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

PRIMARY ALDOSTERONISM STUDIES ON SCREENING, OUTCOME OF ADRENALECTOMY AND FUNCTIONAL PATHOLOGY

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Be not afraid of growing slowly; be afraid only of standing still.
Chinese proverb

Los dueños de la verdad la siguen buscando.
José Narosky

To my family

Thesis defence

Thoraxaulan
Thorax N2:U1
Karolinska University Hospital, Solna

Friday 8th of November, 2013
09:00 am

ABSTRACT

This thesis comprises four studies with the general aim to improve the management of patients with primary aldosteronism (PA).

PA is a hypertensive disorder due to autonomous secretion of aldosterone from one or both adrenal glands. PA exerts negative effects on the cardiovascular system beyond the negative effects of the elevated blood pressure, why it is important to detect PA among patients with hypertension and provide specific treatment.

In PA, aldosterone is raised, leading to reduced renin levels. An elevated aldosterone to renin ratio (ARR) can be used as a screening tool to detect PA. The overall prevalence of PA is 5-15 % among patients with hypertension. In primary care, the frequency is lower, but displays a wide range and is therefore uncertain. In this thesis, the prevalence of PA was studied in a primary care setting by screening with the ARR among 178 patients with hypertension with continued antihypertensive medication. The prevalence was 1.6-3.3 %, why screening among the general hypertensive population would not be awarding. Antihypertensive medication, especially angiotensin-receptor blockers, ACE-inhibitors and thiazide diuretics were found to lower the ARR in patients with essential hypertension. Different cut-off levels for the ARR when screening with or without antihypertensive medication may be of value.

Unilateral PA is usually due to an adrenal adenoma while bilateral PA most often is due to hyperplasia. Unilateral disease can be cured by adrenalectomy, while bilateral disease is treated pharmacologically. Some patients are not cured by adrenalectomy or recur at long-term follow-up, because they have bilateral PA, and will need specific medical treatment. At histopathology evaluation it is sometimes difficult to distinguish between adenoma and hyperplasia. Methods for functional pathology would serve in the postoperative evaluation.

Functional pathology was used to detect the site of aldosterone and cortisol synthesis in adrenal glands from patients operated for PA. The results were related to clinical outcome and long-term follow-up. With situ hybridization mRNA, expressing enzymes for aldosterone and cortisol synthesis, was detected. With immunohistochemistry, specific antibodies against these enzymes were applied. Results from the two methods were never conflicting. Adenomas typically demonstrated evidence of aldosterone synthesis and sometimes also cortisol synthesis. In some adrenals, there were several nodules with demonstration of aldosterone synthesis, suggestive of hyperplasia.

A consecutive series of 120 patients with PA were operated 1985-2010 due to unilateral aldosterone hypersecretion. Adrenalectomy led to cure of PA in 91 % with a median follow-up of 5 years. Also patients not cured benefitted from the operation, as their blood pressure improved. Functional pathology changed the diagnosis in 7 %. All adenoma patients were cured. Their preoperative 24 h- aldosterone values were higher compared to patients with hyperplasia. Among hyperplasia patients, 61 % were cured. Their preoperative aldosterone values were lower than in those not cured. It remains to be seen if they recur in the future.

Conclusions: The low detection rate of PA among hypertensive patients in primary care does not favor general screening. With functional pathology, the site of aldosterone synthesis in adrenals from patients operated for PA was for the first time demonstrated in a clinical setting with a large series of patients. It is suggested that immunohistochemistry with specific antibodies be used in routine postoperative evaluation as an adjunct to routine histopathology.

LIST OF PUBLICATIONS

This thesis is based on the following original studies, referred to in the text by their Roman numerals:

I. **Volpe C**, Wahrenberg H, Hamberger B, Thorén M. Screening for primary aldosteronism in a primary care unit. *Journal of the Renin-Angiotensin-Aldosterone system 2013; 14(3):212-219.*

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*. *Eur J Endocrinol. 2004 Jul; 151(1):73-85.*

III. **Volpe C**, Höög A, Ogishima T, Mukai K, Lu M, Thorén M, Hamberger B. Immunohistochemistry improves histopathologic diagnosis in primary aldosteronism. *J Clin Pathol 2013; 66:351-354.*

IV. **Volpe C**, Hamberger B, Höög A, Mukai K, Calissendorff J, Wahrenberg H, Zedenius J, Thorén M. Primary aldosteronism; long-term follow-up after adrenalectomy and diagnostic value of immunohistochemistry. *Manuscript.*

CONTENTS

1 INTRODUCTION

Hypertension is a highly prevalent risk factor for cardiovascular disease [1] and is estimated to cause 4.5% of the current global disease burden according to the World Health Organization [2]. The overall prevalence of hypertension appears to be around 30-45 % of the general population [1]. In the majority of cases hypertension is primary, but may also be caused by an underlying disorder, i.e. secondary hypertension. The most common form of secondary hypertension is currently considered to be primary aldosteronism (PA) [3].

PA is a disorder with autonomous hypersecretion of aldosterone which leads to sodium and fluid retention, thereby causing hypertension. Plasma renin levels are suppressed by negative feed-back mechanisms [4]. Hypokalaemia may be present, especially in severe or longstanding cases [5].

It is important to detect and treat PA since aldosterone excess may cause cardiovascular complications *per se* in addition to the negative effects of hypertension [6, 7] and it has been shown that surgical or specific medical treatment of PA will not only cure or improve hypertension but will also lower the risk of cardiovascular events [6].

The knowledge that adrenal products are able to increase blood pressure and fluid retention was improved in 1953 when Simson and Tait described a bio-assay for assessment of mineralocorticoid activity, and communicated that adrenal beef extract had mineralocorticoid properties. They suggested that this was due to an unknown compound different from cortisol [8]. Two years later, together with Dr Reichenstein, they reported the constitution of this mineralocorticoid. As they had found the presence of an aldehyde group, they proposed the name aldosterone [9].

In 1953, Litýnski suggested a relation between malignant hypertension and mineralocorticoid secretion from the adrenals [10]. Two years later, Jerome Conn described PA as a new clinical syndrome; a condition with hypertension and hypokalaemia due to an aldosterone-secreting adrenocortical adenoma [11]. It was later clarified that most patients had milder forms of PA with serum potassium levels within the normal range [5, 12]. During many years it was assumed that the prevalence of PA in the hypertensive population was low; 0.5-2 %, but with the introduction of the aldosterone to renin ratio (ARR) as a screening test for PA in the early eighties (see below) [13-15], and with its application also to normokalaemic patients, the detection rates have increased to 5-18 % in the general hypertensive population [12, 16-26]. Frequencies of PA vary between study populations and increase with the severity of the hypertension [19], being highest, 17-23 %, at referral centres treating resistant hypertension [5], although recent studies from different populations with resistant hypertension have shown a lower prevalence, 7.1-11.3 % [27, 28]. The prevalence of PA in non-referral units is lower, 0.7-13 % [19, 22, 23, 29-32], but still uncertain.

2 BACKGROUND

2.1 ANATOMY AND FUNCTION OF THE ADRENAL CORTEX

The adrenals are paired organs that are located near the superior pool of the kidneys. They are composed of an outer layer, i.e. the cortex, and an inner medulla. The cortex and the medulla are of separate embryonic origin and have separate functions. In the cortex, steroid hormones, i.e. mineralocorticoids, glucocorticoids and androgen hormones are synthesized while the adrenal medulla secrets adrenaline and noradrenaline.

The cortex consists of three layers, i.e. the zona glomerulosa, the zona fasciculata and the zona reticularis [33]. In the outer layer, the zona glomerulosa, mineralocorticoids are synthesized. The zona fasciculata has the capacity to synthesize glucocorticoids and in the zona reticularis androgens and possibly glucocorticoids are produced (Fig 1). The steroid hormones are synthesized from cholesterol. Fig 2 shows an overview of the steroidogenic pathways.

The adrenal	Zones	Group of hormones	Principal hormone	Enzyme	Gene
Adrenal cortex	Zona glomerulosa	Mineralo-corticoids	Aldosterone	CYP11B2	CYP11B2
	Zona fasciculata	Gluco-corticoids	Cortisol	CYP17 CYP11B1	CYP17 CYP11B1
	Zona reticularis	Androgens (Gluco-corticoids)	DHEA (Cortisol)	CYP17 17,20 lyase (CYP11B1)	CYP17 (CYP11B1)
Adrenal medulla		Catechol-amines	Adrenaline, Nor-adrenaline		

Fig 1. The adrenal. Zonation, hormones, enzymes and genes.

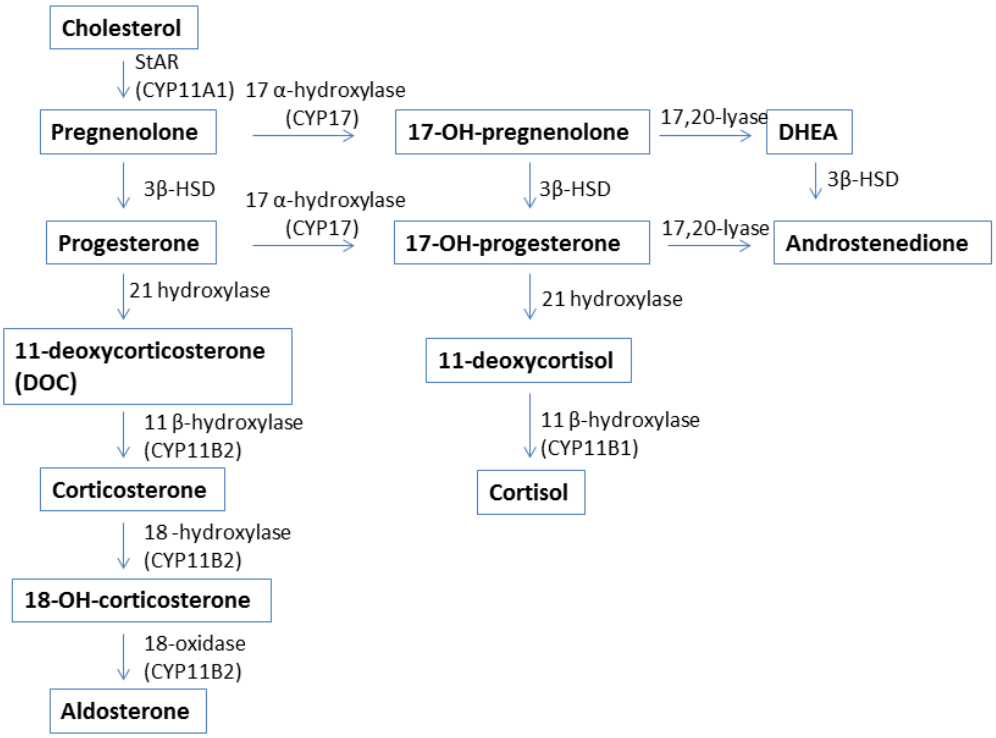

Fig 2. Adrenal steroidogenic pathways. StAR, steroidogenic regulatory protein; 3ß-HSD, 3ß-hydroxysteroid dehydrogenase; OH-, Hydroxy-; DHEA, dehydroepiandrosterone.

2.2 THE RENIN- ANGIOTENSIN-ALDOSTERONE SYSTEM, RAAS

Mineralocorticoids regulate the balance of body fluids and electrolytes, which maintains blood pressure. Aldosterone is the major circulating mineralocorticoid hormone and it is regulated by the renin-angiotensin-aldosterone-system (RAAS). Renin is released from the juxtaglomerular cells in the kidney and catalyses the conversion of angiotensinogen to angiotensin I. Angiotensin I is cleaved by the angiotensin-converting enzyme (ACE) to angiotensin II [34]. Angiotensin II binds to its receptors on the zona glomerulosa cells which stimulates aldosterone synthesis and secretion [35]. Aldosterone exerts its effects by binding to the mineralocorticoid receptors in the distal tubules of the nephron in the kidney. This results in increased concentrations of the epithelial sodium channel, ENaC, and the sodium-potassium pump, i.e. the $Na^{+/}K^{+}$-ATPase, resulting in sodium and water absorption, potassium excretion and volume expansion [36] (Fig 3).

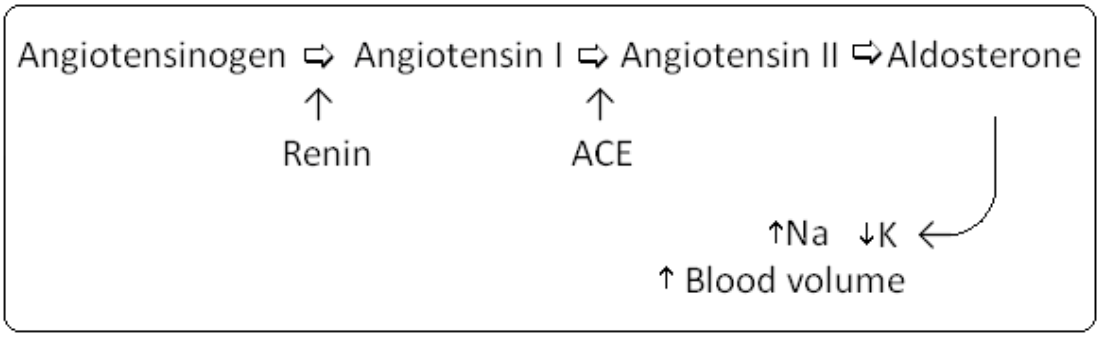

Fig 3. The RAAS-system. Renin is secreted from the juxtaglomerular cells in the kidney and catalyses the conversion of angiotensinogen to angiotensin I. Angiotensin I is cleaved by the angiotensin-converting enzyme (ACE) to angiotensin II, which stimulates aldosterone secretion. Aldosterone increases renal sodium reabsorption in exchange for potassium. Increased sodium reabsorption leads to fluid retention. The level of circulating renin is the rate-limiting factor in this process [34].

Renin is the key regulator in the RAAS-system. Renin release is mainly influenced by factors that affect renal blood flow and glomerular perfusion, by angiotensin II and by aldosterone. Factors that decrease renal blood flow increase plasma renin levels, whereas factors that increase blood pressure decrease renin secretion. Angiotensin II inhibits renin secretion by direct feed-back mechanisms while aldosterone reduces renin by indirect mechanisms through increased sodium re-absorption and plasma volume expansion [34] (Table 1).

Factors that increase plasma renin levels
Upright posture
Salt restriction
Dehydration
Haemorrhage
Renal artery narrowing
Factors that decrease plasma renin levels
Supine posture
High salt intake
Peripheral vasoconstrictors
Angiotensin II
Aldosterone

Table 1. Factors that affect renin release.

Aldosterone synthesis is mainly regulated by angiotensin II and potassium while adrenocorticotropic hormone (ACTH) is a minor stimulatory modulator. High potassium increases aldosterone concentrations whereas low potassium leads to a decrease in aldosterone secretion [34].

2.3 PRIMARY ALDOSTERONISM AND THE RAAS-SYSTEM

In PA, aldosterone production is excessive and relatively autonomous with regard to the RAAS- system. This results in excessive sodium re-absorption, leading to hypertension and suppression of renin and angiotensin II. Urinary loss of potassium and hydrogen ions, exchanged for sodium, may result in hypokalaemia and metabolic alkalosis if severe and prolonged enough [37].

2.4 NON-CLASSIC EFFECTS OF HYPERALDOSTERONISM AND PA

There is compelling data showing that long-standing hyperaldosteronism causes end-organ damage. This was demonstrated indirectly in two landmark studies where treatment with mineralocorticoid receptor blockers compared to placebo reduced cardiovascular mortality and morbidity in patients with severe heart failure [38, 39]. A more recent study reported that among patients who were scheduled for coronary angiography, aldosterone levels were independently associated with cardiovascular mortality even with aldosterone levels within the three upper quartiles of the normal range [40].

The underlying mechanisms for end-organ damage are not fully known. At least in part these effects are mediated through activation of mineralocorticoid receptors in endothelial cells, smooth muscle cells, myocardiocytes and neutrophils [41]. It has been suggested that activation of the mineralocorticoid receptor induces oxidative stress, endothelial dysfunction, inflammation and subsequent fibrosis [42].

In PA, there is a growing body of evidence that hyperaldosteronism exerts negative effects on the cardiovascular system, renal function and metabolism beyond the deleterious effects of hypertension *per se* [43]. A comparison of 124 patients with PA and 465 with primary hypertension documented an increased rate of cardiovascular events in patients with PA [7]. The reported history of stroke, myocardial infarction and atrial fibrillation was 4-fold (12.9 vs. 3.4%), 6-fold (4.0 vs. 0.6%) and 12-fold (7.3 vs. 0.6%) more common, respectively, in PA compared to essential hypertension. Other studies have supported these findings [6, 44, 45]. An association with increased left ventricular mass in patients with PA has also been described [46-48].

Current evidence shows that PA patients have an increased glomerular filtration rate compared to patients with primary hypertension, manifested as higher creatinine clearance and albumin excretion [49, 50]. This may be due to renal adaptation to increased extracellular volume, and counteracts the effects of aldosterone [51]. In the early stages, these changes are reversible [49, 50, 52]. Long-standing hyperaldosteronism seems to cause

renovascular damage, with a reduction of the glomerular filtration rate and progressive renal impairment [53].

Studies on the relationship between the metabolic syndrome and PA show conflicting results. A higher prevalence of the metabolic syndrome among patients with PA compared to primary hypertension has been reported [54], and data from the German Conn Registry found that diabetes mellitus is more prevalent in PA than in primary hypertension [55]. Furthermore, in patients with PA a significant reduction in plasma glucose and insulin levels has been observed after normalizing aldosterone levels [56]. Other studies found evidence for aldosterone excess having direct negative effects on beta-cell function in patients with PA [57, 58]. In contrast, when comparing 460 cases of PA with 1363 cases of primary hypertension, no differences were seen in the prevalence of hyperglycaemia and plasma glucose levels between the groups [59].

2.5 SUBTYPES OF PA

There are different subtypes of PA and the two major are aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH), also referred to as idiopathic hyperaldosteronism (IHA). They account for approximately 95% of cases [60]. The different subtypes and their relative frequency are listed in table 2.

APA, sometimes called Conn adenoma, was the first form of PA to be described, and accounts for approximately 30 –50 % of all PA [61]. It is unilateral and should be suspected especially in younger patients with more severe disease although it can also present with milder derangements.

BAH is the most frequently diagnosed subtype, accounting for 50-70% of the cases [61]. Generally, patients with BAH are older, have milder hypertension, are more often normokalaemic and demonstrate a less severe renin suppression in comparison with patients with APA [62, 63].

Unilateral (primary) adrenal hyperplasia (UAH, PAH) was previously considered to be a rare unilateral form of PA, as hyperplasia has traditionally been considered to be bilateral, and there were few reported cases [64]. UAH has, however, been accepted as a separate entity. The frequency of UAH is not fully known but is reported to be less than 5 % among patients with PA [61].

Adrenocortical carcinomas account for approximately 1% of cases with PA. According to a review of reported cases in the literature, the tumour size varied widely from 2.5 to 15 cm, and signs of malignancy on CT imaging were found in less than half of the patients [65].

2.5.1 Familial forms of hyperaldosteronism

Familial hyperaldosteronism type I (FH-I), also called glucocorticoid-remediable aldosteronism (GRA), is an autosomal dominant inherited disorder caused by a hybrid of the

gene regulating cortisol synthesis (*CYP11B1*) and the one that regulates aldosterone synthesis (*CYP11B2*). The result is a chimeric gene that is regulated by ACTH (which stimulates cortisol synthesis) but encodes for enzymes involved in aldosterone synthesis, thus resulting in the over-production of aldosterone [66]. The reported prevalence is 0.7 % among patients with PA [67]. Genetic testing for GRA is possible, and the recommended treatment is low-dose glucocorticoids [5].

Familial hyperaldosteronism type II (FH-II) is an entity with familial occurrence of APA, BAH or both [68-71]. Patients with FH-II do not display specific clinical or biochemical characteristics and are indistinguishable from sporadic forms of PA and the molecular basis of FH –II is still unknown. The diagnosis is made when ≥2 first- degree members of the same family have confirmed PA [67]. The estimated prevalence is 2.8-6% in adult populations of PA [67, 72].

Familial hyperaldosteronism type III (FH-III) was first described in 2008 in a family with a father and two daughters with severe hyperaldosteronism, suppressed renin levels and childhood- onset of resistant hypertension. All three underwent bilateral adrenalectomy in childhood, and histopathology demonstrated massive adrenal hyperplasia [73]. In 2011, the underlying molecular mechanism in FH-III was identified as a mutation in the gene KCNJ5 [74]. The mutation leads to uncontrolled aldosterone synthesis and cellular proliferation. After the first report, additional KCNJ5 mutations causing FH-III have been described [75-78].

Subtype	Relative frequency
Bilateral adrenal hyperplasia, BAH	50-70%
Aldosterone-producing adenoma, APA	30-50%
Unilateral adrenal hyperplasia, UAH	<5%
Aldosterone-producing adrenal cancer	<0.5%
Familial hyperaldosteronism type I, FH- I (GRA)	<1 %
Familial hyperaldosteronism type II, FH- II	3-6 %
Familial hyperaldosteronism type III, FH- III	<1 %

Table 2. Subtypes of primary aldosteronism. Adapted from Mulatero et al [61].

2.5.2 Functional differences between subtypes of PA

Under normal conditions, the RAAS system is activated with a patient in the upright position in order to maintain blood pressure. This signifies that renin levels increase, which leads to elevation of angiotensin II and aldosterone. In PA, aldosterone production is autonomous and the RAAS-system is under suppression. The response to stimuli that activate the RAAS-system (e.g. upright position) is variable. In APAs about 50 % are unresponsive to the RAAS –system while the rest are angiotensin II-responsive [37, 79]. In BAH the response to angiotensin II is preserved [62], while UAH is unresponsive to changes in the RAAS system

[80]. The functional differences may be used in the subtype classification of PA [37, 62]. See below.

2.6 GENETICS AND PATHOFYSIOLOGY OF PA

The synthesis of aldosterone is initiated by changes in the electrical potential of the cells in the zona glomerulosa of the adrenal cortex [72, 81]. Under normal conditions, potassium channels, calcium channels and ATPases are involved in maintaining hyperpolarization of the cell membrane [82]. Angiotensin II stimulates aldosterone synthesis by binding to the angiotensin II receptor on the cell membrane. Receptor signalling then leads to depolarization of the cell membrane. This causes calcium influx which triggers aldosterone biosynthesis (Fig 4).

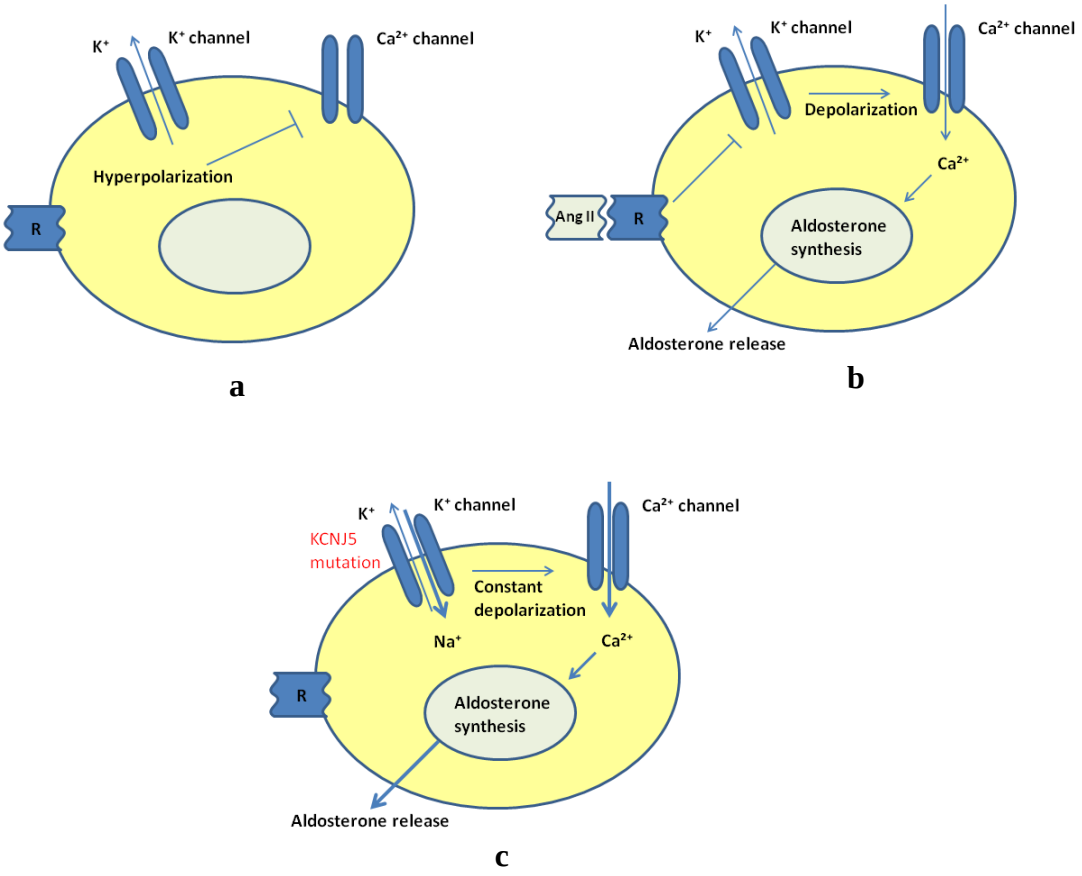

Fig 4. Simplified illustration of the aldosterone-producing cells in the zona glomerulosa under normal conditions and suggested mechanisms for the mutant KCNJ5 potassium channel. **(a)** Under baseline conditions, the cells are hyperpolarized due to high K^+ -conductance which prevents Ca^{2+}-entry into the cell. **(b)** When angiotensin II binds to its receptor, the cells depolarize, Ca^{2+} enters the cells and stimulates aldosterone synthesis. **(c)** The mutant KCNJ5 channel is permeable to Na^+, leading to a constant depolarization of the cell, calcium influx, increased aldosterone synthesis and cell proliferation. R, receptor; Ang II, angiotensin II.

As mentioned above, mutations in the gene KCNJ5 have been identified in adrenal samples from patients with PA [74]. This gene encodes for a potassium channel in the aldosterone-producing cell. Mutations in KCNJ5 affect the ion selectivity of the channel, resulting in sodium entrance into the cell and chronic depolarization of the cell membrane. This causes calcium influx which initiates uncontrolled aldosterone synthesis and cell proliferation (Fig 4). Since the initial publication, tumours removed from patients with APA from different populations have been screened for KCNJ5 mutations. In two studies with > 300 PA patients in each, mutations were present in 34-46 % of APA patients [83, 84]. No mutations were found in hyperplastic tissue surrounding the APAs, neither in patients with UAH (n=9) [83], nor in blood samples from patients with sporadic BAH [84]. On the other hand, KCNJ5 mutations have been found to cause bilateral hyperplasia in one hereditary form of PA, FH-III, as described above.

More recently, somatic mutations in two ATPase genes, *ATP1A1* and *ATP2B3*, have been identified [85]. Mutations of *ATP1A1* lead to depolarization of the zona glomerulosa cells, and mutations of *ATP2B3* increase intracellular calcium levels [85]. The net effect is increased aldosterone synthesis. Among 308 patients with APA, one of the mutations was found in 5.2 % and the other in 1.6 % of the cases. No mutations were identified in patients with FH or in patients with sporadic BAH, and no APA was identified that carried both a KCNJ5 and an ATPase mutation [82].

2.7 DIAGNOSTIC WORKUP OF PA

The diagnostic work-up for PA has traditionally been performed in three steps: screening, confirmation of the diagnosis, and subtype differentiation [86].

2.7.1 Screening

The established screening test for PA is the determination of the aldosterone to renin ratio (ARR) in plasma. The test utilizes the suppressive effect of autonomous aldosterone secretion on renin production, and has been used routinely since the 1980s [14, 15]. In the majority of PA patients renin levels are low, and the diagnosis should be suspected when plasma renin is suppressed and the plasma aldosterone concentration is increased [87].

The ARR is highly dependent on renin levels. The lower limit of detection for renin varies among different renin assays. This can have dramatic effects on the ratio, and the cut-off level for an elevated ratio is therefore laboratory dependent and renin-assay-dependent [60].
As the ARR is so dependent on the renin levels (in fact anyone with suppressed renin will have an increased ARR), it is important to interpret the ratio in light of the aldosterone concentration [80, 87]. Many centres use a lower limit for the aldosterone concentration in conjunction with the ARR for the test to be considered positive [20]. Thus, a high ARR in the presence of an aldosterone level in the mid-normal range or higher is regarded as a positive screening test [80, 87].

Although widely used as a screening tool, the test lacks standardization [60]. In a review of 16 prospective studies of the ARR as a screening test for PA with more than 3000 participants, the cut-off levels for the ratio varied [88]. Moreover, the setting (primary care, referral centre or hospital), the clinical conditions of measurement (diet, antihypertensive medication, body position) varied between studies as well as the tests used to confirm the diagnosis [88]. Ideally, the use of the ARR should be centre-based and adapted to the salt intake in the local population [80].

Several antihypertensive agents interfere with the renin-angiotensin-aldosterone system and may change the ARR (Table 3, Fig 5). There is no consensus on what medication can be accepted during testing, with opinions ranging from maintenance of all anti-hypertensive agents, apart from spironolactone and amiloride [63], to discontinuation of all concomitant medication if possible [89].

Medication	**Effect on aldosterone levels**	**Effect on renin levels**	**Effect on ARR**
ACE inhibitors	↓	↑↑	↓
ARBs	↓	↑↑	↓
Ca^{2+} blockers (DHPs)	↔↓	↑	↓
Diuretics	↔↑	↑↑	↓
Beta-adrenergic blockers	↓	↓↓	↑
NSAIDs	↓	↓↓	↑

Table 3. Medications that may affect the aldosterone to renin ratio. Modified from Funder et al [5]. ARR, aldosterone to renin ratio; ACE angiotensin converting enzyme; ARBs, angiotensin II receptor blockers; DHPs, dihydropyridines; NSAIDs, non-steroidal anti-inflammatory drugs

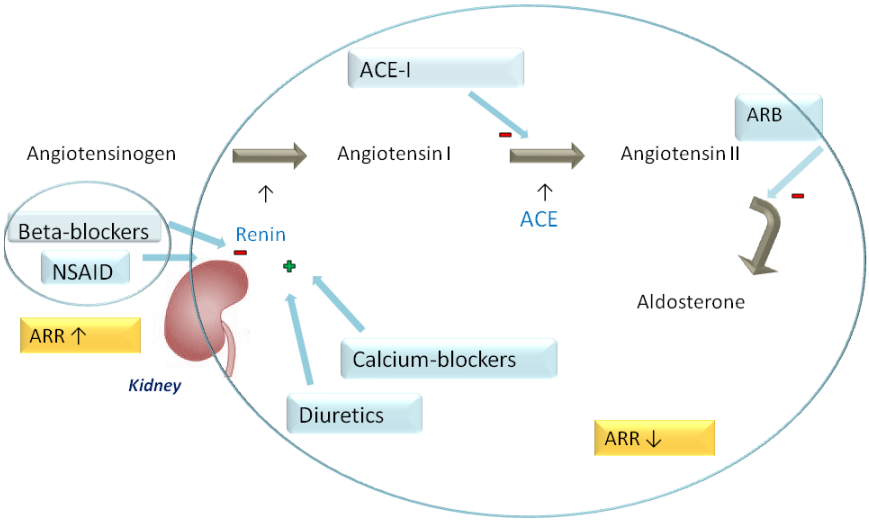

Fig 5. Antihypertensive agents, NSAIDs and their effect on the RAAS-system. NSAID, non-steroidal anti- inflammatory drug; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; ARR, aldosterone-to-renin ratio

2.7.2 Confirmatory tests

The guidelines of the Endocrine Society recognize four tests for confirming PA in patients with an elevated ARR: The fludrocortisone suppression test (FST), the oral sodium loading test, the saline infusion test and the captopril suppression test, but there is currently insufficient evidence to recommend one over the others [5]. The Japanese Endocrine Society recommends that at least two confirmatory tests are performed [90]. The underlying principle for the fludrocortisone suppression test and the two other salt loading tests is that an increase in intravascular volume due to sodium administration should normally decrease renin release and subsequently aldosterone, but in PA the aldosterone secretion is insufficiently suppressed by the high salt load [63].

The fludrocortisone suppression test (FST) has been considered the gold standard test for the confirmation of PA. It comprises the administration of sodium chloride and fludrocortisone acetate, which has a high mineralocorticoid effect. According to some authors this test is the one that most closely resembles the clinical features of PA, as it involves both stimulation of the mineralocorticoid receptor by fludrocortisone and volume expansion due to saline load [86]. Sodium chloride and fludrocortisone are administered orally during the course of 4 days. Plasma aldosterone and renin are measured on day 4. Hypokalaemia is common during the test and potassium levels need to be monitored closely. For many centres this test is impractical. A recent study showed that the FST could be shortened to 3 days and that a lower sodium chloride supplementation was sufficient [91].

In *the saline infusion test* an intravenous (iv) infusion of 2 litres of 0.9 % NaCl solution is administered during 4 hours, after which the plasma aldosterone concentration is measured [5].

In *the oral saline load test* sodium chloride is administrated orally during 4 days followed by measurement of the 24-h urinary aldosterone and sodium excretion. Some authors point out that for patients with a high sodium content in their diet, the administration of sodium chloride tablets is unnecessary [63].

In a comparison between the iv saline infusion test and the FST, with the FST taken as the reference test, Mulatero et al found that the saline infusion test gave a correct diagnosis in 86 of 98 patients (88 %) [92]. Ten per cent of the patients with PA were false negative and 16 % of the patients with primary hypertension were false positive with the saline infusion test. Similar results were found in a recent retrospective review comparing the iv saline infusion test and the FST in patients with APA and primary hypertension [93]. Some patients who underwent both tests had divergent results, and further studies are needed.

It should be noted that confirmatory tests requiring oral or intravenous sodium loading are potentially harmful for patients with severe uncontrolled hypertension, renal insufficiency or congestive heart failure [5].

The rationale for *the captopril suppression test*, also called the captopril challenge test, is that ACE inhibitors (e.g. captopril) inhibit the conversion of angiotensin I to angiotensin II, thereby inhibiting aldosterone production and increasing renin levels in healthy subjects [63]. Plasma renin and aldosterone are measured and the ARR is calculated before and 2 hours after oral administration of captopril. PA is diagnosed if captopril has failed to suppress aldosterone or if the ARR is elevated [86].

There are reports of false negative or equivocal results with the captopril suppression test [94]. Some authors advocate that this test, as it does not include salt administration, could be an alternative for patients where salt load could be harmful [95].

It is important to consider the antihypertensive medication during confirmatory testing. According to the guidelines of the Endocrine Society, antihypertensive agents with minimal or no effect on the RAAS system are preferred for the control of blood pressure during confirmatory testing [5] (Table 3).

2.7.3 Subtype classification

It is important to distinguish between unilateral and bilateral forms of PA, as the former can be cured by adrenalectomy while the latter are usually treated pharmacologically as shown in Table 4. For the subtype classification different radiological imaging techniques and adrenal venous catheterization as well as biochemical tests may be used.

PREFERABLY TREATED SURGICALLY
Unilateral aldosterone hypersecretion (APA, UAH)
Aldosterone- producing adrenocortical carcinoma
Familial hyperaldosteronism type II; FH-II (if unilateral adenoma)
Familial hyperaldosteronism type III; FH-III (if severe)
PREFERABLY TREATED MEDICALLY
Bilateral adrenal hyperplasia; BAH
Familial hyperaldosteronism; FH-I
Familial hyperaldosteronism; FH-II (if bilateral hyperplasia)
Familial hyperaldosteronism; FH-III (if response to medication)

Table 4. Treatment of different subtypes of PA. Modified from Young, W.F., Jr [87].

2.7.3.1 Computed tomography

Computed tomography (CT) gives anatomical information about the adrenal glands and it is a general agreement that it should be performed in all patients. It is important, although, to bear in mind that an adrenal mass on CT can be an APA, a macronodule as part of BAH or a non-hyperfunctioning tumour [60, 96, 97]. Further, a normal CT does not rule out an APA [96]. CT and magnetic resonance imaging (MRI) have comparable diagnostic performance for the detection of APAs [98]. In a review of the literature, CT/MRI was found to misdiagnose the cause of PA in 38 % when adrenal venous sampling (AVS; see below) was used as a reference test for lateralization of aldosterone hypersecretion [99]. Some authors have suggested that adrenalectomy may be performed without other localizing procedures than CT in patients younger than 40 years with an adrenal mass > 1 cm on CT [100]. The age limit has been modified to 35 years though (Young W.F., Jr personal communication 2013). In patients older than 35 years functional tests should be used in order to differentiate between unilateral and bilateral disease before deciding therapy.

2.7.3.2 Adrenal venous sampling

Adrenal venous sampling (AVS) is the most accurate test to differentiate unilateral from bilateral disease in patients with PA [37, 101]. The adrenal veins are catheterized by a percutaneous femoral vein approach. Samples are taken from the inferior vena cava, from a peripheral vein and from both adrenal veins. As the adrenal veins are very small, it can be difficult to place the catheter tip within the vein, especially the right adrenal vein. Frequently, the sample is obtained near the orifice of the vein and may be diluted by blood from other sources. To circumvent this problem, cortisol is measured simultaneously. Comparison of adrenal vein cortisol with peripheral cortisol permits an assessment of the adequacy of the

adrenal vein cannulation. However, there is no agreement on which criteria to use for defining successful cannulation and, in addition, criteria for defining lateralization of aldosterone hypersecretion are not uniform [12, 96, 99, 102-105]. The outlines below refer to routine practice at our unit.

Adrenal vein cortisol should be 3 times higher than in the periphery. Cortisol levels are also used in the calculation of aldosterone to cortisol ratios to correct adrenal venous aldosterone levels for differing degrees of dilution with non-adrenal venous blood. The aldosterone/ cortisol ratios from the right and left adrenal veins are then compared. In order to demonstrate lateralization the ratio should be >3 times higher on one side. Even in case of a distinct preoperative lateralization, not all patients are cured of PA by adrenalectomy [106-110]

2.7.3.3 Adrenocortical scintigraphy in primary aldosteronism

Adrenocortical scintigraphy allows non-invasive assessment of adrenal function and can be used to differentiate between APA and BAH [4]. With this technique, an intravenously injected radiopharmaceutical tracer is accumulated in the adrenal cortex and the uptake is visualized by scanning procedures. One of the most commonly used tracers is a cholesterol analogue, [^{131} I] 6β-iodomethyl-19-norcholesterol (NP-59). NP-59 is attached to low-density lipoprotein (LDL) in plasma and bound to LDL receptors in the adrenal cortex where it is esterified but not further metabolized [111]. Thus, NP-59 is a marker of adrenocortical cholesterol uptake and reflects steroid hormone synthesis in the adrenal.

It has been shown that 50 % of the normal adrenal uptake of NP-59 is ACTH dependent [111]. In order to reduce background radiocholesterol uptake and facilitate visualization of hyperfunctioning lesions, the corticotropin pathway is suppressed with glucocorticoids, usually dexamethasone, during the entire imaging process [112, 113]. In the normal case, the adrenal glands are visualized five days after radiocholesterol administration. Imaging is performed on days 3 and 6. Early breakthrough before the fifth day is compatible with aldosterone hypersecretion (Fig 6). Unilateral uptake is indicative of APA and bilateral uptake indicates BAH [114].

Initially, planar scintigraphic imaging was used. With the introduction of single photon emission computed tomography (SPECT), image resolution improved [115] and when combining with CT (SPECT-CT) the diagnostic performance was further developed [116, 117]. The reported accuracy for detecting APA varies widely with a range from 53 to 94 % according to a review of the literature [118], and a review of our own records showed 79 % [119]. Due to the high radiation dose, the low availability of the tracer and the limited diagnostic accuracy, adrenocortical scintigraphy is more rarely used in the work-up for PA nowadays.

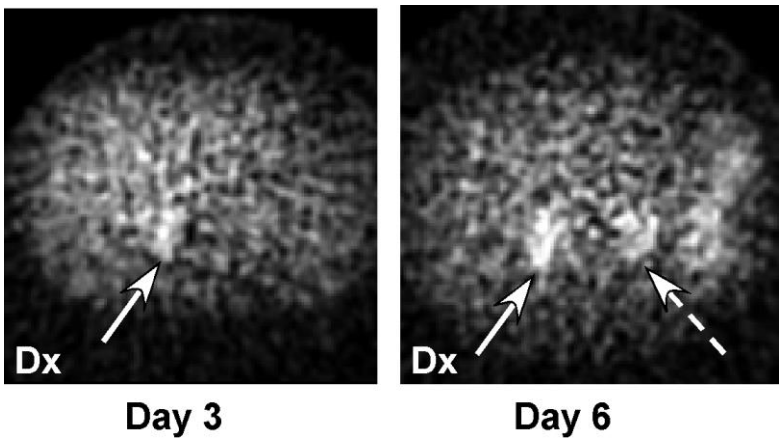

Fig 6. Adrenocortical scintigraphy with SPECT showing a right-sided aldosterone-producing tumour. A right-sided unilateral tracer uptake is visualized on day 3 (arrow, left image). This represents an early break-through compatible with aldosterone hypersecretion. On day 6 both adrenals are visualized (arrows, right image).

2.7.3.4 The posture stimulation test

The posture stimulation test is a biochemical test that may help to distinguish between angiotensin II-responsive and unresponsive forms of PA. It utilizes the differences in response to the RAAS- system between different subtypes of PA. Blood samples are drawn with the patients in the supine and upright positions, and the plasma renin and plasma aldosterone levels are measured.

In a review of 16 reports, the accuracy of the posture stimulation test was 85 % in 246 patients with surgically verified APA [120]. The posture stimulation test, if it shows lack of responsiveness may be consistent with angiotensin II-unresponsive forms of PA. Thus, it may serve an ancillary role for example in those patients for whom AVS was unsuccessful.

2.8 TREATMENT FOR PA

Recovery of cardiovascular, renal and metabolic abnormalities, prevention of cardiovascular complications and impairment of renal function are important goals of treatment in patients with PA in addition to normalization of blood pressure and correction of hypokalaemia. Normalization of circulating aldosterone concentrations or mineralocorticoid–receptor blockade is necessary to prevent the aldosterone-induced tissue damage that occurs independently of blood pressure [121].

2.8.1 Surgery

It is recommended that unilateral minimally invasive adrenalectomy should be offered to patients with unilateral hypersecretion of aldosterone [5]. There are reports that patients with bilateral disease and a clinically predominant nodule also may benefit from unilateral adrenalectomy [122, 123] and this could be an alternative for patients with poorly controlled blood pressure or intolerance to medications.

Almost all patients with unilateral PA improve with respect to blood pressure and serum potassium concentrations postoperatively [5]. Hypertension is cured in 20-72 % [124]. Persistent hypertension after adrenalectomy has been shown to independently correlate positively with age of the patients and duration of the disease [125]. There is also a positive association with a family history of hypertension and the severity of the preoperative hypertension [126]. Further, renal failure, even mild, seems to be associated with residual hypertension after adrenalectomy [127]. A clinical approach to predict blood pressure outcome after surgery is to evaluate the blood pressure response to spironolactone before adrenalectomy [128]. In addition, an aldosteronoma resolution score has recently been proposed to predict outcome after adrenalectomy [129].

2.8.2 Medical therapy

Patients with bilateral PA or patients who are not suited for, or have denied, adrenalectomy should be offered treatment with a mineralocorticoid receptor antagonist, i.e. spironolactone or eplerenone. Amiloride has been used as an alternative to mineralocorticoid receptor antagonists, but there are no clinical studies showing that amiloride protects against the negative effects of aldosterone *per se* [63]. A sodium restricted diet, maintenance of ideal body weight, tobacco avoidance, and a regular aerobic exercise contribute significantly to the success of pharmacological treatment [60].

Patients with BAH frequently require a second antihypertensive agent to achieve good blood pressure control. Hypervolemia is a major reason for resistance to drug therapy, and low doses of a thiazide are effective in combination with the aldosterone receptor antagonist [60]. Calcium channel blockers reduced blood pressure but not aldosterone levels when given to patients with PA according to a review of the literature [130]. When used in combination with spironolactone, the combination offered no additional benefit compared to spironolactone alone. Regarding treatment with ACE inhibitors or angiotensin II receptor blockers for the treatment of PA, few patients have been evaluated, and in general these agents are antihypertensive without major effects on aldosterone excess [5].

In a comparison between the mineralocorticoid receptor blockers spironolactone and eplerenone, the antihypertensive effect of spironolactone was significantly greater in patients with PA [131]. Both drugs were given once daily, but spironolactone and its metabolites have a longer bioactive effect compared to eplerenone. In clinical practice the highest doses of spironolactone given in the study, 225 mg, are seldom used due to adverse effects. Thus, if eplerenone were given twice daily and if the maximum dose of spironolactone were lower, the results would possibly have been different.

2.8.3 Effects of treatment for PA on cardiovascular and metabolic complications.

Evidence of decreased organ damage with treatment for PA has been obtained recently when 54 patients were prospectively followed up for a mean of 7.4 years after adrenalectomy or treatment with spironolactone [6]. Endpoints included myocardial infarction, stroke, any type of revascularization procedure and sustained arrhythmias. The frequency of combined endpoints was comparable in patients with PA and patients with essential hypertension, and

there were no significant differences between patients treated with adrenalectomy and patients treated with spironolactone. The outcome of renal function after treatment of PA was investigated in the same cohort. Increased creatinine clearance as well as albuminuria was reverted by treatment with adrenalectomy or spironolactone, with no significant difference found between mineralocorticoid receptor antagonist treatment and surgery [51] and other studies have reported similar results [49, 132]. Treatment for PA may in fact unmask underlying renal dysfunction [49, 52]. Further, left ventricular mass in patients with PA is reported to be reduced by adrenalectomy or treatment with spironolactone [46, 133]. Finally, treatment with either adrenalectomy or spironolactone restored insulin sensitivity in 47 patients with PA [134].

2.9 QUALITY OF LIFE

In one study including 22 patients with unilateral PA [135] and another including 21 with bilateral PA [136], quality of life (QoL) was assessed before and after treatment. Before treatment QoL was lower than in the general population in both studies. QoL improved after adrenalectomy [135] as well as after initiating medical treatment [136]. Improvement was slower and less complete in medically treated patients.

2.10 COST BENEFIT

In an attempt to compare the costs for adrenalectomy for unilateral PA with the estimated costs of life-long medical treatment for PA, adrenalectomy for surgically amenable PA was significantly less expensive than long-term medical therapy [137].

2.11 HISTOPATHOLOGY EXAMINATION OF THE RESECTED ADRENAL GLAND

2.11.1 Routine histopathology

In PA, preoperatively diagnosed with unilateral hypersecretion, histopathology examination of a resected adrenal gland can show a solitary adrenocortical adenoma, but there is often hyperplasia in the surrounding cortex or there may be multiple small cortical nodules and foci of marked hyperplasia, and sometimes even a second, smaller adenoma [68]. These findings sometimes make it difficult for the pathologist to determine the cause of aldosterone hypersecretion; whether it is an adenoma or hyperplasia that may be bilateral.

2.11.2 Functional histopathology

While routine histopathology demonstrates the anatomy of the adrenal cortex, functional pathology may indicate the site of hormone synthesis, i.e. the function of the cortex. Up to date, it is not possible to detect the aldosterone hormone in the adrenal cortex. Instead, there are methods that indirectly localize aldosterone production: *in situ* hybridization may help localize genes that via mRNA express enzymes responsible for the final steps in aldosterone synthesis. It is also possible to directly identify these enzymes with immunohistochemistry

with specific antibodies. Cortisol is also synthesized in the adrenal cortex and the principles mentioned above are also applicable for the detection of cortisol synthesis.

2.11.2.1 Aldosterone and cortisol synthesis

Aldosterone is synthesized from cholesterol in zona glomerulosa cells within the adrenal cortex [33]. The final steps in aldosterone synthesis are mediated by the steroidogenic enzyme aldosterone synthase (CYP11B2) [138], which has 11β-hydroxylase, 18-hydroxylase and 18-oxidase activity [138, 139]. The enzyme CYP11B2 is encoded by the gene *CYP11B2* [138] (Fig 1 and 7).

Cortisol also derives from cholesterol. In the normal adrenal, cortisol synthesis takes part in the zonae fasciculata and reticularis of the adrenal cortex [33]. Two steps in cortisol synthesis are catalyzed by the enzyme 17 α-hydroxylase (CYP17) and the final step by 11β-hydroxylase (CYP11B1) [139]. These enzymes are encoded by the genes *CYP17* [140] and *CYP11B1* [141] (Fig 1 and 7).

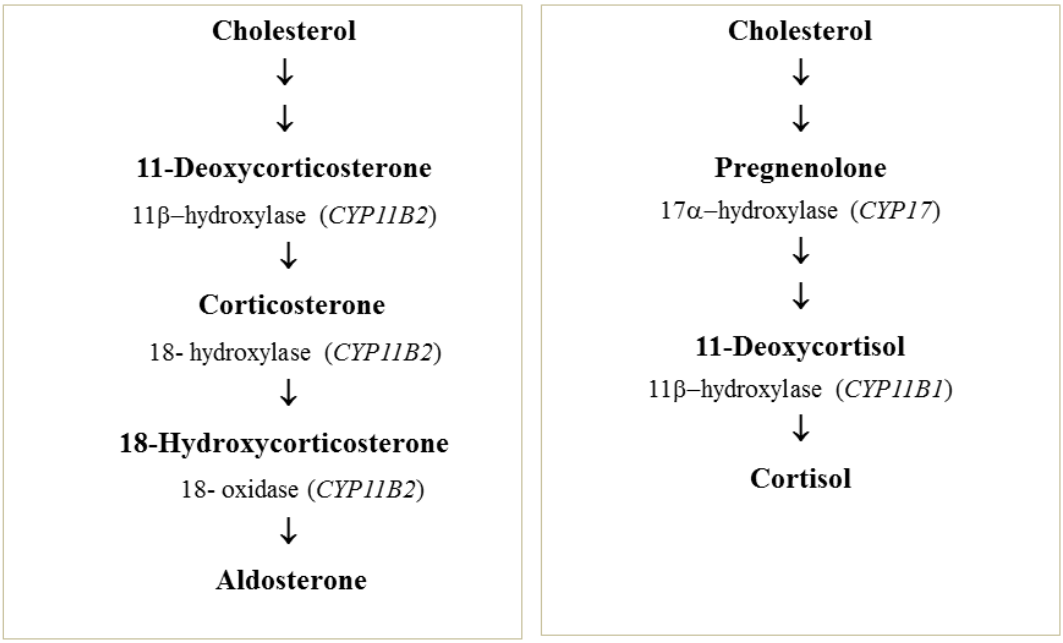

Fig 7. The final steps in aldosterone and cortisol synthesis, respectively, in the adrenal cortex. Intermediate and final products in bold. The enzymes 11β-hydroxylase, 18-hydroxylase and 18- oxidase are also called CYP11B2. 17α–hydroxylase also called CYP17. 11β-hydroxylase also called CYP11B1. The name of the genes encoding for the different enzymes are written in italics.

2.11.2.2 In situ hybridization

Expression of the genes *CYP11B2*, *CYP11B1* and *CYP17* can be demonstrated by *in situ* hybridization. With this technique, radioactively labelled probes are used to detect gene expression in adrenal tissue (Fig 8). The probes consist of sequences of nucleotides that are complementary to tissue mRNA which encodes for the enzymes CYP11B2, CYP11B1 and CYP17, respectively. The probes hybridize with mRNA, and the hybrids are visualized by autoradiography or fluorescence measurements. The site of gene expression in the adrenal cortex is thus localized. When gene expression is demonstrated it is assumed that enzyme synthesis occurs, which indirectly implies aldosterone production [142].

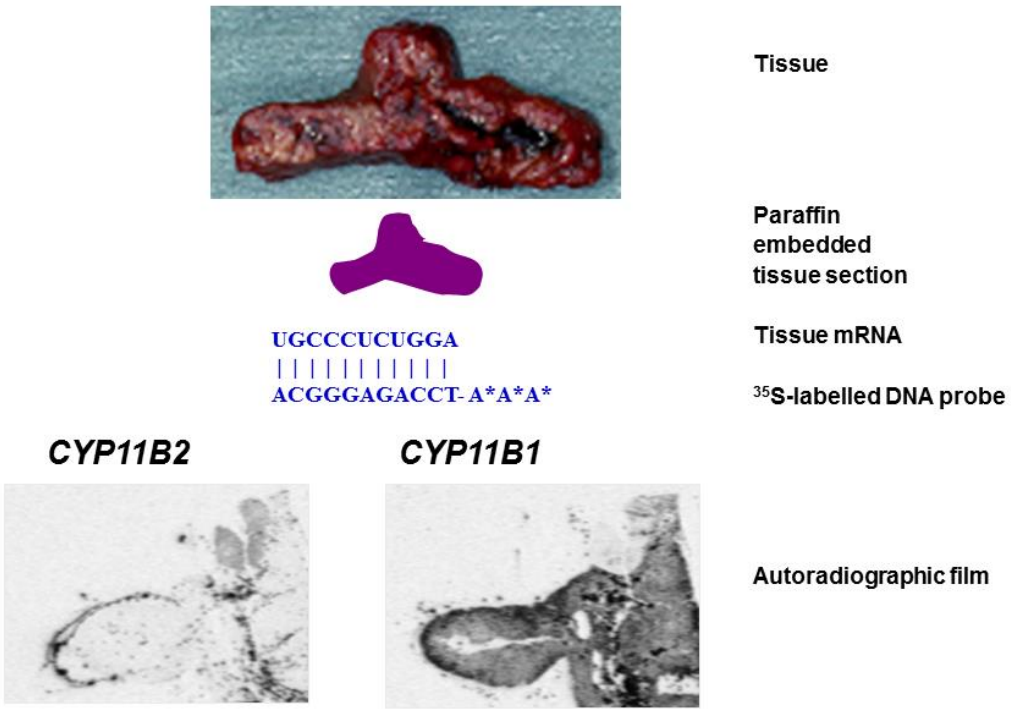

Fig 8. *In situ* hybridization. Adrenal tissue is cut in thin slices and mounted on slides. The tissue is prepared and hybridized with radioactively labelled (A*A*A*) nucleotide probes with DNA-sequences complementary to tissue mRNA. The hybrid is visualized by autoradiography and may be studied in microscope.

2.11.2.3 Immunohistochemistry

Specific antibodies directed against the enzymes CYP11B2 and CYP11B1, respectively, were previously developed [143] but have not until recently been successfully applied to human adrenal tissue [144]. Immunohistochemistry is a method to detect the enzymes by adding antibodies that bind to the enzymes. In brief, in this context, antibodies are added to adrenocortical tissue. Secondary antibodies, coupled to a stain reagent, are added. Binding of the secondary antibodies to the primary antibody-enzyme complex is visualized by staining.

Demonstration of the presence of the enzymes indicates that aldosterone and cortisol, respectively, are produced in the cells were the enzymes are localized [145].

2.11.2.4 Clinical implications for functional histopathology

Methods for functional pathology are not, at present, included in routine postoperative evaluation of patients with PA. Few studies have investigated the clinical utility in larger patient series or compared the results to long-term follow-up.

3 AIMS OF THE THESIS

The general aim of this thesis was to improve the overall management of patients with PA.

The specific aims were

- To study the prevalence of PA in a primary care setting by screening patients with hypertension. (Paper I).

- To establish immunohistochemistry with specific antibodies against enzymes in aldosterone and cortisol synthesis, respectively, as a method for postoperative differentiation between adenoma and hyperplasia in patients with PA. (Paper III).

- To evaluate the clinical utility of functional histopathology for the final diagnosis of PA by means of in situ hybridization and immunohistochemistry. (Papers II and IV).

- To investigate the long- term results of adrenalectomy for PA, with special focus on patients who were not cured by analysing clinical data in conjunction with functional pathology results. (Papers II, III and IV).

4 SUBJECTS AND METHODS

4.1 PATIENTS

4.1.1 Patients with hypertension treated in a primary care centre, Paper I.

Patients with hypertension were recruited from the Skytteholm primary care centre in Solna, Sweden. The inclusion criteria were age 18-70 years and a systolic blood pressure >140 mmHg and/or a diastolic blood pressure >90 mmHg at diagnosis. Exclusion criteria were known secondary hypertension, heart failure, myocardial infarction or transient ischemic attack/stroke within the latest six months, s-creatinine > 130 µmol/l, malignancy or severe illness, and severe mental or psychiatric disturbance with inability to consent to the study. The recruitment and exclusion procedures are shown in Fig 9. Two patients had already been investigated for PA in a specialist clinic; one was diagnosed with PA and the other was not. They were included when the prevalence was calculated. Thus, 235 patients were asked to participate and 182 (77%) accepted; 107 women and 75 men, median age 62 years, range 25-70.

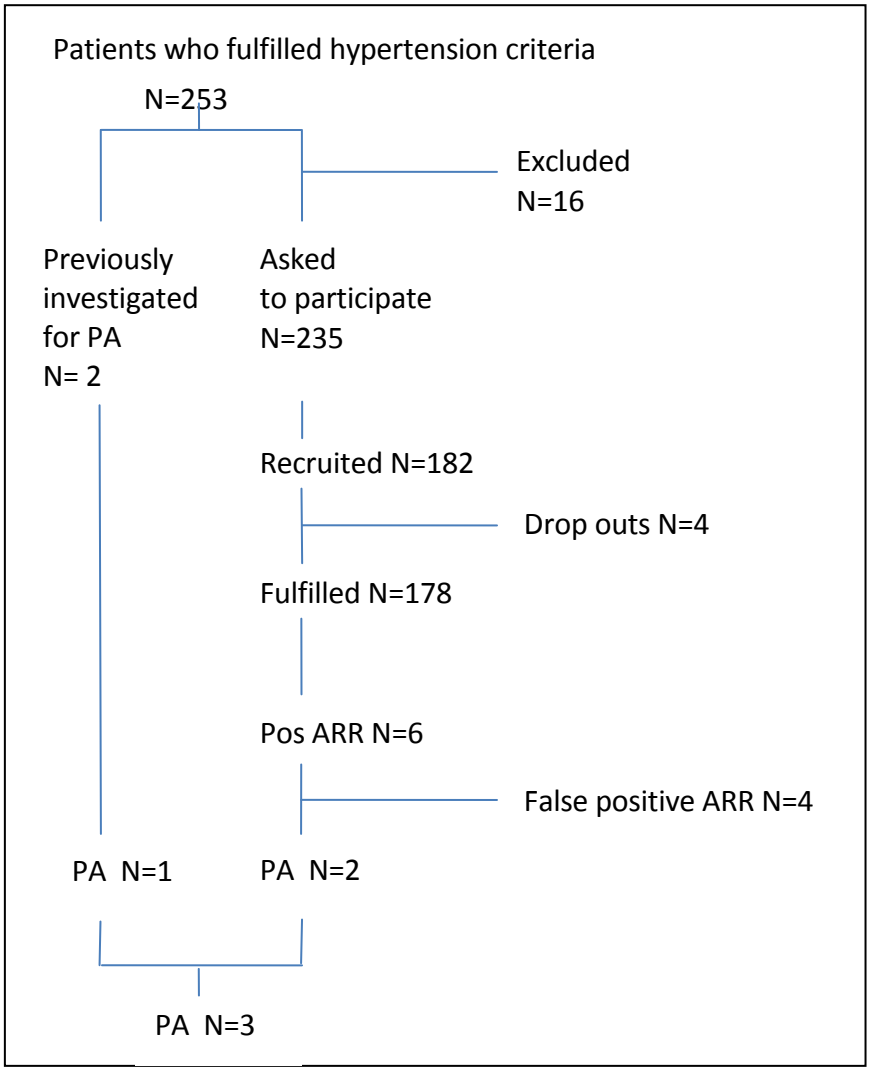

Figure 9. Flow sheet. Recruitment and outcome when investigating for PA.

4.1.2 Patient cohort, operated for PA, Paper II-IV.

The cohort consisted of 120 patients with PA with a supposed aldosterone-producing adenoma who underwent adrenalectomy at the Karolinska University Hospital Solna between 1985 and 2010. Twenty seven patients from the cohort were studied in paper II and 24 patients in paper III. In paper IV the entire cohort was studied.

4.1.3 Paper II

Twenty seven patients who underwent adrenalectomy for PA during the years 1987 - 2001 had clinical data and adrenal tissue sufficient for subtype classification of PA and for *in situ* hybridization of expression of the genes *CYP11B2*, *CYP11B1* and *CYP17*. There were 14 women, aged 16-79, mean 45 years and 13 men aged 42-75, mean 54 years.

4.1.4 Paper III

Twenty-four patients from the cohort of 120 patients were studied. We analysed clinical data and detected the enzymes CYP11B2 and CYP11B1 in adrenal tissue with immunohistochemistry. Patients who were cured of PA, patients who were not cured or had recurred, and patients in whom routine histopathology could not identify a tumour were included. There were 8 women. Median age was 51 (16-71) years.

4.1.5 Paper IV

In order to determine the long-term outcome and establish the final diagnosis, we investigated our cohort of 120 consecutive patients with PA who underwent adrenalectomy for a supposed aldosterone-producing adenoma between 1985 and 2010. There were 60 women. The median age for the cohort was 49 (16-78) years at the time of operation. Functional histopathology with immunohistochemistry and *in situ* hybridization was used when analysing adrenal specimens. Immunohistochemistry was carried out in 38 cases. Results from 24 patients had previously been published (paper III). Data from 27 previously published (paper II) and 31 more recently performed (Ulla Enberg personal communication and ref [146]) *in situ* hybridization analyses were also included.

4.2 METHODS

4.2.1 Study protocol, Paper I

Blood samples were drawn in the morning with the subject in the sitting position after 10 minutes´ rest. Plasma aldosterone, plasma renin and serum electrolytes were analysed and the ARR was calculated. The patients were evaluated with their on-going antihypertensive medication except for amiloride and aldosterone receptor antagonists. The ARR was defined as elevated when it was higher than 50 pmol/ng in the presence of an aldosterone concentration above 350 pmol/l. PA was also considered to be highly unlikely in case of a renin level above the upper normal range, 33 ng/l.

If the ARR was elevated and the patient was treated with a beta-adrenergic blocker and/or a non-steroidal anti-inflammatory drug (NSAID), a new blood sample was taken after withdrawal of this medication. Potassium supplementation was given if necessary. If

additional antihypertensive treatment was needed while the medication was changed, treatment was given with hydralazine or doxazosin or a raised dose of calcium antagonist.

If the ARR was elevated, a new test was performed and the 24-hour urinary aldosterone excretion was measured. Subjects with elevated urinary aldosterone were referred for further diagnostic work-up. Confirmatory tests for PA were either a 24-hour urinary aldosterone measurement during increased oral salt intake or a standard fludrocortisone suppression test.

4.2.2 Patient records and diagnostic evaluation in patients operated for PA, Papers II-IV

4.2.2.1 Clinical work-up and evaluation

Patient records were reviewed in retrospect regarding preoperative work-up, histopathology diagnosis, results from adrenalectomy and final outcome after long-term follow-up. When data were incomplete patients were contacted, and if needed, complementary hormone analyses were performed (papers II and IV).

The diagnostic work-up for PA varied over time. As far as possible, patients had been studied without medication known to interfere with the RAAS-system. In the early period, PA was diagnosed based on the findings of suppressed renin levels and elevated 24-hour urinary aldosterone excretion. Later, the ARR became a standard screening test, and the inability to suppress aldosterone with fludrocortisone or salt load were used as confirmatory tests.

All patients underwent a CT scan or MRI of the adrenals. For lateralization purposes ^{131}iodomethylcholesterol scintigraphy with dexamethasone suppression and/or AVS were performed. For successful selective cannulation at AVS, local cortisol concentrations had to be three-fold higher than in peripheral veins, and for lateralization the aldosterone to cortisol ratio on one side had to be 3 times higher than on the contralateral side.

Originally, patients were operated with open adrenalectomy but from the early nineties laparoscopic adrenalectomy was the method of choice.

Patients were considered to be cured of PA either when the ARR was < 100 pmol/ng and plasma aldosterone was < 400 pmol/l, or when urine aldosterone was < 35 nmol/ 24 h. Patients with normal blood pressure off antihypertensive medication were also considered to be cured. When new blood sampling was desired but could not be carried out for practical reasons, a general assessment was made on clinical grounds.

4.2.2.2 Histopathology

Routine histopathology with hematoxylin-eosin staining was performed, and all adrenal specimens were evaluated by one of two experienced pathologists. An adenoma was defined as a solitary nodule with a capsule or a well-demarcated border to the surrounding tissue, but in some cases it was difficult to decide whether the diagnosis was adenoma or hyperplasia. In many specimens both adenoma and hyperplasia were found.

4.2.2.3 In situ hybridization

After adrenalectomy, adrenal tissue was cut in slices for macroscopic photographing. Selected tissue specimens were subsequently paraffin-embedded.

4.2.2.3.1 Probe preparation

Oligonucleotide probes with sequences complementary to mRNAs encoding for the enzymes CYP11B2, CYP11B1 and CYP17 were synthesized [142] (Fig 8). The gene sequences in the coding regions for *CYP11B2* and *CYP11B1* are 95 % identical [147], why the oligonucleotide sequences were carefully chosen to avoid cross-reactivity. A GAPDH probe was used to ascertain the presence of mRNA in the tissue. A probe with a sequence identical to 17 α-hydroxylase (CYP17) was used as a negative control.

4.2.2.3.2 *In situ* hybridization

Paraffin embedded tissue specimens were cut in slices of 5 µm and mounted on slides. Hybridization solution was added and the slides were exposed to an autoradiographic film for 3 days. Gene expression was studied by visual inspection (Fig 8). In addition, in a number of cases quantification was performed by microdensitometry [148].

Several, but not all slides were subsequently dipped in autoradiographic emulsion and exposed for 4-10 weeks. They were then developed and counterstained with hematoxylin and eosin. Estimation of the mRNA expression was made using light-and dark-field microscopy by assessing the number of silver grains on the cells and the number of cells containing silver grains.

4.2.2.4 Immunohistochemistry

Polyclonal antibodies to the human enzymes CYP11B2 and CYP11B1 were provided by the MUKAI research group [144]. The antibodies were raised in rabbits and purified. CYP11B2 acts in the terminal steps in aldosterone synthesis, while CYP11B1 catalyses the final step in cortisol synthesis. Sections from paraffin blocks were cut at 4 µm thickness and stained with hematoxylin-eosin for diagnostic confirmation. Consecutive sections were stained with CYP11B2 and CYP11B1 antibodies, respectively. The dilution was 1:4000 for CYP11B2 and 1:6000 for CYP11B1. The immunohistochemistry was carried out using Autostainer Bond MAX, Leica Microsystems, Wetzlar, Germany. The antibodies were applied for 1 hour and then visualized with peroxidase enzyme and stained with diaminobenzidine (DAB) as chromogen and counterstained according to the manufacturer´s instruction.

4.2.3 Assays

Aldosterone in serum, plasma and urine was measured by conventional commercially available assays. Plasma renin was measured by conventional assays in paper I, while methods for determination of plasma renin varied during the investigation period in papers II -IV. In paper I the normal range for plasma aldosterone was 190-830 pmol/l and for urinary aldosterone 5.5-35 nmol/24 h and the normal range for renin was 3-33 ng/l.

4.2.4 Statistics

Values were presented as the median and range unless stated otherwise. For paired comparisons, the Student´s T-test was used for data with normal distribution, and for non-normal distribution the Wilcoxon matched pairs test was applied. For comparisons between two independent groups the Mann-Whitney U test was used and, for more than two groups, the Kruskal-Wallis test followed by Dunn´s post hoc test. For nominal data, the Chi^2 –test was employed or, when the number was small (<5), the Fisher´s exact test. Statistical significance was set at $p<0.05$. Statistical analyses were performed using Statistica StatSoft version 8.0 and 11 (Tulsa, OK, USA) and SigmaStat version 2.0.

5 RESULTS AND DISCUSSION

5.1 SCREENING FOR PA IN A HYPERTENSIVE POPULATION IN A PRIMARY CARE SETTING (PAPER I)

The prevalence of PA was investigated by screening patients with hypertension in a primary care centre, by measuring the ARR. The screening was fulfilled by 178 individuals. Six had a positive test (3%). This falls within the range of a large German population-based study that found a positive screening test of 0.2-7.0 % among patients with hypertension [149].

Two of the patients with a positive test were diagnosed with PA. In addition, PA had been confirmed in one of the recruited patients shortly before the investigation was started. Based on these cases the prevalence was 1.6 %. This is the minimum frequency as three patients with a pathological ARR did not complete the study. Thus, the estimated prevalence in the cohort might be between 1.6 and 3.3 %.

This estimate of 1.6 % was lower than the range found in previous studies reporting prevalences from 3.2 to 8.5 % in primary care patients screened without antihypertensive medication [19, 23, 24, 31]. Ito et al reported 3.8 % PA even in individuals with normotension or only mild hypertension [30].

To my knowledge there have been few studies in primary care patients with an unchanged antihypertensive regimen, and the reported prevalence displays a wide variation from 0.7 % to 13 % [22, 29, 32]. Our prevalence falls within this range and may represent a true estimate in the cohort. In a recent review, including patients with hypertension investigated with or without antihypertensive treatment, the mean prevalence of PA was 4.3 % in primary care [150].

It is important to consider the effects of the antihypertensive medication on the ARR. Almost all antihypertensive agents can influence the aldosterone and/or renin levels, which in turn affects the ARR and may lead to false positive or false negative ratios [5]. Our patients took more antihypertensive agents than reported in previous studies. Only 36 % of our patients had no or one agent as compared to 61 % and 75 % on monotherapy in previous reports [22, 32]. Moreover, approximately two thirds of our patients had 2 or more antihypertensive agents.

With the low number found, it was not possible to study the impact of medication on the ARR in PA. It is generally agreed that the ARR in PA patients is less affected by medication compared with that in patient with essential hypertension. In the patients diagnosed with primary hypertension in this study, the more agents used the higher plasma renin levels and the lower ARR levels were observed, while there was no significant change in aldosterone concentrations.

ARBs had a significant stimulatory impact on renin levels and thereby lowered the ARR. Patients on ARBs in monotherapy had significantly higher renin levels compared to hypertensive patients without antihypertensive medication, which resulted in a 73 % lower

ARR for the group of patients treated with ARBs compared with patients without antihypertensive agents.

Subjects treated with ACE inhibitors/ARBs, alone or in combination with other antihypertensive medications, also showed significantly higher renin levels and lower ARR levels compared to subjects without these agents (Fig 10). A similar effect on the renin levels and the ARR was seen in subjects treated with thiazide diuretics compared with those not taking this medication.

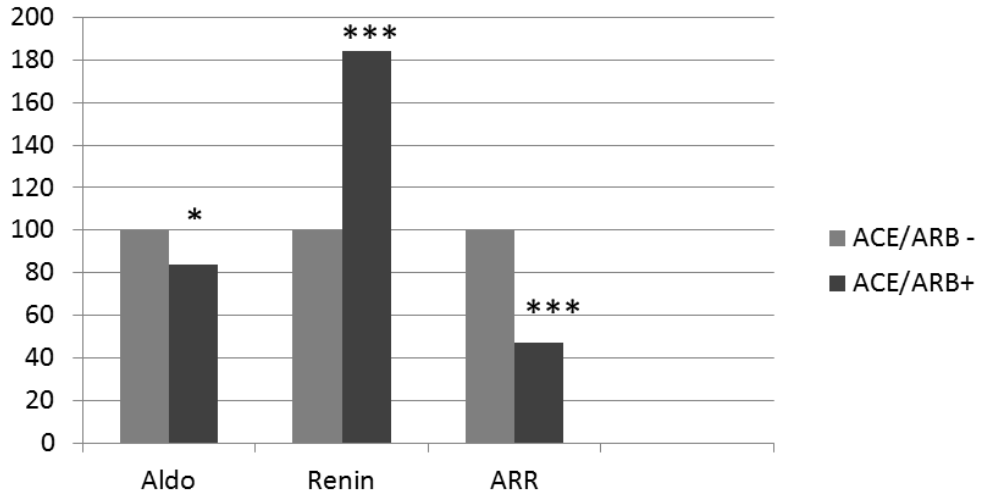

Fig 10. Relative difference (%) in aldosterone, renin and ARR in the group without (n= 70) and with (n=50) treatment with ACE-inhibitors or angiotensin receptor blockers. Patients were treated with monotherapy or combination with other antihypertensive agents. ACE; angiotensin converting enzyme, ARB; aldosterone receptor blocker

Suppression of the ARR due to ongoing antihypertensive therapy has also been reported from other studies [22, 151, 152]. In one of these, a reduction in the ARR of 43 % was seen when the ARB irbesartan was given as single therapy to patients with suspicion of PA, and the ARR became false-negative in 23.5 % [151]. For an ACE inhibitor (fosinopril), a calcium channel blocker (amlodipine), and an alpha-1 adrenergic blocker (doxazosin) the changes in ARR with treatment were -30 %, -17 %, and -5 % respectively. As the doses of antihypertensive medication vary in the different studies, it is difficult to make meaningful comparisons.

There is no consensus in the guidelines as to whether patients should be screened with or without medication but it is generally recommended to discontinue treatment with aldosterone receptor antagonists and amiloride [5]. Interestingly, in a subset of 15 of our patients, the ARR was determined on and off treatment with a low dose of amiloride (2.5-5 mg). Discontinuation resulted in a significant decrease in both aldosterone and renin with an

unchanged median ARR although with wide interindividual variations. The fact that the ARR remained unchanged was probably explained by the low dose of amiloride. Both of the patients diagnosed with PA were on treatment with amiloride. One patient had elevated ARR both with and without amiloride, while the other patient displayed an elevated ARR only after withdrawal of the drug.

Recommendations vary concerning other classes of antihypertensive agents than aldosterone antagonists and amiloride. Some investigators advocate screening without discontinuing therapy [22, 60, 153], others recommend avoidance of beta-adrenergic blockers [152, 154], avoidance of beta-adrenergic blockers and ARBs [151], withdrawal of all agents except for hydralazine, prazosin, and long-acting calcium channel blockers [12], or withdrawal of all agents except for an alpha-receptor blocker (doxazosin) or long-acting calcium channel blockers [89].

In a screening situation it is most important to avoid false negative results which may conceal PA. Negative results are not further investigated in most centres, nor was it done in our study. In a systematic review of 16 reports with 3136 participants, Montori and Young found that only two studies evaluated patients with "negative" ratios with a confirmatory test [88]. Schwartz and Turner found that the optimum cut-off level for the ARR was lower in patients on antihypertensive therapy than in those without therapy [22]. This suggests that different cut-off levels may be preferred in patients on and without medication. With the limited sample size and the large variability of antihypertensive drugs used in different combinations in the present study, this issue could not be further explored.

The cut-off value for the ARR that was selected in this study, 50 pmol/ng, is slightly above the mean of a Swedish series of normal subjects, where the mean and +2SD for the ARR were 40 and 104 pmol/ng [23]. Although suggested cut-off values range from 62 to 150 pmol/ng [23, 155, 156] it is still possible that the cut-off value for the ARR was too high in this context. One of the patients with PA had an ARR of 62 pmol/ng at initial screening. The potential consequence is that mild forms of PA may not have been detected, and that the true prevalence was underestimated in the cohort.

Some investigators advocate a minimum aldosterone level, usually 415 pmol/l, in addition to an elevated ARR for a positive screening test, to avoid a high rate of false positive tests [60, 89]. We used a threshold value for p-aldosterone of >350 pmol/l together with an elevated ratio for the ARR to be considered pathological. There are reports though, of p-aldosterone values as low as 250 pmol/l in primary aldosteronism [19, 24]. In our results, a hypothetical limit for the p-aldosterone value of ≥250 pmol/l would have raised the number of elevated ARRs from 6 to 17. Eight of these 11 additional patients with elevated ARR agreed to deliver new blood and urine samples after withdrawal of their antihypertensive medication. None was diagnosed with PA, which indicates that the original threshold value of > 350 pmol/l for plasma aldosterone was adequate and did not contribute to false negative tests.

As ARBs, ACE inhibitors or diuretics are generally known to raise the renin levels, a low renin level in a patient treated with one of these agents is strongly suggestive of PA [60]. In the present study, 14 patients treated with ACE inhibitors /ARBs had suppressed renin levels

below 5 pg/l. Some of their screening tests could be potentially false negative with the present cut-off levels for the ARR and aldosterone in the study, since no confirmatory tests were performed in these cases. Plasma renin, however, is not always suppressed in PA. The median and range for renin were 3.6 and 0.8-13 ng/l in 30 patients with verified PA, investigated at our clinic. Only 10 had subnormal levels, i.e. < 3 ng/l, 12 were in the range 3-5 ng/l and 8 in the range > 5-13 ng/l (unpublished).

Only one measurement of the ratio was performed in this study. Variations in the endocrine profile in patients with PA have been reported. Tanabe et al found that with repeated testing, only 37 % of 71 patients with PA had a consistent pathologic endocrine profile regarding all three parameters; aldosterone, renin and ARR, and one third of the patients had normal ARR levels on at least one occasion [157]. Jansen et al reported a five-fold difference in ARR values taken under the same conditions [26]. In contrast, Rossi et al reported a good reproducibility of the ARR [158].

In summary, the low detection rate of PA in the present cohort of hypertensive patients, the majority with mild or moderate hypertension, indicates that general screening in such population will not have great implications. This is in contrast to the high frequency of PA found in referral centres treating mostly resistant hypertension.

5.2 *IN SITU* HYBRIDIZATION IN THE POSTOPERATIVE DIFFERENTIATION BETWEEN ALDOSTERONE-PRODUCING ADENOMA AND HYPERPLASIA (PAPER II)

Twenty seven patients who underwent adrenalectomy for PA were investigated by analysing clinical data and by performing *in situ* hybridization. Twenty three (85 %) were cured of PA by adrenalectomy, with a median follow- up of 4 years, range 1-14. Hypertension was resolved in 11 patients (48 %). This is in keeping with reported cure rates of hypertension of 16–72 % in the literature [124]. Four patients improved with respect to urinary aldosterone excretion but were not cured of PA.

In the 23 patients who were cured by adrenalectomy, the histopathology diagnosis was adenoma in 10 cases. In 13 cases it was difficult at histopathologic evaluation to decide whether a nodule was an adenoma or a macronodule as part of a hyperplasia. In this study, a nodule larger than 5 mm was called a dominant nodule and a nodule smaller than 5 mm was called a small nodule.

In situ hybridization was performed on adrenal specimens from all patients. In adrenals from the 23 cured patients, expression of the gene *CYP11B2* indicating aldosterone production was seen in a dominant nodule in 21 patients, as exemplified in Fig 11. In 13 of these, gene expression was also registered in small nodules and/or in the adjacent tissue. Similar findings with *in situ* hybridization were also reported in a more recent study [159]. In addition, in the dominant nodules in most of the adrenals, expression of the genes acting in cortisol synthesis, *CYP11B1* and *CYP17,* was registered. Expression to a various degree of these genes in APAs has been demonstrated previously [142, 160]. In two patients no gene

expression at all could be visualized. This could be due to insufficient sensitivity of the method or lack of representative tissue.

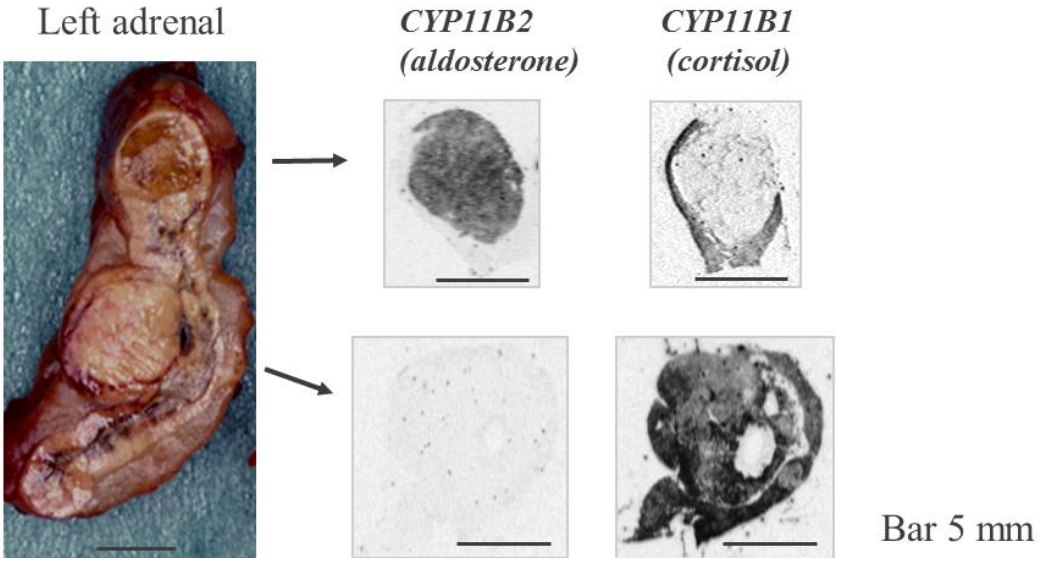

Fig 11. Expression of genes encoding for steroidogenic enzymes in an adrenal with two nodules. Macrophoto (left) and *in situ* hybridization autoradiogram demonstrating expression of *CYP11B2* in the upper nodule indicating aldosterone production, while the lower nodule is expressing *CYP11B1* indicating cortisol production.

Four patients were not cured of PA. In three cases, *in situ* hybridization demonstrated gene expression in nodules and abundantly in the surrounding zona glomerulosa, suggestive of hyperplasia as exemplified in Fig 12. In one patient no gene expression could be visualized. All four patients who were not cured were diagnosed with bilateral adrenal hyperplasia at long term follow-up.

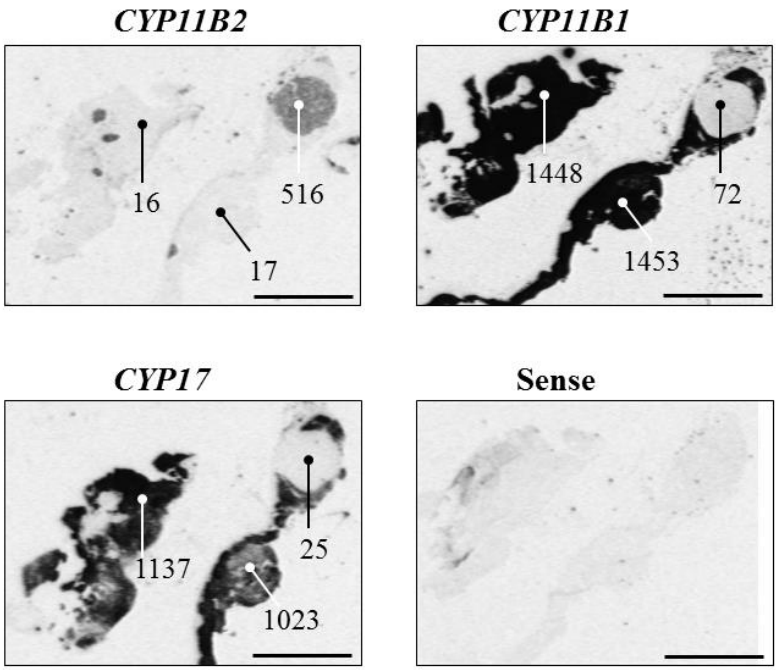

Fig 12. *In situ* hybridization autoradiogram from a patient with primary aldosteronism (Paper II, Fig 4). Several nodules expressing *CYP11B2* (top left) but not *CYP11B1* (top right) or *CYP17* (bottom left), suggesting aldosterone-producing hyperplasia. The numbers represent measurement with microdensitometry. The patient was a 46 year old male who was not cured of PA by adrenalectomy (Patient no 24 Paper II, no 20, Paper IV).

5.3 IMMUNOHISTOCHEMISTRY WITH SPECIFIC ANTIBODIES AGAINST ENZYMES IN THE FINAL STEPS OF ALDOSTERONE AND CORTISOL SYNTHESIS IN PRIMARY ALDOSTERONISM (PAPER III–IV)

Adrenals were studied from patients with PA who had undergone adrenalectomy because of assumed unilateral aldosterone hypersecretion. Immunohistochemistry results, results from routine histopathology and clinical outcome were compared. After routine preparation of adrenal specimens, consecutive sections were stained with hematoxylin-eosin and antibodies against CYP11B2 and CYP11B1, respectively. The immunopattern in the adenomas and in the remaining cortex were then studied.

Typically, the adenomas stained positive for CYP11B2, indicating aldosterone synthesis. Several adenomas also had CYP11B1-positive cells to a varying extent, indicating a capacity for cortisol synthesis. In the cortex, there were CYP11B1-positive nodules, and in some cases CYP11B2-positive aldosterone-producing cell-clusters, APCC, which has been described previously by Mukai and co-workers [144]. Overall, the findings were consistent with the results from Mukai and from a more recent study from the same group [161]. Fig 13 shows a typical aldosterone-producing adenoma.

In some adrenals there was no adenoma, or the adenoma had no immunoreactivity for CYP11B2. Instead, in these adrenals there were several nodules of varying size that showed positive staining for CYP11B2. Some of these nodules were also CYP11B1-positive. These findings were considered to represent aldosterone- producing hyperplasia. Adenomas that lacked reactivity for CYP11B2 were however CYP11B1-positive and were regarded to be non-hyperfunctioning, with a capacity to synthesize cortisol. Fig 14 exemplifies nodular hyperplasia with a CYP11B2-negative adenoma. The present results are comparable with a recent report that described patients without adenoma but with multiple aldosterone-producing cell clusters in the zona glomerulosa, one patient with positive CYP11B2-staining in micronodules and patients without immunoreactivity for CYP11B2 in adenomas [161].

In one adrenal from a patient who was not cured of PA, a CYP11B2-postitive adenoma/large nodule and diffuse CYP11B2- positive hyperplasia of the zona glomerulosa was found. This was considered this to be aldosterone-secreting hyperplasia. Diffuse hyperplasia was reported in adrenocortical tissue from a patient with severe hyperaldosteronism who was not cured with unilateral adrenalectomy, and the authors suggested the possibility of FH-III [161]. Our patient had no family history of PA, but she had been treated with spironolactone for several years before adrenalectomy, which may have contributed to the hyperplasia of the zona glomerulosa.

Immunohistochemistry was also performed in one case with secondary hyperaldosteronism (unpublished) and showed a broad, diffuse CYP11B2-positive hyperplasia of the zona glomerulosa (Fig 15).

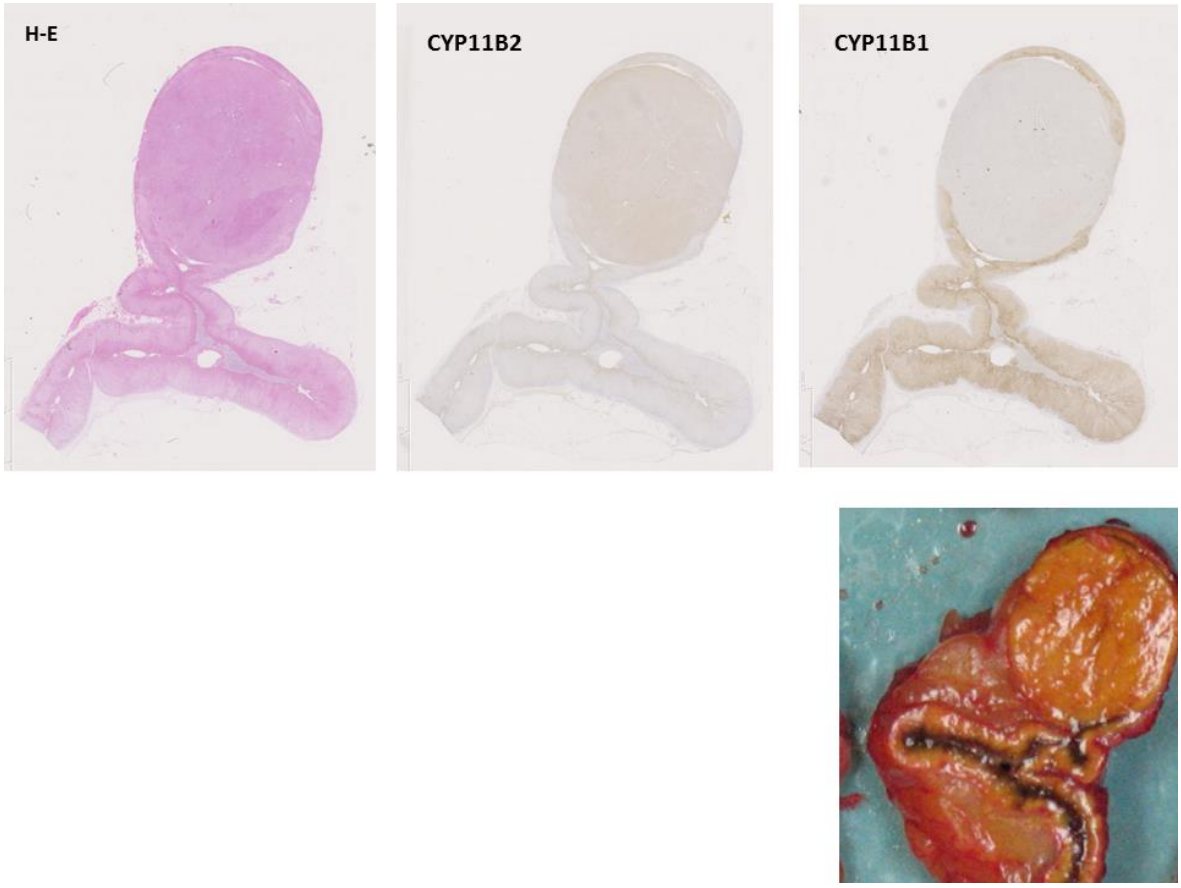

Fig 13 a.

Fig 13 a, b. A case illustrating patients with aldosterone-producing adenoma (APA) (fig 2, Paper IV). Adrenal with H-E staining, immunohistochemistry for CYP11B2 (aldosterone) and CYP 11 B1 (cortisol) and macrophoto of surgical specimen. (a 1,25x, b 5x). The tumour was positive for CYP11B2 but not for CYP11B1, while the cortex was positive for CYP11B1 but not for CYP11B2. The patient was a 42 year old male who was cured of PA by adrenalectomy with a follow-up of 3.5 years. H-E, hematoxylin-eosin.

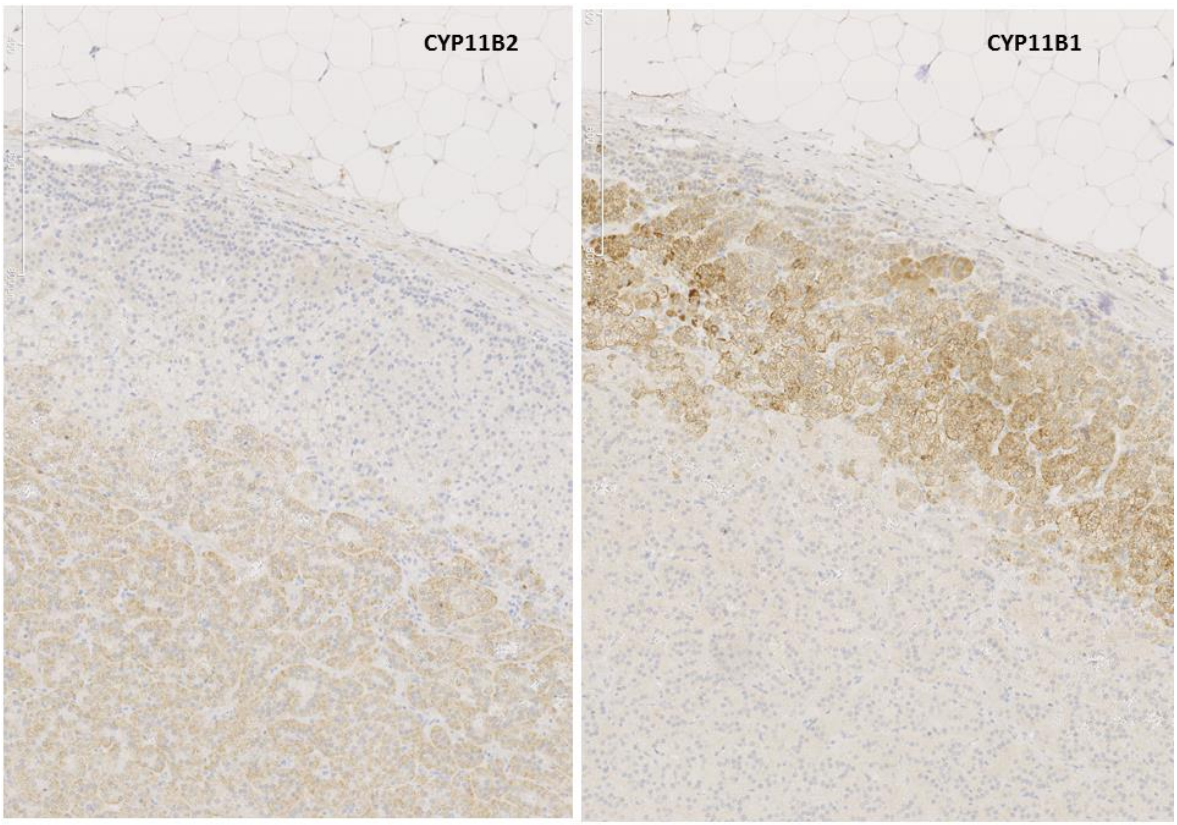

Fig 13b.

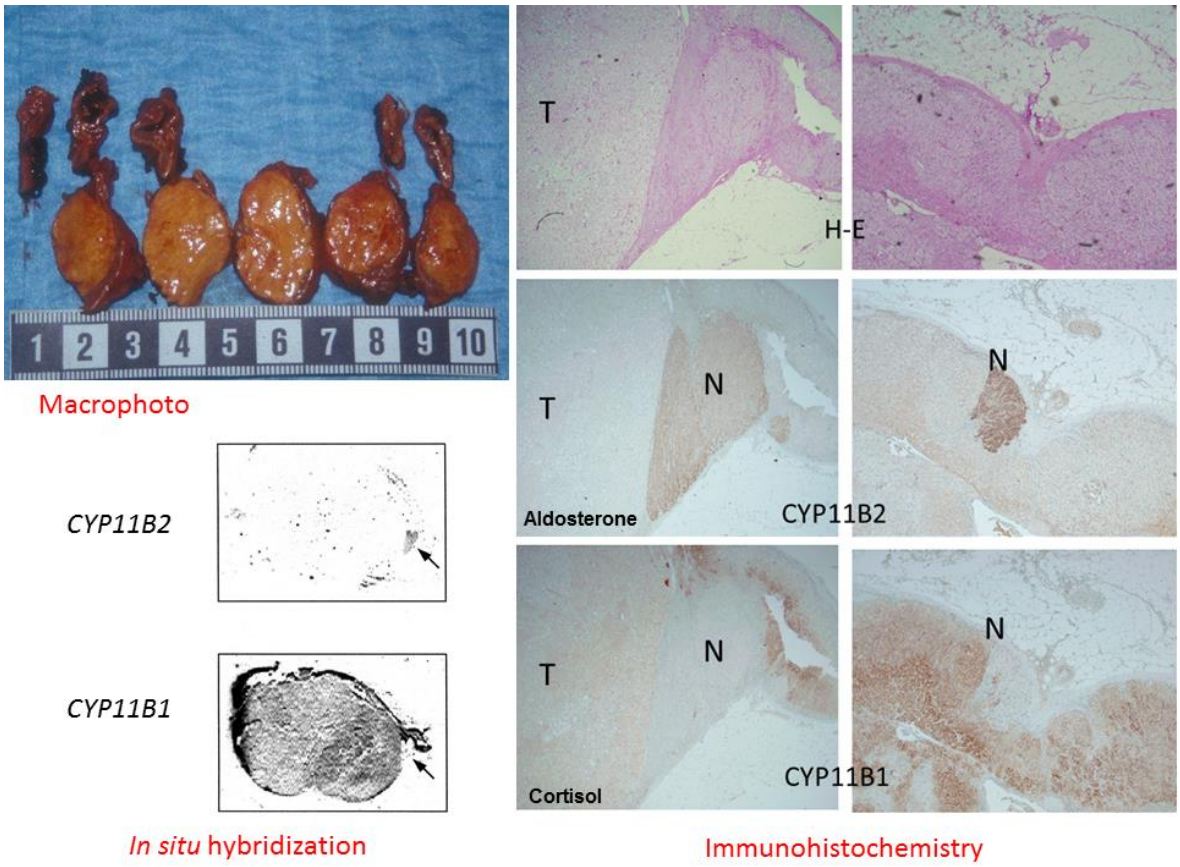

Fig 14. A case illustrating a solitary adenoma without aldosterone production but with aldosterone-producing nodular hyperplasia (Paper III, Fig 2 with addition of macrophoto and *in situ* hybridization). The patient was operated at the age of 62. At follow up 11 years later PA still remained. Macrophoto (upper left) and histopathology (upper right) showed a well circumscribed, yellow 2.5 cm adenoma, and no pathological changes were detected in the surrounding cortex with hematoxylin-eosin staining. Immunohistochemistry showed that the tumour had no CYP11B2 immunoreactivity while there were several cortical nodules with CYP11B2-positive cells (middle right). On consecutive sections these nodules had no CYP11B1-immunoreactivity (bottom right) while the remaining parts of the cortex were CYP11B1-positive. The tumour had a weak but unequivocal CYP11B1-immunoreactivity indicating that it represents a non-hyperfunctioning cortisol-producing adenoma. *In situ* hybridization (bottom left) showed similar finding with no *CYP11B2* in the tumour, but in a small nodule (indicated with arrow), while *CYP11B1* was expressed in the tumour but not in the small nodule (arrow). T, tumour; N, aldosterone-producing nodule; H-E, Hematoxylin-eosin.

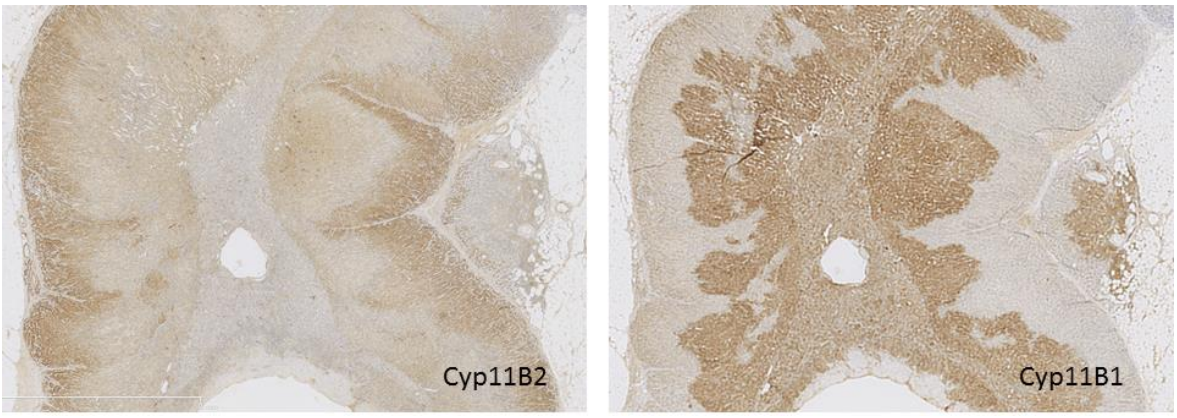

Fig 15. Patient with secondary aldosteronism (unpublished). The patient had high aldosterone and renin levels. CYP11B2 was expressed in the entire zona glomerulosa which was considerably thicker than normal and appears to compress the zona fasciculata expressing CYB11B1.

5.4 FUNCTIONAL HISTOPATHOLOGY IN PRIMARY ALDOSTERONISM (PAPER II-IV)

With functional histopathology with *in situ* hybridization with DNA-probes complementary to tissue mRNA as well as with immunohistochemistry with specific antibodies, the site of aldosterone synthesis in the adrenal cortex was localized.

In adrenal specimens from patients with APA, evidence of aldosterone synthesis in the tumour portions of the adenomas was demonstrated. In addition, it was found that in several of these adenomas, there were cells with the ability to produce cortisol. These findings are consistent with other reports [142, 144, 160-162]. Further, in the nontumour portion of the zona glomerulosa, there were cells with the ability to produce aldosterone, and small aldosterone-producing nodules or cell clusters were also found. These findings are also described by others [144, 159].

It was also demonstrated that in some patients, there was no evidence of aldosterone synthesis in the adenoma, but instead in several smaller nodules or more diffusely in the zona glomerulosa. These results are similar to other reports [144, 161] and are suggestive of aldosterone-producing hyperplasia.

The immunohistochemistry and *in situ* hybridization results were never discordant, although the *in situ* hybridization technique was less sensitive, and negative staining for CYP11B2 with *in situ* hybridization does not exclude the existence of gene expression. Fig 14 demonstrates an adrenal investigated with *in situ* hybridization as well as with immunohistochemistry with specific antibodies.

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5.5 FUNCTIONAL PATHOLOGY AND FINAL DIAGNOSIS IN PATIENTS WITH PA (PAPER IV)

In the cohort of 120 patients operated for PA, functional pathology was performed on adrenal specimens from 83 patients.

Patients were divided into two groups according to histopathology findings. Fig 16 shows an overview over the cohort and the final diagnosis. Among the 120 patients who underwent adrenalectomy, 100 had a histopathology diagnosis of adenoma and 20 were diagnosed with hyperplasia. In the adenoma group, one patient who was cured of PA was found to have hyperplasia when investigated with immunohistochemistry. Among the six adenoma patients who were not cured, all were investigated with functional pathology and five showed hyperplasia. One had an unclear diagnosis, and there is a possibility that the specimen was not representative. Among the 20 patients with histopathology diagnosis hyperplasia, 15 were cured. Thirteen of the cured hyperplasia patients were investigated with functional pathology which confirmed the hyperplasia diagnosis. Among the five patients with histopathology diagnosis hyperplasia who were not cured, functional pathology was performed in four and confirmed the hyperplasia diagnosis. Altogether, with functional pathology the diagnosis was changed in 6 cases (7 %).

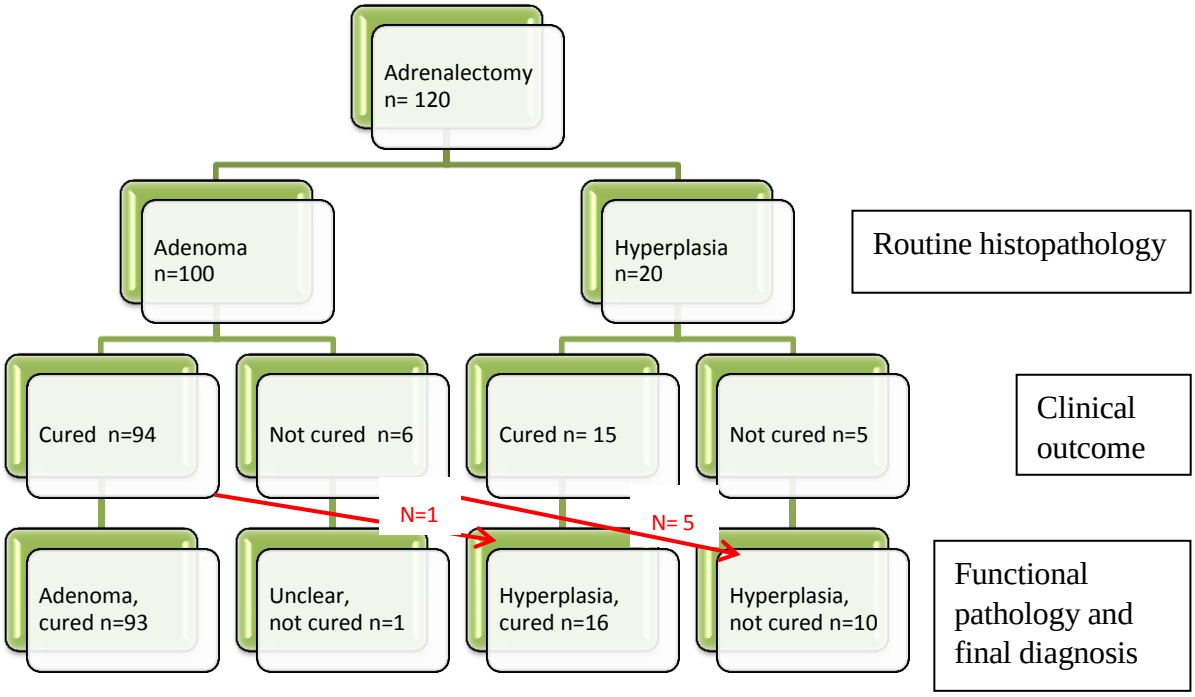

Fig 16. Overview of the cohort, clinical outcome and final diagnosis in patients with PA operated with unilateral adrenalectomy for supposed aldosterone-producing adenoma.

With routine histopathology, it is not possible to determine whether an adenoma is aldosterone-producing or not. Among our patients, functional pathology indicated lack of capacity for aldosterone synthesis in four cases. With routine histopathology these adenomas were supposed to be an APA. Three of these patients were not cured of PA. One patient was cured but may recur in the future and would need long-term follow-up. Further, in many adrenal specimens from patients with PA there are several nodules of various sizes in the zona glomerulosa, and it may be difficult to distinguish nodular aldosterone-producing hyperplasia from adenoma with some degree of focal hyperplasia, which is sometimes present in adenoma [145] patients. Also in these cases functional pathology can facilitate the diagnostic evaluation.

The distinction between adenoma and hyperplasia is important, as adenoma patients are usually cured of PA, while patients with hyperplasia may recur in the future and need long-term follow-up. There is evidence that hyperaldosteronism in PA has deleterious effects on the cardiovascular system [6, 7, 44]. Consequently, it is important to identify hyperplasia patients and give specific treatment if they persist or recur. Thorough post-operative evaluation is mandatory. The *in situ* hybridization technique is not suitable for routine use in a clinical setting, but immunohistochemistry may be appropriate for this purpose. It is therefore suggested that functional histopathology with immunohistochemistry is included in routine histopathology to support the diagnostic evaluation of adrenals from patients with PA.

5.6 LONG- TERM FOLLOW- UP AFTER ADRENALECTOMY AND DIAGNOSTIC VALUE OF IMMUNOHISTOCHEMISTRY: RESULTS (PAPER IV)

In the cohort of 120 patients who underwent adrenalectomy, the overall cure rate of PA was 91 %, with a median follow-up of 5 years. This cure rate was somewhat lower than in other recent reports that showed 98 % and 97 % cure, respectively [163, 164]. In these studies though, only patients who showed adequate lateralization with AVS were included, while patients with equivocal AVS results or patients who had not been investigated with AVS were excluded. In our cohort, which consisted of consecutive patients, also other methods for preoperative lateralisation had been used. Instead, our proportion of cure is similar to that of Zarnegar et al who reported a cure rate of 90 % [106]. Among their patients who were not cured, only 60 % had undergone preoperative AVS. Further, Hennings et al found a cure rate of 93 % with an average follow up similar to ours; 7 (3-22) years [165].

In 42 % of the patients cured of PA in this study, also hypertension was cured, and in the entire cohort 97 % improved their hypertension following adrenalectomy. These findings are in keeping with results from other centres. The reported cure rate of hypertension ranges between 16 % and 72 % [106, 122, 124-126, 129, 166-170] and the mean value of 19 reports was 41 % [124].

Eleven patients (9 %) were not cured of PA by adrenalectomy. Eight had persistent disease and three recurred after 2, 10 and 12 years, respectively. Six of the patients who were not cured had preoperative scintigraphic imaging that indicated unilateral disease. One had a scintigram with bilateral uptake, but was operated based on AVS results that showed lateralization. One AVS, in retrospect, was inconclusive and in two patients AVS showed lateralized aldosterone hypersecretion. One patient was operated due to the size of the adenoma and did not undergo preoperative lateralization procedures. There was no indication of a normal adrenal being removed in the cohort, as all patients improved biochemically or with respect to blood pressure after adrenalectomy, or demonstrated aldosterone- secreting hyperplasia by functional histopathology. Thus, the patients who were not cured, most likely had bilateral hyperplasia.

For subgroup classification, patients were evaluated clinically, biochemically and with routine and functional histopathology. The final diagnosis was APA in 93 (78 %), hyperplasia in 26 (22 %) and unclear in one patient (Fig 16). Preoperative serum potassium levels were lower in the APA group, and urine aldosterone was slightly higher. Similar findings were reported by one group [161], while in two other studies these differences were not observed [164, 169]. The cure rate of hypertension was higher in the APA group (46 %) compared to the hyperplasia group (19 %). However, almost all patients in both groups had improved blood pressure at follow-up and thus benefitted from the operation.

The frequency of hyperplasia in this material agrees with the frequencies reported in the literature, ranging from 16 to 56 % [122, 164, 169, 171-173], and even 74 % [163]. The definition of hyperplasia varies, though, as well as the size of the cohorts and the duration of follow-up. The reported outcome with respect to improvement of blood pressure among patients with hyperplasia ranges from 80 % to 100 % in the literature [122, 163, 169, 171, 173] which is also in accordance with our results.

Among the 26 patients with hyperplasia in our cohort, 16 (62 %) were cured of PA with a median follow up of 4 (2-11) years. Preoperative s-potassium was higher and preoperative p-aldosterone values were lower in cured than in non-cured patients, which indicates a milder disease.

Some of the cured patients may have unilateral adrenal hyperplasia. The prevalence of this entity is still unknown. In the earlier literature there were single case reports [64]. In contrast, Quillo et al reported 21 % with non-single adenoma (NSA) among 167 patients who underwent adrenalectomy for lateralized PA [164]. The cure rate was 100 % with regard to biochemical cure or normotension without antihypertensive medication, with a mean follow up of 13 months (0-185). Hyperplasia implies that several cells in the adrenal can develop nodules with high aldosterone production. It seems unlikely that this ability should be restricted only to one adrenal. Hence, from a biological perspective, it seems logical to believe that if general hyperplasia has developed in one adrenal, it may also exist or develop in the contralateral gland in the future. Patients who undergo adrenalectomy and show hyperplasia at histopathology evaluation are a selected population among hyperplasia patients, as these patients have shown a clear lateralization at preoperative AVS. From a clinical point or view, these patients may have unilateral adrenal hyperplasia and may never

recur. However, patients with hyperplasia with initial remission may recur after many years due to progress of the disease in the remaining adrenal. For instance, one of our patients recurred after 12 years and Walz et al reported a recurrence after 14 years [122]. Thus, it is not excluded that some of our cured patients with hyperplasia may recur, why long-term follow-up is needed.

6 CONCLUSIONS

- We found a prevalence of PA of 1.6-3.3 % when screening among patients with hypertension in primary care with their ongoing antihypertensive medication. It is important to consider the effects of antihypertensive medication on the ARR, as some agents lower the ARR and may give false negative results. Different cut-off levels for the ARR when screening with or without antihypertensive medication may be employed. General screening for PA in the hypertensive population cannot be suggested on the basis of the results from this study.

- Immunohistochemistry with specific antibodies, can localize the enzymes for the final steps of aldosterone and cortisol synthesis in the cortex of adrenal specimens from patients who have undergone adrenalectomy for PA. Presence of the enzymes is indicative of hormone synthesis. Accordingly, it is feasible to distinguish aldosterone-producing adenoma from hyperplasia in the postoperative evaluation of patients with PA.

- In patients who have undergone adrenalectomy for PA, functional histopathology, with *in situ* hybridization or immunohistochemistry with specific antibodies, provides additional information to routine histopathology in the postoperative evaluation. In cases with uncertain histopathology, in cases with patients not cured of PA, or even in cases with apparently clear histopathology findings, functional pathology is of great value in the distinction between aldosterone-producing adenoma and hyperplasia. We found that in most cases, functional pathology supported histopathology findings, but in 7 % functional pathology changed the diagnosis from aldosterone-producing adenoma to hyperplasia. As patients with hyperplasia need long-term follow up and specific treatment, functional pathology may contribute to improved clinical management of PA. We suggest that immunohistochemistry with specific antibodies be included in routine histopathology evaluation.

- In patients with PA who show lateralized aldosterone hypersecretion, adrenalectomy leads to cure of PA in most cases. Most patients who are not cured improve their blood pressure and thus benefit from the operation. Among patients who are not cured or recur at long-term follow-up, routine histopathology sometimes correctly diagnoses hyperplasia but in some cases identifies an adenoma that is incorrectly supposed to be aldosterone secreting. In contrast, with functional pathology, it is possible to localize the site of aldosterone synthesis. We have for the first time demonstrated the pathophysiologic pattern in adrenals from patients with persistent and recurrent disease in a cohort of consecutive patients with PA. A supposed aldosterone-producing adenoma may lack signs of aldosterone production or may show to be part of a nodular hyperplasia. A subset of patients who undergo adrenalectomy for unilateral aldosterone hypersecretion are cured although

histopathology evaluation shows hyperplasia. It remains to be seen if these patients stay cured or if they recur in the future, due to bilateral aldosterone hypersecretion.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Det övergripande syftet med den här avhandlingen var att förbättra omhändertagandet av patienter med primär aldosteronism.

Högt blodtryck, hypertoni, är vanligt förekommande och innebär en ökad risk för hjärt-kärlsjukdom. Man beräknar att 30-45 miljoner människor i världen har högt blodtryck, och enligt Världshälsoorganisationen WHO orsakar högt blodtryck drygt 4,5 % av den totala sjukdomsbördan. Högt blodtryck kan ibland bero på en bakomliggande sjukdom och benämns då sekundär hypertoni. Den vanligaste orsaken till sekundär hypertoni anses vara primär aldosteronism.

Primär aldosteronism orsakas av en överproduktion av hormonet aldosteron. Aldosteron påverkar njurarna till att minska utsöndringen av natrium som i stället stannar i kroppen och binder vätska, vilket leder till blodtrycksstegring.

Forskning har under senare år visat att aldosteron, utöver de blodtryckshöjande effekterna, även tycks ha en direkt skadlig effekt på hjärtkärlsystemet och njurarna utöver de negativa konsekvenserna av själva blodtrycksstegringen. Vidare har aldosteron möjligen ogynnsamma effekter på insulin- och blodsockerbalansen, och det finns även rapporter att patienter med primär aldosteronism har nedsatt livskvalitet jämfört med befolkningen i övrigt. Av dessa skäl ter det sig viktigt att finna patienter med primär aldosteronism och erbjuda behandling, inte bara mot blodtrycket utan även mot aldosteronstegringen.

Hos patienter med högt blodtryck anses primär aldosteronism vara den bakomliggande orsaken i 5-15 % av fallen. Förekomsten är högre i patientgrupper med svårbehandlat blodtryck, och lägre bland patienter i primärvården. Den förstnämnda patientgruppen är väl studerad, medan patienter i primärvården inte har undersökts i lika hög utsträckning. Olika studier har dessutom visat olika resultat, och förekomsten i primärvården har rapporterats vara 0,7–13 %. I en studie som ingår i denna avhandling undersöktes 178 patienter med högt blodtryck på en vårdcentral. Förekomsten av primär aldosteronism var 1,6–3,3 %. Dessa låga siffror motiverar inte utredning av primär aldosteronism hos alla patienter med högt blodtryck i primärvården. I studien framkom också, vilket även är visat tidigare, att olika blodtryckssänkande läkemedel påverkar de prover man kontrollerar vid utredning av primär aldosteronism. Man bör överväga att ha olika normalgränser för prover hos patienter som utreds med, respektive utan blodtrycksmedicinering.

Aldosteron bildas i binjurarna, som är belägna ovanför njurarna. Vid primär aldosteronism sker en överproduktion av aldosteron i den ena eller i båda binjurarna. Överproduktion från den ena binjuren beror vanligtvis på att det bildats en godartad knuta, adenom, som tillverkar aldosteron. När överproduktionen kommer från båda binjurar är orsaken en generell förtjockning, hyperplasi, av det skikt i binjuren där aldosteron bildas. Ensidig överproduktion av aldosteron kan botas genom att man opererar bort den ena binjuren. Överproduktion från båda binjurar behandlas med läkemedel som blockerar aldosteronets effekter.

Det förekommer att patienter inte botas med operation trots att noggrann utredning innan operationen visade tecken till ensidig överproduktion. I en del fall botas patienten först, men får återfall efter några år. Orsaken till att patienter inte botas eller får återfall, är att de har en hyperplasi i båda binjurar. Hyperplasin kan vara asymmetrisk, så att det vid utredning innan operation ter sig som en ensidig åkomma.

Efter operation undersöks den avlägsnade binjuren av en patolog med bedömning i mikroskop, sk histopatologisk undersökning. Många gånger är det då svårt att avgöra om det rör sig om ett adenom, eller om det föreligger hyperplasi. Distinktionen mellan adenom och hyperplasi är viktig, eftersom en patient med hyperplasi kan få återfall och alltså måste följas med regelbundna läkarkontroller, medan en patient med adenom anses vara botad efter operationen. Patienter som inte botas eller får återfall ska erbjudas specifika läkemedel enligt ovan.

Den histopatologiska bedömningen baserar sig på hur binjurens olika celler ser ut och är grupperade. Metoder som i stället kan visa i vilka celler som aldosteron produceras, funktionell patologi, vore av värde som ett komplement till den rutinmässiga histopatologin.
I studier som ingår i denna avhandling, har två olika metoder för funktionell patologi använts vid undersökning av binjurar efter operation. Den ena metoden, *in situ*-hybridisering, visar var de gener finns som är aktiva med att bilda enzym som deltar i produktionen av aldosteron. Med den andra metoden, immunohistokemi, används antikroppar för att detektera detta enzym. Där man finner aktiva gener respektive enzym, kan man anta att aldosteron bildas. Resultat från undersökningarna har ställts i relation mot kliniska patientdata vid långtidsuppföljning efter operation. De båda metoderna visade god överensstämmelse och demonstrerade var i binjuren aldosteron tillverkades vid adenom respektive hyperplasi. *In situ*-hybridisering är en omständlig och tidskrävande metod, medan immunohistokemiska analyser kan göras i apparatur i rutinsjukvård. Histopatologi och funktionell patologi visade aldrig motsägelsefulla resultat. I flera fall, där histopatologin var osäker, kunde man med funktionell patologi stärka diagnostiken och i några fall kunde man med funktionell patologi i stället ändra diagnosen.

Med syfte att undersöka utfallet av operation vid primär aldosteronism vid långtidsuppföljning, studerades en kohort av 120 patienter som opererats i följd under åren 1985-2010. Diagnosen innan operation var ensidig primär aldosteronism. I 91 % av fallen botades patienterna av operation, med en medianuppföljningstid på 5 år. Patienter som inte botades hade ändå nytta av operationen, eftersom deras blodtryck förbättrades. Funktionell patologi ändrade diagnosen i 7 % av fallen.

Slutsats: Den låga förekomsten av primär aldosteronism bland patienter med högt blodtryck i primärvården motiverar inte utredning avseende primär aldosteronism hos alla patienter med högt blodtryck. Med funktionell patologi har platsen för aldosteronproduktion i binjurarna indirekt påvisats hos patienter opererade för primär aldosteronism, och för första gången har dessa metoder applicerats på ett större patientmaterial. Immunohistokemi med specifika antikroppar bör ingå som ett värdefullt komplement i rutindiagnostiken av binjurar från patienter som opererats för primär aldosteronism.

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