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CYP11B2/CYP11B1 Immunophenotyping of 111 Adrenal Glands Excised from Primary Aldosteronism Patients leads to a Novel Classification of the Disease.

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ABSTRACT

Background. Primary Aldosteronism (PA) is due to the presence of an Aldosterone-Producing Adenoma (APA) in almost two thirds of the cases. Due to the lack of a specific antibody for human aldosterone synthase (hCYP11B2), the functional identification of aldosterone-producing sites in the excised adrenal gland has not been possible thus far. To tackle this challenge we used novel monoclonal antibodies for hCYP11B2 and hCYP11B1 to immuno-phenotype a large series of adrenal glands excised from patients with PA, including APA that were diagnosed by the "four corners criteria" and genotyped for KCNJ5 mutations.

Aims. Our aims were to identify the CYP11B2 and CYP11B1 steroidogenic patterns of the PA adrenal glands and to investigate whether immunostaining predicts the biochemical profile and/or the outcome of the patients.

Design and Method. Double immunohistochemistry and immunofluorescence was used to detect CYP11B2 and CYP11B1 in the excised adrenals from PA patients. Quantification of CYP11B2 and CYP11B1 immuno-staining was performed using an observer-independent PC-assisted method specifically developed in our laboratory that takes into account the area fraction and the staining intensity of the markers. The staining intensity was reported as H-score value and evaluated for each potential aldosterone-producing structure, including CYP11B2 positive clusters, APA and nodules.

Results. Based on the CYP11B2/CYP11B1 immunostaining of 111 excised adrenal glands from PA patients we could identify 5 major steroidogenic patterns (the proportion of cases shown in parentheses): Pattern 1: adenoma with uniform CYP11B2 staining and CYP11B1 staining outside the adenoma (16%); Pattern 2: adenoma with diffuse CYP11B2 staining, scattered CYP11B1 staining and some cells co-expressing CYP11B2/CYP11B1 inside the adenoma (46%); Pattern 3: adenoma expressing CYP11B1 and clusters of cells CYP11B2 positive (2%); Pattern 4: no adenoma, multiple clusters of sub-capsular CYP11B2 cells (25%); Pattern 5: multi-nodular adrenal cortex, clusters of sub-capsular CYP11B2 cells (11%).

The rate of pattern 2 differed significantly between mutated and wild-type KCNJ5 tumors (p=.012) and gender (p=.005). CYP11B2 H-score in APAs reflects aldosterone production before surgery (r=0.388, p=.002); by contrast, CYP11B1 did not correlate with the cortisol production. After adrenalectomy, steroidogenic patterns and H-score did not predict different aldosterone values between the patients, but, pattern 3 was prevalently detected in patients who biochemically resolved the PA picture, but still present high blood pressure levels.

Conclusions. Analysis of the excised adrenal gland from patients who were deemed to have an unilateral excess aldosterone production, by using specific antibodies for human CYP11B2 and CYP11B1, unveiled the existence of 5 different steroidogenic patterns. The H-score based on immunophenotyping which takes into account all aldosterone producing sites in the gland, including APA, clusters and nodules, reflected the aldosterone synthesis in the adrenal gland.

Hence surgically-curable PA is far more heterogeneous than previously held, which challenges the classical notion that PA entails only APA or bilateral hyperplasia. CYP11B2 immuno-staining is a promising tool for gaining better understanding in the pathophysiology of PA disease.

RIASSUNTO

Background. Nei centri di riferimento per l'ipertensione arteriosa più del 50% dei casi di iperaldosteronismo primario (PA) sono dovuti alla presenza di un Aldosterone-Producing Adenoma (APA). La surrenectomia determina la cura dall'ipertensione e la correzione del quadro biochimico di PA. La comprensione dei meccanismi che inducono lo sviluppo dell'APA è, pertanto, di fondamentale importanza clinica.

La diagnosi di APA ad oggi è basata sul riconoscimento, all'interno della ghiandola surrenalica, di un nodulo costituito da cellule chiare, senza che si abbiano informazioni funzionali sulla produzione di aldosterone. La diagnosi di APA nel surrene rimosso rimane, quindi, piuttosto incerta. Lo sviluppo recente degli anticorpi monoclonali per l'aldosterone sintetasi (CYP11B2) e l'11β-idrossilasi (CYP11B1) potrebbero permettere di localizzare le cellule responsabili dell'eccessiva produzione di aldosterone.

Scopo. Lo scopo di questo studio è stato di identificare i patterns di espressione degli enzimi steroidogenici CYP11B2 e CYP11B1 nella ghiandola surrenalica da pazienti con PA e indagare se la caratterizzazione immuno-fenotipica possa predire il profilo biochimico e/o l'outcome pressorio di questi pazienti.

Metodi. I surreni rimossi da 111 pazienti operati per PA che al cateterismo delle vene surrenaliche avevano mostrato eccesso di secrezione unilaterale, sono stati studiati mediante immunoistochimica e immunofluorescenza per CYP11B2 e CYP11B1. L'intensità della reazione cromogenica di CYP11B2 e CYP11B1 è stata quantificata utilizzando una metodica sviluppata nel nostro laboratorio che permette di attribuire ad ogni marker analizzato, un valore numerico denominato H-score, che è direttamente proporzionale alla percentuale di area positiva allo staining nella ghiandola e all'intensità di colorazione. Tutte le strutture potenzialmente competenti alla produzione di aldosterone (cluster di cellule CYP11B2 positive, APA e noduli) sono state analizzate per il calcolo dell' H-score corrispondente.

Risultati. L'analisi immunoistochimica nei pazienti PA ha permesso di identificare 5 patterns steroidogenici (la percentuale dei pazienti classificati nei patterns è espressa tra parentesi): pattern 1 comprendeva i surreni con un unico adenoma compatto e positivo per CYP11B2 e cellule positive per CYP11B1 nel tessuto adiacente all'adenoma (16%); nel pattern 2 sono stati classificati gli adenomi composti da cellule positive per CYP11B2 o CYP11B1 e alcune cellule che esprimevano contemporaneamente CYP11B2 e CYP11B1 (46%); i surreni classificati come pattern 3 presentavano un adenoma CYP11B1 positivo e alcuni cluster di cellule CYP11B2 positive (2%); nel pattern 4 non era presente un adenoma riconoscibile ma cluster di cellule CYP11B2 positive (25%); infine, i surreni del pattern 5 non mostravano alcun adenoma ma diversi noduli prevalentemente positivi per CYP11B1 e cluster di cellule CYP11B2 positive (11%).

Al baseline, il pattern 2 si distingueva dagli altri patterns per la maggiore prevalenza di pazienti con una mutazione al KCNJ5 (p=.012) e di genere femminile (p=.005). L'H-score per CYP11B2 negli APA era direttamente proporzionale alla concentrazione di aldosterone (r=0.388, p=.002), mentre l'H-score per CYP11B1 non era correlato con la concentrazione plasmatica di cortisolo. Dopo la surrenectomia, né la classificazione in patterns né l'H-score sono stati in grado di predirre l'outcome biochimico dei pazienti. Tuttavia, i pazienti che dopo surrenectomia mostravano correzione del quadro biochimico ma continuavano ad essere ipertesi erano classificati prevalentemente come pattern 3.

Conclusioni. Gli anticorpi specifici per CYP11B2 e CYP11B1 permettono di identificare cinque patterns immunofenotipici, molto variabili tra loro. La metodica sviluppata dal nostro gruppo per la quantificazione dell'H-score che tiene in considerazione tutte le strutture potenzialmente abili alla produzione dell'aldosterone, ovvero APA, clusters e noduli, fornisce informazioni accurate sulla produzione di aldosterone nei pazienti con PA.

In conclusione, i risultati di questo studio mostrano che le forme di PA siano molto più eterogenee di quanto precedentemente ipotizzato, suggerendo che accanto all'APA classico definito come

nodulo iper-funzionante e all'iperplasia esistano altri fenotipi che includono clusters e/o cellule che esprimono entrambi gli enzimi steroidogenici CYP11B2 e CYP11B1.

L'immuno-staining dei surreni con hCYP11B2 sembra essere un promettente strumento non solo per la diagnostica ma anche per lo studio della fisiopatologia dell'iperaldosteronismo.

INTRODUCTION

High blood pressure (HBP) is a major cardiovascular risk factor for myocardial infarction, congestive heart failure, stroke and end-stage kidney disease. With nearly 1.56 billion people of the world's adult population estimated to develop HBP in the year 2025,[1] this risk factor will reach epidemic proportions, resulting in a major public health problem and also a huge economic burden.

An effective anti-hypertensive therapy can highly reduce or even abolish the BP-related risk of cardiovascular disease; hence, the reduction of BP is of critical relevance today.[2] Secondary hypertension, a term used for the hypertension for which there is an identifiable cause, accounts for at least 40-50% of all hypertensive patients at referral centers.[3,4] The rate of secondary hypertension (HT) was recently reported to be 50% in patients drug-resistant HT.[5] Although this form of hypertension is rare, identification and treatment of the underlying cause might lead to the cure or significant improvement of BP. Thus substantial attention should be made to identify and characterize the secondary causes of hypertension.

Endocrine Hypertension

Endocrine hypertension ranks amongst the most common causes of secondary hypertension. The most prevalent form is primary aldosteronism, that will be discuss in detail in the next section, followed by pheochromocytoma paraganglioma (PPGL), Cushing's syndrome, hypothyroidism, hyperparathyroidism, acromegaly, rare forms of mineralocorticoid hypertension (e.g. apparent mineralocorticoid excess and Liddle's syndrome).

PPGL are neuroendocrine tumors that arise from chromaffin cells of the adrenal medulla and produce, metabolize and usually, but not always, secrete epinephrine, norepinephrine, dopamine and a number of other substances that are responsible for the common symptoms that are associated with this disease, e.g. high heart rate, headache, dyspnea, flushing and anxiety.[6,7] The prevalence of PPGL due to the lack of prospective studies, is not known precisely although it has been reported to occur in 0.05–0.2% of hypertensive individuals.[8,9]

Cushing's syndrome is a condition characterized by a chronic and inappropriately high exposure of tissues to glucocorticoids, which is caused by excessive secretion of ACTH in 80% of cases.[10] In Cushing's syndrome the normal cortisol feedback mechanism of the hypothalamic-pituitary-adrenal axis is disturbed, with loss of circadian rhythm and excess cortisol production resulting in hypercortisolism. Incidence of the disorder ranges from 0.7 to 2.4 per million population per year.[11] In screening studies of obese patients with type 2 diabetes, especially those with poor blood glucose control and hypertension, the reported prevalence of Cushing's syndrome is between 2% and 5%.[12]

Hyperparathyroidism is a common endocrine disorder characterized by oversecretion of parathyroid hormone (PTH), which may be autonomous (independent of serum calcium levels) or the result of a physiological stimulus.[13,14] This disease is generally divided into three types: primary (autonomous), secondary (that results from a chronic stimulus causing PTH secretion) and tertiary (autonomous PTH oversecretion from secondary parathyroid hyperplasia).[15] The incidence of this disorder is closely related to the frequency of routinely obtained biochemical screening tests, socioeconomic issues (i.e. low vitamin D status), dietary habits (i.e. calcium and vitamin D supplementation), as well as the degree of awareness among physicians.[16] Not unexpectedly, these varying conditions affect the rate of this disease in different countries. In Italy, a recent study reported that the mean hospitalization rate is 12.9/100000 inhabitants per year.[17]

Acromegaly is characterized by pituitary somatotroph adenomas that hypersecrete growth hormone (GH), which raised the levels of insulin-like growth factor 1.[18] Common clinical manifestations include hypertension, skeletal and soft tissue overgrowth, particularly in the face and extremities, as well as headache and arthritis.[19] The prevalence of acromegaly is estimated to be 8–24/100.000.[20]

Primary Aldosteronism

Primary Aldosteronism (PA) is the most common endocrine form of secondary arterial hypertension among the patients referred to specialized centers for arterial hypertension[21] and among patients with resistant hypertension.[22] PA comprises a group of disorders characterized by excess

aldosterone production.[23] Aldosterone secretion is normally stimulated by angiotensin II and potassium, whereas in PA, aldosterone production is inappropriately high, relatively independent from the renin angiotensin system and/or potassium and is not suppressed with sodium overload.[24] Overproduction of aldosterone induces high blood pressure values, sodium retention, increased potassium excretion leading to hypokalemia, suppression of plasma renin, and finally cardiovascular damage.[25]

The biochemical picture of PA can be caused by either bilater adrenal hyperplasia (60-65%) or unilateral adrenal hyperplasia (2-3%), aldosterone-producing adenoma (APA) (30-35%), aldosterone-producing adrenocortical carcinoma (<1%), glucocorticoid-remediable aldosteronism (GRA) (familial hyperaldosteronism type I (FH-I)) <1%, familial hyperaldosteronism type II (FH-II) <1%, familial hyperaldosteronism type III (FH-III) <1% and ectopic aldosterone-producing adenoma or carcinoma <1%.[26]

Familial Forms of PA

GRA is characterized by the autosomal dominant transmission of severe early onset aldosteronism and hypertension.[27] This disorder is caused by a chimeric gene deriving from a recombination between the coding sequences of the aldosterone synthase (CYP11B2) and the promoter sequences of 11β-hydroxylase (CYP11B1) genes, both located on chromosome 8.[28] This leads to synthesis of aldosterone no longer regulated by the renin-angiotensin system but, under control of ACTH.[29]

FH-II is a familial form of unknown genetic basis that has been shown to be in linkage with chromosomal region 7p22 in families from different continents.[30] Cases can present clinically with APA or bilateral hyperplasia, and are indistinguishable from patients with sporadic primary aldosteronism.[31]

FH-III was discovered starting from 2011 after the identification of somatic mutations in the KCNJ5 gene encoding the GIRK4 K^+ channel.[32] Choi et al. described a threonine (Thr)-to-alanine

(Ala) substitution at codon 158 in the KCNJ5 gene, resulting in reduced K^+ selectivity, increased Na^+ conductance and cell membrane depolarization. After this report, several germ-line KCNJ5 mutations were independently detected in patients presented with different clinical degrees of PA (G151E, I157S, Y152C, E145Q).[33-36] In 2014, our group also identified one novel mutation in the KCNJ5 gene (c.446insAAC) causing PA with severe drug-resistant HT.[37] Based on the available knowledge of this disease, Lenzini and Rossi proposed a new classification for FH-III as a genetic disease made of two subtypes, one severe (Type A) presenting severe aldosteronism and bilateral massive adrenal hyperplasia requiring bilateral laparoscopic adrenalectomy and one milder (Type B) with much less adrenal hyperplasia and drug-responsive HT (Table 1).[38]

Table 1. Clinical molecular classification of Familial Hyperaldosteronism

Type	Subtypes	Transmission	Gene Mutation	CT Finding	Treatment/ Response
FH-I		Au Dom	CYP11B2/CYP11 B1 Chimeric	BAH or APA	Dexamethasone/Correction
FH-II		Au Dom	Gene Unknown	BAH or APA	MRA or Adrenalectomy/Correction
FH-III	**Type A**	Au Dom	KCNJ5 (T158A, I157S, E145Q)	BAH	MRA/Drug Resistant Hypertension
	TypeB	Au Dom	KCNJ5 (G151E, Y152C)	BAH	MRA/MRA Responsive Hypertension

FH: familial hyperaldosteronism; BAH: bilateral adrenal hyperplasia; APA: aldosterone producing adenoma; MRA: mineralocorticoid receptor antagonist.

Modified from Lenzini and Rossi, Curr Opin Pharmacol. 2015.

Sporadic Cases of PA

The two most common causes of sporadic PA are APA and idiopathic hyperaldosteronism with bilateral adrenal hyperplasia (BAH), which account for more than 90% of clinical cases.[26] The differential diagnosis between APA and BAH is crucially important for the choice of treatment: adrenalectomy can cure or ameliorate hypertension in 55% of patients[39] whereas patients with BAH can be pharmacologically treated with mineralocorticoid receptor antagonists.[21] Currently, the only reliable procedure allowing differentiation of the unilateral from bilateral PA and identification of APA side preoperatively is adrenal venous sampling (AVS).[40-42] In medical centers where AVS is available and strict cutoff values for the lateralization index are used,[43] almost two thirds of PA cases are attributed to an APA whereas one third remains by exclusion classified as bilateral idiopathic forms. The opposite is seen when AVS is unavailable, thus explaining the key role of AVS for subtyping.[44] The diagnosis of APA is usually established when the following *'Four Corners Criteria'*[44] are satisfied: 1) there is evidence of PA at the screening tests, with an inappropriately high aldosterone-to-renin ratio, 2) aldosterone secretion is lateralized at AVS, 3) an adenoma is detected at pathology, and, crucially important, 4) PA is corrected by adrenalectomy.

However, at pathology the identification of an APA may be challenging because of the lack of clearcut criteria to define the aldosterone-producing cells.[45]

Pathologists routinely perform hematoxylin and eosin (HE) staining of the adrenal gland to detect the presence of one or more nodules that are strongly suggestive of APA, and then identify the cell type that is predominant in the nodule(s): compact small cells or large foamy lipid-rich cells. However, a mixture of both cell types can be frequently found in the nodule(s), even in association with atypical cells characterized by nuclear enlargement, nucleoli and/or hyperchromasia. The histological criteria to distinguish between an adenoma and a nodule are not well defined. Benign or malignant neoplasms are most often monoclonal, but clonality determination is not usually done and a clear definition of an adenoma on strict histological characteristics is not forthcoming.[46,47] The presence of multiple nodules in an adrenal cortex often is interpreted as being multiple adenomas or nodular hyperplasia depending on the observer.[48] Most APAs also exhibit zona glomerulosa hyperplasia,[49] but this is open to histological interpretations and, moreover, according to some groups this is seldom seen.[36] Nevertheless, since HE staining provides no information on the functional phenotype, whether the nodule(s) is responsible for the over-production of aldosterone remains uncertain. In the attempt of overcoming such hindrance, an approach based on specific oligonucleotides for the *CYP11B2* for the *in situ hybridization* (ISH) technique was used in some laboratories to ascertain the ability of the nodules found in adrenal specimens from PA patients of synthesizing aldosterone.[50,51] However, being time-consuming and difficult in its implementation, ISH is not feasible for every-day hospital histopathology.

An approach based on immunohistochemistry (IHC) would be strongly desirable as it is faster and less expensive than ISH, suitable for automated systems and, therefore, can be routinely used by most diagnostic pathology services. After a long quest over decades, finally antibodies against human CYP11B2 and 11β-hydroxylase (CYP11B1) have been successfully developed.[52,53] Currently their use is restricted to few research laboratories and not yet commercially attainable,

IHC has remained an impracticable approach for the routine diagnostic work-up of the APA. Commercial antibodies are available from several companies, unfortunately more often than not the immunizing peptide is proprietary and the validation uncertain as some of them are described in the catalogues showing strong staining or western blots in tissues or cell lines that do not express the enzyme. Nonetheless, the development of these antibodies should not only allow unequivocal detection of aldosterone- and cortisol-producing cells, but also may shed new light on the histology of normal adrenal gland and APA. In fact, use of the specific antibodies against CYP11B2 and CYP11B1 unexpectedly unveiled that different patterns of CYP11B1 and CYP11B2 can be associated with what was clinically diagnosed as an APA, thereby questioning the 'classic' view of APA as a single, well demarcated or encapsulated nodule exclusively constituted by cells producing excess aldosterone.

Expression and Detection of CYP11B2 and CYP11B1 in the Adrenal Cortex

In the normal adrenal gland aldosterone production occurs in the outer part of the adrenal cortex, the Zona Glomerulosa (ZG), and it is regulated primarily by Na^+ intake via the RAS, by plasma K^+ concentration, and by ACTH levels.[54] ZG cells are hyperpolarized by K^+ influx through different channels. When a stimulus takes place, the cell membrane depolarizes and this results in enhanced Ca^{2+} intake via L-, N- and T- type Ca^{2+} channels.[55,56] Moreover, mobilization of intracellular Ca^{2+} from the endoplasmic reticulum further increases intracellular Ca^{2+} load,[54] with phosphorylation of transcription factors and activation of *CYP11B2* gene transcription. *CYP11B2* encodes for the enzyme aldosterone synthase, a multifunctional cytochrome enzyme, which first hydroxylates deoxycorticosterone (DOC) at the 11β-position to form corticosterone, then at the 18-position to generate 18-hydroxycorticosterone. The bound 18-hydroxycorticosterone is then finally hydroxylated at the 18-position to generate a germinal diol that spontaneously and rapidly dehydrates to form aldosterone. The aldehyde at C18 of aldosterone exists in equilibrium with its hemiacetal form (Figure 1).

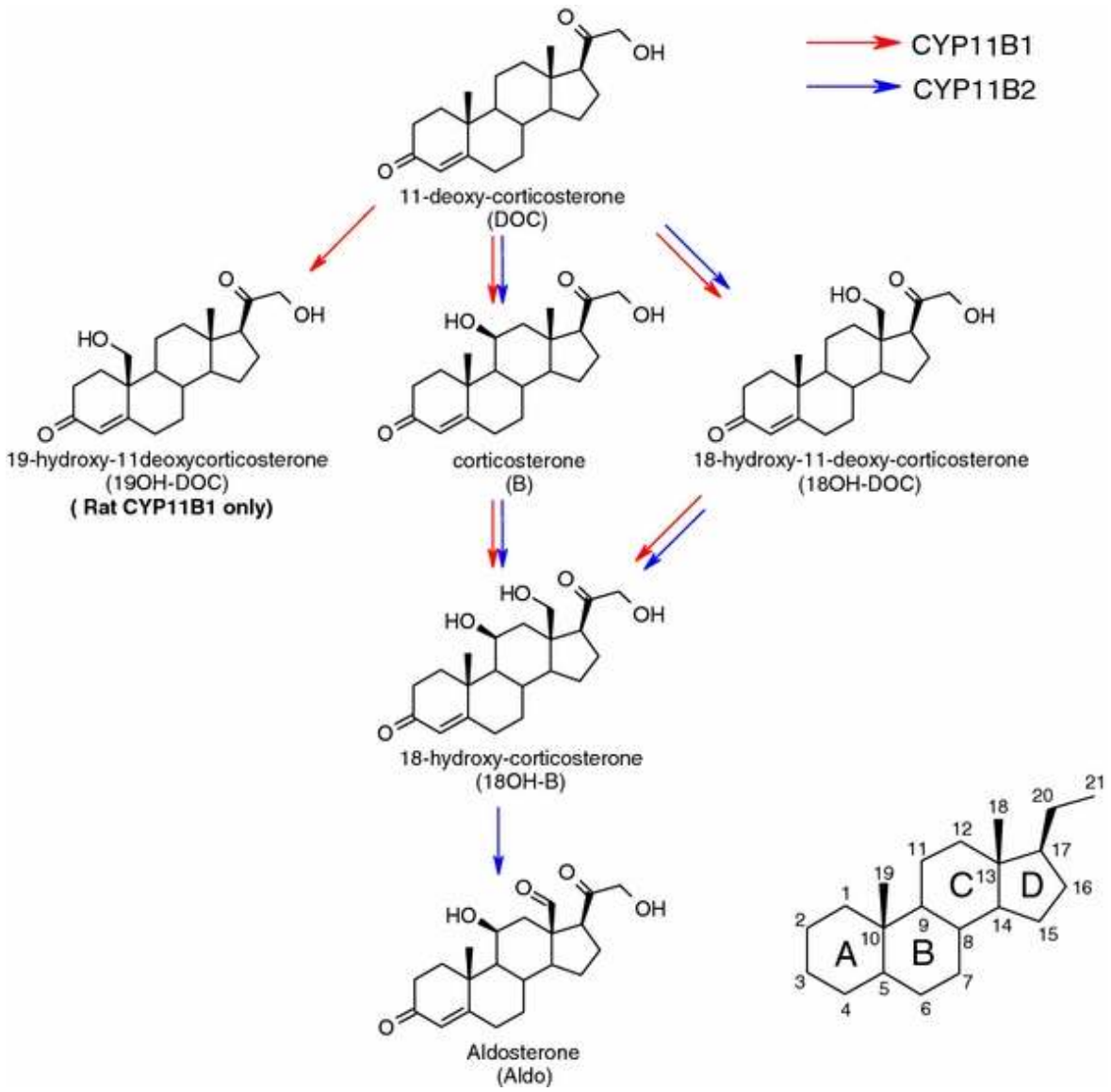

Figure 1. Aldosterone synthesis

Modified from Roumen L, Sanders MP, Pieterse K et al. J Comput Aided Mol Des. 2007

In the cortisol-producing Zona Fasciculata (ZF) cells the enzyme 11β-hydroxylase, which is coded by *CYP11B1* gene, converts 11-deoxycortisol and DOC to generate cortisol and corticosterone. Both CYP11B2 and CYP11B1 are mitochondrial enzymes that share 93% amino acid sequence, making it difficult to generate specific antibodies able to reliably distinguish between the enzymes.[57,58]

Synthesis of Monoclonal Antibodies against CYP11B2 and CYP11B1

Two decades ago, Ogishima et al[52] generated polyclonal antibodies for CYP11B2 and CYP11B1 by synthesizing oligopeptides (figure 2) corresponding to the 80-90 amino acid residues[57] and coupling them to equine myoglobin to immunize domestic rabbits, but only recently reported their use.[59] More recently, monoclonal antibodies against the two human steroidogenic enzymes have been developed in Gomez-Sanchez's laboratory, with whom we collaborate.[53] Five different immunization peptides were designed to cover the area where CYP11B2 and CYP11B1 amino acids sequences diverge. After injecting Swiss-Webster mice for CYP11B2 and Sprague-Dawley rats for CYP11B1 with the conjugated peptides, the spleen cells from the animals with the highest titers and lowest cross-reactivity were fused to modified mouse myeloma SP2-mIL6-hIL21 cells in order to obtain monoclonal antibodies. The antibody against CYP11B2 was obtained from the animals injected with the 41-52 amino acid sequence shown in figure 2 (highlighted in green). This sequence differs from that previously used by Ogishima et al.[52] The antibody against CYP11B1 was achieved from rats inoculated with a peptide corresponding to the 80-90 aa sequence and that used by Ogishima in 1991. ELISA and western blot analysis confirmed the specificity of the two antibodies against CYP11B2 and CYP11B1.[53] Monoclonal antibodies produced from hybridoma cells are of an antibody type against a limited epitope, thus have a clear advantage over polyclonal antibodies that may change from bleeding to bleeding and animal to animal. In addition, their quantities are theoretically unlimited as long as the hybridoma cell line is preserved. Therefore, these novel monoclonal antibodies seem to be the most promising tools to investigate the adrenal disorders.

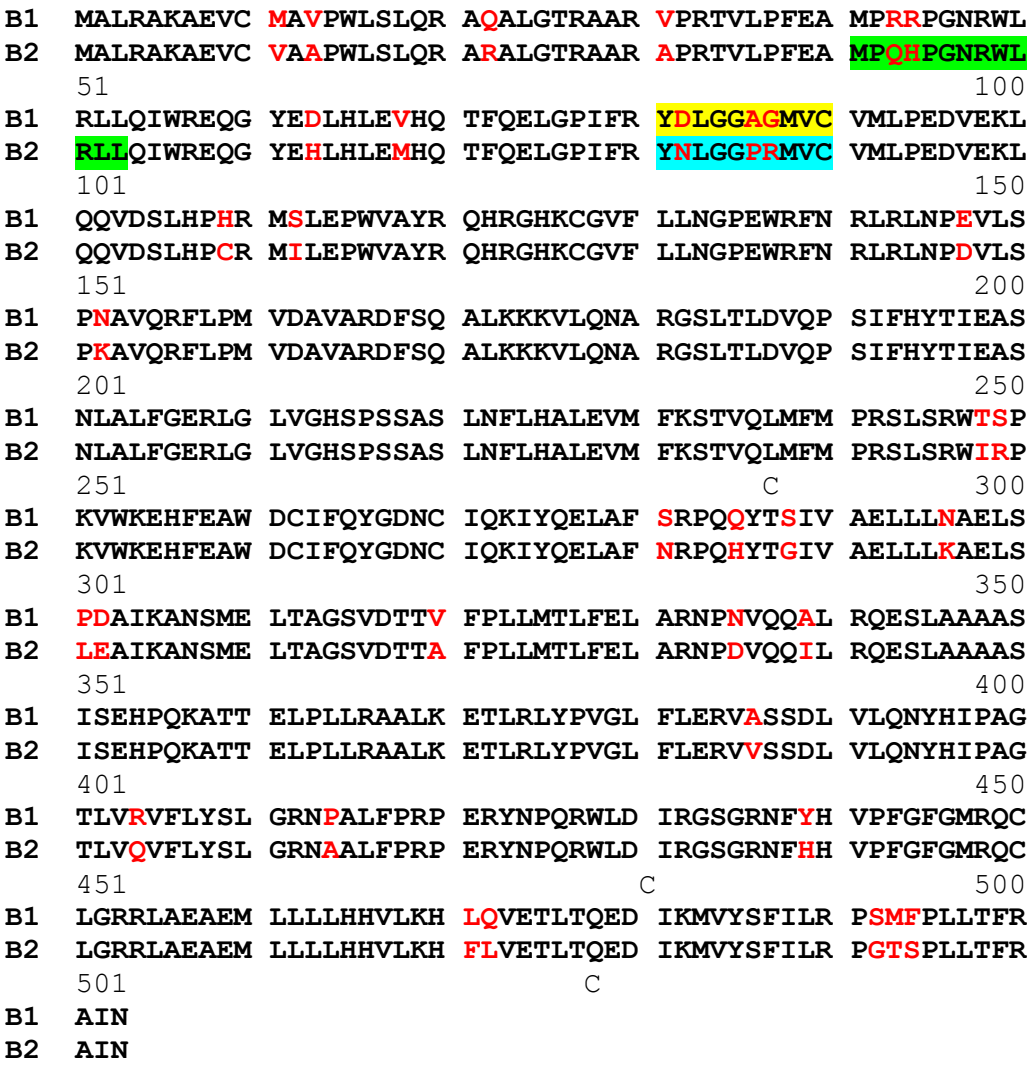

Figure 2. Comparative alignment of the protein sequence between human CYP11B1 and CYP11B2

The red letters indicate the amino acid differences between the sequences. The green highlighted letters are the sequences used by Gomez-Sanchez for synthesis of CYP11B2 peptides. Highlighted in yellow letters are the sequences used by Ogishima and Gomez-Sanchez for synthesis of CYP11B1 peptides, those highlighted in blue are the sequences used by Ogishima for synthesis of CYP11B2 peptides.

Histology of the Normal Human Adrenal Gland

In the classical view three major zones compose the adrenal cortex: the outermost ZG, the midzone ZF and the innermost Zona Reticularis (ZR), with ZG and ZF cells producing aldosterone and cortisol, respectively.[60] The functional zonation in humans was originally presumed based on histology and biochemistry of rodent adrenals,[61-63] with no direct evidence from humans. Only recently, immunostaining of the normal human adrenals with Ogishima's antibodies showed where

cells expressing the enzymes are actually distributed in the human adrenal cortex under normal and pathological conditions. In 2010 Nishimoto et al. revealed two types of CYP11B distribution, termed '*conventional'* and '*variegated'*.[59] *Conventional* distribution shows a zonation similar to that described in rodents: three different zones (ZG, ZF, and ZR) where CYP11B2 can be sporadically detected in the cells of the upper portion of the ZG cords underneath the capsule, whereas CYP11B1 is found in both ZF and ZR. In the *variegated pattern* there are clusters of cells strongly positive for CYP11B2 in the sub-capsular area, with the rest of the cortical area expressing CYP11B1. The clusters, also called aldosterone-producing cell clusters (APCCs),[59] are usually characterized by a width of 200-1300 μm and a depth of 100-500 μm beneath the capsule; occasionally, they are in direct contact with the capsule. APCCs also express 3β-hydroxysteroid dehydrogenase (3βHSD), but not 17β-hydroxylase/17,20-lyase (CYP17), the enzyme required for the synthesis of the cortisol precursor 17α-hydroxypregnenolone, and are surrounded by columnar ZF-like cells forming cords along sinusoids. The cells in sub-capsular areas that are devoid of APCCs lack both CYP11B1 or CYP11B2.

The most recent monoclonal antibodies developed in the Gomez-Sanchez's laboratory confirmed the existence of variable patterns of the human adrenal cortex. The analysis of normal adrenals from 4 adults and one 5 days old infant documented that CYP11B2 immuno-reactive cells exhibited the two patterns: the first characterized by scattered cells, and the other identified by more tightly clustered cells, i.e. APCC. CYP11B1 positive cells extended up to the capsule in many portions of the cortex and were intermingled with CYP11B2 immuno-positive cells.[53] Double staining demonstrated that CYP11B2 and CYP11B1 were mostly expressed in different cells, but double immuno-reactive cells were occasionally found in the sub-capsular region.

Adrenocortical Steroidogenic Zonation with Aging

The adrenal cortex samples from adults had relatively few CYP11B2 immuno-reactive cells close to the capsule when compared to the infant adrenal, which showed many more.[53,59,61-63] The higher plasma levels of aldosterone in infants, compared to normal adults, is presumably due to the low expression of the mineralocorticoid receptor and the partial resistance to aldosterone. Therefore a relationship between cell type distribution and adrenal function was contended.[53,59,61,62,64,65]

This contention is also consistent with the findings obtained from an analysis of 61 surgically- or autopsy-derived adrenals removed from patients ranging from 1 day to 92 years old with no obvious hormone abnormalities. The adult adrenals were found to have less CYP11B2 immuno-reactive cells than those in the infants, suggesting that adrenal zonation changes with aging alongside basal aldosterone levels.[64] It may also reflect the relatively high sodium levels in diet. Homogeneous columns of ZF cells topped with ZG cells mainly constituted the sub-capsular cortex from birth to adolescence, occupying more than half of the adrenal circumference. Starting from adolescence, cells that do not express either CYP11B1 or CYP11B2, but express 3βHSD and P450scc, also known as zona progenitor (ZP) cells, appeared and gradually occupied the upper portion of the cortical cords.[64] After the 40s, ZP cells became the prevailing cell type in the uppermost zone of the cortex, leaving ZG cells organized in scattered clusters. Of importance, the immunohistochemically defined ZG, ZF and ZP cells did not correspond to the HE staining-defined ZG-like or ZF-like cells,[64] thereby suggesting that functional zonation does not correspond to the morphologic zonation and that HE analysis is unable to identify aldosterone-producing clustered cells or an APA.

The functional role of ZP cells is unknown. Many studies have provided evidence for the existence of adrenocortical cells with stem-like capacities across mammalian species and undifferentiated adrenocortical cells with limited or no steroidogenic activity, i.e. ZP cells, thereby suggesting a role in adrenal gland development and functional plasticity.[66-69] According to another hypothesis ZP cells are steroidogenic latent cells that could differentiate in either ZG or ZF according to further

stimulation.[70] More ZG cells seem to be needed at birth and during adolescence than in senescence, probably because plasma volume expansion is a prerequisite for growth. Accordingly, after the reproductive age and a consistently sodium repleted diet, ZG-cell stimulation would decrease and they would lose their ability to synthesize hormones, thereby becoming ZP cells. Similarly, decreased need for gluconeogenesis and HPA activity would decrease the requirement for cortisol synthesis, thus ZF cells would also be transformed to ZP cells. Such a theory, which explains the onset of ZP cells along with ZG/ZF cell involution, is consistent with the higher aldosterone levels measured in the newborns and children than in the adults.[65,71-73] Whether the number of ZG cells, and consequently of ZP cells change, i.e. in response to sodium intake, remains unclear, although evidence from experimental models supports this contention.[70-72,74] Cells that express neither CYP11B1 nor CYP11B2 were found to be more abundant in the adrenals obtained from rats maintained under low stress conditions on a standard or high sodium diet and mingling with cells expressing the CYP11B2 enzyme,[75] and the width of the zona glomerulosa and the number of CYP11B2 positive cells markedly increased in chronically sodium-depleted rats. However, adrenal analysis after manipulation of sodium intake is only possible in experimental animals, but not in humans for obvious reasons. Thus, the role of ZP cells in human adrenals can only be inferred. Moreover, human adrenals are usually obtained from autopsy, often from patients who died after illness that involved stress and/or electrolyte derangements. Adrenal morphology and function under these circumstances by no means reflect normal physiological status.[53]

Adrenal Tissue Adjacent to the APA

The adrenal tissue adjacent to an APA may show either a pattern similar to that observed in the normal adrenal, consisting of diffuse CYP11B1 immuno-reactivity in ZF and ZR[53] and sporadic expression of CYP11B2 in ZG,[59] or a pattern resembling the APA, with APCCs expressing CYP11B2 and 3βHSD but not CYP11B1 or CYP17. The peri-tumoral tissue often shows micro- and/or macro-nodules, with some expressing CYP11B2,[49,59,76] thereby suggesting active aldosterone

production in the area surrounding APAs. These findings were consistent with those found with the ISH analysis, which showed mRNAs of steroidogenic enzymes necessary for aldosterone production (CYP11B1, CYP11B2, HSD3B2, CYP21A2) in sub-capsular micronodules adjacent to APAs.[77] Collectively, these findings suggest that, in contrast with current opinion, a clear boundary between APA and adjacent tissue does not exist; moreover, co-existing sub-capsular micronodules might be the initial foci for the development of APA.

Histology of the APA

The diagnosis of APA includes demonstration of an adenoma, classically depicted as a single and round macronodule in the adrenal gland, consisting of morphologically ZG- or ZF-like cells, or a combination of both.[78] HE staining currently used to reveal the APA and discriminate between ZG- and ZF-like cells cannot provide any information on the function and steroidogenic potential of these cells (figure 3, panel A). Using the Ogishima's antibodies, Nishimoto et al. identified three cell types within the APA (figure 3, panel B): 1) cells positive for CYP11B2 but negative for CYP11B1, 2) cells positive for CYP11B1 but negative for CYP11B2, and 3) cells negative for either CYP11B1 or CYP11B2.[59] No cells showing double staining for CYP11B1 and CYP11B2 were found.[59] In contrast, Nakamura et al. reported that APAs contained not only a mix of CYP11B2 and CYP11B1 positive cells, but also cells expressing both CYP11B enzymes.[79] Immunoreactivity for CYP11B1 tended to be diffuse within the APA, whereas that for CYP11B2 was quite heterogeneous and spotted.[79] CYP11B1 was strongly expressed outside the adenoma, while there was little expression of the CYP11B2 outside the adenoma. CYP11B1, but not CYP11B2, co-localized with CYP17 in most APA cells.[79]

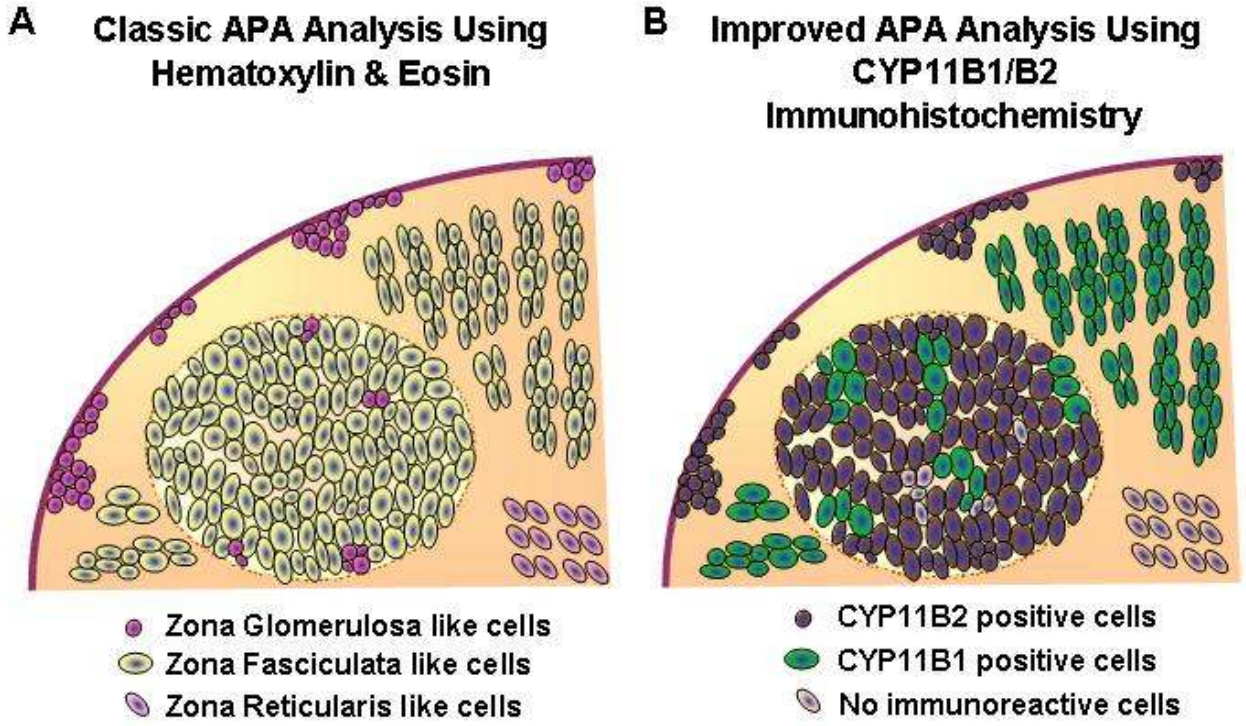

Figure 3. Identification of an APA using either hematoxylin and eosin (HE) (panel A on the left) or antibodies against CYP11B1 and CYP11B2 (panel B on the right).

Panel A. HE allows identification of zona glomerulosa like cells (visualised as pink cells), zona fasciculata like cells (yellow cells) and zona reticularis like cells (light pink cells). The cell phentoypes are shown in different colours (arbitrary chosen) to enable their distinction. Using HE staining ZG-cells appear as small and markedly stained with eosin because of the presence of mitochondria whereas, ZF-cells appear as large and light cells due to the great abundance of lipid drops. The appearance of ZR-cells is quite similar to that of ZG-cells as small pink cells. However, HE does not provide functional information about steroidogenesic capacity. Note that the human adrenal ZG does not form a continuous layer under the capsule as in rodents; the ZG-cells aggregate in clusters. APA cells mostly appear as ZF-cells, with only a few intermingled ZG-cells.

Panel B. IHC using CYP11B1 and CYP11B2 allows identification of cortisol- and aldosterone-producing cells as green cells and brown cells, respectively, this showing that IHC provides functional information on steroid production. The sub-capsular clusters usually show immunoreaction against CYP11B2, whereas cells forming cords exibit immunoreaction against CYP11B1. Both CYP11B1 and CYP11B2 positive cells can be detected in the APA suggesting that there is no perfect matching between ZF-cells, which are commonly detected within the APA with HE, and cells producing aldosterone, i.e. CYP11B2 positive cells. Since neither aldosterone nor cortisol are produced by ZR-cells no specific immunoreactive signal can be detected.

Since the intensity of the immunostaining differed markedly across APAs, some investigators hypothesized that this could reflect the magnitude of the steroidogenesis in the tumor and that a relationship exists between CYP11B2 or CYP11B1 expression, aldosterone production, and the tumor size. Using a very simple semi-quantitative system that assigned a score 1 to dimly immuno-stained APAs and 2 or 3 to well, or very markedly, respectively, stained tumors, Nanba et al. found that the score for CYP11B2 inversely correlated with the tumor size calculated by assuming a spherical shape of the tumor[76].[76] When adjusting the CYP11B2 score for the tumor volume, Nanba found a positive correlation between CYP11B2 staining score and plasma aldosterone concentration (PAC) or the aldosterone-to-renin ratio (ARR). The score negatively correlated with serum K^+ levels, thus leading support to the hypothesis that CYP11B2 score reflects aldosterone synthesis.[76] By exploiting a more complex scoring system, in which the percentage of the stained cells in the APA is multiplied by a factor ranging from 0 to 3 to reflect the intensity of cell immunostaining,[80] Nakamura et al. observed that CYP11B2 H-score in the APA (44.0$\pm$5.1, mean+SE) was not significantly different from that calculated in the normal adrenal gland (58.6$\pm$9.5, mean+SE), but was higher than that measured in the tissue adjacent to the APA (24.5$\pm$4.9, mean+SE).[79] In contrast to Nanba,[76] they found no relationship between intensity of CYP11B1 or CYP11B2 immunostaining and tumor size or age,[79] leaving unclear whether the intensity of immunostaining truly reflects the amount of steroidogenesis. However, experimental evidence with cultured cells and animals suggests that it would.

Because of the not perfectly circular shape of the tumors, Ono et al. determined the precise area of the lesion using the specific software ImageJ.[81] They found that the values differed significantly from the circular area calculated on the basis of the maximum diameter.[81] By analysing a series of 40 APAs they reported that the CYP11B2 H-score was higher in APAs with an area smaller than the median value of 60mm^2, compared to those with an area ≥60mm^2, suggesting that small APAs produce more aldosterone per cell than large tumors consistently with what reported by Nanba et al.[76] However, when CYP11B2 H-score was multiplied by tumor area, the resulting value

correlated positively with PAC.[81] Using different CYP11B1 & CYP11B2 antibodies Monticone et. al. also observed an inverse correlation between intensity of CYP11B2 expression and nodule size, and a positive correlation of CYP11B2 expression corrected for tumor volume with PAC and ARR.[82] They also found that patients not expressing CYP11B1 had lower serum potassium levels compared to patients expressing CYP11B1 in the adrenal nodule, but neither absolute aldosterone nor ARR levels differed between groups.[82]

Somatic Mutations in the Potassium Channel Kir3.4

The recent discovery of somatic mutations in the KCNJ5 gene opened a new window on the molecular mechanisms that control the autonomous aldosterone synthesis in APA. KCNJ5 encodes for the potassium channel Kir 3.4, which allows the selective transport of K^+ through the cell membrane to the extracellular space, keeping the membrane hyperpolarized.[83] Choi et al.[32] first reported that G151R and L168R mutations causing the insertion of a positive charged amino acid into the selectivity filter of the channel resulted in loss of selectivity for K^+, with ensuing leak of Na^+ into the cell, membrane depolarization, increased $[Ca^{2+}]i$, and finally *CYP11B2* gene activation (Figure 4).

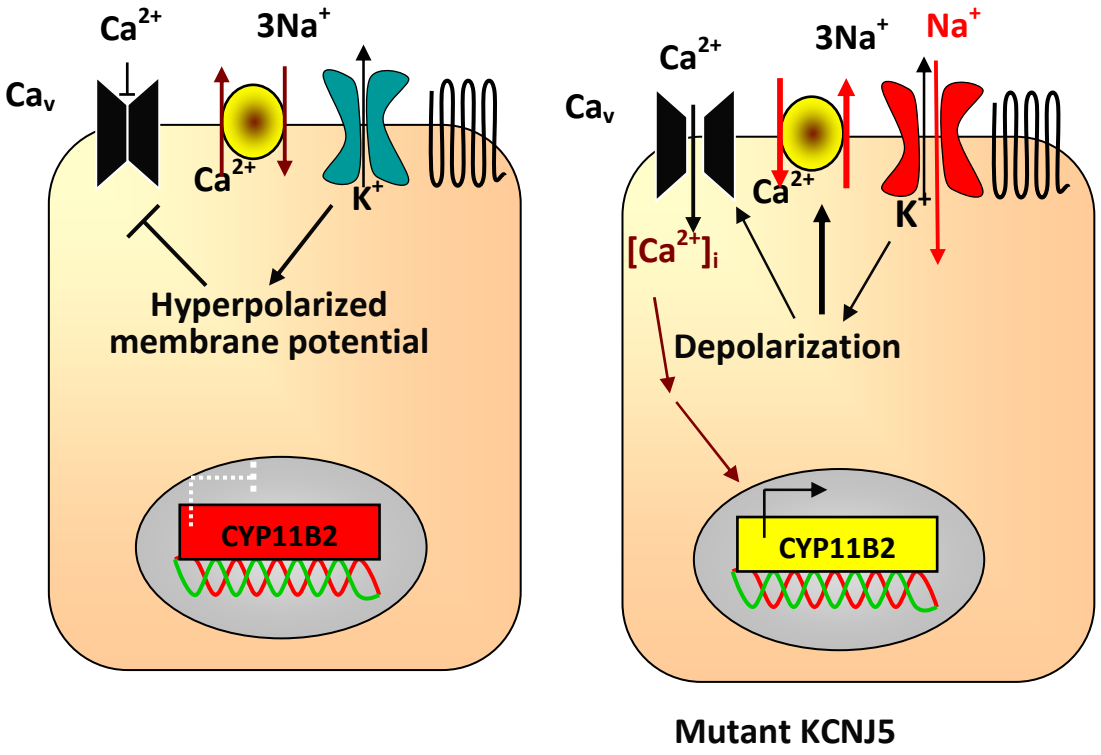

Figure 4. Proposed mechanism underlying aldosterone-producing adenoma and KCNJ5 mutation.

Modified from Choi et al. Science 2011.

KCNJ5 mutated APAs consisted mainly of ZF-like cells with high expression of CYP11B1.[78,82] CYP11B2 immunostaining allowed identification of one case of two CYP11B2 positive nodules in the same APA exhibiting the same KCNJ5 mutation, and one case reporting two nodules carrying two different KCNJ5 mutations (L168A and G151A).[78] Sequencing of APAs with no KCNJ5 mutations led to the discovery of somatic mutation of the sodium/potassium ATPase gene ATP1A1, the calcium ATPase gene ATP2B3 and the voltage-gated Ca^{2+} channel Cav 1.3 gene CACNA1D.[84,85] The loss-of-function mutations in ATPs and Ca^{2+} channels resulted in increased intracellular [Ca^{2+}] load that activates aldosterone secretion via the calcium-calmodulin pathway. ATP1A1 and CACNA1D mutations were found in multinodular glands, whereas ATP2B3 mutation only in solitary adenomas.[78] APAs with the ATP1A1, ATP2B3 and CACNA1D mutation most frequently had a ZG-like phenotype with high expression of CYP11B2.[82] Nodules carrying an

ATP2B3 mutation were smaller than those containing a KCNJ5 mutation and, in contrast to KCNJ5 mutated nodules, did not contain atypical cells.[78]

Clinical Implications of the Antibodies against CYP11B1 and CYP11B2

Since these selective antibodies were made widely available a few years ago several different studies were performed to analyze the CYP11B2/CYP11B1 immuno-histochemical expression and clinical outcome of APA patients.[78,86,87] By analyzing 53 adrenals removed at surgery due to unilateral PA, Dekker et al. observed that at follow-up the patients with a solitary adenomas were cured more often than those with nodular hyperplasia.[78] Volpe et al. retrospectively studied 120 consecutively unilaterally adrenalectomized patients selected on the basis of the AVS or NP59 scintigraphy. In six of their cases (7%), the initial diagnosis was changed from APA to aldosterone-producing hyperplasia after immunohistochemistry investigation; moreover, in 5 of these 6 specimens, the "adenoma" was CYP11B1, not CYP11B2 positive.[87] To date studies searching for a relationship between IHC pattern and clinical outcome have limitations in that they were retrospective, often spanning a long period of time, without uniform diagnostic work-ups for PA. The diagnostic strategies included CT scan, or AVS, or both, but it is well established that CT can be misleading in identifying the side causing lateralized excess aldosterone production in up to 50% of the cases.[88] Therefore it could be that, in some cases, the removed adrenal lacked of nodules with CYP11B2 positive cells simply because of side misclassification.

Due to these limitations, the clinical implications of the different cell type patterns, in terms of prediction of treatment outcome remains to be determined. Obviously, an optimal approach to tackle this question would be by using not just imaging (CT or MR) but also AVS.

After adrenalectomy hypertension is cured in around 50% of patients with APA (range 33–70%) [89] but the remaining patients continue to require antihypertensive drugs to keep their blood pressure in the normal range. Citton et al. failed to identify any evident relationship between the recurrence of PA and classical HE histopathology, also at long-term follow-up.[90] Use of CYP11B2 and

CYP11B1 antibodies markedly enhance the accuracy of the APA diagnosis[86,87] but still leaves unclear whether the steroidogenic phenotype of the APAs and the intensity of immunostaining could give a clue about the clinical outcome of the adrenalectomized patients.

GENERAL AIM

Taking advantage of a novel method to immunohistochemically detect CYP11B1 and CYP11B2, this study was aimed at identifying the CYP11B2/CYP11B1 immuno-phenotypes in the adrenal glands from PA patients undergoing adrenalectomy at 3 referral centers in Italy. Moreover, an ancillary goal was to investigate whether immunostaining predicts the biochemical profile and/or the outcome of APA patients.

SPECIFIC AIMS

After identification of the CYP11B2 and CYP11B1 steroidogenic patterns that best describe the excised adrenal glands from PA patients, the study was specifically aimed at investigating if a specific immuno-phenotype could reflect the clinical profile of PA patients.

In PA patients before adrenalectomy we investigated if

1) aldosterone production differs among patterns;
2) cortisol production differs among patterns;
3) the staining intensity of CYP11B2 in the adrenal gland correlates with the serum aldosterone production;
4) the staining intensity of CYP11B1 in the adrenal gland correlates with the serum cortisol production.

After adrenalectomy, focusing our attention on the biochemical and clinical profile of PA patients we investigated if

1) the steroidogenic CYP11B2/CYP11B1 patterns differ in BP and aldosterone production outcome;
2) the staining intensity of CYP11B2 and CYP11B1 reflect different aldosterone and cortisol production in PA patients.

MATERIAL AND METHODS

Patients

One hundred and eleven adrenal glands removed from PA patients among those referred to the Specialized Centers for Hypertension of the University of Padua (n=64), Rome (n=39) and Pisa (n=8) and 16 normal adrenal glands (NAG) from patients who underwent adrenalectomy for renal carcinoma were processed for IHC. 56 APAs from the 111 adrenal glands removed for PA were genotyped for the most common KCNJ5 mutations. The patients were submitted to adrenalectomy after identification of lateralized excess of aldosterone production according to the current guidelines recommendations.[23] All resected patients had hypertension resistant (defined as blood pressure not controlled by two or more drugs, one of which is diuretic), elevated plasma aldosterone concentration (PAC) and Aldosterone to Renin Ratio (ARR).

The diagnosis was based on the 'four corners' criteria (specified in Table 2)[44] and implementation of lifestyles measures according to the ESH 2013 guidelines.[91]

Tissues were obtained under sterile conditions at surgery, in the operating room the excised adrenal gland was cut into halves according to the size of the APA. Half of the adrenal was sent to the Pathology department for histological study and the remaining tissue was rapidly frozen in liquid nitrogen vapor state for further molecular studies.

An informed written consent was obtained from each patient.

Table 2. Four "corners" criteria

FOUR CORNERS CRITERIA		
Baseline	Biochemical diagnosis of PA	Lateralization of aldosterone secretion at bilaterally selective AVS or NP59 scintigraphy
Follow-UP	Evidence of adrenocortical nodule at histopathology	Cure or improvement of HT, and correction of biochemical picture of PA

APA – DNA Preparation

Genomic DNA was prepared from 56 subject venous blood and tumor tissue by standard procedures (Eurogold DNA Extraction kit, Euroclone, Milan, Italy). Exon 2 KCNJ5 gene was amplified using appropriate primers. PCR was performed on 250ng DNA in a final volume of 50 ul containing 300 nM $MgCl_2$, 400 nM of each primer, 200 μM deoxynucleotide triphosphate, and 2.6U expand high-fidelity enzyme mix (Roche Applied Science, Italy). Purified PCR products underwent direct Sanger sequencing using the ABI Prism Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3700 DNA analyzer (Applied Biosystems). In the patients with mutated APA concomitance of germline kir3.4 mutations was sought for by sequencing DNA from peripheral blood leucocytes or adrenal tissue surrounding the APA.

APA – cDNA Preparation and Genotyping

Mutated APA sample's total RNA was prepared from liquid nitrogen preserved APA tissue using RNAeasy Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. DNA-free RNA was prepared using DNAsel (Ambion, Life Technology, CA). Reverse transcription of 1ug RNA was performed with random examer primers using the Iscript cDNA Synthesis kit (BioRad Laboratories, Milan, Italy) according to the manufacturer's instructions. KCNJ5 cDNA was amplified using intron-spanning primers and sequenced.

High Resolution Melting (HRM) Analysis

Genomic DNA from APA corresponding peripheral blood leukocytes was amplified using SsoFast EvaGreen supermix (BioRad) and primers. Short amplicon covering mutation region of the KCNJ5 gene was amplified using the CFX96 real-time PCR detection system (BioRad), and results were analyzed using the CFX Manager™ and Precision Melt Analysis™ software. The PCR reaction was performed in a 20ul final reaction volume containing 200nmol of each primer and SsoFast ™ EVA Green 5X SuperMix (BioRad). The system amplification protocol was 95°C for 3 min; 50

cycles of 95°C for 10s, 60°C for 30s. Subsequently, a melt curve was generated by heating from 65°C to 95°C with 0.2°C increment. Precision Melt Analysis software was then used to identify areas of stable pre- and post-melt fluorescence from the HRM curve and automatically determined a cluster of each genotype. Some positive (SNP) and negative (wild type) controls were examined in the same PCR and melt reaction to verify the precision of melt analysis. For each cluster, one sample underwent Sanger sequence in order to confirm the false positive sample.

Immunohistochemistry

IHC was performed using CYP11B2 and CYP11B1 antibodies[53] thanks to the collaboration with Prof. Celso Gomez-Sanchez at University of Mississippi Medical Center (Jackson, MS, USA), where the candidate spent six months during the PhD course. Paraffin-embedded adrenal was cut at 5 μm and the sections dried and then melted at 56°C for at least 3 hours. After deparaffination through alcohols, slides were subjected to antigen retrieval using Trilogy (Cell Marque Corp) in autoclave, 15 minutes at 121°C, and then treated with phenylhydrazine 0.1% for 20 minutes to inhibit endogenous peroxidases. Slides were blocked with Tris 0.1 M, goat serum 5%, or horse serum 5%, SDS 0.5% (pH 7.4) for 1 hour, and then incubated with CYP11B2 antibody (hCYP11B2-41-17B clone 1:500 dilution) or both CYP11B1 (CYP11B1-80-7-5 clone 1:200 dilution) and CYP11B2 (hCYP11B2-41-17B clone 1:500 dilution) overnight at 4°C. After washing, slides were incubated with secondary antibodies for 1 hour at room temperature. We used mouse Polink-2 Plus HRP (GBI Labs) for CYP11B2 immunostaining; rat Polink-2 Plus AP (GBI Labs), and ImmPRESS antimouse Ig reagent (Vector Laboratories) for double immunostaining. Slides were developed using diaminobenzidine and HighDef green immunohistochemistry chromogen AP (Enzo Life Sciences). All samples were counterstained with Meyer hematoxylin (Vector Laboratories) before mounting.

Immunofluorescence

Triple immunofluorescence was done using CYP11B1, CYP11B2, and 17α-hydroxylase[92] antibodies. The protocol followed the procedure used for double immunohistochemistry. Primary antibodies incubation was done with a mixture of rat CYP11B1-80-7-5 clone 1/200, mouse CYP11B2-41-17B clone 1/500 and rabbit anti 17α-hydroxylase 1/300 overnight at 4°C. After washing, a combination of highly absorbed antibodies was used, goat anti-mouse IgG Alexa Fluor 488, goat anti-rat IgG Alexa Fluor 594 and goat anti-rabbit IgG Alexa Fluor 647 (Jackson Immunoresearch Inc. Allentown, PA) 1/1000 dilution for 1h at room temperature. The slides were washed and mounted with Vectashield HardSet Mounting Medium with DAPI (Vector Labs, Burlingame, CA). Images were captured using an Eclipse Nikon Microscope with a Rover camera and pseudocolored.

Identification of Patterns Based on IHC using CYP11B2/CYP11B1 Antibodies

The patterns that characterize the normal and overproducing aldosterone adrenal glands were identified using the double IHC with CYP11B2 and CYP11B1 antibodies.[53] Classification of the specimens into so-identified patterns was done independently and blindly by 5 different observers (inter-observer agreement $k>0.85$). When there was discordance or differences in their evaluation, the slides were re-evaluated until a consensus was reached.

Morphometric analysis of CYP11B2 and CYP11B1 expression

Immunoreactivity of CYP11B2 and CYP11B1 staining was assessed semi-quantitatively according to a modified version of the McCarty H score.[80] The procedures were performed by using the ImageJ software,[93] freely available at http://rsb.info.nih.gov/ij/, powered by a routine specifically developed in our laboratory. The routine can be summarized as follows.

1. Image acquisition and pre-processing. From each section all fields corresponding to the APA, nodules or CYP11B2 positive clusters were selected at a primary magnification of 5X and then their bright-field images were acquired in full colors (RGB, 24-bit), processed to correct shading and

finally filed as TIFF. Images were acquired by using a Qwin digital camera (Leica, Wetzlar, Germany).

2. Morphometric assessment of the immunoreactive components. Color thresholding was applied to the images to identify the immunoreactive structures in the stained sections.[94] Pixel colors were represented as hue, saturation, and brightness (HSB) values. Specifically brown-stained structures corresponding to the CYP11B2 positive were identified by selecting the pixels with 'hue' in the red-yellow range and 'brightness' lower than the mean brightness level exhibited by the background minus two standard deviations (thus excluding the unspecific staining). To identify the green-stained components (CYP11B1 positive) the same approach was applied with thresholds for 'hue' in the green-blue range. After interactive editing to remove remaining artifacts as dots or breaks in the slide, the area fraction occupied by each marker in the tissue and the mean brightness value were measured. The product of these two parameters, i.e. area fraction and brightness; was used as the corresponding H-score for CYP11B2 and CYP11B1.

For each adrenals, we quantified the CYP11B2 and CYP11B1 staining intensity in all the potential aldosterone producing structures, including APA, CYP11B2 positive cell clusters and nodules. In addition, we calculated the sum of the CYP11B2 scores or the sum of the CYP11B1 scores of all those structures and defined this score as the total CYP11B2 H-score and the total CYP11B1 H-score, respectively.

Immunoreactivity of CYP11B2 and CYP11B1 staining was assessed by two different researchers with an inter-observer agreement: $r > 0.97$.

Statistical Analysis

All data are presented as mean ± SE. Data were analyzed with SPSS version 23 (IBM, SPSS statistics, Bologna, Italy). After determination of a normal distribution, differences between groups were analyzed with Student's t test for independent samples or one-way ANOVA followed by

Fishers LSD post hoc test for multiple groups. The chi-square test was applied for qualitative variables. Correlations were evaluated by Pearson correlation analysis. Statistical significance was set at $p \leq 0.05$.

RESULTS

Biochemical, Clinical Parameters and Final Diagnosis of Patients Included in the Study

Biochemical and clinical characteristics of the 111 patients who underwent surgery for unilateral excess aldosterone production included in the present study are reported in Table 3.

Based on the four "corners" criteria reported previously, PA patients were sub-classify in according to their final diagnosis as APA, Bilateral form (BAH) being PAC and ARR still elevated after adrenalectomy and, BP Not Cured (BP-NC) when there was biochemical PA picture resolution though not normalization of hypertension after surgery.

Of 111 patients, 94 were diagnosed as APA (n=94), eight patients were diagnosed as BAH and five were diagnosed as BP-NC. For four patients Follow Up (FU) data were not available and so they were excluded from the study.

All patients showed high aldosterone concentration, ARR and BP values at baseline (Table 3). After adrenalectomy APAs exhibited a fall of BP despite a reduction of the number of antihypertensive drugs along with normalization of serum K^+ and ARR. BP-NC patients displayed a decrease in aldosterone concentration and increase in PRA but BP levels still elevated. BAH patients did not show a reduction in PAC and still presented high ARR at FU (Table 3).

Flow Chart of the patients included and excluded in the study

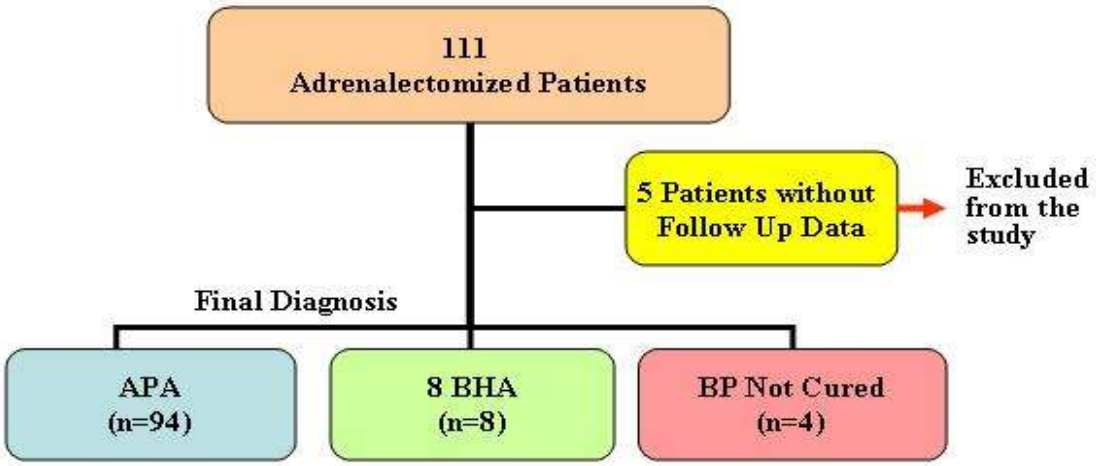

Table 3. Biochemical and clinical characteristics of 111 PA patients included in the study

Diagnosis / Biochemicals	APA (n=94)		BAH (n=8)		BP Not Cured (n=5)		p
Adrenalectomy	**Before**	**After**	**Before**	**After**	**Before**	**After**	
Sex (M/F, %)	52/48		75/25		40/60		NS
Age (yrs)	49±1		55±4		63±5		.01[b]
BMI (kg/m2)	27.4±0.5	26.9±0.5	28.1±1.1	27.9±1.2	31.0±3.8	28.7±3.3	NS
SBP (mmHg)	155±2	131±1	152±4	142±4	170±8	150±6	≤.02[d,e]
DBP (mmHg)	94±1	83±8	94±2	90±4	84±5	80±7	≤.03[d,e]
K^+ (mmol/L)	3.5±0.1	4.3±0.04	3.5±0.3	3.9±0.2	3.8±0.4	4.3±0.5	.005[d]
PAC (ng/dl)	27.8±1.9	11.1±0.7	24.6±3.6	22.0±2.9	24.4±3.8	8.9±1.7	≤.002[d,f]
captoPAC (ng/dl)	22.9±3.3	6.1±0.6	14.7±2.7	18.8±3.9	9.7 (n=1)	7.3 (n=1)	≤.001[d]
PRA (ng/ml*h)	0.4±0.03	1.4±0.2	0.6±0.2	1.3±0.5	0.6±0.2	0.7±0.4	≤.05[a]
captoPRA (ng/ml*h)	0.6±0.1	2.8±0.9	1.1±0.2	1.9±0.8	0.6±0.1	0.2 (n=1)	NS
ARR	108.2±8.7	14.8±1.7	81.1±24.6	42.5±19.8	61.3±26.4	20.2±5.8	≤.001[d]
Cortisol (nmol/L)	325.3±32.1	338.5±16.9	330.0±66.4	312.1±34.9	246.7(n=1)	320.0±7.6	NS
captoCortisol (nmol/L)	357.7±30.4	190.1±16.0	246.7±10.7	240.0±50.1	254 (n=1)	243 (n=1)	NS
Drugs (numbers)	2.2±0.2	1.2±0.1	2.3±0.6	2.3±0.5	2±1.2	NA	≤.03[d,f]

APA, Aldosterone-Producing Adenoma; BAH, Bilateral Hyperplasia; SBP, Sistolic Blood Pressure; DBP, Diastolic Blood Presure; PAC, Plasma Aldosterone Concentration; captoPAC, PAC after captopril; PRA, Plasma Renin Activity; captoPRA, PRA after captopril; ARR, Aldosterone-Renin Ratio; captoCortisol, Cortisol after captopril; Drugs numbers, numbers of drugs before adrenalectomy. NA, Not Available. Mean ± SE.

[a] = APA vs BAH before adrenalectomy; [b] =APA vs Not Cured before adrenalectomy; [c] =BAH vs Not Cured before adrenalectomy. [d] = APA vs BAH after adrenalectomy; [e] =APA vs Not Cured after adrenalectomy; [f] =BAH vs Not Cured after adrenalectomy.

CYP11B2/CYP11B1 Steroidogenic Immuno-Phenotype Patterns

Ninety-four adrenal glands from PA patients diagnosed as APA were processed to double IHC for CYP11B2 and CYP11B1. Five major patterns based on CYP11B2/CYP11B1 immunostaining were identified. Pattern 1 comprises adenomas with uniform CYP11B2 staining and CYP11B1 staining in the zona fasciculata (ZF) of the adjacent normal adrenal gland (n=15, 16%) (figure 5, panels 1-A-B). Pattern 2 includes adenomas with a diffuse CYP11B2 staining but multiple cells positive for CYP11B1 inside the adenoma (figure 5, panels 2-C-D) (n=43, 46%). To evaluate the co-expression of CYP11B2 and CYP11B1 triple IF for CYP11B2/CYP11B1/17α-hydroxylase was performed and, in pattern 2, some cells inside the adenoma showed co-expression of CYP11B2/CYP11B1 (figure 6). Pattern 3 is represented by adenomas that do not express CYP11B2 but CYP11B1 and clusters of sub-capsular cells positive for CYP11B2 (n=2, 2%) (figure 5, panels 3-E-F). Pattern 4 identifies adrenal glands with no evidence of an adenoma; in these samples merely multiple clusters of sub-capsular CYP11B2 cells were present (n=24, 25%) (figure 5, panels 4-G-H). Patter 5 includes multi-nodular adrenal cortex positive prevalently for CYP11B1 with clusters of sub-capsular CYP11B2 cells (n=10, 11%) (figure 5, panels 5-I-L).

Seventeen adrenal glands from patients not diagnosed as APA by the four "corners" criteria were distributed into 5 patterns as follows (the number of cases shown in parentheses): BAH (pattern 1, n=1; pattern 3, n=3; pattern 4, n=1; pattern 5, n=3); BP-NC (pattern 3, n=5) ($p \leq .001$).

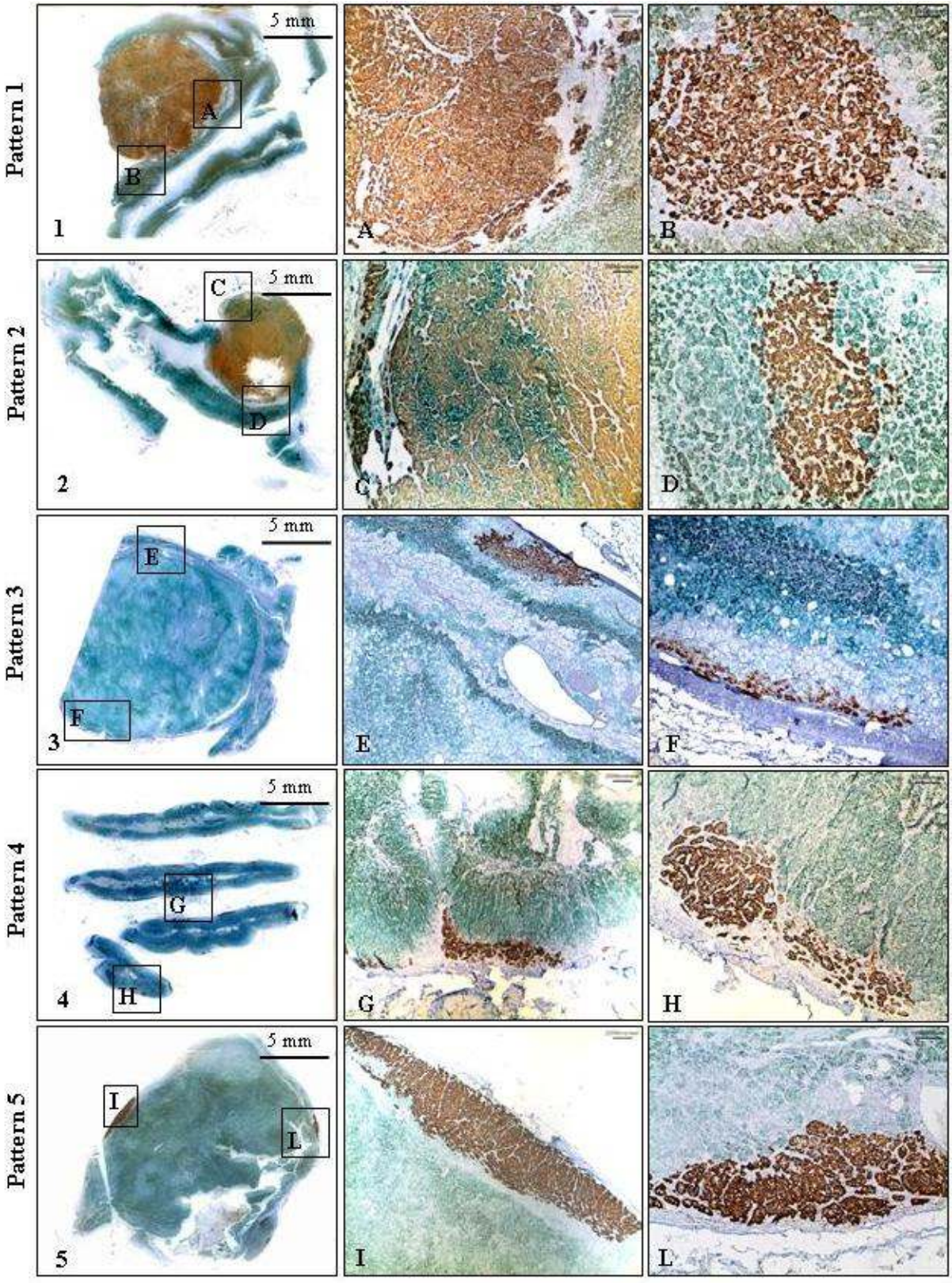

Figure 5. CYP11B2/CYP11B1 steroidogenic patterns

Examples of histological expression of CYP11B2 (brown) and CYP11B1 (green) in APA. Each line represent images at different magnification of the same adrenal gland classified in the five patterns. Nucleus are stained with HE.

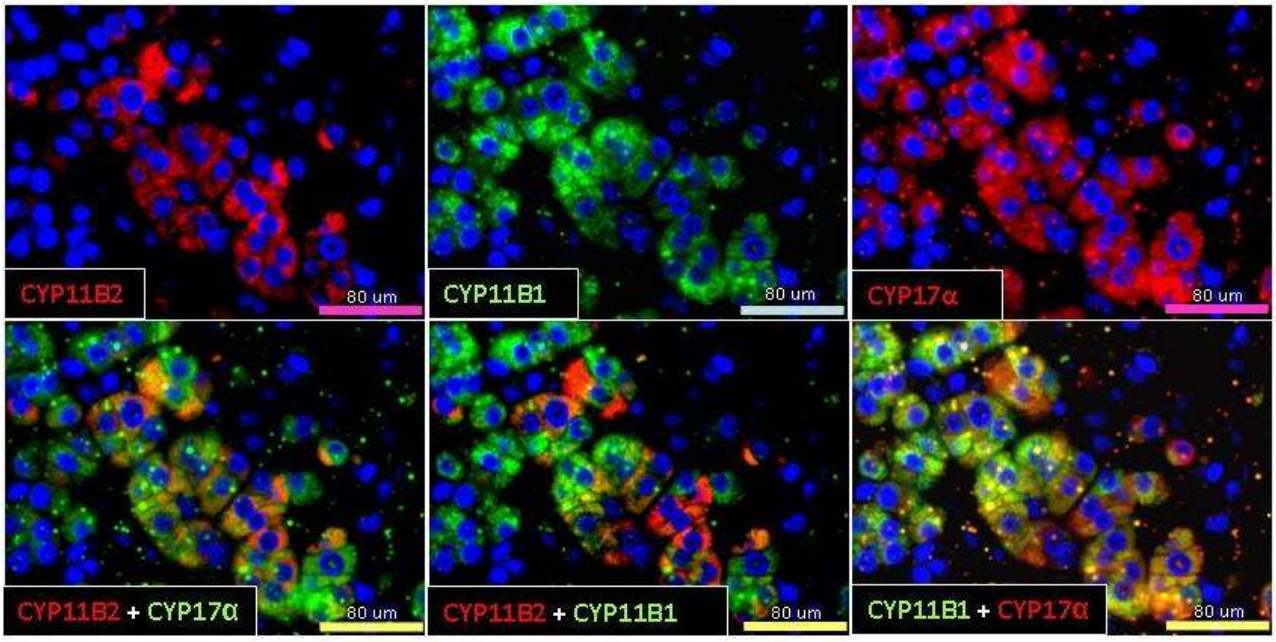

Figure 6. Triple Immunofluorescence for CYP11B2, CYP11B1 and 17α-hydroxylase

Example of triple immunofluorescence for CYP11B2, CYP11B1, and 17α-hydroxylase in the same adrenal gland from a patient classified as pattern 2. Upper panels show images of triple immunofluorescence for CYP11B2, CYP11B1, and 17α-hydroxylase at the same magnification [10X]. Lower panels are the merged image of CYP11B2 (red) and 17α-hydroxylase (green), CYP11B2 (red) and CYP11B1 (green), CYP11B2 (green) and 17α-hydroxylase (red), respectively.

Sixteen NAGs were processed for CYP11B2 and double CYP11B2/CYP11B1 IHC. These adrenals showed the expected strong CYP11B2 immunosignal limited to clusters of sub-capsular Zona Glomerulosa (ZG) cells whereas CYP11B1 staining was present predominantly in ZF cells (figure 7).

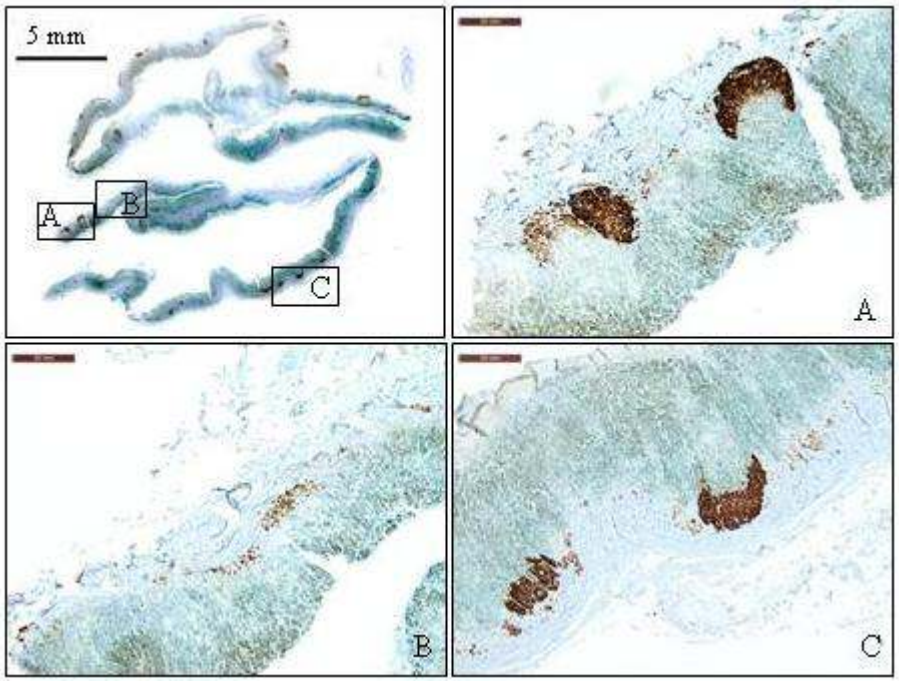

Figure 7. Example of Double IHC for CYP11B2 and CYP11B1 in the Normal Adrenal Gland

Examples of histological expression of CYP11B2 (brown) and CYP11B1 (green) in Normal Adrenal Gland. Panels A-B-C are images at different magnification of the same adrenal gland. Nucleus are stained with HE.

Clinical Characteristics of APA Patients in Steroidogenic Patterns Before Adrenalectomy

The clinical characteristics of APA patients in patterns before adrenalectomy are shown in Table 4. We observed that patients classified into patterns 1 and 2 were younger and had lower BMI than patients in patterns 3, 4 and 5 (46±3 vs 54±6. p≤.03 and 26.7±0.8 vs 30.2±3.0. p≤.03). Of interest, pattern 2 showed prevalence of female gender (64%; p=.005), higher PAC (32.5±3.2) and higher ARR (123.3±14.7) compared to the other patterns. Two cases were classified as pattern 3, they expressed higher K^+ than cases in patterns 1, 2 and 5 (4.5±0.4 vs 3.5±0.2. p≤.03) along lower PAC (17.4±2.5). When captopril stimulation was performed, aldosterone production decreased more in patterns 4 and 5 than in patterns 1 and 2 (13.9±4.3 vs 24.8±4.7).

We performed the genotype analysis for the most common KCNJ5 mutations (G151R, L168R and T158A)[95] in 56 APA cases of the cohort of 94 adrenal glands previously diagnosed as APAs. This analysis revealed that 32% of those patients presented one of the mutations in the K^+ channel GIRK4. Interestingly, nearly 72% of the patients with a known KCNJ5 mutation was classified into pattern 2 (p=.012).

Table 4. Clinical characteristics at baseline of patients included in patterns.

Pattern	**1** (n=15)	**2** (n=43)	**3** (n=2)	**4** (n=24)	**5** (n=10)	p
Sex (M/F, %)	80/20	33/67	50/50	58/42	80/20	.005
Age (yrs)	46±3	46±2	53±13	55±2	54±3	≤.03[a]
BMI (kg/m2)	27.9±0.9	25.5±0.7	31.8±6.2	29.4±0.9	29.3±1.9	≤.03[b]
SBP (mmHg)	157±8	154±3	145±5	155±5	163±9	NS
DBP (mmHg)	95±5	93±2	88±3	94±3	99±7	NS
K^+ (mmol/L)	3.5±0.1	3.4±0.1	4.5±0.4	3.7±0.1	3.4±0.3	≤.03[c]
PAC (ng/dl)	23.5±2.2	32.5±3.2	17.4±2.5	23.5±2.7	23.4±7.5	NS
captoPAC (ng/dl)	21.8±3.5	27.8±5.8	NA	17.3±4.1	10.5±4.4	NS
PRA (ng/ml*h)	0.43±0.11	0.38±0.05	0.30±0.10	0.30±0.05	0.37±0.13	NS
captoPRA (ng/ml*h)	0.91±0.37	0.47±0.06	NA	0.25±0.07	1.01±0.50	NS
ARR	94.4±15.3	123.3±14.7	68.4±31.1	95.9±14.4	90.5±23.7	NS
Cortisol (nmol/L)	288±69	340±47	NA	306±71	320±95	NS
captoCortisol (nmol/L)	481±69	372±40	NA	237±26	280±21	0.02[d]
Drugs (numbers)	2.1±0.3	2.4±0.2	1.0	2.1±0.3	2.3±0.6	NS

SBP, Sistolic Blood Pressure; DBP, Diastolic Blood Presure; PAC, Plasma Aldosterone Concentration; captoPAC, PAC after captopril; PRA, Plasma Renin Activity; captoPRA, PRA after captopril; ARR, Aldosterone-Renin Ratio; captoCortisol, Cortisol after captopril; Drugs numbers, numbers of drugs before adrenalectomy. NA, Not Available. Mean ± SE.

[a] Pattern 4 vs 1, 2. Pattern 2 vs 5.

[b] Pattern 2 vs 3,4,5.

[c] Pattern 3 vs 1, 2, 5.

[d] Pattern 1 vs 4.

Morphometric Analysis of APAs, Clusters and Nodules

Immunoreactivity for CYP11B2 and CYP11B1 was evaluated according to the modified version of the McCarty H-score as specified in the Method section. In each adrenal gland adenomas, clusters and nodules of aldosterone-producing cells were assessed semi-quantitatively for both CYP11B2 and CYP11B1 staining.

H-score quantification for CYP11B2 and CYP11B1 in patterns is reported in Table 5.

The CYP11B2 H-score in APAs resulted higher in pattern 1 and 2 than in pattern 3 (528±131; 571±55 vs 16±13. p=.04). The H-score analysis for CYP11B2 positive clusters showed that NAG clusters presented H-score similar to H-score obtained in clusters of pattern 4 (349±56 and 377±82) and higher (165±32, p≤.020) than that of clusters in pattern 2.

The analysis of the total CYP11B2 H-score in patterns and NAG (Table 5) showed that patterns 1 and 2 presented CYP11B2 H-score two fold higher than pattern 4 and NAG (p≤.05). Interestingly, NAG and adrenal glands classified in pattern 4 showed similar CYP11B2 H-score (377±82 and 349±56).

Concerning total CYP11B1 total H-score, pattern 2, in which the adenoma was double stained for the two steroidogenic enzymes, showed a total higher score than the CYP11B1 positive adenoma of pattern 3, and the multinodular adrenals of pattern 5 (325±32 vs 218±185 and 117±19, respectively. p≤.01).

Table 5. CYP11B2/CYP11B1 H-score values for NAG and pattern of PA patients

CYP11B2 H-score (A.U.)							
	Pattern 1 (n=15)	**Pattern 2** (n=43)	**Pattern 3** (n=2)	**Pattern 4** (n=24)	**Pattern 5** (n=10)	**NAG** (n=16)	**p**
APA	528±131	571±55	16±13	-	-	-	0.038[a]
Clusters	216±55 (n=10)	165±32 (n=24)	95 (n=1)	377±82	215±36 (n=8)	349±56	≤.020[b]
Nodules	-	4.6±4.5 (n=2)	-	-	6.7±2.7	-	NS
Total B2	672±141	663±59	65±62	377±82	179±40	349±56	≤.02[b, c] ≤.05[a, d]
CYP11B1 H-score (A.U.)							
	Pattern 1 (n=15)	**Pattern 2** (n=43)	**Pattern 3** (n=2)	**Pattern 4** (n=24)	**Pattern 5** (n=10)	**NAG** (n=16)	**p**
APA	199±61	295±33	185±151	-	-	-	NS
Clusters	60±16 (n=10)	50±10 (n=24)	15 (n=1)	91±18	83±17 (n=8)	67±12	.05[e]
Nodules	-	23±17 (n=2)	-	-	45±9	-	NS
Total	240±60	325±32	218±185	91±18	117±19	67±12	≤.01[b, f, g]
number of clusters	2.7±0.8	2.2±0.5	1 (n=1)	7.2±1.4	3.3±0.9	9.6±2.0	≤.02[g] ≤.001[b] .029[h]

AU, Arbitrary Units. Mean±SE.
[a] Pattern 2 vs 3
[b] Pattern 2 vs 4 and NAG
[c] Pattern 5 vs 1 and 2
[d] Pattern 1 vs 3, 4 and NAG
[e] Pattern 2 vs 4
[f] Pattern 2 vs 5
[g] Pattern 1 vs 4 and NAG
[h] Pattern 5 vs NAG

The CYP11B2 H-score and Aldosterone Production Before Adrenalectomy

We hypothesized that the H-score for CYP11B2 reflected the aldosterone production. Hence, we investigated if CYP11B2 or CYP11B1 score in APA, nodules, clusters or the total score correlated with the AVS characteristics of patients (Table 6, figure 8). To this aim the correlation of the CYP11B2 and CYP11B1 H-score with PAC in the APA, contralateral side and the inferior vein cava were analysed.

The CYP11B2 H-score detected in APAs correlated directly with PAC (r=0.388, p=.002) and aldosterone concentration in the inferior vein cava (IVC) (r=0.454, p=.006) (figure 8). By contrast the CYP11B1 H-score did not correlate with cortisol production.

The CYP11B2 H-score in the clusters showed a direct correlation with aldosterone concentration in the adrenal vein when this value was normalized for the aldosterone in IVC (r=0.542, p=.001) (figure 8). Interestingly, even nodules prevalently stained for CYP11B1, showed a CYP11B2 H-score that was highly correlated with the ARR (r=0.815, p=.002), albeit not with plasma cortisol level.

Total CYP11B2 H-score was evaluated from the sum of the CYP11B2 staining intensity in all the aldosterone producing structures, including APA, clusters and nodules. It showed a direct correlation with PAC (r=0.335, p=0.002), aldosterone in IVC (r=0.338, p=.0016) and the ratio of the aldosterone in the dominant side normalized for the aldosterone concentration in IVC (r=0.492, p=0.023) (figure 8).

Table 6. Correlation of CYP11B2/CYP11B1 H-score and biochemical parameters

H-score / Biochemicals	CYP11B2 APA	CYP11B1 APA	CYP11B2 Clusters	CYP11B1 Clusters	CYP11B2 Nodules	CYP11B1 Nodules	CYP11B2 Total	CYP11B1 Total
PAC	**.388****	.144	.090	-.049	.368	.298	**.335****	.166
Capto-PAC	.361	.159	-.150	-.210	.350	.483	.297	.152
PRA	.084	-.097	-.141	-.117	-.357	-.146	.066	-.043
ARR	.143	.196	.039	-.066	**.815****	.466	.140	.191
PCC	.175	-.265	.012	.189	-.603	-.260	.192	-.147
Aldo IVC	**.454****	.035	-.131	-.262	-.264	-.079	**.338***	.040
Aldo APA	.117	.231	**.542****	**.554****	.094	-.245	**.458***	.293
LI	.112	-.019	-.019	-.015	.559	.237	.148	.198
CL Index	-.222	-.233	.182	.128	-.253	-.294	-.099	-.177
RAS Index	-.077	-.094	.050	.113	.533	.256	.044	.024

PAC, Plasma Aldosterone Concentration; Capto-PAC, PAC after Captopril stimulation; PRA, Plasma Renin Activity; ARR, Aldosterone-Renin Ratio; PCC; Plasma Cortisol Concentration; Aldo IVC, [Aldosterone] in the inferior vein cava; Aldo APA, [Aldo]/[Aldo IVC] in the adrenal vein of APA side; LI, Lateralization Index, (aldosterone/cortisol $_{\text{APA adrenal vein}}$)/(aldosterone/cortisol$_{\text{contralateral adrenal vein}}$); CL Index, (aldosterone/cortisol $_{\text{contralateral adrenal vein}}$)/(aldosterone/cortisol $_{\text{IVC}}$); RAS Index, (aldosterone/cortisol $_{\text{APA adrenal vein}}$)/(aldosterone/cortisol $_{\text{IVC}}$). ** p≤.01;* p≤.05

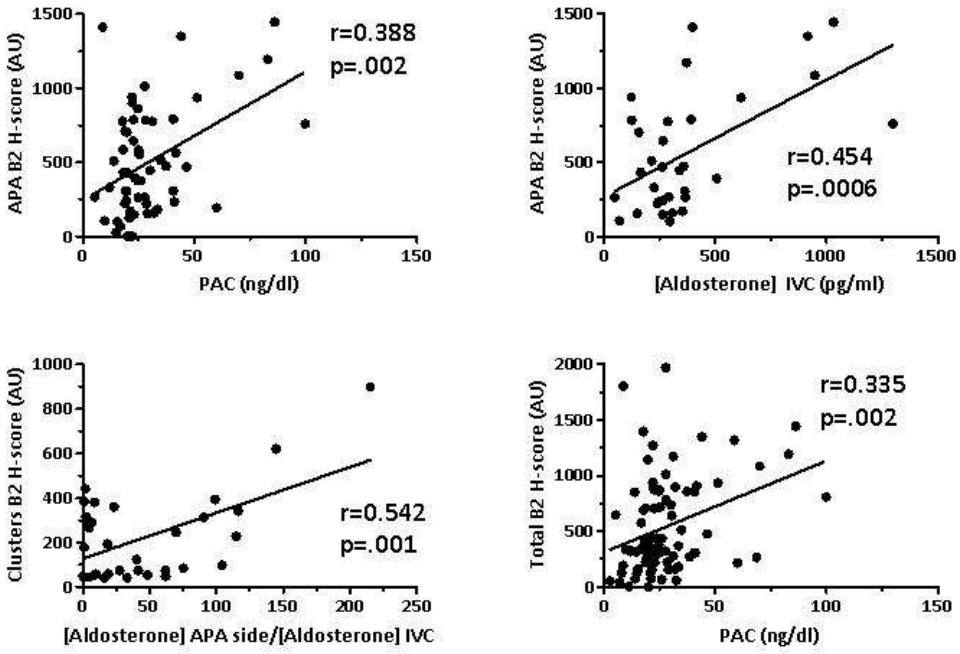

Figure 8. Correlations of CYP11B2 H-score with the aldosterone production

Steroidogenic Patterns and H-score do not Predict the Biochemical Profile After Adrenalectomy

After adrenalectomy, the biochemical profiles of the APA patients classified into steroidogenic patterns did not differ significantly among patterns.

After adrenal surgery all PA patients showed the decrease of aldosterone concentration to normal level and the expected increase of plasma renin activity (PRA). However, no clinical differences were found among steroidogenic patterns.

Concerning the H-score, CYP11B2 expression in clusters correlated with PRA ($r=0.386$, $p=.004$) and CYP11B2 score in nodules correlated inversely with plasma K^+ levels ($r=-0.844$, $p=.001$).

To further understand if different H-scores immuno-phenotypes may predict different outcomes after surgery we performed IHC and measurement of CYP11B2 and CYP11B1 H-score also for the eight patients, who were retrospectively diagnosed as BAH at follow up and the five patients diagnosed as BP-NC. In particular, BAH adrenals presented very heterogeneous immunostaining profiles: one case showed an adenoma positive for CYP11B2, three cases displayed an adenoma prevalently positive for CYP11B1, one case showed no adenoma but multiple clusters of sub-capsular CYP11B2 positive and three patients had multiple nodules positive for CYP11B1 and CYP11B2 cell clusters. BP-NC patients were all classified as pattern 3 (i.e. adenomas prevalently positive for CYP11B1).

Quantification of H-scores in BAH and BP-NC adrenals are reported in Table 7.

No correlation among biochemicals of BAH patients and BP-NC patients and CYP11B2 and CYP11B1 H-score were found, either at baseline or follow up.

Table 7. CYP11B2/CYP11B1 H-score values for BAH, NAG and BP-NC patients

CYP11B2 H-score (A.U.)				
	BAH (n=8)	**BP-NC** (n=5)	**NAG** (n=16)	**p**
APA	11±5 (n=4)	43±27	-	NS
Clusters	423±121	188±38	349±56	NS
Nodules	6.5±6 (n=3)	-	-	NS
Total B2	378±117	194±68	349±56	NS
CYP11B1 H-score (A.U.)				
	BAH (n=8)	**BP-NC** (n=5)	**NAG** (n=16)	
APA	70±36 (n=4)	439±162	-	NS
Clusters	122±48	60±22	67±12	NS
Nodules	44±21 (n=3)	-	-	NS
Total	158±46	487±149	67±12	<.01[a]
Num clusters	6.2±1.9	2.2±0.6	9.6±2.0	NS

BAH, Bilateral Hyperplasia; BP-NC, Blood Pressure Not Cured; NAG, Normal Adrenal Gland; Num clusters: number of CYP11B2 positive clusters. Mean±SE.
[a] BP-NC vs NAG and BAH

DISCUSSION

After 60 years from Conn's first description of APA, only in the last 10 years great strides have been made in our understanding of the genetics and pathophysiology of PA in spite of more than nine thousand studies published on the topic. The current Endocrine Society guidelines for the detection of PA[23] and the *'Four Corners Criteria'*[44] are key for the correct diagnosis of hyperaldosteronism. However, until only recently the histopathology diagnostic criteria for the assessment of the adrenals excised from patients with PA lagged far behind. When a yellow, well-circumscribed tumor is found, it seemed reasonable to assume that this entailed an APA. However, standard histological staining by itself provides no information on the functional phenotype, and therefore whether the nodule is responsible for the over-production of aldosterone remains ambiguous. The only way to identify aldosterone-producing cells is the detection of the final enzymes involved in the biosynthesis of aldosterone, i.e. aldosterone synthase. This steroidogenic enzyme, encoded by the CYP11B2 gene, shares 93% of the aminoacidic sequence with CYP11B1 gene, the enzyme involved in the synthesis of cortisol. This is the reason for the difficulty in generation of specific and not cross-reacting antibodies. Only recently, prof. Gomez-Sanchez from the University of Mississippi Medical Center (Jackson, USA) and prof. Nishimoto from the Keio University (Tokyo, Japan)[53,59] achieved this goal generating antibodies against CYP11B2 and CYP11B1. This is one of the most important advances in the study of the adrenal gland in the last years, and is expected to improve our understanding of the adrenal disorders, as PA.[96]

We benefited from the antibodies developed in the laboratory of prof. Gomez-Sanchez and, in collaboration with him, we identified the steroidogenic immunophenotypes of the adrenal glands in a large cohort of PA patients.

Identification of Five Steroidogenic Patterns in PA Adrenals

By analysing 111 adrenal glands removed from PA we identified five different immunophenotypes. We arbitrarily termed as "patterns", numbering consecutively from 1 to 5. Patterns 1 and 2 comprised the adenomas that could be named "classical APA" because the cases presented a well-circumscribed tumor positively stained for CYP11B2 (figure 5). Differently from pattern 1, pattern 2 showed a mixture of cells positive for CYP11B2 or CYP11B1, with some cells co-expressing both the markers as confirmed by triple immunofluorescence for CYP11B2, CYP11B1 and the ZF enzyme CYP17α (figure 6). This latter gene encodes for the 17α–hydroxylase, an enzyme expressed in the zona fasciculata and in the zona reticularis of the adrenal gland. Co-expression of CYP11B2 and CYP17α clearly indicates that those cells present the enzyme machinery needed to produce both aldosterone and cortisol, and therefore that they exhibit a mixed phenotype with ZG and ZF characteristics.

Clinically, both patterns (in particular pattern 2) are characterized by a high percentage of female and younger patients, with lower BMI than patients of any other pattern (patterns 3, 4 and 5). The results of our study are in agreement with the results of a previous study by Azizan,[97] which reported that 58% of female APAs were characterized by a ZF transcriptional profiles, suggesting a dimorphism for histopathology of APA.

The other patterns (i.e. patterns 3, 4 and 5) showed "non classical" aspect of APA: pattern 3, displayed negative staining for CYP11B2 in the main nodule but strong positivity for CYP11B1, with aldosterone-producing cells confined to clusters in the sub-capsular region (figure 5); pattern 5 showed different nodules prevalently stained for CYP11B1 with few cells positive for CYP11B2 and CYP11B2 cell clusters (figure 5), and pattern 4 was characterized by CYP11B2 cell clusters with no nodule(s) (figure 5).

Aldosterone and Cortisol Production in the Steroidogenic Patterns Before Adrenalectomy

After identification of the CYP11B2 and CYP11B1 steroidogenic patterns of a large cohort of adrenal glands excised for PA, we wondered if aldosterone and cortisol production differs among them.

It was expected that the tumors containing only CYP11B1-positive cells (i.e. pattern 3) were cortisol-producing and non-hyperfunctioning but, on the contrary, cortisol levels did not differed between patterns. Furthermore, unexpectedly the absence of an aldosterone-producing tumor in patterns 3, 4 and 5 was not associated with lower aldosterone production than patterns 1 and 2, showing PAC similar to those of classical APAs. Hence, it is concevaible that aldosterone production in patterns 3, 4 and 5 could be due to the activity of CYP11B2 positive cell clusters that, therefore, could play a major role in the excess aldosterone production in PA.

Clusters of cells expressing CYP11B2 were first identified and named aldosterone-producing cell clusters (APCCs) by Nishimoto's group in 2010,[59] but their role in autonomous aldosterone production and potentially PA remains unknown. In NAGs APCCs are located just below the adrenal capsule and prolonged into cortisol-producing cells, as described in the present study and previous reports.[59,78,82] Considering the low circulating renin levels in PA patients, the aldosterone production by APCC was deemed to be renin-independent. However, the presence of APCC in normal adrenal tissue indicates that APCC are not markers of excess aldosterone production. Whether APCC may represent a precursor of the APA is an intriguing question. Nishimoto and Rainey's at the University of Michigan, by analysing the transcriptome profile of APCC in a series of normal adrenals, detected the presence of CACNA1D and ATP1A1, two somatic mutations that are recurrently observed in APA population.[98] These mutations are known to cause aldosterone over-production in the APA,[99] thereby suggesting a role for APCC in the development of aldosterone-producing tumors, and also explaining the similar PAC value found in the patients with patterns 3, 4 and 5.

The Steroidogenic Patterns identify Different KCNJ5 Genetic Profile

Somatic mutations of the G protein-activated inwardly rectifying K^+ channel Kir3.4, encoded by the KCNJ5 gene, were discovered by Choi et al. in 2011[32] and represented a major advance in the understanding of the pathogenesis of PA. The overall prevalence of KCNJ5 mutations in APA population is very high (around 43%)[100] and therefore, the comprehension of the molecular mechanisms triggered by KCNJ5 and putatively involved in the aldosterone-overproduction is of primary interest.

KCNJ5 mutations are located in, or close to, the selectivity filter,[101] a highly conserved region within this family of channels that allows the selective transport of K^+ over other cations.[32] Mutations of Kir3.4 in *in vitro* experiments resulted in loss of channel selectivity with increase in intracellular sodium, membrane depolarization, and increase in mobilization of calcium, resulting in enhanced expression of the CYP11B2 enzyme and synthesis of aldosterone.[101]

The results of our study showed an interesting correlation between the histological pattern and the genotype. KCNJ5 mutations were detected mostly in adrenal glands with a nodule expressing a mixture of CYP11B1 and CYP11B2 positive cells, classified as pattern 2. This is in agreement with a previous study conducted by Azizan[102] that reported that KCNJ5 mutated tumors presented with a ZF genetic profile associated with a tumor composed mainly of large, CYP11B1 or CYP11B2 and CYP17α positive cells.

A recent meta-analysis of KCNJ5 mutations in APA patients[100] reported that the presence of mutations in the KCNJ5 gene identify patients with higher PAC than wild type. Even though we observed an increased PAC in patients with KCNJ5 mutated tumors, compared to the wild type, this increase was not significant. Such a disagreement could be explained by the small number of genotyped patients in our cohort (n=56) that did not permit to reach the statistical significance. It is also true that our wild-type tumors are probably a heterogeneous group of adenomas carrying other somatic mutations (not investigated in this study) that could increase aldosterone synthesis (e.g.

ATP1A1, ATP2B3 or CACNA1D)[84,85] and other so far unidentified genes that may be involved in regulating and over-producing aldosterone in APAs.

H-score Reflects the Aldosterone Production before Adrenalectomy

Immunohistochemistry (IHC) is one of the most common used technique in the research laboratories, due to its not expensive approach, feasibility of the procedures and warranty of unequivocally identifying the presence of markers in the tissue. The antibodies should be specific and not cross-reacting with epitopes similar to the antigens against which the antibodies were raised. Moreover, an intense and definite chromatic reaction is expected to be developed where the antigen is localized, and the staining intensity should be easily measurable to investigate if specific markers recognized by the antibodies correlate with clinical or biochemical variables.

The availability of the novel antibodies for CYP11B2 and CYP11B1 specific for the target enzymes prompted us to develop a quantification system that permits to quantify the intensity of the immunostaining. The most used quantification scoring system for IHC markers is the McCarty's H-score system[80] in which the percentage of the stained cells is multiplied by a factor ranging from 0 to 3 to reflect the intensity of cell immunostaining. H-score, which has been used by oncologists for years, recently has been also proposed as a tool to investigate the steroidogenesis in the adrenal gland tumors. The main drawback of H-score is the staining intensity operator-dependent assessment, which accounts for the inter-assay variability. Hence, in the present study we developed a modified version of the McCarty H-score to overcome this problem by measuring directly the staining intensity using an observer-independent PC-assisted method specifically created in our laboratory with ImageJ software (detailed explanation in the Methods section). To avoid missing information on the aldosterone synthesis in the whole adrenal gland, in our study, we quantified H-score for each potential aldosterone-producing structure stained with CYP11B2, including APCCs, APA and nodules. The results of our study showed that CYP11B2 score in APA and clusters reflected the aldosterone levels before adrenalectomy. A correlation between the

classic H-score staining of CYP11B2 and CYP11B1 limited to the adenoma was searched previously by three independent groups, but it was found only when the score was normalized for the tumor area or volume.[76,81,82] Hence, the results of our study showed that this modified version of H-score provides more accurate information on the functional activity of CYP11B2 in the adrenal gland and that there is the need to analyse all the structures involved in aldosterone production to obtain a correct clinical correspondence.

By contrast, CYP11B1 H-score did not correlate with the cortisol production, likely because we assessed the CYP11B1 expression in structures that are involved in the aldosterone, not cortisol synthesis, thereby excluding zona fasciculata cells. Whether CYP11B1 H-score evaluated in the whole adrenal gland may provide information on the cortisol production remains unknown.

The Steroidogenic Patterns and H-score do not Predict the Biochemical Outcome of PA Patients

We investigated the biochemicals and BP correction outcome of unilateral adrenalectomy among 111 patients operated for PA in three referral centers for hypertension in Italy. As expected, at follow up all APA cases exhibited a decrease in aldosterone and an increase in renin and K^+ levels, but a surprising finding was that these patients, although showing different steroidogenic patterns, did not show different biochemical pictures after surgery, suggesting that PA disease involved several histological appearances but they are equally resolved by the resection of the aldosterone over-producing adrenal gland. In addition, when we studied the correlations among the staining intensity of CYP11B2 and CYP11B1 of the adrenal glands with biochemical or clinical variables of patients at follow up, no correlation were found. Hence, in our hands H-score was not a predictive tool for the biochemical outcome of adrenalectomized patients.

Furthermore, when we analysed the adrenal glands from patients who underwent surgery because unilateral over-production of aldosterone was found at AVS, but were diagnosed as bilateral

hyperplasia (BAH) retrospectively, we found an high variability of steroidogenic immuno-phenotypes, making difficult to identify a pattern that is characteristic for BAH.

Of interest, when we processed the adrenal glands of five patients who successfully resolved the biochemical PA picture with adrenalectomy, but still presented elevated BP levels at follow up, they showed histological characteristics of patients classified as pattern 3 (i.e. CYP11B1 positive adenoma). This finding suggests that pattern 3 is the pattern that characterizes the patients who take advantage of adrenal surgery for biochemical, but still require medications to control BP levels. Hence, patients who show steroidogenic pattern 3 at histology would require strict follow-up after surgery to achieve optimal blood pressure control.

CONCLUSIONS

The present study revealed that in a large cohort of PA patients who underwent surgery on the basis of the AVS the histological profile of the removed adrenal gland was very heterogeneous.

Five different steroidogenic patterns can be identified with the use of recently developed monoclonal antibodies against the aldosterone synthase and the 11β-hydroxylase. The steroidogenic patterns with a "classical APA" appearance (i.e. patterns 1 and 2) pinpoint patients with biochemical and genetic characteristics that are different from those entailing "non canonical" APA (i.e. patterns 3, 4 and 5).

The modified version of the McCarty H-score developed in our laboratory was observer-independent and took into account not only staining intensity, but also the surface of the stained structures. Moreover, H-score was assessed not only in APA but also in APCCs and nodules, thereby providing accurate information on the aldosterone synthesis in the whole adrenal gland. Hence, the H-score developed by our group could be more accurate in estimating aldosterone production than the classical H-score.

After adrenalectomy, the steroidogenic patterns and the modified H-score failed to predict different clinical outcomes for either APA or BAH patients. However, pattern 3 was mostly found in patients who took advantage of adrenal surgery for correction of the biochemical picture, but did not reach optimal BP control.

In conclusion, based on the results of our study we would like to propose a novel classification of surgical-correctable PA into CYP11B2 and CYP11B1 steroidogenic patterns that could be a useful diagnostic tool to localize aldosterone production sites in the excised adrenal gland and contribute to provide better understanding in the pathophysiology of PA disease.

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