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# FUNCTIONAL AND DIAGNOSTIC ASPECTS ON ADRENOCORTICAL ADENOMA

Ulla Enberg

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**Karolinska  
Institutet**



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From Department of **Molecular Medicine and Surgery**,  
Karolinska Institutet, Stockholm, Sweden

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DIAGNOSTIC ASPECTS ON  
ADRENOCORTICAL  
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**In the memory of my father**

**To my children**



## ABSTRACT

Adrenocortical tumours are frequently detected due to increased use of imaging techniques like computed tomography, magnetic resonance imaging or ultrasound. The majority of the patients have tumours that do not have any overproduction of hormone. The functional adrenocortical adenomas are characterized by overproduction of one of the corticosteroids aldosterone, cortisol or androgen. The most common are aldosteronoma and cortisol producing adenoma. It is not possible to differentiate aldosterone and cortisol producing adenoma by histopathological examination. Thus, the clinical data and determination of steroid hormones in blood and urine form the basis to conclude if the extirpated tumour has been functional.

The aim of the study was to increase our knowledge of the pathophysiology of adrenocortical lesions.

Paper I. An *in situ* hybridisation method was developed to use radioactively labelled oligonucleotide probes on paraffin embedded tissue material to demonstrate the gene expression of the steroid synthesizing enzymes and thereby identify the steroid production of adrenocortical adenoma. *In vitro* release of aldosterone and cortisol from thin slices was compared to the mRNA expression of *CYP11B2*, *CYP11B1* and *CYP17*, which are coding for the specific enzymes needed for the aldosterone and cortisol synthesis. The results indicate that *CYP11B2* expression reflects aldosterone production in the tumour, while *CYP17* and *CYP11B1* reflects cortisol production.

Paper II. The most common forms of primary aldosteronism are unilateral adenoma and bilateral hyperplasia. To attain an optimal treatment there is a demand for thorough functional characterization since adrenalectomy often cures patients with adenoma while patients with hyperplasia are treated medically. To improve the subclassification of primary aldosteronism 27 operated patients were evaluated. *CYP11B2* expression was found in an adenoma from 22 patients. Fourteen of these had additional *CYP11B2* expression in zona glomerulosa. All these 22 patients were cured from hyperaldosteronism. Three patients, who were not cured, had probably bilateral disease. Two adrenal adenomas had no *CYP11B2* expression, but the patients were cured. These results contribute to entirely new possibilities to, postoperatively, determine a correct subclassification of patients with primary aldosteronism.

Paper III. The value of adrenal scintigraphy in lateralisation of aldosterone producing adrenocortical adenoma was investigated in 33 patients, who were evaluated according to cure and compared to the scintigraphic pattern. Twenty-seven patients had scintigraphic images showing a unilateral uptake or a bilateral asymmetric uptake indicating unilateral aldosterone overproduction. Twenty-two of these patients were cured, 3 patients were improved and 2 patients were not cured. Six patients had no uptake at scintigraphy. Adrenal scintigraphy at primary aldosteronism may be a useful complement at the preoperative lateralisation.

Paper IV. The majority of adrenocortical incidentaloma are non-hyperfunctioning adrenal adenoma. Some of them may have autonomous cortisol production not fully restrained by the hypothalamus-pituitary-adrenal-axis. The median ratio of *CYP17* to *CYP11B1* expression for tumours from patients with Cushing's syndrome was significantly higher than the median ratio for the non-hyperfunctioning tumours. In patients with subclinical Cushing's syndrome the tumours have a similar high ratio indicating that these patients may be identified with this method.

# LIST OF PUBLICATIONS

Papers in this thesis will be referred to by their Roman numerals I – IV.

- I. **Enberg U**, Farnebo LO, Wedell A, Gröndal S, Thorén M, Grimelius L, Kjellman M, Bäckdahl M & Hamberger B.  
In vitro release of aldosterone and cortisol in human adrenal adenomas correlates to mRNA expression of steroidogenic enzymes for genes *CYP11B2* and *CYP17*.  
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- II. **Enberg U**, Volpe C, Höög A, Wedell A, Farnebo LO, Thorén M & Hamberger B.  
Postoperative differentiation between unilateral adrenal adenoma and bilateral adrenal hyperplasia in primary aldosteronism by mRNA expression of the gene *CYP11B2*.  
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- III. Volpe C, **Enberg U**, Sjögren A, Wahrenberg H, Jacobsson H, Hamberger B, & Thorén M.  
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Increased ratio of mRNA expression of the genes *CYP17* and *CYP11B1* indicates autonomous cortisol production in adrenocortical tumours.  
Submitted.

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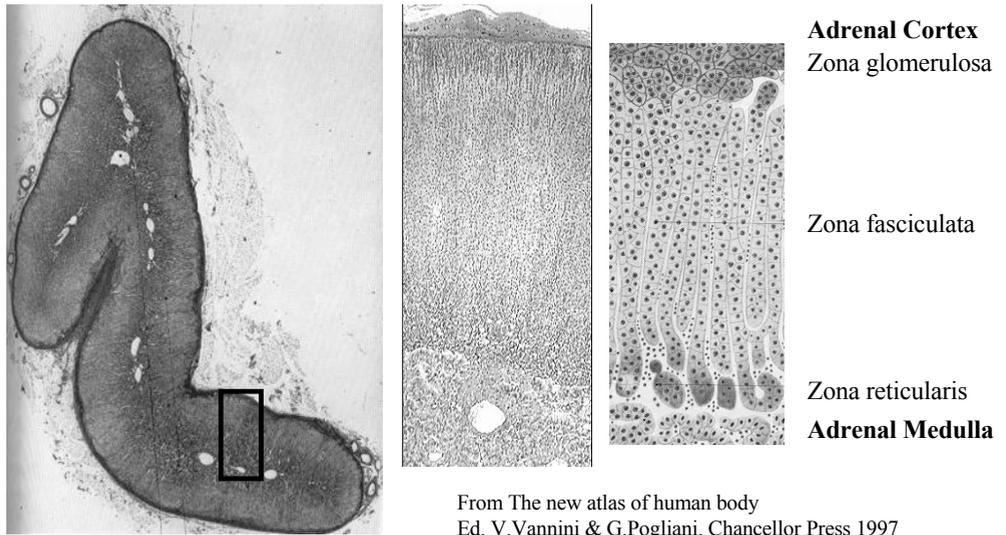
## LIST OF ABBREVIATIONS

|                 |  |
|-----------------|--|
| <i>CYP11B2</i>  | The gene encoding aldosterone synthase   |
| <i>CYP11B1</i>  | The gene encoding 11 $\beta$ -hydroxylase  |
| <i>CYP17</i>    | The gene encoding 17 $\alpha$ -hydroxylase   |
| <i>CYP21</i>    | The gene encoding 21-hydroxylase   |
| <i>GAPDH</i>    | The gene encoding glutaraldehyde-3-phosphate dehydrogenase   |
| ssc             | Cholesterol side-chain cleavage enzyme   |
| 3 $\beta$ -HSD  | 3 $\beta$ -hydroxysteroid dehydrogenase  |
| 21h             | 21-hydroxylase enzyme  |
| 17 $\alpha$ h   | 17 $\alpha$ -hydroxylase enzyme  |
| 11 $\beta$ h    | 11 $\beta$ -hydroxylase enzyme   |
| 18h             | 18-hydroxylase enzyme  |
| 18ox            | 18-oxylase enzyme  |
| 11 $\beta$ HSD2 | 11 $\beta$ -hydroxysteroid dehydrogenase 2 enzyme  |
| ACTH            | Adrenocorticotopic hormone   |
| GR              | Glucocorticoid receptor  |
| MR              | Mineralocorticoid receptor   |
| G-protein       | Guanine binding protein  |
| DNA             | Deoxyribonucleic Acid  |
| RNA             | Ribonucleic Acid   |
| mRNA            | Messenger Ribonucleic Acid   |
| cRNA            | Single stranded Ribonucleic Acid   |
| PA              | Primary aldosteronism  |
| FH I            | Familial hyperaldosteronism type I   |
| FH II           | Familial hyperaldosteronism type II  |
| PRA             | Plasma renin activity  |
| NHF             | Non-hyperfunctioning adenoma   |
| Cushing         | Cushing's syndrome   |
| SCS             | Subclinical Cushing's syndrome   |
| DAX-1           | Dosage sensitive sex reversal-Adrenal hypoplasia congenital, critical region on the X chromosome-1 |
| COUP-TF         | Chicken Ovalbumin Upstream Promoter-Transcription Factor   |

# 1 INTRODUCTION

## 1.1 ADRENAL

The adrenal glands (Fig. 1) are located on the surfaces of each kidney and are composed of two separate endocrine organs secreting specific hormones and regulated by different mechanisms. The outer portion, the adrenal cortex is of mesodermal origin and produces corticosteroids, which are essential for survival. The inner portion, the adrenal medulla secretes catecholamines, is of neuroectodermal origin, and is considered to be a modified sympathetic ganglion.



From The new atlas of human body  
Ed. V.Vannini & G.Pogliani, Chancellor Press 1997

**Figure 1. Adrenal gland.** The adrenal gland has two functionally different parts the adrenal cortex and medulla. Adrenal cortex comprises three distinct layers, zona glomerulosa, which produces aldosterone, and zonae fasciculata and reticularis both synthesizing cortisol. Zona reticularis also produces androgens.

### 1.1.1 Adrenal cortex

During fetal development the adrenal cortex, derived from primitive mesoderm, arises by cell proliferation from the urogenital ridge. Later in gestation the cortex forms two distinct zones, a centrally located fetal zone and an outer rim of definitive cortex, which later forms the adult adrenal cortex. The proliferation is confined to the definitive cortex, suggesting that the same cells give rise to both the fetal and the definitive zones. Growth of the adrenal cortex during fetal development is dependent on pituitary stimulation by ACTH. After birth, the fetal zone involutes and the definitive cortex evolves into three zones of the adult adrenal cortex [1].

### 1.1.2 Functional zonation

The adrenal cortex consists of three distinct functional zones, zonae glomerulosa, fasciculata and reticularis. The outer layer, zona glomerulosa produces the mineralocorticoid aldosterone, while the inner zones, zonae fasciculata and reticularis both have the capacity to synthesize the glucocorticoid cortisol and androgens.

### 1.1.3 Morphology of adrenal cortex

The adrenal is surrounded by a fibrous capsule and beneath are the cells of zona glomerulosa in rounded clusters. The cells in zona fasciculata with abundant cytoplasm contain lipid droplets and are larger than the cells of the other zones. The cells in zona fasciculata are arranged in longitudinal columns stretching between the zona glomerulosa and zona reticularis being the widest layer of adrenal cortex. The thin layer of zona reticularis is composed of irregularly arranged network of cells

## 1.2 HISTORY

The earliest found notation of the adrenal gland is Bartolomeo Eustacchio's 1563 copper-etched depiction of "glandulae Renibus incumentes" [2].

### 1.2.1 Addison's disease – Corticosteroid deficiency

Thomas Addison published in 1849 his description of a lethal clinical syndrome resulting from adrenocortical insufficiency [3] later known as Addison's disease. Symptoms gradually developing in patients with Addison's disease are fatigue, reduced weight, hyperpigmentation, low blood pressure, orthostatism, salt hunger, diarrhea, high fever and finally death.

### 1.2.2 Cushing's syndrome – Cortisol overproduction

During the early 20<sup>th</sup> century the adrenocortical influence on carbohydrate metabolism was investigated. Later the glucocorticoids synthesized by the adrenal cortex were isolated and their structures characterized [4]. Dr Harvey Cushing described a syndrome with symptoms and signs caused by excess glucocorticoids mainly on glucose metabolism and cardiovascular function[5].

### 1.2.3 Conn's syndrome - Aldosterone overproduction

In 1953 Simpson and Tait reported a bioassay with high sensitivity for mineralocorticoid activity [6] and one year later the structure of aldosterone were reported [7]. The same year Jerome W. Conn reported in his presidential address to the Central Society for Clinical Research in Chicago, Illinois of a new clinical syndrome, which he called primary aldosteronism [8]. He described a woman with a history of intermittent muscle spasms, weakness, and paralysis, increased blood pressure, low serum potassium and an alkaline serum. Conn's diagnostic interpretation was that the combination of metabolic alkalosis with hypokalemia and tetany suggested excessive activity of the salt retaining corticoid aldosterone. Conn and a surgeon explored the patient's adrenal glands and found an adrenal tumour and removal reversed the syndrome [9].

## 1.3 ALDOSTERONE

Steroids produced by the adrenal cortex include mineralocorticoids, glucocorticoids and androgens. Aldosterone, the main mineralocorticoid, is synthesized in zona glomerulosa, while zonae fasciculata and reticularis produce the glucocorticoid cortisol. Androgens are mainly produced in zona reticularis.

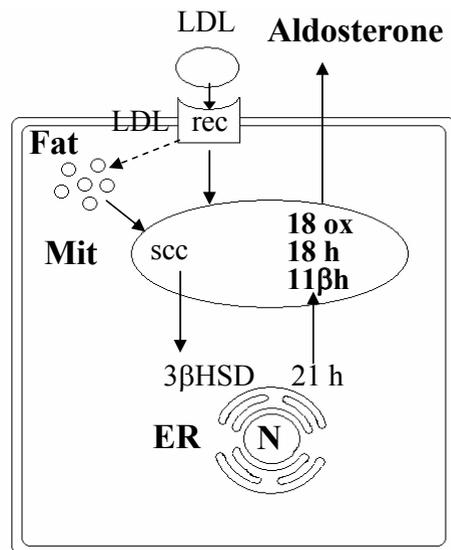
### **Aldosterone synthesis**

The steroid synthesis comprises several enzymatic steps (Fig.2 and 3). The substrate for steroids is cholesterol, which is available either 1) as cholesterol esters in the lipid droplets of the cellular cytoplasm, 2) as low-density lipoprotein particles, which bind to low-density lipoprotein receptors in the plasma membrane and are subsequently taken up by endocytosis or 3) as cholesterol synthesized *de novo* from acetate. Cholesterol is transported to the inner mitochondrial membrane where cleavage by side chain cleavage enzyme to pregnenolone takes place. Pregnenolone leaves the mitochondria and enters the smooth endoplasmic reticulum. From here the synthetic pathway depends on the enzymes expressed by the adrenocortical cells.

### **Aldosterone synthesis**

**Cholesterol**  
**scc**  
 Pregnenolone  
**3 $\beta$ HSD**  
 Progesterone  
**21h (CYP21)**  
 11-Deoxycorticosterone  
**11 $\beta$ h (CYP11B2)**  
 Corticosterone  
**18h (CYP11B2)**  
 18-Hydroxycorticosterone  
**18ox (CYP11B2)**  
**Aldosterone**

### **Cell in zona glomerulosa**



**Figure 2. Aldosterone synthesis.** The final enzymatic steps in the aldosterone synthesis is 11 $\beta$ -hydroxylase, 18-hydroxylase and 18-oxydase all encoded by the *CYP11B2* gene. scc, side chain cleavage enzyme; 3 $\beta$ HSD, 3 $\beta$ -hydroxy steroide dehydrogenase; 21h, 21-hydroxylase; 11 $\beta$ h, 11 $\beta$ -hydroxylase; 18h, 18-hydroxylase; 18ox, 18-oxydase; LDL rec, low-density lipid protein receptor; Mit, mitochondria; ER, endoplasmic reticulum; N, nucleus.

In the aldosterone synthesis (Fig.2) the successive enzymatic steps in endoplasmic reticulum are 3 $\beta$ -hydroxysteroid dehydrogenase to yield progesterone and 21-hydroxylase encoded by the *CYP21* gene to yield 11-deoxycorticosterone. In order to be converted to aldosterone, 11-deoxycorticosterone has to be transferred back to the inner mitochondrial membrane for the final three enzymatic steps, 11 $\beta$ -hydroxylation, 18-hydroxylation and 18-oxydation encoded by a single gene *CYP11B2*. There is no expression of this gene in the mitochondria of zonae fasciculata and reticularis and therefore these cells cannot synthesize aldosterone.

### **Regulation of aldosterone synthesis**

Angiotensin II and serum potassium concentration mainly regulate aldosterone production in zona glomerulosa. Angiotensin II is formed by proteolysis of angiotensinogen produced by the liver. Renin secreted from the juxta glomerular cells in the kidneys cleaves angiotensinogen to the intermediate Angiotensin I. Cleavage of Angiotensin I to Angiotensin II by the angiotensin converting enzyme (ACE) is taking place in the pulmonary circulation. Angiotensin II type 1 receptors in the cell membrane of zona glomerulosa cells, which bind Angiotensin II, are guanine binding protein coupled receptors that activate protein kinase C generating inositol triphosphate and 1,2 diacylglycerol. The inositol triphosphate mobilizes intracellular calcium in turn stimulating aldosterone synthesis. Serum potassium concentration has a direct stimulating effect in glomerulosa cells by increasing intracellular calcium resulting in stimulated aldosterone synthesis. Aldosterone in turn inhibits renin secretion in the kidneys. ACTH secreted by the anterior pituitary has a minor effect on aldosterone synthesis.

### **Mineralocorticoid receptor**

Steroid hormones are fat-soluble and pass through the cellular membrane and bind to their intra cellular receptors. The hormone-receptor protein complex is transported to the nucleus and binds to DNA binding sites stimulating increased transcription of messenger ribonucleic acid followed by subsequent translation of proteins resulting in the cell response.

The mineralocorticoid receptors are found in both epithelial tissues, e.g. kidney and colon, and non-epithelial tissues, e.g. heart and brain. The mineralocorticoid receptor has similar affinity for aldosterone, cortisol and corticosterone. In the epithelium of the kidney distal tubules,  $11\beta$ -hydroxy steroid dehydrogenase 2 ( $11\beta$ HSD2) reduces the receptor stimulation from cortisol by converting cortisol to inactive cortisone allowing aldosterone to activate mineralocorticoid receptors more selectively [10]. Deficiency of  $11\beta$ HSD2 leads to apparent mineralocorticoid excess resulting in cortisol-dependent mineralocorticoid receptor activation resembling aldosterone stimulation causing hypertension and hypokalemia [11]. Prolonged ingestion of licorice which contains glycyrrhetic acid inhibiting  $11\beta$ HSD2, likewise mimicks mineralocorticoid excess causing hypertension [12]. In non-epithelial tissue mineralocorticoid receptors act as glucocorticoid receptors.

## **1.4 PRIMARY ALDOSTERONISM (CONN'S SYNDROME)**

The classical characteristics of primary aldosteronism, which Conn presented [8], are hypertension, hypokalemia and suppressed renin activity due to elevated aldosterone secretion, but Conn also proposed that even normokalemic patients might be present. The patients are often asymptomatic, but symptoms due to hypertension, renal potassium loss and tubular resistance to antidiuretic hormone may present as headache, muscle weakness, polyuria and polydipsia. It is important to diagnose primary aldosteronism in the hypertensive population and provide adequate treatment because elevated aldosterone has negative effects on vascular system and heart which are independent of hypertension increasing the risk to develop left ventricular hypertrophy, chronic kidney disease and endothelial dysfunction [13].

**Table 1. Subtypes of primary aldosteronism.**

| Subtypes of primary aldosteronism   |   |
|---|---|
| Common forms  |   |
| <p><b>Bilateral adrenal hyperplasia</b><br/>50 – 60% of primary aldosteronism</p> | <p><b>Unilateral adrenal adenoma</b><br/>35 –40% of primary aldosteronism</p>   |
| Uncommon forms  |   |
| <p><b>Unilateral adrenal hyperplasia</b><br/>&lt;1% of primary aldosteronism</p>  | <p><b>Familial hyperaldosteronism type I</b><br/>&lt;1% of primary aldosteronism</p>                                      |
| <p><b>Adrenocortical cancer</b><br/>&lt;1% of primary aldosteronism</p>           | <p><b>Familial hyperaldosteronism type II</b><br/>Familial form of APA and/or BAH<br/>&lt;1% of primary aldosteronism</p> |

Primary aldosteronism should be suspected particular in young patients, refractory hypertension, hypokalemia and incidentally detected adrenal mass. Screening for primary aldosteronism with aldosterone to renin ratio was first described by Hiramatsu in 1981 [14]. The screening test is performed on patients after correction for hypokalemia and cessation of interfering medications, e.g. spironolactone, amiloride or  $\beta$ -blockers. In patients, who have increased aldosterone to renin ratio, the absence of aldosterone suppression following saline infusion, oral salt loading or fludrocortisone administration has to be demonstrated to confirm primary aldosteronism.

Initially, the prevalence of primary aldosteronism was considered to be 0.5 – 2% although Conn suggested that it could be as high as 20%, which was strongly opposed at that time. We now know that primary aldosteronism might constitute 5% to 13% among hypertensive patients, thus being the most common form of secondary hypertension [15, 16]. In addition, most patients with primary aldosteronism are normokalemic [17].

After confirmation of the diagnosis subclassification is the next step (Table 1). Primary aldosteronism is caused by autonomous aldosterone secretion either from a unilateral aldosterone producing adenoma or nodular or diffuse hyperplasia of zona glomerulosa in the adrenal cortex. There are also familial variants caused by genetic alterations affecting aldosterone synthesis, familial hyperaldosteronism type I and type II.

Subclassification includes biochemical evaluation, additional examination by imaging techniques and scintigraphy or adrenal venous sampling to determine location of the autonomous aldosterone production for decision of optimal treatment. Adrenocortical adenoma and unilateral hyperplasia are treated by adrenalectomy, while bilateral hyperplasia is primarily treated medically with aldosterone antagonists.

### 1.4.1 Bilateral adrenal hyperplasia

Recently, after the introduction of the screening test for primary aldosteronism, bilateral adrenal hyperplasia is the most common form of primary aldosteronism, about 60% of the patients. Although plasma renin levels are low the aldosterone production can be influenced by up right posture (renin-angiotensin) resembling the response of normal subjects. Medical therapy with aldosterone antagonists e.g. spironolactone or eplerenone is usually effective.

### 1.4.2 Unilateral aldosterone producing adenoma

About 30% of patients with the diagnosis of primary aldosteronism have unilateral aldosterone producing adenoma. There are two described entities of aldosterone producing adenoma exhibiting varying morphology associated with varying responses of aldosterone to angiotensin II. Tumours with predominantly fasciculata-like cells are unresponsive to angiotensin II, whereas those with predominantly glomerulosa-like cells are responsive to angiotensin II [18]. Patients with aldosterone producing adenoma have more severe hypertension, more profound hypokalemia, higher plasma and urinary aldosterone levels than patients with bilateral adrenal hyperplasia [19]. Adrenalectomy is the treatment for patients with aldosteronoma.

### 1.4.3 Unilateral adrenal hyperplasia

Less than 50 cases have been described since Ross in 1965 reported the first case of unilateral adrenal hyperplasia [20]. Thirty of these patients are reviewed by Goh and colleagues [21]. All patients were hypertensive and most of them were hypokalemic. Ten out of 13 patients had a decrease or no change in the plasma aldosterone levels in postural test suggesting a unilateral source of hyperaldosteronism. At a median follow up of 12 months after unilateral adrenalectomy about half of the patients were completely cured of their hypertension and the remaining had improved control. All patients were cured of hypokalemia. Recently, there was a report of cases with unilateral hyperaldosteronism due to multiple adrenocortical micronodules. They all had unilateral hypersecretion of aldosterone by selective adrenal venous sampling and multiple micronodules without an adenoma. These patients had hypertension and suppressed renin and were normokalemic. Postural test failed to increase plasma renin activity [22].

Unilateral adrenal hyperplasia is a rare surgical correctable cause of primary aldosteronism [22, 23]. The existence of unilateral adrenal hyperplasia as a unique pathologic entity is controversial, but outcome of adrenalectomy is favourable. However, extended follow up of operated patients, which could verify the true existence of unilateral hyperplasia is missing.

### 1.4.4 Familial hyperaldosteronism type I and type II (FH I, FH II)

Familial hyperaldosteronism type I (also called Glucocorticoid remediable hyperaldosteronism) is a rare disease with autosomal dominant inheritance, accounting for about 0.5 - 1% of primary aldosteronism [24]. A hybrid gene is formed by fusion of the ACTH-responsive regulatory sequences of *CYP11B1* gene to coding sequences of *CYP11B2* gene presumably generated by unequal meiotic crossing-over [25]. This

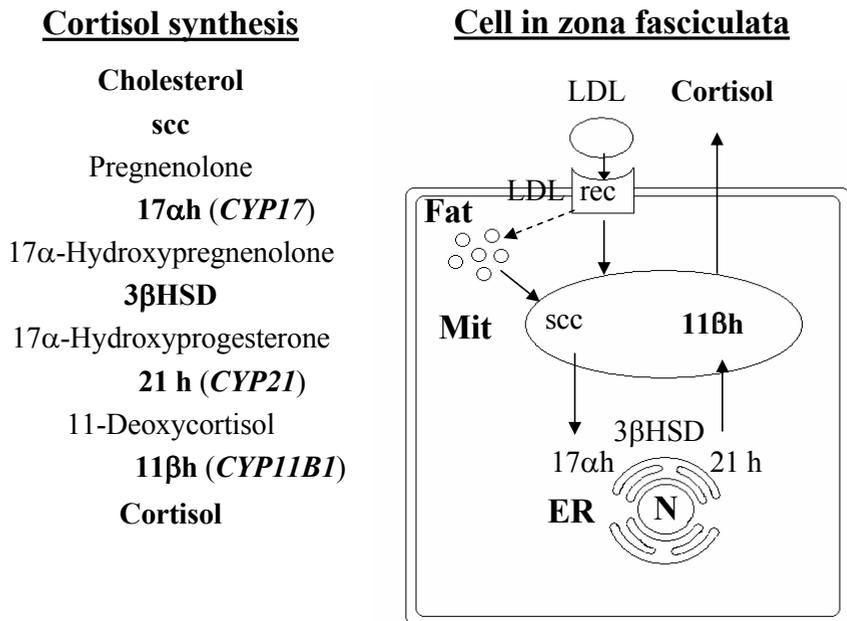
results in ectopic expression of aldosterone synthase activity in the adrenal zona fasciculata under control of ACTH [26]. Thus, dexamethasone treatment inhibiting ACTH stimulation of adrenal cortex is the treatment of choice for familial hyperaldosteronism type I. The diagnosis is confirmed by a genetic test detecting the hybrid gene [27].

Familial hyperaldosteronism type II is a familial variant of primary aldosteronism caused by aldosterone producing adenoma or bilateral hyperplasia or both. Familial hyperaldosteronism type II is clinically, biochemically and morphologically indistinguishable from apparently non-familial primary aldosteronism. It is frequently transmitted in an autosomal dominant fashion and is unlike familial hyperaldosteronism type I not glucocorticoid remediable [28-31].

## 1.5 CORTISOL

### Cortisol synthesis

The initiating step for the synthesis of all steroids is cleavage of cholesterol by side chain cleavage enzyme to pregnenolone (Fig.3). In the cortisol pathway the conversion of pregnenolone to 17 $\alpha$ -hydroxypregnenolone, 17 $\alpha$ -hydroxyprogesterone and 11-deoxycortisol takes place in the endoplasmatic reticulum by 17 $\alpha$ -hydroxylase (encoded by *CYP17*), 3 $\beta$ -hydroxysteroid dehydrogenase and 21-hydroxylase activity (encoded by *CYP21*), respectively.



**Figure 3. Cortisol synthesis.** The most important steps in cortisol synthesis is 17 $\alpha$ -hydroxylase encoded by the gene *CYP17* and the final step 11 $\beta$ -hydroxylase encoded by *CYP11B1* gene. scc, side chain cleavage enzyme; 17 $\alpha$ h, 17 $\alpha$ -hydroxylase; 3 $\beta$ HSD, 3 $\beta$ -hydroxy steroide dehydrogenase; 21h, 21-hydroxylase; 11 $\beta$ h, 11 $\beta$ -hydroxylase; LDL rec, low-density lipid protein receptor; Mit, mitochondria; ER, endoplasmatic reticulum; N, nucleus.

The final step in cortisol synthesis is the conversion of 11-deoxycortisol to yield cortisol by 11 $\beta$ -hydroxylase (encoded by *CYP11B1* gene) which occurs in the inner mitochondrial membrane. Zona glomerulosa cells lack the 17 $\alpha$ -hydroxylase enzyme activity. Thus, synthesis of cortisol occurs only in zonae fasciculata and reticularis.

### Regulation of cortisol synthesis

Corticotrophin-releasing hormone from the hypothalamus stimulates ACTH, which in turn regulates cortisol and androgen synthesis in zonae fasciculata and reticularis. Circulating ACTH binds to membrane receptors of adrenocortical cells inducing an intra-cellular increase in cyclic adenosine monophosphate through G-proteins and adenylate cyclase activation. Cyclic adenosine monophosphate activates protein kinase A, leading to calcium influx, modulation of protein kinase activity and subsequent cortisol production [4]. Cortisol secretion exerts a feedback inhibition on both corticotropine releasing hormone in hypothalamus and ACTH in the pituitary gland.

### Glucocorticoid receptor (GR)

Almost all cells in the human body have glucocorticoid receptors explaining the wide variation of cortisol effects on the body.

### Effector organs of cortisol

Glucocorticoids have multiple effects involving glucose metabolism, cardiovascular function, brain function with increased appetite, increased catabolism of protein especially in skeletal muscle and connective tissue, suppression of immunity and inflammation, and inhibition of osteoblast function [32].

## 1.6 CUSHING'S SYNDROME

Most cases of Cushing's syndrome are iatrogenic, caused by pharmacological treatment with glucocorticoids.

**Table 2. Endogenous Cushing's syndrome.**

### Causes of endogenous Cushing's syndrome

| <b>Etiology</b>                                      | <b>Approximate prevalence</b> |
|--|-------------------------------|
| <b>ACTH-dependent</b>                                |                               |
| ACTH-secreting pituitary adenoma (Cushing's disease) | 70%                           |
| Ectopic ACTH secretion                               | 15%                           |
| Ectopic CRH secretion                                | Very rare                     |
| <b>ACTH-independent</b>                              |                               |
| Adrenal adenoma                                      | 10%                           |
| Adrenal carcinoma                                    | 5%                            |
| Bilateral micronodular hyperplasia                   | Rare                          |
| Macronodular hyperplasia                             | Very rare                     |

From: Clutter, 2001 [32].

Endogenous Cushing's syndrome (Table 2) is caused by excess glucocorticoid secretion either due to a pituitary adenoma (Cushing's disease), a cortisol producing adrenal adenoma or an ectopic ACTH or CRH producing tumour. These patients lack the normal negative feedback between ACTH and cortisol secretion as well as a normal diurnal rhythm of cortisol production. Patients with Cushing's disease retain a partial ACTH dependency whereas the cortisol hypersecretion in adrenocortical or ectopic tumours is ACTH independent. After suspicion of the disorder based on symptoms such as easy bruising, thin skin and proximal muscle weakness the diagnosis of the syndrome is confirmed by hypersecretion of cortisol, reduced or absent suppression of cortisol by dexamethasone suppression and loss of normal diurnal variation of plasma cortisol levels [32].

## **1.7 NON-HYPERFUNCTIONING ADRENAL TUMOUR**

The vast majority of masses found on computed tomography, magnetic resonance imaging or ultrasound are non-hyperfunctioning tumours, i.e. they do not have any excessive hormone production causing symptoms. Some patients with non-hyperfunctioning adrenal adenoma, which have a subtle cortisol production not fully restrained by feedback from the hypothalamic-pituitary-adrenal axes [33-35], have subclinical Cushing's syndrome. They seldom develop overt Cushing's syndrome [36] but they have signs and symptoms of metabolic syndrome with higher prevalence of obesity, hypertension, insulin resistance and/or type 2 diabetes than matched controls or patients with non-hyperfunctioning tumours [35, 37].

## **1.8 MOLECULAR BIOLOGY**

### **1.8.1 DNA– mRNA – Protein**

The chromosomes in the cell nucleus are composed of two complementary DNA strands. Genes are parts of the DNA actively duplicated for further transcription to mRNA and successive translation of protein determining the cell function. mRNA *in situ* hybridisation visualizes mRNA occurrence in the cell but not the protein produced by the cell. At translation the protein synthesized are beginning with the amino acid methionine, while one or several amino-terminal amino acids are frequently cleaved away by proteolytic enzymes to produce functional polypeptide products that have different amino-terminal amino acids from those of the primary translation products. Therefore, the mRNA does not necessarily reflect the true protein produced by the cell.

## 2 AIMS

In the present study the general aim was to increase our knowledge on the pathophysiology of adrenocortical tumours.

The specific aims were

to develop a method to determine the mRNA expression of genes coding for steroidogenic enzymes to identify the steroid production in adrenocortical tumours

to improve the subclassification of patients with primary aldosteronism through evaluation pre and post adrenalectomy combined with long term follow up

to evaluate norcholesterol scintigraphy for the preoperative localization of aldosterone hypersecretion

to characterize tumours with and without cortisol hypersecretion, including tumours causing subclinical Cushing's syndrome

## 3 MATERIAL AND METHODS

### 3.1 PATIENTS

The patients with primary aldosteronism were diagnosed based on the preoperative endocrinological evaluation. They had the classical signs of primary aldosteronism hypertension, hypokalemia (III 80%), suppressed renin activity and excessive aldosterone secretion. Patients with primary aldosteronism were included in paper I – III. In paper I and IV were patients with Cushing's syndrome and non-hyperfunctioning tumours. Controls were patients operated for renal carcinoma (I, II, IV) and pheochromocytoma (I). Two patients were included in paper I and II. Seventeen patients in paper II were referred to in paper III.

### 3.2 METHODS

#### 3.2.1 Patient records

Patient records were thoroughly evaluated and follow up was performed including blood pressure, medication and measurement of sodium, potassium, aldosterone and renin.

#### 3.2.2 *In vitro* steroid release

Thin tissue slices (<0.5 mm) of the adrenocortical tumours were incubated in a Krebs-Ringer bicarbonate medium with glucose, ascorbic acid, and 1% bovine serum albumin. Aldosterone and cortisol in the medium after 1 hour incubation were measured with commercially available radioimmunoassays and the release (nmol/g tissue) was calculated as previously described [38].

#### 3.2.3 *In situ* hybridization

In short (for details see paper II):

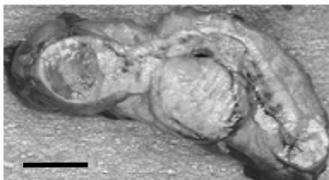
##### 3.2.3.1 *Probe preparation*

Oligonucleotide probes with sequences complementary to mRNAs encoding for the synthesizing enzymes 11 $\beta$ -hydroxylase (*CYP11B1*), 18-hydroxylase (*CYP11B2*), 17 $\alpha$ -hydroxylase (*CYP17*) and glutaraldehyde-3-phosphate dehydrogenase (*GAPDH*) were synthesized. The *GAPDH* probe was used to ascertain the presence of tissue mRNA. A sense probe identical with a sequence of the *CYP17* gene (negative control) was synthesized. The oligonucleotides were 3'prim-end-labelled with a [<sup>35</sup>S]-poly A tail, using terminal deoxynucleotidyl transferase. This procedure generated probes to a specific activity of 2.7–6.3x10<sup>5</sup> c.p.m./ $\mu$ l.

##### 3.2.3.2 *Hybridisation (Fig.4)*

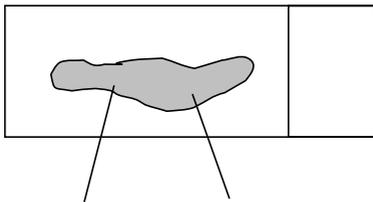
Paraffin-embedded tissue specimens were cut in sections and mounted on slides. The sections were deparaffinized, rehydrated through graded ethanol and treated with Proteinase K in phosphate-buffered saline. Slides were then acetylated in triethanolamine buffer, containing acetic anhydride, washed in sodium citrate buffer, dehydrated through graded ethanol solutions and air-dried. Prior to hybridization, the sections were heated to 60°C for 30 minutes. Hybridization solution, containing

deionised formamide, sodium citrate buffer, Tris-HCl, Denhart's solution, EDTA, dextran sulfate, and RNA from torula yeast, was mixed with denatured salmon sperm DNA, dithiothreitol and 5 ng/ml  $^{35}\text{S}$ -probe or 10 ng/ml for *CYP11B2* probe (II). The mixture was preheated to 42°C for 30 minutes, spread on top of the sections, covered, and put in a humidified chamber for hybridisation at 42°C overnight. The slides were rinsed in sodium citrate buffer, dehydrated through graded ethanol solutions, and air-dried before autoradiography. The slides were exposed to autoradiographic film. After film exposure, the slides were dipped in autoradiographic emulsion, exposed, subsequently developed, and stained with hematoxylin and eosin. Quantification of the autoradiographic film was done by microdensitometry, as previously described [39] and the expression visualized as silver grains were evaluated in both light and dark field microscope.



### Adrenal with tumours

Bar 5 mm



### Tissue section 5 $\mu\text{m}$ on slide

UGCCCUCUGGA

|||  
ACGGGAGACCT- A\*A\*A\*

### mRNA in the tissue

$^{35}\text{S}$ -labelled DNA-probe

**Figure 4. mRNA *in situ* hybridization of adrenal tissue.** *In situ* hybridisation is a reaction where single stranded mRNA sequences in the tissue and a complimentary labelled probe are united to a double stranded hybrid. This hybrid is visualized by autoradiography. *In situ* hybridisation demonstrates the morphological distribution of specific mRNA sequences in the tissue.

### 3.2.4 Statistical methods

Values are presented as mean  $\pm$  S.E.M. ( I - II ), median and range ( III - IV ).

Correlation between groups were carried out with Spearman Rank Order correlations ( I ) and comparison between groups with Mann-Whitney U test ( II - IV ). Two by two table evaluation of diagnostic power and sensitivity, specificity, positive predicted value, negative predicted value and accuracy with corresponding 95% binominal confidence intervals were calculated in paper III.

### 3.2.5 Ethics

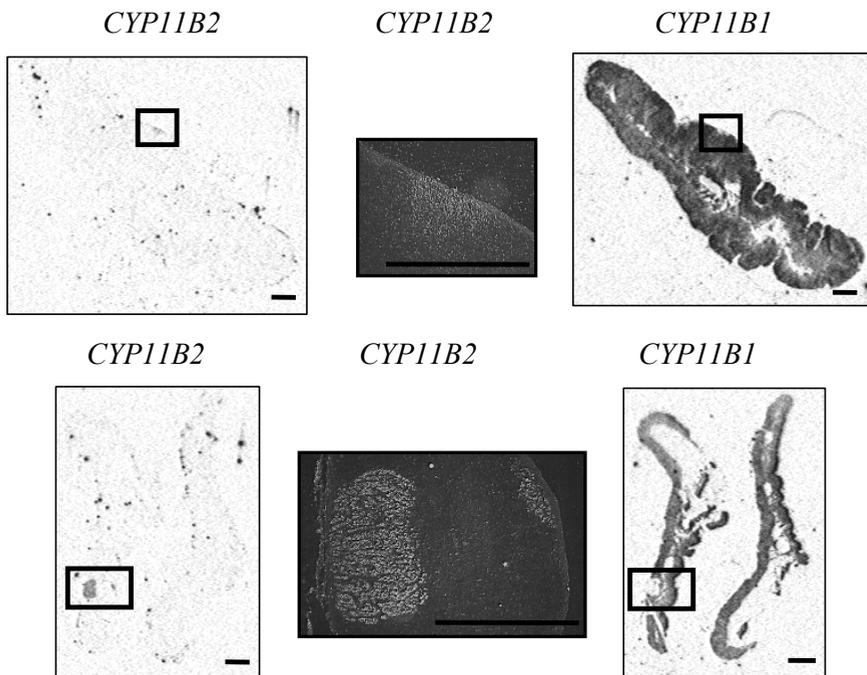
The Medical Ethics Committee of the Karolinska Institute approved the present studies.

## 4 RESULTS AND DISCUSSION

### 4.1 METHODOLOGY (I, II, IV)

In the present study the *in situ* hybridisation technique was developed to visualize the mRNA expression of steroidogenic enzyme genes with oligo nucleotide probes on routinely embedded paraffin sections. The oligo nucleotide probes are normally used on frozen sections, but the frozen material had too poor morphology for differentiation of cells in the separate layers of adrenal cortex. This separation was essential for verification of probe specificity.

The expression of *CYP11B2* should be present in zona glomerulosa only, because glomerulosa cells have the synthetic enzymes needed to produce aldosterone. Furthermore, *CYP11B1* and *CYP17* expression coding for cortisol synthetic enzymes should be found in zonae fasciculata and reticularis in normal adrenal cortex. In addition, carefully chosen oligo nucleotide probes can be more specific than the cRNA probes usually utilized on paraffin sections (see below).



**Figure 5. Normal adrenal controls.** Autoradiograms (left and right) of *in situ* hybridisation in two normal adrenal controls (patients operated for renal carcinoma). Dark-field microscopic magnified picture (middle row). *CYP11B2* gene expression is seen in few zona glomerulosa cells in one of the adrenals (top), while the other adrenal (bottom) has a small nodule with *CYP11B2* expression close to the adrenal capsule. Note that hybridisation with *CYP11B1* probe shows no expression in none of the adrenals in the corresponding area of *CYP11B2* expression. Scale bar 1 mm.

In normal adrenal cortex (Fig.5) we found *CYP11B2* gene expression in zona glomerulosa cells as predicted. One normal adrenal had a small nodule as well (Fig.5). This may represent a normal variant or an early development of primary aldosteronism. Furthermore, *CYP17* and *CYP11B1* expression was present in zonae fasciculata and reticularis. These results show that the method is specific enough to apply to tumour tissue.

Erdmann and colleagues [40] using cRNA probe also found clear expression of *CYP11B1* in zonae fasciculata and reticularis but not in zona glomerulosa in the non-tumour portion of an adrenal gland with an aldosterone producing adenoma. On the other hand, a recent study [23], also using cRNA probes, showed mRNA expression of both aldosterone and cortisol synthetic enzymes in all three zones of normal adrenal cortex, although there were differences of expression in the zones. Intense expression of *CYP11B2* and *CYP11B1* was seen in zona glomerulosa, but there was also *CYP11B2* expression in zonae fasciculata and reticularis indicating cross reactivity of *CYP11B2* and *CYP11B1* probes. With the nucleotide sequences of *CYP11B2* and *CYP11B1* being 95% identical in coding regions [41], the selection of DNA sequences when designing oligo nucleotide probes, including G/C content, is essential and the probes can be more specific (Fig.6) using the shorter oligo nucleotide probes than cRNA probes, since redundant or conserved nucleotide sequences can be avoided during probe design and synthesis [42].

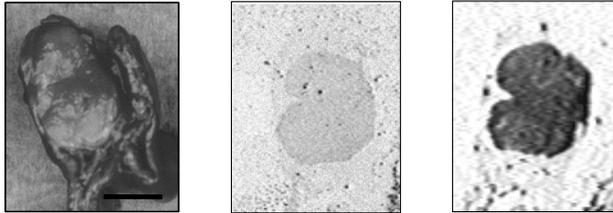
Previously, the expression of *CYP11B2* gene has been difficult to demonstrate probably due to the scarce presence of gene copies. Compared to the amount of aldosterone and cortisol synthesized, the number of mRNA copies of the *CYP11B2* gene would be about 100 times less than that of the *CYP11B1* and *CYP17* genes in normal adrenals.

The *in situ* hybridisation technique needs be developed to be able to use at routine histopathological analysis. This could be achieved with the non-radioactive labelling compound Digoxigen. The advantage with the Digoxigenin system is that sections are developed in only some days instead of several weeks and the probes are stable in contrast to the [<sup>35</sup>S]-radioactive labelled probes. The detection of the signal is performed by immunoassay with anti-Digoxigenin-alkalinephosphatase-antibody conjugate providing a colour reaction, which is a disadvantage, because quantification is more complicated.



**Figure 6. Probe specificity check.** Autoradiographic film of consecutive sections of normal adrenal hybridised with [<sup>35</sup>S]-labelled oligo nucleotide probe complementary to *CYP11B1*. **1.** Labelled *CYP11B1* probe only. **2.** Addition of 20 times unlabelled *CYP11B2* probe. No visible effect on hybridisation result. **3.** Addition of 20 times unlabelled *CYP11B1* probe. No specific hybridisation of labelled probe in presence of abundant unlabelled probe.

In paper II we doubled the concentration of *CYP11B2* probe increasing the sensitivity (Fig.7).



**Figure 7. An adrenal adenoma from a patient with primary aldosteronism.** *In situ* hybridisation autoradiograms of *CYP11B2* expression, middle, paper I and ,right, paper II. Note the difference of intensity of the expression. In paper II the concentration of the probe was doubled. Scale bar 5 mm.

The data obtained by the *in situ* hybridisation technique must be considered semi quantitative due to the risk of degradation of mRNA in archival tissue. However, control experiments with frozen tissue (unpublished) have shown similar results with regard to the expression of steroid synthesizing enzyme genes in normal adrenals.

#### **4.2 CORRELATION BETWEEN *IN VITRO* STEROID RELEASE AND EXPRESSION OF STEROIDOGENIC ENZYME GENES ( I )**

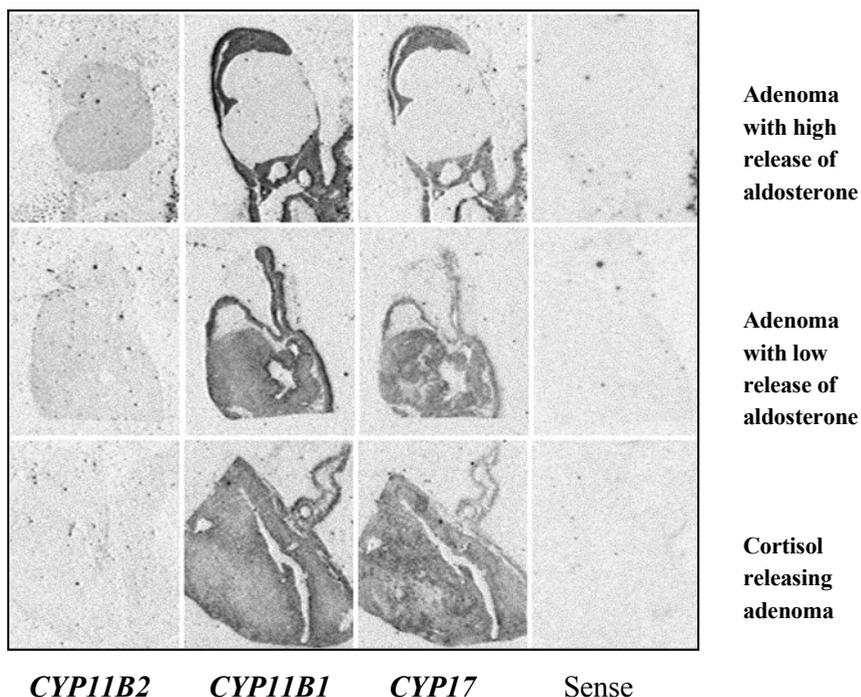
Comparison between steroids released *in vitro* and mRNA expression of steroidogenic enzymes in adrenal adenomas from patients with primary aldosteronism, Cushing's syndrome and non-hyperfunctioning tumours was performed to determine the relationship between steroids synthesized and expression of the enzyme genes.

There was considerable variation in aldosterone release from the aldosteronomas although the two patients with highest release of aldosterone *in vitro* also showed the highest expression of *CYP11B2* gene (Fig.8). All these tumours could also release cortisol and showed variation in *CYP11B1* and *CYP17* expression.

Tumours from patients with Cushing's syndrome, which had no detectable release of aldosterone but high release of cortisol, showed highest expression of *CYP17* and *CYP11B1* but very low *CYP11B2* expression. The non-hyperfunctioning tumours had cortisol release and *CYP11B1* and *CYP17* expression similar to the tumours from patients with Cushing's syndrome. In contrast, the non-hyperfunctioning tumours had low release of aldosterone *in vitro*.

These findings suggest that not only patients with Cushing's syndrome and non-hyperfunctioning tumours but also some patients with aldosteronoma could be expected to suffer from cortisol deficiency in the immediate postoperative period, indicating the need for close follow-up of these patients.

There was a positive correlation between aldosterone and cortisol released *in vitro* and expression of *CYP11B2* ( $r = 0.81$ ,  $p = 0.05$ ) and *CYP17* ( $r = 0.67$ ,  $p = 0.05$ ), respectively. In addition, there was a positive correlation between the expression of *CYP17* and *CYP11B1* ( $r = 0.79$ ,  $p = 0.05$ ). There was also a correlation between *in vitro* release of cortisol and *CYP11B1* ( $r = 0.71$ ,  $p = 0.05$ ) expression as expected, because *CYP11B1* is the gene responsible for  $11\beta$ -hydroxylase, which is the last enzymatic step in the cortisol pathway. These are calculated using Spearman Rank Order correlations. Previous, calculations in paper I was made with the incorrect assumption that all data were normally distributed.



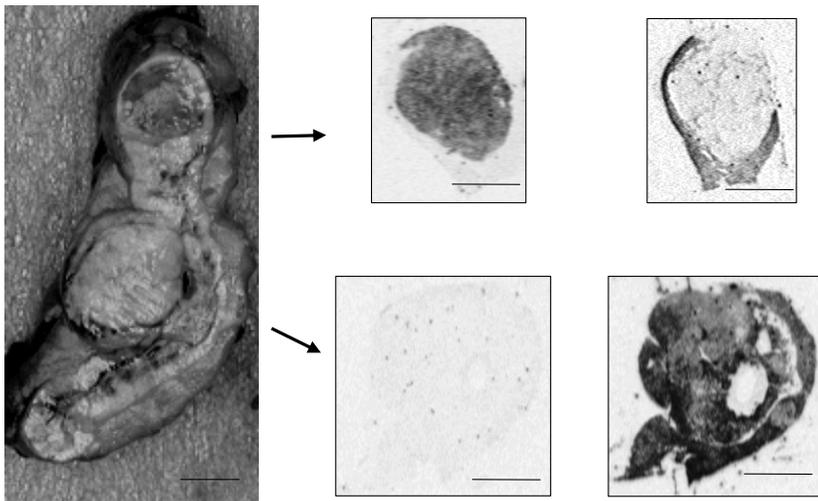
**Figure 8. Adrenal adenoma.** *In situ* hybridisation autoradiograms showing the expression of *CYP11B2*, *CYP11B1* and *CYP17* genes in adrenal adenoma with high (top) and low (middle) release of aldosterone, and release of cortisol (bottom).

In previous studies we have shown that aldosterone and cortisol are released *in vitro* from slices of normal adrenal cortex. This release is due to newly synthesized steroids since addition of a synthesis inhibitor inhibits release [38]. Addition of ACTH, on the other hand, increases steroid release from adrenal slices [43].

This study presents new means for functional characterisation of adrenocortical tumours. The two methods, *in vitro* determination of steroid release and *in situ* hybridisation of mRNA expression of steroidogenic enzyme genes, are complementary in characterizing adrenocortical tumour production.

### 4.3 POSTOPERATIVE DIFFERENTIATION BETWEEN UNILATERAL ALDOSTERONE PRODUCING ADENOMA AND BILATERAL HYPERPLASIA ( II )

Twenty-seven patients subjected to unilateral adrenalectomy for primary aldosteronism were retrospectively evaluated including clinical data, histopathological diagnosis, effect of the operation and long term follow up. The histopathological diagnosis was adenoma (11 pat.), adenoma and/or hyperplasia (15 pat.) or hyperplasia (1 pat.).

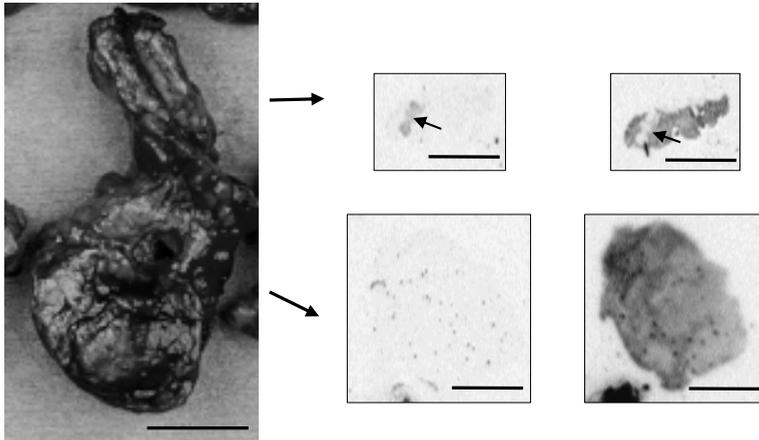


**Figure 9.** An adrenal with two tumours from a patient with primary aldosteronism. *In situ* hybridisation autoradiograms (left) of the tumours revealed one (top) to have *CYP11B2* expression indicating aldosterone production but no *CYP11B1* or *CYP17* (not shown) expression. The other tumour (bottom) had no *CYP11B2* expression but *CYP11B1* and *CYP17* (not shown) expression indicating cortisol production. Scale bar 5 mm.

*CYP11B2* expression, indicating aldosterone production, was found in an adenoma of 22 patients (Fig.9). Fourteen of these tumours had *CYP11B2* expression also in zona glomerulosa and 6 adrenals had small nodules close to the capsule. These 22 patients were cured by adrenalectomy except one, who also had high expression of *CYP11B2* in zona glomerulosa. This patient was initially cured, but increased aldosterone secretion had recurred at follow up 4.5 years later.

Two patients had a mass shown on computed tomography. Analysis of the tumour with *in situ* hybridisation showed no *CYP11B2* expression but *CYP11B1* and *CYP17* expression indicating cortisol production (Fig.10). However these adrenals contained small nodules with *CYP11B2* expression. These small nodules could not be seen on computed tomography but were probably responsible for the overproduction of aldosterone in these cases. These two patients were not cured probably because the correct diagnosis was bilateral hyperplasia. This illustrates that it is difficult to

differentiate between adenoma and hyperplasia and only long term follow up will eventually provide the correct diagnosis.



**Figure 10.** An adrenal adenoma from a patient with primary aldosteronism, first operation. *In situ* hybridisation autoradiograms of the adrenal (right). The adenoma, seen on computed tomography, had only specific expression of *CYP11B1* and *CYP17* (not shown) indicating cortisol production. In the adrenal cortex there was a small nodule (top) with *CYP11B2* expression indicating aldosterone production. The patient was not cured and had a second operation 3 years later. The second adrenal was hyperplastic and had several small nodules with *CYP11B2* expression in the adrenal cortex. These small nodules, not seen by computed tomography, were probably responsible for the aldosterone secretion in this patient. Scale bar 5 mm.

In contrast, there were two patients cured from primary aldosteronism by adrenalectomy showing no *CYP11B2* expression in the adenoma seen on computed tomography. This is difficult to explain but it may be due to insufficient sensitivity of the method or non-representative tissue used for the *in situ* hybridisation.

Cured patients had normal serum potassium and renin levels, and urinary aldosterone was within reference range. Blood pressure returned to normal in 11 patients (48%), while 12 patients were improved but still needed antihypertensive medication. Other studies report a cure rate of hypertension of 41 – 71% in patients operated for primary aldosteronism [44-46]. There was no difference in median duration of hypertension at diagnosis between the patients without (6.3 years) medication and the patients depending on medication (6.5 years), postoperatively.

The 14 cases of adrenocortical adenomas with additional expression of *CYP11B2* in zona glomerulosa and/or small nodules were compared with 8 cases without this expression (unpublished data). The size of the adenomas was similar in the two groups. In the former group the duration of hypertension was longer (median 9 years versus median 4 years, NS), and age at diagnosis was higher (median 50 years versus median 44 years, NS) suggesting that with increasing age and longstanding hypertension the development of small nodules and zona glomerulosa hyperplasia is stimulated.

There have been reports of an intra adrenal renin-angiotensin system, which possibly could be the stimulator in an autocrine or paracrine manner to the increase of zona glomerulosa and formation of small nodules when the aldosterone synthesis in the adrenal becomes autonomous. Confirmation of an existing intra adrenal renin-angiotensin system has been presented in rat and human tissues. Angiotensin I and Angiotensin II are present in cells of zona glomerulosa [47-49]. Angiotensin converting enzyme is also present but the cell type has not been determined. Furthermore, preparations of zona glomerulosa cells incubated with Angiotensin I produce Angiotensin II, and this synthesis is blocked by angiotensin converting enzyme inhibitors. The mRNA for angiotensinogen is found in fibroblast-like cells in the adrenal capsule but not in steroid producing cells [50]. Thus, the zona glomerulosa cells appear to synthesize and secrete Angiotensin II, contain renin and angiotensin converting enzyme in close proximity. Angiotensinogen presumably moves in a paracrine fashion to the cells in zona glomerulosa that produce Angiotensin II [51, 52]. Another interesting finding is that angiotensin responsive aldosterone producing adenomas have increased expression of renin mRNA compared to angiotensin unresponsive aldosterone producing adenomas or normal adrenals. In conclusion this implies a possible influence of intra adrenal renin on aldosterone production in aldosteronomas [53]. However, there are not yet convincing evidence of the physiological role of the intra adrenal renin system in adrenal function.

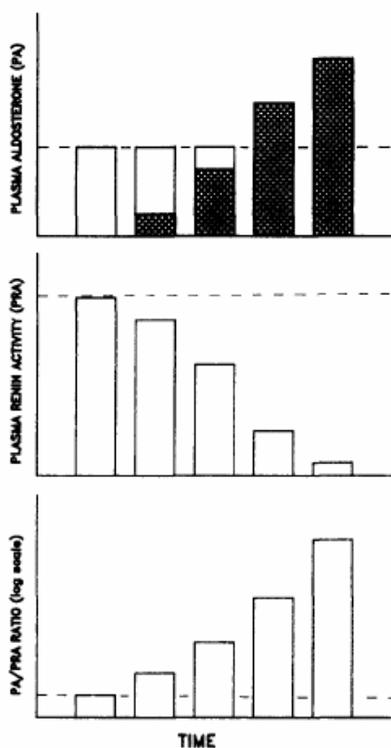
The present study demonstrates a relationship between *in situ* hybridisation expression of *CYP11B2*, *CYP17* and *CYP11B1* and the outcome of adrenalectomy in a clinically well characterized group of patients operated for primary aldosteronism. The results illustrate the value of knowing which part of the adrenal is producing aldosterone for correct subclassification. mRNA *in situ* hybridisation of steroidogenic enzymes can visualize the location of the probable steroid production, unlike the current used histopathological evaluation. In combination with clinical data, endocrinologic evaluation and histopathology the *in situ* hybridisation of steroidogenic enzyme genes provide improved means for correct subclassification postoperatively of patients with primary aldosteronism.

#### **4.4 SCINTIGRAPHY OF PATIENTS WITH PRIMARY ALDOSTERONISM ( III )**

Differentiation before operation between the two major groups of primary aldosteronism, unilateral aldosterone producing adenoma and bilateral hyperplasia, is essential since therapy in the former is surgical and in the latter primarily medical. However, this task is complicated because primary aldosteronism may comprise a pathological continuum between a true solitary adenoma and pure bilateral micronodular hyperplasia with intermediate degrees of unilateral or bilateral macronodular and micronodular hyperplasia [54].

Gordon and colleagues have proposed an interesting model for development of primary aldosteronism [18] possibly correlated to the different forms seen in primary aldosteronism from micro- and/or macro-nodular hyperplasia to adenoma (Fig.11). The

fact that patients with adrenal hyperplasia usually present with milder biochemical and hormonal abnormalities than adrenal adenoma patients supports this assumption.



**Figure 11. Progressive development of primary aldosteronism.**

During the first phase (first column) no biochemical or clinical abnormalities exist and diagnosis must be obtained by genetic screening. During the second phase (second and third columns) biochemical abnormalities appear, consisting of reduced (but still within normal range) plasma renin activity, and frankly raised plasma aldosterone/plasma renin activity ratio. Hypertension, and rarely, hypokalemia may appear in predisposed individuals. During the final phase (fourth and fifth columns), clinical abnormalities of hypertension and hypokalemia are consistently present. The hatched portions of columns indicate autonomous aldosterone production and the interrupted lines the appropriate levels (with regard to prevailing conditions such as dietary sodium) when aldosterone is normally regulated [18].

Scintigraphy of the adrenal gland utilises [ $^{131}\text{I}$ ] norcholesterol (NP59), which binds to specific adrenocortical cell receptors. The hormone receptor complex is transported into the cell cytoplasm entering the steroidogenic synthetic pathway (see above). Thus, the labelled compound visualizes steroid production in the adrenocortical tissue by single-photon emission computed tomography. Before and during the test period suppression of the ACTH dependent synthesis of cortisol is maintained by dexamethasone administration.

In the present study, scintigraphic imaging pattern was correlated to clinical diagnosis, therapy, and outcome, with a mean follow up of 4 years. Norcholesterol scintigraphy

with dexamethasone suppression was performed preoperatively on 33 patients with the diagnosis of primary aldosteronism to determine the side of aldosterone hypersecretion.

Scintigraphy showed lateralized isotope uptake in 27 patients (Fig. 12). Six patients had normal or no isotope uptake. Twenty-two of the operated patients were cured from primary aldosteronism with normalized aldosterone and potassium levels and normalized or improved blood pressure. Three patients were improved having slightly elevated aldosterone concentration but normalized potassium concentration. Two patients showed no improvement. One of the patients, who had no uptake on scintigraphy, had a histopathological diagnosis of an unusual corticomedullary tumour. The other 5 patients were cured and they were all lateralized by adrenal venous sampling before operation.

### Scintigraphy

|          |          |         |
|----------|----------|---------|
| Positive | 25<br>TP | 2<br>FP |
| Negative | 5<br>FN  | 1<br>TN |

TP, true positive; TN, true negative; FP, false positive; FN, false negative.

Sensitivity  $TP/(TP + FN) = 83\%$  (65-94%)

Specificity  $TN/(FP + TN) = 33\%$  (8-91%)

Positive predictive value  $TP/(TP + FP) = 93\%$  (76-99%)

Negative predictive value  $TN/(TN + FN) = 17\%$  (4-64%)

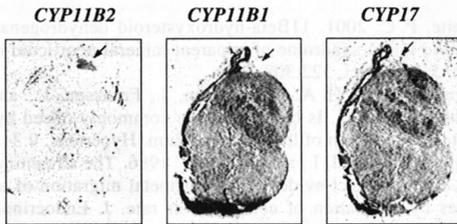
Accuracy  $(TP + TN)/(TP + FP + TN + FN) = 79\%$  (61-91%)

**Figure 12.** Two by two table evaluation of diagnostic power to distinguish between unilateral from bilateral aldosterone hypersecretion by [<sup>131</sup>I] norcholesterol (NP-59) scintigraphy. Performance characteristics with corresponding 95% binomial confidence intervals are given below.

Thus, in 25 of the 33 patients operated (76%) scintigraphy indicated the correct side leading to cure. Accuracy and positive predicted value were 79% and 93%, respectively. A review of the literature reported 53 to 94% accuracy [55].

One of the patients, who was not cured after adrenalectomy, had an adenoma, which has been studied with determination of the mRNA expression of genes coding for adrenocortical steroid synthesizing enzymes using *in situ* hybridisation [56]. This is the only method that can postoperatively determine if an adenoma has the ability to produce aldosterone or not. The adenoma in this patient had expression of *CYP11B1* and *CYP17* genes needed for cortisol production (Fig.13). Only small nodules in the surrounding cortex had *CYP11B2* expression, which is needed for aldosterone synthesis. It is likely that the adenoma had an autonomous cortisol secretion, not suppressed by dexametason. Fifteen of the 17 adrenals analysed with *in situ* hybridisation had *CYP11B2* expression in the adenoma and these patients were cured.

Fourteen patients in this study were judged to have the probable diagnosis of bilateral hyperplasia and were treated with aldosterone antagonists. In these patients the scintigraphy was normal with no side difference. However, although most aldosterone producing adenomas are angiotensin II unresponsive, some share the characteristics of bilateral hyperplasia being angiotensin II responsive. As the patients were not operated and adrenal venous sampling was only performed in a few patients, the presence of small angiotensin II responsive adenomas not detected by computed tomography or magnetic resonance imaging cannot be excluded.



From Enberg et al, 2003 [56].

**Figure 13. Autoradiogram of *in situ* hybridisation in an adrenal adenoma from a patient with primary aldosteronism.** No expression of *CYP11B2*, indicating aldosterone production, was found in the adenoma, but there were *CYP11B1* and *CYP17* expression indicating cortisol production. In the cortical region there was a small nodule with *CYP11B2* expression (arrow).

Adrenal venous sampling is considered the golden standard in lateralizing aldosterone hypersecretion [57] although it is an invasive alternative and associated with a risk of vein rupture [58-61]. Furthermore, cannulation of both adrenal veins is needed for a reliable result [54]. In expert hands the accuracy is very high [59]. Identification of a unilateral adrenal cause of aldosterone hypersecretion is essential for proper treatment of patients with primary aldosteronism, because removal of an aldosteronoma produces long term cure of both hypertension and hypokalemia and prevents the need for life-long costly antihypertensive therapy. Patients cured for hypertension and hypokalemia are reported to be approximately 69% of 694 cases [54, 62-64] but long term follow up has not usually been obtained.

In conclusion, a functional test should be performed on patients with primary aldosteronism for subclassification and lateralization of the side of aldosterone hypersecretion. Norcholesterol scintigraphy can serve as a compliment to adrenal venous sampling for this purpose.

#### 4.5 AUTONOMOUS CORTISOL PRODUCTION IN ADRENOCORTICAL TUMOURS ( IV )

Increasing number of adrenal masses is detected by computed tomography, magnetic resonance imaging, or ultrasound due to more common use and improved sensitivity of imaging techniques. The majority of these lesions are non-functioning, or preferably non-hyperfunctioning [43], but all patients found with these lesions need a thorough clinical work up to exclude autonomous function or possible malignant tumours, which

both need to be operated. In this study we tried to identify tumours with subtle autonomous cortisol production among the non-hyperfunctioning tumours.

Thirty-seven adrenal tumours were studied, including non-hyperfunctioning tumours, adenomas from patients with Cushing's syndrome and pheochromocytomas, the latter as negative controls. mRNA *in situ* hybridisation of the genes *CYP11B1* and *CYP17* coding for steroidogenic enzymes, needed for cortisol synthesis, was performed and patient records evaluated. From the preoperative diagnostic work up and postoperative follow-up 21 patients probably had non-hyperfunctioning tumours and 2 patients were considered to have subclinical Cushing's syndrome. For comparison 12 patients confirmed to have overt Cushing's syndrome were included.

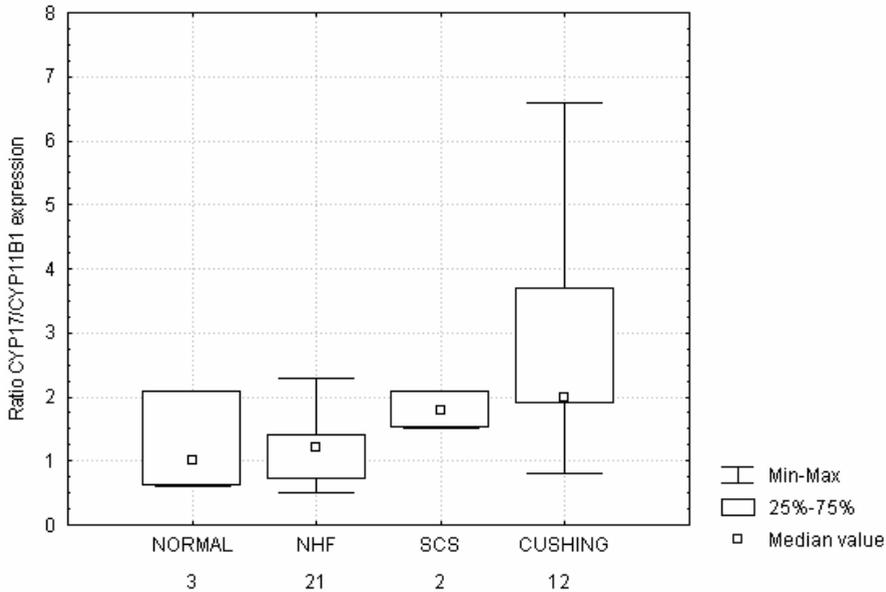
Ten of the 21 non-hyperfunctioning tumours had atrophy of zonae fasciculata and reticularis. In adenoma from patients with Cushing's syndrome the adjacent cortex was either atrophic, compressed or hyperplastic.

Previously we have presented a positive correlation between *in vitro* release of cortisol and *CYP11B1* and *CYP17* expression. Consequently, *CYP17* and *CYP11B1* expression presumably indicate cortisol production. The ratio of the expression of *CYP17* and *CYP11B1* was used to compare different experiments. With support from previous studies (see below) the assumption was that *CYP17* gene was overexpressed.

Several studies have shown increased expression of *CYP17* expression to be present in cortisol producing adenomas from patients with Cushing's syndrome [65-67]. Furthermore, Shibata and colleagues [67] showed that the orphan nuclear hormone receptors DAX-1 (dosage sensitive sex reversal-adrenal hypoplasia congenital, critical region on the X chromosome-1) and COUP-TF (chicken ovalbumin upstream promoter-transcription factor), known to be transcriptional repressors of *CYP17* gene, were significantly down regulated in tumours from patients operated for Cushing's syndrome probably contributing to increased cortisol synthesis. These findings suggest a possible role of these nuclear receptors in the dysregulation of glucocorticoid synthesis.

In the present study, there was a significant difference in the median ratio *CYP17* to *CYP11B1* expression between tumours from patients with Cushing's syndrome and the non-hyperfunctioning tumours (Fig. 14). Two patients were considered to have subclinical Cushing's syndrome, one of them with the histopathological diagnosis of pigmented black adenoma similar to one of the patients with Cushing's syndrome. Black adenoma is usually associated with Cushing's syndrome [68].

In this study we could clinically and by steroid synthesizing enzyme expression identify two patients with probable subclinical Cushing's syndrome. Two more patients had tumours with ratios close to the median ratio for patients with Cushing's syndrome indicating that these patients also could have subclinical Cushing's syndrome. Although they had no obvious clinical signs of cortisol deficiency after adrenalectomy their adrenals had cortical atrophy. This might indicate inhibition of ACTH stimulation on adrenal cortex in these patients.



**Figure 14. Ratio *CYP17* to *CYP11B1* expression in adrenocortical tumours and normal adrenals.** The median ratio of the expression of *CYP17* to *CYP11B1* for tumours from patients with Cushing's syndrome, including patients with bilateral hyperplasia, was significantly ( $p = 0.0002$ ) higher than the median ratio for the non-hyperfunctioning tumours (NHF). The ratios for tumours from patients with subclinical Cushing's syndrome (SCS) were close to the median ratio for tumours from patients with Cushing's syndrome, while the ratios for normal adrenals was close to the median ratio for the non-hyperfunctioning tumours.

The occurrence of subclinical Cushing's syndrome among incidentally discovered adrenal adenomas is reported in the literature to be 12 – 16% [69-71]. Subclinical Cushing's syndrome is associated with high occurrence of hypertension, diabetes mellitus, elevated lipids and diffuse obesity. This emphasizes the importance of careful endocrine evaluation in all patients with apparently non-functioning adrenal adenoma [33].

## 5 CONCLUSIONS

A method was developed to determine the mRNA expression of the steroidogenic enzyme genes *CYP11B2*, *CYP11B1* and *CYP17*. Radioactive oligonucleotide probes were hybridised on paraffin sections and expression was visualized by exposure to autoradiographic film. In addition, the sections were dipped in autoradiographic emulsion, exposed and successively developed for evaluation on cellular level.

The release of aldosterone and cortisol was correlated to the steroidogenic enzyme gene expression suggesting that *CYP11B2* expression indicate aldosterone production and *CYP17* and *CYP11B1* expression indicate cortisol production.

Subclassification of patients with primary aldosteronism was performed by thorough evaluation pre and post adrenalectomy. A few patients recurred several years after operation. This emphasizes the importance of long-term follow up.

The present method is reliable in differentiating between adrenal adenoma and bilateral hyperplasia and in identifying tumours, which are probably not producing aldosterone.

It is suggested that a functional test should be performed before operating on a patient with primary aldosteronism. Norcholesterol scintigraphy predicted the correct side of aldosterone production in 76% of patients with primary aldosteronism. Scintigraphy can serve as a compliment to adrenal venous sampling.

In tumours from patients with Cushing's syndrome the median ratio between the expression of the *CYP17* and *CYP11B1* genes is significantly higher than the median ratio in the non-hyperfunctioning tumours. Also in patients with subclinical Cushing's syndrome the tumours have a similar high ratio indicating that these patients may be identified with this method.

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