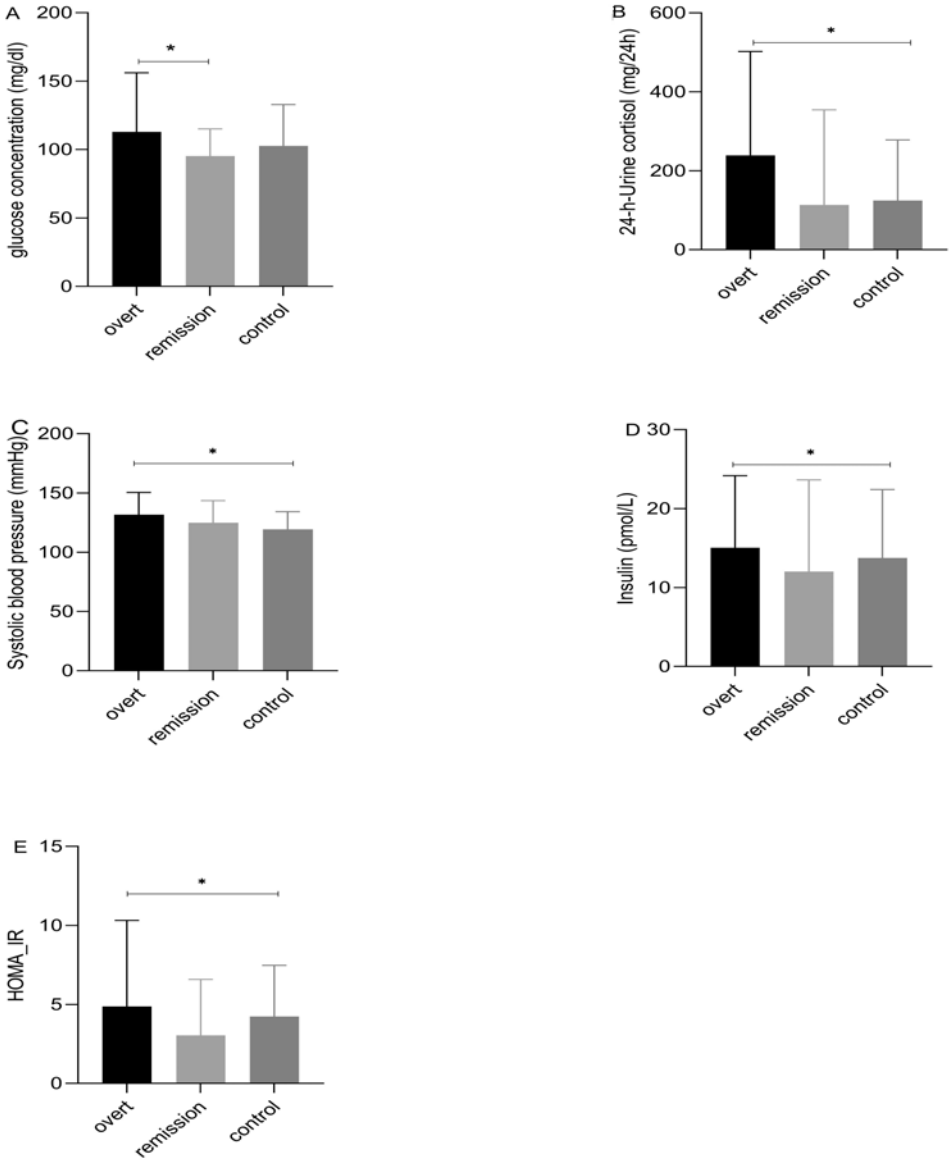

Figure 2. Clinical features of participants. Positive significant differences were observed for glucose concentartion, 24-h-urine cortisol, systolic blood pressure insulin and HOMA among overt CS, remission CS and the control group. (A) Glucose concentrations in the overt CS subgroup are higher than those of patients with CS in remission (p = 0.0037). (B) The overt CS group presents with increased level of 24-h-urine cortisol than patients in remission and healthy controls ($p < 0.0001$). (C) Systolic blood pressure of patients with overt CS is higher than in patients in remission and healthy controls (p = 0.0326). (D) Insulin was higher in patients with

overt CS (p = 0.0086). (E) Homeostatic model assessment-insulin resistance (HOMA-IR) was also higher in patients with overt CS (p = 0.0050), date shown as median with 95% CI; p-value <0.05 (*) by one-way ANOVA.

Table 1. Characteristics of participants

	overt CS		remission CS		control		#P-value
	N	Mdn-IQR	N	Mdn-IQR	N	Mdn-IQR	
*Age, y	29	59 (53-68)	90	48 (38 5-64 5)	26	31 5 (26 - 36)	0 9310
Body composition							
Weight, Kg	28	80 (74-90)	89	76 (61-84)	25	89 (71 - 117)	0 0710
Body fat mass, %	13	34 7 (24 5-42 3)	48	35 8 (30 2-38 5)	15	32 8 (45 5 - 8 7)	0 0970
Waist-arm-ratio	29	3 06 (2 93-3 27)	91	2 81 (2 72-3 07)	26	2 96 (2 65 - 3 24)	0 3630
Waist-hip-ratio	29	0 89 (0 81-0 92)	91	0 82 (0 78-0 92)	26	0 87 (0 75-0 93)	0 8810
Blood pressure							
*RR-sys, mmHg	28	127 (118-146)	87	122 (110-132)	25	117 33 (108 33 - 129 33)	0 0326
RR-dia, mmHg	28	80 (74-82 7)	87	79 2 (72-84 3)	25	77 67 (73 - 83)	0 1110
Serum biochemistry							
*Glucose, mmol/L	29	106 (97-121)	89	88 (84-101)	24	99 5 (87 5 - 102)	0 0037
HbA1c, mg/dL	28	5 8 (5 4-6 6)	90	5 45 (5 2-5 9)	25	5 3 (5 1 - 5 7)	0 0740
Cholesterin, nmol/L	27	198 (179-215)	90	192 (177-210)	24	206 5 (188 - 226)	0 8660
TG, nmol/L	27	119 (104-165)	90	125 (94-165)	24	126 (72 5 - 210 5)	0 8970
HDL, mmol/L	27	56 (50-63)	90	57 (48 5-69)	24	52 (41 - 69 5)	0 7170
LDL, mmol/L	27	107 (96-138)	90	106 (88 5-123)	22	124 5 (101 - 132)	0 8610
*24hUFC, µg/24h	27	158 (81 3-218)	72	43 3 (8-62)	25	93 (62 - 115)	< 0 0001
*Insulin, pmol/L	27	11 1 (10-19 8)	87	8 9 (4 7-14)	24	15 8 (9 1 - 24 5)	0 0086
*HOMA-IR	27	3 05 (2 85-5 18)	86	2 07 (1-3 53)	24	3 93 (1 99 - 6 19)	0 0050

n: number, Mdn: median, IQR: interquartile range, RR-sys: blood pressure systolic, RR-dia: blood pressure diastolic, HbA1c: glycated hemoglobin, TG: triglyceride, HDL: high-density lipoprotein, LDL: low-density lipoprotein, 24h-UFC: 24-hour urinary free cortisol, HOMA-

IR: homeostatic model assessment - insulin resistance. #: P-value between overt group and remission group, *p*-value < 0.05 (*).

Table 2. Characteristics of participants II

	ACTH-dependent CS		ACTH-independent CS		control		$P-value
	N	Mdn-IQR	N	Mdn-IQR	N	Mdn-IQR	
*Age, y	79	59 (53-68)	41	48 (38 5-64 5)	26	31 5 (26 - 36)	0 5800
Body composition							
Weight, Kg	79	80 (74-90)	41	76 (61-84)	25	89 (71 - 117)	0 9500
Body fat mass, %	79	34 7 (24 5-42 3)	41	35 8 (30 2-38 5)	15	32 8 (45 5 - 8 7)	0 8320
Waist-arm-ratio	79	3 06 (2 93-3 27)	41	2 81 (2 72-3 07)	26	2 96 (2 65 - 3 24)	0 0220
Waist-hip-ratio	79	0 89 (0 81-0 92)	41	0 82 (0 78-0 92)	26	0 87 (0 75-0 93)	0 2370
Blood pressure							
*RR-sys, mmHg	79	127 (118-146)	41	122 (110-132)	25	117 33 (108 33 - 129 33)	0 8620
RR-dia, mmHg	79	80 (74-82 7)	41	79 2 (72-84 3)	25	77 67 (73 - 83)	0 7590
Serum biochemistry							
*Glucose, mmol/L	79	106 (97-121)	41	88 (84-101)	24	99 5 (87 5 - 102)	0 3220
HbA1c, mg/dL	79	5 8 (5 4-6 6)	41	5 45 (5 2-5 9)	25	5 3 (5 1 - 5 7)	0 8380
Cholesterin, nmol/L	79	198 (179-215)	41	192 (177-210)	24	206 5 (188 - 226)	0 2080
TG, nmol/L	79	119 (104-165)	41	125 (94-165)	24	126 (72 5 - 210 5)	0 6700
HDL, mmol/L	79	56 (50-63)	41	57 (48 5-69)	24	52 (41 - 69 5)	0 1850
LDL, mmol/L	79	107 (96-138)	41	106 (88 5-123)	22	124 5 (101 - 132)	0 3300
*24hUFC, µg/24h	79	158 (81 3-218)	41	43 3 (8-62)	25	93 (62 - 115)	0 2750
*Insulin, pmol/L	79	11 1 (10-19 8)	41	8 9 (4 7-14)	24	15 8 (9 1 - 24 5)	0 9380
*HOMA-IR	79	3 05 (2 85-5 18)	41	2 07 (1-3 53)	24	3 93 (1 99 - 6 19)	0 7630

n: number, Mdn: median, IQR: interquartile range, RR-sys: blood pressure systolic, RR-dia: blood pressure diastolic, HbA1c: glycated hemoglobin, TG: triglyceride, HDL: high-density lipoprotein, LDL: low-density lipoprotein, 24h- UFC: 24- hour urinary free cortisol, HOMA -

IR: homeostatic model assessment - insulin resistance. $: P-value between ACTH-dependent CS subgroup and ACTH-independent CS subgroup, *p*-value < 0.05 (*).

4.2 E47 gene expression

To determine cortisol dependent differences of E47 mRNA expression we compared E47 gene expression in 120 patients with CS and 26 healthy controls with physiological baseline cortisol levels. E47 gene expression in whole blood samples was significantly lower in patients with overt CS (n = 29) compared to patients in remission (n = 91; $p = 0.0474$), well showed no differece with healthy control (n = 26, $p = 0.1870$). No statistical difference was observed between E47 expression in patients with CS in remission and healthy controls ($p < 0.9999$). Comparing E47 mRNA expression before and after surgery, E47 mRNA expression was demonstrated for matched samples of each individual patient, for which both pre-surgery and post-surgery blood samples were available ($p = 0.0166$, n = 14) (Figure 4).

Figure 3.

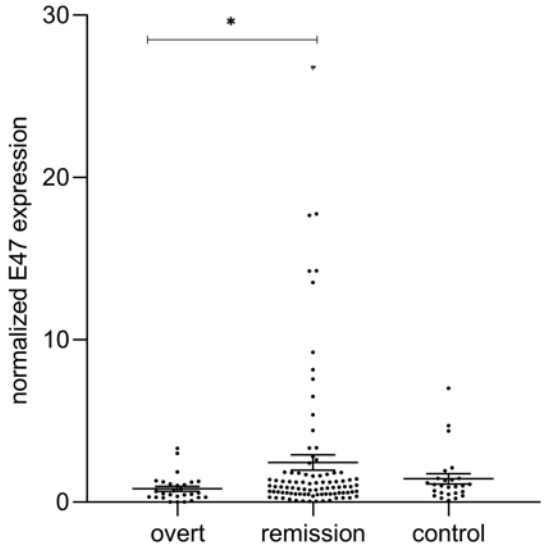

Figure 3. E47 gene expression in patients with CS correlates with disease status. E47 transcript levels in whole blood samples were normalized to endogenous expression of β-Actin in overt CS (n = 29), CS in remission (n = 91) and control

subjects (n = 26), Comparison between these three groups showed a statistical difference (p = 0.0479). Normalized E47 transcript levels in whole blood samples of patiens with CS in remission are increased compared to overt CS (p = 0.0474), while no difference can be observed compared to control subjects (p = 0.1870), there was also no statistical difference between remission group and healthy control (p < 0.9999), data are shown as median with 95% CI; p-value <0.05 (*) by Kruskal-Wallis test with Dunn's multiple comparison test.

Figure 4.

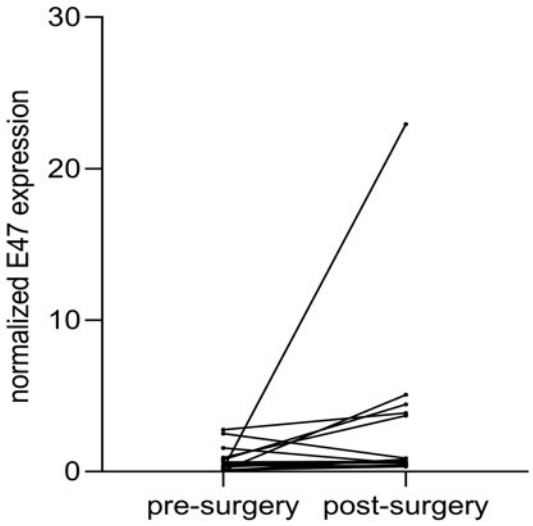

Figure 4. E47 mRNA expression pre- and post-surgery. Comparing pre- and post-surgery E47 mRNA expression in each individual patient, E47 expression is increased in the same individual patients post-surgery (n = 14, p = 0.0353); p-value <0.05 (*) by Wilcoxon matched-pairs signed rank test.

In order to analyze ACTH-dependency of the observed effect on E47 mRNA expression in patients with endogenous cortisol excess, we performed E47 gene analysis in ACTH-dependent (n = 79) and -independent subgroups (n = 40), respectively. Comparing differences in E47 gene expression in overt CS, no statistically significant difference was observed between ACTH-dependent (n = 16) and -independent subtypes of CS (n = 13). E47 gene expression showed

non-significant change compared to healthy controls (n = 26, p = 0.3825) (Figure 5A). No statistically significant difference of E47 mRNA expression was detected between ACTH-dependent (n = 63) or -independent CS patients (n = 27) in remission and healthy controls (n = 26, p = 0.9593) (Figure 5B).

To further understand the dynamic changes of E47 gene expression, we observed that administration of 1 mg dexamethasone (n = 23) led to non-significant change in E47 mRNA expression (Figure 6A). In 12 patients with CS, administration of ACTH resulted in a significant decrease of E47 mRNA expression 30 minutes after i.v. injection compared to baseline measurements (p = 0.0015) (Figure 6B).

Figure 5.

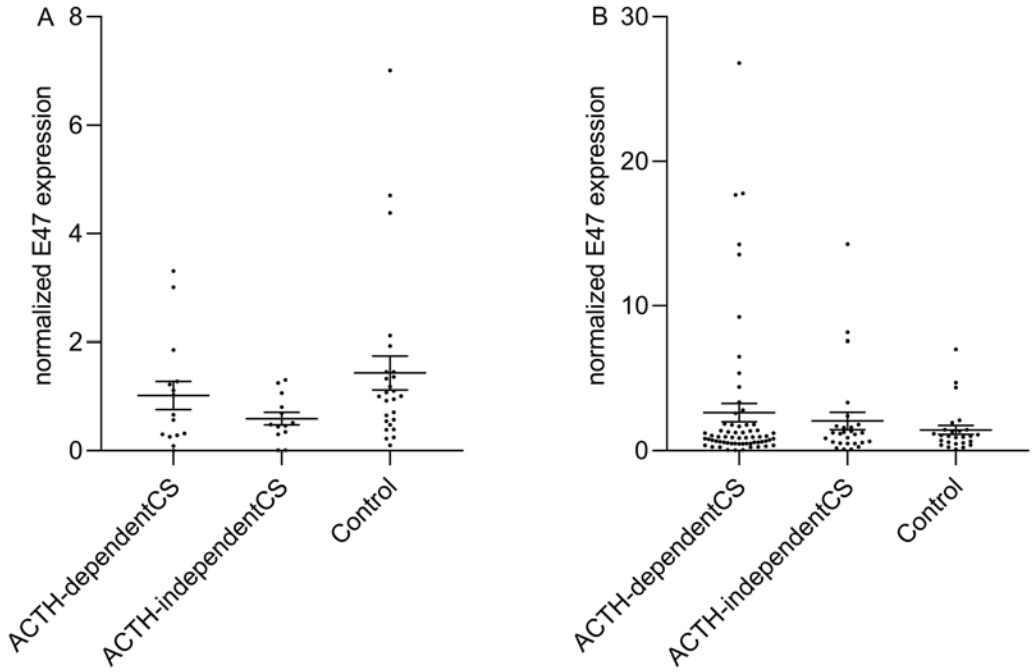

Figure 5. E47 mRNA expression in both ACTH-dependent and -independent subgroups compared to controls. (A) No significant difference in E47 mRNA expression was observed between ACTH-dependent (n = 16) or -independent (n = 13) overt CS and controls (n = 26, p = 0.3825). (B) There is no statistically significant difference in E47 mRNA expression in ACTH-dependent (n = 63) or -independent (n = 27) CS patients in remission and controls (n = 26, p = 0.9593). p-value <0.05 (*) by

Mann-Whitney test.

Figure 6.

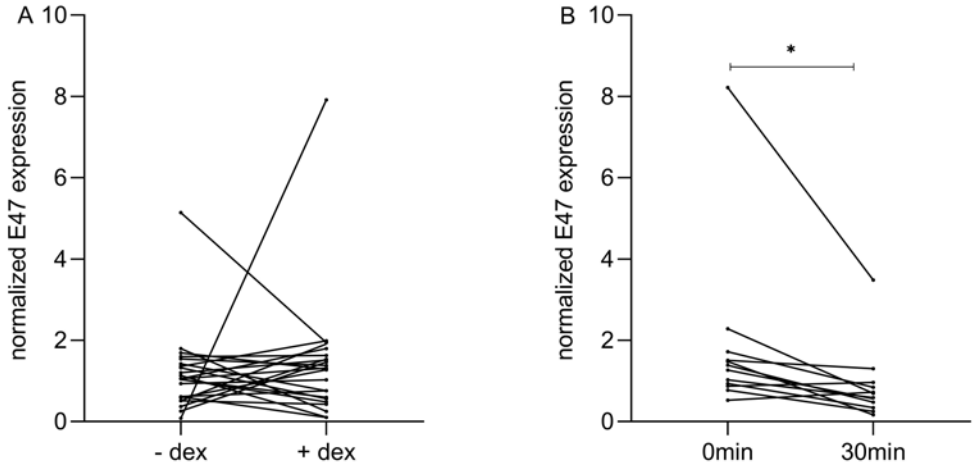

Figure 6. E47 mRNA expression in Dexamethasone suppression test and ACTH stimulation test. (A) E47 mRNA expression showed no statistical difference pre- or post-administration of 1mg dexamethasone (n = 23, $p = 0.2902$). (B) E47 mRNA expression was significantly reduced 30 minutes after i.v. injection of Synacthen® (n = 12); p-value < 0.05 (*) by Wilcoxon matched-pairs signed rank test ($p = 0.0015$).

4.3 Correlation of E47 gene expression and clinical parameters

We further examined the correlation between clinical parameters and E47 mRNA expression. In the subgroup of patients with CS in remission (total n = 90), E47 gene expression showed a positive correlation with total serum cholesterol ($p = 0.0036$, r = 0.3022), LDL ($p = 0.0157$, r = 0.2527) measurements and waist-arm-ratio ($p = 0.0138$, r = 0.2616) (Figure 7A-C) in the subgroup of patients in remission. No positive correlations were found both in the subgroup of patients with overt CS (total n = 27). Although no statistically significant correlation was detected, we saw the same trends of positive correlation between serum cholesterol and LDL levels, as well as waist-arm-ratio and E47 gene expression in the subgroup of patients with overt CS (Figure 7D-F).

Figure 7.

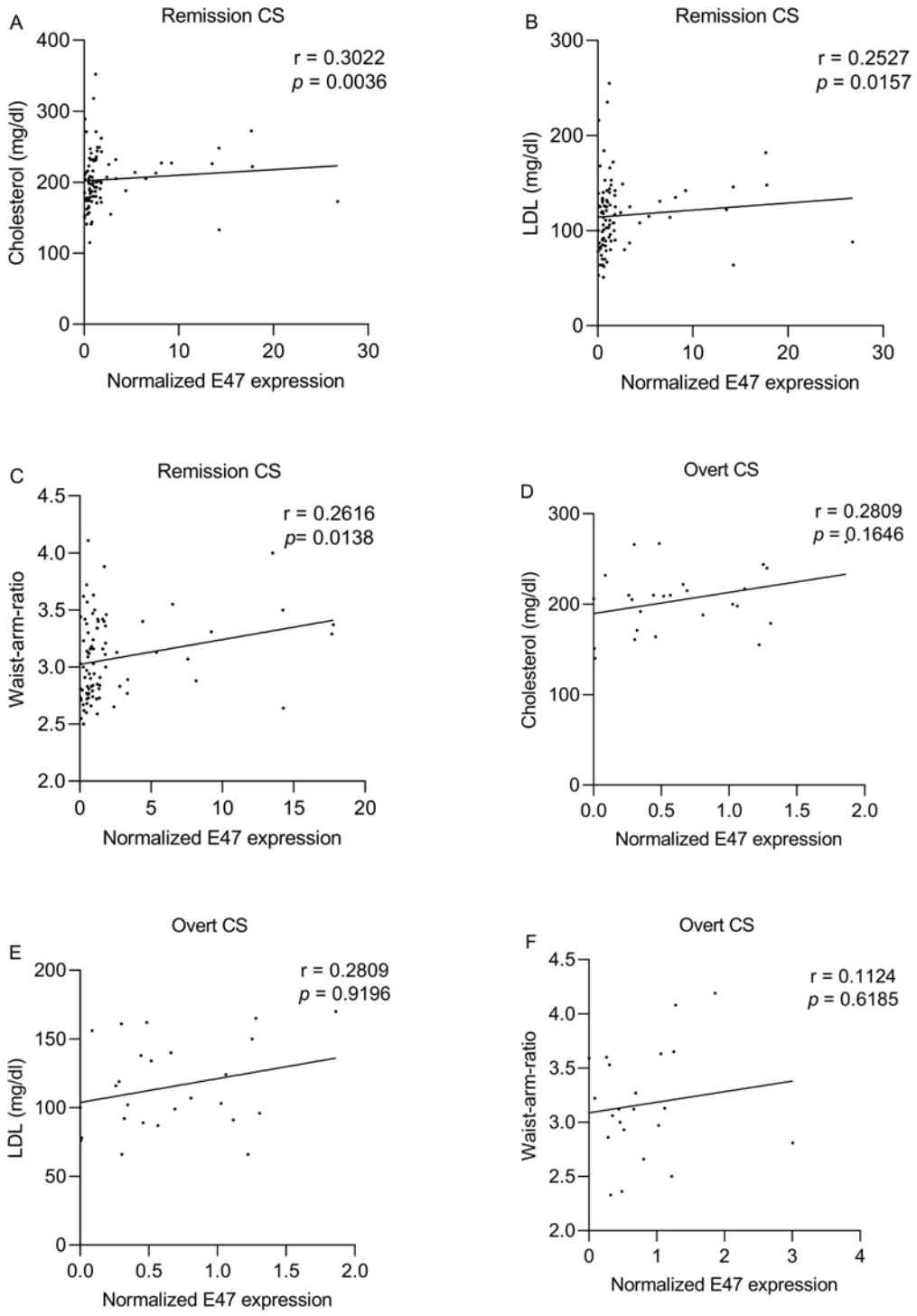

Figure 7. Correlation of E47 gene expression and different clinical parameters in patients with overt CS and CS in remission. (A-C) E47 gene expression shows positive correlation with total serum cholesterol (p = 0.0036, n = 90, r = 0.3022), serum LDL (p = 0.0157, n = 90, r = 0.2527) and waist-arm-ratio (p = 0.0138, n = 90, r

= 0.2616). (D-F) E47 gene expression shows a visual, but not significant correlation with total serum cholesterol (p = 0.1646, n = 27, r = 0.2809), serum LDL (p = 0.9196, n = 27, r = 0.2809) and waist-arm-ratio (p = 0.6185, n = 23, r = 0.1124), p-value< 0.05 considered significant as determined by nonparametric Spearman correlation.

5. Discussion

This is the first study investigating the role of E47 in a human disease model and also the first study assessing E47 gene expression in patients with endogenous glucocorticoid excess. In total we included 120 female patients with CS and 26 healthy female controls in our analysis of E47 gene expression under different conditions (in overt and in remission) as described above. In an attempt to translate the findings from mice to humans, we investigated the expression and correlation of E47 with metabolic adverse effects in patients with endogenous Cushing syndrome. Patients with CS suffer from multiple severe metabolic changes caused by an excessive GC secretion due to an adrenal, pituitary or ectopic tumor. A myriad of undesirable metabolic side effects limit the therapeutic use of GC. Therefore, a better understanding of the regulation of GR-mediated metabolic target genes is crucial to develop safer drugs.

According to our research findings, the clinical characteristics of the trial participants consistent with their disease status. Positive significant differences were observed for glucose, 24-h-urine cortisol, insulin, HOMA and systolic blood pressure among overt CS, remission CS and the control group. Glucose concentrations in the overt CS subgroup are higher than those of patients with CS in remission (Figure 2A). Regarding 24-h-urine cortisol measurements, the overt CS group presents with increased level than patients in remission and healthy controls (Figure 2B). Systolic blood pressure of patients with overt CS is higher than in patients in remission and healthy controls (Figure 2C). Insulin and HOMA- IR were both higher in patients with overt CS(Figure 2D, 2E). These positive statistics results were all consistent with the clinical features of CS. In conclusion, CS patients in overt who have increased glucocorticoids with associated symptoms such as increased blood glucose, increased insulin and increased blood pressure than patients in remission or normal controls. Other serum biochemistry were included as well, such as HbA1c, lipid metabolism indicators (cholesterin, TG, HDL, LDL). But we didn’t see significant differeces between patients in overt Cushing and in remission. It is important to mention that our

small sample size of the overt subgroup (n = 29) may have contributed to such results. A larger sample size would provide a more accurate picture of serum biochemistry in CS patients.

In results of our experiments, positive associations between E47 gene expression and their corresponding cholesterol, low density lipidemia protein were revealed female patients with CS in remission (Fig. 7A, Fig. 7B). Besides, among female patients with overt CS (Fig. 7D, Fig. 7E), they kept the same trend but not significant. One possible reason was that, the lack of significance may be due to the too small sample size of those patients with overt CS.

The altered lipid metabolism and hyperglycemia observed in CS also contribute to the development of obesity (129). In particular, patients suffer from central obesity and show changes body composition with a redistribution of body fat, increased abdominal visceral adipose tissue and reduced peripheral subcutaneous adipose fat deposition (129). Central obesity is further characterized by a decrease in muscle mass, resulting in thinning of arms and limbs (130). BMI therefore often is not a sensitive marker to distinguish differences in body composition caused by CS. In our research, we also did not observe any correlation of BMI and E47 expression in our patient cohort. As a more representative and sensitive parameter for visceral obesity we investigated waist-arm-ratio and waist-hip-ratio. Because CS patients were typically clinically characterised by central obesity with reduced muscle mass in the extremities, waist-to-arm circumference ratios are generally higher than in normal health condition. We could show that E47 mRNA expression in blood correlated positively with waist-arm-ratio in patients with CS in remission (Fig. 7C). In contrast, no significant correlation between waist-arm-ratio and E47 gene expression could be detected in patients with overt CS (Fig. 7F). This might also be explained by a significantly smaller group size of patients with overt CS. An increased waist-arm-ratio due to an increase in waist circumference and a decrease in arm circumference of CS patients can be seen with a relatively high expression of E47. We therefore infer that the low expression state of E47 seems to correspond to a state that favours fat metabolism and is protective of the body.

We did not observe a correlation of E47 expression with waist-hip-ratio in either patient group. This could be explained by the fact that the waist-arm-ratio might be a more specific and suitable marker as measurement of the arm and waist is more reliable among CS patients and less dependent on the investigator.

Of note, as hyperglycemia in CS is not only caused by GC effects on the liver, but by a combination of GC effects on the liver but also muscle, adipose tissue and pancreas, it might be difficult to detect a modulation of glucose concentrations by E47 in vivo. Standardized tests as an oral glucose tolerance test might be able to detect these modulatory effects.

As already mentioned above, E47 has been described as a transcription factor cooperating with GR in the regulation of hepatic glucose and lipid metabolism in mice (121). Loss of E47 protected mice from GC-induced hyperglycemia, hyperlipidemia and hepatic steatosis. These mice showed lower glucose concentrations, less lipid accumulation in the liver and circulation and did not develop hepatic steatosis. They use CHIP- suquencing profile with using E47 antibody in mice livers, then concluded that E2A and GR partially overlap and share a large number of binding sites. After 3 weeks of feeding Dexamethasonein in their drinking water, wild-type mice showed slightly hyperglycemia, but blood glucose levels of E47 knockout mice were remarkably lower than wild-type, after the injection of the glucose for 1.5 hours. The pyruvate tolerance test showed that E47 -/- mice respond to Dex with a decrease in hepatic glucose production. Meanwhile, no differences in basal glucose or pyruvate tolerance were found in untreated E47-/- animals (121). Our results are consistent with the E47 mouse experiment, where the E47 gene was positively associated with glucose and lipid metabolism. When E47 gene expression is reduced, blood glucose and lipids are less likely to be elevated by high glucocorticoids. This phenomenon suggested that reduced E47 activity may prevent the development of hyperlipidaemia, at least reduced productions of cholesterol and LDL based on the results of our research.

Until now, many studies have been performed to declear the relationship between GCs and GRs. In 2000, the first investigation researching GR down-regulation was

held on CS patients. In that study, experimenters used a whole-cell dexamethasone binding assay to clarify the characteristics of GR in peripheral mononuclear blood leukocytes (PBML) from 13 patients with endogenous Cushing's syndrome and 15 control participants. In addition, cortisol concentrations were check in order to find out the possible relationship between serum cortisol levels and receptor characteristics. They showed that the GR was not down-regulated in peripheral mononuclear blood leukocytes. However, the CS patients' whole cell dexamethasone ligand binding affinitiy was significantly decreased compared with controls (124). They gave a possible explanation that high cortisol cause downregulation of receptor number and decreased ligand affinity. This unexplained decrease in ligand affinity may partially protect cells from high cortisol levels. This unexplained decrease in ligand affinity may partially protect act a role in protecting cells from high cortisol levels. Coherent with our experimental results, we found there was a high variability in individual E47 expression levels in whole blood both in CS patients and controls. Furthermore, E47 gene expression was not significantly different between patients with overt CS and controls. But in patients with overt CS E47 was expressed significantly higher compared to those patients who were in remission (Fig. 3).

Moreover, we try to look for how the E47 gene expression in pre-surgery and their corresponding post- surgery condition with Cushing's syndrome in remission (n = 14). E47 mRNA expression was significantly lower in patients with CS pre-surgery compared to matched samples of the individual patients post-surgery with remission of Cushing's disease (Figure 4). This result is likewise consistent with the above findings. E47 gene expression was inversely correlated with GC concentration.

In order to analyze ACTH- dependency of the effect on E47 mRNA expression, and analyzed within the group of patients with ACTH-dependent CS whether normalized E47 expression is altered. E47 gene expression in overt CS or in remission CS, there was no difference observed among ACTH- dependent, - independent subtypes of CS and healthy controls (Fig. 6A, Fig. 6B). This result suggests that E47 expression is not related to the ACTH dependence or not, but only to CS disease status.

Next, to confirm this GC-dependent downregulation of E47 and the dynamics of E47

in humans, we exposed participants to a short Synacthen test. ACTH strongly stimulates the release of downstream Cortisol. Upon ACTH stimulation, E47 mRNA expression was significantly downregulated within 30 minutes compared to basal mRNA expression (Fig. 7B). This is again in line with the observation that E47 expression was decreased in patients with overt CS compared to those in remission. In next step, the duration of blood collection could be extended in our subsequent trials to better characterize the dynamic changes of E47. Interestingly, in the dexamethasone depression test, we didn't see difference of E47 gene expression in bloods between pre- and post drug exposure (Fig. 7A). We can only speculate why we did not observe an effect upon GC exposure in the dexamethasone suppression test but a clear suppression of E47 following ACTH stimulation. It could be that the used dose of dexamethasone of 1 mg was too low as stimulus compared to a maximum stress stimulation by 250 µg ACTH. Also, we could have missed the time window of E47 downregulation. A study comparing healthy female volunteers taking oral contraceptives, as oral contraceptives cause an increase in total plasma cortisol concentrations, resulting in a false positive result for 1 mg-DST, the research found that 11% of subjects had a false positive value after 1 mg DST (131). This also suggests that 1 mg of dexamethasone is somethime not effective enough to suppress hypercortisolism. We may complete our follow-up study on the high dose dexamethasone suppression trial to search the dynamic of the E47 gene expression following cortisol suppression.

In our study, no direct correlation was seen between 24h-UFC, baseline cortisol concentrations or AUC of saliva cortisol and peripheral blood E47 mRNA expression. Furthermore, we could not observe a correlation with fasting glucose concentrations, nor insulin or HOMA index or HbA1c. Therefore, how E47 gene expression is specifically regulated in glucocorticoid excess states needs to be further investigated. we were only able to analyze E47 gene expression in peripheral blood from patients with CS, but we are not able to comment on hepatic E47 gene expression in patients with CS and whether alterations affect hepatic glucose production. One may only speculate that there is no linear correlation with glucocorticoid concentration but a

ceiling effect may occur. The down-regulation of GR mRNA may be associated with decreased receptor affinity, shorter receptor half-life and reduced mRNA abundance (125). Previous in vitro trials have shown that after 24h treatment with dexamethasone, GR-mRNA levels increased in the peripheral blood cells obtained from healthy volunteers (126). However, for the chronic hyperglucocorticoid condition, levels of both GR mRNA and protein were greatly downregulated after 2 weeks of dexamethasone treatment (127). As GR and E47 co-regulate some metabolic enhancers and inhibitors, E47 may partly play the same role as GR.

The secretion of glucocorticoids is regulated by the pituitary gland and has a pulsatile, and circadian, rhythm, with a peak at 8 a.m and a gradual decline between 10 a.m and 12 p.m, returning to full normal at 12 p.m at night (4). However, this is not the case in CS patients. Patients with Cushing's syndrome are short of this natural circadian rhythm of serum cortisol concentrations. The CS patients stay in a chronic high GC state. The complexity of glucocorticoid action makes the study of the E47 gene in this hyper GC state also extremely challenging. So whether E47 protein expression, protein activity and sequence elements binding ability are affected in patients with chronic hypercortisolism needs be further probed. Interestingly, Cannizzo et al. reported an upregulation of E47 in the thymus of cattle experimentally treated with low doses of DEX for 40 days in 2008 (128). Combining the findings of our experiments, E47 gene expression may have tissue variability under hyperglucocorticoid states.

In summary, we were inspired by the novel role of E47 in the metabolic gene regulation of GCs by hepatocytes in mouse experiments and wanted to investigate the role played by this gene in patients with endogenous hyperglucocorticoidemia. We evaluated the expression of E47 gene in whole blood of 120 female patients with endogenous glucocorticoid excess and 26 female healthy controls. We conclude that individual expression levels of E47 were highly variable in both CS patients and controls. E47, a GC-dependent gene, is downregulated in the presence of elevated endogenous glucocorticoid levels and may provide protection against glucocorticoid-dependent comorbidities. We deduce that this decreased expression

of E47 mRNA in CS patients who under a hyperglucocorcoid may be a protected form of negative feedback. E47 shares only a portion of its target genes with GR and therefore regulates GC responses in a definite manner. As this function is conserved at several promoters and enhancers in humans, in combination with the relevant literature mentioned above, we can speculate that differences in E47 expression levels may influence the sensitivity to GC in patients in different situations. Downregulation of E47 mRNA may protect ligands from being triggered by persistent signals in the presence of hypercortisolism or other forms of GC overload. Not only have we demonstrated novel ways in which E47 may eliminate some of the side effects of GC treatment, but we also propose new directions for targeting CS drug therapy. In the end, this shared set of dysregulated target genes (responsible for protective effects) that bind to GR and E47 may provide new inspirations for the development of new drug treatment curves that maintaining their potent anti-inflammatory properties.

6. Outlook

Endogenous glucocorticoid excess leads to serious damage to health and affects almost all systems of the body. It is therefore essential to understand the mechanisms of glucocorticoid regulation in detail in order to provide more targeted treatment. Until now, the mechanism causing side-effects when using glucocortioid treatment is not clearly understood. The aim of this experiment was to discover the expression of E47 in the peripheral blood of patients with endogenous hypercortisolism. E47 has been described as a transcription factor cooperating with GR in the regulation of hepatic glucose and lipid metabolism in mice (121). Loss of E47 protected mice from GC-induced hyperglycemia, hyperlipidemia and hepatic steatosis.

It would be highly interesting to include male patients in to the study and to increase the sample size as we have seen quite a large variation of E47 expression. Effects in a living system of the human body might only be detectable in substantially larger cohorts.

A limitation of our study is that the mean age between the patients with CS was different from that of the controls In order to avoid any bias due to age, age-matched controls would be ideal.

In order to investigate the dynamics of changes of E47 gene expression over time, it would also be interesting to perform a low dose ACTH stimulation test over several hours. It might also be interesting to analyse E47 expression after dexamethasone suppression in patients with CS.

As in E47 knockout mice experiments, by performing ChIP sequencing in mouse livers. They found E47 and the GR partially overlapped and shared various binding sites. E47 co-occupies a subset of GR-bound enhancers and promoters in mice livers, at target genes governing hepatic glucose and lipid metabolism (121).

E47 and GR are partly co-modulators in regulating downstream gene expressions, we can also search GR gene expression profile of these participants from our cohort in the mean time. This allows a clearer picture of the expression of GR and E47 in the peripheral blood of the same individual.

As E47 has been demonstrated in mouse experiments as a transcription factor involved in regulating glucose and lipid metabolism in the mice liver (121). It would be highly interesting to compare E47 expression in the liver of patients with CS to healthy controls. But of course this tissue is not available. It would be interesting to carry out in vitro experiments with human liver cell lines or other related cell line. Then treated at different concentrations of glucocorticoids to examine the expression of E47 and GR genes and proteins.

7. Conclusion

Our data offer new insights into the regulation of GC-induced adverse effects. We found that there was a high variability in individual E47 expression levels both in CS patients and controls. We also found that there was a positive correlation between E47 mRNA expression and cholesterol as well as LDL. Higher expression levels of E47 correlated with higher concentrations of cholesterol and LDL.. E47 rather seems to be another GC-dependent gene that is down-regulated in situations with high exposure to GC, as in CS potentially to give some protection from side effects. In particular, our results contribute to a better understanding of the complex mechanisms of the pathophysiology of GC-induced dyslipidemia. Our results support the idea that the previously described findings in mice are also relevant to human physiology. E47 acts as a GC-dependent gene that is downregulated in glucocorticoid excess, potentially offering protection from glucocorticoid-induced comorbidities mediated by the liver.

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I hereby declare, that the submitted thesis entitled

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