Local Control of Steroid Hormone Biosynthesis

Johannes Hofland

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Local Control of Steroid Hormone Biosynthesis

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These hormones still belong to the physiologist and to the clinical investigator as much as, if not more than, to the practicing physician.

Philip S. Hench
Nobel Prize Lecture 1950

Awarded for "for the discoveries relating to the hormones of the adrenal cortex, their structure and biological effects"

Voor Pa

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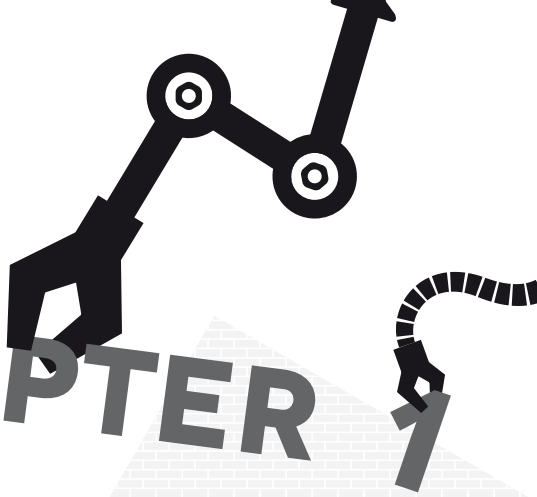


CHAPTER 1

General Introduction

Partly based on:

Inhibins and activins: Their roles in the adrenal gland and the development of adrenocortical tumors.

Johannes Hofland & Frank H. de Jong

Molecular and Cellular Endocrinology, *2011,* Epub June 22nd

GENERAL INTRODUCTION

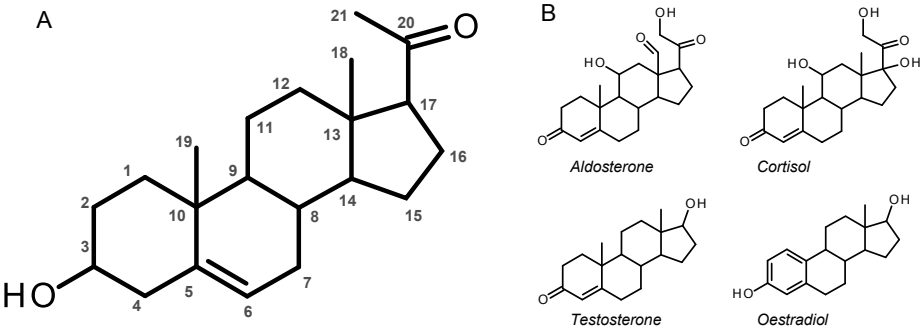
1 STEROIDS

Steroids are essential for vertebrate physiology during pre- and postnatal life. Whereas the skeleton structure of cyclopenta[α]phenanthrene rings is common to all steroid molecules, differences occur in methyl- of ethylgroups attached to the four rings or the oxidation state of the carbon atoms in the rings (Figure 1).[1] Endogenous production of steroids is realized in steroidogenic tissues. From these tissues, steroid molecules can be secreted into the circulation to act in an endocrine fashion. By binding to receptors in target tissues they manipulate gene transcription, influencing a wide variety of cellular functions. Steroids can also exert local effects in the steroidogenic tissues after secretion into the extracellular space (paracrine or autocrine) or directly within the cell in which they are produced (intracrine).

1.1 Steroid hormones

In humans, the main groups of steroid hormones consist of sex steroids, mineralocorticosteroids and glucocorticosteroids (Table 1). The sex steroids testosterone, oestradiol and progesterone are predominantly produced in the gonads and are crucial for sexual development, sex behavior and reproduction.[2] They can bind to the androgen receptor (AR), estrogen receptor (ER) and progesterone receptor (PR), respectively, in peripheral target tissues.[3] The major postnatal production of these steroids commences at the start of puberty and declines after menopause in females.

Figure 1: Steroid structure



(A) Pregnenolone, containing the common cyclopenta[α]phenanthrene structure. Numbers refer to carbon atoms in the steroid molecule. Nomenclature of the steroidogenic reactions is derived from the number of the affected carbon atom. (B) Molecular structure of several active steroid hormones.

Table 1: Principal steroid molecules in man

	Mineralocorticoids	Glucocorticoids	Sex steroids
Steroids	Aldosterone	Cortisol	Testosterone (I), oestradiol (II), progesterone (III)
Origin	Zona glomerulosa	Zona fasciculata	Gonads Placenta Zona reticularis
Function	Blood pressure Cardiovascular effects	Protein, carbohydrate, lipid and nucleic acid metabolism Stress and immune modulation	Sexual development and behavior Reproduction
Receptor	Mineralocorticoid receptor (MR)	Glucocorticoid receptor (GR)	I: Androgen receptor (AR) II: Estrogen receptor (ER) III: Progesterone receptor (PR)
Syndrome	Conn	Cushing	I) Virilization II) Feminization
Symptoms	Hypertension Hypokaliemia Metabolic alkalosis	Moon facies, buffalo hump Diabetes mellitus Hypertension Amenorrhea Striae, easy bruising Osteoporosis Prone to infections Depression, psychosis	I) Secondary male sexual characteristics II) Secondary female sexual characteristics

Aldosterone is the principal mineralocorticoid and can bind to the mineralocorticoid receptor (MR). Its main effects include the induction of expression of the epithelial sodium channel (ENaC) and the Na^+/K^+ ATPase in the distal convoluted tube and collecting duct of the nephron, thereby regulating Na^+ reabsorption and K^+ excretion. Through this mechanism, aldosterone is one of the key determinants of blood pressure.[4] The main glucocorticoid in man is cortisol, which regulates physiology in a multitude of tissues such as the immune system, brain, cardiovascular system and metabolic tissues through binding of the glucocorticoid receptor (GR).[5] Corticosterone also possesses glucocorticoid activity and is the predominant glucocorticoid in rodents. The other steroids depicted in Figure 2 are also present in the steroidogenic organs and in the circulation. These are classically labeled as steroid precursors, since they need to be converted into the active steroid hormones in their target tissues. Quantitatively, dehydroepiandrosterone-sulfate (DHEA-S) is the most abundant steroid metabolite in the human circulation; its serum levels are in the micromolar range. Whether DHEA-S exerts direct effects (i.e. without conversion into other steroids) has been an area of controversy: a recent study has found a direct effect of DHEA-S on neutrophil leukocyte function, although a specific receptor has yet to be found.[6]

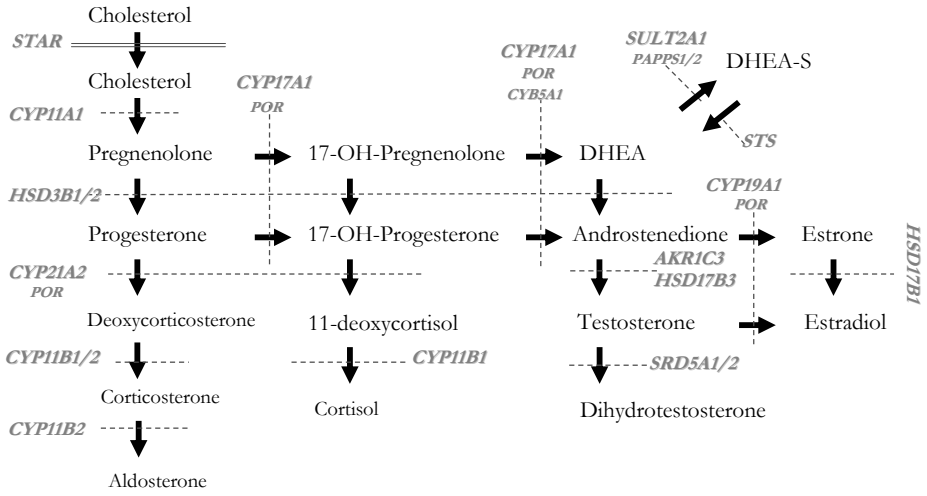
1.2 Steroidogenesis

Steroidogenesis is the biological process that involves sequential enzymatic reactions leading to the conversion of the common steroid precursor cholesterol into active steroid

hormones. Steroidogenesis is realized by steroidogenic enzymes that belong to the families of cytochrome P450 (CYP) enzymes and hydroxysteroid dehydrogenases (HSD) and catalyze different conversions of the steroid molecules (Figure 2).[7-9] Steroidogenic reactions and enzymes are named according to the position of the affected carbon atoms in the cyclopenta[a]phenanthrene structure as indicated in Figure 1. The type and quantity of steroidogenic enzymes in tissues as well as the presence of co-enzymes control the formation of steroid hormones in tissues throughout the body.

Cholesterol, either taken up from the circulation through LDL- or HDL-receptor[10] or produced *de novo* from acetic acid, has to be transported across the outer mitochondrial membrane for the first step of steroidogenesis to take place. Upon phosphorylation, the Steroid Acute Regulatory protein (StAR) actively translocates cholesterol over the mitochondrial inner membrane into the matrix where CYPscc (encoded by *CYP11A1*) can cleave the side chain of cholesterol, thus forming pregnenolone.[11] This process is the quantitative acute regulator of steroidogenesis and can be rapidly activated by the gonadotropins or adrenocorticotropin (ACTH) through cyclic AMP (cAMP)-mediated StAR phosphorylation.[12]

Figure 2: Human steroid biosynthetic pathway



Biologically active steroid molecules are all derived from the common precursor cholesterol. After cholesterol transport across the mitochondrial membrane, a series of sequential enzymatic reactions, involving cytochrome P450 (CYP) or hydroxysteroid dehydrogenase (HSD) enzymes, convert cholesterol into active steroid hormones. Genes coding for these enzymes are shown in grey, whereas the co-factors necessary for these reactions are depicted in lower case letters. The bioactive steroid hormones include progesterone (binds to the PR), deoxycorticosterone and aldosterone (MR), corticosterone and cortisol (GR), testosterone and dihydrotestosterone (AR) and finally oestradiol (ER).

Pregnenolone can subsequently be converted into mineralocorticoids, glucocorticoids or sex steroids, depending on the presence of the steroidogenic enzymes (Figure 2). The pivotal enzyme that determines the type of steroids produced is P450c17 (*CYP17A1*), which possesses both 17-hydroxylase and 17,20-lyase activities.[13-14] Addition of a hydroxyl group to carbon atom 17 by P450c17 in a reaction mediated by the NADPH-dependent electron donor P450 oxidoreductase (*POR*), allows for cortisol production. The 17,20-lyase activity of P450c17 creates C19-steroids and is dependent on the presence of POR as well as of the allosteric factor cytochrome b5 (*CYB5A1*).[15] Another enzyme activity necessary for production of bioactive steroid hormones is that of 3β-HSD (encoded by iso-enzymes *HSD3B1* and *HSD3B2*), which converts Δ^5-3-hydroxysteroids into Δ^4-3-ketosteroids.[16] Corticosteroid production is completed through subsequent reactions involving 21-hydroxylase (*CYP21A2*) followed by 11β-hydroxylase (*CYP11B1*) for glucocorticoids or aldosterone synthase (*CYP11B2*) for mineralocorticoids.[9]

The androgens testosterone and dihydrotestosterone (DHT) are formed from androstenedione through 17β-HSD (*HSD17B3* and *AKR1C3*) followed by 5α-reductase (*SRD5A1* and *SRD5A2*). Estrogen formation is realized through conversion of (precursors of) androgens by aromatase (*CYP19A1*).[17] Finally, DHEA-S is formed from DHEA by sulfotransferase (*SULT2A1*) in a reaction that involves 3′-phosphoadenosine-5′-phosphosulfate synthase (*PAPSS1* and *PAPSS2*).[18]

The relevance of steroidogenic enzymes in human physiology is evident from disorders caused by mutations in the genes encoding CYP or HSD enzymes or co-factors which lead to steroid deficiencies. Mutations in the most prevalently affected gene, *CYP21A2*, lead to congenital adrenal hyperplasia in approximately 1 in 16,000 live births.[19-20] Patients are characterized by varying degrees of mineralocorticoid and glucocorticoid deficiency in combination with elevated levels of ACTH and sex steroids. Mutations in other steroidogenic enzyme (*CYP11B1*[21], *CYP11B2*[22], *CYP17A1*[23-24], *HSD3B2*[25], *CYP11A1*[26-27], *HSD17B3*[28], *SRD5A2*[29]), transporter (*STAR*[30]) or co-factor (*POR*[31-32], *CYB5A1*[33], *PAPSS2*[34]) genes can also lead to steroid hormone deficiencies and clinical syndromes.

2 STEROIDOGENIC TISSUES

De novo steroidogenesis, i.e. steroid hormone production from cholesterol, was previously believed to be confined to the endocrine cells of the gonads, placenta and adrenal cortex. These tissues were found to secrete steroid hormones into the circulation and were thus termed the classic steroidogenic tissues. More recently, the detection of steroidogenic enzymes in peripheral target tissues has led to the insight that *de novo* steroidogenesis

or conversion of steroid hormone precursors can also occur in a para-, auto- or intracrine fashion.

2.1 Classic steroidogenic tissues

From the start of puberty, pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) induces sex steroid production in the gonads.[35] In the testis, Leydig cells form testosterone from cholesterol and secrete the androgen into the circulation.[36] The ovarian follicles produce estrogens through the cooperation of granulosa and theca cells. Androgens have to diffuse from theca cells, that lack aromatase expression, to granulosa cells, that lack P450c17, to be completely conversed.[37] During the second half of the menstrual cycle the corpus luteum secretes progesterone and oestradiol. The ovarian production of steroids varies according to the stage of the menstrual cycle, whereas testicular steroidogenesis remains continuously at the same level for a certain age. Sex steroids can also be produced by the placenta although it lacks P450c17 expression. The placenta can thus produce progesterone as well as convert C19 steroids which are synthesized mainly the fetal adrenal cortex into androgens and estrogens.[38]

The corticosteroids, as their name implies, originate from the adrenal cortex.

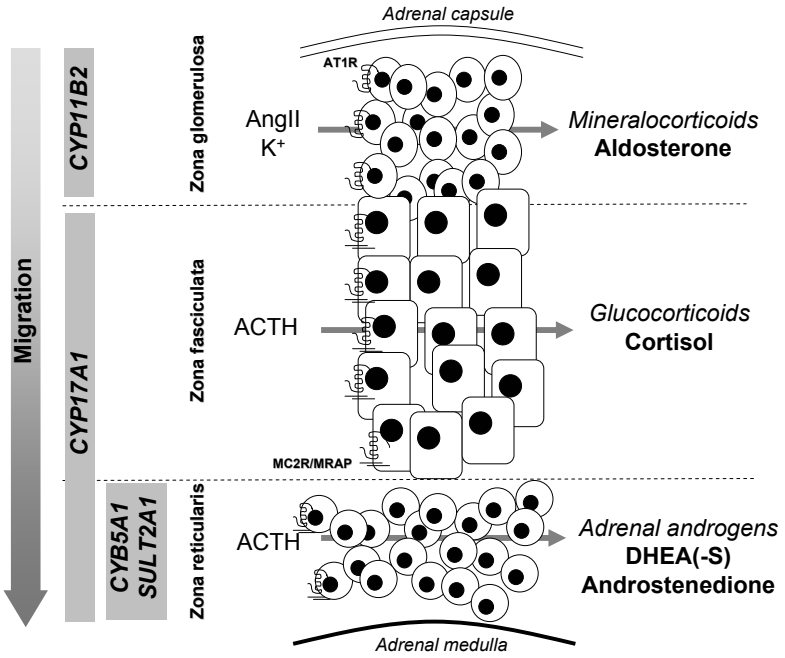
2.1.1 The adrenal cortex - physiology

The adrenal glands are composed of a steroidogenic cortex surrounding a neuro-endocrine medulla that secretes catecholamines. The adrenal cortex comprises three histologically and functionally different layers (Figure 3). Adrenocortical cells proliferate in the outer zone and subsequently migrate centripetally to the adrenal medulla. At the border of the medulla the adrenocortical cells go into apoptosis.[39-41]

The outer zona glomerulosa retains the adrenocortical progenitor cells and cells that secrete aldosterone. The mineralocorticoid-producing cells are positive for *CYP11B2* and negative for *CYP17A1* expression, thus facilitating aldosterone production.[42-43] Whereas the type II 3β-HSD was thought to be indispensable for all adrenocortical steroid production, a recent report suggested that aldosterone production might be specifically mediated by the type I 3β-HSD.[44] Aldosterone production, as part of the renin-angiotensin II-aldosterone system (RAAS, Figure 4), is mainly stimulated by angiotensin II (AngII), through activation of its type I receptor (AT1R), and potassium ion concentrations.[45-47]

The large, middle layer of the adrenal cortex, termed the zona fasciculata, is composed of cortisol-secreting cells. Cortisol exerts a negative feedback action in the hypothalamic-pituitary-adrenal (HPA, Figure 4) axis where its production is controlled by pituitary-derived ACTH. The fasciculata cells have abundant expression of the ACTH receptor, which is also known as the melanocortin type II receptor (MC2R).[43] For proper localization and signaling of the MC2R, these cells also express the trafficking protein and ACTH co-receptor melanocortin 2 receptor associated protein (MRAP).[48] ACTH signaling induces

Figure 3: Functional zonation of the human adult adrenal cortex



Adrenocortical cells migrate centripetally from the adrenal capsule to the medulla. During migration the cells display different expression patterns related to steroidogenesis. The zona glomerulosa is positive for *CYP11B2* while negative for *CYP17A1*, thus facilitating aldosterone production. The presence of the AngII type I receptor (*AT1R*) makes these cells susceptible to AngII-induced aldosterone synthesis. The inner zones harbor positive *CYP17A1* and *MC2R/MRAP* expression, leading to ACTH-regulated production of glucocorticoids and adrenal androgens. Adrenal androgens can be produced in the zona reticularis because of the presence of cytochrome b5 and sulfotransferase.

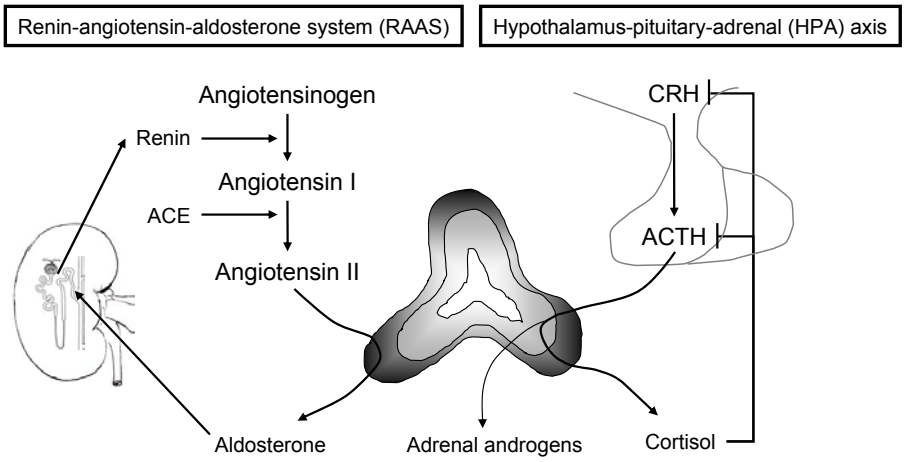
StAR phosphorylation and stimulates the expression of steroidogenic enzymes required for cortisol synthesis, mainly *CYP17A1*, *CYP21A2* and *CYP11B1*.[49-50]

The inner zona reticularis secretes adrenal androgens, such as androstenedione, DHEA and DHEA-S. The production of these steroids, also controlled by ACTH, is possible due to local expression of *CYB5A1* and *SULT2A1*.[51-52] Circulating adrenal androgens become detectable at adrenarche approximately at the age of 6 years when the zona reticularis develops.[53]

2.1.2 The adrenal cortex - pathology

Besides disorders in the steroidogenic enzymes, adrenal cell dysfunction can also be caused by damage or aberrant cellular growth. In 1855 Thomas Addison first described a patient with progressive destruction of the adrenal cortex leading to adrenal insufficiency. This disorder, now termed Addison's disease, is prevalent in 125 per million persons in the

Figure 4: The adrenal cortex as part of the RAAS and the HPA axis



On the left: The production of aldosterone in the zona glomerulosa is controlled by renin and angiotensin II. The juxtaglomerular cells in the kidney secrete renin, an enzyme that cleaves angiotensinogen into angiotensin I, in response to blood pressure fluctuations. The angiotensin-converting enzyme (ACE) subsequently produces angiotensin II that is capable of stimulating the adrenocortical cells in the outer zone to produce aldosterone. Aldosterone stimulates a.o. sodium reabsorption in the kidney, leading to an increase in blood pressure. On the right: the hypothalamus secretes corticotropin-releasing hormone (CRH) into the portal system of the pituitary gland. CRH subsequently binds to the cells in the anterior pituitary and stimulates the release of ACTH into the circulation. ACTH stimulates adrenocortical cells in the zonae fasciculata and reticularis to produce cortisol and adrenal androgens. Besides the widespread effects of cortisol, the glucocorticoid also exerts negative feedback on the production of CRH and ACTH in the hypothalamus and pituitary gland, respectively.

general population and was found to be caused by auto-antibodies against the 21-hydroxylase enzyme.[54-56]

Overproduction of adrenal steroids is most commonly caused by adrenocortical hyperplasia, adenomas or carcinomas. Primary hyperaldosteronism, also known as Conn's syndrome, can arise from AngII-independent bilateral hyperplasia of the zona glomerulosa or aldosterone-producing adenomas (APA).[57] This syndrome is characterized by hypertension, often therapy-resistant and accompanied by hypokaliemia. Increased cortisol production, leading to Cushing's syndrome, mostly arises from augmented stimulation by an ACTH-secreting pituitary adenoma, which is also known as Cushing's disease.[58] Patients with Cushing's syndrome can suffer from a multitude of physical symptoms because of the widespread distribution and effects of the GR. These symptoms include moon facies, skin changes, diabetes mellitus, hypertension, osteoporosis, frequent infections and psychiatric diseases (Table 1). ACTH-independent Cushing's syndrome, accounting for 29% of endogenous glucocorticoid overproduction, can be caused by atypical control of steroidogenesis or by adrenocortical tumors.[59]

The former situation applies to macro- and micronodular hyperplasia of the adrenal cortex. ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) is caused by augmented or ectopic expression of G-protein coupled receptors on adrenocortical cells, which aberrantly couple physiological concentrations of hormones different from ACTH to cortisol production.[60] Known ectopic stimuli include arginine-vasopressin (AVP)[61], gastric inhibitory polypeptide (GIP)[62-63], LH[64-65], catecholamines (through the β-adrenergic receptor)[66] and serotonin (5-HT).[64] Besides stimulation of steroidogenesis, these stimuli lead to hyperplasia of the adrenocortical cells. Origin of the aberrantly expressed receptors on the cells of the adrenal cortex is thus far unknown.

Micronodular hyperplasia on the other hand occurs in the context of primary pigmented nodular adrenocortical disease (PPNAD), either isolated or as part of Carney complex.[67] The majority of these patients suffer from mutations in molecules of the cAMP/protein kinase A (PKA)-pathway that lead to constitutive activation of PKA catalytic subunits. In the adrenal cortex, this predisposes to the development of ACTH-independent adrenocortical hyperplasia, which is usually accompanied by mild or subclinical forms of Cushing's syndrome.[68-70] Cortisol production in PPNAD appears to be influenced by a local glucocorticoid feed-forward loop through increased GR expression or coupling of the GR to the PKA subunits.[71-73]

Adrenocortical adenomas are the most common cause of adrenal enlargement and are found in up to 7% of all abdominal imaging studies.[74] Adenomas can either be clinically non-functional, i.e. not produce increased amounts of biologically active steroids, or functional, leading to hyperaldosteronism, Cushing's syndrome and rarely virilization or feminization. Changes in expression levels of steroidogenic enzymes or transcription factors regulating steroidogenesis contribute to the development of clinical hormone overproduction.[75] Furthermore, aberrant expression of eutopic or ectopic hormone receptors that could influence steroidogenesis have been described to occur in a minority of adrenocortical adenomas.[76-78] Adenomas larger than 4 cm, due to the risk of malignancy, or functional tumors need to be resected and carry an excellent prognosis.[79] Adrenalectomy is the operation of choice, although adrenal-sparing resection of the adenoma appears a feasible alternative.[80]

Adrenocortical carcinomas (ACCs) are rare with an estimated incidence of 1-2 per million per year.[59, 81] Prognosis is poor, with overall 5-year survival rates of 16-44%.[82] Detection of ACC is often late; the average tumor size exceeds 10 cm.[83] Fifty to sixty percent of ACCs are hormonally functional, with hypercortisolism and hyperandrogenism, either isolated or in combination, as the most common features.[84] Factors involved in tumor formation in the adrenal cortex remain largely unknown, although associations have found been with *TP53* mutations[85], IGF-II overexpression[86] and Wnt/β-catenin pathway activation.[87] Adrenocortical tumorigenesis is triggered by gonadectomy in ferrets and certain inbred

strains of mice,[88] suggesting a stimulatory role of the gonadotropins in tumor formation in the adrenal cortex.

According to the current adrenal incidentaloma guidelines, measurement of DHEA-S is advised for assessment of malignancy risk.[79, 89] In addition, radiological findings such as Hounsfield units above 10 without a significant decline during the wash-out phase are suspicious for ACC.[90-91] Radical operative resection is the only clinical prognostic factor known to relate to survival.[84, 92] More recently, transcriptome analysis has revealed new molecular prognostic markers, but their diagnostic value has to be confirmed in prospective studies.[93-95] Mitotane (o,p'DDD) therapy can be given in advanced stages of disease although its efficacy remains controversial,[96] whereas ACC is relatively resistant to conventional chemotherapy. Targeted therapy through monoclonal antibodies directed against the IGF receptor is currently under investigation in clinical trials. In case of unrefractory hormone secretion, steroidogenic enzyme blockers, such as ketoconazole, metyrapone or etomidate[97-98] can be administered.

2.2 Peripheral target tissues

Steroidogenic enzymes have also been detected outside of the gonads, placenta and adrenal cortex. In most cases, this applies to enzymes with 17β-HSD, aromatase or 5α-reductase activity.[99] Through the presence of these enzymes peripheral target tissues can control local sex steroid bio-availability and action. This form of steroid conversion in target cells, also referred to as intracrinology, is crucial for sexual development but also important for hormone-dependent growth in breast cancer and prostate cancer.[100-102]

De novo steroidogenesis was also reported to occur in peripheral tissues. The human skin was even found to possess all functional components of the HPA axis.[103] Recent findings also suggested the presence of *de novo* androgen production in prostate cancer.

2.2.1 The prostate

Prostate cancer (PC) is the most common non-skin cancer in males, affecting an estimated 903.000 men in 2008 worldwide. Despite the indolent nature of PC compared to other prevalent carcinomas, PC-related mortality is still estimated at 258.000 men per year, making it the third most common cause of cancer-related death in men from developed countries.[104]

Since androgens are pivotal for PC development and growth, the cornerstone of PC treatment entails androgen ablation through chemical castration.[105] Blocking the LH secretion through LH-releasing hormone (LHRH) analogues effectively inhibits testicular testosterone production and leads to undetectable serum levels of this androgen. However, after an initial response with disease regression or stabilization, eventually all men will suffer from progression, in a state previously termed hormone independence.[106]

Multiple cellular pathways contribute to this process, of which the AR plays a prominent role. The AR is still present and activated in the majority of these PCs, implying continued androgen dependence.[107-108] These tumors can therefore better be designated as castration-resistant PC (CRPC) instead of hormone- or androgen-independent PC. Furthermore, androgens have been detected in PC samples of castrated patients, consistent with intracrine production of sex steroids.[107, 109-110] Ubiquitous inhibition of P450c17 has resulted in significant anti-tumor effects in patients with CRPC, thereby proving the relevance of continued AR activation as well as offering new medical treatment options for PC.[111]

Recent studies also detected the presence of steroidogenic enzymes required for *de novo* androgen synthesis in patients' CRPC samples and PC cell lines.[112] Liquid chromatography-mass spectrometry tracer studies also showed the capacity of these cells to produce testosterone *de novo* from acetic acid.[113] Whether the contribution of *de novo* steroidogenesis is relevant in comparison to conversion of adrenal androgen precursors, still present in serum of castrated men, remains to be determined.

3 REGULATORS OF STEROIDOGENESIS

Whereas steroid hormone production is mainly influenced by circulating pituitary hormones (ACTH, LH, FSH) or angiotensin II, further fine-tuning of steroidogenesis is accomplished by local growth factors and cytokines.[114-118] Among these local regulators, activins and inhibins, members of the transforming growth factor-β (TGF-β) superfamily of growth and differentiation factors, have been shown to exert effects on multiple components of steroid hormone biosynthesis.[119]

3.1 Activin and inhibin

Inhibin A and B are dimeric peptide hormones that were named after their inhibitory effect on pituitary FSH secretion.[120-121] Both are composed of an inhibin α-subunit (*INHA*) linked to an inhibin βA- (*INHBA*) or βB-subunit (*INHBB*, Figure 5), respectively. *INHA* expression and consequently inhibin formation is confined to classic steroidogenic tissues.[122-123] Circulating inhibin, derived from Sertoli and granulosa cells, acts through antagonism to its counterpart activin (Figure 6).[124-125] Activin is a dimer of inhibin β-subunits, which are widely expressed throughout tissues.[126-127] Activin binds to a tetramer of the activin type II and I receptors, thereby initiating phosphorylation of the type I receptors by the type II receptors.[128] The signal is then relayed to the nucleus by a complex of phosphorylated Smad proteins, which subsequently influences gene transcription (Figure 6).[129] Activin can have profound effects on steroid production in steroidogenic tissues, whereas the

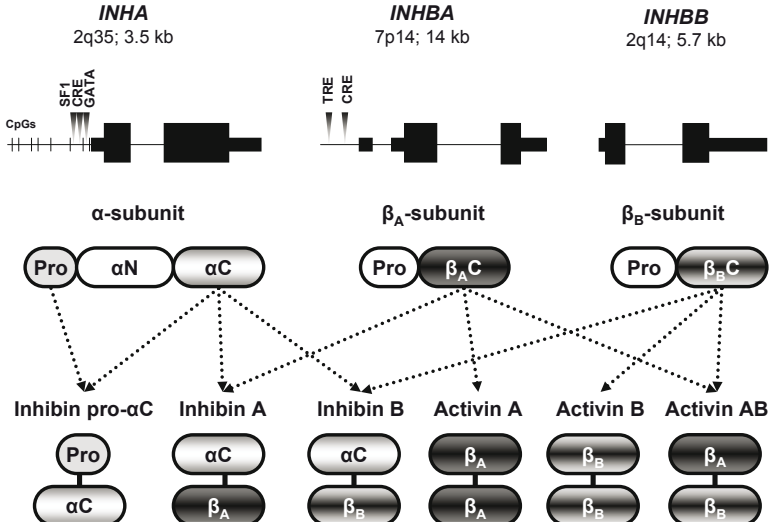
steroidogenic effects of inhibin have been shown to be minimal or absent. Both peptides have also been implicated in tumorigenesis in steroidogenic tissues.[130]

3.2 Activin and inhibin in the adrenal cortex

Incubation of human adrenal cells with activin A has been shown to affect steroidogenesis.[131-134] Activin suppressed *CYP17A1* transcription in a dose- and time-dependent manner. The activin-induced downregulation of *CYP17A1* was accompanied by an impaired production of cortisol and of the adrenal androgens androstenedione, DHEA and DHEA-S.[131-132, 134-135] Above that, activin A induced the expression of *STAR*, *CYP11A1* and *CYP11B2* leading to increased secretion of aldosterone.[133] Inhibin β-subunits and the activin type IB receptor appear to be preferentially expressed at the outer adrenocortical regions, possibly contributing to the regulation of aldosterone production.[131, 136]

The inhibin α-subunit on the other hand is expressed in the zona reticularis, under control of ACTH.[134, 136-137] The effects of inhibin A and B on adrenocortical steroidogenesis are minimal. Ratios between activin A and inhibin A/B protein secretion in primary cultures of adrenal cells did not reflect the relative mRNA levels of inhibin α- and β-subunits detected

Figure 5: Structures of inhibin and activin

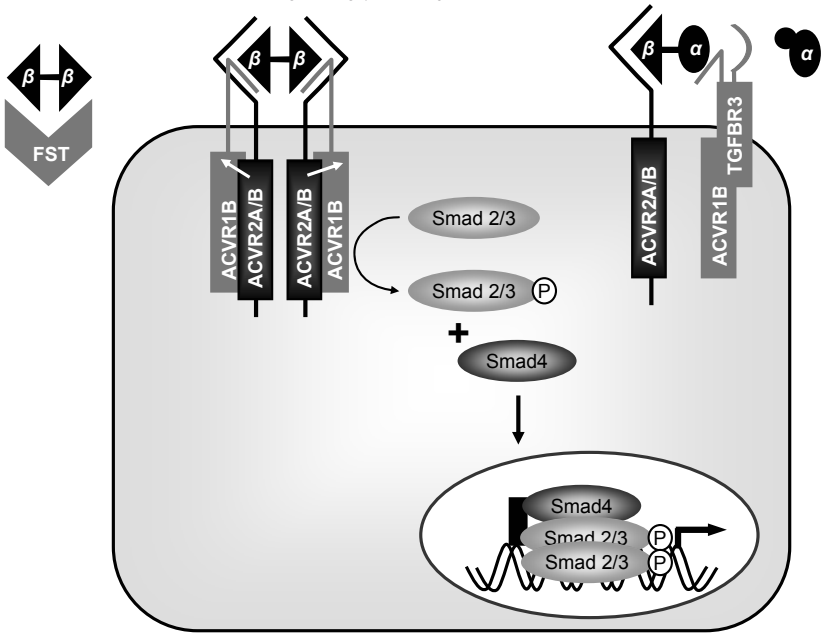


The *INHA* gene codes for the inhibin α-subunit, which is composed of three regions. Expression of the *INHA* gene is strictly controlled by a region of 100-200 bp upstream from the transcription start site, which harbors steroidogenic factor-1 (*SF1*) and GATA binding sites and two cAMP response elements (CRE). The promoter region also contains multiple CpG dinucleotides. The inhibin βA-gene promotor includes a CRE and an activated protein-1 binding site (12-O-tetradecanoylphorbol-13-acetate [TPA]-response element, TRE).
The mature αC region of the α-subunit peptide can be linked with the mature regions of the inhibin β-subunits (either βA or βB) to form inhibin A or B. In the absence of β-subunits, the free inhibin α-subunit or pro-αC is formed. Activins are formed through di-sulfide linkage between mature inhibin β-subunits.

in adrenocortical tissues.[122, 138] Since the subunits show a differential expression pattern in the adrenal cortex, the free α-subunit, also known as inhibin pro-αC,[139] could be the most abundant peptide secreted by the adrenal cortex.

Intriguingly, knockout of *Inha* in mice unequivocally leads to the development of sex-cord stromal tumors.[140] After early gonadectomy 99% of these mice developed adreno-cortical carcinomas instead,[141] implicating the inhibin α-subunit as a tumor suppressor with gonadal and adrenocortical specificity. Absence of inhibin α-subunit protein has been described in a minority of non-functional human ACCs[136, 142] and several mutations in *INHA* have been detected in a selected group of childhood adrenocortical tumors with *TP53* mutations.[143] Surprisingly, *INHA* appears to be overexpressed in another subset of functional ACCs and can even be used as an immunohistochemical marker to distinguish adrenocortical tumors from other neoplasms.[136, 142, 144-146] Due to these conflicting data, the precise role of inhibin in human ACC development and progression is currently unclear.

Figure 6: The activin and inhibin signaling pathway



Activin signals through a tetrameric receptor complex of activin type IB (*ACVR1B*) and II (*ACVRIIA* or *ACVRIIB*) receptors. Phosphorylation of the type IB receptor upon binding of activin recruits intracellular receptor-specific Smad2 or Smad3 proteins. A heteromeric complex of phosphorylated Smad2/3 with common-mediator Smad4 enters the nucleus and binds to Smad response elements in gene promoter regions. Through interaction with other co-activators and co-repressors the activin-induced phosphorylated Smad complex controls gene transcription. Inhibins bind a co-receptor termed betaglycan (*TGFBR3*) and antagonize activin signaling by concurrently occupying the activin type II and type I receptors; no signal arises from this assembly. Follistatin (*FST*) is an extracellular high-affinity activin-binding protein that also prevents activin receptor activation.

3.3 Activin and inhibin in the prostate

Activin is locally expressed within prostatic tissue and PC.[147-148] Activin is involved in the regulation of prostate morphogenesis[149], but its physiological function in the adult prostate remains unknown. Most studies have investigated PC cells, where activin was found to inhibit growth through the induction of apoptosis.[150-151] In contrast, activin also induces expression of the *AR* and increases cell migration in bone metastasis, pleading for an adverse effect on PC progression.[152] Moreover, there is a loss of activin antagonism in PC due to abolished and downregulated expression of the inhibin α-subunit and the inhibin co-receptor betaglycan, respectively.[153-154] Whether activin participates in the progression towards castration-resistance and if it also affects intraprostatic steroidogenesis remains to be investigated.

4 AIMS

Whereas there is a vast expanse of literature on the effects of (biological or synthetic) steroids, little is known of the relevant factors that locally regulate steroidogenic enzyme expression and activity and consequently steroid hormone production. This refers to physiological control of steroidogenesis in the classic steroidogenic tissues and the peripheral target tissues as well as to aberrations of regulatory pathways during tumor development and progression.

The main aim of this thesis is to further elucidate the role of factors involved in fine-tuning of steroidogenesis and the way in which these factors are integrated in complex systems regulated by local and circulating hormones.

The following research aims were defined:

- To unravel regulatory mechanisms of ACTH-sensitivity in health and disease (chapter 2)
- To examine which of the 3β-HSD iso-enzymes is responsible for aldosterone synthesis in the zona glomerulosa (chapter 3)
- To study the regulation and effects of the activin/inhibin system in adrenocortical physiology (chapters 4)
- To compare pituitary and aberrant control of steroidogenesis in ACTH-independent macro- and micronodular hyperplasia (chapters 5 and 6)
- To study the presence of components of the activin/inhibin system in adrenocortical tumors (chapter 7)
- To analyze serum concentrations of inhibin pro-αC (chapter 8) and intratumoral *INHA* (epi-)genetic changes in patients with adrenocortical carcinoma (chapter 9)

- To investigate the occurrence and contribution of *de novo* steroidogenesis versus intracrine conversion of adrenal androgens precursors in (castration-resistant) prostate cancer (chapter 10)
- To study the regulation and effects of the activin/inhibin system in prostate cancer (chapter 11)

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PART I

PHYSIOLOGY OF THE ADRENAL CORTEX

CHAPTER 2

Melanocortin 2 receptor associated protein (MRAP) and MRAP2 in human adrenocortical tissues: regulation of expression and association with ACTH responsiveness

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ABSTRACT

Background:

Adrenocorticotropin (ACTH) stimulates adrenocortical steroid production through the melanocortin 2 receptor (MC2R). MC2R trafficking and signaling are dependent on the melanocortin 2 receptor accessory protein (MRAP). The MRAP-homolog MRAP2 also transports the MC2R to the cell surface but might prevent activation. We studied regulatory pathways of *MRAP* and *MRAP2* and their contributions to ACTH responsiveness in human adrenal tissues.

Methods:

Tissue expression levels of *MRAP*, *MRAP2* and *MC2R* were studied in 32 human adrenocortical samples. Regulation of these mRNAs was investigated in 43 primary adrenal cultures, stimulated with ACTH, forskolin, angiotensin(Ang)II, phorbol-12-myristate-13-acetate (PMA) or dexamethasone. Induction of cortisol, cAMP and ACTH-responsive genes following treatment with ACTH was related to *MRAP*, *MRAP2* and *MC2R* expression levels.

Results:

MRAP and *MRAP2* levels were lower in adrenocortical carcinomas (ACCs) than in other adrenal tissues ($P<0.001$). Patient ACTH and cortisol levels were associated with adrenal levels of *MRAP* and *MC2R* in adrenal hyperplasia samples ($P<0.05$), but not in tumors. ACTH induced expression of *MRAP* 11±2.1-fold and *MC2R* 20±3.8-fold in all adrenal tissue types (mean±SEM, both $P<0.0001$), whereas AngII augmented these mRNAs 4.0±1.2-fold and 12.6±3.2-fold ($P<0.0001$) in all but ACCs. *MRAP2* expression was suppressed by forskolin (-24%, $P=0.013$) and PMA (-22%, $P=0.0007$). There was no association between levels of *MRAP*, *MRAP2* or *MC2R* and ACTH sensitivity *in vitro*, measured by the induction of cortisol, cAMP or gene expression.

Conclusions:

MRAP and *MC2R* expression is induced by ACTH and AngII, which would facilitate cell surface receptor availability. Physiological expression levels of *MRAP*, *MRAP2* and *MC2R* were not limiting for ACTH sensitivity *in vitro*.

INTRODUCTION

The hypothalamic-pituitary-adrenal axis is essential for adaptation to internal and external stressors.[1] Adrenal glucocorticoid production is controlled by adrenocorticotropin (ACTH). Circulating ACTH binds to the $G\alpha_s$ protein-coupled melanocortin 2 receptor (MC2R) in the adrenal cortex, leading to the formation of cAMP and the activation of protein kinase A (PKA). This in turn induces rapid phosphorylation of the steroid acute regulatory protein (StAR) that facilitates transport of cholesterol into the mitochondria for conversion into active steroid hormones.[2] In addition, ACTH induces the transcription of multiple steroidogenic enzymes and thus ensures short- and long-term stimulation of steroidogenesis.[3-4]

The MC2R is the smallest G protein-coupled receptor known to date and belongs to a family of melanocortin receptors (types 1 to 5) that bind to various derivatives of proopiomelanocortin (POMC), especially α-MSH.[5] ACTH stimulates *MC2R* expression in the long-term,[6-7] but also acutely decreases MC2R presence at the cell surface by causing its internalization.[8] Mutations in *MC2R* lead to familial glucocorticoid deficiency (FGD), a potentially lethal syndrome characterized by undetectable serum cortisol levels combined with highly elevated ACTH levels and ACTH unresponsiveness.[9] Only about 25% of FGD is caused by mutations in the *MC2R* gene, suggesting that additional mechanisms are involved in ACTH signaling.

A family segregation study in FGD patients revealed that mutations in a gene termed melanocortin 2 receptor associated protein (*MRAP*) could also cause abrogated ACTH signaling.[10] MRAP was found to be a MC2R-trafficking protein crucial for the translocation of the receptor from the endoplasmatic reticulum to the cell surface.[10] Moreover, MRAP facilitated signaling of the MC2R.[11-13] Loss of function of MRAP thus prevents membrane expression of MC2R and completely prevents ACTH signaling. Interestingly, MRAP forms a unique anti-parallel homodimer in close proximity to the MC2R.[14-15] The accessory protein can also interact with other melanocortin receptors, particularly MC5R, but exerts negative effects on their signaling.[16-17] Expression of *MRAP* was recently shown to be predominantly present in the zona fasciculata in the rat adrenal gland,[18] consistent with its facilitating role in glucocorticoid production. mRNA levels of *MRAP* were found to be upregulated by ACTH and cAMP in murine Y1 adrenocortical cells[19] and normal human adrenal cells.[4]

MRAP2, a protein with 39% amino acid homology to MRAP, was found to share the MC2R-trafficking function.[17] Since MRAP2 is not capable of rescuing ACTH signaling in FGD patients with *MRAP* mutations,[10] MRAP2 does not appear to play a major supportive role in adrenocortical ACTH signaling. On the contrary, *MRAP2* overexpression caused suppression of MC2R activation and positive effects on signaling have only been detected at supraphysiological levels of ACTH.[18-19] However, it is unclear if these effects observed *in*

vitro might have functional consequences *in vivo*. Furthermore, although it is now known that the expression of *MRAP2* is restricted to the adrenal gland and brain tissue,[17] the factors that regulate *MRAP2* expression remain to be determined.

Adrenocortical tumors have an altered responsiveness to ACTH *in vivo*, which could partly be explained by modified expression levels of the MC2R.[20-24] It is also possible that MRAP and MRAP2 modulate ACTH responsiveness in the adrenal, and that this control mechanism is dysregulated in adrenal tumors. For this reason, we have studied the expression of *MC2R*, *MRAP* and *MRAP2* in both normal and pathologic human adrenal tissues and determined the ACTH responsiveness of primary cells from these tissues *in vitro*. *MRAP* and *MC2R* levels were found to be potently stimulated by ACTH and AngII, whereas fluctuations in expression levels of the (co-)receptors were not related to ACTH sensitivity in these cells. Furthermore, we detected a dysregulation of MRAPs in adrenal tumors.

MATERIALS AND METHODS

Tissue collection

Samples of adrenal tissues were obtained from patients operated between 2007 and 2011. Normal adrenal samples were obtained at nephrectomy due to renal cell carcinoma or adrenalectomy due to adrenal cyst. Samples of hyperplastic adrenal tissues were collected at bi-adrenalectomy because of incurable Cushing's disease or ectopic ACTH secretion or at adrenalectomy because of ACTH-independent macronodular adrenal hyperplasia (AIMAH).[25] Tissue samples of adrenocortical adenomas and carcinomas were also obtained following adrenalectomy. Adrenocortical carcinomas were diagnosed as such if the van Slooten index exceeded 8 during pathological evaluation.[26] This study was approved by the Medical Ethics Committee of the Erasmus MC and all patients gave written informed consent. Fasting cortisol and ACTH levels, cortisol after 1 mg dexamethasone overnight screening test (DST) and 24 hour cortisoluria were measured during routine clinical diagnostic evaluation by chemiluminescence-based immunoassays (Immulite 2000, Siemens, Deerfield, IL, USA).

Cell culture

Adrenal or intratumoral samples were dissected shortly after resection. Parts of the samples were stored in TissueTek and kept at -80 °C until the isolation of RNA. If sufficient material was available, the tissues were minced into small pieces and taken up in DMEM/F12 medium containing 5% fetal calf serum (FCS) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) for the development of primary cell cultures as previously described.[27] In short, minced tissue was washed twice with culture medium before

incubation with 2.5 mg/ml type 1 collagenase (Sigma-Aldrich, St. Louis, MO, USA) for two hours at 37 °C. After obtaining single cell suspensions, cells were washed and separated from cellular debris by centrifugation through a Ficoll gradient. Thereafter, lipid-laden cells were counted and plated in 24-well plates at 100,000 cells per well.

After attachment for at least 24 hours in medium containing 5% FCS, media were replaced with serum free medium. The next day, $ACTH_{1-24}$ (10 ng/ml, Novartis, Basel, Switzerland), the PKA stimulator forskolin (FSK, 10 μM), angiotensin II (AngII, 100 nM), the protein kinase C (PKC) stimulator phorbol-12-myristate-13-acetate (PMA, 5 nM) or dexamethasone (1 μM, all Sigma-Aldrich) were added in culture medium to quadruplicate wells. The supernatants were removed after an incubation period of 48 hours and the plates were snap-frozen on dry ice and stored at -80 °C until further processing. Supernatant cortisol and cAMP levels were measured by Immulite and radioimmunoassay (Beckman Coulter, Woerden, The Netherlands), respectively.

mRNA measurements

Hematoxylin and eosin-stained slides of frozen tissue samples were checked for tissue composition and the presence of extensive necrosis or fibrosis. Representative viable tissue sections were cut by microtome and used for RNA isolation. Isolations from frozen tissue and plated cells were performed with TriPure reagent (Roche, Penzberg, Germany). After RNA quantification by spectrophotometry, cDNA was created from 1 μg of RNA by reverse transcription using Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, Leiden, the Netherlands) as reported before.[28] The equivalent of 20 ng of RNA was used in a quantitative polymerase chain reaction (PCR) for the detection of hypoxanthine phosphoribosyltransferase 1 (*HPRT1)*, *MC2R*, *MRAP*, *MRAP2*, cytochrome P450 11β-hydroxylase (*CYP11B1)*, cytochrome P450 17-hydroxylase (*CYP17A1)*, inhibin α-subunit (*INHA)* and cytochrome P450 21-hydroxylase (*CYP21A2)* mRNAs in duplicate. *MRAP* (Hs01588793_m1, which measures both known transcript splice variants), *MRAP2* (Hs00536621_m1) and *CYP21A2* (Hs00416901_g1) assays were purchased from Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). A FAM-TAMRA duo-labeled probe was used for the detection of *HPRT1*, *CYP11B1*, *CYP17A1* and *INHA* (methods and sequences in [28-29]), whereas FastStart Universal SYBR green master mix (Roche) was used for the *MC2R* assay (forward primer: CCCAGAAAGTTCCTGCTTCA, reverse: TCTTCAGGATCTTTTCTTCCTTG). The expression levels of the housekeeping gene *HPRT1* were not affected by incubation with any of the secretagogues. Expression was calculated relative to that of *HPRT1* using the ΔCt method.

Statistics

All statistical analyses were performed in GraphPad Prism (GraphPad software, La Jolla, CA). mRNA expression levels were log-converted before analysis. Analysis was performed by t-test or one-way analysis of variance, followed by Tukey's multiple comparison test. Associations were analyzed by Pearson's correlation coefficient and linear regression. All tests were calculated as two-tailed and significance was assumed at a P-value below 0.05.

RESULTS

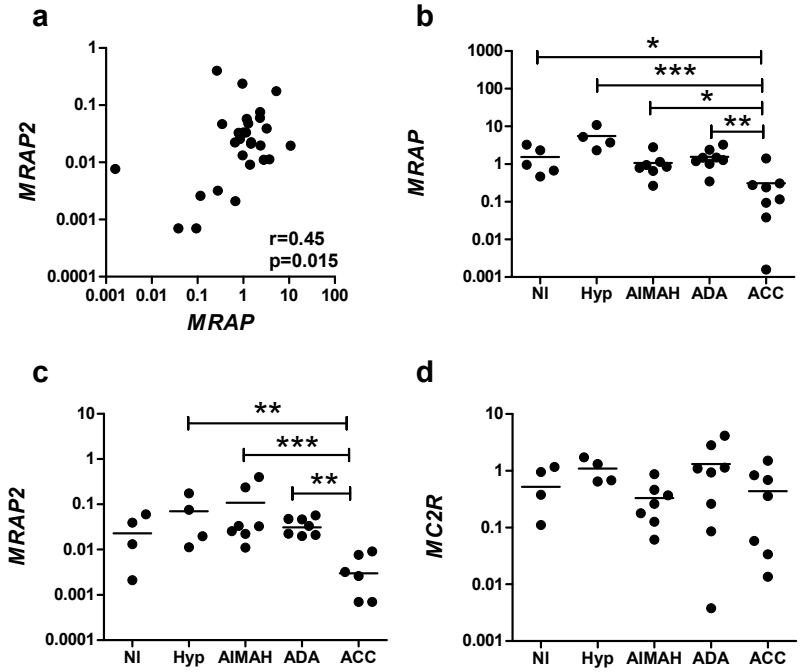
MRAPs and MC2R in patient samples

Basal levels of *MRAP*, *MRAP2* and *MC2R* mRNAs were studied in tissue samples of normal adrenal glands (n=5), ACTH-dependent hyperplasia (n=4), AIMAH (n=7), adrenocortical adenomas (ADA, n=8) and carcinomas (ACC, n=8). Of the adenomas, four tumors secreted cortisol (one concomitantly with aldosterone), one aldosterone and two sex steroids, whereas one adenoma was non-functional. The carcinomas produced cortisol in one patient, sex steroids in another and a combination of both in four patients, whereas the other two ACCs were non-functional.

Overall, *MRAP* expression levels (Ct range 24-36) exceeded those of *MRAP2* (range 31->40) 95±24-fold (relative to *HPRT1*, mean±SEM), although the expression levels of these mRNAs were correlated within individual samples (r=0.45, P=0.015, Figure 1a). *MRAP* mRNA expression levels in ACTH-dependent adrenal hyperplasia, AIMAH and adrenal adenomas were not significantly different from levels in normal adrenal tissues. The main finding was that *MRAP* and *MRAP2* gene expression levels were uniformly suppressed to near undetectable levels in ACC samples compared with normal and the other pathologic samples (Figures 1b and c, P<0.001), whereas mean *MC2R* expression was not significantly altered (Figure 1d).

Patient serum steroid and plasma ACTH levels were not available for the normal adrenal samples. When analyzing the other adrenal samples, only *MRAP* expression was significantly correlated with plasma ACTH levels in all patients prior to operation (r=0.42, P=0.039, data not shown). There was no association between adrenal expression levels of *MRAP*, *MRAP2* or *MC2R* and fasting cortisol, cortisol after DST or 24 hour cortisoluria. However, when analyzing the hyperplasia subgroups (ACTH-dependent and –independent) separately there were clear associations between *MRAP* expression and fasting cortisol (r=0.79, P=0.0036) and ACTH levels (r=0.69, P=0.020, Figure 2 upper panel). *MC2R* levels were also correlated with fasting cortisol (r=0.66, P=0.026), cortisol after DST (r=0.85, P=0.0037) and ACTH levels (r=0.67, P=0.025), but only in the combined hyperplasia group. There was no association between the levels of *MRAP*, *MRAP2* or *MC2R* expression with serum cortisol or plasma ACTH levels in the adrenal adenoma and

Figure 1: Basal expression levels of *MRAP*, *MRAP2* and *MC2R* in 32 human adrenal samples



(a) Association between *MRAP* and *MRAP2* levels in tissues of adrenal origin. Quantitative mRNA levels of *MRAP* (b), *MRAP2* (c) and *MC2R* (d) in patient samples of normal adrenal glands (NI), ACTH-dependent hyperplasia (Hyp), ACTH-independent macronodular adrenal hyperplasia (AIMAH), adrenocortical adenomas (ADA) and carcinomas (ACC). *MRAP2* and *MC2R* expression levels were not detectable in four and two tissue samples, respectively, and are not shown because of the logarithmic scale. Levels are expressed as arbitrary units, relative to *HPRT1*. *P<0.05, **P<0.01, ***P<0.001

carcinoma samples (Figure 2, lower panel). Similarly, expression levels of *MRAP*, *MRAP2* or *MC2R* did not relate to clinical steroid secretion of the adrenocortical tumors (aldosterone, cortisol or sex steroids, data not shown).

Regulation of *MRAP, MRAP2* and *MC2R* expression

We successfully developed primary cell cultures from 43 adrenocortical samples of varying pathological entities. Since previous studies revealed that *MRAP* and *MC2R* expression in Y1 and human normal adrenal cells were stimulated by ACTH,[4, 19] we studied regulation of expression of *MRAP*, *MRAP2* and *MC2R* in these cultures by ACTH as well as by AngII and dexamethasone. Furthermore, direct adenylyl cyclase and PKC stimulation was performed by the addition of FSK and PMA, respectively.

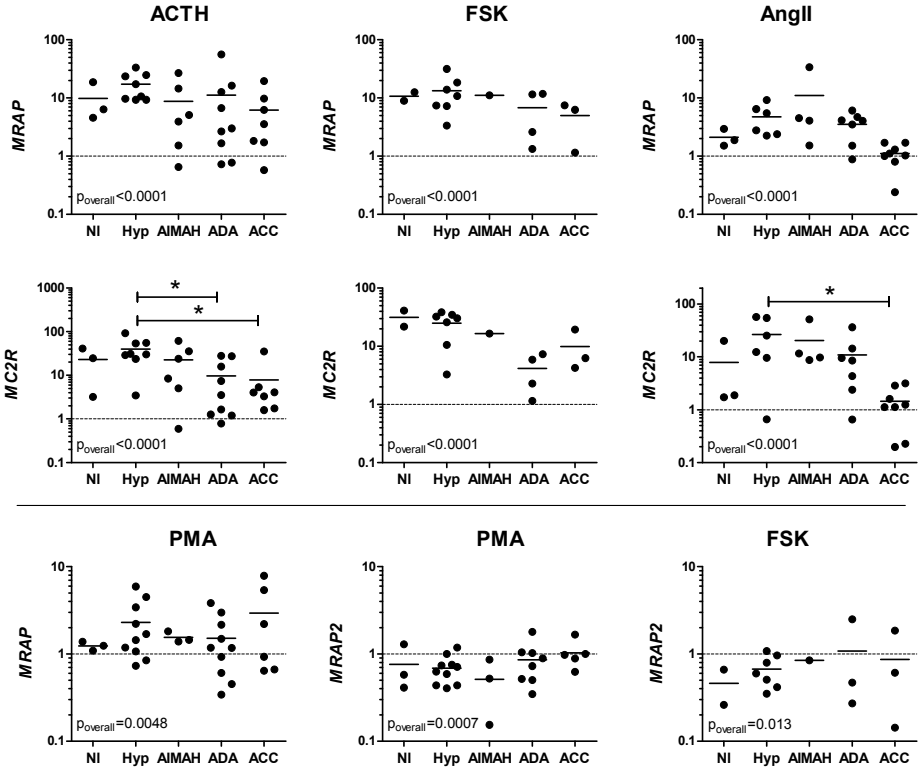
ACTH and FSK both significantly stimulated expression of *MRAP* and *MC2R* in primary cultures (Figure 3, P<0.0001), while FSK suppressed *MRAP2* expression to 0.76±0.15-fold (mean±SEM, P=0.013). ACTH stimulated *MRAP* 11±2.1-fold without differences between the various tissue types. *MC2R* expression was increased 20±3.8-fold following the ad-

Figure 2: Associations between adrenal *MRAP* and *MC2R* expression and patient ACTH and cortisol levels

Upper panel: In patients with either ACTH-dependent or ACTH-independent hyperplasia, there were significant correlations between *MRAP* levels and fasting cortisol and ACTH levels. *MC2R* levels were also associated with patient fasting cortisol and ACTH levels as well as cortisol levels after DST in these samples. Lower panel: The strong associations found in hyperplasia samples did not apply to adrenocortical adenomas and carcinomas. A.U.: arbitrary units, relative to *HPRT1*.

dition of ACTH; this induction was larger in ACTH-dependent hyperplasia, compared to that in adrenocortical adenomas and carcinomas (both P<0.05, Figure 3). AngII also stimulated *MRAP* and *MC2R* expression to 4.0±1.2-fold (P<0.0001) and 12.6±3.2-fold (P<0.0001, Figure 3), respectively. This inductive effect was apparent in all groups with the exception of ACCs. Addition of PMA to cultures of adrenocortical cells stimulated *MRAP* expression to 2.0±0.3-fold (P=0.005) and concurrently decreased expression of *MRAP2* 0.78±0.07-fold (P=0.0007, Figure 3), while having no effect on *MC2R* transcription (data not shown, P>0.05). Dexamethasone incubation in a subset of samples (n=7) did not influence expression levels of *MRAP*, *MRAP2* or *MC2R* (data not shown, P>0.05).

Figure 3: Regulation of *MRAP*, *MRAP2* and *MC2R* expression in primary adrenocortical cell cultures



Quantitative mRNA expression of components of the MC2R receptor complex was studied in adrenal cultures of normal adrenal glands (NI), ACTH-dependent hyperplasia (Hyp), ACTH-independent macronodular adrenal hyperplasia (AIMAH), adrenocortical adenomas (ADA) and carcinomas (ACC). The induction or inhibition following 48 hours incubation with 10 ng/ml ACTH, 10 μM FSK, 100 nM AngII or 5 nM PMA is plotted, stratified by type of adrenal tissue. Values are relative to control condition, bar represent mean. $P_{overall}$ represents paired t-tests on the difference in expression values of all groups combined between the treatment and the control condition. Differences in induction levels between types of tissues were analyzed by ANOVA, followed by Tukey's multiple comparison tests, and plotted in the figure, if significant. *P<0.05.

MRAPs, MC2R and ACTH responsiveness

The AIMAH patients all underwent *in vivo* testing for hormonal stimuli according to Lacroix *et al.*[30] We found no association between the maximal increase in serum cortisol following 250 µg $ACTH_{1-24}$ intravenously and adrenal levels of *MRAP*, *MRAP2* or *MC2R* mRNAs in these 7 patients (data not shown).

In vitro cortisol levels after the 48 hour incubation period were detectable in 21/43 (49%) primary cultures. Overall, ACTH stimulated supernatant cortisol levels 5.4±0.64-fold (P=0.0081). The induction of cortisol following the addition of ACTH was not significantly associated with basal expression levels of *MRAP*, *MRAP2* or *MC2R* (P>0.05, data not shown). Supernatant cAMP levels were also measured in a subset of cultures. These levels were undetectable after 48 hours in 67% (10 out of 15) of cultures in unstimulated conditions and 33% (5/15) of cultures following ACTH treatment (data not shown). There was no relation between the expression levels of *MRAP*, *MRAP2* or *MC2R* and the ACTH-induced cAMP levels in the supernatant of the 10 adrenal cell cultures with detectable cAMP (P>0.05, data not shown).

CYP11B1, *CYP17A1*, *CYP21A2, INHA*, and *MRAP* are the five most differentially ACTH-regulated genes in adult adrenocortical cells.[4] The proteins of the first three genes are key steroidogenic enzymes of cortisol production, whereas the inhibin α-subunit is presumably involved in adrenocortical cell proliferation and can serve as a tumor marker for ACC.[31-32] We measured the induction of the above mentioned genes by ACTH in a variety of adrenocortical primary cultures as an indicator of ACTH responsiveness. Average induction of *CYP11B1*, *CYP17A1*, *INHA*, *CYP21A2* and *MRAP* by ACTH after 48 hours was 43±26-fold (mean±SEM), 10±2.1-fold, 25±8.6-fold, 14±4.2-fold and 11±2.0-fold, respectively. Regression analysis uncovered no associations between the unstimulated levels of *MRAP*, *MRAP2* or *MC2R* and the induction of any of these five genes or the combination thereof (P>0.05, data not shown).

Nine primary cell cultures (1 normal, 3 hyperplasia, 1 AIMAH, 2 adenomas and 2 carcinomas) were separately incubated with both ACTH and FSK. We calculated the ACTH-induced stimulation of gene expression relative to that by FSK as a measure of MC2R-related signaling potential. When comparing these ratios to the expression levels of the MC2R-MRAP complex we found a negative correlation between *MRAP2* and the ACTH/FSK-induction ratio of *CYP21A2* (r=-0.70, P=0.036), but this failed to reach significance following Bonferroni correction for the five genes tested (0.05/5). The average ratio of all five genes studied in these nine samples was not associated with *MRAP2* expression (r=-0.46, P>0.05). *MRAP* and *MC2R* expression levels were also not associated with the ACTH/FSK induction ratio.

DISCUSSION

ACTH is the principal regulator of adrenal cortisol production and signals through the MC2R in a cAMP/PKA-dependent pathway. The discovery of the MC2R accessory proteins has uncovered new insights into G protein-coupled signaling. Adequate MRAP expression is obligatory for cell surface localization and activation of the MC2R,[10-11] whereas MRAP2 appears to inhibit ACTH signaling.[19] Most studies on this subject have used overexpression systems in models devoid of endogenous *MC2R* or *MRAP* expression or mouse Y1 cells.[10-17, 33] The role and effects of these accessory proteins in human primary adrenal disease have not been explored thus far, partly because of the lack of a suitable antibody to the co-receptors. Furthermore, regulation of endogenous levels of these proteins remains largely unknown. We now show that *MRAP*, concurrent with *MC2R*, is positively regulated by ACTH and AngII in human adrenal tissue and that adrenal *MRAP* and *MC2R* levels are correlated with high ACTH and cortisol production states in patients with ACTH-dependent and ACTH-independent adrenal hyperplasia. No clear relationship was found between physiological levels of *MRAP, MRAP2* or *MC2R* and ACTH responsiveness in adrenal cells.

ACTH binding to the MC2R induces a rapid conformational change in the MC2R-MRAP complex and leads to the activation of adenylyl cyclase.[14] Within minutes after the binding of ACTH, the MC2R is internalized to endocytic vesicles through a clathrin-dependent pathway.[8] In that manner, ACTH decreases cell surface expression of its receptor and thus ACTH responsiveness.[8] By increasing the transcription of *MC2R* and its accessory protein *MRAP* ACTH stimulates expression of the MC2R-MRAP complex at the plasma membrane and would be expected to improve signaling in its target tissue, the adrenal cortex.[7] Moreover, the absence of concomitant stimulation of *MRAP2* by ACTH, or even suppression of *MRAP2* expression as seen following direct adenylyl cyclase stimulation by FSK, would prevent the additional formation of MRAP2-MC2R complexes that signal poorly in response to ACTH.[18-19] Furthermore, *MRAP* mRNA expression in the adrenal cortex markedly exceeds that of *MRAP2*. Although mRNA expression levels are not uniformly representative of protein levels, this excess would predispose to the formation of functional MC2R-MRAP complexes.

Consistent with their reduced responsiveness to ACTH, adrenocortical carcinomas showed lower levels of *MRAP* mRNA compared to all other types of adrenal tissue. The ACTH-dependent hyperplasia samples, which had been chronically stimulated by ACTH *in vivo*, showed the highest expression levels of *MRAP*. Interestingly, the ACTH responsiveness, as measured by the induction of *MC2R* by ACTH, was higher in adrenal hyperplasia, compared to adenomas and carcinomas. Adrenocortical carcinomas have an impairment in their cAMP/PKA pathway due to decreased expression of cAMP response element-binding protein (CREB) and inducible cAMP early repressor isoforms[34-35] which could contribute to a reduced stimulation of *MRAP* following ACTH. The lower *MRAP* levels in ACCs

could be expected to decrease ACTH responsiveness, but since we found no relation between mRNA expression of *MRAP* and ACTH responsiveness, this remains speculative. Surprisingly, *MC2R* expression in ACCs was comparable to that in other adrenal tissues, whereas a previous study, with a larger sample size, detected lower *MC2R* mRNA levels in ACC by Northern blot.[20] Our findings suggest that ACCs show a divergent regulatory control of *MRAP* and *MC2R* expression. *In situ* hibridization and immunohistochemistry studies could provide further insight into *MRAP, MRAP2* and *MC2R* presence and function in adrenal tumors.

Correlation analysis between clinical data and adrenal *MRAP* and *MC2R* levels revealed associations between ACTH and cortisol production with *MRAP* and *MC2R* levels, but only in patients with adrenal hyperplasia. The increased cortisol secretion is a result of the elevated ACTH levels and in AIMAH of other hormonal factors that stimulate G protein-coupled receptors and cAMP formation.[25] The relationship between cortisol and ACTH levels and adrenal mRNA levels indicate that ACTH/PKA is also a major regulator of *MRAP* and *MC2R* transcription in adrenal hyperplasia *in vivo*. This was recently also observed in patients with Cushing's disease who showed decreased sensitivity to exogenous ACTH in the first week after successful surgical resection of ACTH-producing pituitary adenomas.[36] In adrenocortical tumors however, there appears to be an uncoupling between control of steroidogenesis and MRAP-MC2R levels.

The main stimulator of aldosterone production, AngII, also increased the expression of *MC2R* and *MRAP*. This confirms that AngII increases ACTH responsiveness in AngII type 1 receptor (AT1R)-positive cells.[7] Since ACTH is responsible for approximately 10% of aldosterone production, this could be an important physiological link between AngII- and ACTH-controlled mineralocorticoid production. The ACCs showed no response to AngII, consistent with the absent or minimal AT1R levels present in these tumors.[37] AngII-induced *MRAP* expression could be PKC-dependent since the addition of PMA showed a similar effect. PMA did not increase *MC2R* expression, possibly linking the induction of *MC2R* by AngII to the Ca^{2+}-dependent pathway of AT1R-dependent signaling. This regulatory mechanism could add to the differential expression of the receptor and its accessory protein, as stated above.

On the other hand, adrenal *MRAP2* expression was not found to be affected by AngII. PMA did reduce *MRAP2* expression, implying that other AT1R downstream pathways such as the Ca^{2+}-dependent pathway simultaneously inhibit *MRAP2* transcription following AngII signaling. *MRAP2* levels were also decreased in ACC and correlated with the levels of *MRAP*. The decreased *MRAP2* expression could be speculated to result from the tumor formation itself or factors overexpressed in ACC, such as IGF-II.[38]

The effects of ACTH, i.e. increased steroidogenesis, would be expected to be dependent on the expression levels of components of the MC2R complex. However, we were unable to find a direct relationship between expression levels of *MRAP*, *MRAP2* or *MC2R* with *in*

vivo or *in vitro* induction of cortisol, cAMP or ACTH-responsive gene expression following the administration of ACTH. Physiological mRNA levels of *MRAP* and *MC2R* were thus not limiting for the ACTH effect. For *MRAP* this was previously also found in Y1 cells, in which overexpression of *MRAP* did not increase the ACTH-induced cAMP production over that of endogenous levels of MRAP.[19]

When the ACTH response was corrected for maximum possible cAMP (FSK) response of the cells, an inverse association between ACTH responsiveness of *CYP21A2* expression and *MRAP2* was uncovered in adrenal cells *in vitro,* but this failed to reach statistical significance after Bonferroni correction. Combined with the absence of associations of *MRAP2* levels with the ACTH-induced stimulation of cortisol, cAMP or the other gene expression levels studied, *MRAP2* levels within the physiological range also do not appear to inhibit ACTH responsiveness *in vitro*. Although it has been shown that overexpression of *MRAP2* suppressed ACTH signaling via the MC2R in one study,[19] which was not confirmed in two other reports,[17-18] the low levels of *MRAP2* currently encountered in various human adrenal tissues do not negatively affect ACTH sensitivity.

In conclusion, we found that *MRAP* and *MC2R* are positively regulated by ACTH and AngII in human adrenocortical tissues. *In vivo* cortisol and ACTH levels were associated with adrenal levels of *MRAP* and *MC2R*, consistent with their regulation *ex vivo*. We found no association between the ACTH-induced stimulation of cortisol, cAMP or ACTH-responsive genes and expression levels of *MRAP*, *MRAP2* or *MC2R*, suggesting that physiological levels of the ACTH (co-)receptors are not limiting for ACTH responsiveness in adrenocortical cells.

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CHAPTER 3

Expression and gene variation studies deny association of human 3β-hydroxysteroid dehydrogenase type 1 gene (*HSD3B1*) with aldosterone production or blood pressure

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ABSTRACT

Background:

Recent evidence suggests that type I 3β-hydroxysteroid dehydrogenase (3β-HSD), encoded by *HSD3B1*, could be involved in aldosterone production and that genetic variation in *HSD3B1* is associated with blood pressure. In the current study, we studied whether *HSD3B1* is expressed in the human adrenal cortex and affects the risk of hypertension.

Methods:

Expression of *HSD3B1* and *HSD3B2* was investigated in various adrenocortical tissues (n=15) and in primary adrenal cell cultures (n=5) following the stimulation with adrenocorticotropin and angiotensin II (AngII). Six tagging SNPs within the *HSD3B1* gene were studied for association with blood pressure and hypertension in a meta-analysis of four Dutch cohorts (n=9814).

Results:

HSD3B1 expression was minimal or absent in adrenocortical tissues, including 6 aldosterone-producing adenomas. In contrast to the ubiquitously expressed *HSD3B2* mRNA, *HSD3B1* levels were not stimulated by adrenocorticotropin or AngII. No variants in the *HSD3B1* gene were associated with blood pressure or the occurrence of hypertension.

Conclusions:

We found no evidence that *HSD3B1* is involved in aldosterone synthesis in the human adrenal cortex or that genetic variation in *HSD3B1* affects blood pressure or hypertension, indicating that all adrenocortical steroidogenesis is dependent on the type II 3β-HSD.

INTRODUCTION

The renin-angiotensin-aldosterone system (RAAS) is an important regulator of blood pressure. Angiotensin II (AngII) binds to its type 1 receptor in the zona glomerulosa (ZG) of the adrenal cortex, leading to the stimulation of aldosterone production.[1] The mineralocorticoid aldosterone controls blood pressure primarily by increasing sodium reabsorption in the distal convoluted tube and collecting duct of the nephron.[2]

Aldosterone is produced in the adrenal cortex from cholesterol through sequential steroidogenic enzymatic reactions.[3] First, cholesterol is transformed into pregnenolone by cytochrome P450 (CYP) side chain cleavage. The 3β-hydroxysteroid dehydrogenase (3β-HSD)/Δ^5-Δ^4 isomerase enzymes subsequently produce progesterone from pregnenolone through oxidation and isomerization. Aldosterone is formed from progesterone by four additional reactions, involving hydroxylation at carbon atoms 21, 11 and 18, respectively, followed by oxidation of the C18 hydroxyl group. The latter three reactions are catalyzed by the CYP11B2 enzyme, expression of which is limited to the ZG.[4] Steroidogenesis in the other adrenocortical zones and gonads is diverted towards glucocorticoids and sex steroids by 17-hydroxylase/17,20-lyase, encoded by *CYP17A1,* and also requires the 3β-HSD enzymes.

The human genome contains two 3β-HSD enzymes that share 94% sequence homology: type I (*HSD3B1*) and type II (*HSD3B2*).[5] The type II enzyme was considered responsible for all adrenocortical and gonadal steroid production, whereas *HSD3B1* was thought to be expressed in the placenta and in peripheral tissues, but not in the adrenal cortex.[6] However, recent developments have indicated that the type I 3β-HSD might be the enzyme leading to aldosterone formation in the ZG. First, it was shown that increased expression of type VI 3β-HSD caused hypertension in circadian clock-deficient *Cry*-null mice through stimulated production of aldosterone in the murine ZG.[7] Murine *Hsd3b6* was linked through sequence homology to human *HSD3B1*, expression of which was subsequently shown to be enriched in the human ZG. *HSD3B2* expression on the other hand was relatively low in ZG cells.[7]

Secondly, common variants and mutations in *HSD3B1* have been associated with blood pressure increase and essential hypertension in humans.[8-10] Although these genetic studies were all performed in relatively small groups of hypertensive subjects, these findings suggested that *HSD3B1* instead of *HSD3B2* is responsible for mineralocorticoid production. On the other hand, two large genome wide association (GWA) studies on blood pressure and hypertension showed associations between common variants in the *CYP17A1* gene and high blood pressure,[11-12] suggesting that these variants lead to mild forms of enzyme deficiency.[13]

In order to investigate whether *HSD3B1* plays an important role in aldosterone production in the human ZG, we studied expression levels of both 3β-HSD enzymes in human

adrenocortical tissues as well as genetic associations between *HSD3B1* and blood pressure in large study cohorts of Caucasian origin.

MATERIALS & METHODS

RNA analysis

Patient material

Tissue samples were collected from patients who underwent adrenalectomy at the Erasmus Medical Center, between 1994 and 2009. Samples from normal adrenal glands were obtained from radical nephrectomies due to renal cell carcinoma (n=9). Adrenocortical tumor samples were collected from patients after adrenalectomy because of Conn's syndrome (n=7), Cushing's syndrome (n=2) or suspicion of pheochromocytoma (n=1). For measurement of RNA representative tissue samples were snap-frozen and stored at -80 °C until further processing. For primary culture purposes, adrenal tissues were taken up in DMEM-F12 culture medium containing 5% fetal calf serum (FCS, Invitrogen, Carlsbad, CA, USA).

This study was approved by the Medical Ethics Committee of the Erasmus Medical Center and informed consent was obtained from all patients.

Primary culture

Isolated adrenocortical cells were obtained by treating the tissue samples with type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) and removing debris by centrifugation through a Ficoll gradient.[14] Cell viability and type were checked by microscopical evaluation with trypan blue. Lipid-laden cells were identified as adrenocortical cells and plated at a density of 100.000 cells per well in DMEM-F12 containing 5% FCS and allowed to attach overnight. The next day medium was changed to serum free and 24 hours later cells were incubated with vehicle, 10 ng/ml $ACTH_{1\text{-}24}$ (Novartis, Basel, Switzerland) or 100 nM AngII (Sigma). After 48 hours of incubation the supernatant was removed from the cells and plates were frozen on dry ice and stored at -80 °C.

Steroidogenic enzyme measurement

Total RNA was isolated from frozen tumor tissue and plated cells using Trizol reagent (Invitrogen). Subsequently, reverse transcription reactions were performed as previously described.[15] Twenty ng of RNA was used in duplicate in quantitative polymerase chain reaction (qPCR) for *HPRT1*, *HSD3B1*, *HSD3B2*, *CYP11B1* and *CYP11B2*. Primer and dual-labeled probe sequences and qPCR have been reported previously.[16] Assays displayed no cross-reactivity with the homologous DNA sequences in related genes. Positive controls

consisted of normal adrenal gland (*CYP11B1* and *HSD3B2*), Conn adenoma (*CYP11B2*) and placenta (*HSD3B1*) and yielded threshold cycles (Ct) below 25.

DNA analysis

Study populations

The Rotterdam Study I (RS-I), Rotterdam Study II (RS-II) and Rotterdam Study III (RS-III) are prospective population-based cohort studies. The RS-I comprises 7,983 subjects aged 55 years or older. Participants completed an interview at home and at the research centre, where participants were subsequently examined. Baseline data were collected between 1990 and 1993. In 1999, inhabitants who turned 55 years of age or moved into the study district since the start of the study were invited to participate in an extension of the Rotterdam Study (RS-II), 3,011 participated. In 2006 a further extension of the cohort was initiated in which 3,932 subjects were included (RSIII), aged 45 years and older, living in the Ommoord district. The rationale and design of the RS have been described in detail elsewhere.[17]

The Erasmus Rucphen Family (ERF) Study is a large family-based cohort study, including over 3,000 participants descending from 22 couples living in the Rucphen region, the Netherlands, in the 19th century. The rationale and design of the ERF Study have been described elsewhere.[18-19] All descendants were invited to visit the regional clinical research centre where they were examined and a fasting blood sample was drawn. All participants filled out a questionnaire on risk factors. The participants included in these analyses consisted of the first series of participants.

The Medical Ethics Committee of Erasmus Medical Center approved the studies and written consent was obtained from all participants.

Genotyping

All RS participants with available DNA were genotyped using Illumina Infinium II HumanHap BeadChips (RS-I and RS-II) or using Illumina Human 610 Quad array at the Department of Internal Medicine, Erasmus Medical Center following manufacturer's protocols. Participants with call rate < 97.5%, excess autosomal heterozygosity, sex mismatch, or outlying identity-by-state clustering estimates were excluded. After quality control 5,974 RS-I participants, 2,157 RS-II participants and 2,082 RS-III participants were included. Of these, 4742 RS-I participants, 1760 RS-II participants and 2072 RS-III participants had successful blood pressure measurements.

In ERF, all DNA samples were genotyped on four different platforms (Illumina 6k, Illumina 318K, Illumina 370K and Affymetrix 250K), which were then merged and imputed to 2.5 million SNPs hapmap using build 36 HapMap (release 22) CEU populations as a

reference cohort. After quality control 1240 participants with genotyping and blood pressure measurements were included for these analyses.

TagSNP selection was based on linkage disequilibrium (r^2>0.8) by using the international HapMap Project.[20] (http://www.hapmap.org).

Blood pressure measurements

Two seated blood pressure measurements were obtained of the right brachial artery with a random zero sphygmomanometer for RS-I and RS-II subjects and with an automated device for RS-III and ERF subjects. The subject had been seated for at least five minutes. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were obtained and the averages of these two measurements were used for analysis. For participants who were taking anti-hypertensive medication we added 10 mmHg to observed SBP values and 5 mmHg to DBP values. Hypertension was defined as SBP≥140 or DBP≥90 mmHg or the use of antihypertensive medication at the time of assessment.

Statistical analyses

The mRNA levels were quantified by calculating expression relative to housekeeping gene *HPRT1* using the δ-Ct method. Differences between groups of tissues were analyzed by Kruskal-Wallis test and post-hoc Dunn's multiple comparison test. Effects of incubations were analyzed after log transformation using Student's t-test with Bonferroni correction. Statistical significance was assumed at P<0.05.

Individual SNP analyses were conducted within each cohort using an additive genetic model. Regression models were fitted for systolic and diastolic blood pressure (separately) and hypertension, adjusting the associations for age, age², sex and body mass index. Within study associations were combined by using an inverse-weighted variance meta-analysis.

A threshold of p < 0.008 was used to indicate statistical significance for genetic testing to correct for multiple testing with Bonferroni method (0.05/6). GenABEL was used for individual SNP analyses. METAL was used for meta-analyses.

RESULTS

mRNA studies

HSD3B expression in adrenal tissues

Patient tissues were divided into 3 groups: normal whole adrenal glands (n=6), non-aldosterone secreting adenomas (1 non-functional, 2 cortisol-secreting) and aldosterone-secreting adenomas (n=6). Expression of both 3β-HSD types, *HSD3B1* and *HSD3B2*, as

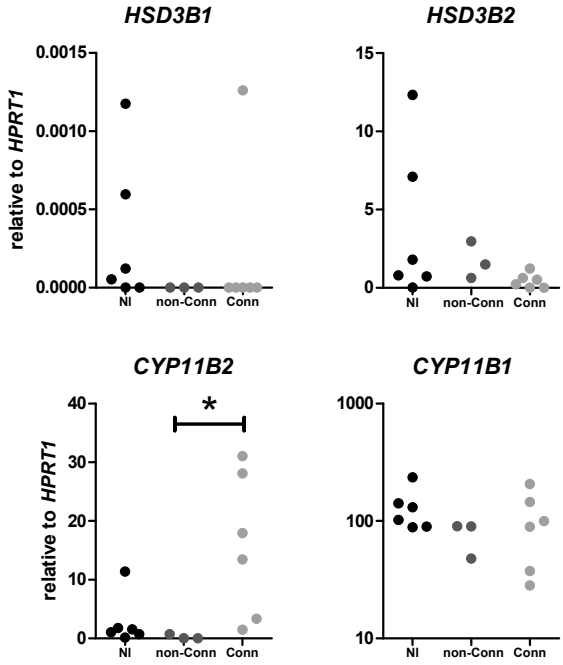
well as the enzymes responsible for the final conversion into cortisol and aldosterone, *CYP11B1* and *CYP11B2* respectively, was studied (Figure 1). *HSD3B1* expression was positive in 4 normal adrenal glands and one Conn adenoma, although at low levels (Ct range: 34.8-38.4).

Moreover, expression of *HSD3B1* was not increased in Conn adenomas compared to normal adrenals or non-aldosterone-producing adenomas. *HSD3B2* mRNA was positive in all tissues except for one Conn adenoma, which was negative for both 3β-HSD enzymes. *CYP11B1* was highly expressed in all tissues studied, whereas *CYP11B2* was most abundantly but not exclusively expressed in the Conn adenomas. Conn adenomas harbored a significantly higher expression of *CYP11B2* compared to non-Conn adenomas (P=0.011). *HSD3B1* expression levels were not associated with other steroidogenic enzyme levels, age, sex or tumor size.

Primary cultures

Normal adrenal glands (n=3) and Conn adenomas (n=2) were used for purposes of primary culture in order to test whether expression of both types of 3β-HSD was regulated by key stimulators of aldosterone and cortisol production. Basal expression of *HSD3B1* was pres-

Figure 1: Expression of steroidogenic enzyme mRNAs in adrenal tissues



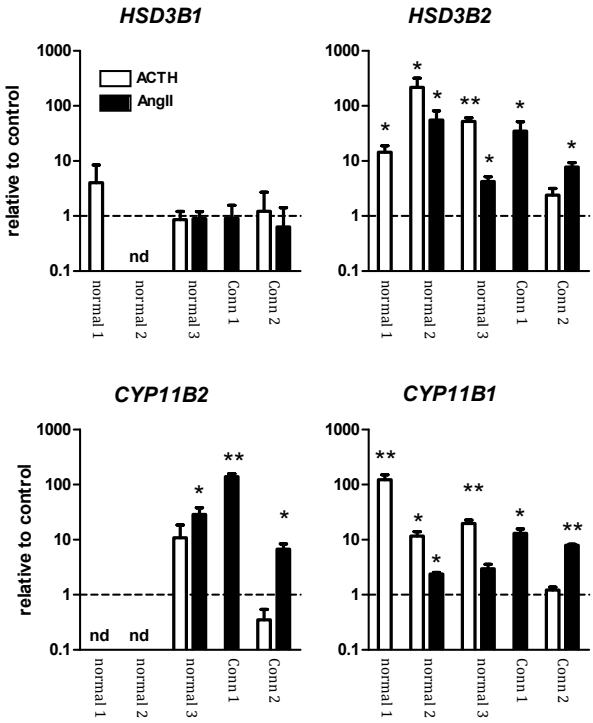
Quantitative analysis of enzymes deemed responsible for aldosterone (*HSD3B1* and *CYP11B2*) and for cortisol production (*HSD3B2* and *CYP11B1*) in normal adrenal glands (NI), non-aldosterone secreting adrenocortical adenomas (non-Conn) or Conn adenomas. * P<0.05

ent in two normal adrenals and both Conn adenomas. *HSD3B2* expression was positive in all samples studied and more abundant than *HSD3B1* expression: approximately 300-fold higher expression in normal adrenals and 100-fold higher in Conn adenomas. Incubation with ACTH or AngII did not increase *HSD3B1* expression in normal adrenal glands or Conn adenomas (Figure 2). In contrast, both ACTH and AngII potently stimulated the expression of the steroidogenic enzymes *HSD3B2*, *CYP11B1* and *CYP11B2* in primary cultures of adrenocortical cells (Figure 2).

Genetic analysis

The total sample size for this analysis was 9814 (RSI, n=4742; RSII, n=1760; RSIII, n=2072; ERF, n=1240). Characteristics of the study sample are presented in Table 1. The mean age of study participants varied from 48.3 years to 67.6 years.

Figure 2: Steroidogenic enzyme expression after stimulation of primary adrenocortical cells with ACTH or AngII



Effects of ACTH (10 ng/ml) and AngII (100 nM) incubation on steroidogenic enzyme mRNA levels of in primary cultures of three normal adrenal glands and two Conn adenomas. Data presented as mean ± SEM. nd: not detectable * P<0.05, ** P<0.01, compared to control condition.

Table 1: Baseline characteristics of the study participants

	RS-I (n=4742)	RS-II (n=1760)	RS-III (n=2072)	ERF (n=1240)
Age, y	67.6	63.9	56.0	48.3
Gender male, %	39.6	44.5	43.8	38.9
Body mass index (kg/m²)	26.2	27.2	27.7	26.7
Systolic blood pressure, mmHg	139	143	135	139
Diastolic blood pressure, mmHg	74	79	85	80
Use of antihypertensive, %	18.3	21.6	20.7	22
Hypertension, %	53	59.6	47.2	51

For analysis we selected 6 tagging SNPs in *HSD3B1.* Function and allele frequencies of these SNPs are described in Supplementary Table 1. Within cohort analyses were combined by meta-analysis and results for all SNPs are presented in Table 2 for systolic blood pressure, Table 3 for diastolic blood pressure and Table 4 for hypertension.

The T allele of rs4986952 increased systolic blood pressure in ERF (Effect 10.47 mmHg; standard error (SE) 3.80; P=0.00579), increased systolic blood pressure in RS-II (Effect 8.07 mmHg; SE 3.20; P=0.012) and increased diastolic blood pressure in ERF (Effect 4.64 mmHg; SE 2.02; P 0.021). The meta-analysis showed that the T allele increased the systolic blood pressure with 3.1 mmHg (P=0.013, Tables 2 and 3). However, p-values do not pass the pre-specified threshold for multiple testing. All other SNPs in *HSD3B1* were not associated with systolic, diastolic blood pressure or hypertension (Tables 2, 3 and 4).

DISCUSSION

In this study we found minimal levels of *HSD3B1* expression in the human adrenal cortex and no association between genetic variation in the *HSD3B1* gene with systolic and diastolic blood pressure or hypertension in three large population-based studies and one family-based study. These findings plead against a significant role of *HSD3B1* in the production of aldosterone in the ZG.

Since the discovery of two different 3β-HSD enzymes that can both convert Δ^5 steroids into Δ^4 steroids,[5, 21] it was commonly thought that the type II enzyme was responsible for all adrenocortical and gonadal steroidogenesis. The type I enzyme is mainly present in placenta and peripheral tissues, including liver, mammary gland and skin.[6] This theorem was supported by the discovery of patients with congenital adrenal hyperplasia (CAH) and male pseudohermaphroditism due to 3β-HSD deficiency. Sequence analysis showed that these patients harbored mutations in *HSD3B2*, whereas the *HSD3B1* gene was not affected.[22-23] This syndrome presents with a salt-wasting phenotype in case of *HSD3B2* mutations leading to a complete abrogation of 3β-HSD activity in the gonads and the

Table 2: SNPs in the *HSD3B1* gene associated with systolic blood pressure

SNPs	Coded allele	Allele freq	RS-I			RS-II			RS-III			ERF			Meta-analysis		
			Beta	Se	Pval	Beta	Se	Pval	Beta	Se	Pval	Beta	Se	Pval	Beta	Se	Pval
rs4986952	T	0.037	3.04	1.86	0.102	**8.07**	**3.20**	**0.012**	-2.69	2.46	0.275	**10.47**	**3.80**	**0.00579**	**3.14**	**1.27**	**0.013**
rs6428829	A	0.297	-0.03	0.49	0.949	1.46	0.77	0.057	-0.34	0.62	0.586	1.16	0.88	0.190	0.30	0.32	0.346
rs6203	T	0.427	-0.38	0.49	0.431	-1.46	0.75	0.051	0.38	0.61	0.535	0.17	0.87	0.850	-0.29	0.32	0.345
rs1047303	C	0.002	3.69	8.46	0.663	-9.26	18.63	0.619	8.37	27.44	0.760	-1.06	20.50	0.959	1.62	6.98	0.816
rs10754400	G	0.345	0.40	0.49	0.419	1.40	0.77	0.069	-0.71	0.62	0.252	0.43	0.83	0.607	0.28	0.32	0.377
rs11581942	C	0.014	0.20	2.66	0.939	-2.91	3.76	0.439	4.72	3.26	0.148	1.61	5.31	0.761	0.78	1.71	0.649

Table 3: SNPs in *HSD3B1* gene associated with diastolic blood pressure

SNPs	Coded allele	Allele freq	RS-I			RS-II			RS-III			ERF			Meta-analysis		
			Beta	Se	Pval	Beta	Se	Pval	Beta	Se	Pval	Beta	Se	Pval	Beta	Se	Pval
rs4986952	T	0.037	-0.32	0.97	0.746	1.08	1.68	0.521	-2.09	1.44	0.146	**4.64**	**2.02**	**0.021**	0.08	0.68	0.903
rs6428829	A	0.297	-0.34	0.26	0.182	0.55	0.40	0.175	-0.25	0.36	0.491	0.44	0.47	0.344	-0.05	0.17	0.766
rs6203	T	0.427	-0.04	0.26	0.883	-0.54	0.39	0.164	0.28	0.36	0.435	-0.001	0.46	0.997	-0.06	0.17	0.742
rs1047303	C	0.002	0.16	4.44	0.971	-10.60	9.74	0.277	-14.92	16.01	0.351	2.87	10.94	0.792	-1.87	3.69	0.612
rs10754400	G	0.345	0.08	0.26	0.747	0.55	0.40	0.174	-0.43	0.36	0.236	0.21	0.44	0.641	0.07	0.17	0.679
rs11581942	C	0.014	-0.14	1.40	0.920	3.24	1.97	0.099	1.98	1.90	0.298	3.10	2.83	0.273	0.02	0.92	0.983

Table 4: SNPs in *HSD3B1* gene associated with hypertension

SNPs	Coded allele	Allele freq	RS-I			RS-II			RS-III			Meta-analysis		
			OR	Se	Pval	OR	Se	Pval	OR	Se	Pval	OR	Se	Pval
rs4986952	T	0.037	1.09	0.18	0.605	1.65	0.4	0.139	0.93	0.29	0.789	1.13	0.14	0.388
rs6428829	A	0.297	0.93	0.05	0.148	1.03	0.08	0.680	1.04	0.07	0.622	0.98	0.04	0.511
rs6203	T	0.427	0.98	0.05	0.640	0.94	0.08	0.461	0.97	0.07	0.678	0.97	0.03	0.377
rs1047303	C	0.002	2.01	0.84	0.404	0.80	1.89	0.906	1.12	3.09	0.970	1.68	0.75	0.488
rs10754400	G	0.345	0.97	0.05	0.529	1.03	0.08	0.668	0.99	0.07	0.918	0.99	0.04	0.740
rs11581942	C	0.014	1.09	0.25	0.730	1.63	0.39	0.208	1.61	0.38	0.208	1.05	0.19	0.789

adrenal cortex. Less severe forms are characterized by residual *in vitro* enzyme activity.[22, 24] *HSD3B2* thus appears the only 3β-HSD in the human adrenal gland and gonads responsible for steroidogenesis.

Therefore, the findings in a recent study in mice that showed that type VI 3β-HSD contributes to hypertension in circadian clock-deficient *Cry*-null mice[7] were unexpected. This observation was extrapolated to the presence of *HSD3B1* in the ZG in two human adrenocortical tissues through micro-dissection. Moreover, genetic evidence on a possible link between type I 3β-HSD and aldosterone has been reported, since several studies in hypertensive subjects showed an association between *HSD3B1* and hypertension (Supplementary table 2).

In the present study, we could not confirm the findings by Doi *et al.* in a series of adrenocortical tissues, including aldosterone-producing adenomas. Similar to the previous study[7] we used a probe and primers[16] that were specific for the two different iso-enzymes to prevent cross-reaction due to the high sequence homology. *HSD3B1* expression was low to absent in the adrenal cortex and was not regulated by the main tropic hormones that stimulate adrenocortical steroidogenesis, ACTH and AngII. In contrast, aldosterone synthase (*CYP11B2*) was enriched in Conn adenomas and potently stimulated by AngII. *HSD3B2* mRNA was ubiquitously expressed, also in Conn adenomas, and was induced by both ACTH and AngII. Although we did not microdissect our tissue samples, the absence of significant *HSD3B1* expression in Conn adenomas as well as the lack of AngII effects on *HSD3B1* plead against a pivotal role of this enzyme in aldosterone production.

An alternative cause for the reported increase in *HSD3B1* expression in the ZG[7] could also relate to the presence of other adrenocortical cells, such as adrenal stem and progenitor cells.[25] Aldosterone-producing cells were recently found to constitute only a small proportion of the ZG,[4] and thus 3β-HSD type I could be localized in non-aldosterone-producing cells. This hypothesis is consistent with our findings that *HSD3B1* is not upregulated in Conn adenomas nor induced by AngII. Alternatively, adrenocortical expression of type I 3β-HSD could still be differentially regulated by peripheral clock genes, instead of by the tropic hormones, and be involved in hypertension associated with changes in circadian rhythm.[26] However, the role of *HSD3B1* in aldosterone production would at most be supplementary as it does not rescue 3β-HSD activity in *HSD3B2* mutant patients with a salt-wasting phenotype. Furthermore, *HSD3B1* does not appear to be involved in primary hyperaldosteronism.

Further determination of the role of type I 3β-HSD in aldosterone production could be obtained by simultaneous immunostaining of *CYP11B2* and *HSD3B1* proteins, but due to the high sequence homology there are currently no specific antibodies that adequately distinguish between the two types of 3β-HSD. Alternatively, specific knockdown of the minimal amounts of *HSD3B1* in primary adrenocortical cells could provide conclusive proof on the role of this enzyme in aldosterone production.

Twin and family studies previously indicated that a substantial proportion of blood pressure variance is due to the effect of genes, with heritability estimates ranging from 30 to 60%.[27-28] Despite this high heritability and considerable knowledge about pathways that are critical to blood pressure regulation in heart, kidneys and vessels, there is limited consistent evidence of genetic loci influencing blood pressure regulation. The steroidogenic enzymes involved in the production of aldosterone form candidate genes for genetic linkage to blood pressure. Rosmond *et al.*[8] were the first who showed an association between *HSD3B1* gene, blood pressure and hypertension. The T→C Leu^{338} variant, rs6203, of *HSD3B1* was shown to be associated with increased systolic and diastolic blood pressure in 263 men. In addition, the C allele was significantly more frequent in grade 1 hypertensive subjects (n=39). Shimodaira *et al.* subsequently demonstrated in 275 essential hypertension patients of Japanese origin that again rs6203 was associated with hypertension and that a second SNP in *HSD3B1,* rs1047303, was also associated with hypertension.[9] Moreover, these two SNPs were associated with higher plasma aldosterone levels. Variants in the *HSD3B1* gene were also associated with blood pressure, plasma aldosterone and potassium in a cohort of 729 newly discovered and never treated hypertensive patients.[10] In contrast, Speirs *et al.*[29] published a study with 168 essential hypertensive patients and 312 normotensive controls that did not confirm the results of the previous studies; no association was demonstrated between rs6203 and hypertension.

The present study, also including rs6203 and rs1047303, does not support the evidence of association between the *HSD3B1* gene and blood pressure. Compared to previous studies that showed an association between *HSD3B1* gene and blood pressure, our cohort size was more than ten times larger. On the other hand, in the ERF cohort with the youngest participants we did find a significant association (after Bonferroni correction) between rs4986952 and systolic blood pressure. The previously reported associations were also found in cohorts of persons with a mean age of 50 years,[8-9] although this effect would have to be confirmed in a meta-analysis. In the older cohorts and our meta-analysis however we found no relation between *HSD3B1* genotype and blood pressure. Unfortunately, we have no data on aldosterone levels of the subjects in our cohort. Our results refute the previously reported associations[8-10] between *HSD3B1* and blood pressure. Genetic association between steroidogenic enzymes and blood pressure has thus far only been replicated for *CYP17A1.*[11-12]

To conclude, through genetic and expression level analyses we found no relation between *HSD3B1* and aldosterone production, blood pressure or hypertension. Therefore, it is unlikely that *HSD3B1* plays an important role in aldosterone synthesis. Consistent with the phenotype of *HSD3B2* mutants, these studies support *HSD3B2* as the pivotal enzyme responsible for all adrenocortical steroidogenesis.

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Supplementary Table 1: Tagging SNPs in *HSD3B1*

SNP ID	Gene map locus	Position	Function
rs4986952	1p12	120054192	Missense Arg → Ile
rs6428829	1p12	120054741	Intron region
rs6203	1p12	120057158	Synonymous Leu[338]
rs1047303	1p12	120057246	Missense Thr → Asn
rs10754400	1p12	120057982	Near gene
rs11581942	1p12	120059946	Near gene

Supplementary Table 2: Previous reported SNPs in the literature

	SBP	DBP	HTN
Shimodaira *et al.* [9]			
- rs3765945			NS
- rs3088283	NA	NA	NS
- rs6203			CC genotype OR = 2.33 (p=0.031*)
- rs1047303			AC + CC genotype OR = 1.50 (p=0.027)
Tripodi *et al.* [10]			
- rs2236780	Effect AA genotype: + 3.9mmHg (p<0.05)	Effect AA genotype: + 2.7mmHg (p<0.05)	
- rs3765945	Effect CC genotype: + 3.5mmHg (p<0.01)	Effect CC genotype: + 2.0mmHg (p<0.05)	NA
- rs6203	NS	Effect TT genotype: -2.5mmHg (p<0.05)	
- rs1047303	Effect CC genotype: + 5.3mmHg (p<0.05)	NS	
Speirs *et al.* [29]			
- rs6203	NS	NS	NS
Rosmond *et al.* [8]			
- rs6203	Effect CC genotype: + 8.8 mmHg (p<0.05)	Effect CC genotype: + 5.4mmHg (p<0.05)	C allele more frequent in the hypertensive Group (p=0.018)

NA: not available; NS= non significant; SBP: systolic blood pressure; DBP: diastolic blood pressure; HTN: hypertension

* male subjects.

CHAPTER 4

Protein kinase C-induced activin A switches adrenocortical steroidogenesis to aldosterone by suppressing *CYP17A1* expression

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Submitted

ABSTRACT

Background:

Functional zonation of the adrenal cortex is a consequence of the zone-specific expression of P450c17 (*CYP17A1*) and its cofactors. Activin and inhibin peptides are differentially produced within the adrenocortical zones and have been implicated in steroidogenic control. We investigated whether activin and inhibin function as intermediates in human functional adrenocortical zonation.

Methods:

Regulation and effects of activin-signaling pathway components were studied in the adrenocortical carcinoma cell lines and human primary adrenocortical cell cultures.

Results:

Inhibin βA-subunit mRNA and activin A protein levels were increased up to 1900-fold and 49-fold, respectively, after protein kinase C (PKC) stimulation through angiotensin II or PMA. PKA stimulation through adrenocorticotropin or forskolin increased expression of the inhibin α-subunit and betaglycan, both inhibitors of activin action. Activin A decreased *CYP17A1* expression and function in the cell lines and in primary adrenal cell cultures with the exception of adrenocortical carcinomas. Inhibition of activin signaling during PKC stimulation through silencing of the inhibin βA-subunit or blocking of the activin type I receptor opposed the PMA-induced downregulation of *CYP17A1* expression and function.

Conclusions:

Activin A acts as a PKC-induced paracrine factor involved in the suppression of *CYP17A1* in the zona glomerulosa and could thereby contribute to functional adrenocortical zonation.

INTRODUCTION

The human adrenal cortex is composed of three histologically and functionally different layers. Just under the capsule, the zona glomerulosa is responsible for the production of mineralocorticoids, mainly aldosterone, which is regulated by angiotensin II (AngII) and potassium. The middle zona fasciculata produces the glucocorticoid cortisol, a process controlled by adrenocorticotropic hormone (ACTH). In the inner zona reticularis, adrenocortical cells mainly produce adrenal androgens, such as dehydroepiandrosterone (DHEA), DHEA-sulfate (DHEA-S) and androstenedione.[1] Functional differences between the adrenocortical zones arise from the presence or absence of steroidogenic enzymes or their co-factors.[2] Most importantly, the enzyme cytochrome P450c17 (encoded by *CYP17A1*) executes the switch between the production of mineralocorticoids, glucocorticoids and adrenal androgens through its 17-hydroxylase and 17,20-lyase activities.[3] *CYP17A1* expression is absent from the zona glomerulosa, thereby facilitating aldosterone production, whereas it is present within the two inner adrenocortical zones.[4] The zona reticularis develops at adrenarche and is characterized by the expression of cytochrome b5[5] (encoded by *CYB5A1*), a co-factor necessary for the 17,20-lyase reaction of P450c17, resulting in the formation of adrenal androgens.[6]

According to the migration theory adrenocortical cells proliferate in the zona glomerulosa, migrate inwards through the three zones and go into apoptosis at the border of the medulla.[7] Adrenocortical cells thus switch steroidogenic capacity depending on their location during migration.[2] Factors controlling these processes are largely unknown, but one of the factors known to regulate expression of *CYP17A1* is activin A.[8-9]

Activins, members of the transforming growth factor-beta (TGF-β) family, are homo- or heterodimeric peptides of inhibin β-subunits. Both activin A (βA-βA dimer) and B (βB-βB) are expressed within the adrenal cortex, but the inhibin α-subunit is also present,[10-11] leading also to the possible formation of inhibin A (α-βA), inhibin B (α-βB) or inhibin pro-αC (α).[12] Activin can regulate steroidogenic enzyme expression and steroid production as well as induce apoptosis in the adrenal cortex.[8-10, 13-14] The physiological role of activin in the adrenal cortex remains unknown. The role of its antagonist inhibin in adrenocortical physiology is even more obscure, since it has failed to show consistent effects on steroidogenesis.[8-10] On the other hand, the inhibin α-subunit has been implicated in adrenocortical tumor formation in murine models[15-16] and can function as a tumor marker in patients.[17]

In the current study we investigated whether activin can act as an auto- or paracrine factor in the intra-adrenal control of steroidogenesis. Using human adrenocortical carcinoma cell lines and primary cultures of adrenal cells from patients with various adrenocortical conditions, we studied regulation and effects of the activin-signaling pathway in the human adrenal cortex.

MATERIALS AND METHODS

Cell culture

Human adrenocortical carcinoma cell lines H295R (ATCC-LGC, Wesel, Germany) and HAC15 (an ACTH-responsive and cortisol-producing clone of H295R,[18] courtesy of Prof. W.E. Rainey), were grown in DMEM/F12 containing penicillin and streptomycin (Invitrogen, Carlsbad, CA USA) and 5% fetal calf serum (FCS) or 10% cosmic calf serum (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Cells were maintained in 75 cm^2 culture flasks under conditions of 37 °C and 5% CO_2. When confluency was reached, cells were trypsinized and plated in 24 well plates at a density of 100.000 cells per well. Cells were allowed to attach overnight before the medium was changed to serum free DMEM/F12 (H295R) or 0.1% cosmic calf serum (HAC15) and secretagogues were added the next day in quadruplicate. All cell culture experiments were performed in triplicate and results are shown as mean results of the three separate experiments.

Primary adrenal cell cultures were obtained from adrenalectomy samples of patients operated within the Erasmus MC, between 2007 and 2010. This study was approved by the local Medical Ethics Committee and written, informed consent was obtained from all participants. Samples included normal adrenals, obtained at nephrectomy because of renal cell carcinoma, hyperplastic adrenals, because of incurable Cushing's disease or ectopic ACTH secretion, and adrenocortical adenomas and carcinomas. Shortly after resection, adrenal tissue samples were dissected and primary single-layer cell cultures were prepared as previously described.[19] Viable lipid-laden cells were counted after isolation and plated at a density of 100.000 cells per well in DMEM/F12 containing 5% FCS. Cells were treated as described above for H295R cells. Experiments could only be run once for each primary culture.

Activin A (R&D systems, Abingdon, UK), FST (Peprotech, Rocky Hill, NJ, USA), inhibin A (courtesy of Prof. T.K. Woodruff), $ACTH_{1-24}$ (Novartis, Basel, Switzerland), SB-505124, Angiotensin II (AngII), phorbol 12-myristate 13-acetate (PMA) and forskolin (FSK, all from Sigma-Aldrich, St. Louis, MO, USA) were dissolved in culture medium and added to the cells in the designated concentrations. After an incubation period of 6-48 hours supernatants were removed and stored at -20 °C, whereas plated cells were frozen on dry ice and stored at -80 °C until RNA isolation.

Hormone measurements

Progesterone, androstenedione and cortisol levels were measured by chemiluminescence-based immunoassays (Immulite 2000, Siemens, Deerfield, IL, USA). Aldosterone levels were measured by radioimmunoassay (coat-a-count RIA, Siemens). Inhibin A, B and pro-αC (Diagnostic Systems Laboratories, Webster, TX, USA) and activin A levels (R&D systems) were measured with enzyme-linked immunometric methods. Supernatant

hormone levels at time of the addition of secretagogues were concurrently measured and substracted from the hormone levels at the end of the incubation period.

mRNA measurements

RNA isolation, total RNA measurement, reverse transcriptase reaction and quantitative polymerase chain reaction (qPCR) of the cholesterol transporter, steroidogenic enzymes and co-factors, activin-related genes and the housekeeping gene *HPRT1* were performed as previously described.[20-21] mRNA expression levels were calculated relative to that of *HPRT1*, of which the expression was shown beforehand not to be influenced by the different culture conditions.

Silencing

For knockdown of *INHBA* expression an shRNA construct (TRCN0000059267) from the TRC-Hs1.0 library (Thermo Fisher Scientific) was used. This shRNA targets bps 1026-1046 (CTCTGGCTATCATGCCAACTA) in the coding region of human *INHBA*. BLAST analysis revealed no other targets in the human transcriptome. tGFP (Lentiviral (LV) vector with turbo GFP insert) and Mock (no LV-shRNA) were utilized as transduction controls, whereas a non-targeting shRNA vector (Scramble, SHC002) was used as a negative control for the assay. A modified Trono lab protocol was used for the production of the LV vectors and has been described previously.[22] One day prior to LV transduction, H295R cells were seeded into 24-well plates in medium containing 5% FCS. Transduction consisted of overnight LV incubation followed by medium replacement. Fluorescence microscopy revealed that >95% of cells transduced with a tGFP-containing vector expressed GFP after six days of culture. Therefore, medium of H295R cells was changed to serum free six days after LV transduction and further secretagogues were added the next day.

Statistics

Data analysis of results from multiple groups was performed with a paired one-way analysis of variance followed by Dunnett multiple comparisons test or Newman-Keuls test. Data pertaining to two groups were analyzed by paired t-tests. mRNA expression levels were logarithmically transformed before analysis. All tests were calculated as two-tailed and statistical significance was assumed at $p<0.05$.

RESULTS

Presence of activin and inhibin

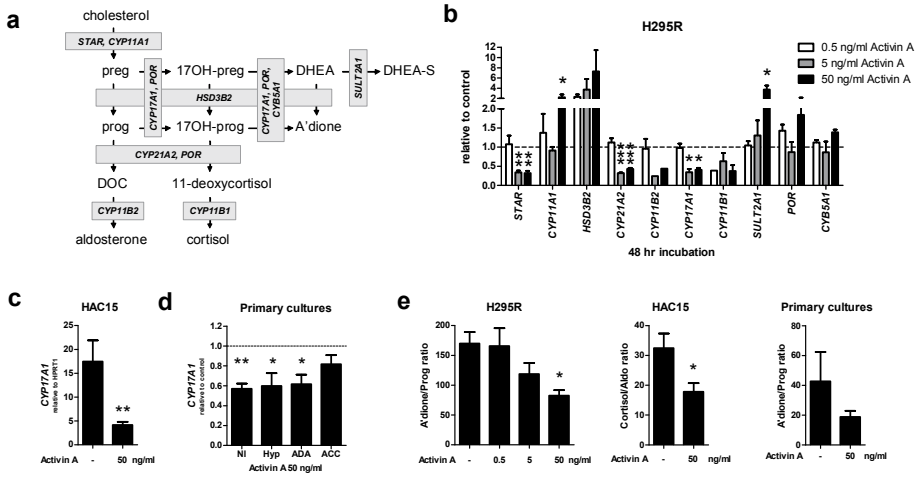
Primary cultures, obtained from one normal and three hyperplastic adrenal tissues, secreted activin A (range: 196-1710 ng/l), inhibin B (range: 9-534 ng/l) and inhibin pro-αC

(range: 23-316 ng/l) after 72 hours in serum free conditions. Inhibin A on the other hand was not detectable in supernatants of untreated cells (<2 ng/l), indicating that most of the inhibin βA-subunits produced in the adrenal cortex do not link to the α-subunit to form inhibin A, but are released as activin A. This suggests that both subunits are not expressed within the same cells[12] and is compatible with previous immunohistochemical studies that show that inhibin βA-subunit is predominantly present in the zona glomerulosa whereas the α-subunit is located in the inner adrenocortical zones.[10]

Exogenous activin A regulates steroidogenesis

Addition of activin A to cultured human adrenocortical cell line H295R altered mRNA expression levels of the cholesterol transporter and steroidogenic enzymes (Figure 1a) at 24 (data not shown) and 48 hours (Figure 1b). At 5 ng/ml activin A significantly suppressed expression of *STAR* (p=0.0006), *CYP21A2* (p<0.0001) and *CYP17A1* (p=0.013), which persisted at a higher dosage. At the 50 ng/ml dose activin A also augmented

Figure 1: Effect of activin A on human adrenocortical steroidogenesis



(a) Adrenocortical steroidogenesis. Preg: pregnenolone, prog: progesterone, DHEA: dehydroepiandrosterone, A'dione: androstenedione, DOC: deoxycorticosterone. STAR: steroid acute regulatory protein, CYP: cytochrome P450, POR: cytochrome P450 oxidoreductase, HSD: hydroxysteroid dehydrogenase, SULT: sulfotranferase. (b) Steroidogenic enzyme and co-factor mRNA expression, measured by qRT-PCR, in the human adrenocortical cell line H295R after 48 hours of activin A incubation under serum free conditions. Effects of 48 hour activin A incubation on *CYP17A1* expression in adrenocortical cell line HAC15 (c) and primary cultures (d) composed of normal adrenal (NI, n=4), adrenocortical hyperplasia (Hyp, n=8), adenoma (ADA, n=9) or carcinoma (ACC, n=9). (e) Activin A also affected steroid hormone concentrations in supernatants of H295R, HAC15 and primary cultures. Progesterone and androstenedione were measured in H295R and primary cultures as an estimate of P450c17 function since these steroids were detectable in these models. In HAC15 both aldosterone and cortisol were present under basal conditions and were influenced by activin A. *P<0.05, **P<0.01, ***P<0.0001, compared to control. Data presented as mean + SEM.

mRNA expression of P450-side chain cleavage (*CYP11A1*, p=0.033) and sulfotransferase (*SULT2A1*, p=0.019). Inhibin A (100 ng/ml) and FST (200 ng/ml) did not significantly alter steroidogenic enzyme mRNA levels in H295R cells (data not shown).

Since activin decreased *CYP17A1*, a factor absent from the zona glomerulosa, we focused further on this effect. Activin A at 50 ng/ml also decreased the expression of *CYP17A1* in HAC15 cells after 48 hours (Figure 1c, p=0.0067). In primary cultures, obtained from human adrenal tissues, activin A suppressed *CYP17A1* expression in normal adrenal glands (n=4, p=0.009), adrenocortical hyperplasia (n=8, p=0.015), and adenomas (n=9, p=0.0014), but in carcinoma samples the effect was not significant (n=9, p=0.066, Figure 1d).

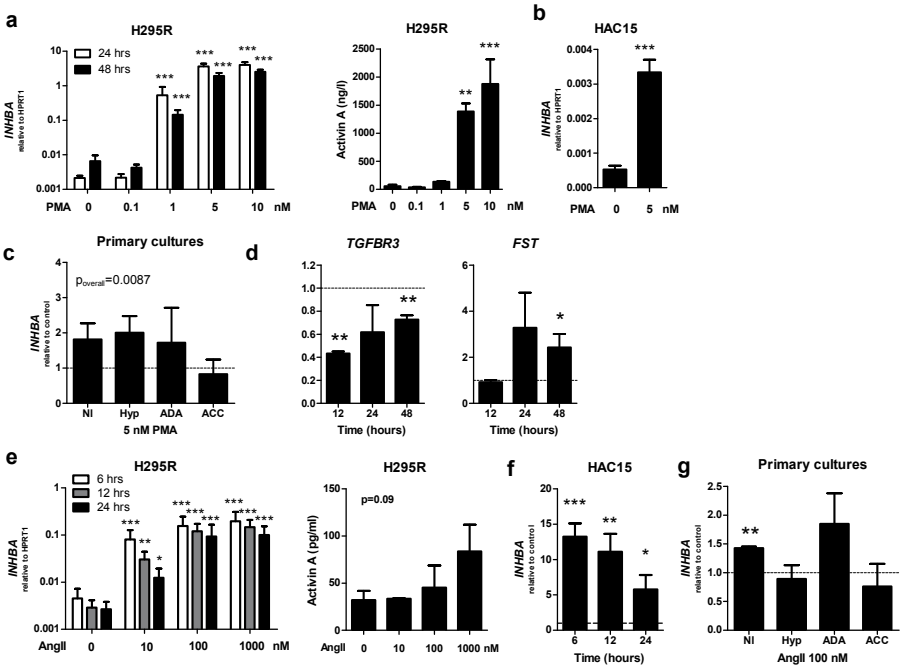
As an estimate of P450c17 function we concurrently measured supernatant steroid levels. Due to the very low cortisol levels in H295R and possible cross-reactivity with the high 11-deoxycortisol levels (detected in micromolar range, data not shown) combined with the effects of activin on other steroidogenic enzymes, we used progesterone and androstenedione as a measure of P450c17 activity. Activin A dose-dependently increased progesterone levels by maximally 53% (p=0.021) and simultaneously decreased androstenedione levels by maximally 17% (p=0.004, data not shown). Taken together, the androstenedione to progesterone ratio decreased dose-dependently after activin A incubation (Figure 1e, p=0.015).

Since HAC15 cells secrete both cortisol and aldosterone in relevant amounts, we investigated whether activin can affect the glucocorticoid to mineralocorticoid ratio in these cells. Indeed, activin A decreased the secretion of cortisol (-30%, p=0.026) while simultaneously increasing aldosterone secretion (+15%, p=0.043, data not shown) in HAC15 cells. Thereby, activin suppressed the production of cortisol relative to that of aldosterone (Figure 1e, -46%, p=0.015). Primary cultures of normal and hyperplastic adrenal tissues showed a large variability in steroid production capacity. Although 10 out of 12 primary cultures showed a decrease in androstenedione to progesterone ratio after activin incubation, the overall decrease was not significant due to variability between steroid production in different adrenocortical cultures (Figure 1e, p=0.07).

Dependence of activin-signaling pathway components on protein kinase A and C

Protein kinase C (PKC) has been reported as a potent regulator of *INHBA* expression in several cell types, including the adrenal cortex.[8, 11] We confirmed that *INHBA* mRNA levels are induced up to 1900-fold by the addition of the PKC stimulator PMA in H295R cells (Figure 2a, p<0.0001). This was accompanied by a mean 49-fold upregulation of activin A protein in the supernatant of H295R cells. Also in HAC15 cells, PMA increased the expression of *INHBA* mRNA (Figure 2b, p=0.0045). In primary adrenal cell cultures basal *INHBA* mRNA levels were much higher than those in the cell lines. Nonetheless, PMA stimulated

Figure 2: Regulation of activin-signaling molecules in the adrenal cortex by PKC and AngII



The PKC stimulator PMA influenced expression of *INHBA* mRNA and supernatant concentrations of activin A (a) in H295R after 24-48 hours. These effects were confirmed in HAC15 (b) and primary cultures of adrenal cells (n=25, c). Expression of other components of the activin signalling pathway betaglycan (*TGFBR3*) and follistatin (*FST*) were regulated by PKC stimulator PMA (5 nM) in H295R cells (d). The physiological adrenocortical PKC stimulator AngII dose- and time-dependently increased *INHBA* expression and activin A supernatant levels after 24 hours (e) and *INHBA* expression in HAC15 (f) and normal adrenal cell cultures (g). *P<0.05, **P<0.01, ***P<0.0001, compared to control. Data presented as mean + SEM.

INHBA expression by a mean of 195% in normal and hyperplastic adrenals (n=10, p=0.036) and 228% in all primary cultures (n=25, p=0.0087, Figure 2c).

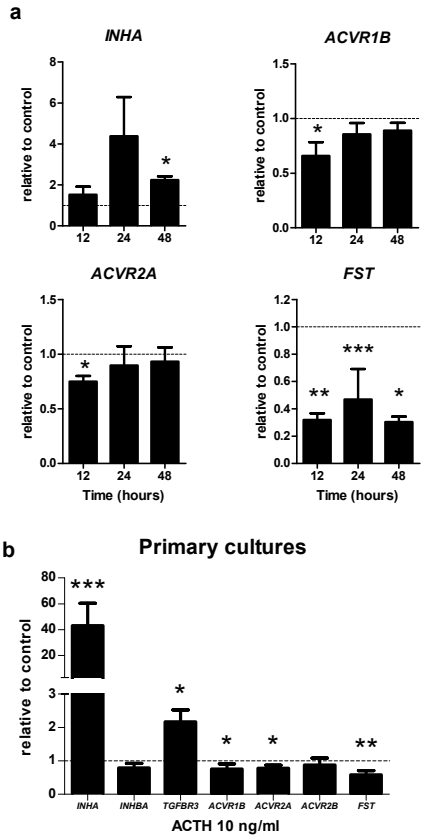
Additionally, PMA lowered the transcription of *TGFBR3* mRNA, significantly after 12 and 48 hours in H295R cells (Figure 2d, p=0.018). It also led to an increase in *FST* mRNA after 24 hours (p=0.0036), presumably due to increased activin signaling. Expression of *INHA*, *ACVR1B*, *ACVR2A* and *ACVR2B* was present but not affected (data not shown). *INHBB* mRNA levels were either very low or undetectable in all models studied and its expression was not modulated by protein kinase C stimulation (data not shown).

The production of aldosterone in the zona glomerulosa is dependent on AngII-induced PKC activation.[23] Incubation of H295R cells with AngII led to an increase of *INHBA* expression (maximally 50-fold at 12 hours, p<0.0001) and a trend towards increased activin A protein (261%, p=0.085, Figure 2e). This increase was also present in HAC15 cells after

6-24 hours and in 3 normal adrenals after 48 hours (Figs 2f, p<0.05 and 2g, p=0.0025, respectively), but not in primary cultures of other origin (p>0.05).

Glucocorticoid and adrenal androgen production is mainly regulated by the ACTH-stimulated cyclic-AMP/protein kinase A pathway. Since H295R cells lack ACTH responsiveness,[18] we used forskolin as a PKA stimulator. Results of these experiments revealed that expression levels of *INHA*, *FST*, *ACVR1B* and *ACVR2A* were affected by protein kinase A stimulation (Figure 3a). Since these responses to forskolin were relatively small, effects of ACTH in primary cultures of normal and hyperplastic adrenal glands were also investigated. ACTH led to an increase in the expression of *INHA* and *TGFBR3*, whereas *ACVR1B*, *ACVR2A* and *FST* levels were significantly downregulated in primary cultures (Figure 3b).

Figure 3: Regulation of activin-signaling molecules in the adrenal cortex by FSK and ACTH



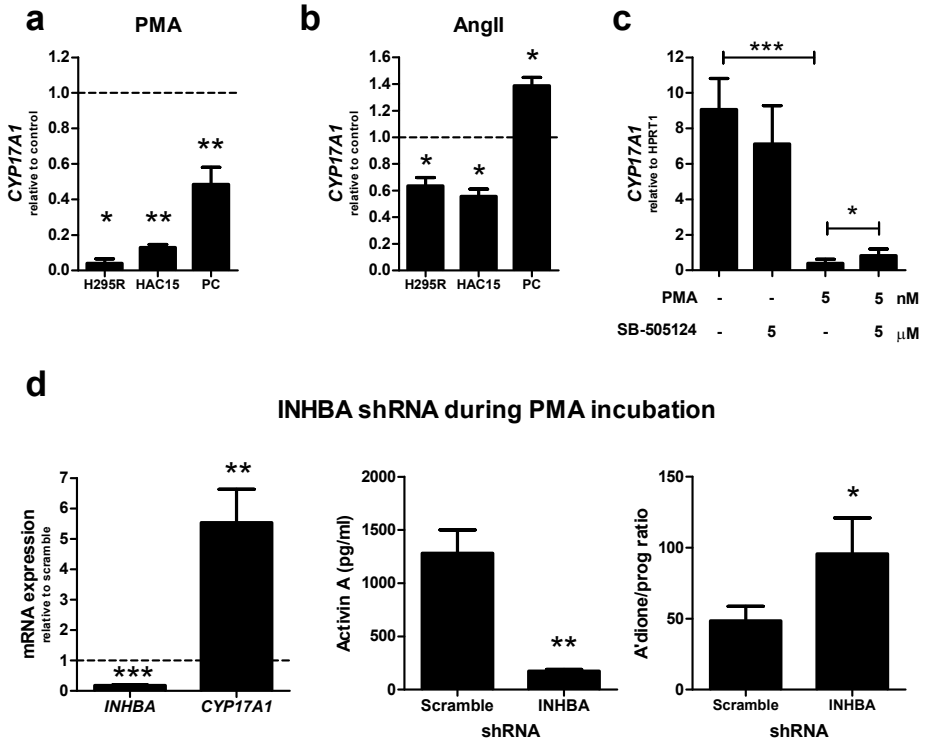
(a) Expression of the inhibin α-subunit (*INHA*), activin receptors type IB (*ACVR1B*) and type IIA (*ACVR2A*) and follistatin (*FST*) were regulated by the PKA stimulator FSK (10 μM) in H295R cells. (b) PKA stimulation by ACTH in 11 primary adrenocortical cell cultures also affected expression of *INHA*, activin receptors and *FST* after 12-48 hours. *P<0.05, **P<0.01, ***P<0.0001, compared to control. Data presented as mean + SEM.

Overall, stimulation of protein kinase C appeared to potentiate activin-signaling, whereas protein kinase A appeared to decrease activin-signaling potential.

Activin A is a PKC-stimulated intermediate in *CYP17A1* downregulation

Similarly to the effect of activin, PMA also suppressed *CYP17A1* expression in H295R and HAC15 cells and in primary cultures (Figure 4a). AngII displayed more diverse effects, leading to *CYP17A1* downregulation in H295R and HAC15, whereas expression was augmented in primary cultures (Figure 4b). However, since the AT1R is expressed within aldosterone-producing cells specifically,[24] AngII-induced PKC stimulation could contribute to the absence of *CYP17A1* from the zona glomerulosa. Because PMA as well as AngII

Figure 4: Role of activin A in the downregulation of *CYP17A1* by protein kinase C



Effects of PMA (a, 5nM) and AngII (b, 100 nM) on *CYP17A1* expression in different adrenocortical cell lines and primary cultures after 48 hours. (c) Inhibition of activin type I receptor with SB-505124 did not affect basal levels of *CYP17A1* in H295R, but did oppose the PMA-induced downregulation of *CYP17A1*. (d) LV transduction of H295R cells with shRNA against *INHBA* mRNA led to a decrease in *INHBA* expression and activin A protein production during 48 hours incubation with 5 nM PMA. This knockdown of activin A led to an increase in *CYP17A1* expression and function, as measured by an augmented androstenedione to progesterone ratio. *P<0.05, **P<0.01, ***P<0.0001, compared to control or scramble unless otherwise indicated. Data presented as mean + SEM.

also led to increased expression of *INHBA*, activin could be an intermediate in PKC-related inhibition of *CYP17A1*.

To investigate this hypothesis, we tested whether inhibition of the activin receptor during PKC stimulation would affect *CYP17A1* expression. SB-505124, an activin receptor type I inhibitor, did not significantly alter the basal expression of *CYP17A1* in H295R. Whereas PMA potently inhibited *CYP17A1* to 4.0% of baseline, the co-incubation of SB-505124 with PMA doubled *CYP17A1* expression to 9.2% ($p=0.019$, Figure 4c). Since SB-505124 could possibly affect signaling of other TGF-β family members, H295R was also transduced with lentivirus containing shRNAs against *INHBA* mRNA. Compared to cells transduced with a scramble shRNA, cells with *INHBA*-shRNAs had a 82% decrease in both *INHBA* mRNA ($p<0.0001$) and activin A protein after 48 hours of PMA incubation ($p=0.001$, Figure 4d). The specific *INHBA* knockdown resulted in an opposite effect of the PMA-induced *CYP17A1* downregulation. This counteraction was accompanied by an increase in the androstenedione to progesterone ratio (Figure 4c), representing increased *CYP17A1* activity after blockade of the activin A induction by PMA.

DISCUSSION

Many factors are known to regulate adrenocortical steroidogenesis.[25-27] The factors contributing to functional zonation of the adrenal cortex are however largely unknown. Mineralocorticoid production in the zona glomerulosa is made possible by the absence of cytochrome P450c17, which, if present, diverts steroid production towards glucocorticoids and adrenal androgens.[28] The relevance of P450c17 activity for aldosterone-mediated effects has consistently been shown by associations between genetic variation in *CYP17A1* and blood pressure.[29-30] Therefore factors controlling the presence or absence of *CYP17A1* expression could have clinical implications. This study shows that activin A, a paracrine factor produced in the adrenal cortex, is increased by PKC stimulation and can inhibit *CYP17A1* expression and function, both in cell lines and primary adrenal cell cultures. Blocking of the activin effects during PKC stimulation leads to augmented *CYP17A1*, proving that activin A is an intermediate in PKC-controlled expression of *CYP17A1*.

AngII, being a part of the renin-angiotensin-aldosterone system, is the main physiological regulator of aldosterone production. By binding to the AT1R, AngII induces calcium influx and the stimulation of intracellular diacylglycerol; the latter subsequently activates protein kinase C (PKC). The accumulation of activated PKC isoforms is thought to be responsible for the sustained or chronic phase of steroidogenesis in the zona glomerulosa of the adrenal gland.[23] Adrenal expression of the *AT1R* is confined to the zona glomerulosa and radiolabeled AngII specifically binds to adrenocortical cells in that zone.[24, 31] AngII addition to cultured adrenal cell lines led to increased levels of aldosterone as well as corti-

sol.[32] However, in the presence of PKA stimulation, AngII decreased 17-hydroxylase activity in a PKC-dependent manner.[33] AngII effects on *CYP17A1* expression differ between model systems and experimental conditions, but in the human adrenal cortex AngII-stimulated cells lack *CYP17A1* expression.[4, 31] It is therefore likely that an AngII-stimulated factor suppresses *CYP17A1* expression within zona glomerulosa cells. Direct PKC stimulation by PMA did inhibit *CYP17A1* expression in H295R and HAC15 cells as well as in primary cultures (Figure 4a), suggesting that AngII-induced PKC activation is involved in the regulation of *CYP17A1* in these cells. In all models we studied, PMA concurrently induced *INHBA* expression in a time- and dose-dependent manner, leading to higher activin A secretion (Figure 2).

Activin A influenced the expression levels of several steroidogenic enzymes and thus affected adrenocortical steroid production at multiple levels. Since activin A was regulated by PKC and shared its inhibitory effects on *CYP17A1* expression we focused on the absence of P450c17 in the zona glomerulosa. It has previously been shown that phorbol esthers inhibit *CYP17A1* expression in the adrenal cortex.[34-35] The mechanisms causing this suppression were unknown. Through receptor inhibition and *INHBA* knockdown we now show that part of this downregulation is through activin A induction. Since the *CYP17A1* inhibition could only be partially opposed, other factors are likely to be involved in this pathway as well. A possible candidate could be c-fos, which was found to have a similar role in theca cell-like tumor cells.[36] C-fos was also potently upregulated by AngII and increased aldosterone production in bovine adrenocortical cells, although *CYP17A1* was not studied in this cell model.[37]

Interestingly, the effects of activin A were relatively small in primary cultures of ACC. This would imply that ACCs become resistant to activin, possibly through decreased receptor expression.[21] Furthermore, the PMA-induced decrease in *CYP17A1* was small compared to that in the cell lines. This could relate to the endogenously high levels of activin A levels in primary cultures and to the relatively small increase in the *INHBA* expression after PMA stimulation, especially in ACC.

The inhibin α-, βA- and βB-subunits can assemble into multiple mature peptides through the formation of di-sulfide bridges.[8] Primary cultures of normal adrenocortical cells secreted activin A, inhibin B and inhibin pro-αC whereas inhibin A levels were undetectable. Adrenocortical production of inhibin pro-αC and to a lesser extent inhibin B is compatible with the reports that these peptides can be elevated in serum of patients with adrenocortical tumors.[17, 38] The finding that activin A and inhibin pro-αC were amply produced without concomitant secretion of inhibin A indicates the different cell types in which these subunits are expressed, e.g. zona glomerulosa for the inhibin βA- and reticularis for the α-subunit.[10, 39] Thus it appears that activin A is predominantly produced in the aldosterone-producing cells and that it exerts its para- or autocrine functions here. Importantly, the zona glomerulosa is composed of different cell types,[40] among which the

adrenocortical progenitor cells.[41] Activin A might also affect proliferation and apoptosis in these cells.

The fact that most of the inhibin α-subunit in the adrenal is secreted as inhibin pro-αC might explain why no direct effect of inhibin in the adrenocortical steroidogenesis has been detected. Besides inhibition of activin action at the receptor level by occupation of the activin type II receptor,[42] the α-subunit could also antagonize activin formation by binding to the β-subunit, provided that both subunits are synthesized within the same cell. More recently, inhibin A was found to antagonize TGF-β signaling potential through the internalization of betaglycan.[43] The ACTH-induced stimulation of *INHA* and *TGFBR3* expression and inhibition of activin receptor expression could be a counterregulatory mechanism to prevent activin-related *CYP17A1* inhibition. Interestingly, *Cyp17a1* recurrence in primary pigmented adrenocortical nodular lesions in adrenal-specific *Prkar1a* knock-out mice was accompanied by an upregulation of *Inha* expression.[44]

The activin-inhibin signaling pathway thus appears to be involved in the fine-tuning of steroidogenesis after AngII or ACTH signaling. Whereas activin relays AngII signals in the zona glomerulosa, ACTH upregulates inhibin components, possibly to prevent activin signaling in the zona reticularis and fasciculata. This could lead to a gradient of activin signaling across the adrenal cortex, a process similar to that of the morphogen function of activin in Xenopus development.[45] Such a gradient would enable zone-specific expression patterns of steroidogenic enzymes and allow adrenocortical cells to change steroidogenic capacity, e.g. mineralocortoid, glucocorticoid or adrenal androgen production, during migration towards the medulla. This mechanism can explain the absence of *CYP17A1* in the zona glomerulosa, but the presence of *CYB5A1* or *SULT2A1* in the inner zones cannot currently be explained on basis of an activin-signaling gradient.

In conclusion, we show that in the adrenal cortex protein kinase A and C have opposing effects on components of the activin-signaling pathway. Inhibin βA-subunits, mostly present in the outer cortex, are regulated by AngII through PKC and are involved in the absence of *CYP17A1* expression in the zona glomerulosa, thus facilitating aldosterone production. ACTH, on the other hand, decreases activin-signaling potential in the inner zones of the human adrenal cortex, possibly in order to prevent *CYP17A1* downregulation. Activin A therefore is involved in relay of the signals from AngII and ACTH to steroidogenesis in the adrenal cortex.

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PART II

DISEASES OF THE ADRENAL CORTEX

In vivo and *in vitro* studies in ACTH-independent macronodular adrenocortical hyperplasia reveal prevalent aberrant responses to hormonal stimuli and coupling of arginine-vasopressin type 1 receptor to 11β-hydroxylase expression

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Manuscript in preparation

ABSTRACT

Background:

Adrenal Cushing's syndrome can be caused by ACTH-independent macronodular adrenocortical hyperplasia (AIMAH), characterized by bilateral adrenal enlargement and aberrant responses to eutopic or ectopic hormonal stimuli. We have investigated the *in vivo* and *in vitro* prevalence of responsiveness, in terms of cortisol production and steroidogenic enzyme expression, to these stimuli in a large cohort of AIMAH patients.

Methods:

In vivo cortisol responses to hormonal stimuli were studied in 35 patients with ACTH-independent bilateral adrenal enlargement and (sub-)clinical hypercortisolism. These stimuli were also applied to AIMAH and other adrenal cell cultures to investigate cortisol secretion and steroidogenic enzyme mRNA expression. In addition, arginine-vasopressin (AVP) receptor mRNAs were investigated in adrenal tissues.

Results:

Positive serum cortisol responses to stimuli were detected in 27/35 patients tested, with multiple responses within individual patients occurring for up to five stimuli. AVP and metoclopramide were the most prevalent hormonal stimuli in AIMAH patients *in vivo*. Catecholamines induced short-term cortisol production more in AIMAH than in cells of other adrenal origin. Short- and long-term incubation with AVP increased cortisol secretion in cultures of AIMAH cells. AVP also increased steroidogenic enzyme mRNAs, among which an aberrant induction of *CYP11B1*. Vasopressin type 1a receptor was the only AVPR expressed and was higher in AIMAH tissue compared to adrenocortical carcinomas. *AVPR1A* expression was correlated to the AVP-induced stimulation of *CYP11B1*.

Conclusions:

Multiple hormonal signals can simultaneously induce hypercortisolism in AIMAH. AVP is the most prevalent eutopic signal, presumably caused by an aberrant link between *AVPR1A* and *CYP11B1.*

INTRODUCTION

Patients with Cushing's syndrome (CS) can be divided into groups with adrenocorticotropin (ACTH)-dependent and ACTH-independent disease.[1] The latter group forms the minority and is most frequently caused by neoplasms of the adrenal cortex, i.e. adenomas and carcinomas. In rare cases ACTH-independent CS can be the consequence of primary adrenocortical hyperplasia. Micronodular hyperplasia or primary pigmented nodular adrenocortical hyperplasia (PPNAD) occurs in the context of Carney syndrome and is frequently associated with mutations in the cyclic AMP (cAMP)/protein kinase A (PKA) pathway.[2] On the other hand, ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) is characterized by multiple bilateral nodules consisting of hyperplastic adrenal cells that lead to autonomous (over)production of cortisol. The pathogenesis of AIMAH is thought to involve proliferation of adrenocortical cells and an increase in steroidogenesis through signaling by several aberrant eutopic or ectopic hormone receptors.[3]

Key regulators of normal adrenocortical steroidogenesis are ACTH for glucocorticoids and adrenal androgens, whereas mineralocorticoids are principally regulated by angiotensin II (AngII) and plasma potassium concentrations.[4] Nonetheless, steroid production can also be physiologically controlled by other endocrine, paracrine and autocrine signals.[5] In AIMAH patients there is an exaggerated or ectopic response to stimulation by hormonal signals. Receptors for these hormones, eutopically or ectopically expressed on adrenocortical cells and activated by endogenous hormones, stimulate the cAMP/PKA pathway leading to subclinical or clinical CS.[3] Aberrant hormonal responses and receptor expressions in AIMAH have been well documented for glucose-dependent insulinotropic polypeptide receptor (GIPR, ligand: GIP[6-8]), α4-, β1- and β2-adrenergic receptor (α_4-AR and $\beta_{1/2}$-AR, ligands: catecholamines[9-10]), vasopressin type 1A and 2 receptors (AVPR1A and AVPR2, ligand: arginine-vasopressin (AVP)),[11-12] luteinizing hormone receptor (LHR, ligands: LH, human chorionic gonadotropin (hCG)[13-14]) and serotonin type 4 receptor (5-HT4R, ligand: serotonin (5-HT) [15-16]). Other possible aberrantly expressed receptors include the AngII type I receptor (AT1R, ligand: AngII[17]), glucagon receptor[18-19] and thyrotropin receptor (TSHR, ligand: TSH).

Diagnostic protocols for AIMAH include administration of the various hormonal ligands or stimuli to patients. Results are considered positive obtained if the hormone increased serum cortisol concentrations by more than 50%, whereas partial responses have been arbitrarily defined as a stimulation between 25% and 50%.[3] The relevance of these effects has been shown by the results of administration of antagonists to the expressed receptors,[9, 13, 20] confirmation of stimulatory effects of hormones on primary AIMAH cells *in vitro* and the detection of the hormone receptors on AIMAH cells. Furthermore, these effects have been described in adrenocortical adenomas and carcinomas, which in certain

circumstances can also overexpress receptors responsive to endocrine and/or paracrine signals.[20]

Whereas the presence of aberrant receptors has been firmly established, much is unknown about the cause of receptor expression and downstream signals coupling receptor activation to stimulation of cell growth and steroidogenesis.[21-22] Recent findings do suggest a role for mutations of *TP53* in a minority of patients with AIMAH.[23]

Most evidence on AIMAH pathophysiology has been collected from case reports, small case series and reviews; only three centers have reported on larger groups of 16, 18 and 32 AIMAH patients, respectively.[10, 19, 22] Furthermore, no large series have systematically related clinical data to *in vitro* findings.

This study describes the largest *in vivo* and *in vitro* evaluation of patients with AIMAH so far. We performed *in vivo* stimulation tests with ACTH and ligands for multiple hormone receptors. Furthermore, AIMAH and other adrenocortical tissues obtained from adrenalectomies were used for primary cultures in order to investigate effects of clinically relevant hormonal stimuli on short- and long-term glucocorticoid production and expression levels of steroidogenic enzyme mRNAs.

MATERIALS & METHODS

Patients

All patients that presented to the Erasmus MC between 1994 and 2011 with ACTH-independent bilateral adrenal enlargement with (sub-)clinical CS were included in the study. Adrenal enlargement could consist of bilateral hyperplasia or bilateral adenomas, as detected by computer tomography or pathological evaluation. Clinical CS was defined by the presence of clinical symptoms and at least two positive tests for hypercortisolism: the absence of a cortisol diurnal rhythm, increased 24 h urinary free cortisol excretion and/or a failure to suppress cortisol levels below 50 nmol/l after 1 mg dexamethasone overnight. Subclinical CS was defined by one positive test for hypercortisolism.[24] Patient adrenal samples were collected for *in vitro* studies from adrenalectomy due to renal cell carcinoma (n=3), AIMAH (n=19), ACTH-dependent hyperplasia (n=11), adrenocortical adenoma (n=11) or carcinoma (n=4). The study was approved by the Medical Ethics Committee of the Erasmus MC and all patients gave informed consent.

Clinical studies

All patients were admitted for measurement of baseline hormonal levels and subsequent evaluation of eutopic and ectopic stimuli: 250 µg synacthen (Novartis, Basel, Switzerland) iv, 100 µg luteinising hormone releasing hormone (LHRH, Ferring, Hoofddorp, The Netherlands) iv, 200 µg thyrotropin releasing hormone (TRH, Ferring) iv, a 2 hour upright

posture test, 1 mg metoclopramide (Pharmachemie, Haarlem, The Netherlands) orally, 10 IU AVP (Ferring) im, 1 mg glucagon (Novo Nordisk, Alphen aan den Rijn, The Netherlands) iv, intravenous salt loading (NaCl 3% at 0.1 cc/kg/min), and a standard mixed meal (116 g carbohydrates, 27 g proteins, 14 g fat). From 2002 on, the intravenous salt loading test was replaced by intramuscular injection of AVP.[3] In case of subclinical hypercortisolism in combination with a severe phenotype (difficult to treat hypertension, diabetes or severe osteoporosis) or clinical CS patients underwent bilateral laparoscopic adrenalectomy and were put on lifelong glucocorticoid and mineralocorticoid replacement.

Tissue processing

Adrenal tissue was collected following bilateral adrenalectomy. Parts of the tissue were snap-frozen and stored at -80 °C until the isolation of RNA. Other tissue parts were cut into small pieces and put into DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 5% fetal calf serum (FCS), penicillin and streptomycin (Invitrogen). Subsequently, tissues were prepared for primary adrenal cell cultures using collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) as previously described.[25] Adrenal cell viability was checked with trypan blue and always exceeded 90%. Cells were either put into 5 ml tubes at 2.000.000 cells per ml for short-term incubations and, in case of sufficient cell yield, plated in 24 well plates at 100.000 cells per ml for long-term culture.

Short-term incubation

Following placement in the tubes incubations were performed in quadruplicate in a 2 ml volume containing 5% FCS with the following secretagogues: vehicle, $ACTH_{1-24}$ (50-500 pg/ml, Novartis), GIP (10^{-7} M, Sigma), metoclopramide (10^{-6} M), hCG (100 mIU/ml, Organon, Oss, The Netherlands), epinephrine (10^{-6} M, Centrafarm, Etten-Leur, The Netherlands), norepinephrine (10^{-6} M, Centrafarm), glucagon (10^{-7} M, Novo Nordisk, Alphen aan den Rijn, The Netherlands), AVP (pitressin, 10^{-7}-10^{-8} M, Monarch Pharmaceuticals, Bristol, TN, USA), desmopressin (10^{-8} M, Ferring), angiotensin II (10^{-8} M, Sigma) or TSH (10^{-8} M, Genzyme Europe BV, Naarden, The Netherlands). The PKA stimulator forskolin (FSK, 10^{-6} M, Sigma) was added as a positive control. Depending on the cell yield following isolation, secretagogues were selected on the basis of positive *in vivo* responses obtained in individual AIMAH patients. After the addition of hormones, tubes were covered with parafilm and incubated in a rocking water bath at 37 °C. After two hours, tubes were centrifuged and supernatants were removed and stored at -20 °C until the measurement of cortisol. For comparison, cells from 1 normal adrenal gland, 10 ACTH-dependent hyperplasia samples and 8 adrenocortical adenomas were also investigated using this protocol.

Long-term cell culture

Cells were allowed to attach overnight and the medium was changed to serum free DMEM/F12 with antibiotics the next day. After 24 hours the following hormonal stimuli were added in quadruplicate: ACTH (10 ng/ml), GIP (10^{-7} M), metoclopramide (10^{-6} M), hCG (100 mIU/ml), norepinephrine (10^{-6} M), glucagon (10^{-6} M), AVP (pitressin, 10^{-7}-10^{-8} M), desmopressin (10^{-8} M), angiotensin II (10^{-7} M) or TSH (10^{-8} M). Cells were cultured at 37 °C for 48 hours. Subsequently, supernatant were removed and stored at -20 °C, whereas the attached cells were snap-frozen on dry-ice and stored at -80 °C until the isolation of RNA. Forty-eight hour effects of the hormones on cortisol secretion and steroidogenic enzyme mRNA expression were also studied in cells from 3 normal adrenal glands, 1 ACTH-dependent hyperplasia, 3 adenomas and 4 carcinomas.

Cortisol measurement and quantitative mRNA analysis

Serum and supernatant cortisol levels were measured using a chemiluminescence-based method (Immulite, Siemens Diagnostics, Deerfield, IL, USA). RNA was isolated from plated cells and frozen adrenal tissue samples and reverse-transcribed as previously described.[26] The assays for the measurement of mRNA expression of the housekeeping gene *HPRT1*, steroid acute regulatory protein (*STAR*) and steroidogenic enzymes (cytochrome P450 side chain cleavage [*CYP11A1*], 3β-hydroxysteroid dehydrogenase type 2 [*HSD3B2*], 17-hydroxylase/17,20-lyase [*CYP17A1*], 21-hydroxylase [*CYP21A2*] and 11β-hydroxylase [*CYP11B1*]) were as reported.[27] For the measurements of expression of the vasopressin receptors, we used SYBR green-based assays for *AVPR1A* (F: TTTGTGATCGTGACGGCTTACA, R: GGTGATGGTAGGGTTTTCCGA) and *AVPR1B* (F: CAGCAGCATCAACACCATCT, R: CCATGTAGATCCAGGGGTTG) and purchased the *AVPR2* assay (Hs00181055_m1) from Applied Biosystems (Nieuwerkerk aan den IJssel, The Netherlands). Positive controls for the assays for the vasopressin receptors consisted of adrenal gland, kidney and pituitary gland. Quantitative PCR was performed in a 12.5 µl mixture containing PCR mix, primers and 20 ng cDNA. PCR efficiency exceeded 90% for all assays used. mRNA levels were calculated relative to that of *HPRT1*, expression of which was shown to be stable under the conditions used, on the basis of the ΔCt-method.

Data analysis and statistics

A full response was defined by a more than 50% increase of serum cortisol following the administration of the hormonal stimulus. Responses between 25-50% were termed partial. Analyses were performed using GraphPad Prism (GraphPad software, version 5.01) and SPSS (version 17.0). Group comparisons were made with Kruskal-Wallis followed by Dunn's multiple comparison tests. Differences between 2 groups were analyzed by Mann-Whitney U or Wilcoxon signed rank tests. Spearman's correlation coefficient was used for analysis of association between variables. Multiple testing was adjusted by

Bonferroni correction. All tests were calculated as two-tailed and a P-value below 0.05 was considered to be statistically significant.

RESULTS

In vivo studies

A total of 35 patients with ACTH-independent (sub-)clinical CS and bilateral adrenal hyperplasia underwent *in vivo* evaluation of cortisol responses to eutopic and ectopic hormonal stimuli. Patient characteristics are summarized in Table 1. ACTH administration increased serum cortisol 5.2±0.9-fold compared to baseline (mean±SEM, P<0.0001); positive responses were demonstrated in 28 out of 29 patients tested. Of the other hormonal stimuli tested, AVP gave the highest percentage response: 52% of patients displayed a >50% increase in cortisol levels, whereas 29% had a partial response. The other prevalent responses were obtained with upright posture, metoclopramide, mixed meal, salt loading, LHRH, glucagon and TRH, see Table 2. Mean induction by these stimuli is indicated in Figure 1. Overall significant induction of serum cortisol in the AIMAH patients was found for LHRH, upright posture, metoclopramide, AVP, glucagon and salt loading.

Of the 35 patients investigated, 27 (77%) patients showed a minimum of one full response to any administrated stimulus. One, 2, 3 or 4 positive responses were demonstrated in 10, 9, 5 and 1 patients, respectively. Two patients concurrently reacted to 5 aberrant stimuli at *in vivo* testing (Table 2). Of the 26 patients that underwent an upright posture test and either salt loading or direct stimulation with AVP 9 patients (35%) had negative responses to both tests, 8 patients (31%) had positive responses to both tests and 9 patients had discordant results. Of these, 5 patients (19%) had only a positive stimulation of serum

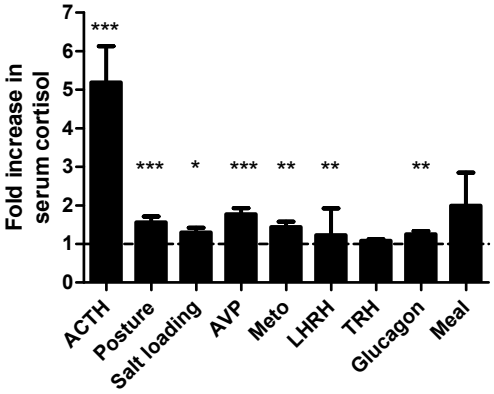
Table 1: Characteristics of AIMAH patients evaluated *in vivo*

Total	35
Male/female	9/26
Age (mean±SD)	56.1±9.8
Adrenalectomy	22 (63%)
Diameter adrenal (mm, mean±SD)	
- left	40.5±16.1
- right	37.0±16.3
Hypercortisolism	
- subclinical	19 (54%)
- clinical	16 (46%)
Hypertension	28 (80%)
Diabetes	12 (34%)
Bone loss	
- osteopenia	9 (26%)
- osteoporosis	10 (29%)

Table 2: *In vivo* response to aberrant/ectopic stimuli in patients with AIMAH

Stimulus	n tested	Partial response (25-50%) n (%)	Full response (≥50%) n (%)
$ACTH_{1-24}$	30	-	29 (97%)
Posture	26	4 (15%)	13 (50%)
Salt loading	6	1 (17%)	1 (17%)
AVP	21	6 (29%)	12 (52%)
Metoclopramide	29	3 (10%)	7 (24%)
LHRH	30	5 (17%)	5 (17%)
TRH	29	2 (7 %)	3 (10%)
Glucagon	26	4 (15%)	4 (15%)
Mixed meal	32	2 (6%)	6 (18%)
Multiple responses			n
0			8
1			10
2			9
3			5
4			1
5			2

Figure 1: *In vivo* induction of cortisol production following administration of hormonal stimuli



Patients were tested for the presence of aberrant expression of eutopic or ectopic hormone receptors by the administration of hormonal stimuli in various tests indicated on the X-axis. Data are represented as mean+SEM. *P<0.05, **P<0.01, ***P<0.001 compared to basal level (Wilcoxon signed rank). Meto: metoclopramide.

cortisol to the upright posture test. The morning cortisol levels were inversely associated with the cortisol responses to $ACTH_{1-24}$ (r=-0.514, P=0.004) and a standard mixed meal (r=-0.516, P=0.004). These ACTH and mixed meal responses were also highly correlated (r=0.966, P<0.001). The patients who responded positively to LHRH were less likely to have hypertension than those who failed to respond (25% vs. 80%, P=0.022). There were no other significant relations between responsiveness to the different stimuli and clinical characteristics in the AIMAH patients. As expected, midnight serum cortisol levels, cor-

tisol following dexamethasone overnight and cortisoluria were all higher in patients with clinical CS compared to subclinical CS (all P<0.001). However, there were no differences in the degree of cortisol induction following the hormonal stimuli between patients with subclinical and clinical CS.

Short-term effects on steroidogenesis

Primary cultures were successfully created from the resected adrenal tissues of 17 out of 22 (77%) operated patients. Short-term (2h) incubations were performed in all cultures (Table 3). Incubation with ACTH (500 pg/ml) in 13 cultures gave a partial response in 13% and full response in 62%. Augmented supernatant cortisol levels were found in 3 out of 7 cultures (43%) incubated with 1 μM metoclopramide. In comparison, short-term AVP incubation gave a full response in only 1 out of 4 (25%) primary cultures. In this model, hCG, norepinephrine, glucagon, desmopressin, angiotensin II and TSH did not augment cortisol secretion above 50% in any of the cultures investigated, as summarized in Table 3. The mean induction of cortisol *in vitro* was significant after 50 and 500 pg/ml ACTH (3.14±0.81-fold, P=0.0039 and 3.19±1.06-fold, P=0.0005, respectively), FSK (3.44±1.36-fold, P=0.0029) and norepinephrine (1.16±0.04-fold, P=0.016), but not for the other stimuli, see Figure 2. Overall associations between hormonal effects obtained *in vivo* and *in vitro* were not significant for any of the pathways investigated (Table 3).

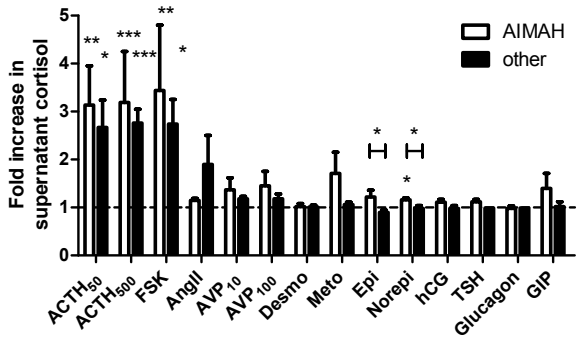
Short-term incubations were also performed with cells from 19 other adrenal tissues, consisting of 1 normal adrenal gland, 10 ACTH-dependent hyperplasias and 8 adrenocorti-

Table 3: Two hour *in vitro* cortisol responses to hormonal stimuli in primary adrenal cell cultures

	AIMAH					Non-AIMAH		
	dose	n	Partial response (%)	Full response (%)	Correlation with *in vivo* test	n	Partial response (%)	Full response (%)
$ACTH_{1-24}$	50 pg/ml	9	2 (22%)	6 (67%)	0.30	5	1 (20%)	4 (80%)
$ACTH_{1-24}$	500 pg/ml	13	4 (31%)	8 (62%)	0.07	18	1 (6%)	16 (89%)
FSK	1 μM	11	-	9 (82%)	0.29	9	-	7 (78%)
AngII	10 nM	2	-	-	n.a.[#]	2	1 (50%)	1 (50%)
AVP	10 nM	6	2 (33%)	1 (17%)	-0.72[†]; -0.50[#]	4	1 (25%)	-
AVP	100 nM	4	1 (25%)	1 (25%)	-1.00[†]; -0.80[#]	3	1 (33%)	-
Desmopressin	10 nM	5	-	-	-0.80	3	-	-
Metoclopramide	1 μM	7	-	3 (43%)	0.71	14	3 (21%)	-
Epinephrine	1 μM	7	1 (14%)	1 (14%)	-0.50[#]	5	-	-
Norepinephrine	1 μM	7	1 (14%)	-	-0.15[#]	5	-	-
hCG	100 IU/ml	10	3 (30%)	-	0.60	4	-	-
TSH	10 nM	4	-	-	-0.32	1	-	-
Glucagon	100 nM	4	-	-	0.50	1	-	-
GIP	10 nM	5	2 (40%)	1 (20%)	0.32[¶]	4	1 (25%)	-

[¶] standard mixed meal, [#] upright posture test, [†] AVP im, n.a.: not applicable

Figure 2: Short-term (2 hour) *in vitro* induction of cortisol following addition of hormonal stimuli



Primary cell cultures from AIMAH tissues (white bars) or from normal adrenals, ACTH-dependent adrenocortical hyperplasia or adenomas (other, black bars) were incubated in the presence of specific hormone receptor agonists indicated on the X-axis. Desmo: desmopressin, meto: metoclopramide, epi: epinephrine, norepi: norepinephrine. Supernatant cortisol levels were measured 2 hours after incubation. Concentrations used have been summarized in Table 3. Data are represented as mean+SEM. *P<0.05, **P<0.01, ***P<0.001 compared to basal level (Wilcoxon signed rank) or between pathological entities (Mann-Whitney U test).

cal adenomas. Results are also summarized in Table 3 and Figure 2. When comparing the mean effects, only the responses to epinephrine (1.22 vs. 0.90, P=0.023) and norepinephrine (1.16 vs. 0.97, P=0.023) were significantly higher in AIMAH cultures than in the non-AIMAH cultures. Cortisol levels were not stimulated in any of the primary cultures of non-AIMAH origin following the incubation with hCG, epinephrine, norpepinephrine, glucagon, desmopressin or TSH (Table 3).

Long-term effects on steroidogenesis

Of the total of 17 primary adrenal cell cultures 7 AIMAH tissues yielded a sufficient number of cells to also perform concurrent 48 hour incubations with several hormonal stimuli. Supernatant cortisol levels were detectable in all cultures of AIMAH cells. Table 4 shows the responses obtained for cortisol after 48 hours. Again AVP (86%) and metoclopramide (50%) were the stimuli that most often led to >50% increases in supernatant cortisol levels. The mean induction of cortisol was only significant following the addition of ACTH (4.55±1.51-fold, P=0.016) and 100 nM AVP (2.21±0.32-fold, P=0.016), see Figure 3; the sample sizes for the other groups were small.

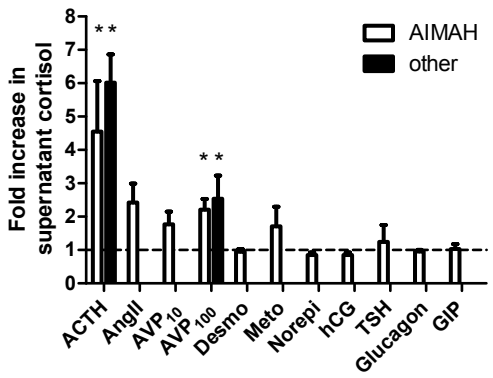
Again, there was a poor overall correlation between effects obtained *in vivo* and *in vitro,* Table 4. There was one significant correlation between cortisol induction by upright posture and AngII, but this was only available for 3 patients. Similarly, there were significant associations between the 2 and 48 hour incubations for both metoclopramide and desmopressin (both n=3).

Table 4: Forty-eight hour *in vitro* cortisol responses to hormonal stimuli in AIMAH cells

	AIMAH					
	dose	n	Partial response n (%)	Full response n (%)	Correlation with *in vivo* test	Correlation with t=2 hr
$ACTH_{1-24}$	10 ng/ml	7	-	6 (86%)	-0.39	0.20
AngII	100 nM	4	-	4 (100%)	1.00**#	n.a.
AVP	10 nM	3	-	2 (67%)	0.50†; n.a.#	n.a.
AVP	100 nM	7	1 (14%)	6 (86%)	-0.18†; -0.086#	-0.50
Desmopressin	10 nM	5	-	-	-0.67	1.00**
Metoclopramide	1 µM	4	-	2 (50%)	0.60	1.00**
Norepinephrine	1 µM	4	-	-	-0.50#	-0.50#
hCG	100 IU/ml	4	-	-	-0.60	-0.50
TSH	10 nM	2	-	1 (50%)	n.a.	n.a.
Glucagon	1 µM	4	-	-	-0.20	n.a.
GIP	100 nM	2	-	-	-1.00¶	n.a.

**P<0.01, ¶ standard mixed meal, # upright posture test, † AVP im, n.a. not applicable

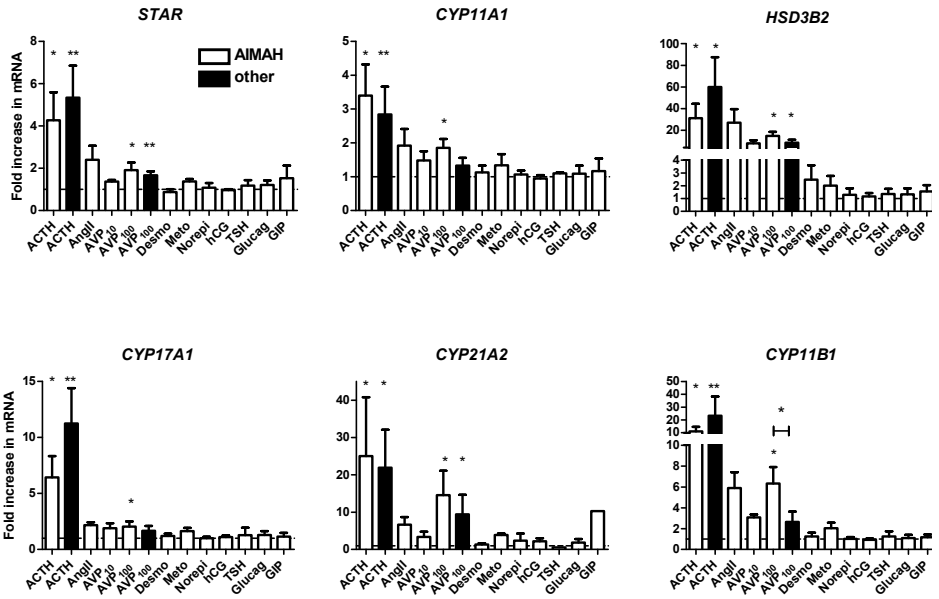
Figure 3: Long-term (48 hour) *in vitro* induction of cortisol following addition of hormonal stimuli



Primary cell cultures from AIMAH tissues (white bars) or from normal adrenals, ACTH-dependent adrenocortical hyperplasia, adenomas or carcinomas (other, black bars) were incubated in the presence of specific hormone receptor agonists depicted on X-axis. Desmo: desmopressin, meto: metoclopramide, norepi: norepinephrine. Concentrations used are summarized in Table 4. Data are represented as mean+SEM. *P<0.05, compared to basal level (Wilcoxon signed rank).

Furthermore, we evaluated the effects of the hormonal stimuli on mRNA levels of the cholesterol transporter *STAR* and of the steroidogenic enzymes *CYP11A1*, *HSD3B2*, *CYP17A1, CYP21A2* and *CYP11B1* (Figure 4). ACTH (n=7) stimulated the expression of the cholesterol transpoter and all steroidogenic enzymes studied in the AIMAH cultures. AVP at 100 nM (n=5) also significantly increased mRNA levels of all enzymes. Although stimulatory effects were also found for the other secretagogues, especially for AngII, 10 nM AVP and metoclopramide, these effects were not significant, in part due to the small sample size (n≤4).

Figure 4: Long-term (48 hour) *in vitro* induction of *STAR* and steroidogenic enzyme mRNAs following addition of hormonal stimuli



Primary cell cultures from AIMAH tissues (white bars) or from normal adrenals, ACTH-dependent adrenocortical hyperplasia, adenomas or carcinomas (other, black bars) were incubated in the presence of hormones depicted on X-axis. Desmo: desmopressin, meto: metoclopramide, norepi: norepinephrine, glucag: glucagon. Concentrations used are summarized in Table 4. The mRNA levels of steroid acute regulatory protein (*STAR*), cytochrome P450 (CYP)-side chain cleavage (*CYP11A1*), 3β-hydroxysteroid dehydragenase type 2 (*HSD3B2*), *CYP17A1*, *CYP21A2* and *CYP11B1* were studied by quantitative RT-PCR. Data are represented as mean+SEM. *p<0.05, **p<0.01, compared to basal level (Wilcoxon signed rank) or between pathological entities (Mann-Whitney U test).

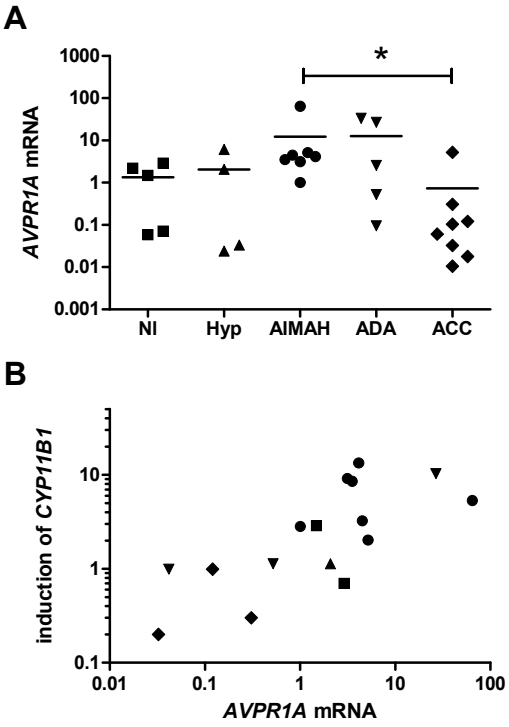
Since AVP was the most prevalent stimulus leading to responses *in vivo* we focused further on AVP and concurrently measured AVP effects in adrenal cells of non-AIMAH origin (e.g. 3 normal adrenals, 1 ACTH-dependent hyperplasia, 3 adenomas and 4 carcinomas). Although cortisol levels were below assay detection levels (28 nmol/l) in the media of cultures from one adenoma and 3 carcinomas, AVP at 100 nM significantly increased cortisol secretion in the remaining 7 primary adrenal cell cultures (2.54±0.69-fold, P=0.016). AVP induced a >50% increase in supernatant cortisol levels in 5 out of 7 (71%) of non-AIMAH adrenal cultures, whereas the other two cultures showed a partial response to stimulation with AVP. The effects on steroidogenic enzyme mRNAs obtained after the addition of AVP were comparable between cultures from AIMAH cells and other adrenocortical tissues, with the exception of *CYP11B1*. Forty-eight hours of culture in the presence of AVP stimulated *CYP11B1* expression 6.34±1.57-fold in AIMAH cells, compared to a 2.66±0.97-fold induction in cells of non-AIMAH adrenal origin (P=0.033). ACTH responsiveness with

respect to stimulation of cortisol or steroidogenic enzyme mRNAs did not differ between AIMAH and non-AIMAH cultures (Figures 2-4).

Vasopressin receptor expression

Expression of mRNA of vasopressin receptors (*AVPR1A*, *AVPR2*, *AVPR1B*) was studied in a panel of adrenocortical tissues, consisting of 7 AIMAHs, 5 normal adrenals, 4 ACTH-dependent hyperplasias, 5 adenomas and 8 carcinomas. The vasopressin type 3 receptor (V_3, *AVPR1B*) was not detectable in any of the adrenocortical tissues studied, whereas the type 2 receptor (V_2, *AVPR2*) mRNA was detectable in 10 out of the 29 samples, distributed among the five types of tissues, but at very low levels (Ct values>38). *AVPR1A* (V_1) mRNA was readily detectable in all samples and was found to be higher in AIMAH samples compared to the adrenocortical carcinomas (P=0.037, Figure 5A). V_1 levels in the 7 AIMAH patients studied were not associated with clinical characteristics or *in vivo* cortisol induction

Figure 5: Vasopressin receptor expression in adrenal tissues



(A) Vasopressin type 1a receptor (V_1, *AVPR1A*) mRNA expression in human adrenocortical tissues, measured by qRT-PCR. *P<0.05, Kruskal-Wallis test. V_2 and V_3 receptor expression levels were extremely low or undetectable. (B) Significant correlation between *AVPR1A* expression levels and the induction of *CYP11B1* by 100 nM AVP after 48 hours in cultures of primary cells of adrenal tissues (r=0.76, P=0.006). Corresponding icons for individual tissue groups are depicted in Figure A and B.

following upright posture or AVP administration P>0.05). Furthermore, *AVPR1A* mRNA was not correlated with the induction of cortisol production following the addition of 100 nM AVP after 2 or 48 hours. On the other hand, there were significant relations between *AVPR1A* levels in AIMAH and non-AIMAH tissues and the *in vitro* induction of *CYP11B1* by 100 nM AVP (r=0.76, P=0.006, Figure 5B), but not for the other steroidogenic enzymes.

DISCUSSION

ACTH-independent CS due to macronodular hyperplasia has been linked to the presence of aberrantly expressed eutopic and ectopic receptors on adrenocortical cells. In the current study, encompassing the largest cohort of AIMAH patients described thus far, we have systematically measured hormonal responses in patients with bilateral adrenal disease and ACTH-independent (sub-)clinical CS. These effects were also investigated in a significant series of *in vitro* studies on both short-term and long-term steroidogenesis.

Our data show that ACTH is still the most potent stimulus for steroidogenesis in AIMAH. Both *in vivo* and *in vitro* ACTH stimulated cortisol production in almost all patients. The induction of cortisol production was comparable in primary cultures of AIMAH and non-AIMAH origin. We have previously demonstrated that *MC2R* levels are equal in normal adrenals, ACTH-dependent hyperplasia, AIMAH, adrenocortical adenomas and carcinomas (Chapter 2). Negative feedback at the hypothalamic and pituitary levels causes undetectable levels of ACTH in most AIMAH patients. Since ACTH increases adrenal expression of the MC2R and ACTH sensitivity,[28-29] lower levels of ACTH signaling would be expected. Possibly, other GPCR-coupled pathways in AIMAH cells stimulate cAMP formation and thereby *MC2R* expression. On the other hand, low plasma ACTH levels can be detected in a subset of patients; here ACTH could still play a role in causing hypercortisolism.

The most prevalent exaggerated responses to hormonal stimulation *in vivo* were to AVP and upright posture, confirming the findings of the previous large AIMAH study.[19] Although the first studies were unclear on which vasopressin receptor was causative for this response,[11, 30] several studies have now demonstrated the presence of the eutopically expressed type V_1 receptor.[12, 31] Using a quantitative approach, we found that the levels of the type V_2 and V_3 receptors were (near to) undetectable. Furthermore, *in vitro* studies revealed no effects of desmopressin, a selective vasopressin type 2 receptor agonist. Although no overall significant differences in *AVPR1A* mRNA levels were detected between normal, ACTH-dependent hyperplasia and AIMAH in this and other studies,[12, 31] individual patients could still overexpress the V_1 receptor as we and other have previously described.[30, 32-33] *In vitro* comparisons of AVP effects showed an equal induction of cortisol secretion in AIMAH and non-AIMAH cells, questioning whether the response to AVP indeed represents an aberrant effect. AVP also induced mRNA expression of the

cholesterol transporter StAR and four steroidogenic enzymes uniformly in these two groups, suggesting that AVP has a physiological effect on adrenocortical steroidogenesis. However, the selective stimulation of *CYP11B1*, a key enzyme in cortisol synthesis, in AIMAH by AVP suggests a novel molecular mechanism underlying the coupling between the V_1 receptor and steroidogenesis in AIMAH. This suggestion is supported by the association between *AVPR1A* levels and the AVP-induced expression of 11β-hydroxylase. These findings in AIMAH might open up new opportunities for medical treatment with selective V_1 receptor antagonists, such as relcovaptan.[34] Adrenocortical carcinomas seem to have an impaired response to AVP, possibly due to decreased expression of the type V_1 receptor, which was found in the current and a previous study.[31]

The upright posture test was positive in half of the patients studied *in vivo*. Of the 11 patients with a positive response to upright posture, 7 reacted also positively to AVP intramuscularly. The other 4 patients could have reacted aberrantly to surges in catecholamines or AngII.[3] Interestingly, in the short-term *in vitro* experiments we found a difference in responsiveness to both epinephrine and norepinephrine between AIMAH and non-AIMAH cells. In contrast to AVP and 5-HT, this would suggest the presence of ectopic adrenergic receptors. Previous studies revealed that this could be related to adrenocortical expression of β_1-, β_2- or α_4-adrenergic receptors.[9-10]

The other prevalent hormonal response in AIMAH patients was that to metoclopramide, an agonist of the serotonin type 4 receptor (5-HT4R). Physiologically, 5HT4R is expressed in the adrenal gland and can affect cortisol production in an autocrine or paracrine manner.[35-36] In the present study, 34% of patients had a >25% stimulation of serum cortisol levels following metoclopramide, which was lower than the 56% observed in the French study.[19] *In vitro*, this response was also found for a large set of AIMAH samples but also to a lesser extent in controls. Moreover, there was no significant difference in response between AIMAH and non-AIMAH cells *in vitro*, questioning whether there is truly an aberrant response to 5-HT in AIMAH patients.

Other hormonal stimuli can lead to a stimulation of serum cortisol in a minority of AIMAH cases. For LHRH, TRH, glucagon and GIP we have found that this constitutes 10-22% of patients. Interestingly, the patients that responded to LHRH had a lower prevalence of hypertension than the non-responders. The pathophysiology behind this observation is unclear. *In vitro*, we found no large effects of these stimuli on cortisol production besides in individual cases.

The clinical description of AIMAH cases has often been coupled to *in vitro* investigations on patient tissue samples. In this manner, direct stimulating effects of the hormones can be reproduced. We have evaluated *in vitro* responses in 17 primary cultures of AIMAH tissues. Taken together, there is a poor correlation between clinical and experimental responses to individual stimuli. This is in contrast to the previous studies in which common effects were obtained *in vivo* and *in vitro*. Possible causes include publication bias of those patients

in which effects could be replicated *in vitro* and the concentrations of stimuli used or the experimental set-up. With respect to the latter cause, we also found clear differences and an overall lack of association between short-term and long-term effects of the stimuli on cortisol concentrations. Associations across different experimental set-ups were only detected in the experiments with metoclopramide. These conclusions should however be drawn with caution due to the small sample sizes of cultures in some of the short- and long-term experiments. Besides the rarity of the disease, the low percentage of patients being operated due to clinical disease and the multitude of testable hormonal stimuli hamper large *in vitro* studies with all of the possible secretagogues in AIMAH patients.

The presence of eutopic or ectopic hormone receptor expression is thought to underlie the occurrence of AIMAH. Responses to the different hormonal stimuli were not associated with *in vivo* hormonal activity since patients with clinical and subclinical CS showed comparable results. This is a confirmation of the French study,[19] in which they stated that this finding suggests that clinical and subclinical CS represent a continuum of disease rather than two separate entities. Exaggerated responses to hormonal stimuli have been reported for multiple cases and case series, although the responses in healthy individuals have not been investigated for all hormones. It is therefore uncertain to what extent *in vivo* responses are the result of aberrant expression patterns of hormone receptors. The current *in vitro* data obtained in the non-AIMAH tissues suggest significant effects of AVP, AngII and possibly 5-HT. Unfortunately, there are no systematic comparisons for *in vivo* responses between healthy individuals and AIMAH patients, which could shed more light on the definition of an aberrant response. The arbitrarily used criterion of >50% elevation in serum cortisol could also be modified if effects in healthy individuals would be identified.

In conclusion, multiple hormonal responses frequently occur in AIMAH patients, with AVP and 5-HT most commonly leading to aberrant eutopic responses. AVP induces steroidogenic enzyme expression in both AIMAH and non-AIMAH adrenocortical tissues. However, in AIMAH there appears an aberrant coupling of normal levels of *AVPR1A* to the induction of *CYP11B1* expression that may be involved in the pathogenesis of AVP-mediated cortisol overproduction. Catecholamines appear to represent the most prevalent ectopic response in AIMAH patients. Furthermore, there are poor overall correlations between *in vivo* and *in vitro* studies of AIMAH patients and tissues.

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Regulation of steroidogenesis in a primary pigmented nodular adrenocortical disease-associated adenoma leading to virilization and subclinical Cushing's syndrome

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Submitted

ABSTRACT

Background:

Primary pigmented nodular adrenocortical disease (PPNAD) can lead to steroid hormone overproduction. Mutations in the cAMP-protein kinase A (PKA) regulatory subunit type 1A (PRKAR1A) are causative for PPNAD and its related syndrome Carney complex. Steroidogenesis in PPNAD can be modified through a local glucocorticoid feed-forward loop. The regulation of steroidogenesis in a case of PPNAD with virilization was investigated.

Methods:

A 33-year old woman presented with primary infertility due to hyperandrogenism. Elevated levels of testosterone and subclinical ACTH-independent Cushing's syndrome led to the discovery of an adrenal tumor, which was diagnosed as PPNAD. *In vivo* evaluation of aberrantly expressed hormone receptors showed no steroid response to known stimuli. Genetic analysis revealed a PRKAR1A protein-truncating Q28X mutation. After adrenalectomy steroid levels normalized and the patient conceived. Tumor cells were cultured and steroidogenic responses to ACTH and dexamethasone were measured and compared to those in normal adrenal and adrenocortical carcinoma cells. mRNA levels of 17β-hydroxysteroid dehydrogenase (HSD) types 3 and 5 and the glucocorticoid and androgen receptors were quantified in PPNAD, normal adrenal and adrenal adenoma tissues. Proteins of 17β-HSD types 3 and 5 and β-catenin were stained in the PPNAD tissue by immunohistochemistry.

Results:

Isolated PPNAD cells, analogous to normal adrenal cells, showed both increased steroidogenic enzyme expression and steroid secretion in response to ACTH. Dexamethasone did not affect steroid production in the investigated types of adrenal cells. 17β-HSD types 3 and 5 and steroid receptor mRNAs were expressed in PPNAD tissues, but not upregulated compared to other adrenocortical samples.

Conclusions:

PPNAD-associated adenomas can cause virilization and infertility by adrenal androgen overproduction. This may be due to different steroidogenic control than what has been described in PPNAD without large adenomas.

INTRODUCTION

Primary pigmented nodular adrenocortical disease (PPNAD) constitutes a rare cause of adrenocortical hyperplasia and adrenocorticotropin (ACTH)-independent Cushing's syndrome. PPNAD can occur sporadically or in conjunction with other tumors in Carney complex.[1] Known genetic causes of PPNAD and Carney complex are mutations in components of the cAMP-protein kinase A (PKA) pathway: *PRKAR1A*[2], *PDE11A*[3] and *PDE8B.*[4] The net effect of these mutations is increased activity of the PKA catalytic subunits.[2] Aberrant cAMP-PKA signaling in the adrenal cortex leads to hyperplasia, the formation of multiple pigmented nodules, and the sporadic formation of a large tumor. The latter has been linked to mutations in *CTNNB1*, which leads to constitutive activation and nuclear translocation of its product β-catenin.[5] Other manifestations of Carney complex include lentiginosis, myxomas, and pituitary, thyroid and testicular tumors.[6]

PPNAD is the most common endocrine manifestation of Carney complex[7] and can lead to ACTH-independent Cushing's syndrome.[8] Testosterone hypersecretion from PPNAD has also been described in two female patients.[9] In addition, the autonomous cortisol production by the adrenal nodules reacts paradoxically to dexamethasone administration in 69% of patients during the course of Liddle's test,[8] due to increased glucocorticoid receptor (GR) expression[10] and possible specific interactions between the GR and the PKA catalytic subunits.[11]

Macronodular adrenal hyperplasia with autonomous cortisol production can be associated with aberrant expression of one of several eutopic and ectopic G-protein coupled receptors (GPCRs) which are functionally coupled to steroidogenesis.[12-13] This includes receptors for arginine-vasopressin (AVP), gastric inhibitory polypeptide and luteinising hormone (LH)[12] that, when activated by their ligands, stimulate cortisol production. Eutopic or ectopic GPCR expression does not appear to play a major role in controlling steroidogenesis in PPNAD, unlike in macronodular hyperplasia,[14] although clinical testing for aberrant responses has only been reported for two PPNAD patients.[10]

Here we describe a patient presenting with virilization and subclinical Cushing's syndrome due to PPNAD and a single adenoma that developed in her right adrenal. We performed several *in vivo* stimulation tests to screen for eutopic or ectopic stimuli that could possibly regulate the peculiar hypersecretion of cortisol and androgens. To further obtain insight into the regulation of steroidogenesis in this single tumor, *in vitro* studies were performed in which we examined the effects of ACTH and dexamethasone on steroidogenic enzyme expression and steroid production. In addition, mRNA expression of the testosterone-producing enzymes 17β-hydroxysteroid dehydrogenase (HSD) types 3 and 5 as well as of the glucocorticoid and androgen receptors was measured in PPNAD tissue as well as in normal, hyperplastic and adenomatous adrenal tissues. Both 17β-HSD enzymes also stained in the virilizing PPNAD tissue using immunohistochemistry.

PATIENT AND METHODS

Clinical case

A 33-year old Caucasian woman was referred to our department because of primary infertility and hyperandrogenism. The patient had been investigated for infertility for several years. Two years before referral, fertility screening showed no abnormalities in the patient or her partner. Six intra-uterine insemination sessions and an in vitro fertilization attempt did not result in pregnancy. The patient was then referred to the department of gynecology of our hospital for a second opinion; here laboratory analysis showed an increased serum level of testosterone.

The patient had menarche at the age of 13 years. Soon thereafter, she started using oral contraceptives because of facial acne and hirsutism. Seven years before presentation the patient stopped oral contraceptive use and regained regular menstrual cycles. She noticed increased and coarse hair on her face, abdomen and upper legs with concomitant frontotemporal hair loss. During the past years libido had increased and her clitoris grew larger. Her past medical history and family history were unremarkable nor did she take any medication or hormonal preparations.

Upon physical examination, the patient displayed a female phenotype with overt hirsutism and a male pattern baldness. Her extremities and torso were covered with multiple lentigines; clitoromegaly was confirmed upon pelvic examination. Endocrinological evaluation showed increased levels of testosterone, 17-hydroxyprogesterone (17-OHP) and a suppressed ACTH level (Table 1). Morning and midnight cortisol levels were 263 and 246 nmol/l, respectively. Cortisol and androgen levels were not adequately suppressed after the overnight 1 mg dexamethasone test. Abdominal CT subsequently showed a nodular enlargement in the right adrenal (19x14 mm). Hounsfield-units measured 45 at basal, rising to 135 after intravenous administration of contrast. MRI confirmed the right adrenal nodule (Figure 1A) with increased signal on the T2-weighted image, which enhanced after intravenous gadolinium administration. No signal loss was observed during the wash-out phase.

The patient was tested for aberrant hormone receptor expression by measuring cortisol, 17-OHP, androstenedione and testosterone at several time points following: LH-releasing hormone (100 µg intravenously), thyrotropin-releasing hormone (200 µg iv), glucagon (1 mg iv), metoclopramide (10 mg iv) and AVP (10 IU intramuscularly) administration, a standard mixed meal (116 g carbohydrates, 27 g proteins, 14 g fat) and an upright posture test.[15] The patient failed to show an increase of steroid levels of >50% after stimulation by the above mentioned procedures.

The patient underwent an open right-sided adrenalectomy because of the suspicion of adrenocortical cancer. Post-operative testing showed non-detectable testosterone, dehydroepiandrosterone (DHEA)-sulfate and ACTH levels, whereas 17-OHP and andro-

Table 1: Serum hormone levels

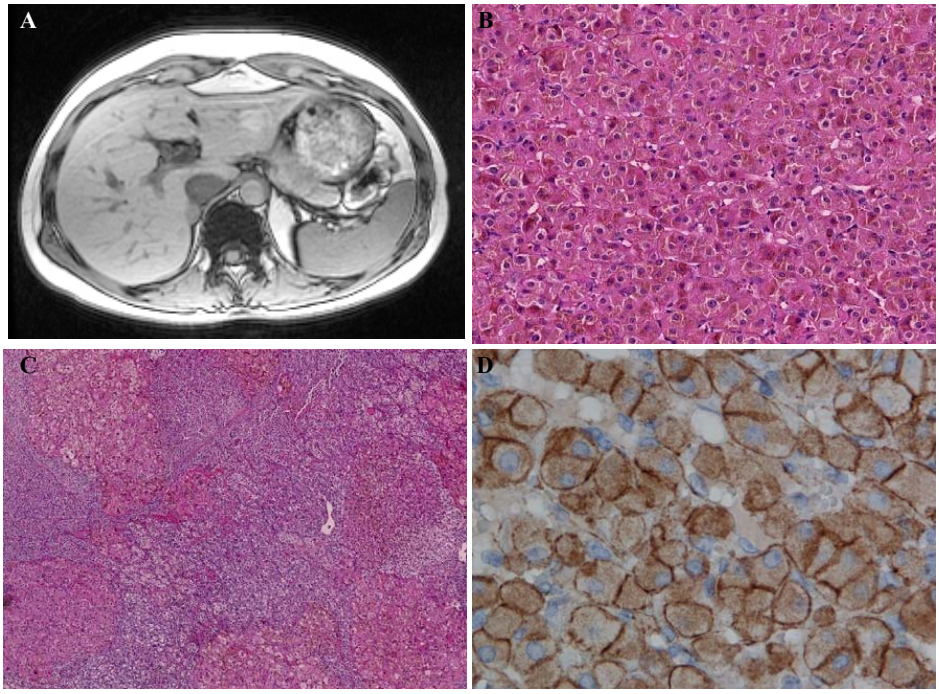
			Pre-operative			Post-operative	
	unit	*reference values*	*at presentation*	*after 250 µg synacthen*	*1 mg dex overnight*	*1 week*	*27 months*
cortisol	nmol/l	200-800	263	419	298	226	337
testosterone	nmol/l	0.5-3	9.7	10.1	7.5	<0.1	0.4
17(OH)progesterone	nmol/l	0.5-10	30	31	25.9	3.1	2.2
androstenedione	nmol/l	2-15	14	17.4	14.5	1.14	<1.05
11-deoxycortisol	nmol/l	0-50	40	48		21	
DHEA	nmol/l	1.4-25	7.2	12.5		4.1	5.3
DHEA-sulfate	umol/l	0.8-10	1.00	1.01		<0.41	0.52
estradiol	pmol/l	100-1000	256			279	
ACTH	pmol/l	0-11	<1.1			<1.1	11.7
LH	U/l	2-8	4.4			1	
FSH	U/l	1-8	9.2			1.4	
urine cortisol	nmol/day	0-850	455				

stenedione concentrations were markedly diminished (Table 1). Pathological examination of the right adrenal showed a “black”, round adenoma that measured approximately 2 cm in diameter. Microscopically the tumor consisted of eosinophilic cells with extensive granular pigmentation (Figure 1B). The tumor was embedded within a hyperplastic cortex peppered with multiple other pigmented nodules (Figure 1C). The histopathologic picture of the remaining cortex was consistent with that of PPNAD.[1] Immunohistochemistry of β-catenin, using a mouse monoclonal antibody (#610154, diluted 1:200, BD Biosciences[16]), displayed a membranous and cytoplasmic staining in both the adenoma and the surrounding cortex (Figure 1D).

Leukocyte DNA sequencing, performed as published elsewhere,[17] revealed a previously reported PRKAR1A protein-truncating Q28X mutation,[18] confirming the diagnosis of PPNAD. Separate sequencing of microdissected adenoma and PPNAD tissues, using methods[19] and primers[5] previously reported, subsequently showed no mutations in exon 3 of *CTNNB1*.

Screening of the patient for pituitary tumors and for cardiac myxoma was negative, whereas the patient did suffer from several non-functional benign thyroid nodules and fibro-adenomas in both breasts. No family members showed signs of Carney complex-associated disease. Six months after operation, the patient reported to be 7 weeks pregnant. At 39 weeks pregnancy she successfully gave birth to a healthy boy. Endocrinological evaluation 2 years after operation showed a normal HPA-axis and normal levels of androgens (Table 1).

Figure 1: Radiological and microscopical characteristics of the PPNAD



(A) Abdominal T1-weighted MRI imaging revealed the presence of a hyperintense lesion in the right adrenal (arrow). Photomicrographs of the resected right adrenal: (B) Large eosinophilic cells comprising the large black node. Several areas with increased intracellular pigmentation can be appreciated (magnification 200x). (C) Several smaller nodules were found dispersed in the remaining adrenal cortex (magnification 50x). (D) Immunohistochemical staining for beta-catenin showed membrane and cytoplasmic staining of the adenoma (magnification 800x).

Control samples

Patient samples for *in vitro* examination were also obtained from one normal adrenal cortex obtained at radical nephrectomy due to renal cell carcinoma, from two patients with adrenocortical hyperplasia due to metastasized ACTH-producing neuro-endocrine tumors and from four patients with adrenocortical carcinoma. Analysis of the normal and hyperplastic adrenal samples were combined in the group designated as “normal”.

Three other patients with histologically proven PPNAD and clinical Cushing’s syndrome were identified and adrenal samples were subsequently used for mRNA analysis. These encompassed a 33-year old female (PPNAD2), a 24-year female (PPNAD3) and a 24-year old male (PPNAD4). PPNAD2 also showed no significant increases in cortisol production following *in vivo* testing for aberrantly expressed GPCRs. None of the PPNAD patients underwent the full Liddle’s test, but subjects PPNAD3 and PPNAD4 did undergo a 7 mg intravenous dexamethasone test, which increased cortisol levels by 19% and 13%, respectively. Other samples for mRNA analysis of the 17β-HSD types 3 (*HSD17B3*) and

5 (*AKR1C3*) and steroid receptors included normal adrenals (n=7), ACTH-dependent hyperplasia (n=10), and clinically non-functional (n=3), cortisol- (n=6) and aldosterone-secreting (n=6) adrenocortical adenomas.

Informed consent was obtained from all patients prior to operation. The study was approved by the medical ethical committee of the Erasmus MC and the DNA studies were completed under an approved *Eunice Kennedy Shriver* National Institute of Child Health & Human Development clinical protocol.

Tissue processing

Shortly after resection, adrenal tissue samples were snap-frozen and kept at -80 °C for mRNA analysis or put in DMEM-F12 (Invitrogen) containing 5% fetal calf serum (FCS), penicillin and streptomycin (Invitrogen) for purposes of primary culture. A monolayer culture was obtained by treating the tissue with collagenase (Sigma-Aldrich) as described previously.[20] After allowing the cells to attach overnight the medium was changed to serum free medium. The next day cells were incubated with vehicle, 10 ng/ml $ACTH_{1-24}$ (Novartis, Basel, Switzerland) or 1 µM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). After 48 hours of incubation the supernatants were removed and stored at -20 °C; cells were simultaneously snap-frozen and stored at -80 °C. Hormone measurements were performed as previously reported.[19]

mRNA and protein analysis

RNA was isolated from plated cells and homogenized frozen adrenal tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA measurement, reverse transcriptase reactions and quantitative polymerase chain reactions (qPCR) were performed as described previously.[21] The qPCR was performed in a 12.5 µl volume for *HPRT1, STAR, CYP11A1, HSD3B2, CYP17A1, CYP21A2, CYP11B1,*[21] *AKR1C3, HSD17B3* and steroid receptors *GR* and *AR* (Assay on Demand, Hs00366267_m1, Hs00970002_m1, Hs00230818_m1 and Hs00907242_m1, Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Expression levels were calculated relative to that of the housekeeping gene *HPRT1*, expression of which was stable between incubations and the different groups of tissues, using the delta-Ct method.

Immunohistochemistry of HSD17B3 and AKR1C3 protein was performed with methods equal to that of β-catenin staining, using monoclonal antibodies purchased from Sigma (HPA015307, 1:30) and Abcam (Cambridge, UK, ab49680, 1:3000), respectively.

Statistics

All data of steroid hormone levels and mRNAs were analyzed using paired t-test or one-way ANOVA with post-hoc Newman-Keuls multiple comparison test. Logarithmic conver-

sion was applied to obtain normality if necessary. Statistical significance was assumed at P<0.05.

RESULTS

Primary cultures

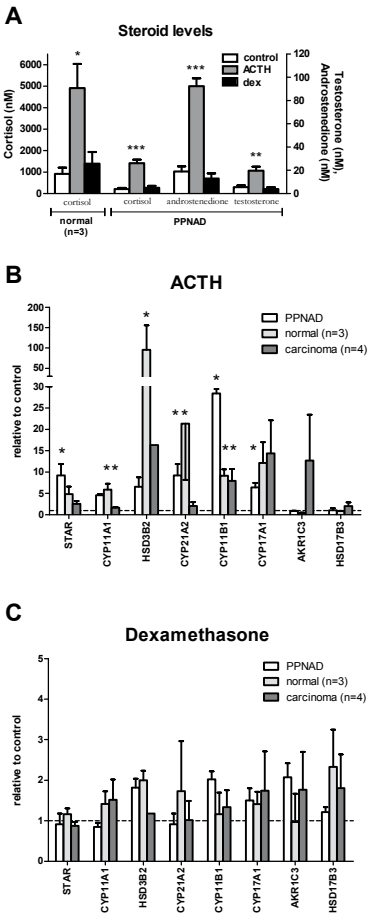
Primary cultures were obtained from patient's adrenal tissue, one normal adrenal, two hyperplastic adrenals and four adrenocortical carcinomas. Forty-eight hour incubation with ACTH of the patient's tumor cells led to an increase in cortisol (5.3-fold), androstenedione (4.9-fold) and testosterone (3.6-fold) levels (Figure 2A). The increase in cortisol secretion following ACTH stimulation in normal adrenal tissue was 6.8-fold. Dexamethasone incubation did not alter steroid levels compared to vehicle controls. All steroidogenic enzymes studied were present in cells from the PPNAD. Of the human 17β-HSD isoforms that are known to produce testosterone, *AKR1C3* mRNA expression levels were 68-fold higher than that of *HSD17B3* in the cultured PPNAD cells. mRNA analysis revealed that ACTH increased the expression of *STAR, CYP17A1, CYP21A2* and *CYP11B1* in the PPNAD (Figure 2B), whereas dexamethasone had no significant effect on steroidogenic enzyme mRNAs (Figure 2C). Also in normal and carcinomatous adrenals, ACTH stimulated steroidogenic enzyme expression whereas dexamethasone did not affect these mRNAs.

17β-HSDs and steroid receptors

The mRNA levels of *HSD17B3* and AKR1C3 were measured in the fresh frozen tissue samples of patient's tumor, three other PPNAD samples, normal adrenals and adrenocortical adenomas. Overall, adrenal *AKR1C3* levels were higher than those of *HSD17B3*. Within the PPNAD group, PPNAD1 had the increased levels of *AKR1C3* and *HSD17B3* (Figure 3, depicted as square). Compared to all other adrenal samples, the virilizing PPNAD had the highest expression of *HSD17B3*. Of the different adrenal tissues, non-functional adenomas had higher levels of *AKR1C3* mRNA expression compared to cortisol-producing adenomas (P<0.05, Figure 3). Immunohistochemistry of both 17β-HSDs revealed specific, cytoplasmic staining in the PPNAD tissue. Both proteins showed a heterogeneous staining pattern among the large adenoma, the smaller nodules and the remaining cortex. Positive staining of AKR1C3 was more prevalent and more intense than that of HSD17B3 (Figure 3).

Because of the paradoxical rise in cortisol after dexamethasone described in cases of PPNAD and the hyperandrogenism of the current PPNAD case we also measured GR and AR mRNAs in these samples. GR and AR mRNAs were expressed in all samples studied. GR expression in PPNAD did not differ from normal adrenals, adrenocortical hyperplasia or adenoma samples. There were no significant differences between AR expression levels in any of the tissues that were studied (Figure 3).

Figure 2: Steroid and steroidogenic enzyme levels in primary cultures of PPNAD and other adrenal lesions



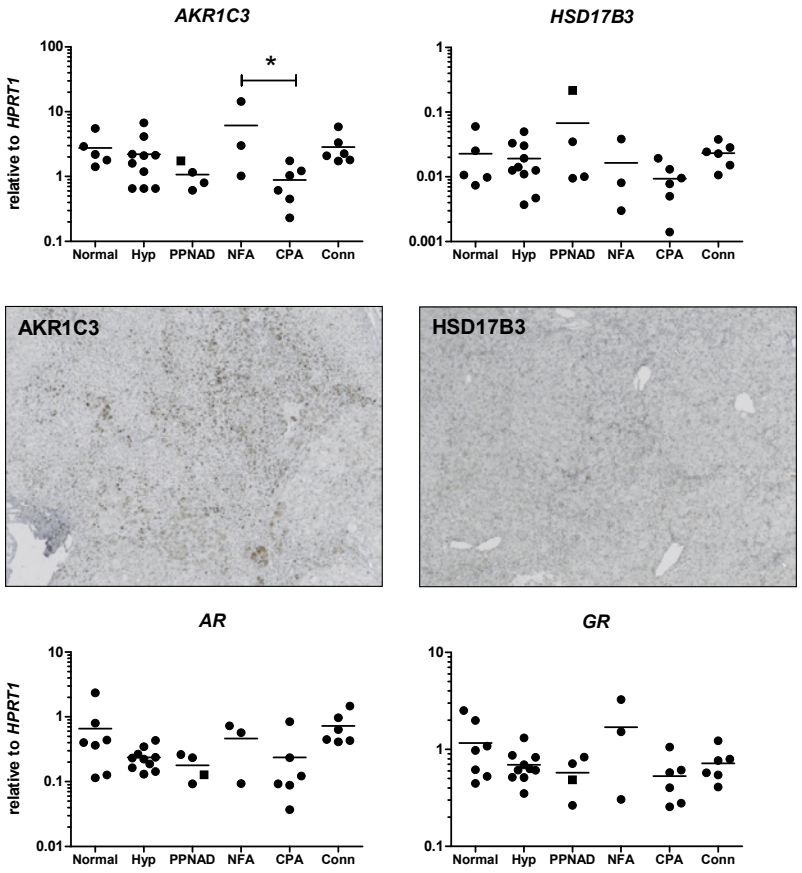
(A) Cortisol, androstenedione and testosterone levels in the supernatant of primary cultures following 48 hours of ACTH or dexamethasone incubation. mRNA levels of cholesterol transporter and steroidogenic enzymes in primary cultures of the PPNAD, normal adrenals (n=3) and adrenocortical carcinomas (n=4) following 48 hours of ACTH (B) or dexamethasone (C) incubation. * P<0.05, **P<0.01, *** P<0.001 compared to control. Data are expressed as mean ± SEM

DISCUSSION

Since the first description of Carney complex in 1985,[1] the genetic basis of the disease has been elucidated for the majority of the patients.[22] However, the factors controlling the development of clinically significant PPNAD in individual patients remain largely unknown.

A patient presenting with PPNAD with primary infertility has not been described previously. The high serum levels of androgens and androgen precursors, combined with the

Figure 3: 17β-HSDs and steroid receptors in adrenal tissues



AKR1C3, *HSD17B3*, *GR* and *AR* mRNA expression in normal adrenal glands (n=7), adrenocortical hyperplasia (Hyp, n=10), PPNAD (n=4), and non-functional (NFA, n=3), cortisol-producing (CPA, n=6) and aldosterone-producing (Conn, n=6) adrenocortical adenomas. The virilizing PPNAD is depicted as square. Expression is calculated relative to that of the housekeeping gene *HPRT1*. Bar denotes mean. * P<0.05. In the middle: immunohistochemistry of AKR1C3 and HSD17B3 showed a cytoplasmic, but heterogeneous staining pattern in the PPNAD-associated adenoma. The remaining cortex and smaller nodules showed a similar pattern, with AKR1C3 as the predominant 17β-HSD (magnification 50x).

absence of a cortisol diurnal rhythm and a suppressed ACTH, led to the discovery of an adrenal tumor with atypical radiological features. Since such high androgen levels have not been previously reported for PPNAD and the patient's left adrenal showed no abnormalities an adrenocortical carcinoma was suspected and an open adrenalectomy was subsequently performed. Removal of the tumor led to normalization of serum androgen levels and a successful pregnancy within a few months. The steroid-secreting PPNAD-associated adenoma was the cause of the patient's primary infertility, since the serum

steroid profile normalized after unilateral adrenalectomy and despite the remaining left adrenal presumably also affected by PPNAD.

Steroid overproduction in PPNAD usually results in a mild, ACTH-independent form of Cushing's syndrome.[8] As is evident from our case other steroids can also be produced in PPNAD, leading to a very different clinical presentation. Recent studies in adrenal cortex-specific *Prkar1a* knockout mice showed that PKA R1α loss is sufficient to drive PPNAD development and autonomous steroidogenesis. ACTH responsiveness remained intact in these mice.[23] Human adrenal cells with mutated *PRKAR1A* remain cAMP-responsive and increase PKA activity even more compared to non-mutated cells since *PRKAR1A* mutations lead to constitutive PKA activity.[2, 9] *In vivo* and *in vitro* studies of our patient showed that the PPNAD cells still possessed the ability to respond to ACTH; production of cortisol, androstenedione and testosterone increased after exposure to ACTH. The ACTH responsiveness was comparable between normal adrenocortical and the patient's PPNAD cells. The ACTH effect as measured by steroidogenic enzyme mRNA expression levels showed an augmented and diminished response for *CYP11B1* and *HSD3B2*, respectively, making it possible that there was an aberrant regulation of *CYP11B1* and *HSD3B2* in the patient's PPNAD cells.

Testosterone is mainly formed from androstenedione by 17β-HSD types 3 and 5, encoded by *HSD17B3* and *AKR1C3*, respectively.[24] Whereas HSD17B3 is the predominant testosterone-forming enzyme in the testis, AKR1C3 mainly ensures testosterone formation in the peripheral tissues, such as the adrenal cortex[25] and prostate gland.[26] Our patient showed particularly high mRNA levels of *HSD17B3* compared to all other adrenal tissues, but this could not be confirmed by immunohistochemistry. *AKR1C3* expression in this PPNAD was increased compared to the other PPNAD samples, but not compared to other adrenal tissues. Given the heterogeneous staining pattern and the higher expression levels compared to *HSD17B3*, *AKR1C3* forms the principal candidate for the cause of hyperandrogenism in our patient, presumably due to mass effect in the dominant nodule.

In vivo studies in our original patient and PPNAD2 did not show evidence for ectopic expression of hormone receptors known to aberrantly control adrenal function in macronodular hyperplasia. This finding is consistent with the two other cases described previously.[10] It therefore appears unlikely that aberrant GPCR-signaling stimuli regulate increased adrenocortical steroidogenesis in human PPNAD cells.

The paradoxical rise in serum cortisol levels following the Liddle's test implicated that glucocorticoids can locally regulate adrenocortical steroidogenesis in the majority of PPNAD. Dexamethasone incubation in primary cells from our patient's PPNAD did not stimulate steroid secretion or steroidogenic enzyme mRNA levels, comparable to the effects in normal and malignant adrenal cells. This implies that the overproduction of cortisol and androgens was present without stimulatory effects of the GR. Although the constitutively activated cAMP-PKA pathway could be sufficient cause for the hormonal syndrome in

the patient, it could also be speculated that mechanisms different from glucocorticoid action are involved in steroidogenic control within PPNAD-associated adenomas. In addition, using qPCR we could not show an increased expression of GR mRNA in PPNAD samples from four patients, two of whom showed slightly augmented cortisol levels after dexamethasone *in vivo*. This finding suggests that in at least some PPNAD tissues aberrant coupling of the GR to the cAMP-PKA pathway[11] instead of GR overexpression[10] may be the culprit for the dexamethasone-induced rise in cortisol production. Other steroid receptors, such as the progesterone and estrogen receptors, have also been detected in PPNAD tissue.[27] The finding that *AR* mRNA is present in PPNAD is novel, although the *AR* appears to be expressed similarly in the various adrenal tissues that were examined in the present study.

In conclusion, we described a novel clinical presentation for PPNAD, a female with primary infertility due to a virilizing adenoma formed in the context of PPNAD. Successful pregnancy ensued upon adrenalectomy. This tumor also had a unique steroid secretion profile *in vitro*, suggesting that defects of the PKA pathway may also affect secretion of additional steroids, apart from glucocorticoids. The steroidogenic regulation in this PPNAD-associated adenoma may be different from that in PPNAD without adenomas.

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Expression of activin and inhibin subunits, receptors and binding proteins in human adrenocortical neoplasms

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ABSTRACT

Background:

The growth and differentiation factors activin and inhibin can affect tumor formation and steroid production in the adrenal cortex. These factors bind to type I (Alk-4), type II (ActRIIA, ActRIIB) and type III (betaglycan) receptors or to the activin-binding protein follistatin. Quantitative expression of these activin-related mRNAs was measured in different types of adrenocortical tissues and tumors in order to study the relationship with tumorigenesis.

Methods:

28 human adrenocortical samples from normal and hyperplastic adrenals and from adrenocortical adenomas and carcinomas were collected after surgery for purpose of study. Using quantitative RT-PCR, we investigated the expression of inhibin α-, βA- and βB-subunits, follistatin, betaglycan, ActRIIA, ActRIIB, and Alk-4 in the adrenocortical tissues. The expression of cytochrome P450c17 (*CYP17A1*) mRNA was also measured to investigate its association with inhibin and activin subunit expression.

Results:

All genes studied were expressed in all tissues, with the exception of the inhibin α-subunit in one hyperplastic adrenal and three adrenocortical carcinomas. Expression of inhibin βA-subunit, follistatin, betaglycan, ActRIIA, ActRIIB, and *CYP17A1* differed between non-tumorous adrenals and carcinomas. These differences together with correlation analysis indicate parallel regulation of the expression of *CYP17A1*, the inhibin α-subunit, ActRIIA, ActRIIB, betaglycan, and follistatin.

Conclusions:

Expression of activin and inhibin subunits, receptors and binding proteins is affected by tumor formation in the adrenal gland and may play a role in tumorigenesis.

INTRODUCTION

Adrenal tumors are common among the general population, with a prevalence of 0.35-4.36% of so-called incidentalomas in patients undergoing a CT scan for other reasons than adrenal mass suspicion.[1] Autopsy studies suggest a prevalence of incidentalomas of about 2.1%.[2] Most of these tumors are non-secretory adrenal adenomas (74.0%), but a minority consists of hypersecretory adenomas (14.8%) or carcinomas (4.0%).[3] Patients with functional adenomas of the adrenal cortex can present with Cushing's syndrome, Conn's syndrome, virilization, or combined hormone excess syndromes. Adrenocortical carcinomas are hypersecretory in approximately 50% of the cases.[4] Familial adrenocortical tumorigenesis has been linked with Li-Fraumeni syndrome (LFS; OMIM #151623), Beckwith-Wiedemann syndrome (OMIM #130650), and Carney complex type I (CNC1; OMIM #160980), with several gene mutations, e.g. in G protein coupled receptors and p53, and with overexpression of certain adrenocortical-specific factors, e.g. steroid acute regulatory protein.[4-5]

Tumor formation in the adrenal cortex has also been linked with the glycoproteins inhibin and activin since Matzuk *et al.*[6] showed that inhibin α-subunit knock out mice developed adrenocortical tumors with 99% penetrance after gonadectomy, which prevented early death of the animals due to ovarian or testicular tumors. Inhibins were first discovered to regulate FSH release from the pituitary gland.[7] The molecules antagonize the action of their counterparts: the activins. Like other members of the TGF-β superfamily of growth and differentiation factors, activins and inhibins are dimeric glycoproteins.[8] Whereas inhibin is composed of an α- and a β-subunit, activin is made up of two β-subunits. One α-subunit and two different β-subunits (βA and βB) make up inhibin A (αβA), inhibin B (αβB), activin A (βAβA), activin B (βBβB) or activin AB (βAβB). Activin binds to type II (ActRIIA and ActRIIB) and type I (Alk-4) receptors. This assembly transfers a signal into the cell where receptor-specific, common-mediator and inhibitory Smads relay the signal and influence gene expression.[9] Inhibin can bind to the type III receptor betaglycan and subsequently also to ActRIIA or ActRIIB,[10] blocking activin signaling.[11] The activin-binding protein, follistatin, also inhibits the actions of activin.[12] Activin and inhibin have been extensively studied in the ovary and testis, where both may influence follicle development and spermatogenesis. Throughout the human body activins exert many functions in tumorigenesis, wound healing, erythropoiesis, tissue differentiation, mesoderm induction, and bone growth.[8, 13] Extrapituitary functions of inhibins are still largely unclear.[13]

Expression and production of inhibin and activin, their subunits, receptors, binding and signaling proteins have been described in the adrenal cortex.[14-20] It has been shown that the production of activin and inhibin is regulated by ACTH and 8-BrcAMP[18] and that activin can influence adrenocortical steroidogenesis.[19, 22-23] The tumor suppressor role of

inhibin in gonadectomized inhibin α-subunit knock out mice[6] has not always been supported by studies in human adrenocortical adenomas and carcinomas.[15-16, 21, 24-25]

The goal of this study was to investigate the presence of the activin-signaling pathway in the normal adrenal cortex and in adrenocortical tumors in detail. We quantified the expression of inhibin and activin subunits, their receptors and binding proteins and of the steroidogenic enzyme cytochrome P450c17 (encoded by *CYP17A1*) in normal, hyperplastic and tumorous adrenocortical tissues. We performed real-time RT-PCR in several groups of adrenal neoplasms in order to gain insight into the differential expression of these genes and to obtain an indication for a possible role of these proteins in tumor formation and steroidogenesis in the adrenal cortex.

MATERIALS AND METHODS

Patient material

Samples of normal adrenal tissues and adrenocortical neoplasms were obtained from patients undergoing abdominal surgery at the Erasmus MC, Rotterdam, from 1991-2004. Normal adrenal tissues were collected after nephrectomy or pheochromocytoma surgery. These tissues were used under the guidelines, which had been approved by the Medical Ethics Committee of the Erasmus MC. After extirpation samples were immediately frozen in liquid nitrogen and stored at –80 ºC by the Erasmus MC Tissue Bank until the extraction of RNA. Malignancy was assumed whenever metastases were present or a van Slooten index greater than 8 was found.[26]

RNA isolation

RNA was isolated from one term placenta, the endometrium tumor cell line ECC-1 (courtesy of dr. J. Foekens), 5 normal adrenal glands, 5 hyperplastic adrenals resulting from pituitary ACTH-secreting adenomas, 4 adrenocortical adenomas and 14 adrenocortical carcinomas. Total RNA was extracted from frozen (–80 ºC) samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol with some modifications. The RNA pellet was dissolved in 25 µl sterile water. Incubation at 55 to 60 ºC was omitted. Samples were frozen overnight at –20 ºC. RNA was measured by spectrophotometry and OD 260/280 ratios > 1.6 were obtained for all samples.

Quantitative RT-PCR

For the reverse transcription reaction 1.0 µg of sample RNA was mixed with 1.25 µl (25µM) 10x concentrated hexanucleotide mix (Roche Applied Science, Penzberg, Germany), and 1.25 µl (200nM) oligo(dT)15 (Promega Benelux B.V., Leiden, The Netherlands) in an eppendorf tube in duplicate. In order to obtain samples for standard curves, reactions were

performed with 1.0, 0.3, 0.1, 0.03 and 0.01 µg RNA of placenta, ECC-1 (inhibin βB-subunit) and a hyperplastic adrenocortical sample (*CYP17A1*). The volume was made up to 15.0 µl with sterile water. Subsequently the mix was placed in a heat block for 5 minutes at 70 ºC and directly placed on ice to unfold all secondary loops in the RNA. Then a 10 µl mix was added containing 0.5 µl (10mM) dNTPs (Amersham Biosciences, UK), 5.0 µl 5x RT-buffer (Promega), 0.625 µl (40U/µl) rRNasin (Promega), 1.0 µl (200U/µl) Moloney Murine Leukemia Virus (M-MLV, Promega) and 2.88 µl sterile water. To check for the presence of genomic DNA an RT-minus reaction for each sample was made by replacing the M-MLV with 1.0 µl sterile water. The RT reaction was realized by placing the reaction mix successively in a water bath at 37 ºC for 45 minutes, in a water bath at 42 ºC for 15 minutes and in a heat block at 94 ºC during 5 minutes. The tubes were put on ice immediately and 100 µl of sterile water was added to the synthesized cDNA.

Primers and probes for the PCR reaction (Table 1) were designed using Primer Express software (PE Biosystems, Foster City, CA, USA). Most primers were obtained from Invitrogen, the glyceraldehydephosphate dehydrogenase (*GAPDH*) and *CYP17A1* primers and most HPLC purified probes from Eurogentec (Liege, Belgium). The primers and the HPLC-purified probe for hypoxanthine ribosyl transferase (*HPRT1*) were purchased from Biosource (Nivelles, Belgium). Quantitative PCR reactions were performed in a 25 µl volume, consisting of 12.5 µl Taqman Universal PCR Master Mix (Roche), 7.5 pmol forward primer, 7.5 pmol reverse primer, 5.0 pmol probe, 5.0 µl cDNA sample and sterile water. The reactions were performed in an ABI Prism 7700 Sequence Detector (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) as follows: 2 minutes at 50 ºC, 10 minutes at 95 ºC and 40 cycles of 15 seconds at 95 ºC and 1 minute at 60 ºC. Analysis of PCR results was carried out with ABI 7700 Prism software (Applied Biosystems). Threshold cycle (Ct) was calculated as the cycle at which the emitted photon energy of the reporter passed the background energy plus 10 times its standard deviation. Parallelism of the curve for standard and adrenal tissue was proven beforehand. RNA levels were calculated relative to expression of a housekeeping gene, *GAPDH* or *HPRT1*, according to the following formula:

$$\text{Arbitrary units} = 2^{-(\text{Ct gene} - \text{Ct housekeeping gene})}$$

Statistical analysis

Data analysis of quantitative RT-PCR experiments was performed first using Kruskall-Wallis tests. Differences between individual groups were subsequently analyzed by Mann-Whitney U tests. Correlations between gene expressions were performed with Spearman's test. SPSS 11.0 for Windows was utilized for analysis. All tests were calculated as two-tailed. Statistical significance was assumed at $P<0.05$. The Bonferroni-Holm correction was applied to the correlation analyses to allow for multiple testing.

Table 1: Primer and probe sequences used in quantitative real-time RT-PCR

Gene	Amplicon size (bp)	Primers 5′ - 3′	Probe 5′ FAM - 3′ TAMRA
α-subunit *(INHA)*	258	CCGAGGAAGAGGAGGATGTCT CGGTGACAGTGCCAGCAG	TGACTTCAGCCCAGCTGTGGTTCCA
βA- subunit *(INHBA)*	165	CCTCGGAGATCATCACGTTTG GGCGGATGGTGACTTTGGT	CTGACAGGTCACTGCCTTCCTTGGAAATCT
βB-subunit *(INHBB)*	215	ACGGCCGCGTGGAGAT GGACGTAGGGCAGGAGTTTCA	TCCGAAATCATCAGCTTCGCCGA
Betaglycan *(TGFBR3)*	189	ACCCCCAACTCTAACCCCTACA GCCAATACTGTTAGGACAATAATTTTC	TCCTGATCTTGAAGTGCAAAAAGTCTGTCAACTG
Follistatin *(FST)*	105	GAGGAGGACGTGAATGACAACA TCCACAGTCCACGTTCTCACA	CCCCCGTTGAAAATCATCCACTTGAAGAG
Alk-4 *(ACVR1B)*	220	CATCATTGTTTTCCTTGTCATTAACTATC CTTGCCAATAATCTCTTGTAAAACGA	AGCGCACAGTGGCCCGAACC
ActRIIA *(ACVR2A)*	98	TTCTCGCTGTACTGCTGCAGAT CTTCCTGCATGTCTTCAAGAGATG	TGGCCAATTTCCTCCTCAAATGGCA
ActRIIB *(ACVR2B)*	160	TCAGCACACCTGGCATGAAG AGTTCGTTCCATGTGATGATGTTC	ACAAGGGCTCCCTCACGGATTACCTCA
HPRT1[45]	109	TGCTTTCCTTGGTCAGGCAGTAT TCAAATCCAACAAAGTCTGGCTTATATC	CAAGCTTGCGACCTTGACCATCTTTGGA
GAPDH	70	ATGGGGAAGGTGAAGGTCG TAAAAGCAGCCCTGGTGACC	CGCCCAATACGACCAAATCCGTTGAC
CYP17A1[46]	63	TCTCTGGGCGGCCTCAA AGGCGATACCCTTACGGTTGT	TGGCAACTCTAGACATCGCGTCC

RESULTS

Samples were divided into groups based on the type of autonomous tumor formation. Non-neoplastic tissues consisted of normal and hyperplastic adrenal cortex samples. Benign and malignant neoplastic tissues constituted two groups of adrenocortical adenomas and carcinomas respectively. Patient characteristics of all samples are summarized in Table 2.

Slopes of the standard curves in the real-time RT-PCR experiments were between –3.0 and –3.7, correlation coefficients were above 0.92 and the Y-intercepts at 1.0 ng RNA were below Ct 36. Each sample yielded a Ct-value for all of the genes studied (Ct ≤ 36), except for the inhibin α-subunit in one hyperplastic adrenal and three carcinomas. Expression of the housekeeping genes *GAPDH* and *HPRT1* showed a significant correlation in the 28 samples (r=0.652; P<0.001). We choose to normalize all values of measured expression in our samples relative to expression of the most commonly used housekeeping gene *GAPDH*.

All genes studied by RT-PCR were expressed in all groups of tissues. Results are shown in Table 3. Overall analyses by Kruskall-Wallis tests showed significant differences between non-tumorous, adenomatous and carcinomatous adrenals for follistatin (P=0.030), betaglycan (P=0.011), ActRIIA (P=0.036), ActRIIB (P=0.012) and *CYP17A1* (P=0.001). Expression of inhibin βA-subunit showed a trend towards differences between the groups of tissues (P=0.094). These differences in mRNA expressions of inhibin βA-subunit (P=0.048), follistatin (P=0.011), betaglycan (P=0.003), ActRIIA (P=0.026), ActRIIB (P=0.003), and *CYP17A1* (P<0.001) were all based on the decreased expression in the adrenocortical carcinomas compared to the normal and hyperplastic adrenals (Mann-Whitney U tests).

Table 2: Characteristics of the 28 patients, of whom adrenocortical samples were studied by quantitative RT-PCR

	Normal and hyperplastic adrenals	Adrenocortical adenoma	Adrenocortical carcinoma
Number of samples	10	4	14
Sex			
- Female	6	3	9
- Male	4	1	5
Age at operation (mean ± SEM)	50.1 ± 5.2	42.0 ± 4.8	53.1 ± 2.4
Maximal tumor diameter in cm (mean ± SEM)	-	6.4 ± 1.8	10 ± 1.7
Left adrenal	4	2	6
Right adrenal	3	2	8
Bi-lateral	3	0	0

Table 3: Results of real-time RT-PCR experiments in normal adrenal tissues and adrenocortical tumors

	Normal and hyperplastic adrenal cortex	Adrenocortical adenoma	Adrenocortical carcinoma
n	10	4	14
α-subunit	0.19 ± 0.085	0.19 ± 0.12	0.094 ± 0.035
βA- subunit	0.16 ± 0.013[a]	0.17 ± 0.030	0.13 ± 0.044[a]
βB-subunit	0.089 ± 0.036	0.018 ± 0.012	0.034 ± 0.0059
Follistatin	0.13 ± 0.030[b]	0.24 ± 0.18	0.046 ± 0.013[b]
Betaglycan	0.48 ± 0.16[c]	0.26 ± 0.11	0.098 ± 0.027[c]
Alk-4	0.034 ± 0.014	0.013 ± 0.0054	0.013 ± 0.0034
ActRIIA	0.34 ± 0.077[d]	0.31 ± 0.11	0.13 ± 0.025[d]
ActRIIB	1.1 ± 0.21[e]	1.0 ± 0.42	0.43 ± 0.069[e]
CYP17A1	4783 ± 769[f]	6475 ± 4533	492 ± 188[f]

Values are relative to mRNA expression of *GAPDH,* x1000 and are shown as means ± SEM. Values indicated by the same letters are significantly different (P<0.05), Mann-Whitney U tests.

No differences were detected for the other genes studied or between the adenomas and any of the other two groups.

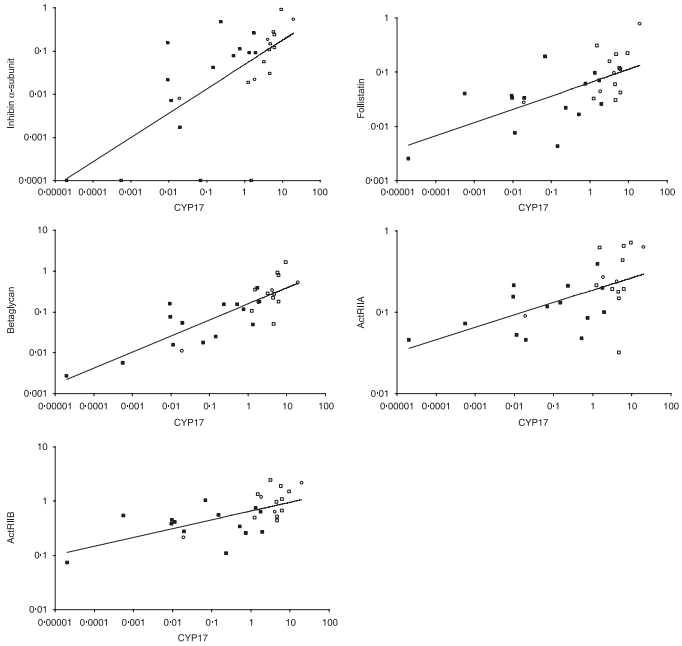
We found several significant correlations between the levels of individually coupled gene expression in the 28 adrenocortical samples. These are depicted in Table 4. Relevant correlations between expressions of *CYP17A1* and other genes are shown in Figure 1.

Table 4: Correlations between mRNA expression of genes of the activin-signaling pathway and *CYP17A1* in human normal and tumorous adrenocortical samples (n=28)

	Inhibin α-subunit	Inhibin βA-subunit	Inhibin βB-subunit	Follistatin	Betaglycan	Alk-4	ActRIIA	ActRIIB	CYP17A1
Inhibin α-subunit	-	0.129	0.160	0.352	0.724	0.532	0.508	0.244	0.663
Inhibin βA-subunit	0.515	-	0.265	0.372	0.299	0.109	0.370	0.383	0.198
Inhibin βB-subunit	0.415	0.173	-	0.010	0.264	0.430	0.063	0.020	0.187
Follistatin	0.067	0.051	0.958	-	0.673	0.264	0.663	0.762	0.610
Betaglycan	**<0.001**	0.122	0.175	**<0.001**	-	0.719	0.718	0.604	0.802
Alk-4	0.004	0.579	0.022	0.174	**<0.001**	-	0.473	0.419	0.545
ActRIIA	0.006	0.053	0.751	**<0.001**	**<0.001**	0.011	-	0.695	0.563
ActRIIB	0.210	0.044	0.920	**<0.001**	**0.001**	0.027	**<0.001**	-	0.634
CYP17A1	**<0.001**	0.314	0.340	**0.001**	**<0.001**	0.003	**0.002**	**<0.001**	-

On the upper right side the correlation coefficients are shown, while on the lower left the P-values of the corresponding correlations are depicted. Statistically significant P-values are printed in bold.

Figure 1: Relationships between the expression of *CYP17A1* mRNA and the mRNAs coding for the inhibin α-subunit (r=0.663), follistatin (r=0.610), betaglycan (r=0.802), ActRIIA (r=0.563), and ActRIIB (r=0.634) in 28 adrenocortical samples



Values are relative to GAPDH, x1000. For inhibin α-subunit four samples showed no expression; because of logarithmic scaling these samples were give the value 0.0001 in the graph. □ non-tumorous adrenals ○ adenomas ■ carcinomas.

DISCUSSION

Since the first detection of activin and inhibin in the human adrenal gland,[20] it has been found that these proteins can exert effects upon adrenal function. Several studies have uncovered correlations between activin expression and adrenocortical steroidogenesis.[14, 18-20, 23] Other reports illustrated the relation between activin and inhibin and adrenal neoplasms.[6, 16, 21, 24-25, 27-30] In the present study, we measured the expression of mRNA encoding activin and inhibin subunits in neoplasms of the human adult adrenal cortex together with the expression of their receptors and binding proteins by quantitative RT-PCR in order to investigate possible functional differences and their interrelationships.

Expression of the inhibin- and activin-related genes and *CYP17A1* was normalized on the basis of *GAPDH* because its variance is smaller than that of *HPRT1*.[31] The results of our experiments on the expression of activin and inhibin subunits are in accordance with other reports showing predominantly non-quantitative evidence for the presence of mRNA of these proteins in the adrenal cortex, both *in vitro* and *in vivo*.[18-20, 23, 27, 32]

Of the various mRNAs we measured in the adrenal cortex, the α-subunit of inhibin has been most extensively studied. Using inhibin α-subunit knock-out mice Matzuk *et al.*[6] showed the potential of the α-subunit as a tumor suppressor with primarily gonadal specificity and secondary adrenocortical effects after gonadectomy. This association could not be confirmed in human adrenocortical benign and malignant neoplasms. Instead, α-subunit expression seems to be upregulated in certain adrenocortical adenomas or carcinomas, but this is not consistent between studies.[16, 21, 24-25, 33] Our study showed no differences in inhibin α-subunit expression between the groups of non-tumorous adrenals, adenomas, and carcinomas. Only two other studies[21, 25] have investigated mRNA expression of α-subunit in a semi-quantitative manner in adrenocortical tumors; like the results of our study these authors did not find differences between the different types of tumors.

Strikingly, we found several samples in which no inhibin α-subunit expression could be detected. Detection of mRNAs of the other genes in these samples indicates that this loss of expression of α-subunit is not due to breakdown of mRNA. Pelkey *et al.*[33] and Munro *et al.*[16] reported loss of α-subunit protein in adrenocortical adenomas and carcinomas before. We found loss of inhibin α-subunit expression in three adrenocortical carcinomas and one hyperplastic adrenal. Thus a subgroup of the adrenocortical neoplasms could have developed as a consequence of loss of inhibin α-subunit, which could resemble the *Inha* knock-out model described by Matzuk *et al.*[6]

Differences in inhibin βA- and βB-subunits in adrenal neoplasms have not been studied in great detail. Munro *et al.*[16] did not find significant changes in βA- and βB-subunits between the normal adrenal cortex and tumors after performing immunohistochemistry. In contrast to this previous study, our study detected a decrease of inhibin βA-subunit mRNA expression in carcinomas compared to non-tumorous adrenals. These results however must be cautiously interpreted due to the small difference in expression and the absence of a significant difference in overall analysis. In the adrenocortical carcinoma cell line H295R treatment with activin A led to an increased rate of apoptosis.[19] Possibly, the anti-apoptotic role of activin A is lost which could have added to the process of tumorigenesis in the carcinoma samples. The inhibin βB-subunit does not seem to play any discriminative role between the types of adrenocortical tissues due to the absence of a difference in expression.

Qualitative adrenal expression of ActRIIA, ActRIIB, Alk-4, betaglycan and follistatin mRNAs was shown earlier by Väntinnen *et al.*[18] and Suzuki *et al.*[23] in normal human fetal and adult adrenals and the H295R cell line. We were the first to study the expression levels of these mRNAs in different types of adrenocortical neoplasms.

In our study carcinomas were found to have decreased expression levels of *CYP17A1*, follistatin, betaglycan, ActRIIA and ActRIIB compared to normal and hyperplastic adrenals. Correlation analysis of our real-time RT-PCR data revealed significant relations between the expression levels of *CYP17A1* on the one hand, and follistatin, betaglycan and the

activin type II receptors on the other. Inhibin α-subunit expression also had significant correlation with *CYP17A1* and betaglycan expression. The expression patterns in the groups of tissues combined with the correlations between mRNA levels suggest that expression of these genes is regulated in parallel. ACTH controls the cAMP concentration in the adrenocortical cell and thereby regulates expression of *CYP17A1* through cAMP-responsive sequences.[34] The regulation of expression of the inhibin α-subunit, follistatin and recently of betaglycan, ActRIIA, and ActRIIB mRNAs have been investigated in gonadal cells,[35-38] where PKA stimulation also increases the expression of these five proteins. In contrast Aloi *et al.*[39] detected inhibition of ovarian ActRIIA expression after administration of gonadotropins in hypophysectomized rats.

The activity of the ACTH/cAMP/PKA pathway differs in the groups between which differences in expression of activin-related genes and *CYP17A1* were detected. First, hyperplastic adrenals have been exposed to increased ACTH concentrations and thus to high cAMP signaling. Secondly, significantly lower concentrations of cAMP response element-binding protein (CREB), an important transcription factor in the cAMP pathway, have been found in adrenocortical carcinomas compared to adenomas.[40] And finally, altered cAMP signaling was shown by Peri *et al.*[41] in human adrenocortical carcinoma samples and by Groussin *et al.*[42] in the H295R cell line due to decreased ACTH receptor expression and loss of expression of CREB and of inducible cAMP early repressor isoforms (ICERs). The carcinomas thus show lower expression of PKA regulated genes when compared with normal and hyperplastic adrenals. We speculate that *CYP17A1*, inhibin α-subunit, follistatin, ActRIIA, ActRIIB, and betaglycan in the adrenal cortex and its malignant tumors are collectively controlled by cAMP, under influence of ACTH. This relationship likely also applies to the adrenocortical adenomas, although no definitive conclusion can be made due to the small number of adenoma samples in our study. The subsequent effects of these differential expression patterns in the different types of adrenocortical tissues require further study.

In conclusion, we detected expression of inhibin α-, βA- and βB-subunits, follistatin, ActRIIA, ActRIIB, Alk-4, and betaglycan in normal and hyperplastic adrenals, adrenocortical adenomas and carcinomas, indicating full potential for activin and inhibin signaling in these tissues. The inhibin α-subunit was not detected in three carcinoma samples and inhibin βA-subunit mRNA was found slightly decreased in the carcinomas, suggesting that tumor formation in carcinomas might have been caused by loss of expression of the α-subunit or decreased βA-subunit expression. Significant differences in expression between groups were detected for *CYP17A1* and several genes in the activin/inhibin signal transduction pathway, suggesting involvement of changes in the activity of the cAMP signal transduction pathway. This study suggests that inhibin and activin signaling is dependent on tumor status of the adrenal cortex and may itself play a role in tumor formation.

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Serum inhibin pro-alphaC is a tumor marker for adrenocortical carcinomas

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ABSTRACT

Background:

The insufficient diagnostic accuracy for differentiation between benign and malignant adrenocortical disease and lack of sensitive markers reflecting tumor load emphasize the need for novel biomarkers for diagnosis and follow-up of adrenocortical carcinoma (ACC). Since the inhibin α-subunit is expressed within the adrenal cortex, the role of serum inhibin pro-αC as tumor marker for ACC was studied in patients.

Methods:

Regulation of adrenal pro-αC secretion was investigated by adrenocortical function tests. Serum inhibin pro-αC levels were measured in controls (n=181) and patients with adrenocortical hyperplasia (n=45), adenoma (ADA, n=32), ACC (n=32) or non-cortical tumors (n=12). Steroid hormone, adrenocorticotropin (ACTH) and inhibin A and B levels were also estimated in patient subsets.

Results:

Serum inhibin pro-αC levels increased by 16% after stimulation with ACTH (P=0.043). ACC patients had higher serum inhibin pro-αC levels than controls (medians 733 versus 307 ng/l, P<0.0001) and patients with adrenocortical hyperplasia, ADA or non-adrenocortical adrenal tumors (148, 208 and 131 ng/l, P=0.0003). Inhibin pro-αC measurement in ACC patients had a sensitivity of 59% and specificity of 84% for differentiation from ADA patients. ROC analysis displayed areas under the curve of 0.87 for ACC versus controls and 0.81 for ACC versus ADA (P<0.0001). Surgery or mitotane therapy was followed by a decrease of inhibin pro-αC levels in 10/10 ACC patients tested during follow-up (P=0.0065).

Conclusions:

Inhibin pro-αC is produced by the adrenal gland. Differentiation between ADA and ACC by serum inhibin pro-αC is limited, but its levels may constitute a novel tumor marker for ACC.

INTRODUCTION

Tumors of the adrenal gland are frequently detected on abdominal imaging studies.[1-2] Although the majority of adrenal neoplasms constitutes non-functional adenomas, a subset of patients presents with syndromes of hormonal excess or with malignancy.[3-5] Adrenocortical carcinomas (ACCs) are rare tumors accompanied by a poor prognosis, especially in the presence of metastases.[6-8] Determinants such as tumor size, imaging phenotype on CT or MRI and serum adrenal steroid levels, particularly those of dehydroepiandrosterone-sulfate (DHEA-S), have been applied in order to differentiate ACC from other adrenal neoplasms. These determinants, however, have limited sensitivity.[9-10] Thus, there is a clear need for additional diagnostic tools for differentiation between ACC and its benign counterparts. In addition, given the limited value of steroid hormones as tumor markers in patients with established ACC, the availability of a reliable serum marker could improve the diagnostic follow-up after surgery or medical therapy with mitotane or other chemotherapeutic agents.

Inhibins are dimeric peptide hormones belonging to the transforming growth factor-β superfamily of growth and differentiation factors.[11] The inhibin α-subunit (*INHA*) has been implicated in adrenocortical tumorigenesis since gonadectomized *Inha* knock-out mice develop adrenocortical carcinomas.[12] Several forms of the inhibin α-subunit are known to be produced by the human gonads. The inhibin α-subunit precursor contains three regions: a pro-region, an N-terminal region and the mature C-terminal region, called αC. In the presence of the inhibin βA- or βB-subunit, the αC-region can be linked to the β-subunit in order to form inhibin A or B, respectively.[13] During assembly the αC-region can also bind to the pro-region, forming the "free" inhibin α-subunit molecules pro-αC and pro-αNαC.[13-14] These peptides are the most abundant serum inhibin forms and are thought to arise predominantly in the absence of inhibin β-subunits.[14-15] Like the mature inhibins A and B, inhibin pro-αC has been linked to various forms of ovarian cancer.[16-18]

The only detectable serum inhibin form in post-menopausal women is inhibin pro-αC suggesting the existence of an extragonadal source of this free α-subunit form.[19] Since physiological expression of *INHA* is confined to the ovary, testis, placenta and adrenal cortex [20-21] we hypothesized that serum inhibin pro-αC can be derived from the adrenal cortex. Therefore we studied whether *in vivo* stimulation or inhibition of adrenocorticotropin (ACTH), the physiological regulator of adrenocortical *INHA* expression,[22] alters serum inhibin pro-αC levels. Furthermore, given the role of the various inhibin forms as tumor markers in ovarian cancer and reports of *INHA* overexpression in human adrenocortical tumors[23-25] we investigated the possibility to use serum inhibin pro-αC as a tumor marker for adrenocortical neoplasms.

MATERIALS AND METHODS

Patient material

In order to obtain reference values for serum inhibin pro-αC levels blood was collected from healthy blood bank donors. The total reference group included 111 men and 70 women, age range 20-70 years. For the study of *in vivo* regulation of adrenocortical inhibin pro-αC secretion serum specimens were collected from patients who were evaluated for hypothalamus-pituitary-adrenal (HPA)-axis abnormalities with 250 µg of synthetic $ACTH_{1-24}$ (tetracosactide, before and after 30 minutes) intravenously, 750 mg metyrapone every four hours orally (serum taken before and after 24 hours) or before and after an oral dose of 1 mg dexamethasone (DST) overnight. Samples were included in the study if HPA-axis responsiveness was within normal ranges, i.e. cortisol levels after tetracosactide or DST higher than 550 nmol/l or lower than 50 nmol/l, respectively, or 11-deoxycortisol levels greater than 350 nmol/l after metyrapone stimulation.

Serum samples were collected from patients that presented with an adrenal tumor or hyperplasia between 1999 to 2009 in the three participating centers. Samples were stored at -20 °C. Tumors were classified on the basis of histopathological evaluation. Adrenocortical tumors were designated as carcinomas if the van Slooten index was >8[26] or if a metastasized adrenal tumor was detected. In 10 ACC patients serum samples were also collected shortly after adrenal surgery or after starting mitotane therapy for metastasized disease. The study was conducted under the guidelines that had been approved by the Medical Ethics Committee of the Erasmus Medical Center.

Determination of hormone levels

Inhibin pro-αC, A and B levels were measured by commercially available enzyme-linked immunometric assay (Diagnostic Systems Laboratory, Webster, TX, USA). Serum levels of cortisol, progesterone, androstenedione, DHEA-S and ACTH were measured using fluorescence-based immunoassays (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA). Testosterone and estradiol levels were measured by coated tube radioimmunoassay (RIA, Siemens), DHEA levels by RIA (DSL) and 17-hydroxyprogesterone and 11-deoxycortisol levels were estimated using previously described in-house RIAs.[27] Local laboratory age- and sex-specific reference values were adopted for steroid levels. Intra- and interassay variability for the inhibin pro-αC assay were smaller than 8% and 10% respectively. For the DHEA-S assay variation coefficients were smaller than 9% within assays and 11% between assays. Local assay references levels are summarized in Supplementary Table 1.

Table 1: Patient characteristics

	Hyperplasia[¶]	Adenoma (ADA)	Carcinoma (ACC)	Other[†]
Number of patients – no.	45	32	32	12
- Male – no.	11	13	9	5
- Pre-menopausal women – no.	18	12	6	1
- Post-menopausal women – no.[#]	16	7	17	6
Clinical syndrome				
- Cushing's syndrome – no.	45	10	6	0
- Virilization – no.	0	2	4	1[‡]
- Both – no.	0	1	9	0
- Feminization – no.	0	0	2	0
- Conn's syndrome – no.	0	3	1	0
- Non-functional – no.	0	16	10	11
Age – yr*	45.9 ± 15.2	50.7 ± 15.7	52.5 ± 20.5	55.9 ± 15.2
Tumor size – cm*	n.a.	4.5 ± 2.3[§]	10.9 ± 4.7[§]	n.a.
ENSAT 2008 classification[6]				
- I	n.a.	n.a.	4	n.a.
- II			6	
- III			5	
- IV			17	
Van Slooten index*	n.a.	2.7 ± 2.7	16.9 ± 5.8	n.a.

[*] Values expressed as mean ± SD, [#] Four prepubertal girls were analyzed as post-menopausal for comparison of age-related inhibin pro-αC levels, [¶]Group composed of patients with Cushing's disease (n=25), ectopic ACTH secretion (n=17) or AIMAH (n=3), [†]Group composed of patients with adrenal tumors of primary non-cortical origin: pheochromocytoma (n=6), metastasis (n=3), ganglioneuroma, cyst and lymphoma (all n=1), [§] Statistically significant difference, P=0.008, [‡] Patient with a testosterone-secreting ganglioneuroma, n.a.: not applicable

Statistical analysis

Normality of reference values was checked by D'Agostino and Pearson omnibus normality test. After log-transformation normality was obtained for all reference groups and 95% confidence intervals were calculated. Effects of adrenocortical function tests and tumor removal were evaluated by paired Student's t-tests. Differences between groups of patients were estimated by Student's t-test or one-way ANOVA followed by post-hoc Tukey's multiple comparisons test. Pearson's correlation coefficients were calculated for associations between hormone levels; here multiple testing was accounted for by Bonferroni correction. Analyses were performed with GraphPad Prism (version 5.01, GraphPad software, La Jolla, CA, USA) and SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). Statistical significance was assumed at a two-sided P-value lower than 0.05.

RESULTS

Reference values of serum inhibin pro-αC

Blood donor samples were divided into groups of men (n=111), pre-menopausal (n=36) and post-menopausal women (n=34). Age of 50 years was used as cut-off between

pre- and post-menopause. In the post-menopausal group three outliers (>4 SD from mean) were excluded from further analysis. Overall, the median pro-αC value was 307 ng/l (range: 17-1007 ng/l). Reference values of serum inhibin pro-αC were calculated as the 95% confidence intervals of the remaining samples and were as follows: 196-685 ng/l for men, 36-780 ng/l for pre-menopausal women and 15-83 ng/l for post-menopausal women.

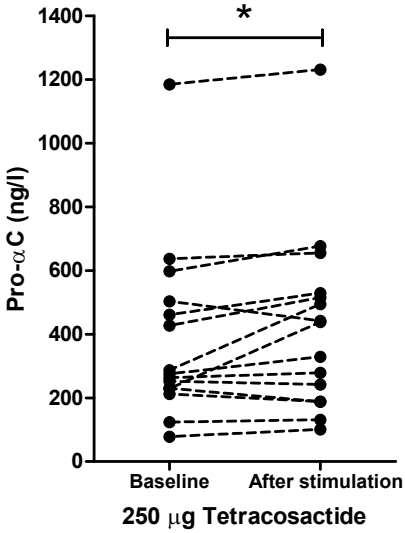
In vivo tests of adrenocortical function

Since expression of the inhibin α-subunit is regulated by ACTH, we evaluated whether *in vivo* manipulation of serum ACTH levels could affect serum inhibin pro-αC levels. Short-term adrenocortical stimulation through intravenous administration of tetracosactide in 15 subjects increased serum inhibin pro-αC levels by 16% (P=0.043), see Figure 1. Long-term ACTH stimulation through metyrapone (n=15, +38%, P=0.15) and overnight ACTH inhibition with dexamethasone (n=8, -16%, P=0.17) did not significantly alter serum levels of inhibin pro-αC (data not shown).

Adrenocortical pathology

Serum samples were obtained from patients with adrenocortical hyperplasia (n=45), adenoma (ADA, n=32), ACC (n=32) or non-adrenocortical adrenal neoplasms (n=12). Patient demographics and tumor characteristics have been summarized in Table 1. Patients were

Figure 1: Effect of *in vivo* ACTH stimulation on serum levels of inhibin pro-αC



Samples were collected before and 30 minutes after 250 μg tetracosactide intravenously in order to measure serum inhibin pro-αC levels in 15 patients with a normal HPA axis. *P<0.05, paired t-test

also divided based on gender and menopausal status. Female subjects were classified as post-menopausal when last menstruation was more than 1 year ago or serum FSH level was above 30 IU/l. When clinical data on menstrual cycle or FSH levels were missing, age above 50 years was considered to represent post-menopausal status. With respect to analysis of pro-αC levels, values from four pre-pubertal girls (ages at diagnosis: 11 months, 3, 6 and 9 years) were analyzed relative to levels in post-menopausal controls since it has been demonstrated that serum inhibin pro-αC reference values of these subgroups are comparable.[28]

Results of serum hormone measurements are shown in Table 2. Serum inhibin pro-αC levels were higher in patients with ACC than in controls (P<0.0001) and also higher when compared to patients with adrenal hyperplasia, ADA or non-adrenocortical adrenal neoplasms (P=0.0003, Figure 2A). Inhibin A was either not detectable or within normal ranges in all patients tested. Serum inhibin B levels were not different between patient groups, but were elevated in three ACC patients: 491 ng/l in a male patient (reference: <400 ng/l), 194 ng/l in a 6-year old girl and 55 ng/l in a post-menopausal woman (both reference: <10 ng/l). Patients with ACTH-dependent adrenal hyperplasia had higher morning cortisol (Figure 2B, P=0.0003) and ACTH (P=0.005) levels compared to patients with ADA and ACC. Serum steroid levels were not significantly elevated in ACC compared to ADA, but morning cortisol, androstenedione and DHEA-S levels did show a pattern similar to that of inhibin pro-αC (Figure 2).

When compared to their gender- and age-specific reference values 4 out of 9 men (44%), 3 out of 3 children (100%), 4 out of 6 pre-menopausal women (67%), and 8 out of 14 post-menopausal women (57%) with ACC had increased serum levels of inhibin pro-αC. High levels of serum inhibin pro-αC (i.e. 2.5 and 7.1 times the upper reference limit) were demonstrated in two out of four patients with ACC ENSAT stage 1. Overall sensitivity of inhibin pro-αC serum levels for ACC was 59%, compared to 45% for DHEA-S levels (P=0.26). Among 29 patients with ACC in whom both inhibin pro-αC and DHEA-S concentrations were measured, 12 patients had concomitantly elevated levels of both hormones, 4 had only elevated pro-αC levels and one patient had only an increased level of DHEA-S.

When comparing ACC to ADA patients, the specificity of the results of the pro-αC and DHEA-S assays were 84% and 92% respectively (P=0.36). Positive predictive values of pro-αC and DHEA-S for the differentiation between ACC and ADA were 79% and 87%, respectively (P=0.56). Combining both measurements increased the positive predictive value to 92% (P=0.57). Negative predictive values were 68% for pro-αC, 60% for DHEA-S and 60% for the combination of both markers (P=0.71). The highest level of inhibin pro-αC in a patient with ACC was 121 times the age- and sex-specific upper reference value, whereas elevations of androstenedione and DHEA-S were maximally 29 and 10 times the age- and sex-specific upper reference levels respectively.

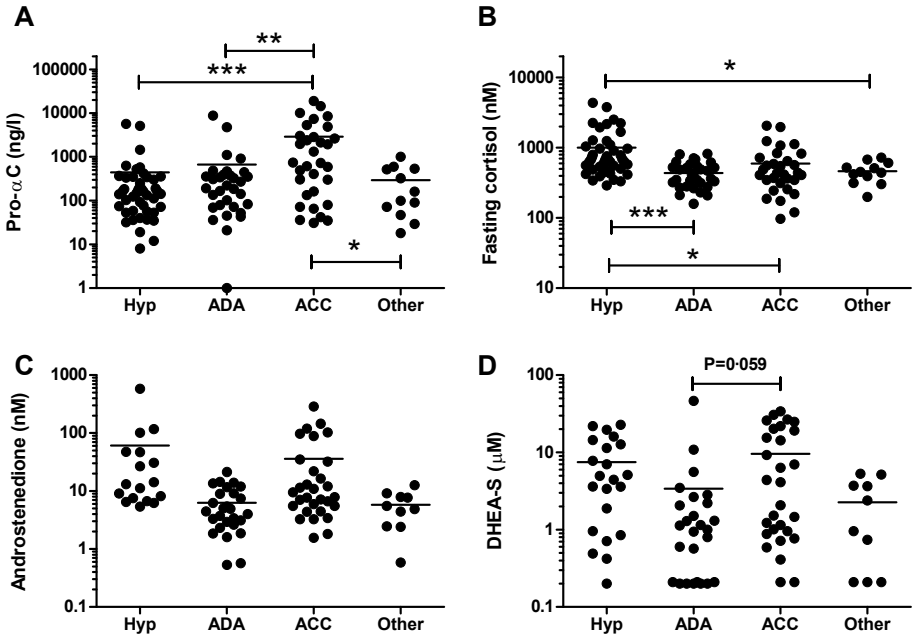
Table 2: Hormonal evaluation of patients with adrenal hyperplasia or neoplasms*

	Hyperplasia	ADA	ACC	Other	P-value†
Inhibin pro-αC					
- ng/l	148 (8-5632)[a]	208 (0-8730)[a]	733 (31-18957)	131 (18-1005)[a]	P=0.0003
- elevated level: no./ total no. (%)	7/45 (16%)	5/32 (16%)	19/32 (59%)	3/12 (25%)	
Inhibin A					
- ng/l	0 (0-0)	0 (0-2)	0 (0-5)	n.d.	P=0.229
- elevated level: no./ total no. (%)	0/3 (0%)	0/13 (0%)	0/8 (0%)		
Inhibin B					
- ng/l	96 (62-202)	216 (15-399)	150 (5-419)	142 (52-165)	P=0.454
- elevated level: no./ total no. (%)	1/5 (20%)	1/13 (5%)	3/9 (33%)	1/3 (33%)	
Morning cortisol					
- nmol/l	679 (290-4348)	417 (159-821)[b]	436 (97-2050)[b]	447 (199-723)	P=0.0003
- elevated level: no./ total no. (%)	15/45 (33%)	2/31 (6%)	7/30 (23%)	0/12 (0%)	
Midnight cortisol					
- nmol/l	638 (146-5116)	260 (42-617)[b]	318 (62-1661)	206 (81-800)	P=0.027
ACTH					
- nmol/l	9.70 (0.55-217)	1.85 (0.55-4.40)[b]	0.98 (0.55-16.00)[b]	3.60 (1.30-13.30)	P=0.0051
Cortisol after DST#					
- nmol/l	547 (94-4525)	114 (14-579)	339 (14-1760)	41 (29-332)	P=0.101
- elevated level: no./ total no. (%)	32/32 (100%)	10/11 (91%)	12/15 (80%)	1/3 (33%)	
Cortisoluria					
- nmol/24 hour	2520 (73-76258)	1047 (272-1953)	716 (163-4709)	763 (288-1325)	P=0.029
- elevated level: no./ total no. (%)	38/44 (86%)	8/15 (53%)	10/25 (45%)	3/7 (43%)	
Progesterone					
- nmol/l	2.1 (0.3-32.8)	0.7 (0.3-71.3)	2.3 (0.3-12.6)	0.6 (0.3-1.4)	P=0.359
- elevated level: no./ total no. (%)	4/11 (36%)	1/11 (9%)	7/19 (37%)	0/8 (0%)	
17OH-Progesterone					
- nmol/l	2.2 (0.5-16.1)	2.2 (0.2-71.3)	3.5 (0.8-31.0)	1.7 (0.8-3.6)	P=0.447
- elevated level: no./ total no. (%)	2/14 (14%)	2/13 (15%)	5/24 (21%)	0/9 (0%)	
11-deoxycortisol					
- nmol/l	29 (22-490)	23 (14-56)	34 (5-819)	24 (10-37)	P=0.222
- elevated level: no./ total no. (%)	3/7 (43%)	1/11 (9%)	7/23 (30%)	0/8 (0%)	
Androstenedione					
- nmol/l	13.1 (5.4-581.0)	4.5 (0.5-21.3)	8.7 (1.6-287.0)	5.2 (0.6-12.6)	P=0.070
- elevated level: no./ total no. (%)	7/17 (41%)	4/27 (15%)	13/30 (43%)	1/10 (10%)	
DHEA					
- nmol/l	20.8 (5.2-126.2)	7.7 (0.7-39.7)	22.9 (5.3-197.6)	16.0 (5.6-49.0)	P=0.087

DHEA-sulfate					
- µmol/l	4.7 (0.2-22.9)	1.1 (0.2-46.5)	4.1 (0.2-33.9)	1.7 (0.2-5.3)	P=0.034
- elevated level: no./ total no. (%)	10/22 (45%)	2/26 (8%)	13/29 (45%)	0/10 (0%)	
Estradiol					
- pmol/l	147 (1-772)	101 (5-456)	170 (14-19787)	88 (5-146)	P=0.453
Testosterone					
- nmol/l	2.7 (0.8-14.1)	1.2 (0.1-10.1)	1.8 (0.3-24.6)	14.1 (0.3-22.1)[c]	P=0.025

[*] Values expressed as medians and ranges. Elevated level is positive if levels exceed local age- and sex-specific reference values, described in Supplementary Table 1. [†]One-way ANOVA of all groups. Post-hoc Tukey's multiple comparisons test revealed statistically significant differences compared to ACC[a], to hyperplasia[b] or to ADA[c]. [#]DST: 1 mg dexamethasone overnight suppression test. n.d.: not determined

Figure 2: Serum hormone levels in patients with adrenal pathology



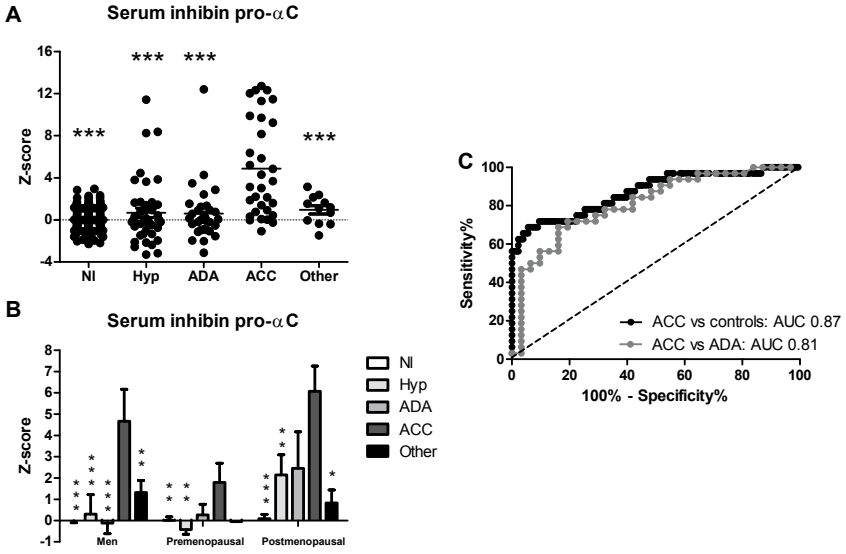
Levels of inhibin pro-αC (A), morning cortisol (B), androstenedione (C) and DHEA-S (D) were studied in patients with adrenocortical hyperplasia (Hyp), adrenocortical adenoma (ADA), adrenocortical carcinoma (ACC) and non-cortical adrenal tumors (other). *P<0.05, **P<0.01, ***P<0.001, one-way ANOVA followed by post-hoc Tukey's multiple comparison test

Receiver operating characteristics (ROC) analysis of inhibin pro-αC in ACC patients versus controls showed areas under the curve (AUCs) of 0.93, 0.75 and 0.88 for men, pre-menopausal and post-menopausal women, respectively. After Z-score transformation based on gender and menopausal status, inhibin pro-αC levels remained highly significantly elevated in ACC patients compared to all other groups (P<0.0001, Figure 3A). Within male subjects, the pro-αC Z-scores were higher in ACC subjects than in all other subject groups (P<0.0001). For the female subjects, the ACC patients had higher

Z-scores compared to the control and adrenal hyperplasia groups (P=0.004 for premenopausal; P<0.0001 for postmenopausal), but not relative to the adenomas (Figure 3B). The combined ROC analysis of the Z-scores showed an AUC of 0.87 (P<0.0001, Figure 3C) for the differentiation between ACC patients and control subjects. ROC analysis of serum inhibin pro-αC levels in ACC patients versus ADA patients yielded AUCs of 0.91, 0.70 and 0.67 for the three groups respectively; overall analysis after Z-score transformation gave an AUC of 0.81 (P<0.0001, Figure 3C).

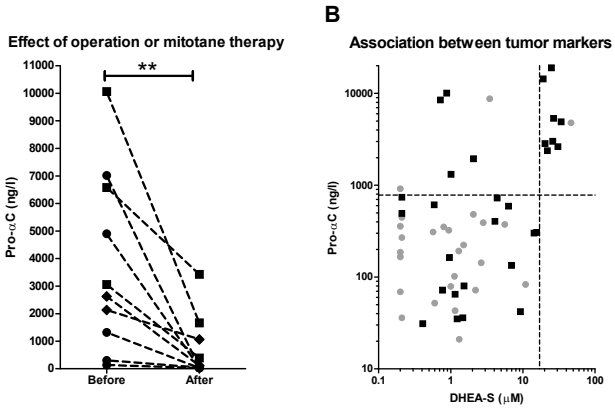
Treatment of ACC led to a decrease in serum inhibin pro-αC levels in all 10 patients tested (P=0.007, Figure 4A). Serum inhibin pro-αC and steroid concentrations restored to normal values in all 5 patients who underwent radical ACC resection, although two patients did subsequently develop lymph node metastases within one year after operation. Three patients underwent tumor reductive surgery which led to a reduction of pro-αC levels in all. The presence of residual disease in these patients was accompanied by postoperative inhibin pro-αC levels that were still elevated compared to reference values. Serum steroid levels were normal in two out of the three patients after incomplete resection. In addition, a decrease of pro-αC levels was also observed in two patients with metastasized ACC, 5 and 7 months after the initiation of mitotane. Mitotane therapy also diminished DHEA-S and other adrenocortical steroid levels in both patients, similar to the decline detected

Figure 3: Inhibin pro-αC levels after adjustment for gender and menopausal status



(A) Inhibin pro-αC levels after Z-score-transformation in normal subjects (NI) and patients with adrenocortical hyperplasia (Hyp), adrenocortical adenoma (ADA), adrenocortical carcinoma (ACC) and non-cortical adrenal tumors (other). ***P<0.001, compared to ACC. (B) Mean Z-scores of inhibin pro-αC levels, stratified by gender and menopausal status. ***P<0.001, **P<0.01, *P<0.05, compared to ACC (C) ROC analysis of Z-scores of serum inhibin pro-αC levels. Patients with ACC (n=32) were compared to control subjects (n=178) or patients with ADA (n=32)

Figure 4: Association of inhibin pro-αC levels with ACC treatment and DHEA-S levels



(A) Treatment of ACC through radical resection (circles), incomplete resection (squares) or mitotane therapy (checkers) led to a normalization or reduction of serum inhibin pro-αC levels in 10 out of 10 patients. **P<0.01, paired t-test. (B) Association (r=0.45, P<0.0001) between serum inhibin pro-αC and DHEA-S levels in patients with ADA (grey circles, n=26, r=0.46, P=0.02) or ACC (black squares, n=29, r=0.41, P=0.03). Dotted line indicates maximum upper reference values: 780 ng/l for inhibin pro-αC and 17 μmol/l for DHEA-S.

for inhibin pro-αC. This was reflected by radiological regression of multiple hepatic and pulmonary metastases in one patient, but was accompanied by progression of pulmonary and retroperitoneal lesions in the other.

In ACC patients no significant relation was found between inhibin pro-αC levels and age, tumor size or van Slooten index. In a combined group of patients with ADA or ACC, we found that pro-αC levels were correlated with serum levels of DHEA-S (r=0.454, P<0.0001, Figure 4B), morning fasting cortisol (r=0.391, p=0.002) and midnight cortisol (r=0.656, P=0.002). Inhibin pro-αC levels were higher in patients with tumors causing steroid hormone overproduction compared to those with clinically non-functional tumors: 3202 ± 4841 versus 805 ± 1787 ng/l (mean ± SD, P=0.0065) in patients with and without hypercortisolism and 4591 ± 5450 versus 842 ± 1950 ng/l (P<0.0001) in patients with and without hyperandrogenism, respectively.

DISCUSSION

After inhibin α-subunit expression was discovered in the human adrenal cortex,[22] adrenal glands have been found to secrete inhibin-like immunoreactivity into the circulation under the influence of ACTH.[29-30] Extracts of adrenal tumors were also found to contain inhibin-like immunoreactivity, which appeared to be increased in Cushing adenomas.[29] Subsequently, it was suggested that serum inhibin assays could also be used in the diagnosis of patients with adrenocortical pathology.[31-32] This is the first study demonstrating

that serum levels of inhibin pro-αC are elevated in a subset of patients with ACC and may serve as a tumor marker for ACC.

In current clinical practice, imaging studies and assessment of the steroid hormone profile are important diagnostic tools for the preoperative differentiation between benign and malignant adrenocortical tumors. Due to overlap of tumor characteristics this may be difficult, especially for tumors with a diameter between 4 and 6 cm and non-steroid secreting tumors.[2-5] In view of the increasing incidence of adrenal incidentalomas on abdominal imaging studies, additional diagnostic markers are needed for differentiation between various pathological entities. Assessment of steroid levels can be helpful as serum tumor markers in order to monitor the response to treatment in patients with ACC, but not all ACCs are hormonally functional.[33-37] Furthermore, correlation with tumor burden has not been shown for adrenal androgens and the usefulness of steroid levels as adrenal tumor markers is restricted by the relatively low elevation above reference values.

The inhibin α-subunit is expressed in the zona reticularis of the human adrenal cortex, with some extension into the zona fasciculata.[25] Adrenocortical inhibin β-subunit expression is low and exhibits a different zone-specific distribution pattern,[23, 25] thereby reducing the possibility of formation of mature inhibin A or B.[13] Inhibin pro-αC may therefore be expected to be the predominant inhibin form secreted by the adrenal cortex. In men and pre-menopausal women the gonads are the main source of serum inhibin pro-αC, but the presence of inhibin pro-αC in serum of postmenopausal women suggests that the adrenal cortex also significantly contributes to its production.[19] In spite of the gonadal contribution ACTH can still modulate serum pro-αC levels. A similar response has been described for total inhibin-like immunoreactivity in hypogonadal men.[29] On the other hand, we did not observe a change in serum inhibin pro-αC levels after chronic ACTH stimulation, as occurs in patients with pituitary or ectopic ACTH production or after metyrapone administration during 24 hours. This could suggest the presence of adaptive mechanisms under long-term ACTH stimulation.

Following the discovery that the adrenal gland can secrete inhibin pro-αC and the role of inhibin forms as tumor markers for ovarian cancer we now demonstrate that the majority of patients with ACC also have increased serum levels of pro-αC. The tumor suppressor role of the inhibin α-subunit, as detected in murine models,[12] therefore does not apply to a subset of human ACCs. Serum levels of the inhibin pro-αC peptide were substantially higher in patients with ACC than in patients with benign adrenocortical disorders. The pro-αC form of inhibin thus constitutes a novel and specific serum tumor marker for ACC. In contrast, serum inhibin A and B levels did not differ between patient groups, although three ACC patients did have increased serum levels of inhibin B, as was described in two case reports before.[38-39]

In contrast to inhibin pro-αC, serum cortisol and androgen levels, including DHEA-S, were not significantly different between ACC and ADA. Sensitivity and specificity of the

inhibin pro-αC assay was comparable to that of DHEA-S. Although the current study was not designed to detect significant differences in predictive values between these two diagnostic tests, inhibin pro-αC could have a more favorable sensitivity in contrast to a higher specificity of DHEA-S. The combined measurement of inhibin pro-αC and DHEA-S increased the positive predictive value for the detection of ACC to 92%, making concomitant elevation of both serum markers highly suspicious of malignancy. This suggests that the combined measurement of both serum markers could have additional diagnostic value. Inhibin pro-αC was increased in 25% of ACC patients with normal serum DHEA-S levels, making it the only serum tumor marker in these patients. Inhibin pro-αC measurement appears to be most discriminating in pediatric ACC patients, all of whom showed increased pro-αC levels, and in male subjects with adrenal enlargement. The discriminative power of inhibin pro-αC was found to be reduced in women, who form the largest subset of patients with ACC. As a consequence, the result of measurement of inhibin pro-αC, has a low overall sensitivity at 59%. Nonetheless, the magnitude of differences in serum pro-αC levels between groups, particularly in male and pediatric subjects, underscore the potential diagnostic value of serum inhibin pro-αC as a serum marker for ACC.

Serum inhibin pro-αC levels appear to reflect tumor burden, falling drastically to normal values after radical surgery and also decreasing after tumor-reductive therapy. Although not correlated with tumor size in the entire group of patients, these levels seem suitable as a tumor marker for individual treatment success. The serum pro-αC levels detected are higher than those of adrenal androgens when compared to their reference values, possibly leading to a broader range of sensitivity during follow-up.

The limitations of this study include the sample size of the patients with ACC. Using this multicenter approach we obtained serum samples from 32 ACC patients, which, given the rare tumor incidence,[40] comprises a large group. Controls were obtained from blood bank samples, leading to a predominance of male subjects which is not representative of the gender-specific distribution of ACC.[7] However, the currently described reference levels are highly comparable to the previously published reference values of the inhibin pro-αC assay,[15] thereby validating this approach. The negative predictive value of inhibin pro-αC for the differentiation between ADA and ACC is moderate at 68%. This finding indicates that a normal serum pro-αC level is not informative in the presence of radiologically suspicious adrenal tumors and should not influence clinical decision making. Given that patients with ENSAT stage I ACC also displayed increased pro-αC levels suggests that the presence of elevated levels in patients with adrenal tumors of clinically uncertain behavior, such as small tumors, could reflect malignancy. This might constitute an additional argument for surgical intervention instead of surveillance.

This study was primarily designed to describe the characteristics of inhibin pro-αC as a serum marker for ACC. Future studies in larger patient groups comparing the predictive values, clinical applicability and costs of serum inhibin pro-αC and DHEA-S and also diag-

nostic tools such as urinary steroid profiles by gas chromatography/mass spectrometry[41] are needed to determine the optimal test in patients with an adrenocortical disorder. With regard to follow-up we only studied inhibin pro-αC levels after tumor surgery or chemotherapy. Whether pro-αC levels are also indicative for tumor recurrence or growth should be assessed in prospective studies, but the effect of tumor reduction on the inhibin α-subunit levels seems promising in this respect.

In conclusion, we describe serum inhibin pro-αC as a novel serum tumor marker for adrenocortical carcinoma. Inhibin pro-αC is secreted by the adrenal cortex and its levels are increased in serum of ACC patients. Measurement of inhibin pro-αC, although hampered by a moderate sensitivity, might be a helpful diagnostic tool to discriminate between ACC and benign adrenal neoplasia in patients with normal steroid levels. Serum inhibin pro-αC has a high positive predictive value in combination with serum DHEAS levels and might serve as a tumor marker for ACC during treatment follow-up.

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Supplementary Table 1: Local serum references values of studied hormones

Hormone	Group / age	Male	Female
Inhibin pro-αC (ng/l)	All	196-685	
	Premenopausal		36-780
	Postmenopausal		15-83
Inhibin A (ng/l)	All	<10	
	Premenopausal		<150
	Postmenopausal		<10
Inhibin B (ng/l)	All	150-400	
	Premenopausal		10-200
	Postmenopausal		<10
Morning cortisol (nmol/l)	All	200-800	200-800
Cortisol after DST (nmol/l)	All	<50	<50
Cortisoluria (nmol/24 hr)	All	<850	<850
Progesterone (nmol/l)	All	0.5-2.0	
	Premenopausal		<0.5-70
	Postmenopausal		<0.5-2.0
17OH-Progesterone (nmol/l)	All	<10	<10
11-deoxycortisol (nmol/l)	All	<50	<50
Androstenedione (nmol/l)	< 2 yrs	0.35-1.2	0.35-1.2
	2-4 yrs	0.35-1.4	0.35-1.4
	4-6 yrs	0.35-2.6	0.35-2.6
	6-8 yrs	0.7-3.8	0.7-3.8
	8-10 yrs	1.0-3.8	1.0-3.8
	10-12 yrs	1.4-5.2	1.4-5.2
	>13 yrs	2.0-10	
	Premenopausal		2.0-15
	Postmenopausal		1.0-10
DHEA-sulfate (μmol/l)	<9 yrs	0.4-1.4	0.4-1.4
	10-19 yrs	0.8-17.0	1.4-10.0
	20-29 yrs	7.0-17.0	1.5-10.0
	30-39 yrs	4.0-14.0	1.2-7.0
	40-49 yrs	2.5-14.0	0.8-7.0
	50-59 yrs	2.0-8.0	0.7-5.0
	60-69 yrs	1.0-8.0	0.3-4.0
	>70 yrs	1.0-5.0	0.3-3.0

Methylation and common genetic variation in the inhibin alpha-subunit (*INHA*) promoter affect its expression in human adrenocortical carcinomas more than *INHA* mutations

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ABSTRACT

Background:

Adrenocortical carcinoma (ACC) is a rare, but highly malignant tumor of unknown origin. Inhibin α-subunit (*Inha*) knockout mice develop ACCs following gonadectomy, whereas *INHA* expression varies widely in human ACCs. We investigated whether genetic or epigenetic changes in *INHA* causing loss or variation of *INHA* expression are present in human ACC.

Methods:

Analyses of *INHA* sequence, methylation and mRNA expression were performed in human adrenocortical tissues. Serum inhibin pro-αC levels were measured in ACC patients.

Results:

INHA genetic analysis in 37 ACCs revealed 13 novel, heterozygous mutations. Of the 6 coding bases affected, four were synonymous and two missense mutations: S72F and S184F. The minor allele of rs11893842 at -124 bp was observed at a low frequency (24%) in ACC samples and was associated with decreased *INHA* mRNA levels: 0.0047±0.0019 (arbitrary units, mean±SEM) for AA, compared to 0.0026±0.011 for AG/GG genotypes (P=0.034). Methylation of four *INHA* promoter CpGs at -285, -241, -203 and -149 was aberrantly increased in five ACCs (47.7±3.9%), compared to normal adrenals (18.4±0.6%, P=0.0052), whereas the other 14 ACCs studied showed diminished promoter methylation (9.8±1.1%, P=0.020). Promoter methylation at these CpGs was inversely correlated with *INHA* mRNA levels in ACCs (r=-0.591, p=0.020), but not with serum inhibin pro-αC levels.

Conclusions:

Aberrant methylation of and genetic variation in the *INHA* promoter occur in human ACCs and are associated with decreased *INHA* expression. *INHA* gene mutations appear to play only a minor role in ACCs.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare malignancy with a poor survival rate.[1-2] The occurrence of ACC has a female preponderance and a bimodal distribution with an increased incidence in children and in adults over 60 years.[3] Familial ACC occurs in the context of genetic syndromes, such as Beckwith-Wiedeman syndrome[4] and Li-Fraumeni syndrome.[5] Mutations in genes underlying these disorders have also been linked to sporadic ACC formation, especially in the case of *TP53*.[6] The most frequent alteration found in ACC is overexpression of the maternally imprinted IGF-II locus.[7] More recently, mutations in the Wnt/β-catenin pathway have been shown to occur during adrenocortical tumor progression.[8] Genetic causes and the role of chromosomal aberrations in adrenocortical tumorigenesis remain largely unknown.

The inhibin α-subunit (encoded by *INHA*) forms inhibin A or B by coupling to the inhibin βA- or βB-subunits, respectively, and its expression is limited to the gonads, placenta and adrenal cortex. The principal effect of circulating inhibin A and B is inhibition of local activin-induced follicle-stimulating hormone (FSH) secretion in the pituitary gland.[9] In a murine knockout model, the inhibin α-subunit was found to have a tumor suppressive role for gonadal tissue[10] and, after gonadectomy, for the adrenal cortex.[11] Ninety-nine percent of *Inha* -/- mice developed adrenocortical steroid-secreting carcinomas after gonadectomy.[11] Pathways involved in this effect include the differentiation into granulosa cell-like cells with expression of fetal or gonadal markers such as *Gata4*, *Lhr, Fshr* and *Cyp17a1*.[12] *Inha*-related carcinogenesis in mice has also been attributed to decreased activin signaling potential and aberrant expression and effects of TGF-β2.[13-14]

In man, the evidence for *INHA* as an adrenocortical tumor suppressor is conflicting. Several mRNA and protein analysis studies have shown lack of *INHA* expression in a proportion of patients with ACC as well as *INHA* overexpression in another subset.[15-19] Recently, we reported that serum levels of the free peptide form of the α-subunit, inhibin pro-αC, were increased in patients with adrenocortical carcinomas and that these levels can be utilized as a tumor marker;[20] inhibin pro-αC levels may be useful for the differentiation between malignant and benign adrenocortical tumors as well as for follow-up of individual patients. Although the majority of ACC patients showed increased serum levels of inhibin pro-αC a subset of patients had normal levels, possibly representing the tumors that do not express *INHA*.[20]

Several DNA alterations are known to influence gene expression and can be disrupted during tumorigenesis. Next to the genetic changes leading to aberrant or loss of expression, epigenetic alterations, such as chromatin remodeling and CpG methylation, also frequently occur in cancer and affect gene transcription.[21] Methylation of CpG islands in gene promoter regions can result in transcriptional silencing and loss of gene expression due to interference with the binding of transcription factors.[22]

In the current study we investigated causes of aberrant *INHA* expression in human adrenocortical carcinomas. Sequencing of the *INHA* gene was undertaken to search for mutations that could affect gene function or expression levels. Furthermore, quantitative analysis of *INHA* promoter methylation was performed in order to study whether methylation of CpGs contributes to the differences in expression. Together these analyses were coupled to intratumoral mRNA levels of the inhibin α-subunit and serum concentrations of inhibin pro-αC.

MATERIALS & METHODS

Sample collection

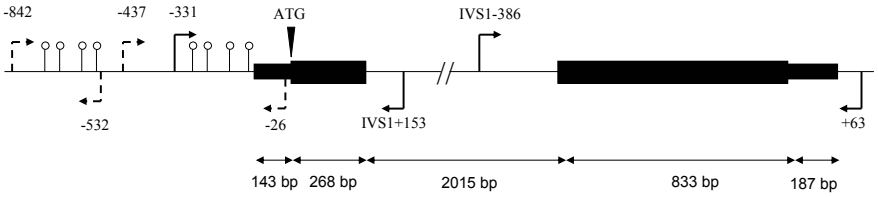
Paraffin-embedded tissue blocks were collected from the pathological archives of the Erasmus MC. Tissue samples originated from patients operated between 1991 and 2010 in the Erasmus MC. The diagnosis of adrenocortical carcinoma was made if the van Slooten index exceeded 8.[23] Tumor staging was categorized according to the European Network for the Study of Adrenal Tumors (ENSAT) staging system.[1] Haematoxylin and eosin-stained slides were evaluated by a pathologist and sections with a high percentage of viable tumor cells were microdissected for further analysis. From 2007 and onwards, samples were obtained in a prospective study of adrenal tumors. These also included adrenal tissues from patients who underwent adrenalectomy due to renal cell carcinoma, adrenal hyperplasia, adenoma and carcinoma. Tumor sections were gathered from viable tumor parts and snap-frozen in liquid nitrogen or dry ice shortly after resection. Pre-operative serum levels of inhibin pro-αC were measured in patient subsets using an enzyme-linked immunometric assay (Diagnostic Systems Laboratory, Webster, TX, USA). The study was performed according to the Dutch regulations on the use of residual tissues, approved by the Medical Ethics Committee of the Erasmus MC and informed consent was obtained from all participants.

DNA sequencing

DNA was isolated with a DNA mini kit (Qiagen, Venlo, The Netherlands) according to manufacturer's protocol and dissolved in H_2O. Its concentration was measured using a Nanodrop dispenser (Thermo Fisher Scientific, Waltham, MA, USA).

The inhibin α-subunit gene, located at 2q35, is composed out of two exons (Figure 1). For DNA analysis of paraffin-embedded tissues primer pairs were selected to amplify regions of 200-250 bp. For freshly frozen tissues regions up to 500 bp could be amplified. Primer pairs and location are summarized in Table 1. Primers covered the coding region, up to -331 bps from the ATG start site (containing the cAMP binding, SF-1 response and

Figure 1: The human inhibin α-subunit (*INHA*) gene



Located at 2q35, *INHA* is composed of two exons separated by a 2 kb intron. The coding sequence is composed of 1101 bps. The regions sequenced in this study are indicated by the continuous arrows. The areas investigated for methylation are depicted by the dashed arrows; CpG dinucleotides successfully characterized are shown as open circles.

GATA elements at -151 to -112 bps[24-25]) and at least 153 bps of intron adjacent to exon-intron boundaries (Figure 1).

PCR amplication was performed in a 30 µl volume of 0.05 U/µl FastTaq polymerase (Roche Applied Science, Almere, The Netherlands), 1 ng/µl DNA, 250 nM forward and reverse primers (Biolegio, Nijmegen, the Netherlands), 200 µM dNTPs (Amersham Biosciences, Uppsala, Sweden) and buffer containing $MgCl_2$ (Roche) in a GeneAmp 9700 (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) under the following conditions: 7 minutes at 95 °C, followed by 40 cycles of 1 minute intervals at 95 °C, 56-63 °C and 72 °C, ending with 10 minutes at 72 °C. PCR products were purified by High Pure PCR Product Purification Kit (Roche).

Both forward and reverse PCR primers were used in a separate sequence reaction with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Three µl of purified PCR product was used with 500 nM of primer in a reaction of 1 minute at 96 °C and 25 cycles of 30 seconds at 96 °C, 15 seconds at 50 °C, and 4 minutes at 60 °C. The sequence reaction products were purified with the use of the Dye-Ex 96 Purification Kit (Qiagen) and Micro-Bio-Spin Purification Columns (Bio-Rad, Veenendaal, The Netherlands). Sequence detection was performed using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencher software (Genes Codes Corporation, Ann Arbor, MI, USA) was used for DNA analysis.

Methylation analysis

One µg of DNA, obtained from frozen samples, was treated with bisulfite using the EZ DNA methylation kit (Zymo research, Irvine, CA, USA), dissolved in 100 µl H_2O and stored at -80 °C. Bisulfite-treated DNA in the promotor region of *INHA* was amplified by PCR while a T7 promotor was introduced in the reverse primer. Using software provided by Sequenom (Hamburg, Germany) two primer sets were constructed that covered multiple CpG dinucleotides upstream of the *INHA* start site (Figure 1); sequences are described in Table 1.

Table 1: Primer sequences (5' to 3')

sequence	forward	position
INHA	TGTGTGTAGGGAGAAGGTGTT	-331
INHA	GGAAGACTGGATGAGAAGGG	-134
INHA	TTCTTGCTGCTGACCCC	22
INHA	CTGGTGGCCACATCCCTG	IVS1-89
INHA	AGAGTGCAGCCCATCATT	IVS1-116
INHA	CCATCCATGTAGACACCATTC	IVS1-386
INHA	GCACAGCAGCCTCCAATA	422
INHA	TCCCCTCTGTACCTGCTCA	600
INHA	ATGCCAACTGCCACAGAGTA	776
INHA	CGGATGGAGGTTACTCTTTCA	1031
	reverse	
INHA	CACCCACCCTCTTCTACC	-99
INHA	GCCAGAACAAGTTCCCG	92
INHA	TGCTTTTTCTCAAAGTCATCC	IVS1+45
INHA	GGGAGACAGAAGCATAAGGA	IVS1+153
INHA	GGGGCTCAGAGCTATTGG	451
INHA	CGGTGACAGTGCCAGCAG	477
INHA	GACATCAGGGGAGTTGAGC	713
INHA	AAACTGGGAGGGTACACGAT	857
INHA	GAGAAGGTTGGGCACTGTCT	1077
INHA	AGATCTGACAGTCCCATGCTC	69 bp 3' of *INHA*
methylation	**forward**	
INHA	AGGAAGAGAGGTTGTTTGGTTTTTTGTTTTTAGGA	-842
INHA	AGGAAGAGAGTTGATGTTATTTTTGGATGTGTTTG	-437
	reverse	
INHA	CAGTAATACGACTCACTATAGGGAGAAGGCT AACCTTCTAAAACCCCTTTCAATAA	-532
INHA	CAGTAATACGACTCACTATAGGGAGAAGGCT TAATAAAAAACTCACACCCTACCCC	-26
mRNA	**forward**	
INHA	CCGAGGAAGAGGAGGATGTCT	221
HPRT1	TGCTTTCCTTGGTCAGGCAGTAT	293
GAPDH	ATGGGGAAGGTGAAGGTCG	1
	reverse	
INHA	CGGTGACAGTGCCAGCAG	477
HPRT1	TCAAATCCAACAAAGTCTGGCTTATATC	545
GAPDH	TAAAAGCAGCCCTGGTGACC	70
	probe (FAM-TAMRA labeled)	
INHA	TGACTTCAGCCCAGCTGTGGTTCCA	377
HPRT1	CAAGCTTGCGACCTTGACCATCTTTGGA	489
GAPDH	CGCCCAATACGACCAAATCCGTTGAC	47

The PCR was performed in a 5 µl volume containing 0.05 U/µl HotStar Taq polymerase (Qiagen), 200 µM dNTPs, 200 nM of both primers, 1 µl of bisulfite-treated DNA, buffer and H_2O. After 10 minutes at 95 °C, 35 cycles were performed of 30 seconds at 95 °C, 30

seconds at 53 °C and 45 seconds at 72 °C. The reaction ended with a 7 minute annealing step at 72 °C. After confirmation of PCR product on a 2% agarose-containing gel, the product was treated with 2 µl Shrimp Alkaline Phosphotase for 20 minutes at 37 °C and 5 minutes at 85 °C.

Next, *in vitro* transcription was performed in triplicate in a 5 µl mixture containing T7 R&DNA polymerase, T-specific cleavage mix, DTT, RNase A, PCR product and buffer (Sequenom). The resulting fragments were diluted with H_2O and 6 mg of CLEAN resin was added for 10 minutes to remove sodium and potassium ions. This mixture was dispensed on a SpectroCHIP with the MassARRAY Nanodispenser instrument (Sequenom). Quantitative methylation was detected by a MassARRAY epiTYPER (Sequenom) and analysis was performed using accompanying software.

Assays standard curves were constructed by assaying mixtures of prediluted DNA containing 0% to 100% methylation with intervals of 10%. Methylation analysis was successful for 8 CpGs in the *INHA* promoter: located 149, 203, 241, 285, 558, 599, 719 and 751 bps upstream of the *INHA* start site.

mRNA analysis

Total RNA was isolated from frozen adrenocortical tissues by Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase and quantitative polymerase chain reaction of *INHA* and housekeeping genes *HPRT1* and *GAPDH* was performed in duplicate as previously described.[15] Sequences of primers and probes sequences have been indicated in Table 1. Expression levels of *INHA* were calculated relative to that of the average threshold cycle (Ct) of *GAPDH* and *HPRT1* using the delta-Ct method.

Statistical analysis

Analyses of differences between groups were performed with Chi-Square tests, one-way analyses of variance followed by Tukey's multiple comparison tests or t-tests using Graphpad Prism software (Graphpad Inc, La Jolla, CA, USA). mRNA levels were log-converted before analysis. Correlations were analyzed by Pearson's correlation coefficient. All tests were calculated as two-tailed and a P-level below 0.05 was considered statistically significant.

RESULTS

Sequence analysis

The *INHA* sequence was analyzed in 37 unique adrenocortical carcinoma tissues (12 fresh frozen, 25 paraffin-embedded). In 35 out of 37 (95%) tumor samples the coding region of *INHA* could be completely sequenced; genetic analysis was thus far unsuccessful for

bps -100 to 20 in two paraffin-embedded tissues due to insufficient PCR product. Results from the sequence analyses have been summarized in Table 2.

In total, sequencing of the *INHA* gene in 37 ACCs revealed 13 novel mutations in 10 ACCs. One ACC harboured three heterozygous mutations (-77G>A, -63A>G and –56G>T) in the 5'UTR, whereas a heterozygous mutation directly after the stop codon (*1G>A) was detected in another ACC. We located three intronic mutations, located 179, 72 and 9 bps upstream of the intron 1-exon 2 border. In the coding region of *INHA* we detected 4 synonymous nucleotide changes (Gly16Gly, Ser245Ser, Ala25Ala and Val195Val) and 2 missense mutations, in 5 distinct ACCs. The latter both comprised heterozygous C→T mutations, at bps 215 and 552, leading to serine to phenylalanine changes at amino acids 72 and 184, respectively.

In our series, the –124A>G SNP, rs11893842, was present in ACCs with a minor allele frequency (MAF) of 24%, compared to 44% in reference population (www.1000genomes.org[26]). Furthermore, the minor allele of the intronic SNP IVS1-87G>A (rs116399602) was present in 16% of ACC samples, seemingly higher than in healthy individuals (2.8% MAF). Rs7588807 (IVS1-314G>T) was only measured in the subset of frozen DNA samples. The minor T allele was previously reported to occur in 48% in control subjects; the ACCs showed a lower MAF of 29%. Rs35118453 (-16C>T) and rs12720063 (532C>T) were present at low frequency, comparable to reference[26]: 9% and 12%, respectively. No other known SNPs in the *INHA* gene were detected in our series.

Methylation analysis

The first series in which *INHA* promoter methylation was investigated, encompassed DNA from 3 normal adrenal glands and 12 ACCs. For the 4 CpG dinucleotides 558, 599, 719 and 751 bps upstream of the *INHA* start site low methylation ratios were obtained in all samples tested: 4.4±1.1%, 4.5±0.8%, 1.7±0.7% and 2.5±0.6% (mean±SEM), respectively. Furthermore, there were no differences between normal adrenal tissues and ACCs (data not shown). The CpGs in proximity to the start site were methylated to a higher degree in a subset of samples; therefore we analyzed an additional 7 ACCs with the downstream primer pair only.

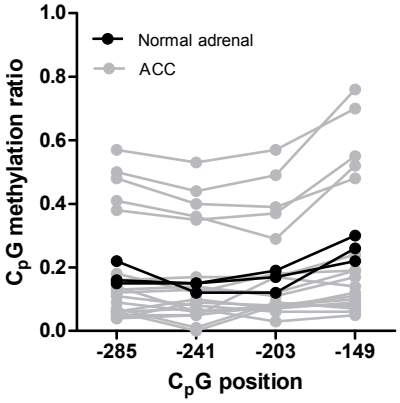
Results of the methylation analysis of CpGs at -285, -241, -203 and -149 are depicted in Figure 2. Five out of the 19 ACCs had aberrantly high methylation rates of all four proximal CpGs investigated in the *INHA* promoter. Average methylation ratio of these ACCs was 47.7±3.9%, compared to 18.4±0.6% for normal adrenals (P=0.0052) and 9.8±1.1% for the other ACCs (P<0.0001). The difference in methylation between normal adrenals and the other ACCs was also statistically significant (P=0.020). The percentage of *INHA* promoter methylation in the ACC samples was not associated with tumor characteristics, such as hormonal overproduction, van Slooten index or ENSAT stage (data not shown).

Table 2: Mutation analysis of *INHA* in 37 human adrenocortical carcinomas

	Clinical characteristics					Type	Mutations‡			SNPs**				
patient	Sex	Age†	cushing	androgens	ENSAT					rs11893842	rs35118453	rs7588807¶	rs116399602	rs12710063
1	F	33	+	+	4	paraffin	-77G>A	-63A>G	-56G>T	G	T			
2	F	9	+	+	4	paraffin				G				
3	M	62	-	-	4	frozen				G				
4	F	57	-	-	2	frozen				A/G		T		
5	F	38	-	-	2	frozen						T		
6	M	44	-	-	4	frozen				A/G		T		
7	F	51	-	-	4	frozen				A/G		G/T	G/A	C/T
8	M	43	-	-	4	frozen							A	
9	F	56	-	-	2	frozen				G				
10	F	54	-	-	2	frozen				G	C/T			T
11	F	61	-	-	4	frozen	Ser184Phe	Val195Val					G/A	
12	M	68	-	-	2	frozen								
13	F	74	+	-	4	frozen				A/G				
14	F	65	-	-	4	frozen								
15	F	69	+	+	4	paraffin	Ser245Ser						A	
16	F	40	-	-	2	paraffin	*1G/A							
17	M	54	-	+	2	paraffin	Gly16Gly							
18*	F	58	-	-	2	paraffin				G				
19	M	52	+	+	2	paraffin	Ala25Ala							C/T
20	F	38	+	+	2	paraffin								
21*	F	52	+	+	2	paraffin	IVS1-179G>T							
22	M	38	+	+	2	paraffin							G/A	
23	F	33	+	+	4	paraffin								
24	F	35	-	+	2	paraffin	Ser72Phe						A	
25	F	53	-	-	2	paraffin							A	
26	F	56	+	+	2	paraffin								
27	M	42	-	-	4	paraffin					C/T			
28	F	69	-	-	4	paraffin								
29	M	41	+	-	4	paraffin					T			T
30	F	4	-	+	1	paraffin								
31	F	4	-	+	1	paraffin	IVS1-72C>A							
32	F	39	-	-	4	paraffin								
33	F	27	+	-	1	paraffin								
34	F	76	-	-	2	paraffin				G	C/T		G/A	C/T
35	F	68	+	+	3	paraffin								C/T
36	F	64	+	-	2	paraffin								
37	F	68	-	-	2	paraffin	IVS1-9T>C							

*bps -100 to 20 are missing in the current analysis**,** †expressed in years**,** ‡all detected mutations were heterozygous, **only heterozygotes and minor alleles have been indicated, ¶this SNP was only studied in frozen tissue samples

Figure 2: *INHA* methylation analysis in adrenocortical tissues



Quantitative methylation analysis of four CpG dinucleotides in the *INHA* promoter was performed in 3 normal adrenals and 19 ACCs. Individual CpGs are indicated on the x-axis by the bp number located 5' from the ATG start site. 0 indicates no methylation of DNA whereas 1 indicates that all DNA tested in the tissue sample is methylated. Individual data points are composed of a mean of triplicate measurements.

Expression analysis

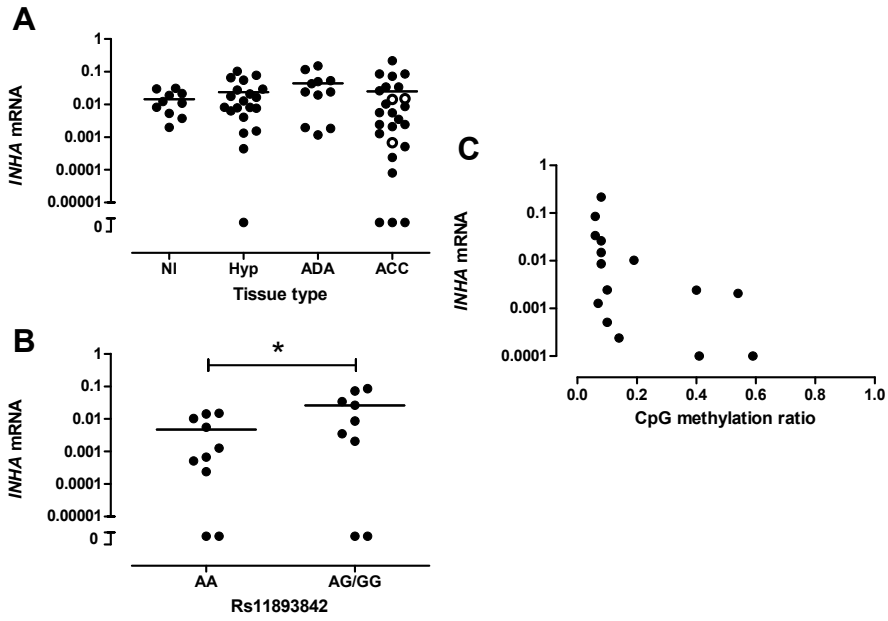
INHA mRNA expression levels were measured in normal adrenal (n=10), adrenocortical hyperplasia (n=20), adenoma (ADA, n=11) and ACC (n=25) tissues. As previously described[15] 3 ACC samples showed no *INHA* mRNA whereas the other ACCs demonstrated a wide range of expression from 0.000080 to 0.22 arbitrary units (A.U.). Overall, there were no significant differences between *INHA* expression in all groups investigated (Figure 3a). Also, mRNA levels of *INHA* in ACCs were not related to tumor characteristics (data not shown).

Of the five ACC samples with mutations in *INHA* exons, three were analyzed for expression; these samples all showed normal *INHA* mRNA levels (Figure 3a, open circles). When stratified for the five separate SNPs, only the rs11893842 gene variation was associated with changes in *INHA* mRNA: mean expression in tissues with the AA genotype was 0.0047±0.0019 A.U., compared to 0.0026±0.011 A.U. for the AG/GG genotypes (P=0.034, Figure 3b).

Combined mRNA and methylation analyses were available for 15 tissues. Overall, there was a significant negative association between the average methylation ratio of the proximal CpG island and *INHA* mRNA expression (Figure 3c, r_s=-0.591, P=0.020).

Serum inhibin pro-αC levels were available in a subset of patients. There were no significant relations between serum inhibin pro-αC Z-scores on one hand and methylation ratio (n=9, r_s=-0.10, P=0.81) or mRNA expression (n=13, r_s=-0.47, P=0.11) on the other (data not shown). Inhibin pro-αC levels were only available for one ACC patient with *INHA* mutations: this premenopausal female patient with three mutations in the 5'UTR of *INHA* had highly increased inhibin pro-αC levels at 3000 ng/l (normal<780 ng/l).

Figure 3: *INHA* mRNA analysis in adrenocortical tissues



(A) Quantitative *INHA* mRNA analysis was comparable in normal adrenals (Nl, n=10), adrenocortical hyperplasia (Hyp, n=20), adenomas (ADA, n=11) and carcinomas (ACC, n=25). Three ACCs (patient no. 11, 15 and 17 in Table 2) harbouring mutations in *INHA* are displayed as open circles. Bar represents mean. (B) Variation in rs11893842 (-124A>G) was associated with changes in *INHA* gene expression. Bar represent mean, *P<0.05. (C) Negative association between promoter methylation of the *INHA* gene and *INHA* mRNA expression (r=-0.591, P=0.020).

DISCUSSION

The inhibin α-subunit has been implicated in adrenocortical tumorigenesis since gonadectomized *Inha* -/- mice developed adrenocortical carcinomas with a high penetrance.[11] Loss of *INHA* expression has been detected in a small subgroup of human ACCs,[15-17, 19, 27-28] pleading against a significant tumor suppressor role of *INHA* in human adrenocortical carcinogenesis. This is the first study showing that the large variation in *INHA* expression in ACCs is at least partly caused by methylation and common genetic variation of the *INHA* promoter and is not due to mutations of the gene.

In the murine knockout model, *Inha* is thought to predispose adrenocortical cells to endure a phenotypic switch to gonadal-like cells.[11-12, 14] This is hypothesized to be caused by increased availability of the TGF-β type III receptor betaglycan leading to augmented TGF-β2 signaling.[13] The concomitant rise in circulating gonadotropin levels could ensure proliferation through a cyclin D2-dependent pathway.[29] Whether local knockdown of *INHA* in human adrenocortical cells would suffice for adrenocortical tumorigenesis is

unknown. Since ACCs have predominance in postmenopausal women who have increased gonadotropin levels, this mechanism could occur in this subgroup of patients.

Previous expression studies have revealed that the inhibin α-subunit is not expressed in a small subset of ACCs.[15-17, 19, 27-28] In contrast, *in vivo* studies have revealed increased levels of the inhibin α-subunit in serum of patients with ACC.[20, 30-31] These findings are likely to be a consequence of the wide variation in intratumoral expression levels, in the current study over a 1000-fold. Causes of the loss of or variation in *INHA* expression were unknown.

One previous study has investigated *INHA* mutations in ACCs. Longui *et al.*[18] studied pediatric ACCs patients with germline *TP53* mutations and found 3 heterozygous *INHA* mutations in 6 out of 46 (13%) patients. Of these three novel mutations, one (G227A) was subsequently shown to be a SNP (rs12720061) and did not occur in our ACC cohort. Implications of the other two mutations (P43A and A257T) are unknown; there were not found in the current investigation of sporadic ACCs. Interestingly, the previous study found loss of heterozygosity (LOH) in the vicinity of the *INHA* gene in eight out of nine ACCs studied,[18] suggesting that LOH could cause decreased expression levels. Furthermore, comparative genomic hybridization analyses of human ACCs described sporadic chromosomal loss of the *INHA* region at 2q33-36, with a predominance in childhood tumors.[32-34] On the other hand, the inhibin α-subunit was previously shown to be overexpressed in pediatric ACCs.[20]

In the current study, two novel heterozygous missense mutations were detected. The serine to phenylalanine substitutions at amino acids 72 and 184 might affect the activity of the resulting inhibin α-subunit, but since the function of inhibin in the human adrenal gland is unknown,[35] it is difficult to investigate the consequences of potentially altered activity. The tumor harbouring the S184F mutation expressed normal levels (0.015 A.U.) of *INHA* mRNA, but translation, function or protein degradation could still be affected. The mutations located 179, 72 and 9 bps upstream of the intron-exon border might lead to alternative splicing of the *INHA* gene. Importantly, no homozygous mutations were detected, pleading against total knockout of *INHA* leading to ACC formation. Unfortunately, we had only one patient with *INHA* mutations and concomitantly available serum inhibin pro-αC levels. Here, high serum levels were found despite three mutations in the promoter region. Given the low frequency of *INHA* mutations in sporadic and familial ACCs,[18] these mutations might only be involved in adrenocortical tumorigenesis in a small subset of ACC patients.

Several common *INHA* SNPs were detected in our patients. Minor alleles of rs11893842 and rs758807 were found to occur in ACC patients at lower frequencies than previously reported in healthy cohorts, which might suggest that the major alleles of these SNPs play a role in oncogenesis. Intriguingly, the minor allele of rs11893842, located in the promoter region in close proximity to crucial regulatory sequences,[24-25] was associated with lower levels of *INHA* mRNA expression. The decreased frequency of this SNP in ACC samples appears to contradict the hypothesis that *INHA* is a tumor suppressor in human ACC.

Methylation of promoter regions of tumor suppressor genes is a common mechanism involved in carcinogenesis.[22] Since prevalent *INHA* mutations could not be detected, decreased *INHA* expression in ACCs could be caused by increased methylation of the *INHA* promoter. Methylation of follistatin, involved in the activin/inhibin signaling pathway as an activin antagonist, was previously found in the human ACC cell line H295R.[36] We now show that a subset of human ACCs (26%) has an increased methylation ratio of several CpGs in the *INHA* promoter, in contrast to the majority of ACCs that show a decreased methylation of the *INHA* promoter compared to normal adrenal tissue. This wide range of methylation presumably accounts for part of the wide *INHA* expression range, as shown by the inverse relationship between *INHA* promoter methylation and *INHA* mRNA expression.

The levels of methylation of the *INHA* promoter and *INHA* mRNA expression were not associated with serum inhibin pro-αC levels. Furthermore, we previously found no association between pro-αC levels and tumor stage or size.[20] Therefore, these levels could be primarily dependent on (post-)translational modifications, tumor cell activity, peripheral degradation or clearance from the circulation.

In conclusion, aberrant methylation and common genetic variation within the promoter region of the *INHA* gene affect *INHA* mRNA expression in human ACC. These genetic and epigenetic *INHA* changes could contribute to human adrenocortical tumorigenesis, similar to the murine *Inha* knockout model. The importance of *INHA* mutations in the pathophysiology of ACC appears to be minimal.

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PART III

PROSTATE CANCER

CHAPTER 10

Low expression of enzymes for *de novo* steroid biosynthesis suggests limited role for intratumoral steroidogenesis in prostate cancer

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ABSTRACT

Background:

Current treatment of disseminated prostate cancer (PC) through androgen-deprivation therapy eventually leads to castration-resistant PC (CRPC) in all patients. Intratumoral androgen production could sustain local androgen levels and thereby contribute to tumor progression despite suppressed serum androgen concentrations. The present study investigated if PC and CRPC tissue is capable of intratumoral androgen synthesis.

Methods:

Steroidogenic enzyme mRNAs were quantified in hormonally manipulated human PC cell lines and xenografts as well as in human samples of normal prostate, locally confined and advanced PC, local non-metastatic CRPC and of lymph node metastases.

Results:

Overall, the majority of samples showed low or absent mRNA expression of steroidogenic enzymes required for *de novo* steroid synthesis. Simultaneous but low expression of the enzymes *CYP17A1* and *HSD3B1*, essential for the synthesis of androgens from pregnenolone, could be detected in 19 of 88 patient samples. Out of 19 CRPC tissues examined only 5 samples expressed both enzymes. Enzymes that convert androstenedione to testosterone (T) (*AKR1C3*) and T to dihydrotestosterone (DHT) (*SRD5A1*) were abundantly expressed. *AKR1C3* expression was negatively regulated by androgens in the experimental models and was increased in CRPC samples. Expression of *SRD5A1* was upregulated in locally advanced cancer, CRPC and lymph node metastases.

Conclusions:

The potential contribution of intratumoral *de novo* steroid biosynthesis to intraprostatic androgen levels appears smaller than that of circulating adrenal androgens. Therefore, blockade of specific enzymes that disrupt adrenal androgen production and its intraprostatic conversion into DHT, such as *CYP17A1* inhibition, would form relevant therapeutic options in patients with CRPC.

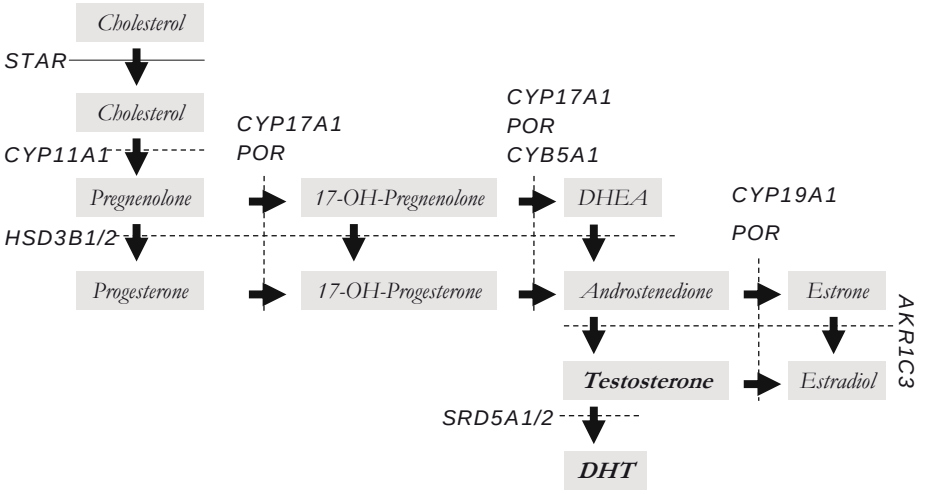
INTRODUCTION

Despite current early detection methods and surgical and radiotherapeutical treatment options, many prostate cancer (PC) patients still present with unresectable stages of disease.[1] Since 1941 the mainstay of treatment of advanced PC is focused on suppression of intraprostatic testosterone (T) and dihydrotestosterone (DHT) actions. Nowadays, androgen-deprivation therapy is based on lowering the luteinizing hormone (LH)-induced testicular testosterone production through LH-releasing hormone agonists (chemical castration) with or without anti-androgens that block the androgen receptor (AR). Growth inhibition is initially achieved in the majority of patients. However, eventually all patients develop hormone-refractory or castration-resistant prostate cancer (CRPC), marked by a rise in prostate-specific antigen (PSA) and progression of the tumor.[2] Second line treatment with docetaxel chemotherapy combined with prednisone is only temporarily effective with a small survival benefit.[3] Due to this untreatable stage of disease, prostate cancer is still estimated to be the cause of 28,660 deaths in the United States in 2008 alone.[4]

Several hypotheses underlie the occurrence of CRPC, such as the selective outgrowth of androgen-independent clonal cell populations caused by activation, suppression or fusion of genes and AR signaling pathway-related causes, encompassing ligand-independent AR activation, AR hypersensitivity due to AR overexpression and ligand promiscuity due to AR mutations.[5-6] More recently, intratumoral conversion of adrenal androgens and *de novo* steroid synthesis have been brought forward as potential causes of tumor progression.[7-9] The presence of active AR in CRPC samples and reported high intratumoral T and DHT concentrations in CRPC patients with castrate serum androgen levels support the concept of intratumoral conversion of steroidal precursors.[8, 10] Recent publications have put renewed emphasis on this intratumoral steroidogenesis by demonstrating the previously unknown expression of steroidogenic enzymes in normal prostate and PC tissue[8-9, 11-12] as well as a differential expression pattern between the various tumor types and the normal prostate gland.[8-9] Furthermore, conversion of the radiolabeled steroid precursor acetic acid into DHT has been shown to occur *in vitro* in LNCaP cells and *ex vivo* in CRPC cells.[7] Potential upregulation of steroidogenic enzymes in CRPC and the resulting local T and DHT production may account for the observed intratumoral androgens in levels sufficient to activate the AR.[7-8, 10, 13] Consequently, such a mechanism would require new therapeutic modalities that aim to block intratumoral steroidogenesis.

De novo steroid production has been thought to be confined to a few organs: the gonads, the adrenal cortex and the placenta. Steroidogenesis is initiated by the transport of cholesterol through the mitochondrial membrane, for which the steroid acute regulatory protein (*STAR*) is obligatory (Figure 1). Once cholesterol has entered the mitochondria it can be converted into pregnenolone by desmolase, also known as cytochrome P450 side-

Figure 1: Simplified scheme of the classical steroid biosynthetic pathway



The depicted cholesterol transporter, steroidogenic enzymes and co-factors are necessary for *de novo* synthesis of androgens from cholesterol and were investigated in this study. STAR: steroid acute regulatory protein, CYP: cytochrome P450, HSD: hydroxysteroid-dehydrogenase, POR: P450 oxidoreductase, CYB5: cytochrome b5, AKR: aldo-keto-reductase, SRD: 5α-reductase, DHT: 5α-dihydrotestosterone

chain cleavage (*CYP11A1*). The Δ5-steroid pregnenolone can subsequently be converted through 17-hydroxylase and 17,20-lyase activities (both encoded by *CYP17A1*, together with co-factors cytochrome P450 oxidoreductase [*POR*] and cytochrome b5 [*CYB5A1*]), and through 3β-hydroxysteroid dehydrogenase activity (by either of the iso-enzymes *HSD3B1* or *HSD3B2*) to form androstenedione.[14-15] Further metabolism of androstenedione can take place in peripheral tissues, such as the male reproductive tract. Here several 17β-hydroxysteroid dehydrogenases are able to convert androstenedione to T, of which the type 5 enzyme (*AKR1C3* or *HSD17B5*) is the most important isoform in the prostate gland.[16] Androstenedione and T can be aromatized into estrogens by *CYP19A1*. The two 5α-reductase isoforms, *SRD5A1* and *SRD5A2*, are able to metabolize T to DHT. The type 2 reductase is thought to be the most important isoform for intraprostatic conversion.[16]

In order to study the capability of human PC to synthesize androgens *de novo* as well as to convert adrenal androgens locally, we measured steroidogenic enzyme expression in a large set of hormonally manipulated experimental models of prostate cancer and in patient material from normal prostate, local prostate cancer, lymph node metastases and trans-urethral resection of the prostate (TURP) tissues. In all samples the expression of key enzymes required for *de novo* synthesis of androgens was low or absent and was not affected by androgen ablation therapy. The enzymes required for T and DHT production from androstenedione were abundantly expressed and their expression was affected by androgen levels and could be related to prostate cancer progression.

MATERIALS AND METHODS

In vitro cultures and tumor-bearing mice

The investigated cell lines and xenografts have been characterized previously,[17-21] as summarized in Table 1. The prostate cancer cell lines LNCaP and PC346C were grown in DMEM/F12, VCaP and DuCaP in RPMI 1640, all in presence of 5% fetal calf serum (FCS) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), until the start of the experiment, followed by two consecutive 36 hour periods in androgen deprived medium containing 5% dextran-coated charcoal-stripped FCS. Subsequently, cells were incubated with 1 nM of the synthetic androgen R1881 (NEN, Boston, MA, USA) or ethanol vehicle. After 8 hours, the medium was removed and cells were frozen and stored at -80 °C.

Thirteen established xenografts of prostate carcinomas were grown in nude mice. The xenografts were designated as being androgen-dependent, androgen-responsive, androgen-independent or androgen-unresponsive on basis of their (in-)ability to proliferate in castrated nude mice.[17] Xenografts were collected from intact or 7-14 days castrated male mice, snap-frozen and stored at -80 °C.

Patient samples

Patient samples were collected from patients operated within the Erasmus MC, between 1984 and 2001, after approval from the local Medical Ethics Committee. Tissues included

Table 1: Investigated PC cell and xenografts models

	Androgen dependence	AR status	Origin
Cell lines			
- LNCaP	Responsive	AR+	Lymph node
- VCaP	Responsive	AR+	Bone
- DuCaP	Responsive	AR+	Dura
- PC346C	Responsive	AR+	PC346
Xenografts			
- PC82	Dependent	AR+	Prostate
- PC295	Dependent	AR+	Lymph node
- PC310	Dependent	AR+	Prostate
- PC346	Responsive	AR+	TURP
- PC346B	Responsive	AR+	TURP
- PC374	Responsive	AR+	Skin
- PC133	Independent	AR-	Bone
- PC135	Independent	AR-	Prostate
- PC324	Independent	AR-	TURP
- PC339	Independent	AR-	TURP
- PC346I	Unresponsive	AR+	PC346
- PC346BI	Unresponsive	AR+	PC346B
- PC374F	Unresponsive	AR+	PC374

samples from radical prostatectomy specimens of locally confined prostate carcinoma, from lymph node dissection of metastases and TURP resection of locally advanced prostate cancer (TURP) or of CRPC (Table 2). CRPC samples were obtained through TURP of patients with urinary obstruction due to locally progressive disease during androgen deprivation therapy. A second series of 4 locally advanced PC (TURP) and 9 CRPC samples was collected in order to verify primary data (Table 2). Sections from tumor areas and normal tissues were snap-frozen in liquid nitrogen and stored at -80 °C. H&E-stained slides of the frozen sections were scored independently by two pathologists for percentage of tumor tissue and Gleason score. In each slide the percentages of normal epithelial, stromal and tumor nuclei were scored. Abundant presence of inflammatory cells was recorded. Normal prostate was defined as benign prostate tissue containing more than 60% glands. Tumor tissue was used for subsequent analyses if >70% of cells were tumor.

RNA isolation and quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

RNA from prostate specimens, xenografts and cell lines was isolated using RNAbee reagent as described by the manufacturer (Tel-Test Inc., Friendswood, TX, USA). The reverse transcriptase reaction was performed with 1 µg RNA and oligo T12 primer and preincubated for 10 minutes at 70 °C. First strand buffer, DTT, dNTPs, RNAsin and Moloney murine leukemia virus reverse transcriptase (MMLV RT, Promega Benelux B.V., Leiden, The Netherlands) were added and incubated for 1 hour at 37 °C. After this, the reaction was kept for 10 minutes at 90 °C and samples were immediately frozen thereafter.

Gene expression in the cell lines and xenografts was analyzed in a ABI Prism 7900 Sequence Detection System. Primer and probe sequences are depicted in Supplementary

Table 2: PC patient characteristics

	Normal prostate	Locally confined prostate cancer	Lymph node metastases	Locally advanced prostate cancer	Castration-resistant prostate cancer
No. patients, unique	17	11	16	21	10
age at diagnosis, median (range)	62 (54-72)	62 (56-70)	66 (52-71)	63 (46-68)	62 (53-68)
Gleason score, n					
6		6	0	3	1
7		3	4	4	5
8		1	9	13	3
9-10		1	3	1	1
epithelium in tissue, average % (range)	72 (60-80)	83 (70-100)	90 (60-100)	87 (70-100)	88 (70-100)
cancer in tissue, average % (range)	0	89 (70-100)	100	98 (90-100)	97 (90-100)

Table 1. Each assay was tested beforehand for human cDNA specificity and did not detect human DNA or murine cDNA equivalents. PCR efficiency was checked by cDNA dilution curves and efficiency exceeded 90% for all assays. The real time PCR reaction was performed in a volume of 12.5 µl, containing 20 ng cDNA, 2x Taqman Universal Master Mix (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), 300 nM primers, 100 nM probe (Biolegio, Nijmegen, The Netherlands) and H_2O. In case of validated Taqman Gene Expression Assays (Applied Biosystems) a 1:50 volume of the primer-probe mix was used. Positive controls consisted of cDNA of human placenta (*HSD3B1*, *CYP19A1*), normal prostate gland (*SRD5A2*), normal adrenal cortex (*HSD3B2*) or the steroid-secreting adrenocortical cell line H295R (other assays). Expression was calculated relative to the average of threshold cycles (Cts) of two housekeeping genes, *HPRT1* and *GAPDH*, using the ΔCt-method. Ct values >40 were considered as no expression.

The patient samples were analyzed likewise in an ABI Prism 7500 FAST Sequence Detection System using a total of 15 µl reaction volume. Other human tissue and cell line controls were added to compare the prostate samples to tissues with presumably low mRNA expression of steroidogenic enzymes. Because of the low expression of *HSD3B1* and *CYP17A1* mRNA, additional assays were performed using commercially obtained primer-probe combinations (Applied Biosystems). PCR efficiency of these assays was 100% (r^2>0.995) in the Ct range 26.0-36.7 and 22.3-39.6 for *HSD3B1* (own design and Applied Biosystems, respectively) and 22.7-38.5 and 23.4-37.4 for *CYP17A1*. Expression was calculated relative to the expression of the housekeeping genes *GAPDH* and *HMBS*. For the quantitation of *AR* and *HMBS* mRNA the reaction mix included SYBR Green PCR Master Mix (Applied Biosystems) and a dissociation stage was added to the PCR program to check for assay specificity.

Statistics

The effect of hormonal manipulation on steroidogenic enzyme expression in the cell lines and xenografts was analyzed using paired Student's t-tests on relative values. Expression in patients samples was analyzed using Kruskal-Wallis tests and post-hoc Dunn's multiple comparison tests for multiple groups or Mann-Whitney U-tests for two groups. Correlation between gene expressions was calculated using Spearman's correlation coefficient with a Bonferroni-Holm correction for multiple testing. Analyses were carried out using GraphPad Prism Version 5.01 (GraphPad Software, San Diego, CA, USA). P<0.05 was considered to be statistically significant.

RESULTS

Cell lines and xenografts

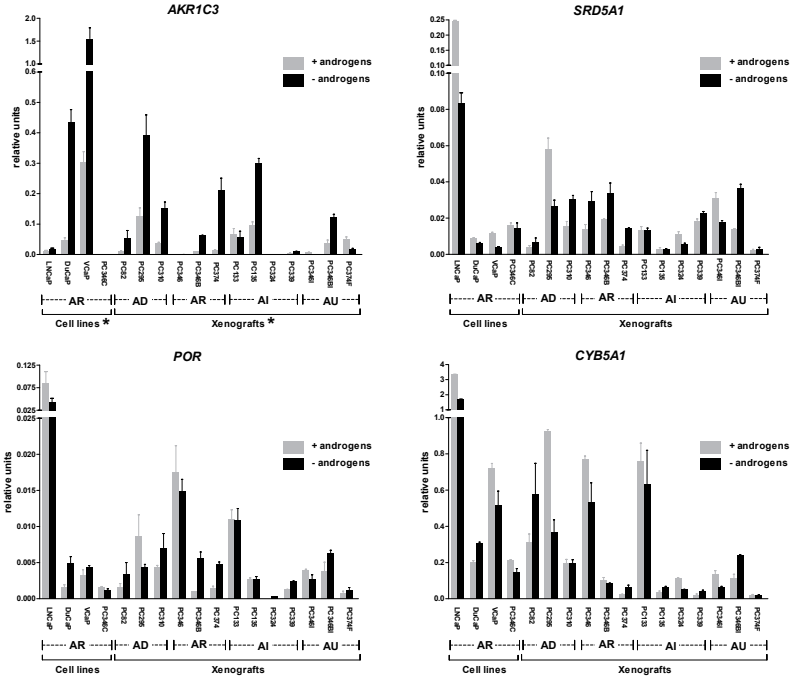
The mRNA expression of *STAR*, *CYP11A1*, *HSD3B1*, *HSD3B2*, *CYP17A1*, *POR*, *CYB5A1*, *AKR1C3*, *CYP19A1*, *SRD5A1* and *SRD5A2* was measured in 4 different cell lines, which were treated with R1881 or vehicle, and in 13 different xenografts, grown in intact or castrated male mice. Absolute Ct values of these assays are shown in Supplementary Table 2. Quantitative RT-PCR for *HSD3B2* displayed Ct values of ≥40 in all cell lines and xenografts studied. Analysis of the *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1*, *CYP19A1* and *SRD5A2* assays in all samples yielded Ct values in the higher ranges of 35.3-≥40, 30.8-≥40, 34.5-≥40, 34.1-≥40, 30.7-≥40 and 34.1-≥40, respectively, indicating very low expression. The expression of *POR*, *CYB5A1*, *AKR1C3* and *SRD5A1* was more pronounced with Ct values in the range of 20-30 in all samples studied. The positive controls displayed appropriate Ct values in these assays ranging from 18.7-27.0. In Figure 2, expression of the 4 above mentioned mRNAs relative to housekeeping genes is shown. R1881 treatment suppressed *AKR1C3* expression in all cell lines (P=0.022). Intratumoral *AKR1C3* expression was increased in castrated tumor-bearing mice as compared to tumors grown in intact mice (P=0.034). R1881 treatment of cell lines or castration of tumor-bearing mice did not significantly alter the expression of *SRD5A1*. Hormonal manipulation also did not affect the mRNA expression of the ubiquitously present co-factors *POR* and *CYB5A1* in these cell lines and xenografts.

Patient materials

We measured the expression of the steroidogenic enzymes *CYP11A1*, *CYP17A1*, *HSD3B1*, *HSD3B2*, *AKR1C3* and *SRD5A1* and the *AR* in the first series of 75 individual patient samples. Absolute Ct values are shown in Supplementary Table 3. *CYP11A1* mRNA was detectable in 65 of 75 samples. When calculated relative to the housekeeping genes, the normal prostate tissue displayed higher expression levels of *CYP11A1* than the four prostate carcinoma groups (P<0.01, Figure 3a). Ct values for *HSD3B2* were again ≥40 in all samples studied. Expression of *HSD3B1* and *CYP17A1* varied between Ct values 36.4-≥40 and 36.0-≥40 respectively. More importantly, all but 4 of the 75 patient samples did not show concomitant expression of *CYP17A1* and *HSD3B1*, essential for *de novo* androstenedione and testosterone production. These levels were in the same range as found in other non-steroidogenic tissues, such as liver, fat and leucocytes (Supplementary Table 3). Of the proven CRPC samples none was positive for both enzymes.

Because of the importance of *HSD3B1* and *CYP17A1* for *de novo* production of androgens we also measured their expression in the patient samples using commercially available certified RT-PCR assays (Applied Biosystems). Results have been included in Supplementary Table 3 and Figure 4. Again the samples yielded high Ct values for *HSD3B1*

Figure 2: mRNA expression of the steroidogenic enzymes and co-enzymes *AKR1C3*, *SRD5A1*, *POR* and *CYB5A1* in hormonally manipulated prostate cancer cell lines and xenografts

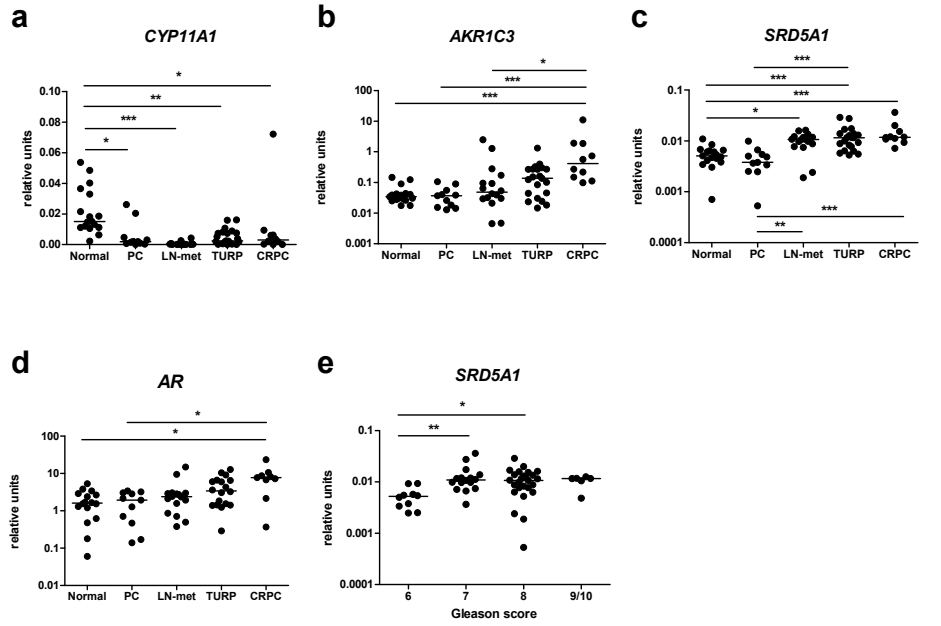


Four cell lines were treated with ethanol vehicle or 1 nM R1881 for 8 hours. Thirteen xenografts were obtained from control or castrated male nude mice. mRNA expression was calculated relative to the average of housekeeping genes *HPRT1* and *GAPDH*. AD: Androgen-dependent, AR: Androgen-responsive, AI: androgen-independent, AU: androgen unresponsive. *P<0.05 relative values of treated versus control, using a paired t-test. Data presented as means and range of duplicates.

and *CYP17A1* although these assays were slightly more sensitive than the previous sets, range 32.9-≥40 and 31.5-≥40 respectively. No significant differences were found between the expression of *HSD3B1* (Figure 4a) or *CYP17A1* (Figure 4b) in the various groups of prostate tissues. Using these primer-probe sets 13 out of the 75 tissues (5 normal prostate glands, 2 local prostate cancers, 3 lymph node metastases, 2 TURP samples and 1 CRPC sample) showed positive expression for both enzymes, all with Ct values in the range of 33.8-38.2 (Figure 4c). In the additional TURP and CRPC tissues very low expression of both *HSD3B1* and *CYP17A1* mRNAs was obtained as well (Supplementary Table 3). Relative expression of these samples has been added to Figure 4a and 4b. Two locally advanced PC and four CRPC samples were positive for both *HSD3B1* and *CYP17A1* (Figure 4c, gray symbols).

The mRNAs for *AKR1C3* and *SRD5A1* were detectable at substantially lower Ct values than obtained for *HSD3B1* and *CYP17A1*, ranging from 24.2-33.6 and 28.2-36.0 respectively, in all samples tested in the first series. In Figure 3b-c expression of these genes is indi-

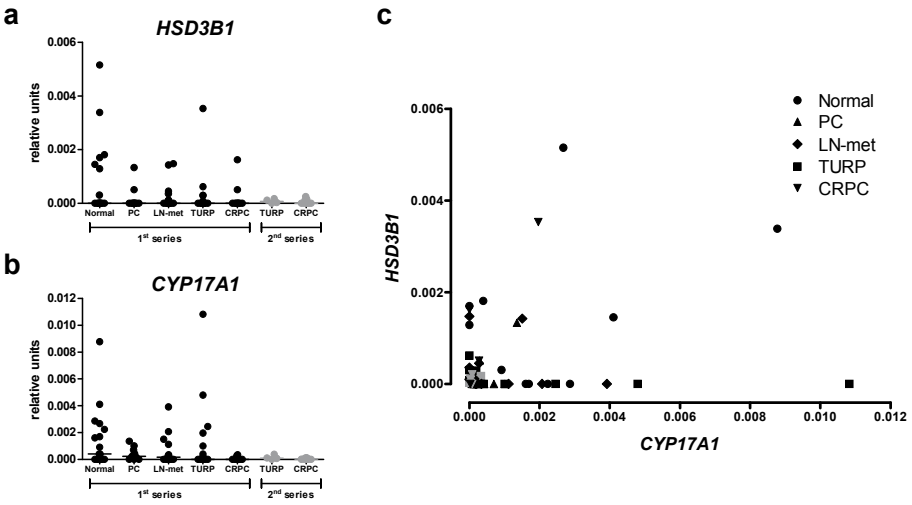
Figure 3: (a-d) Quantitative mRNA expression of the steroidogenic enzymes *CYP11A1*, *AKR1C3*, *SRD5A1* and the androgen receptor (*AR*) in normal prostate gland, local prostate carcinoma (PC), lymph node metastases (LN-met), locally advanced PC (TURP), and CRPC samples. (e) Association between *SRD5A1* mRNA expression and Gleason score in the tumor samples



mRNA expression was calculated relative to the average of the housekeeping genes *HMBS* and *GAPDH*. Analyses were performed using Kruskall-Wallis test and post-hoc Dunn's multiple comparison test.
*** P<0.001, ** P<0.01, *P<0.05. Bars represent means. Note: log scale in Figures b-e.

cated relative to that of housekeeping genes. *AKR1C3* expression was significantly higher in CRPC samples compared to normal prostate, local PC and lymph node metastases. *SRD5A1* mRNA was increased in lymph node metastases and both CRPC and non-CRPC TURP samples compared to normal prostate and local PC. *SRD5A1* was associated with Gleason score: tissues with a Gleason score of 6 had lower *SRD5A1* mRNA expression levels compared to higher Gleason scores (Figure 3e). There was no association between *AKR1C3* or *SRD5A1* expression and the development of metastases or PSA progression during hormonal therapy. mRNA expression of the *AR* was detectable in all patient samples except for one normal prostate sample, three TURP samples and one CRPC. When these samples were excluded from further analysis, the AR expression was increased in CRPC compared to normal prostate and locally confined prostate cancer (P<0.05, Figure 3d). mRNA expressions of *AR* and *AKR1C3* (r=0.387; P=0.001) were significantly correlated. *AKR1C3* and *SRD5A1* mRNA expressions were also correlated in these patient samples (r=0.486; P<0.001), but when corrected for *AR* expression this relation was no longer significant (partial r=0.041; P=0.869).

Figure 4: Quantitative mRNA expression of *HSD3B1* and *CYP17A1* in the different types of samples



The second series only comprised samples from locally advanced PC (TURP) or CRPC. These results have been depicted in gray. (a-b) mRNA expression was calculated relative to the average of the housekeeping genes *HMBS* and *GAPDH*. *P<0.05. Bars represent means. (c) Absence of concomitantly positive mRNA expression of *CYP17A1* and *HSD3B1* in majority of human samples from normal prostate gland, local prostate carcinoma (PC), lymph node metastases (LN-met), locally advanced PC and CRPC samples. Black symbols represent the first series of samples tested, whereas the second series is depicted by gray symbols. The 45 investigated tissues that displayed Ct values ≥40 for both enzymes are not depicted here. Moreover, *HSD3B2* expression was absent in all samples

DISCUSSION

In this study we quantified steroidogenic enzyme mRNA expression in a panel of experimental models of human PC and in human normal and tumorous prostatic tissues. In the majority of PC cases, simultaneous expression of all enzymes necessary for *de novo* synthesis of androgens from cholesterol to T could not be detected. The enzymes which convert androstenedione to T and T to DHT were ubiquitously expressed and their levels were affected by endocrine therapy and related to the state of tumor progression.

In the experimental models for human prostate cancer we detected Ct values in the range of ≥34 for *STAR*, *CYP17A1*, *HSD3B1*, *HSD3B2* and *SRD5A2* indicating very low mRNA copy numbers. Incubation with the synthetic androgen R1881 or castration of tumor-bearing mice did not alter the mRNA levels of these steroidogenic enzymes. In patient samples we again investigated *de novo* androgen synthesis. Whereas *HSD3B2* was negative in all samples, expressions of *CYP17A1* and *HSD3B1* excluded each other in the vast majority of samples, implying that enzymatic transformation of pregnenolone into androstenedione cannot occur in these tumor cells. Only in a subset of patients could levels of

both enzymes be detected simultaneously albeit at low levels (Figure 4c). These studies were conducted using a validated quantitative approach in samples which were evaluated for prostate tissue or tumor content, whereas positive controls for our assays, i.e. adrenal cortex, placenta or normal prostate gland, showed readily detectable amplification of gene product. Detected Ct values were in the same range as in other non-steroidogenic tissues. Moreover, in the patient samples expression of the key enzymes *HSD3B1* and *CYP17A1* was tested with two different sets of primers and probes and confirmed in a second series of samples of locally advanced PC and CRPC. Overall, in this large series of experimental models and patient samples of PC our findings plead for a limited role of intratumoral steroidogenesis.

We realize that our study is limited by the lack of distant metastatic PC tissues and that our conclusions are therefore limited to local PC, a limited number of CRPC and lymph node metastases. Also, it should be realized that the quantitative mRNA levels determined in this study may not automatically reflect protein levels; modifications in (post-) transcriptional processes could still possibly lead to protein levels of the steroidogenic enzymes which might be sufficient for androgen production.

Recent studies on steroidogenic enzymes in prostate cancer showed evidence of the presence of *STAR*, *HSD3B1*, *HSD3B2* and *CYP17A1* expression in normal prostate or metastasized prostate carcinoma using immunohistochemistry, micro-array or quantitative RT-PCR.[8-9, 22] Importantly, these studies have only depicted relative expression values. The simultaneous expression of *HSD3B1* and *CYP17A1* in metastases as reported by Montgomery *et al.*[8] could only be confirmed in a limited number of TURP samples in our series, depending on the assay used. Possibly this discrepancy is due to the fact that these authors investigated soft tissue metastases, in which upregulation or local expression could have played an important role. We have not studied those types of tissue, but detected simultaneous very low expression of *HSD3B1* and *CYP17A1* also in a limited number of normal prostate or locally confined prostate carcinoma tissues. *HSD3B1* expression may result from its presence in basal cells, that constitute a small population of the normal prostate.[22] This could explain the higher percentage of positive expression in normal prostate samples compared to tumor tissues. Moreover, in contrast to Montgomery *et al.*[8], we used a probe-based assay with intron-spanning primers which could have added to the specificity of our detection method.

Interestingly, *CYP11A1* was detectable in the majority of normal prostate tissues whereas its expression was strongly reduced in the tumor samples. Although this could lead to the formation of pregnenolone in these cells, pregnenolone itself does not possess biological activity and requires further metabolic transformation by *CYP17A1*. The expression of *CYP11A1* could possibly be related to the *CYP11A1* driven conversion of other substrates, such as 7-dehydrocholesterol and vitamin D3.[23] Vitamin D has been shown to exert effects on prostate cancer progression.[24] Aromatase (*CYP19A1*) could only be located in a few

xenografts and castration resulted in a small increase of its expression (data not shown). This is in agreement with the observation by Hiramatsu *et al.*[25] that aromatase immunoreactivity was only detectable in stromal cells adjacent to carcinoma cells.

The present results indicate that intraprostatic production of DHT could occur starting from androstenedione due to the presence of *AKR1C3* and *SRD5A1*. The expression of *AKR1C3* was negatively affected by androgens in both cell lines and xenografts. Furthermore, *AKR1C3* expression was increased in CRPC compared to normal prostate, locally confined prostate cancer and lymph node metastases, which is in line with previous immunohistochemical studies[11, 26] and micro-array data.[9] The upregulation of intratumoral *AKR1C3* expression in patients receiving hormonal therapy in addition to the presence of (over-)expressed *AR* constitutes a plausible cause for the development of CRPC. The substrate for the *AKR1C3*-encoded enzyme would most likely be adrenal androstenedione, which is present in serum in the nanomolar range. *In vivo* studies using radiolabeled DHEA and androstenedione have detected intraprostatic levels of labeled T and DHT although extraprostatic 3β-hydroxysteroid dehydrogenase activity cannot be ruled out in this setting.[27] Steroid conversion into androgens has also been shown for androstenedione, DHEA and DHEA-sulfate in isolated prostatic tissue.[27-28] The adrenocortical production of adrenal androgens therefore remains a crucial therapeutic target. Specific AKR1C3 inhibition for CRPC could also form a new therapeutic target: non-steroidal anti-inflammatory drugs, selective COX-2 inhibitors and steroid carboxylates have been proven to suppress this enzyme activity.[29]

During tumor progression in prostate tissue there is a switch of iso-enzymes for 5α-reductase from SRD5A type II to SRD5A type I.[9, 26, 30-31] We could confirm these findings in our experimental models and found higher *SRD5A1* levels in metastasized and hormone-refractory tumors. The association between *SRD5A1* expression and Gleason score is in line with the observation that patients with Gleason score 7 to 10 prostate cancer had a smaller decline of intraprostatic DHT concentrations during hormonal therapy compared to patients with Gleason score 6 or less.[32] However, it must be noted that in CRPC samples the Gleason score is less reliable and could give an erroneously high value. The hypothesis that *SRD5A1* is the main enzyme responsible for the local production of DHT in prostate cancer has important implications for the use of enzyme inhibitors in progressive disease. Based on these findings, treatment with finasteride, a selective type II 5α-reductase inhibitor, is likely to be less efficacious than treatment with an inhibitor of both types of enzymes, such as dutasteride.[12]

Overall, we detected high Ct values for steroidogenic enzymes responsible for *de novo* androgen synthesis indicating low copy numbers whereas the enzymes responsible for the final conversions into T and DHT were readily detected. The very high Ct values detected in our samples question the importance of these mRNAs in CRPC development. From our data it is difficult to extrapolate whether the very low expression of *STAR*, *HSD3B1* and

CYP17A1 detected in our collection of PC samples are clinically relevant and also apply to distant metastatic lesions. Recently, Attard *et al.* showed the efficacy of administration of the CYP17A1 inhibitor, abiraterone acetate, in patients with CRPC.[33] The percentage of patients with PSA decline was comparable to that of similar patients receiving low-dose dexamethasone, inhibiting adrenal androgen production, in another study.[34] This implies that the result of specific CYP17A1 inhibition is comparable to that of the blockade of adrenocortical steroid production and therefore suggests that intratumoral *CYP17A1* plays a limited role in CRPC development compared to that of adrenal androgens. The limited role of intratumoral steroid conversion is further underlined by the absence of concomitant expression of *HSD3B1* or *HSD3B2* and *CYP17A1* in 69 out of the 88 patient samples, suggesting potential relevance only in a subset of patients. It appears most likely that the major source of intratumoral androgens after androgen-deprivation therapy is blood-derived androstenedione, which because of its nanomolar concentration is an important substrate for the highly expressed enzymes *AKR1C3* and *SRD5A1* providing significant conversion into T and DHT in the prostate cancer cells.

To study if intratumoral *de novo* steroid synthesis may still play a role in a subset of CRPC patients, adrenocortical blockade should first be administered. This setting can be mimicked in the *in vitro* models of cells grown in medium supplemented with charcoal-treated FCS and in the xenografts in castrated nude mice since the murine adrenal cortex is incapable of producing DHEA or androstenedione due to its lack of *CYP17A1* expression.[35] Our study indicates that the expression of *CYP17A1* and *HSD3B1* is only detectable at very low levels in the androgen-responsive cell lines and two androgen-dependent xenografts. It must also be stated that through utilization of the recently discovered "backdoor pathway" of steroidogenesis,[36] as was shown in small amounts for LNCaP cells,[7] precursor 17-OH-progesterone could be converted into DHT in the samples in which *CYP17A1* mRNA was detected. Although the increased *SRD5A1* expression would be beneficial to this pathway, *CYP17A1* levels are detected at a low range and *HSD3B1* would still be necessary for de novo steroid synthesis.

We confirmed previous studies on *AR* expression in CRPC by showing expression of *AR* in the majority of samples.[37-38] The reported correlation of the AR expression with expression of *AKR1C3*[26] could also be confirmed. However we could not replicate the previously reported correlation with *SRD5A1* expression reported by the same authors.

In conclusion, enzymes for *de novo* synthesis of androgens are not highly expressed in the studied tissue samples of normal prostate gland, locally confined PC, lymph node metastases and TURP from locally advanced PC and CRPCs, nor in experimental models of human PC. During tumor progression *SRD5A1* expression rises whereas *AKR1C3* expression increases during hormone ablation therapy, thus giving the prostate tumor an opportunity to convert circulating steroids of adrenal origin to T and DHT locally and thereby to progress during hormonal therapy. Therefore, adjuvant treatment modalities

should be directed to block adrenal androgen production. Additionally, the production of adrenal androgens as well as the putative presence of intratumoral *de novo* steroid biosynthesis in a subset of CRPCs may require additional inhibition of intratumoral *AKR1C3* or *SRD5A1* activity to disrupt the conversion of (adrenal) steroid precursors into active T and DHT and consequently AR pathway activation.

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Supplementary Table 1: Primer and probe sequences

Design (Label)	Gene	RefSeq	Amplicon size (bp)	Primers 5′ - 3′	Probe
Own (5'FAM – 3'TAMRA)	*STAR*	NM_000349.2	177	CTACTCGGTTCTCGGCT CACATCTGGGACCACTTT	GAAGGAGAGTCAGCAGGACAATG
	CYP11A1	NM_000781.2	143	CTTCTTCGACCCGGAAAATTT CCGGAAGTAGGTGATGTTCTTGT	CCCAACCCGATGGCTGAGCAA
	HSD3B1	NM_000862.2	151	AGGAGGGTTTCTGGGAC TCCTTCCAGCACTGTCA	AGGAGATCAGGGTCTTGGACAA
	HSD3B2	NM_000198.2	274	TCTAAGTTACGCCCTCTTCT AAGTACAGTCAGCTTGGTCC	AGGAGATCAGGGCCTTGGACAA
	CYP17A1	NM_000102.3	63	TCTCTGGGCGGCCTCAA AGGCGATACCCTTACGGTTGT	TGGCAACTCTAGACATCGCGTCC
	POR	NM_000941.2	144	TGGCCGAAGAAGTATCTC CAGAGGAGGTCAATGTCT	TTCAGCATGACGGACATGATT
	CYB5A1	NM_148923.2	144	ATGGCAGAGCAGTCGGACGA TCAGTCCTCTGCCATGTATAG	AGGATGTCGGGCACTCTACAGAT
	CYP19A1	NM_000103.3	110	CCTGCAACTACTACAACCG GTGCTTCATTATGTGGAACA	CTCCAGAGATCCAGACTCGCAT
	HPRT1	NM_000194.2	109	TGCTTTCCTTGGTCAGGCAGTAT TCAAATCCAACAAAGTCTGGCTTATATC	CAAGCTTGCGACCTTGACCATCTTTGGA
	GAPDH	NM_002046.3	70	ATGGGGAAGGTGAAGGTCG TAAAAGCAGCCCTGGTGACC	CGCCCAATACGACCAAATCCGTTGAC
Own (SYBR)	*AR*	NM_000044.2	414	TGACTCCGTGCAGCCTATTG ATGGGAAGCAAAGTCTGAAG	
	HMBS	NM_000190.3	139	CATGTCTGGTAACGGCAATG GTACGAGGCTTTCAATGTTG	
Applied Biosystems (5'FAM)	*HSD3B1*	NM_000862.2	72	Hs00426435_m1	
	CYP17A1	NM_000102.3	72	Hs01124136_m1	
	AKR1C3	NM_003739.4	112	Hs00366267_m1	
	SRD5A1	NM_001047.2	120	Hs00602694_mH	
	SRD5A2	NM_000348.3	83	Hs0016583_m1	

Supplementary Table 2: Ct values of steroidogenic enzymes and housekeeping genes in prostate cancer cell lines and xenografts

Sample	Dependence	Androgens	*HPRT1*	*GAPDH*	*StAR*	*CYP11A1*	*HSD3B1*	*HSD3B2*	*CYP17A1*	*POR*	*CYB5A1*	*AKR1C3*	*CYP19A1*	*SRD5A1*	*SRD5A2*
Cell Lines		(+ R1881, - control)													
LNCaP	**AR**	**+**	**24,1**	**18,0**	**35,8**	**30,8**	**35,9**	nd	nd	**24,6**	**19,3**	**27,4**	nd	**23,0**	nd
		-	**24,1**	**18,5**	**35,7**	**36,6**	**38,3**	nd	nd	**25,9**	**20,6**	**27,0**	**36,7**	**24,9**	nd
VCaP	**AR**	**+**	**23,2**	**18,2**	nd	**36,4**	**34,5**	nd	**34,1**	**30,0**	**23,0**	**25,1**	nd	**27,5**	**35,5**
		-	**24,7**	**19,7**	nd	**36,7**	**35,8**	nd	**35,8**	**29,9**	**24,0**	**23,4**	nd	**29,6**	nd
DuCaP	**AR**	**+**	**25,1**	**19,3**	nd	nd	nd	nd	**34,5**	**30,4**	**22,6**	**23,9**	nd	**28,6**	nd
		-	**26,3**	**19,6**	nd	nd	nd	nd	**35,8**	**30,8**	**23,9**	**22,3**	nd	**30,8**	nd
PC346C	**AR**	**+**	**23,0**	**16,6**	nd	nd	nd	nd	nd	**29,1**	**22,0**	**36,4**	nd	**25,7**	nd
		-	**22,3**	**15,9**	nd	nd	nd	nd	nd	**28,9**	**21,9**	**34,3**	nd	**25,3**	nd
Xenografts		(+ intact, - castrated)													
PC82	**AD**	**+**	**23,4**	**17,2**	nd	**32,9**	**35,9**	nd	nd	**29,6**	**22,0**	**26,8**	nd	**28,3**	nd
		-	**26,5**	**21,3**	nd	nd	nd	nd	nd	**32,2**	**24,7**	**28,2**	nd	**31,2**	nd
PC295	**AD**	**+**	**21,9**	**18,7**	nd	**36,6**	**36,3**	nd	nd	**27,2**	**20,4**	**23,3**	nd	**24,4**	nd
		-	**24,0**	**19,0**	nd	nd	nd	nd	**36,6**	**29,3**	**22,9**	**22,9**	nd	**26,7**	**38,5**
PC310	**AD**	**+**	**22,9**	**18,8**	nd	nd	nd	nd	nd	**28,7**	**23,2**	**25,5**	nd	**26,9**	**35,0**
		-	**23,9**	**20,1**	nd	nd	nd	nd	**35,5**	**29,2**	**24,4**	**24,7**	**36,3**	**27,1**	nd
PC346	**AR**	**+**	**23,3**	**19,0**	nd	**35,1**	nd	nd	nd	**27,0**	**21,5**	**37,5**	nd	**27,3**	nd
		-	**23,8**	**19,8**	nd	nd	nd	nd	nd	**27,9**	**22,7**	**33,8**	**37,1**	**26,9**	nd
PC346B	**AR**	**+**	**22,0**	**18,1**	nd	nd	nd	nd	nd	**29,9**	**23,4**	**26,7**	nd	**25,8**	nd
		-	**22,6**	**17,7**	nd	nd	nd	nd	nd	**27,7**	**23,7**	**24,1**	**34,1**	**25,0**	nd
PC374	**AR**	**+**	**21,1**	**16,9**	nd	nd	nd	nd	nd	**28,5**	**24,3**	**25,1**	nd	**26,7**	nd
		-	**21,1**	**17,9**	nd	nd	nd	nd	nd	**27,2**	**23,5**	**21,7**	nd	**25,6**	nd
PC133	**AI**	**+**	**23,5**	**18,9**	**35,9**	nd	nd	nd	nd	**27,7**	**21,6**	**25,1**	nd	**27,5**	nd

		-	**23,0**	**18,5**	**35,3**	**36,7**	nd	nd	nd	**27,3**	**21,5**	**25,0**	nd	**27,0**	nd
PC135	**AI**	+	**21,5**	**18,4**	nd	**36,3**	nd	nd	nd	**28,5**	**24,8**	**23,4**	nd	**28,4**	nd
		-	**22,4**	**19,5**	nd	nd	nd	nd	nd	**29,5**	**25,0**	**22,7**	nd	**29,5**	nd
PC324	**AI**	+	**22,2**	**18,5**	nd	nd	nd	nd	**34,8**	nd	**23,5**	**33,0**	**35,5**	**26,9**	nd
		-	**21,3**	**17,9**	nd	**36,9**	nd	nd	**36,2**	**31,5**	**23,9**	**34,3**	**33,9**	**27,0**	nd
PC339	**AI**	+	**22,7**	**17,0**	nd	nd	nd	nd	nd	**29,4**	**25,4**	**27,8**	**33,2**	**25,6**	nd
		-	**22,5**	**18,1**	nd	nd	nd	nd	nd	**29,0**	**24,9**	**27,0**	**30,7**	**25,8**	nd
PC346I	**AI**	+	**21,8**	**17,7**	nd	**36,6**	nd	nd	nd	**27,7**	**22,6**	**26,9**	nd	**24,7**	**34,1**
		-	**21,0**	**16,8**	nd	nd	nd	nd	**37,3**	**27,5**	**22,9**	**29,8**	nd	**24,7**	**35,6**
PC346BI	**AI**	+	**20,8**	**16,9**	nd	**35,3**	nd	nd	**36,7**	**27,0**	**22,0**	**23,6**	nd	**25,0**	**34,7**
		-	**21,7**	**18,5**	nd	**36,7**	nd	nd	nd	**27,4**	**22,2**	**23,1**	nd	**24,9**	**34,2**
PC374F	**AI**	+	**20,6**	**17,5**	nd	nd	nd	nd	nd	**29,5**	**25,1**	**23,4**	nd	**27,8**	nd
		-	**20,3**	**17,1**	nd	nd	nd	nd	nd	**28,4**	**24,4**	**24,7**	nd	**27,1**	nd
Positive controls															
H295R					**23,1**	**20,9**			**19,5**						
Normal adrenal cortex								**22,3**							
Placenta							**23,0**						**18,7**		
Normal prostate gland															**27,0**

nd: not detectable, Ct ≥ 40.

Supplementary Table 3: Ct values of steroidogenic enzymes and housekeeping genes in patient samples

Patient	Tissue type	*PBGD*	*GAPDH*	*CYP11A1*	*HSD3B1**	*HSD3B1*†	*HSD3B2*	*CYP17A1**	*CYP17A1*†	*AKR1C3*	*SRD5A1*	*AR*
First series												
1	Normal	**25,1**	**26,1**	**31,7**	**37,7**	**35,7**	nd	nd	nd	**30,1**	**33,4**	nd
2	Normal	**23,2**	**24,3**	**32,6**	nd	**32,9**	nd	**37,5**	**31,6**	**28,8**	**31,0**	**23,4**
3	Normal	**24,5**	**24,6**	**29,5**	nd	nd	nd	**37,4**	nd	**29,3**	**31,4**	**25,6**
4	Normal	**25,0**	**25,2**	**31,1**	nd	nd	nd	nd	nd	**29,8**	**32,3**	**29,2**
5	Normal	**24,8**	**25,1**	**30,7**	**38,8**	nd	nd	nd	nd	**29,5**	**32,9**	**24,3**
6	Normal	**24,0**	**23,9**	**29,7**	**38,0**	**34,5**	nd	nd	**36,7**	**29,0**	**32,3**	**23,2**
7	Normal	**24,3**	**24,5**	**31,7**	nd	**35,6**	nd	nd	**34,1**	**29,6**	**32,1**	**24,1**
8	Normal	**25,0**	**25,7**	**30,9**	nd	nd	nd	**36,8**	**36,1**	**28,1**	**32,7**	**23,4**
9	Normal	**25,3**	**26,6**	**32,5**	**37,0**	nd	nd	nd	nd	**29,0**	**32,5**	**24,5**
10	Normal	**24,2**	**25,7**	**31,4**	**39,3**	**34,7**	nd	nd	**35,6**	**30,2**	**32,3**	**23,2**
11	Normal	**25,5**	**25,6**	**29,8**	**38,7**	**36,3**	nd	nd	nd	**29,0**	**36,0**	**24,9**
12	Normal	**23,6**	**24,5**	**28,4**	**39,8**	nd	nd	nd	**36,1**	**29,1**	**31,6**	**23,3**
13	Normal	**23,1**	**24,5**	**28,4**	**38,9**	nd	nd	**37,0**	**33,9**	**29,6**	**31,5**	**22,5**
14	Normal	**24,5**	**25,7**	**31,3**	nd	nd	nd	nd	**35,2**	**29,7**	**32,7**	**23,6**
15	Normal	**22,8**	**22,7**	**29,2**	nd	nd	nd	nd	nd	**28,6**	**30,0**	**23,4**
16	Normal	**24,9**	**25,2**	**29,8**	nd	nd	nd	nd	**34,8**	**30,3**	**33,1**	**27,5**
17	Normal	**23,6**	**24,5**	**30,4**	nd	**36,7**	nd	**37,3**	**35,1**	**28,9**	**32,2**	**21,6**
18	PC	**23,8**	**25,2**	**32,3**	nd	nd	nd	**37,0**	**36,2**	**28,0**	**31,2**	**23,0**
19	PC	**23,4**	**23,0**	nd	nd	nd	nd	nd	**34,8**	**29,2**	**34,1**	**25,8**
20	PC	**22,9**	**21,4**	**32,7**	nd	nd	nd	**37,0**	**36,1**	**26,8**	**29,4**	**21,8**
21	PC	**24,2**	**25,1**	**34,1**	nd	**36,8**	nd	nd	**36,8**	**27,9**	**32,2**	**22,9**
22	PC	**23,6**	**23,6**	**32,5**	nd	nd	nd	nd	**34,5**	**28,2**	**31,3**	**22,6**
23	PC	**25,1**	**25,0**	**30,3**	nd	nd	nd	nd	nd	**31,3**	**33,7**	**25,5**
24	PC	**23,7**	**24,5**	**29,7**	**38,4**	nd	nd	**37,5**	**34,8**	**28,9**	**32,3**	**22,4**

25	PC	24,2	25,1	36,3	nd	nd	nd	nd	nd	30,4	32,7	23,0
26	PC	22,2	23,6	33,0	nd	nd	nd	36,6	36,5	29,0	31,5	25,8
27	PC	24,1	24,7	32,7	nd	nd	nd	37,3	nd	28,5	32,0	23,3
28	PC	22,6	23,6	32,2	nd	34,8	nd	nd	35,6	28,4	31,2	24,2
29	LN-met	25,5	26,2	36,3	nd	nd	nd	nd	nd	27,7	32,3	24,8
30	LN-met	23,9	25,4	36,6	nd	35,8	nd	nd	36,5	28,9	33,7	24,8
31	LN-met	24,3	25,9	33,0	nd	nd	nd	36,0	34,2	27,7	31,6	24,4
32	LN-met	21,4	21,8	nd	37,3	nd	nd	nd	36,1	29,4	28,2	20,1
33	LN-met	23,8	25,5	nd	nd	34,2	nd	nd	34,1	30,2	31,2	23,2
34	LN-met	22,5	23,7	nd	39,0	35,9	nd	nd	nd	30,8	29,4	21,5
35	LN-met	24,2	24,4	35,1	nd	35,6	nd	39,4	nd	28,8	31,1	23,0
36	LN-met	24,2	24,0	35,6	nd	nd	nd	nd	33,2	29,1	31,1	25,5
37	LN-met	23,5	24,4	32,6	37,8	nd	nd	37,0	35,5	27,4	30,6	24,9
38	LN-met	26,0	25,1	nd	nd	nd	nd	37,2	nd	24,2	31,5	21,6
39	LN-met	23,6	24,2	34,6	nd	nd	nd	nd	nd	23,5	30,6	20,6
40	LN-met	23,1	24,1	36,2	nd	37,1	nd	nd	36,8	27,5	29,8	22,0
41	LN-met	22,6	24,5	37,2	nd	nd	nd	36,9	36,2	28,7	30,0	24,1
42	LN-met	22,6	24,3	nd	nd	nd	nd	36,4	35,5	28,5	30,5	22,5
43	LN-met	23,5	25,4	34,5	37,5	nd	nd	nd	nd	29,1	30,4	23,2
44	LN-met	23,1	23,6	36,3	nd	33,1	nd	nd	nd	26,8	32,0	21,8
45	TURP	23,5	23,7	30,7	37,8	nd	nd	nd	nd	28,1	31,1	21,9
46	TURP	22,6	23,0	36,2	nd	nd	nd	nd	31,5	28,9	29,2	22,1
47	TURP	23,7	26,5	34,2	nd	nd	nd	nd	nd	27,8	31,7	21,4
48	TURP	27,2	29,0	35,8	nd	35,5	nd	nd	36,4	33,6	34,5	nd
49	TURP	23,5	23,2	nd	nd	nd	nd	nd	36,4	28,7	30,9	21,7
50	TURP	23,0	24,4	32,5	39,0	nd	nd	36,1	nd	25,6	29,9	21,6
51	TURP	24,4	25,5	33,0	nd	nd	nd	nd	nd	26,3	30,8	21,8
52	TURP	26,3	27,0	32,6	nd	nd	nd	nd	36,8	28,5	33,1	26,2
53	TURP	25,3	26,1	32,3	nd	nd	nd	36,2	35,2	27,4	32,5	nd
54	TURP	27,3	27,7	33,4	nd	nd	nd	nd	37,3	30,3	33,7	nd

55	TURP	**23,8**	**24,1**	**34,2**	nd	**34,1**	nd	nd	nd	**25,8**	**31,0**	**23,6**
56	TURP	**23,6**	**25,7**	**31,5**	nd	nd	nd	nd	**36,5**	**29,1**	**31,9**	**26,4**
57	TURP	**24,9**	**25,9**	**32,3**	nd	nd	nd	nd	**35,4**	**28,0**	**30,5**	**24,8**
58	TURP	**24,2**	**25,7**	**32,1**	nd	nd	nd	**35,7**	nd	**30,0**	**30,1**	**22,4**
59	TURP	**24,8**	**26,0**	**34,5**	nd	nd	nd	nd	**36,6**	**28,4**	**31,6**	**22,7**
60	TURP	**24,2**	**25,2**	**35,8**	nd	**36,2**	nd	nd	**36,7**	**28,0**	**30,5**	**21,3**
61	TURP	**24,0**	**25,9**	**34,0**	nd	nd	nd	nd	nd	**24,5**	**31,3**	**24,5**
62	TURP	**22,6**	**23,1**	**35,3**	nd	nd	nd	nd	nd	**24,7**	**30,1**	**20,2**
63	TURP	**23,9**	**24,1**	nd	nd	nd	nd	nd	nd	**25,6**	**30,9**	**21,2**
64	TURP	**23,7**	**25,5**	**31,7**	nd	nd	nd	nd	**35,5**	**30,4**	**31,4**	**23,7**
65	TURP	**26,7**	**26,7**	**35,4**	nd	nd	nd	nd	nd	**30,3**	**34,1**	**24,8**
66	CRPC	**25,5**	**25,6**	**33,6**	**38,8**	**36,5**	nd	nd	nd	**22,1**	**32,0**	**22,5**
67	CRPC	**24,3**	**25,4**	**32,2**	nd	**33,8**	nd	nd	**34,6**	**28,2**	**31,3**	**26,3**
68	CRPC	**25,2**	**25,8**	nd	nd	**36,7**	nd	nd	nd	**24,5**	**32,0**	**20,9**
69	CRPC	**24,0**	**23,1**	**32,4**	nd	nd	nd	nd	nd	**26,3**	**29,2**	**20,4**
70	CRPC	**24,2**	**23,9**	**27,8**	**36,4**	nd	nd	nd	nd	**24,5**	**28,8**	**20,6**
71	CRPC	**25,7**	**26,2**	**37,7**	nd	nd	nd	nd	**35,4**	**29,1**	**32,3**	nd
72	CRPC	**24,5**	**25,1**	nd	nd	**36,5**	nd	nd	nd	**27,0**	**31,2**	**23,7**
73	CRPC	**23,6**	**24,3**	**32,7**	nd	nd	nd	**37,1**	nd	**24,8**	**31,1**	**21,0**
74	CRPC	**24,9**	**25,5**	**32,6**	nd	nd	nd	nd	**36,2**	**24,3**	**31,2**	**22,3**
75	CRPC	**24,3**	**24,6**	**31,2**	nd	nd	nd	nd	nd	**26,3**	**31,2**	**21,7**
Second series												
1	TURP	**23,3**	**21,2**		nd	**37,5**	nd	nd	nd			
2	TURP	**25,6**	**22,6**		nd	**37,4**	nd	**36,2**	**37,8**			
3	TURP	**26,8**	**25,1**		nd	**38,2**	nd	**38,8**	**37,3**			
4	TURP	**25,1**	**23,7**		nd	nd	nd	**36,2**	**37,4**			
5	CRPC	**26,5**	**22,3**		nd	nd	nd	**37,3**	**39,5**			
6	CRPC	**27,6**	**24,0**		nd	nd	nd	nd	nd			
7	CRPC	**25,4**	**21,9**		nd	**36,9**	nd	**39,1**	**37,8**			
8	CRPC	**26,2**	**22,6**		nd	nd	nd	**36,0**	nd			

9	CRPC	**23,8**	**21,6**	nd	**38,0**	nd	**38,5**	**37,1**	
10	CRPC	**26,0**	**23,1**	nd	nd	nd	**36,4**	**37,6**	
11	CRPC	**24,0**	**20,4**	nd	nd	nd	**36,7**	**37,2**	
12	CRPC	**23,2**	**20,1**	nd	**34,5**	nd	**37,8**	**37,3**	
13	CRPC	**26,2**	**22,7**	nd	**36,4**	nd	**38,1**	**37,7**	
Other human controls									
Liver		**24,8**	**19,7**	**32,8**	**36,9**	**32,9**	nd	**31,5**	**32,4**
Fat		**25,7**	**20,8**	**32,9**	nd	**37,5**	nd	**37,0**	nd
Heart		**27,5**	**20,7**	**35,0**	nd	nd	nd	**38,1**	**38,5**
Leucocytes		**34,6**	**28,6**	nd	nd	nd	nd	**38,7**	nd
HEK (embryonic kidney cell line)		**27,7**	**23,4**	nd	nd	nd	nd	**36,9**	**38,0**
AN3CA (endometrium carcinoma cell line)		**22,6**	**17,8**	nd	nd	**39,4**	nd	**35,8**	**37,4**
K562 (chronic myeloid leukemia cell line)		**22,5**	**18,3**	nd	nd	nd	nd	**33,4**	**35,5**

nd: not detectable, Ct ≥ 40.

CHAPTER 11

Activin A stimulates local testosterone production and growth in human prostate cancer through intracrine androgen conversion

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ABSTRACT

Background:

Local androgen synthesis in prostate cancer (PC) may contribute to the development of castration-resistant PC (CRPC), but pathways controlling intratumoral steroidogenic enzyme expression in PC are unknown. The effects of activin, a factor involved in the regulation of PC growth and steroidogenic enzyme expression in other steroidogenic tissues, on intratumoral steroidogenesis was studied in PC.

Methods:

Activin A effects and regulation of the activin-signaling pathway molecules were studied in the PC cell lines LNCaP, VCaP and PC-3 and in 13 individual PC xenograft models. Also, expression levels of inhibin βA- and βB-subunits (*INHBA* and *INHBB*) and of the activin antagonist follistatin were quantitated in patient PC tissues.

Results:

Activin A induced the expression and enzyme activity of 17β-hydroxysteroid dehydrogenases *AKR1C3* and *HSD17B3* in LNCaP and VCaP. Inhibition of endogenous activin A action in the PC-3 cell line decreased *AKR1C3* and *HSD17B3* levels and consequently testosterone synthesis. In return, androgens suppressed *INHBA* expression in both VCaP cells and the PC xenograft models. The anti-proliferative effects of activin A were opposed by physiological concentrations of androstenedione in LNCaP cells. In patient PC tissues, expression levels of *INHBA* were increased in CRPC samples and correlated with *AKR1C3* levels. Moreover, a high ratio of activin subunits to follistatin was associated with a worse metastasis-free survival in patients.

Conclusions:

Activin A is controlled by androgens in prostate cancer models and regulates local androgen production. Activin thus seems to mediate (residual) intratumoral androgen levels and could form a novel therapeutical target in CRPC.

INTRODUCTION

Prostate cancer (PC) is dependent on androgens for its development and growth. Treatment of advanced stages of PC is targeted at decreasing serum levels of testosterone and dihydrotesterone (DHT), androgen conversion or action through luteinizing hormone-releasing hormone (LHRH) agonists, 5α-reductase inhibitors and anti-androgens.[1] Hormonal therapy of PC can delay but not prevent tumor progression, inevitably giving rise to castration-resistant prostate cancer (CRPC) in all patients.[2] Although multiple androgen receptor (AR)-dependent and AR-independent pathways are known to contribute to castration resistance,[3] recent emphasis has been on residual intratumoral androgen levels after castration. Despite undetectable serum androgen levels, intratumoral levels of T and DHT in CRPC are comparable to those in prostatic tissues of eugonadal men.[4-5] Relevance of continued AR activation in CRPC has recently been shown in clinical trials in which CRPC patients treated with the steroidogenic enzyme inhibitor abiraterone acetate or the novel pure AR antagonist MDV3100 showed increased survival rates.[6-7] These findings may be explained by the presence of intratumoral steroidogenic enzymes, which can either convert adrenal androgen precursors into testosterone through 17β-hydroxysteroid dehydrogenase (17β-HSD) types 3 and 5, encoded by *HSD17B3* and *AKR1C3* respectively, or synthesize androgens *de novo* from cholesterol.[8-11] Pathways involved in the regulation of the expression and the activity of steroidogenic enzymes in PC and thereby local androgen levels and growth are largely unknown.

Activins are members of the transforming growth factor-beta (TGF-β) superfamily of growth- and differentiation factors.[12] Activins are homo- or heterodimers of inhibin β-subunits (inhibin βA [*INHBA*], βB [*INHBB*], βC or βE); the most commonly studied dimers include activin A (βA-βA), activin B (βB-βB) and activin AB (βA-βB). Activin binds to a complex of type I (*ACVRIB*) and type II receptors (*ACVRIIA* and *ACVRIIB*), that is phosphorylated upon ligand binding and ultimately affects gene expression in the nucleus through actions of intracellular Smad proteins.[13] Inhibins are composed of an inhibin α-subunit (*INHA*) and a β-subunit and block the actions of activin by binding to its type II receptors,[14] a process which is mediated by the TGF-β type III receptor betaglycan (*TGFBR3*).[15] Follistatin (*FST*) is an extracellular glycoprotein capable of binding and antagonizing activin.[16]

Activins are pluripotent peptides that can influence (patho-)physiological processes in a wide variety of tissues.[17] One of these functions is the modulation of expression levels of steroidogenic enzymes and steroid production in steroidogenic tissues.[18-21] Activin expression, especially that of activin A, and its signaling components have also been detected in prostate tissue.[22-24] Activin A has been found to decrease cell growth through the induction of apoptosis in the androgen-responsive LNCaP PC cell line, but not in androgen-independent PC-3 cells.[25-26] Others reported enhanced AR and prostate-

specific antigen (PSA) expression and cell migration following activin A incubation in LNCaP cells.[27-28] Patient PC samples have lost or impaired expression of *INHA*, *TGFBR3* and *ACVRIB* compared to normal prostatic tissue, whereas serum activin A levels were found to be increased in patients with PC bone metastases.[23, 29-31]

Since the importance of intraprostatic steroidogenic enzymes has recently been re-established and activin A is known to regulate steroidogenic enzyme expression in gonadal and adrenocortical tissue, we studied the effects of activin A on steroidogenic enzymes in PC. We found that activin A upregulates expression of the 17β-HSD iso-enzymes inducing local conversion of androstenedione to testosterone. The anti-proliferative effects of activin were consequently counteracted in the presence of physiological levels of androstenedione. In addition, the ratio of inhibin βA- and βB-subunits to follistatin was upregulated in human PC tissue samples and inversely associated with metastasis-free survival.

MATERIALS AND METHODS

Cell cultures

PC cell lines LNCaP, VCaP and PC-3 (previously characterized[11, 32]) were grown in T75 culture flasks containing RPMI 1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum (FCS) and penicillin and streptomycin (Invitrogen). Cells were trypsinized once- or twice-weekly and plated in 5% dextran-coated charcoal (DCC)-treated medium in 24-well plates at a density of 150.000 cells per well or in a 96-well plate at 10.000 cells per well for the growth assays. For VCaP flasks and plates were pre-coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. Cells were allowed to attach overnight and the next day incubations with vehicle control, activin A (R&D systems, Abingdon, UK), the activin type 1B receptor inhibitor SB-505124 (Sigma), androstenedione or R1881 (NEN Life Sciences, Boston, MA, USA) were performed. After the incubation period supernatants were removed and stored at -20 °C until the measurement of hormones. Plated cells were frozen on dry-ice and stored at -80 °C until RNA isolation. Cell culture experiments were all performed in triplicate.

Tumor-bearing mice

Thirteen PC xenografts were grown in nude mice as previously described; characteristics have been reported before.[11] Tumor tissues were obtained from male mice 7-14 days after castration or from eugonadal controls. Samples were snap-frozen and stored at -80 °C until further processing.

RNA isolation, reverse transcriptase and quantitative polymerase chain reaction

RNA was isolated from cells and tissues using TriPure (Roche Applied Science, Almere, The Netherlands). Specifications of the reverse transcriptase reaction, quantitative polymerase chain reaction and primer and probe sequences have been published before.[11, 33-34] The assays were specific for human cDNA and PCR efficiency always exceeded 90%.

Steroid and activin measurements

Testosterone and activin A levels were measured in supernatants of cell cultures in quadruplicate. Testosterone concentrations were estimated by radio-immunoassay (coat-a-count RIA, Siemens, Deerfield, IL, USA). Since androstenedione had a cross-reactivity of ~4% in the testosterone RIA, DCC medium containing 1 µM of androstenedione was tested in each assay and cross-reaction was consequently subtracted from results in the experimental conditions. Activin A levels were measured by the human Activin A DuoSet enzyme-linked immunosorbent assay (R&D systems).

Proliferation assay

LNCaP and VCaP cells were grown in 200 µl DCC medium for 7-10 days after incubations in duplicate. Numbers of metabolically active cells were measured by the addition 30 µl of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) to the medium and incubation at 37 °C. After four hours supernatant was removed and the end-product formazan was dissolved in buffered DMSO, containing one-tenth of PBS with 0.1 M glycine, 0.1 M NaCl, pH 10.5. Absorbance at 595 nm was measured in Victor 1420 multilabel counter (Wallac) and values of wells containing only DCC medium were substracted from the results.

Patient samples

Normal prostate and PC samples were collected from patients operated in the Erasmus MC between 1984 and 2001. Tissue samples were composed of normal prostate (Nl), local PC (PC) and lymph node metastasis (LN-met), obtained at radical prostatectomy, or recurrent locally advanced PC resected through trans-urethral resection of prostate (TURP) which was either confirmed as castration-resistant growth (CRPC) or not (TURP). Patient description and tissue processing have been described in a previous report.[11]

Statistics

Analysis of cell culture studies was based on the collective results of triplicate experiments using paired one-way analysis of variance with post-hoc Dunnett's multiple comparison test or a paired t-test. mRNA expression data were logarithmically converted before analysis. Paired t-tests were also used for analysis of expression levels in xenograft

samples. Patient samples were analyzed by Kruskall-Wallis and post-hoc Dunn's multiple comparisons test. Associations between expression levels were studied by Spearman's correlation coefficient. Survival comparisons were performed by Kaplan-Meyer analysis. All tests were calculated as two-tailed and statistical significance was assumed at P<0.05. Data were analyzed using Prism GraphPad (version 5.01, GraphPad software, La Jolla, CA, USA).

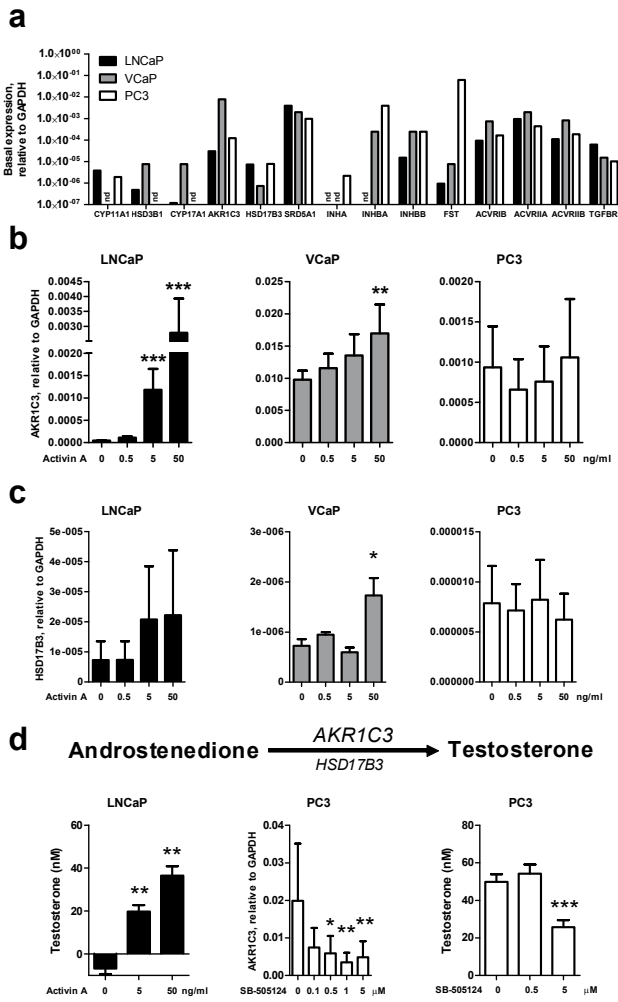
RESULTS

Activin A augmented 17β-HSD enzyme expression and activity

LNCaP, VCaP and PC-3 were chosen as model systems for the study of activin effects on steroidogenic enzymes because these cell lines differentially express levels of cytochrome P450 (CYP) side-chain cleavage (*CYP11A1)*, 3β-hydroxysteroid dehydrogenase type 1 (*HSD3B1),* CYP 17-hydroxylase/17,20-lyase (*CYP17A1), AKR1C3, HSD17B3,* 5α-reductase type 1 *(SRD5A1),* inhibin α-subunit *(INHA), INHBA, INHBB, FST, ACVRIB, ACVRIIA, ACVRIIB* and *TGFBR3* mRNAs (Figure 1A). Incubation of the androgen-responsive LNCaP, lacking endogenous activin A, and VCaP, expressing moderate levels of activin A, with activin A dose-dependently increased the expression of *AKR1C3* (P<0.0001 and P=0.02, respectively), but not in the high activin A-expressing androgen-independent PC-3 cell line (P>0.05, Figure 1B). In LNCaP, *AKR1C3* expression was increased 26-fold at 5 ng/ml of activin A and 62-fold at 50 ng/ml. *AKR1C3* was the most dominant 17β-HSD enzyme expressed, but *HSD17B3* transcription was also activated by activin A in VCaP cells (P<0.05), with variable induction in LNCaP and no effect in PC-3 cells (Figure 1C). Consequently, incubation of LNCaP cells with activin A affected the 17β-HSD enzyme activity, as culturing of cells with androstenedione resulted in increased levels of testosterone when pre-incubated with activin A for 24 hours (P=0.003, Figure 1D). Expression levels of *CYP11A1, HSD3B1, CYP17A1* and *SRD5A1* were not affected by activin A in the different cell lines (P>0.05, data not shown). Activin A incubation at 50 ng/ml increased the transcription of AR mRNA 3.1-fold in LNCaP (P<0.01) and 1.5-fold in VCaP cells (P<0.05, data not shown). PSA expression levels were also augmented to maximally 2.8- and 3.5-fold in these cell lines, respectively (P<0.05, data not shown). The androgen-independent PC-3 cells lack AR and PSA expression.

Incubation of PC-3 cells, which highly express activin A, with SB-505124, an inhibitor of the activin type I receptor, dose-dependently decreased *AKR1C3* (P=0.007, Figure 1D) and *HSD17B3* (P=0.03, data not shown) mRNA expression. Likewise, conversion of androstenedione to testosterone was decreased in PC-3 cells after 24 hours pre-incubation with SB-505124 (P=0.006, Figure 1D).

Figure 1: Activin A effect in different PC cell lines

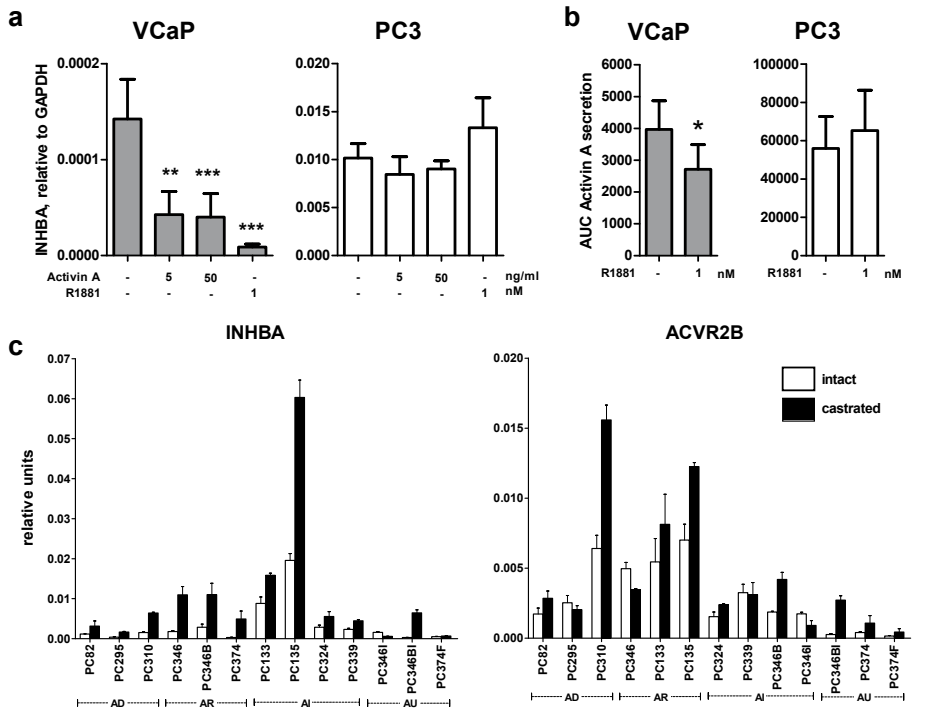


Basal mRNA expression levels of steroidogenic enzymes, activin subunits, follistatin and activin receptors in the three investigated PC cell lines (a). nd: not detectable. Activin A dose-dependently induced the mRNA expression of *AKR1C3* (b) and *HSD17B3* (c) after 24 hours in androgen-responsive cell lines LNCaP and VCaP. Activin A incubation did not influence expression of the type 3 and 5 17β-HSDs in the androgen-independent PC-3 cells. (d) Local testosterone production from its precursor androstenedione is effectuated by 17β-HSD types 3 and 5, encoded by *HSD17B3* and *AKR1C3*, respectively. Pre-incubation with activin A for 72 hours led to an increased conversion of 1000 nM androstenedione into testosterone during 24 hours in LNCaP. Since androstenedione had a cross-reactivity of ~4% in the testosterone assay these were substracted from the assay result, leading to negative results for testosterone in LNCaP in the absence of activin A. Inhibition of endogenous activin signaling by SB-505124 decreased *AKR1C3* expression and local androstenedione to testosterone conversion dose-dependently in PC-3 cells, * P<0.05, ** P<0.01, ***P<0.001, compared to control.

Regulation of activin subunits, activin receptors and of follistatin by androgens

In the androgen-responsive cell line VCaP, the synthetic androgen R1881 significantly suppressed *INHBA* mRNA expression (P<0.001, Figure 2A). This was accompanied by a decrease in activin A concentrations in the supernatant during 72 hours of R1881 incubation (P=0.02, Figure 2B). R1881 did not alter *INHBA* mRNA and activin A protein levels in the androgen-independent, high activin A-expressing PC-3 cells (P>0.05). Androgen treatment did not affect expression levels of *INHBB*, *FST*, *ACVR1B*, *ACVR2A*, *ACVR2B* and *TGFBR3* in all three cell lines investigated (data not shown).

Figure 2: Regulation of activin expression in PC cell lines and xenografts



Incubation with 1 nM R1881 decreased *INHBA* expression after 24 hours in the androgen-responsive VCaP after 24 hours, but not in androgen-independent PC-3 (a). In LNCaP cells *INHBA* mRNA expression was not detectable. Activin A also decreased mRNA expression of its own subunits in VCaP. Consistent with *INHBA* mRNA expression, supernatant activin A concentrations during 72 hours of R1881 incubation in VCaP were also decreased (b). *P<0.05, **P<0.01, ***P<0.001, compared to control. (c) In PC xenografts grown in nude mice, castration led to an overall increase in *INHBA* expression (P=0.041) and a trend towards increased *ACVRIIB* levels (P=0.057). Expression levels in xenografts were calculated relative to that of housekeeping genes *HPRT1* and *GAPDH*. AD = androgen-dependent, AR = androgen-responsive, AI = androgen-independent, AU = androgen-unresponsive xenografts.[11]

In androgen-ablated, castrated xenografts higher intratumoral *INHBA* expression levels were observed as compared to tumors from intact mice (P=0.041), further confirming that *INHBA* expression is suppressed by androgens *in vivo* (Figure 2C). This effect was comparable to that previously reported for *AKR1C3* expression.[11] Xenograft samples also expressed mRNAs for *INHBB*, although at a lower level than that of *INHBA*, allowing for activin AB or activin B formation (data not shown). Expression of *INHA* and *TGFBR3* was low or undetectable in xenograft models (data not shown), which is in line with previous reports on impaired expression of these proteins in PC.[29-30] Expression levels of *FST* were present but low, while the type I and II receptors of activin (*ACVRIB*, *ACVRIIA* and *ACVRIIB*) were readily detectable in all xenograft samples (data not shown). A trend towards higher expression in xenografts from castrated mice compared to intact controls was observed for *ACVRIIB* mRNA levels (P=0.057, Figure 2C). Overall, all xenografts models showed a higher potential for activin signaling after castration.

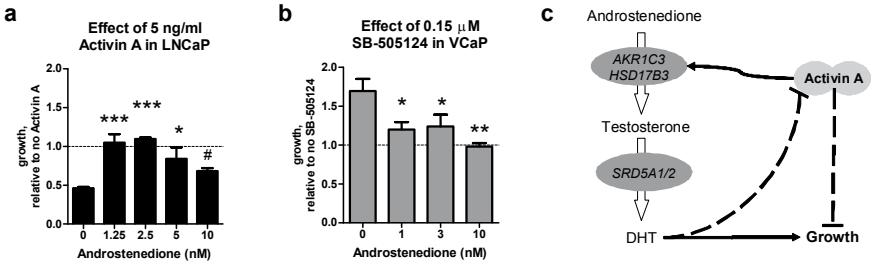
Activin increased androstenedione-induced proliferation

Activin A has been reported to have an apoptosis-inducing effect in PC cells grown in DCC-treated medium.[25-26] Since activin A increased *AKR1C3* expression in LNCaP and VCaP cells, we tested whether activin A-induced growth inhibition would persist in the presence of androstenedione at physiological concentrations observed in CRPC patients (1-10 nM). The anti-proliferative effect of activin A in the activin A-devoid LNCaP dissipated when co-incubated with androstenedione in a concentration range of 1.25 to 5 nM (P=0.008, Figure 3A). At 10 nM of androstenedione the additive effect of co-incubation with the androgen precursor diminished (P<0.05). In activin A-expressing VCaP cells, incubation with SB-505124 in steroid-deprived DCC medium had a growth-stimulatory effect compared to vehicle control. However, when incubated in the presence of physiological concentrations of androstenedione this proliferative effect was blocked (P=0.04, Figure 3B). Activin thus appears to have an AR-independent apoptotic effect as well as an 17β-HSD (thus androgen-dependent) proliferative effect on PC cells (Figure 3C).

Patient samples

Expression levels of *INHBA* and *INHBB* and the activin antagonist *FST* were measured in a panel of tissue samples consisting of locally confined PC, normal adjacent prostate, lymph node metastases, locally advanced PC and CRPC. Patient characteristics have been summarized before.[11] Overall *INHBA* expression exceeded *INHBB* expression by approximately 30-fold (data not shown). *INHBA* expression was higher in CRPC than in normal prostate samples (P<0.01, Figure 4A), whereas *FST* expression was lower in lymph node metastases (P<0.001) and TURP samples (P<0.01, Figure 4B) compared to normal. Within PC samples, FST was lower in tissues containing 100% epithelial cells compared to those containing 70% epithelial cells, consistent with the finding that follistatin is mostly

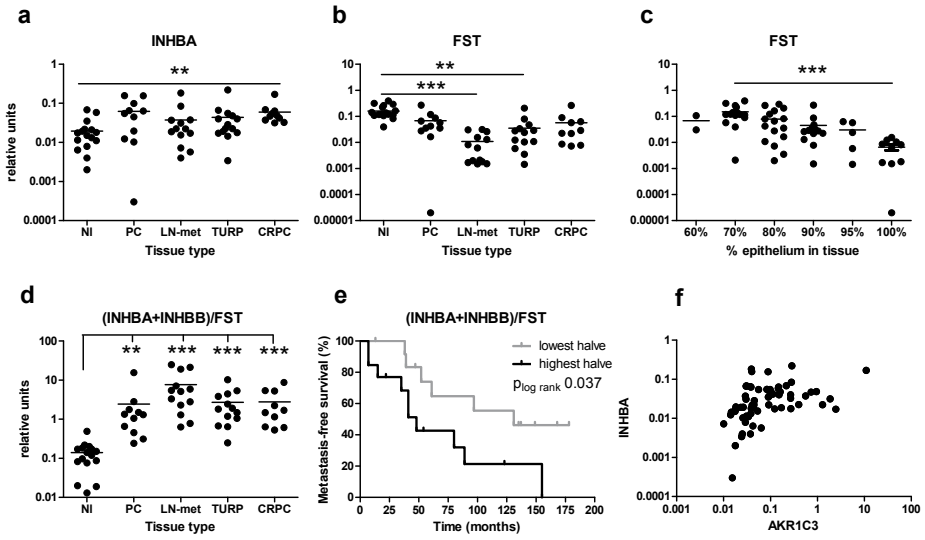
Figure 3: Effect of the activin-signaling pathway on PC growth



LNCaP cells were grown in 96-well plates and analyzed for metabolically active cell number with MTT assay 7 days after incubation with activin A and/or androstenedione. The anti-proliferative effect of Activin A in LNCaP in DCC medium decreased when co-incubated with 1.25 to 5 nM of androstenedione (a). At 10 nM of androstenedione the activin-induced proliferation benefit decreased. In VCaP cells, inhibition of activin-signaling by incubation with SB-505124 for 10 days led to increased proliferation in steroid-deprived medium (b). At 1 to 10 nM concentrations of androstenedione this proliferation effect was opposed. * P<0.05, ** P<0.01, ***P<0.001 compared to control; # P<0.05 compared to 2.5 nM. (c) Proposed model for regulation and effects of activin A in PC cells. Expression of activin A is inhibited by androgen action. When PC cells are androgen-deprived, activin A expression is increased. Activin A has an androgen-independent apoptosis-inducing effect,[26] but simultaneously stimulates *AKR1C3* and *HSD17B3* expression. This latter effect leads to an increase in androgen-dependent growth in the presence of androstenedione due to increased conversion into testosterone. Together, these two effects of activin A on PC growth neutralize each other in the physiological ranges of androstenedione concentrations. Continuous line indicates stimulation, dashed line indicates inhibition.

expressed in stromal cells of the prostate (P<0.001, Figure 4C).[35] The ratio of activin subunits to follistatin ([*INHBA*+*INHBB*]/*FST*) was higher in all PC tissue groups compared to normal (P<0.01, Figure 4D), pointing towards increased activin signaling in PC. This ratio was associated with metastasis-free survival, since the PC samples with a high *INHBA* and *INHBB* to *FST* ratio had a worse outcome than those with a low ratio: median time to the occurrence of metastases was 48 months, compared to 131 months (Figure 4E, P=0.037) for samples within the lowest half of expression. There were also trends towards worse PC-specific overall survival associated with high *INHBA* and *INHBB* to *FST* ratio (P=0.079) and lower metastasis-free survival associated with low *FST* levels (P=0.071). *INHBA* levels were correlated with *AKR1C3* levels previously measured in the same samples[11] (r=0.529, P<0.0001, Figure 4F), which is in line with the finding that activin regulates *AKR1C3* expression. *FST* expression was significantly correlated with *CYP11A1* expression (r=0.588, P<0.001, data not shown), presumably due to the stromal-specific expression of both proteins.

Figure 4: Expression of activin subunits and follistatin in human prostate and PC samples



Expression of the inhibin βA- (*INHBA*) and βB-subunits (*INHBB*) and follistatin (*FST*) was studied in human samples of normal prostate (NI), local prostate cancer (PC), lymph node metastases (LN-met), locally advanced PC (TURP), and CRPC (a,b). Expression was calculated relative to that of housekeeping genes *HMBS* and *GAPDH*. *FST* expression was dependent on the percentage of epithelium observed during microscopical evaluation of the tissue (c). A ratio of the activin subunit expression to follistatin expression ((*INHBA+INHBB*)/*FST*) was highly significantly elevated in PC compared to normal prostatic tissue (d). **P<0.01, ***P<0.001. (e) Patient samples with a high (*INHBA+INHBB*)/*FST* ratio had a worse metastasis-free survival: median survival was 48 months, compared to 131 months (P=0.037). (f) Significant associations between expression levels of *INHBA* and *AKR1C3* (upper, r=0.529, P<0.0001).

DISCUSSION

Activin has been implicated in PC development and growth with both oncogenic as well as tumor-suppressive properties.[25-28, 31] Here we show evidence that activin A is a mediator of local androgen production in PC through the regulation of 17β-HSD expression. In the presence of physiological concentrations of androgen precursors (like androstenedione) this regulation counteracts the anti-proliferative effects of activin. Furthermore, activin A expression is upregulated in patient CRPC samples and metastasis-free survival benefit is associated with low activin subunits to follistatin levels.

Development and progression of castration-resistant disease could be driven by persisting intratumoral androgens in concentrations sufficient to activate the AR.[4, 6] Castration leads to increased levels of *AKR1C3 in vitro* and *in vivo,*[11] which could sustain tumor growth through the activation of conversion of serum androstenedione into testosterone. Determination of the pathways involved in regulating steroid synthesis or conversion in PC could lead to novel markers for castration-resistance and possible treatment targets.

The inhibin βA- and βB-subunits are expressed in the epithelial cells of the prostate, whereas follistatin is mainly present in the stromal compartment,[35] suggesting the presence of a paracrine system for activins and follistatin within the prostate gland. In the PC cell lines LNCaP and VCaP, with undetectable or low expression of *INHBA* respectively, incubation with exogenous activin A increased the expression of 17β-HSD types 3 and 5, encoded by *HSD17B3* and *AKR1C3*. As a consequence, local androstenedione to testosterone conversion was also augmented. Incubation of PC-3 cells, which endogenously express high levels of activin A, with an inhibitor of the activin type I receptor yielded the opposite effect. The AR-independent, apoptosis-inducing function of activin A was counteracted when co-incubated in the presence of physiological levels of androstenedione, indicating that the regulation of 17β-HSD by activin is significant. Other studies examining the activin A effect on PC growth[25-26] utilized steroid-deprived medium that lacks adrenal androgen precursors which are present in serum of castrate patients. Steroids such as androstenedione can significantly attenuate the effect of relevant factors tested, in this case activin A, due to altered potential of intratumoral androgen production. Since the relevance of intracrine steroid conversion in CRPC has recently been established,[6, 10, 36] the usage of DCC medium as a model for CRPC has its drawbacks.

In human PC samples there is an upregulation of *INHBA* expression in combination with a downregulation of *FST* expression, leading to an overall increase in activin signaling. Moreover, reports on impaired inhibin action potential, through decreased or absent expression of either *INHA* or *TGFBR3,*[29-30] support this hypothesis.

Increased *INHBA* expression in CRPC appears to be a direct consequence of medical castration, since we now show in both *in vitro* and *in vivo* models that *INHBA* expression is suppressed by androgens. This coincides with increased 17β-HSD levels in CRPC patient samples and xenograft models after castration.[10-11] Therefore, activin A may serve as a bypass mechanism of the castration-induced loss of AR activation promoting the development of CRPC through increased *AKR1C3* expression. The correlation found between the expression levels of *INHBA* and *AKR1C3* in the patient samples forms an additional argument in favour of this mechanism.

This study thus adds new complexities to the effects of activin A in PC. Since the activin subunits to FST ratio is increased in PC and metastasis-free survival is better in patients with a low ratio, activin A appears to have a more oncogenic than tumor-suppressive role in CRPC patients. The augmented intracrine androgen production due to activin together with the findings that activin A increases *AR* expression[27] and cell migration[28] could account for the metastasis-free survival impairment related to higher activin A-signaling potential. Whether inhibition of ubiquitous activin A actions would indeed also favour PC growth *in vivo* is subject to future studies. Several compounds are currently available which inhibit the activin-signaling pathway, such as SB-505124, inhibin, follistatin and the soluble activin type II receptor,[37] and would be suitable for these investigations.

In conclusion, activin A regulates 17β-HSD expression in PC and thereby stimulates local androgen production from steroid precursors. This effect opposes the previously described anti-proliferative effects of activin A in PC. Levels of activin A are increased in CRPC samples, are associated with local *AKR1C3* levels and its ratio to *FST* is inversely related to metastasis-free survival. Activin A therefore is an important paracrine factor that could play a role in the development and progression of CRPC.

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CHAPTER 12

General Discussion

GENERAL DISCUSSION

1 STEROIDS

In the past years, the field of steroid biosynthesis, thought to contain relatively few unresolved mysteries, has been revived. Recent exciting developments include the elucidation of a backdoor pathway to androgens,[1] new clinical syndromes due to mutations in co-receptors and co-factors involved in steroidogenesis,[2-6] insight into the regulation of steroid hormone production in adrenal hyperplasia,[7] advanced mass-spectrometric methods for estimating steroid levels,[8] and the implementation of novel steroidogenic enzyme blockers in breast and prostate cancer.[9-10] The studies described in this thesis were specifically conducted to further clarify control of steroidogenesis in two types of tissue, namely the human adrenal cortex and human prostate gland.

2 STEROIDOGENESIS IN THE ADRENAL CORTEX

Pleiotropic signals from endocrine, paracrine and autocrine factors regulate the production of steroid hormones in the adrenal cortex. The principal stimulating endocrine signals arise from ACTH, AngII and potassium ion concentrations.

2.1 ACTH

ACTH activates the MC2R on adrenocortical cells to stimulate cAMP production and subsequent PKA activation, resulting in the stimulation of cAMP-response elements in promoter regions of genes encoding steroidogenic enzymes followed by the transcription of these genes. PKA also directly phosphorylates StAR on serine 195 to facilitate cholesterol transfer from the outer to the inner mitochondrial membrane.[11]

The presence of the MC2R on the adrenocortical cell surface is required for the production of glucocorticoids and adrenal androgens.[12] Translocation of the receptor from the endoplasmatic reticulum to the membrane and effective MC2R signaling are dependent on the presence of the co-receptor MRAP. The factors regulating expression of *MRAP* expression and its related protein homolog *MRAP2* in human adrenocortical cells were previously unknown. As described in chapter 2, we found that the cAMP/PKA pathway controls expression of both *MRAP* and *MC2R*. Since the receptor complex is internalized upon ligand binding,[13] a concomitant signal is necessary for continued receptor function. MRAP2, which has been shown to be capable of trafficking the MC2R to the cell surface but to have negative or no effects on signaling,[14-15] was found to be regulated in a different,

possibly PKC-dependent, manner. Since the regulation of these two accessory proteins is regulated by different pathways, effective MC2R renewal and functioning is guaranteed.

ACTH sensitivity is impaired in adrenocortical carcinomas. Although decreased *MC2R* expression was previously detected,[16-17] our current findings point towards a decrease in the expression of *MRAP* rather than that of *MC2R*. In contrast, both *MC2R* and *MRAP* levels were associated with HPA axis activity *in vivo* in normal and hyperplastic adrenal samples, which would imply that these levels are controlled by ACTH. Since *MC2R* and *MRAP* expression levels were not correlated with *in vitro* ACTH sensitivity, this would plead for an ACTH effect on these expression levels rather than for dose-limiting effects of *MRAP* or *MC2R* on ACTH action. On the other hand, *MRAP2* expression might have a negative influence on ACTH signaling, although this was not consistently observed across several readouts of ACTH responsiveness.

2.2 Angiotensin II

The main function of AngII is stimulation of the AT1R, a G_q protein-coupled receptor, and the consequent production of mineralocorticoids. Receptor activation instigates Ca^{2+} influx, thought to mainly regulate short-term aldosterone production, and activates PKC, needed for chronic steroidogenic enzyme stimulation.[18] We described novel crosstalk between the AngII and ACTH pathways (Chapter 2) since the former hormone stimulated both *MC2R* as well as *MRAP* expression. This finding might indicate a positive effect of AngII on glucocorticoid production. On the other hand, the *AT1R* is almost exclusively expressed in the zona glomerulosa,[19] where cortisol cannot be produced due to the absence of P450c17.[17] The AngII-induced stimulation of *MC2R* and *MRAP* could serve to increase ACTH sensitivity in aldosterone-producing cells, as was previously reported.[20] ACTH is also partly responsible for mineralocorticoid production in the zona glomerulosa through local binding to the MC2R.[17]

Although AngII did not affect expression of *MRAP2*, direct PKC stimulation decreased *MRAP2* levels by 22%. This effect could serve to decrease ACTH sensitivity, but other GPCRs than the AT1R would be culprit to stimulation of PKC in this setting. Therefore, the physiological relevance of this observed PKC effect remains doubtful.

Study on the origin of circadian fluctuations in hypertension in mice led to the observation that *Hsd3b6* was responsible for aldosterone production in the murine zona glomerulosa.[21] Based on sequence homology it was subsequently postulated that human *HSD3B1*, instead of the type II enzyme *HSD3B2*, catalyzes the conversion of pregnenolone to progesterone in the human zona glomerulosa. The human *HSD3B2* knockout phenotype already provided evidence to refute this hypothesis.[22]

Our studies, which are described in chapter 3, have now found minimal to absent levels of *HSD3B1* expression in human adrenal tissues in the absence of an increase of *HSD3B1*

mRNA levels in Conn adenomas. Moreover, AngII had no effect on *HSD3B1* expression in human adrenocortical cells, whereas *HSD3B2* was potently stimulated by both ACTH and AngII. Genotype analysis in a large cohort of community-dwelling persons also revealed no association between *HSD3B1* and a common endpoint for lifelong aldosterone exposure, i.e. hypertension. These findings contrast with previously published associations in studies between *HSD3B1* and blood pressure,[23-25] possibly due to the small sizes or younger age of these latter cohorts. Thus, aldosterone synthesis is still most likely mediated by the type II 3β-HSD.

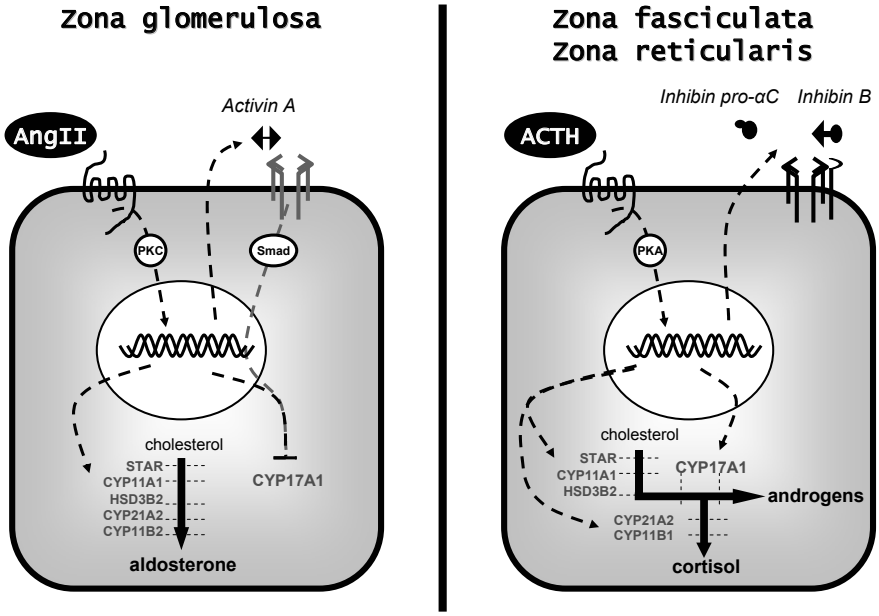
Interestingly, genetic variation in *CYP17A1* has consistently been associated with hypertension in genome wide association (GWA) studies.[26-27] Whether this effect is gluco- or mineralocorticoid related is unknown. Based on the phenotype of patients with congenital adrenal hyperplasia due to *CYP17A1* mutations[28] and effects of pharmacological P450c17 inhibition,[29] augmented production of corticosterone would be the most likely mechanism behind these observations.

Adrenocortical cells proliferate just under the adrenal capsule and migrate towards the adrenal medulla, where the cells go into apoptosis.[30-31] Whereas the zona glomerulosa AT1R-containing cells specifically produce mineralocorticoids, at the boundary with the zona fasciculata the migrated cells start to express *CYP17A1*[17] whilst loosing *CYP11B2* expression,[32] thus leading to glucocorticoid formation. The local factors responsible for this phenotypic switch of adrenocortical cells were completely unknown.

Activin and inhibin have also been shown to exhibit zone-specific expression patterns. Based on the first study on inhibin subunits in the adrenal cortex,[33] the inhibin α-subunit is preferentially located in the inner zones, whereas the inhibin βA-subunit resides mostly in the zona glomerulosa. In chapter 4, we described that this zone-specific expression is due to the specific stimulation of *INHBA* expression by AngII in the zona glomerulosa and of *INHA* by ACTH in the zonae fasciculata and reticularis. This would be expected to lead to a gradient of activin A on the outside and inhibin pro-αC near the medullary border. The lack of increased serum levels of inhibin A in patients with adrenocortical disease, reported in chapter 8, pleads against adrenocortical coupling of the inhibin α- and βA-subunits. This was confirmed *in vitro* by the measurement of inhibins and activins in supernatants of adrenocortical cells (Chapter 4).

We have shown that activin A functions as a mediator in the PKC-induced downregulation of *CYP17A1* (Chapter 4 and Figure 1). Activin A constitutes the first plausible paracrine factor regulating zone-specific steroidogenesis, since *CYP17A1* expression is not present in (the vicinity of) activin-producing cells. The formation of inhibin B in the inner zones, as a consequence of concomitant PKA stimulation by ACTH, could lead to counterregulation of activin A by occupation of the activin type II receptor.[34] Recent evidence suggests that the free inhibin α-subunit can bind the activin type 1B receptor and thus also inhibit

Figure 1: Regulation and effects of activin and inhibin in the adrenal cortex



In the zona glomerulosa AngII-induced PKC stimulation augments *INHBA* mRNA and activin A protein expression. AngII also stimulates expression of steroidogenic enzymes necessitated for aldosterone production. Activin A is at least partially responsible for the PKC-induced downregulation of *CYP17A1* which prevents glucocorticoid and adrenal androgen formation. In the inner adrenocortical zones ACTH induces steroidogenic enzymes, a.o. *CYP17A1*, that ensure cortisol and androgen production. ACTH also stimulates inhibin B and pro-αC expression. These peptides could counteract the effects of activin A, specifically the inhibition of *CYP17A1* expression.

activin signaling.[35] Our hypothesis is supported by the strong correlation between *INHA* and *CYP17A1* expression levels observed in adrenocortical samples (Chapter 7). Furthermore, the receptors and downstream factors of the activin and inhibin signaling pathway also show an adrenocortical zone-specific distribution pattern compatible the above-mentioned presence of the different peptides.[36] The activin/inhibin gradient constitutes a novel physiological mechanism in the human adrenal cortex which warrants further investigation. We did not measure activin B and activin AB levels, but these would be expected to be low since expression of the inhibin βB-subunit was much lower than that of the βA-subunit (Chapters 4 and 7).

In adrenocortical carcinomas, the effects of activin A were diminished as a result of decreased expression of activin receptors (Chapters 4 and 7). Whether this is a consequence of proliferation of zona reticularis cells, that express low levels of activin receptors,[36] or is resultant to knockdown of expression during tumorigenesis is unknown. Since activin also induces apoptosis in adrenocortical cells,[33, 36-38] tumor cells could escape activin-mediated apoptosis as a result of diminished activin receptor expression.

2.3 Adrenal hyperplasia

Besides the tropic factors ACTH and AngII, many other factors have been implied to change steroidogenic enzyme expression or activity in the adrenal gland.[39-41] The most common causes of non-ACTH mediated adrenocortical stimulation include other G-protein coupled receptors that also induce PKA or PKC stimulation. Although only recently recognized,[42] ACTH-independent macronodular hyperplasia (AIMAH) is more common than previously thought.[7] Our study in chapter 5 constitutes the largest reported group of patients with AIMAH to date.

Apart from ACTH, the adrenal glands of AIMAH patients are stimulated by (in decreasing order of frequency) AVP, upright posture, metoclopramide, a mixed meal, LHRH, glucagon and TRH. The *in vivo* responsiveness of AIMAH to aberrant and ectopic stimuli in our study was comparable to that in another large French study of AIMAH patients,[43] with the exception of metoclopramide and glucagon. In our series, stimulation of serum cortisol levels following these two stimuli were obtained in a smaller subset of patients. AVP increased cortisol production in primary cultures of AIMAH as well as of other adrenal tissues, confirming a physiological role of the eutopically expressed AVP type 1A receptor in adrenocortical steroidogenesis.[44-45] Interestingly, although increased levels of *AVPR1A* could not account for the effects of AVP in our series, there was an aberrant coupling of AVP to expression of *CYP11B1* in AIMAH cells. This constitutes a possible pathophysiological mechanism for the development of (subclinical) Cushing's syndrome in these AVP-responsive patients.

We are the first to report on a large, consecutive patient group from whom adrenal cells were studied *in vitro*. After collecting the data of all cultures, we found an overall lack of association between clinical and experimental responses to hormonal stimuli in individual samples. This would appear to be in contrast to the previous case reports and small case series;[46-50] this discrepancy might be the consequence of publication bias.

Causes of AIMAH remain undetermined although the first mutations have been identified in a minority of cases[51]. *In vivo* aberrant or ectopic responses to hormonal stimuli have also been described for adrenocortical tumors, especially aldosterone-secreting adenomas.[52-56] We did not study *in vivo* responses in patients with ADA or ACC during the current investigations, but were able to detect some exaggerated responses in ADAs and ACCs *in vitro* (Chapter 5). Our *in vitro* cohort unfortunately did not contain aldosterone-producing adenomas, mainly because of the small size of Conn adenomas and the resulting low cell yield after single cell isolation methods. Overall, the pathophysiology underlying AIMAH elegantly illustrates the possible impact of non-ACTH mediated stimulation of steroidogenesis.

The precise mechanisms regulating steroid production in micronodular hyperplasia, as seen in PPNAD, is currently unelucidated. The underlying mutations in the cAMP/PKA

pathway, e.g. in *PRKAR1A*, are expected to convey stimulation of steroidogenesis, but it is unknown whether this is sufficient to account for the clinical syndrome of increased steroid production. Several different profiles of steroid production have been reported in PPNAD before.[57-58] The currently presented PPNAD case in chapter 6 was the first patient with primary infertility due to an androgen- and cortisol-producing adrenal nodule.

Previous studies have implicated a feed-forward loop entailing glucocorticoids as cause for the hypercortisolism,[46, 59] but this did not seem to pertain to our patients. Possibly the mass effect of *AKR1C3* expression in the dominant nodule has led to the hyperandrogenism in this patient. *In vivo*, we found no evidence of other aberrantly expressed receptors causing the increased androgen levels.

The adrenal glands of conditional *Prkar1a* knockout mice showed a recurrence of *Cyp17a1* expression and production of cortisol,[60] which is normally absent from murine adrenal glands.[61] Interestingly, the adrenal glands also harbored increased expression of *Inha* and *Fst*. This was extrapolated to human PPNAD tissues, which showed high levels of inhibin α-subunit protein on immunohistochemistry.[60] Whether this resembles the common expression of fetal adrenal markers, combined regulation of PKA-stimulated genes (Chapter 7) or possible stimulatory effects of the inhibin α-subunit on activin-suppressed *Cyp17a1* expression (Chapter 4) remains to be determined and warrants further investigation.

Alternatively, impairment of activin signaling in micronodular adrenocortical hyperplasia might also be Smad3-dependent. Knockout of *PRKAR1A* in human and *Prkar1a* in murine adrenocortical cells through silencing led to a decrease in *Smad3* expression and consequently an impaired apoptotic response to TGF-β.[62] Since activin and TGF-β signal through a common pathway, Smad3 inhibition could also affect activin actions in cells deficient for *Prkar1a*. This impaired activin responsiveness could have contributed to the recurrence of murine adrenocortical *Cyp17a1*.

2.4 Adrenocortical tumors

2.4.1 Pathogenesis

Research into the pathophysiology of adrenocortical tumors has been hampered by the rarity of this disease and by the advanced stages of disease in which these tumors are most commonly detected. For this reason, much is unknown about the natural history and progression of ACC. The first genetic associations in human ACC were with mutations in the tumor suppressor *TP53*.[63-64] More recent discoveries on factors involved in ACC pathogenesis include the overexpression of *IGF-2* at the imprinted 11p15 region[65-66] and activation of the Wnt/β-catenin pathway.[67-68]

One of the first murine models to develop adrenocortical tumors was the gonadectomized *Inha* knock-out mouse. These findings were difficult to confirm in human ACC;

conflicting results have been described (Chapter 7 and [69-74]). Subsequent studies showed that the combination of gonadectomy and *Inha* knockout leads to aberrant proliferation of subcapsular granulosa cell-like cells in the adrenal cortex, which under the influence of elevated gonadotropin levels derange into carcinomas[75-78] (Figure 2a). This could be applicable to a minority of human ACCs. Especially child ACCs have been shown to share characteristics with gonadal tumors.[79-80] On the other hand, these pediatric tumors specifically have increased pro-αC levels (Chapter 8) which would represent expression of gonadal markers rather than a tumor-suppressive role of *INHA*.

A minority of ACC samples showed loss of or low *INHA* expression levels (Chapters 7 and 9). This was found to be associated with increased methylation of CpGs 5' of *INHA* or the occurrence of common genetic variation in the promoter rather than mutations in the *INHA* gene. Loss of heterozygosity of the *INHA* region could also contribute to decreased *INHA* expression.[81]

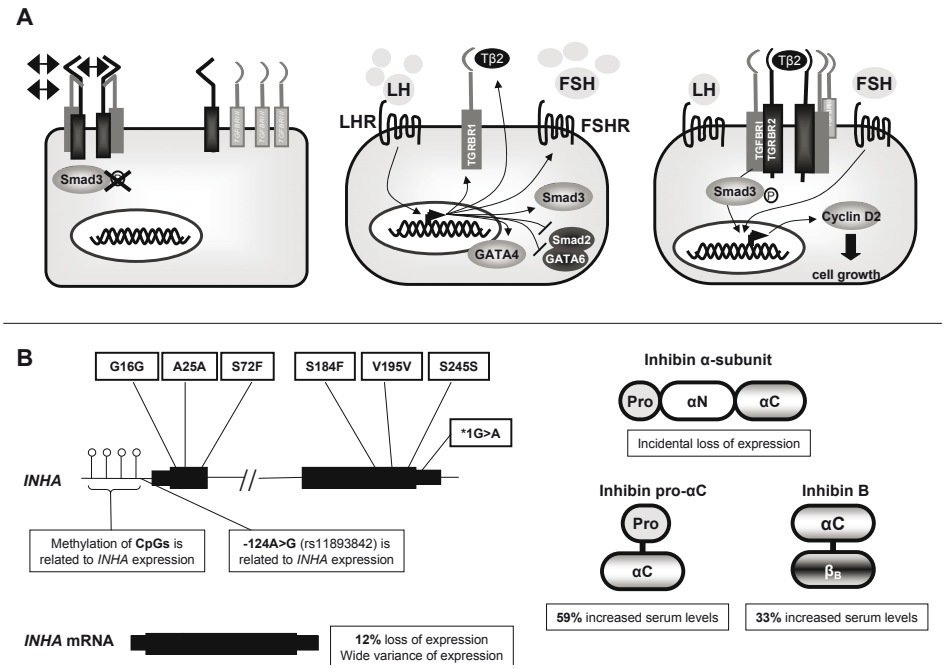
Two *INHA* mutations were previously described by Longui *et al.* in *TP53*-related pediatric ACC in a Brazilian population.[81] These mutations were not found in our cohort of 37 sporadic ACC patients (Chapter 9). Methylation of the *INHA* promoter thus appears a more common cause of *INHA* expression knockdown. The cause of the increased methylation ratio in a subset of ACCs is unknown, although epigenetic changes are common features during tumorigenesis.[82]

Although increased activation of the ACTH/cAMP/PKA pathway can lead to adrenal hyperplasia and the formation of benign nodules, this pathway does not appear to play a major role in adrenocortical carcinogenesis.[83] No activating mutations of the MC2R could be detected in ACCs.[84] Contrarily, ACCs are characterized by decreased ACTH sensitivity.[85] Impaired expression of *MRAP* (Chapter 3) and other factors downstream of the MC2R[86-88] lead to decreased expression of ACTH-regulated genes, such as *CYP17A1* and *TGFBR3* (Chapter 7). Although correlations between expression levels of these genes, *FST* and the activin type II receptors were detected in adrenocortical tissues (Chapter 7), the latter mRNAs were not stimulated by ACTH in primary adrenal cell cultures (Chapter 4), implying divergent regulation of expression.

2.4.2 Diagnosis

INHA shows a bimodal effect, being upregulated in the majority of ACCs (chapter 8). The detection of inhibin pro-αC in serum of postmenopausal women, despite the limited expression profile of *INHA*, has led to our study on the free inhibin α-subunit in serum of patients with adrenocortical tumors. Here, we have shown that serum inhibin pro-αC is a novel tumor marker for human ACCs. Inhibin B was also elevated in a subset of ACC patients. These and other inhibin α-subunit changes detected in human ACC have been summarized in Figure 2b.

Figure 2: Inhibin α-subunit changes in murine and human adrenocortical tumors



(A) Effects of *Inha* -/- on activin/inhibin-signaling in murine adrenocortical cells. Left: Although circulating activin levels are upregulated in *Inha* -/- mice, activin is unable to stimulate Smad3 phosphorylation, presumably due to decreased levels of the activin receptors. The loss of inhibin prevents internalization of the TGF-β type III receptor betaglycan, leading to increased cell surface expression of this co-receptor. Middle: The combination of *Inha* -/- and gonadectomy, marked by augmented serum levels of LH and FSH, induces the development of gonadal-like cells with increased expression of gonadal markers GATA4, FSHR and Smad3, whereas Smad2 and GATA6 expression levels are reduced. LH also stimulates expression of TGF-β2 and its type I receptor. Right: Concomitant with the augmented cell surface expression of betaglycan due to the absence of inhibin, TGF-β2 increases Smad3-dependent signaling and growth. This is possibly accomplished through an FSHR-dependent increase in cyclin D2 expression. (B) Inhibin α-subunit genetic, epigenetic, mRNA and protein changes observed in human ACCs. Heterozygous mutations detected in the coding regions and 3' UTR are indicated above the *INHA* gene. Methylation at CpGs -285, -241, -203 and -149 and common genetic variation at -124 of the *INHA* promoter in ACC samples was associated with *INHA* mRNA levels. *INHA* mRNA and protein expression are lost in a minority of ACC cases, as detected by quantitative PCR and immunohistochemistry. In serum of patients with ACC increased levels of inhibin pro-αC or inhibin B can be detected.

The characteristics of inhibin pro-αC as a tumor marker for ACCs are comparable with those of DHEA-S, with a non-significant trend towards increased sensitivity. This sensitivity could be more beneficial for patient follow-up, also given the high levels above reference ranges. In our series, we found a decrease of serum inhibin pro-αC levels in 10 out of 10 ACC patients treated with surgery or mitotane. Residual disease in 5 patients after irradical resection or during mitotane therapy was reflected by persistently elevated levels of serum inhibin pro-αC. Recently, GC/MS measurement of urine steroid metabolites has

given an AUC at ROC of 0.97 for the differentiation between ADA and ACC.[89] This would appear to be a superior technique, but is also more costly and labor-intensive than the inhibin pro-αC ELISA. The development of LC/MS-MS on patient serum or urine samples seems promising in this respect. This technique allows high-throughput simultaneous determination of multiple steroids.[8]

The current gold standard for discrimination between benign and malignant adrenocortical disease is pathological evaluation using the Weiss or van Slooten scores[90] or the presence of metastases. The coming years will show whether implementation of novel techniques, such as quantitative PCR and micro-array, have additional value for the prediction of clinical outcomes. A two gene signature has been found to accurately discern between ADA and ACC.[91] Other micro-array studies have also provided gene signatures in order to distinguish malignant from benign disease.[92-94] This could have significant effect on the necessity for patient follow-up. However, this would not be helpful for the initial evaluation for operative resection nor tumor recurrence. In this setting, serum or urinary tumor markers such as steroids and inhibin pro-αC would be more beneficial.

2.4.3 Treatment

Treatment of adrenocortical tumors is also rapidly evolving, despite its rare incidence. New surgical therapies encompass adrenal-sparing surgery and laparoscopy instead of an open procedure.[95-96] Success rates for adrenal cancer are poor, among others because of the advanced stages of disease upon first detection. Mitotane is a feasible option as palliative treatment[97]; the first prospective trial encompassing adjuvant mitotane therapy is presently recruiting patients (the ADIUVO trial, NCT00777244, www.clinicaltrials.gov).

Several chemotherapy and monoclonal antibody regimens have been described in small studies and are without large effect.[98-101] Only recently, the first randomized, multicenter international trial has been executed. A benefit on secondary endpoints was shown for a regimen containing doxorubicin, etoposide, cisplatin and mitotane, compared to streptozocin and mitotane.[102] Overall survival is still poor in stage IV disease,[103] although survival rates in stage II disease appear to have improved in the last decades.[104]

New targets are needed, not only for growth control but also for accompanying clinical syndromes. Concerning tumor growth, IGF-II has been shown to be highly increased in ACC tissues.[65-66] The first studies with IGF-II receptor inhibitors[105] are currently ongoing (NCT00924989 and NCT00778817). Published targets related to activin are BMP2 and BMP5, expression of which was shown to be decreased in ACC. Incubation with BMP2 and BMP5 decreased proliferation and steroid production in human adrenocortical cells.[106] Moreover, BMP5 treatment was shown to decrease adrenocortical tumor growth in a murine xenograft model.[107]

New therapies for blocking steroidogenesis in Cushing's syndrome (due to metastasized adrenocortical carcinomas, Cushing's disease or ectopic ACTH production) have barely evolved over the last decades. The currently used enzyme blockers include ketoconazole, metyrapone and etomidate.[108] Ketonazole has been most commonly used to treat hypercortisolism, but this anti-fungal drug comes with significant side-effects.[109-110] We have recently found that the related drug fluconazole could be a feasible alternative.[111] Although higher serum levels are needed to block steroidogenic enzymes, this drug has much less side-effects than ketoconazole. In case of refractory disease the progesterone receptor antagonist and partial antagonist of the GR, mifepristone, can also be administrered.[112] In case of the patients with AIMAH successful treatment has already been reported with antagonists to the ectopically or aberrantly expressed receptors.[113-114] These receptors could form treatment targets for inhibiting steroid production in adrenal tumors that have been described to show exaggerated responses to hormonal stimuli.[52-53, 55-56]

Finally, a more promising adrenal-blocking drug for treating hypercortisolism could be the novel potent P450c17 inhibitor, abiraterone acetate,[115] used in the treatment of castration-resistant prostate cancer (see below). Currently, there are no registered studies with abiraterone acetate in patients with Cushing's syndrome.

3 STEROIDOGENESIS IN PROSTATE CANCER

Steroidogenesis in prostate cancer is still a rapidly evolving field, despite the fact that the first observations of positive effects of castration were reported already in 1941 and successful treatment ensued with LHRH agonists.[116-117] In castration-resistant disease, previously termed hormone-refractory or androgen-independent PC, the AR is still activated.[118-120] Although several bypass pathways can induce growth in CRPC, the continued presence of residual androgens requires targeting. In the past, reports have already described additional effects of ketoconazole or glucocorticoids on PC growth in the adjuvant setting.[121-123] These studies however showed no survival benefit, which has prevented widespread implementation of these drugs in patients with CRPC. Additional androgen blockade through androgen receptor antagonists[124] did offer a small benefit, which can postpone chemotherapy in patients with castration-resistance.[125]

The only treatment option for patients with CRPC is chemotherapy in the form of doxetaxel with prednisone which offers a modest increase in survival compared to standard of care.[126] Despite the promise of full androgen blockade with anti-androgens and 5α-reductase inhibitors, the AR was still activated, showing that these blocks were not complete. Possible bypass mechanism include AR mutations, AR upregulation and stimulation of type I instead of type II 5α-reductase.[127]

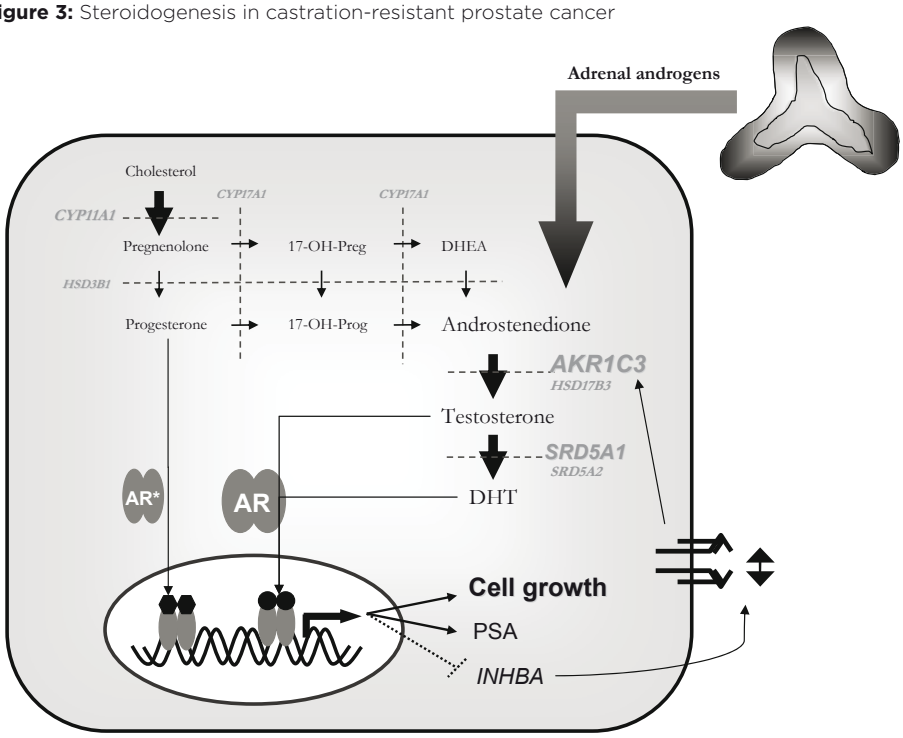
Renewed evidence that despite chemical castration androgens are still present in CRPC[128] has inspired new trials with novel and more potent steroidogenic enzyme inhibitors and anti-androgens. Treatment of CRPC patients with abiraterone acetate, a P450c17 inhibitor that potently inhibits circulating levels of androgens and glucocorticoids,[29] resulted in a decrease of intratumoral androgens, PSA, tumor growth[129-131] and eventually increased survival in a multicenter, randomized, phase III trial.[9] Furthermore, a novel antagonist of the AR without agonist effects, MDV3100, also had significant anti-tumor activity in a phase I-II trial in patients with CRPC.[132] These studies have ultimately proven that residual androgen activity plays a role in CRPC, but have not shown the source of the androgens.

The first clue to this source was provided by a micro-array study in which differences in gene expression between PC and CRPC were investigated.[133] Here, *AKR1C3* mRNA was found to be one of the most upregulated genes in CRPC. Subsequent studies also showed the presence of steroidogenic enzymes that could convert acetic acid into androgens.[119, 134] Our study in chapter 10 showed that the latter enzymes are present at very low concentrations and their expression was not stimulated in CRPC tissues. However, *AKR1C3* was again the gene most upregulated in castration-resistant samples, thereby making the adrenal androgens DHEA and androstenedione the most relevant precursors for residual testosterone and DHT in CRPC. Interestingly, this is compatible with the finding that tumor response to abiraterone acetate was associated with serum DHEA, DHEA-S and androstenedione levels before treatment.[129] Other studies have since then shown that the steroidogenic enzymes could be present and lead to AR activation in certain models.[119, 135-136] A recent investigation of bone marrow biopsies containing CPRC before and after treatment with abiraterone acetate investigated AR and P450c17 protein expression by immunohistochemistry.[131] Although quantitative expression measurements were not performed in this study, intratumoral P450c17 levels were associated with local testosterone levels and response to treatment.

In order to unravel the relative contributions of *de novo* steroidogenesis and adrenal androgen conversion in CRPC, we undertook a study to investigate the effects of physiological levels of androgen precursors on AR targets and PC growth.[137] Here, we found that *de novo* steroidogenesis could lead to AR activation and growth in a subset of cell lines. Adrenal androgen conversion, however, occurred in all cell lines and was much more potent than *de novo* steroidogenesis. Thus, although both pathways have been shown to be present in CRPC, adrenal androgen conversion, instigated by *AKR1C3*, appears most relevant (Figure 3).

Factors that regulate expression and activity of steroidogenic enzymes in prostate cancer have scarcely been described. Recently, two reports have shown relations between steroidogenesis in PC cells and growth factors IL-6 and insulin.[138-139] Since serum IL-6 levels are elevated in patients with CRPC[140] and this cytokine specifically induced *AKR1C3* ex-

Figure 3: Steroidogenesis in castration-resistant prostate cancer



The low levels of *HSD3B1/2* and *CYP17A1* expression in CRPC tissues decrease the potential for *de novo* steroidogenesis from cholesterol. On the other hand, augmented expression of *AKR1C3* and *SRD5A1* can convert circulating adrenal androgens, unaffected by chemical castration, into testosterone and DHT. These steroids can activate the AR leading to cell growth, expression of PSA and inhibition of *INHBA* expression. Upon androgen withdrawal, *INHBA* levels are increased and activin A augments expression of *AKR1C3*, stimulating testosterone production and giving rise to CRPC. In case of the T877A-mutated AR (AR*) other steroids, such as progesterone, can also directly stimulate the AR and lead to castration resistance.

pression in PC cell lines,[139] IL-6 is the first endo- or paracrine factor described to regulate steroidogenic enzyme expression in CRPC.

We have focused on activin A, which was previously shown to possess both oncogenic as well as tumor-suppressive properties in PC. Our investigations, detailed in chapter 11, revealed that activin A shared this *AKR1C3*-inducing effect, and was stimulated following castration. In this manner, activin A could be an intermediate between castration and castration-resistance due to the conversion of intratumoral androstenedione (derived from the adrenal cortex) to testosterone conversion (Figure 3). The association between the ratio of activin subunits to follistatin and the decreased metastasis-free survival suggests a negative effect of activin on CRPC evolution. Intriguingly, the first murine studies with activin pathway inhibitors are already under investigation. Since activin was found to be a growth factor that negatively affects bone remodeling,[141-142] models have been employed

in order to study therapeutical options of activin inhibition for osteoporosis.[143-144] These models could also be employed for the study of activin effects in PC.

4 GENERAL REMARKS

Most studies described in this thesis have used estimations of mRNA expression levels. Advantages of the employed PCR techniques include the simultaneous precise quantification of mRNA in large amounts of samples. The main negative sequela of mRNA studies can be the lack of association with protein levels, as for instance shown in chapter 9 for *INHA* mRNA and serum inhibin pro-αC. The processes of translation as well as trafficking, dimerisation (especially in case of inhibin subunits), phosphorylation and protein breakdown could lead to the absence of a relation between the levels of mRNA and those of protein. To validate the mRNA findings in several of our studies, we have chosen to investigate protein levels mostly through ELISA or through measuring protein effects, such as steroid conversion in the case of steroidogenic enzymes.

The quantification of steroids was performed with immunofluorescent methods and radio-immunoassay. Main disadvantage of these techniques is assay cross-reactivity between individual steroids. This could have confounded some results, such as the measurement of testosterone after incubation with androstenedione in chapter 11. More accurate estimation of steroids might be obtained by measurement through LC-MS/MS. This technique has recently been established to accurately and sensitively determine steroid content in various media and is expected to be the gold standard for steroid quantification in future experiments. Our current and future experiments on steroid conversions are incorporating this promising technique.

The currently presented studies encompassed mostly human studies, since there are large differences between the adrenal glands of murine and human models. The mouse adrenal gland lacks *Cyp17a1* and a discernible zona reticularis.[61, 145] This has prompted some researchers to study evolutionary related species, such as the rhesus macaque.[146] For this reason, we have chosen to specifically study human adrenal samples. These combined studies form the largest reported collection of *in vitro* primary adrenal cell cultures obtained from patient tissues.

Also for prostate cancer we have employed only human PC cell lines and xenografts in murine models. As stated in chapter 11, the utilization of murine models or dextran-coated charcoal treated medium is not representative for the situation in serum of CRPC patients, since their serum contains adrenal androgens in the nanomolar range, levels sufficient to drive AR-determined effects[137].

5 FUTURE DIRECTIONS

The studies described in this thesis open up new hypotheses that warrant ensuing investigations. In conjunction with newly developed techniques these could shed new light on adrenocortical and prostate cancer (patho)physiology.

5.1 Adrenal cortex

Functional studies in the three separate steroidogenic cell types have been hampered by the human adrenocortical zonation and by adrenomedullary cells that penetrate the adrenal cortex.[147] Ideally studies of the differential zonal distribution of components of the activin-signaling pathway should be performed in isolated cell populations, for instance after laser-microdissection[148] or immunomagnetic bead isolation.[149] In the latter case, AT1R antibody-covered beads have been utilized to successfully isolate zona glomerulosa cells, which could further aid in studying activin effects in these cells specifically. As illustrated in chapter 9, the micro-dissection of tumor tissues can prevent contamination with normal adrenal cells, and will reduce bias. This could further increase insights in receptor expression in AIMAH and tumor-specific differences in activin-related genes.

Recent discoveries on *INHA* upregulation in micronodular hyperplasia[60] and effects of inhibin on TGF-β2/Smad3-dependent signaling[77] form exiting prospects. Since these pathways were also found to be altered in mRNA studies of human ACCs (Chapter 7 and [92]), further elucidation of the way by which the inhibin α-subunit and TGF-β2 could affect cyclin D2 expression and growth in adrenocortical cells is warranted. The recently successfully employed techniques of adrenal cortex-specific knockout of genes in murine models[150-151] could facilitate these investigations. Despite the interspecies differences, it would be intriguing to employ conditional knockin of *Fst* or knockout of *Inhba* in the murine adrenal cortex in order to study effects on *Cyp17a1* expression and aldosterone and corticosterone production. These murine models could also be used for the study of *Hsd3b6*. Although the association with *HSD3B1* in humans could not be confirmed, the role of 3β-HSD in the circadian rhythm and hypertension in shift workers, long-distance transmeridian travelers and individuals with sleep disorders would also be intruiging to study.

The studies on *INHA* and inhibin pro-αC have increased insights into the role of the inhibin α-subunit in human ACC. Since loss of heterozygosity, promoter CpG methylation and SNPs cannot fully explain the mRNA level changes seen in adrenocortical carcinomas, future studies should also encompass histone-acetylation investigations. From a clinical point of view, a replication study in a large set of ADA and ACC samples is necessary to confirm the findings currently presented. Furthermore, associations of inhibin pro-αC levels with tumor recurrence and patient survival need to be investigated. Ideally, other markers, such as adrenocortical steroid measurements by conventional and mass-spec-

trometric methods, should be included. In the future new markers could be found through proteomics or circulating tumor cells. The realization of a European collaboration on the study of adrenal tumors (ENSAT; www.ensat.org) is a promising development that will hopefully stimulate adrenal research.

The roles of other TGF-β family members have been studied to a lesser extent, although recent findings suggest a possible therapeutic role for BMP analogues.[106] The interplay between these molecules and activin/inhibin in the processes of transdifferentiation and tumorigenesis requires additional study. Furthermore, genetic analysis of downstream factors of the activin-signaling pathway, extensively studied in other types of cancer,[152] could further aid in better understanding activin and inhibin dysregulation during adrenocortical tumor formation. The development of exome and full genome sequencing[153] will lead to a rapid developing plethora of underlying mutations for all human cancers, most of which are probably currently unknown.

For AIMAH the challenge will reside in the elucidation of the cause of the aberrant and ectopic expression of receptors. Furthermore, by elucidating the additional responsible receptors these should form therapeutical targets for treatments of hypercortisolism or hyperaldosteronism in AIMAH, PPNAD or possibly even adenomas and carcinomas. In this respect, it will be interesting to investigate whether the stimulation of activin production by AngII could be blocked by angiotensin receptor blockers and whether this will affect *CYP17A1* expression.

In search for diseases characterized by high circulating activin A levels, lipopolysaccharide (LPS) was found to induce activin secretion in sheep and rats.[154-155] The activin surge following LPS administration predated the increase in serum levels of other well-studied cytokines, such as TNF-α and IL-6. Patients with septicaemia showed increased levels of activin A and follistatin, compared to control subjects[156] and inhibition of activin by the administration of follistatin significantly improved survival in mice injected with a sublethal dose of LPS. In the latter study, all surviving mice had lower activin A levels than the mice that succumbed to the endotoxaemia.[157] Since relative adrenal insufficiency is a common cause of morbidity and mortality in patients with septicaemia[158] and activin A exerts negative effects on cortisol production, we are currently investigating associations between activin A levels, steroid levels and clinical outcomes in patients admitted at the ICU with septicaemia.

5.2 Prostate cancer

The most important goal in the immediate future for studies on steroidogenesis in prostate cancer is the selection of those patients that are susceptible for P450c17 or AR blockade. New pathways leading to resistance will have to be investigated. Recently, Cai *et al.* have shown that *AR* mutations and *CYP17A1* upregulation could participate in resistance to P450c17 inhibitors, in this case ketoconazole.[135] Similarly, increased utili-

zation of the backdoor pathway has been described to occur following treatment with abiraterone acetate.[29] The relative contributions of adrenal androgen conversion versus *de novo* steroidogenesis have to be ascertained by including more patient samples before and after therapy. Circulating tumor cells appear to be a developing source of tissue that is easily accessible.[159]

Pathways that control steroidogenic enzyme expression in PC tissue are currently incompletely understood. The insight that 17β-HSD type 5 is the main enzyme responsible for residual androgens in CRPC should further prompt research to develop inhibitors. The only currently available non-specific inhibitor is indomethacine, which was indeed shown to decrease tumor growth.[135] New, more specific inhibitors are needed. The herein described studies on activin would suggest an effect of activin-signaling inhibitors, which should be investigated further.

Summarizing, these studies in the adrenal cortex and prostate cancer reveal novel insights into mechanisms involved in the local control of steroid hormone biosynthesis. Furthermore, they show that the field of steroidogenesis still poses many unresolved clinical, translational and basic mysteries that could introduce new markers for the diagnosis and treatment of rare and prevalent diseases.

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SUMMARY

Steroid hormones influence a plethora of physiological and pathophysiological processes. The principal steroids in man can be grouped into sex steroids (testosterone, estradiol and progesterone), mineralocorticoids (aldosterone) and glucocorticoids (cortisol) based on their effects in target tissues. The production of steroid hormones is mainly realized within the classic steroidogenic tissues, i.e. the gonads, placenta and adrenal cortex. These organs secrete steroids into the circulation, where they are transported to their target tissues, e.g. the reproductive tract, skin, brain, kidney, heart, fat, and liver. Here, they exert their effects after binding to their specific receptors.

Steroidogenesis, the process of steroid hormone biosynthesis, is accomplished by sequential enzymatic reactions that change the chemical structure of the common steroid precursor cholesterol. These reactions are catalyzed by enzymes and co-factors that control the intracellular production and conversion of steroid molecules. Changes in the activity or level of steroidogenic enzymes influence the levels of locally available (para- or autocrine) or circulating (endocrine) levels of steroid hormones.

The first sections of this thesis centre on steroidogenesis in the adrenal cortex. Adrenal progenitor or stem cells divide near the adrenal capsule and migrate centripetally whilst consecutively producing aldosterone, cortisol or adrenal androgens, depending on their localization in each of the three different zones of the gland. This is a result of the zone-specific expression patterns of steroidogenic enzymes and their co-factors.

The adrenocortical production of cortisol and adrenal androgens is predominantly regulated by adrenocorticotropin (ACTH), a pituitary-derived hormone that potently induces steroidogenic enzyme expression. ACTH binds to the melanocortin 2 receptor (MC2R) leading to the formation of cyclic AMP (cAMP) and the activation of protein kinase A (PKA). The presence and activity of the MC2R in the membrane of adrenocortical cells is highly dependent on co-expression of the recently discovered melanocortin 2 receptor associated protein, MRAP.

In **chapter 2** we describe the first studies on the regulation of *MRAP* expression in various human adrenocortical tissues. ACTH, known to induce internalisation of the MC2R upon binding, potently stimulated the mRNA expression of *MRAP* and *MC2R*. MRAP2, a protein homologous to MRAP that is also capable of MC2R trafficking but abrogates its function, was found to be regulated in a different manner. These regulatory mechanisms could serve to ensure continuous cell surface expression and activity of the MC2R-MRAP complex. *MRAP* and *MC2R* levels in hyperplastic adrenal glands were related to patients' peripheral ACTH and cortisol levels, reflecting the control of expression of this receptor complex by ACTH. This relationship was lost in patients with adrenocortical tumors. We found no association between *in vitro* ACTH responsiveness and *MRAP*, *MRAP2* or *MC2R*

expression, suggesting that physiological fluctuations in levels of these (co-)receptors do not modulate ACTH sensitivity.

The other main regulator of adrenocortical steroidogenesis is angiotensin II (AngII) which is one of the factors in the renin-angiotensin-aldosterone system. The principal function of AngII is the stimulation of aldosterone production in the outer zona glomerulosa of the adrenal cortex. The biosynthesis of aldosterone is dependent on the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) for the conversion of pregnenolone to progesterone and the concomitant absence of P450c17, encoded by *CYP17A1*, that diverts steroidogenesis towards production of glucocorticoids and adrenal androgens.

Recent genetic and expression studies suggested that the 3β-HSD type I iso-enzyme (*HSD3B1*) might be responsible for aldosterone production, whereas all adrenocortical steroidogenesis was previously believed to be enabled through the activity of the type II 3β-HSD (*HSD3B2*). In **chapter 3**, we studied the presence of *HSD3B1* and *HSD3B2* in human adrenal tissues. We were unable to detect sufficient levels of *HSD3B1* in adrenocortical samples, among which aldosterone-producing tumors. Moreover, *HSD3B1* expression, in contrast to that of the highly expressed *HSD3B2*, was not stimulated by AngII. Our genetic analysis study also did not reveal a relationship between single nucleotide polymorphisms (SNPs) in the *HSD3B1* gene and blood pressure, a validated endpoint for aldosterone effects. Based on these results, aldosterone production would still appear to be primarily dependent on the type II 3β-HSD.

Apart from ACTH and AngII, many other endo-, para- and autocrine signals are known to affect steroidogenic enzyme expression in the adrenal cortex. The locally produced growth factor activin A was previously shown to affect these levels in adrenocortical cells. Activin A, the homodimer of two inhibin βA-subunits (*INHBA*), showed a zone-specific expression with high levels in the zona glomerulosa. The expression pattern was opposite to that of the inhibin α-subunit (*INHA*) which forms the activin antagonists inhibin A or inhibin B when combined with the inhibin βA-subunit or βB-subunit, respectively. Studies described in **chapter 4** revealed that activin A is induced by AngII in human adrenocortical cells and is an intermediate in the AngII- and protein kinase C (PKC)-induced down-regulation of *CYP17A1* expression. Therefore, AngII stimulation in the zona glomerulosa can lead to increased local activin A levels that impair *CYP17A1* expression in an autocrine fashion and thus preserve the production of aldosterone in that zone. The expression of the inhibin α-subunit, regulated by ACTH, in the inner adrenocortical zones could serve to prevent activin A-induced *CYP17A1* suppression leading to glucocorticoid and adrenal androgen production in these zones.

Overproduction of adrenocortical steroids is often the result of adrenal hyperplasia or tumors. Adrenocortical hyperplasia is most frequently caused by increased ACTH stimulation, but can also occur due to primary adrenal disease. ACTH-independent macronodular adrenal hyperplasia (AIMAH) is caused by aberrant expression of eutopic or ectopic G protein-coupled receptors on adrenocortical cells that stimulate steroidogenic enzyme levels. In **chapter 5** we report *in vivo* and *in vitro* studies in the largest described AIMAH patient group thus far. Although still ACTH-responsive, these patients also show augmented serum cortisol concentrations after the administration of arginine-vasopressin (AVP), serotonin or catecholamines and to a lesser extent also of luteinizing hormone-releasing hormone, thyrotropin-releasing hormone, glucagon or gastric inhibitory polypeptide. Significant stimulation of serum cortisol by multiple hormonal stimuli frequently occurred in individual patients. These findings were confirmed in *in vitro* studies, although intra-individual responses *in vivo* and *in vitro* correlated poorly. The most prevalent hormonal stimulus, AVP, was found to aberrantly increase expression of the steroidogenic enzyme responsible for the final conversion into cortisol, *CYP11B1*. Although AVP type 1A receptor levels did not differ between AIMAH and other adrenal tissues, aberrant coupling of this receptor to *CYP11B1* constitutes a novel mechanism that could underlie the hypercortisolism in AVP-dependent AIMAH patients.

Primary pigmented nodular adrenal hyperplasia (PPNAD), a form of micronodular adrenal hyperplasia, is frequently associated with mutations in downstream factors of ACTH, particularly in the PKA type Iα regulatory subunit (*PRKAR1A*). These mutations lead to constitutional activation of catalytic PKA subunits that subsequently drives adrenocortical hyperplasia and steroidogenesis. **In chapter 6** we describe a female PPNAD patient with primary infertility due to increased serum testosterone levels. This unique serum steroid profile for PPNAD dissolved after unilateral adrenalectomy and the patient subsequently conceived. Genetic analysis revealed a protein-truncating mutation in *PRKAR1A*. Previously reported glucocorticoid feed-forward loops in PPNAD could not be confirmed in this and three additional PPNAD cases. Of the testosterone-forming 17β-HSDs investigated, the type 5 was expressed most abundantly in the PPNAD-associated adenoma, making *AKR1C3* the most plausible cause for her hyperandrogenism.

Tumors in the adrenal cortex are frequently characterized by the overproduction of aldosterone, cortisol or adrenal androgens leading to clinical syndromes: respectively Conn's syndrome, Cushing's syndrome or virilization. Factors contributing to derailed steroidogenesis in adrenocortical adenomas and carcinomas are largely unknown. The seldom occurring adrenocortical carcinoma is characterized by a poor prognosis and novel treatment options are urgently needed.

In **chapter 7** we studied the expression levels of components of the activin-signaling pathway in adrenal tumors. We detected decreased mRNA expression levels of *INHBA*, the activin antagonist follistatin, activin type II receptors and the inhibin co-receptor betaglycan in adrenocortical carcinomas, compared to non-tumorous adrenals. This could have been the consequence of impaired ACTH/PKA signaling in adrenocortical carcinomas. Furthermore, *INHA* mRNA was not detectable in three out of fourteen adrenocortical carcinomas. This latter finding could reflect the potential tumor suppressive role of the inhibin α-subunit in the adrenal cortex that was previously described to occur in 99% of *Inha -/-* mice after gonadectomy.

On the other hand, *INHA* can also be overexpressed in human adrenocortical tumors. In **chapter 8** we studied serum levels of the free inhibin α-subunit, inhibin pro-αC, in patients with adrenal tumors and detected increased concentrations in the majority of the patients with adrenocortical carcinomas. These levels were also higher in carcinoma patients than in patients with adrenal hyperplasia or other adrenal tumors. Serum inhibin pro-αC levels differentiated carcinomas from adenomas. This novel tumor marker could aid clinical decision making, particularly for patients with small adrenal tumors in the absence of increased serum steroid levels. Inhibin pro-αC levels could also be implemented for patient follow-up since 10 out of 10 ACC patients showed decreased serum concentrations following treatment.

Since we detected a wide variation in *INHA* expression in adrenocortical carcinomas, we investigated whether mutations in or promoter methylation of the *INHA* gene affect gene expression and serum inhibin pro-αC levels in these patients. As shown in **chapter 9**, we found 4 synonymous and 2 missense heterozygous *INHA* mutations in 37 adrenocortical carcinomas, suggesting that *INHA* mutations do not play a major role in human adrenocortical carcinomas. Expression levels of *INHA* were related to CpG methylation as well as a SNP located in the *INHA* promoter. Since we also found hypermethylation of the *INHA* promoter in 26% of carcinomas, methylation could be a mechanism through which adrenocortical carcinomas express low or absent levels of *INHA*. Whether this contributes to tumorigenesis remains unknown.

Besides in the classic steroidogenic tissues, levels of steroidogenic enzyme expression can also be detected in peripheral target tissues. Intracellular steroid hormone bioavailability and bioactivity in the target tissues can be regulated through local expression of these enzymes. Prostate cancer, a hormone-sensitive malignancy, has been found to utilize local conversion of sex steroids to ensure stimulation of the androgen receptor and thus tumor growth. The third section of this thesis focuses on steroidogenesis within prostate cancer cells. Recent evidence suggests the presence of steroidogenic enzymes responsible for *de novo* steroidogenesis in prostate cancer. The intracrine production of androgens in

prostate cancer could render cells resistant to medical treatment by chemical castration. The castration-resistant disease state does not respond to other medical therapies and inevitably progresses to death.

In **chapter 10** we investigated the effects of androgen deprivation on steroidogenic enzyme expression levels in human prostate cancer cell lines, xenografts and patient tissues. Enzymes required for *de novo* steroid synthesis were absent or expressed at low levels in prostate cancer samples. Also they were not induced by androgen deprivation. In contrast, steroidogenic enzymes needed for the conversion of adrenal androgens into testosterone and the more potent dihydrotestosterone were expressed at high levels. Moreover, the type 5 17β-HSD enzyme, encoded by *AKR1C3*, was potently stimulated by androgen deprivation in the cell lines and xenografts. *AKR1C3* levels were increased in castration-resistant prostate cancer samples compared to normal prostatic tissue, local hormone-dependent prostate cancer and lymph node metastases. This induction of *AKR1C3* could cause the intracrine conversion of androstenedione, derived from the adrenal cortex, to testosterone and underlie the resistance to medical castration treatment.

Since we found effects of activin A on steroidogenic enzyme expression in adrenocortical tissues, we investigated in **chapter 11** whether activin A affects these enzyme levels in prostate cancer. Activin A was previously found to cause apoptosis in prostate cancer cells, but also to enhance expression of the androgen receptor and cell migration. In our study we found that activin A potently stimulated expression of *AKR1C3* and thereby local conversion of androstenedione into testosterone. This intracrine mechanism opposes the apoptotic effect of activin A in the presence of physiological concentrations of androstenedione. Expression of *INHBA* and production of activin A were suppressed by androgens *in vitro* and a ratio of activin subunits to follistatin was higher in prostate cancer compared to normal prostate tissue. Furthermore, this ratio was inversely associated with metastasis-free survival in patients with prostate cancer. Activin A therefore forms an intermediate between androgen deprivation and the stimulation of *AKR1C3* leading to castration-resistant prostate cancer. Because of this effect, activin A forms a novel target for the treatment of castration-resistant disease.

Finally, in **chapter 12** we discuss the results described in this thesis as well as their current and possible future implications for our understanding of local control of steroid hormone biosynthesis.

SAMENVATTING

Steroïdhormonen beïnvloeden vele fysiologische en pathofysiologische processen in het lichaam. De belangrijkste steroïden in de mens kunnen op basis van hun effecten in doelweefsels worden onderverdeeld in geslachtshormonen (testosteron, oestradiol en progesteron), mineralocorticoïden (aldosteron) en glucocorticoïden (cortisol). Steroïdhormonen worden hoofdzakelijk geproduceerd binnen de klassieke steroïdogene weefsels, dat wil zeggen de gonaden, de placenta en de bijnierschors. Deze organen scheiden de steroïden af in de bloedsomloop die hen transporteert naar hun doelweefsels, zoals voortplantingsorganen, huid, nieren, hersenen, hart, vet en lever. Steroïden beïnvloeden het functioneren van deze organen nadat ze aan hun specifieke receptoren gebonden zijn.

Steroïdogenese is het proces van steroïdhormoon biosynthese: een aantal opeenvolgende enzymatische reacties die de chemische structuur van het gemeenschappelijk voorlopersteroïd cholesterol veranderen. Deze reacties worden gekatalyseerd door steroïdogene enzymen en co-factoren die de omzetting van steroïdmoleculen reguleren. Veranderingen in de activiteit of het niveau van de steroïdogene enzymen beïnvloeden de lokaal beschikbare (para- of autocriene) of circulerende (endocriene) niveaus van steroïdhormonen.

Het eerste secties van dit proefschrift behandelen de steroïdogenese in de bijnierschors. Bijnier voorlopercellen of stamcellen delen net onder het bijnierkapsel en migreren richting het bijniermerg waarbij ze achtereenvolgens aldosteron, cortisol en bijnierandrogenen produceren in de drie verschillende zones van de bijnierschors. Dit wordt mogelijk gemaakt door zone-specifieke expressie patronen van steroïdogene enzymen en hun co-factoren.

De productie van cortisol en bijnierandrogenen wordt voornamelijk geregeld door corticotropine (ACTH), een hormoon afkomstig uit de hypofyse, dat de expressie van de steroïdogene enzymen krachtig stimuleert. Binding van het ACTH aan de melanocortine 2 receptor (MC2R) leidt tot de vorming van cyclisch AMP (cAMP) en de activering van proteïne kinase A (PKA). De aanwezigheid en activiteit van de MC2R in het membraan van bijnierschorscellen is sterk afhankelijk van co-expressie van het recent ontdekte melanocortine 2 receptor geassocieerde eiwit MRAP.

In **hoofdstuk 2** beschrijven we de eerste studies over de regulatie van *MRAP* expressie in verschillende humane bijnierweefsels. ACTH, waarvan bekend is dat het internalisering van de MC2R bewerkstelligt na binding, verhoogt de expressie van *MRAP* en *MC2R* mRNAs. De productie van MRAP2, een eiwit dat homoloog is aan MRAP en ook in staat is om MC2R te binden maar de functie van MC2R remt, bleek op een andere manier geregeld te worden. Deze regelmechanismen spelen een rol bij het zekerstellen van de expressie en activiteit van het MC2R-MRAP complex op het celoppervlak. *MRAP* en *MC2R*

niveaus in hyperplastische bijnieren waren gerelateerd aan de perifere spiegels van ACTH en cortisol. Deze relatie werd niet gevonden in patiënten met bijnierschorstumoren. Wij vonden geen relatie tussen ACTH gevoeligheid van bijniercellen in kweek en niveaus van *MRAP*, *MRAP2* of *MC2R* wat suggereert dat fysiologische fluctuaties in expressieniveaus van deze (co-)receptoren de ACTH gevoeligheid niet moduleren.

Naast ACTH is angiotensine II (AngII) een belangrijke factor in de regulatie van de steroïdogenese in de bijnierschors. AngII maakt deel uit van het renine-angiotensine-aldosteron systeem. De belangrijkste functie van AngII is de stimulatie van aldosteronproductie in de zona glomerulosa, de buitenste zone van de bijnierschors. De vorming van aldosteron is afhankelijk van de expressie van 3β-hydroxysteroid dehydrogenase (3β-HSD) voor de omzetting van pregnenolon in progesteron en van het gebrek aan P450c17, gecodeerd door *CYP17A1*, dat de steroïdogenese in de richting van glucocorticoïden en geslachtshormonen leidt.

Met betrekking tot 3β-HSD suggereren recente genetische en expressie studies dat het type I iso-enzym (*HSD3B1*) mogelijk verantwoordelijk is voor de productie van aldosteron, terwijl voorheen werd gedacht dat de vorming van alle steroïdhormonen in de bijnierschors mogelijk werd gemaakt door het type II 3β-HSD (*HSD3B2*). In **hoofdstuk 3** onderzochten we de aanwezigheid van *HSD3B1* en *HSD3B2* in humane bijnierweefsels. We detecteerden lage tot afwezige expressie van *HSD3B1* in bijnierschorsmonsters, waaronder ook aldosteron-producerende tumoren. Bovendien werden de mRNA niveaus van *HSD3B1*, in tegenstelling tot het hoog tot expressie gebrachte *HSD3B2*, niet gestimuleerd door AngII. In een genetische studie bleek ook geen relatie te bestaan tussen "single nucleotide polymorfisms" (SNPs) in het *HSD3B1* gen en bloeddruk, een gevalideerd eindpunt voor effecten van aldosteron. Op basis van deze resultaten verwachten wij dat de aldosteronproductie in de bijnier in de eerste plaats afhankelijk is van het type II 3β-HSD.

Het is bekend dat naast ACTH and AngII vele andere endo-, para- en autocriene signalen de expressie van steroïdogene enzymen in de bijnierschors kunnen beïnvloeden. Van de lokale groeifactor activine A werd al eerder aangetoond dat het de niveaus van deze enzymen in bijnierschorscellen beïnvloedt. Activine A, bestaande uit twee inhibine βA-subunits (*INHBA*), toonde een zone-specifieke expressie met hoge niveaus in de zona glomerulosa. Deze localisatie is tegengesteld aan die van de inhibine α-subunit (*INHA*), die samen met de βA-subunit of βB-subunit de activine-antagonisten inhibine A of inhibine B, respectievelijk, kan vormen en die voornamelijk in de binnenste zone van de bijnierschors tot expressie komt. Uit de studies beschreven in **hoofdstuk 4** werd duidelijk dat activine A niveaus in menselijke bijnierschorscellen worden gestimuleerd door AngII via stimulatie van het proteïne kinase C (PKC). Ook werd duidelijk dat activine A betrokken is bij de PKC-geïnduceerde onderdrukking van *CYP17A1* expressie. Dit leidt tot het model dat

AngII de lokale activine A niveaus in de zona glomerulosa stimuleert, die op hun beurt de expressie van *CYP17A1* remmen en daarmee de productie van aldosteron in die zone mogelijk maken. De expressie van *INHA*, gecontroleerd door ACTH, in de binnenste zones van de bijnierschors zou ertoe kunnen leiden dat de *CYP17A1* expressie en de daarmee samenhangende productie van glucocorticoïden en bijnierandrogenen gegarandeerd zijn.

Overproductie van bijniersteroïden wordt frequent veroorzaakt door bijnierhyperplasie of bijnierschorstumoren. Bijnierhyperplasie is meestal het gevolg van toegenomen stimulatie door ACTH, maar kan ook optreden als gevolg van een primaire ziekte in de bijnier. ACTH-onafhankelijke macronodulaire bijnierhyperplasie (AIMAH) wordt veroorzaakt door verhoogde of ectopische expressie van G eiwit-gekoppelde receptoren op bijniercellen die de steroïdogenese stimuleren. In **hoofdstuk 5** beschrijven we *in vivo* en *in vitro* studies in de grootste beschreven patiëntengroep met AIMAH tot nu toe. Hoewel deze patiënten nog steeds reageerden op ACTH met toegenomen serum cortisol spiegels, toonden zij ook verhoogde serum cortisol niveaus na de toediening van arginine-vasopressine (AVP), serotonine of catecholamines en in mindere mate ook van luteïniserend hormoon-vrijmakend hormoon, thyrotropine-vrijmakend hormoon, glucagon of gastric inhibitory polypeptide. Significante stijgingen van het serum cortisol op meerdere hormonale stimuli traden ook frequent op in individuele patiënten. Deze bevindingen werden bevestigd in *in vitro* studies, hoewel de intra-individuele *in vivo* en *in vitro* reacties slecht gecorreleerd bleken. De meest voorkomende hormonale stimulus, AVP, bleek de expressie van het steroïdogene enzym dat verantwoordelijk is voor de uiteindelijke omzetting van voorlopersteroïden in cortisol, *CYP11B1*, buiten proportie te verhogen. Hoewel de niveaus van de AVP type 1A receptor in AIMAH en andere bijnierweefsels niet verschilden, kan deze afwijkende koppeling van de AVP receptor met *CYP11B1* ten grondslag liggen aan het hypercortisolisme in AVP-afhankelijke AIMAH patiënten.

Primaire gepigmenteerde nodulaire bijnierhyperplasie (PPNAD), een vorm van micronodulaire bijnierhyperplasie, is vaak geassocieerd met mutaties in de downstream factoren van ACTH, met name in de PKA type Ia regulerende subunit (*PRKAR1A*). Mutaties in dit gen leiden tot een constitutionele activatie van PKA katalytische subunits die vervolgens de ontwikkeling van bijnierschorshyperplasie en steroïdogenese stimuleren. In **hoofdstuk 6** beschrijven we een vrouwelijke PPNAD casus met een primaire onvruchtbaarheid als gevolg van verhoogde serum testosteron niveaus. Dit voor PPNAD unieke serum steroïdprofiel verdween na eenzijdige bijnierextirpatie en leidde vervolgens tot een succesvolle zwangerschap. Genetische analyse toonde een eiwit-truncerende mutatie in *PRKAR1A*. Eerder gepubliceerde glucocorticoïd feed-forward loops in PPNAD konden niet worden bevestigd in deze en drie additionele PPNAD patiënten. Van de enzymen die testosteron produceren kwam het type 5 17β-HSD (*AKR1C3*) het meest tot expressie in het adenoom

van de PPNAD. Dit enzym is daardoor waarschijnlijk de oorzaak van haar verhoogde androgeenspiegels.

Tumoren in de bijnierschors worden vaak gekenschetst door de overproductie van aldosteron, cortisol of bijnierandrogenen, leidend tot klinische syndromen: respectievelijk syndroom van Conn, syndroom van Cushing of virilisatie. Factoren die bijdragen aan de ontspoorde steroïdogenese in bijnieradenomen en -carcinomen zijn grotendeels onbekend. Het zeldzame bijnierschorscarcinoom wordt gekenmerkt door een slechte prognose en nieuwe behandelingsopties zijn dringend noodzakelijk.

In **hoofdstuk 7** hebben we de expressieniveaus van de componenten van de activine signaleringsroute bestudeerd in bijniertumoren. We hebben geconstateerd dat er in bijnierschorscarcinomen verlaagde mRNA expressieniveaus zijn van *INHBA*, de activine antagonist follistatine, activine type II receptoren en de inhibine co-receptor betaglycan, in vergelijking tot normale bijnieren. Dit is waarschijnlijk een gevolg van dysfunctie van de ACTH/PKA pathway in bijnierschorscarcinomen. Bovendien was het mRNA van *INHA* niet detecteerbaar in drie van de veertien bestudeerde bijnierschorscarcinomen. Deze laatste bevinding zou de mogelijke tumor-onderdrukkende rol van de inhibine α-subunit in de bijnierschors kunnen weerspiegelen; deze werd eerder beschreven in *Inha -/-* muizen na chirurgische resectie van de gonaden.

Aan de andere kant kunnen humane bijnierschorstumoren *INHA* ook tot overexpressie brengen. In **hoofdstuk 8** bestudeerden we serumniveaus van de vrije inhibine α-subunit, inhibine pro-αC, in patiënten met bijnierschorstumoren. We detecteerden verhoogde serumspiegels van inhibine pro-αC in de meerderheid van de patiënten met bijnierschorscarcinomen. Deze niveaus waren ook hoger dan bij patiënten met een bijnierhyperplasie of andere tumoren van de bijnier. Serum inhibine pro-αC bleek hierdoor bijnierschorscarcinomen te kunnen onderscheiden van de andere bijnierafwijkingen. Deze nieuwe tumor marker zou de klinische besluitvorming kunnen ondersteunen, met name bij patiënten met kleine tumoren in de bijnier zonder verhoogde serumniveaus van steroïden. Inhibine pro-αC niveaus kunnen ook worden geïmplementeerd in de follow-up van patiënten met bijnierschorscarcinomen aangezien 10 uit 10 onderzochte patiënten met bijnierschorscarcinomen verminderde serumspiegels hadden na behandeling.

Na de ontdekking van de grote variatie in *INHA* expressie in bijnierschorscarcinomen onderzochten we vervolgens of mutaties in of promotormethylatie van het *INHA* gen de expressie en serum inhibine pro-αC niveaus bij patiënten met deze tumoren beïnvloeden. Zoals weergegeven in **hoofdstuk 9**, vonden we vier synonieme en twee missense heterozygote puntmutaties in het *INHA* gen in 37 verschillende bijnierschorscarcinomen. Dit suggereert dat *INHA* mutaties geen belangrijke rol spelen bij de vorming van bijnierschor-

scarcinomen in mensen. De expressie van *INHA* was wel gerelateerd aan CpG methylering en een SNP in de *INHA* promotor. Aangezien wij ook toegenomen methylatie van de *INHA* promotor vonden in 26% van de carcinomen, zou methylering een mechanisme kunnen vormen waardoor een deel van bijnierschorscarcinomen lage of afwezige niveaus van *INHA* hebben. Of dit bijdraagt aan het ontstaan van de tumoren is nog onbekend.

Behalve in de klassieke steroïdogene weefsels komen de steroïdogene enzymen ook tot expressie in een aantal niet klassiek-endocriene weefsels. De biologische beschikbaarheid en activiteit van steroïdhormonen in de doelwitweefsels kunnen gereguleerd worden door middel van lokale expressie van deze enzymen. In prostaatkanker, een hormoon-gevoelige tumor, is lokale omzetting van geslachtshormonen aangetoond; deze omzetting leidt tot stimulatie van de androgeenreceptor en daardoor tot groei van de tumor. Het derde deel van dit proefschrift richt zich op steroïdogenese binnen prostaatkankercellen. Recente studies suggereren de aanwezigheid van steroïdogene enzymen die verantwoordelijk zijn voor *de novo* steroïdogenese in prostaatkanker. De resulterende intracriene productie van androgenen in prostaatkanker zou kunnen leiden tot resistentie tegen de medische behandeling met chemische castratie. Deze castratie-resistente ziekte is ongevoelig voor andere medische behandelingen en leidt onvermijdelijk tot de dood.

In **hoofdstuk 10** hebben we de effecten van androgeenonttrekking op de expressie van steroïdogene enzymen onderzocht in humane prostaatkanker cellijnen, xenotransplantaten en patiëntweefsels. De enzymen die nodig zijn voor *de novo* steroïdsynthese waren afwezig of kwamen in geringe mate tot expressie in monsters van prostaatkanker; ze werden niet gestimuleerd door androgeendeprivatie. Steroïdogene enzymen die nodig zijn voor de omzetting van bijnierandrogenen in testosteron en het meer potente dihydrotestosteron kwamen op een hoog niveau tot expressie. Bovendien werd het type 5 17β-HSD enzym, gecodeerd door *AKR1C3*, krachtig gestimuleerd na androgeendeprivatie in de prostaatkanker cellijnen en xenotransplantaten. *AKR1C3* niveaus waren verhoogd in castratie-resistente prostaatkanker in vergelijking met normaal prostaatweefsel, lokale hormoon-gevoelige prostaatkanker en lymfkliermetastasen. Deze inductie van *AKR1C3* kan leiden tot toegenomen intracriene omzetting van androsteendion, afkomstig uit de bijnierschors, in testosteron en ten grondslag liggen aan de resistentie tegen de behandeling middels castratie.

Aangezien wij effecten van activine A op de expressie van steroïdogene enzymen in bijnierweefsels vonden, onderzochten we in **hoofdstuk 11** of activine de expressieniveaus van deze enzymen in prostaatkanker beïnvloedt. Er werd eerder al beschreven dat activine A apoptose in prostaatkankercellen veroorzaakt, maar tevens de expressie van de androgeenreceptor en celmigratie verhoogt. In onze studie vonden we dat activine A de expressie van *AKR1C3* in prostaatkankercellen en daarmee de lokale omzetting van an-

drosteendion in testosteron sterk verhoogde. Dit intracriene mechanisme heft het apoptotische effect van activine A op in de aanwezigheid van fysiologische concentraties van androsteendion. De productie van activine A werd *in vitro* onderdrukt door androgenen en de verhouding tussen de activine subunits en het activine-bindend eiwit follistatine was hoger in prostaatkanker vergeleken met normaal prostaatweefsel. Bovendien was deze verhouding omgekeerd geassocieerd met de metastase-vrije overleving in patiënten met prostaatkanker. Activine A vormt daarmee een intermediair tussen androgeendeprivatie en de stimulatie van *AKR1C3* welke leidt tot castratie-resistente prostaatkanker. Omwille van dit effect vormt activine A een nieuw doelwit voor de behandeling van castratie-resistente ziekte.

Tenslotte worden in **hoofdstuk 12** de resultaten beschreven in dit proefschrift bediscussieerd, alsmede de huidige en mogelijke toekomstige gevolgen van deze resultaten voor onze inzichten in de lokale regulatie van steroïdhormoon biosynthese.

LIST OF PUBLICATIONS

1 **J Hofland**, MA Timmerman, WW de Herder, RHN van Schaik, RR de Krijger & FH de Jong. Expression of activin and inhibin subunits, receptors and binding proteins in human adrenocortical neoplasms. *Clinical Endocrinology* (2006) **65**, 792-799

2 **J Hofland**, FH van Nederveen, MA Timmerman, E Korpershoek, WW de Herder, JW Lenders, AA Verhofstad, RR de Krijger & FH de Jong. Expression of activin and inhibin subunits, receptors and binding proteins in human pheochromocytomas, a study based on mRNA analysis and immunohistochemistry. *Clinical Endocrinology* (2007) **66**, 335-340

3 **J Hofland*,** WM van Weerden*, NF Dits, J Steenbergen, GJ van Leenders, G Jenster, FH Schröder & FH de Jong. Evidence of limited contributions for intratumoral steroidogenesis in prostate cancer. *Cancer Research* (2010) **70**, 1256-1264

4 W Chai*, **J Hofland***, PM Jansen, IM Garrelds, R de Vries, AJ van den Bogaerdt, RA Feelders, FH de Jong & AHJ Danser. Steroidogenesis vs. steroid uptake in the heart: do corticosteroids mediate effects via cardiac mineralocorticoid receptors? *Journal of Hypertension* (2010) **28**, 1044-1053

5 JW de Groot, TP Links, AP Themmen, LH Looijenga, RR de Krijger, PM van Koetsveld, **J Hofland**, G van den Berg, LJ Hofland & RA Feelders. Aberrant expression of multiple hormone receptors in adrenocorticotropin-independent macronodular adrenal hyperplasia causing Cushing's syndrome. *European Journal of Endocrinology* (2010) **163**, 293-399

6 **J Hofland** & FH de Jong. Inhibins and activins: roles in adrenal stem cells and the development of adrenocortical cancers. *Molecular and Cellular Endocrinology* (2011) Epub Jun 22

7 **J Hofland**, RA Feelders, R van der Wal, MN Kerstens, HR Haak, WW de Herder & FH de Jong. Serum inhibin pro-alphaC is a tumor marker for adrenocortical carcinomas. *European Journal of Endocrinology* (2011) **166,** 281-289

8 PM Jansen*, **J Hofland***, AH van den Meiracker, FH de Jong & AHJ Danser. No direct effect of renin and prorenin on aldosterone synthesis in the human adrenocortical cell lines H295R and HAC15. *Journal of the Renin-Angiotensin-Aldosterone System* (2012) Epub Mar 6

9 **J Hofland**, PJ Delhanty, J Steenbergen, LJ Hofland, PM van Koetsveld, FH van Nederveen, WW de Herder, RA Feelders & FH de Jong. Melanocortin 2 receptor associated protein (MRAP) and MRAP2 in various human adrenocortical tissues: regulation of expression and association with ACTH responsiveness. *Journal of Clinical Endocrinology and Metabolism* (2012) Epub Jan 25

10 **J Hofland**, WM van Weerden, J Steenbergen, NFJ Dits, G Jenster & FH de Jong. Activin A stimulates local testosterone production and growth in human prostate cancer through intracrine androgen conversion. *Pending major revisions*

11 J Kumagai, **J Hofland**, S Erkens-Schulze, NFJ Dits, J Steenbergen, G Jenster, CH Bangma, Y Homma, FH. de Jong & WM van Weerden. Induction of androgen receptor-regulated prostate cancer cell proliferation by testosterone precursors is mediated by the presence of steroidogenic enzymes and androgen receptor mutation. *Pending major revisions*

12 **J Hofland**, WW de Herder, L Derks, LJ Hofland, PM van Koetsveld, RR de Krijger, A Horvath, CA Stratakis, FH de Jong & RA Feelders. Regulation of steroidogenesis in a primary pigmented nodular adrenocortical disease-associated adenoma leading to virilization and subclinical Cushing's syndrome. *Submitted*

13 **J Hofland**, J Steenbergen, LJ Hofland, PM van Koetsveld, H Eijken, FH van Nederveen, G Kazemier, WW de Herder, RA Feelders & FH de Jong. Protein kinase C-induced activin A switches adrenocortical steroidogenesis to aldosterone by suppressing CYP17A1 expression. *Submitted*

14 GC Verwoert*, **J Hofland***, N Amina, FUS Mattace-Raso, EJG Sijbrands, JCM Witteman, CM van Duijn, FH de Jong & AHJ Danser. Expression and gene variation analyses deny association of human 3β-hydroxysteroid dehydrogenase type 1 gene (*HSD3B1*) with aldosterone production and blood pressure. *Manuscript in preparation*

15 **J Hofland**, LJ Hofland, PM van Koetsveld, J Steenbergen, WW de Herder, RR de Krijger, FH van Nederveen, MO van Aken, JWB de Groot, TP Links, FH de Jong & RA Feelders. In vivo and in vitro studies in ACTH-independent macronodular adrenocortical hyperplasia reveal prevalent aberrant responses to hormonal stimuli and possible coupling of arginine-vasopressin type 1 receptor to 11β-hydroxylase. *Manuscript in preparation*

16 **J Hofland**, J Steenbergen, JM Voorsluijs, MMPJ Verbiest, RR de Krijger, LJ Hofland, PM van Koetsveld, AG Uitterlinden, RA Feelders & FH de Jong. Methylation and common genetic variation in the inhibin alpha-subunit (*INHA*) promoter affects its expression in human adrenocortical carcinomas more than *INHA* mutations. *Manuscript in preparation*

17 M Rodenburg, **J Hofland**, C van Noord, LE Visser, A Dehghan, M Barbalic, AHJ Danser, A Hofman, J Witteman, E Boerwinkle, AG Uitterlinden, FH de Jong & BHC Stricker. Sex-specific differences in local androgen metabolism in the heart as indicator for the risk of myocardial infarction. *Manuscript in preparation*

18 PM van Koetsveld, G Vitale, RA Feelders, M Waaijers, DM Sprij-Mooij, RR de Krijger, EJM Speel, **J Hofland**, SWJ Lamberts, WW de Herder & LJ Hofland. Interferon-beta is a potent inhibitor of cell growth and cortisol production in vitro and sensitizes human adrenocortical carcinoma cells to mitotane. *Manuscript in preparation*

19 R van der Pas, LJ Hofland, J Steenbergen, **J Hofland**, FH de Jong, P van Koetsveld & RA Feelders. Fluconazole inhibits human adrenocortical steroidogenesis in vitro. *Manuscript in preparation*

* joint first authors

CURRICULUM VITAE

Hans Hofland was born on August 30th 1982 in The Hague, The Netherlands.

After obtaining his high school degree at the Vrijzinning Christelijk Lyceum in 2000, he studied Medicine at the Erasmus University Rotterdam. He passed his first year and fourth year exams both *cum laude*, followed by completing his medical degree *cum laude* at the end of 2006. From 2001 to 2006 he worked in the nursing staff at the Endocrinology ward of the Erasmus MC.

In 2004 he executed his graduation research project, entitled "Activin and inhibin in adrenal neoplasms", at the Endocrine Laboratory of the Department of Internal Medicine, Erasmus MC under the supervision of prof. dr. F.H. de Jong. For this research he was awarded the Gerrit-Jan Mulder prize for best graduation research at the Erasmus MC in 2005.

After obtaining his medical degree the author started his career as a resident in training to clinical researcher (AGIKO) with the currently presented PhD project at the section of Endocrinology of the Department of Internal Medicine at the Erasmus MC, under supervision of prof. dr. F.H. de Jong, prof. dr. W.W. de Herder and dr. R.A. Feelders. In the beginning of 2009 he commenced his specialisation in Internal Medicine at the Maasstad Hospital, Rotterdam, supervised by dr. M.A. van den Dorpel. In 2010 he returned to the Endocrine Laboratory of the Erasmus MC to complete his PhD project. During this project the author received three travel grants, one poster award and one abstract award and was involved as co-investigator in an awarded Erasmus MC Grant.

From Januari 1st 2011 he has resumed his residency Internal Medicine at the Maasstad Hospital in Rotterdam. In 2014 he will start his fellowship in Clinical Endocrinology at the Erasmus MC.

PHD PORTFOLIO

	Year	Hours
Research skills		
Course Molecular Diagnostics, Rotterdam	2007	24
Basic and Translational Endocrinology, Rotterdam	2007	60
Get out of your lab days, Rotterdam	2007	16
Classical Methods of Data-analysis (NIHES), Rotterdam	2007	150
Radioactivity safety course, Rotterdam	2007	10
ZonMw workshop on writing grant proposal, The Hague	2010	15
Clinical courses		
33e Erasmus Endocrinologie cursus, Noordwijkerhout	2007	28
Rotterdamse Internistendag	2008, 2011	16
Dutch Internal Medicine Days, Maastricht	2009	15
Advanced Life Support, Rotterdam	2009	10
Communicatie rondom donatie, Rotterdam	2009	5
Communicatie, Desiderius	2009	8
Video training on the job, Rotterdam	2011	15
Local presentations:		
3-weekly presentation at research group	2007-2008, 2010	120
Annual presentation at research department	2007-2008, 2010	60
Clinical presentations	2009, 2011	60
Oral presentations at meeting/conferences:		
Dutch Adrenal Network, Amsterdam	2007	20
International Congress of Endocrinology, Rio de Janeiro, Brazil	2008	45
International Congress of Endocrinology, Rio de Janeiro, Brazil	2008	45
Science Days Internal Medicine, Antwerp, Belgium	2009	27
NAI Regional Meeting Internal Medicine, Rotterdam	2010	15
Dutch Endocrine Meeting, Noordwijkerhout	2010	35
Regional Endocrinology Meeting, Rotterdam	2010	20
The 52nd Meeting of the Endocrine Society, San Diego, USA	2010	65
The European Congress of Endocrinology, Rotterdam	2011	65
Dutch Internal Medicine Days, Maastricht	2011	35
Regional Endocrinology Meeting, Rotterdam	2011	20
Dutch Endocrine Meeting, Noordwijkerhout	2012	35
Abstracts/posters at meeting/conferences:		
Molecular Medicine Day, Rotterdam	2007	17
Science Days Internal Medicine, Goes	2007	25
Molecular Medicine Day, Rotterdam	2008	17
Science Days Internal Medicine, Antwerp	2008	25
The 51th Meeting of the Endocrine Society, Washington DC, USA	2009	55
Dutch Internal Medicine Days, Maastricht	2010	25
Science Days Internal Medicine, Antwerp	2010	25
Adrenal 2010, San Diego, USA	2010	45
The European Congress of Endocrinology, Rotterdam	2011	15
Other meetings/conferences attended:		
Dutch Adrenal Network	2008, 2010	10

Symposium congenital adrenal hyperplasia, Nijmegen	2008	5
Opening Radboud Adrenal Center, Nijmegen	2010	5
Teaching activities:		
Junior Med school, 2008	2008	5
Workshop Molecular Techniques	2007	5
Workshop thyroid: basic	2007-2008, 2010	10
Workshop thyroid: clinical	2007-2008, 2010	15
Workshop adrenal	2007-2008, 2010	15
Lecture Endocrine Hypertension	2010	10
Supervision student research technician	2008	60
Supervision of medical students	2009, 2011	30
Total hours		**1458**
Total ECTS		**52**

DANKWOORD

Steroïdogenese is een proces dat bewerkstelligd wordt door een aantal kernenzymen en co-factoren en positief beïnvloed wordt vanuit velerlei andere signalen; hiermee is het vergelijkbaar met promoveren. Promotieonderzoek is ook een proces van samenwerking tussen verschillende factoren waarbij vanuit allerlei kanten hulp, ideeën en suggesties komen zodat uiteindelijk een mooi eindresultaat kan worden bereikt. Ik ben dan ook veel dank verschuldigd aan de vele mensen binnen Inhiberia (intracrien), de sectie Endocrinologie (paracrien) en daarbuiten (endocrien) die me geholpen hebben dit project succesvol af te ronden.

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En toen kwam er een olifant met een lange snuit en die blies het verhaaltje uit.