Adrenal Histopathology in Primary Aldosteronism
Is It Time for a Change?

Francesca Gioco,* Teresa Maria Seccia,* Elise P. Gomez-Sanchez, Gian Paolo Rossi, Celso E. Gomez-Sanchez

• Online Data Supplement

Primary aldosteronism (PA) entails a group of disorders characterized by excess aldosterone production, relatively independent from the renin–angiotensin system.1 Because PA is the most common form of secondary hypertension and patients with PA have higher cardiovascular morbidity and mortality than age- and sex-matched patients with essential hypertension and the same degree of blood pressure elevation,2,3 early recognition of PA is of utmost importance to cure the high blood pressure and prevent deleterious cardiovascular effects. The 2 major PA subgroups are the unilateral forms, mainly aldosterone-producing adenoma (APA), and bilateral forms, mostly adrenal zona glomerulosa (ZG) hyperplasia, which require surgical or medical treatment, respectively. Adrenal vein sampling (A VS) is the best available method to distinguish between unilateral and bilateral aldosterone hypersecretion.4,5 In medical centers where AVS is available and strict cutoff values for the lateralization index are used,6 almost two thirds of PA cases are attributed to an APA, whereas one third remain classified as bilateral idiopathic forms. The opposite is seen when AVS is unavailable.10 Hence, the availability of AVS greatly affects the recognition of unilateral forms of PA that can be successfully treated with adrenalectomy.

After surgery, demonstration of an adenoma at pathology is needed to reach a conclusive diagnosis of the only PA subtype that can be unequivocally diagnosed, eg, APA. After the introduction of the 4 corner criteria,10 an APA can be diagnosed when (1) there is evidence of PA at the screening tests, with an inappropriately high aldosterone/renin ratio and confirmation as needed; (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 until recently no clear criteria define the aldosterone-producing cells.11 Pathologists routinely perform hematoxylin and eosin (HE) staining of the adrenal gland to detect the presence of at least 1 nodule that is 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 (Figure A). 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 hyperchromasia. The histological distinction between an adenoma and a nodule is not clear cut. 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.12,13 The presence of multiple nodules in an adrenal cortex often is interpreted as being composed of multiple adenomas or nodular hyperplasia.14 Most APAs also exhibit ZG hyperplasia,15 but this is open to histological interpretations as some groups describe that this is seldom seen.16 Nevertheless, because HE staining provides no information on the functional phenotype, whether the nodule(s) is responsible for the overproduction 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 technique was used in some laboratories to ascertain the ability of the nodules found in adrenal specimens from patients with PA of synthesizing aldosterone.17,18 However, in situ hybridization is not feasible for everyday hospital histopathology, being time-consuming and difficult in its implementation.

An approach based on immunohistochemistry would be strongly desirable as it is faster and less expensive than in situ hybridization and suitable for automated systems, and therefore, it can be routinely used by most diagnostic pathology services. After a long quest, the antibodies against human CYP11B2 and 11β-hydroxylase (CYP11B1) have been recently developed. However, because their use is currently restricted to few research laboratories and not yet commercially attainable, immunohistochemistry has remained an
impracticable approach for the routine diagnostic workup of the APA. Commercial antibodies are available from several sources but more often than not the immunizing peptide is proprietary, and the validation is suspect 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 not only allows unequivocal detection of aldosterone and cortisol producing cells but also sheds new light on the histology of normal adrenal gland and APA. In fact, the use of the specific antibodies against CYP11B2 and CYP11B1 unexpectedly unveiled that different patterns of CYP11B1 and CYP11B2 can be associated with an APA, thereby questioning the classical view of APA as a single, well-demarcated or encapsulated nodule exclusively constituted by cells producing excess aldosterone. Hence, in this review, we report how the antibodies against CYP11B2 and CYP11B1 have been developed and discuss how the use of these antibodies is improving our knowledge of PA pathophysiology.

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 ZG, and it is regulated primarily by the Na⁺ intake via the renin–angiotensin system, plasma K⁺ concentration, and adrenocorticotrophic hormone levels. ZG cells are hyperpolarized by K⁺ influx through different channels; however, when a stimulus takes place, the cell membrane depolarizes, and this results in enhanced Ca²⁺ intake via L-, N-, and T-type Ca²⁺ channels. Moreover, mobilization of intracellular Ca²⁺ from the endoplasmic reticulum further increases intracellular Ca²⁺ load, with phosphorylation of transcription factors and activation of CYP11B2 gene transcription. CYP11B2 encodes for the enzyme aldosterone synthase, a multifunctional cytochrome enzyme, that first hydroxylates deoxycorticosterone at position 11β to form corticosterone and then at position 18 to generate 18-hydroxycorticosterone. The bound 18-hydroxycorticosterone is then finally hydroxylated at position 18 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.

In zona fasciculata (ZF) cells, the enzyme 11β-hydroxylase, which is coded by CYP11B1 gene, converts 11-deoxycortisol and deoxycorticosterone to generate cortisol and corticosterone. Both CYP11B2 and CYP11B1 are mitochondrial enzymes that share 93% of amino acid sequence, making it difficult to generate specific antibodies able to distinguish between the enzymes. Until now, only 2 groups have successfully achieved this goal. Two decades ago, Ogishima et al generated polyclonal antibodies for CYP11B2 and CYP11B1 by synthesizing oligopeptides (Figure S1 in the online-only Data Supplement) corresponding to the 80- to 90-amino acid residues and coupling...
them to equine myoglobin to immunize domestic rabbits, but only recently, they reported their use. More recently, monoclonal antibodies against the 2 human steroidogenic enzymes have been developed in the laboratory of Gomez-Sanchez et al. Five different immunization peptides were designed to cover the area where CYP11B2 and CYP11B1 amino acid 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 to obtain monoclonal antibodies. The antibody against CYP11B2 was obtained from the animals injected with the 41- to 52- amino acid sequence shown in Figure S1 that differs from the sequence previously used by Ogishima et al. The antibody against CYP11B1 was achieved from rats inoculated with a peptide corresponding to the 80- to 90-amino acid sequence and that used by Ogishima et al in 1991. ELISA and western blot analysis confirmed the specificity of the 2 antibodies against CYP11B2 and CYP11B1. Monoclonal antibodies produced from hybridoma cells are against a limited epitope; thus, they have a clear advantage compared with 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.

**Histology of the Normal Human Adrenal Gland**

In the classical view, 3 major zones compose the adrenal cortex: the outermost ZG, the midzone ZF, and the innermost zona reticularis, with ZG and ZF cells producing aldosterone and cortisol, respectively. The functional zonation in humans was originally presumed based on histology and biochemistry of rodent adrenals, with no direct evidence from humans. Only recently, immunostaining of the normal human adrenals with antibodies generated by Ogishima et al 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 2 types of CYP11B distribution, termed conventional and variegated. Conventional distribution shows a zonation similar to that described in rodents: 3 different zones (ZG, ZF, and zona reticularis) 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 zona reticularis. In the variegated pattern, there are clusters of cells strongly positive for CYP11B2 in the subcapsular area, with the rest of the cortical area expressing CYP11B1. The clusters, also called aldosterone-producing cell clusters (APCCs), are usually characterized by a width of 200 to 1300 μm and a depth of 100 to 500 μm beneath the capsule, but occasionally, they are in direct contact with the capsule. APCCs also express 3β-hydroxysteroid dehydrogenase but not 17β-hydroxylase/17,20-lyase, the enzyme required for the synthesis of the cortisol precursor 17α-hydroxyprogrenolone, and are surrounded by columnar ZF-like cells forming cords along sinusoids. The cells in subcapsular areas that are devoid of APCCs lack both CYP11B1 and CYP11B2.

The most recent monoclonal antibodies developed in the laboratory of Gomez-Sanchez et al confirmed the existence of variable patterns of the human adrenal cortex. The analysis of normal adrenals from 4 adults and one 5-day old infant documented that CYP11B2 immunoreactive cells exhibited the 2 patterns: the first characterized by scattered cells and the other identified by more tightly clustered cells, that is, APCC. CYP11B1-positive cells extended up to the capsule in many portions of the cortex and were intermingled with CYP11B2 immunopositive cells. Double staining demonstrated that CYP11B2 and CYP11B1 were mostly expressed in different cells, but double immunoreactive cells were occasionally found in the subcapsular region.

**Adrenocortical Steroidogenic Zonation With Aging**

The adrenal cortex samples from adults had relatively few CYP11B2 immunoreactive cells close to the capsule when compared with the infant adrenal, which showed many more. The higher plasma levels of aldosterone in infants compared with normal adults is presumably because of 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. Such contention is 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 immunoreactive cells than those in the infants, suggesting that adrenal zonation changes with aging along with basal aldosterone levels. It may also reflect the relatively high-sodium levels in diet. Homogeneous columns of ZF cells topped with ZG cells mainly constituted the subcapsular 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 3β-hydroxysteroid dehydrogenase and P450sc, also known as zona progenitor (ZP) cells, appeared and gradually occupied the upper portion of the cortical cords. 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, thereby suggesting that functional zonation does not correspond to the morphological 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, that is, ZP cells, thereby suggesting a role in adrenal gland development and functional plasticity. According to another hypothesis, ZP cells are steroidogenic latent cells that could differentiate in either ZG or ZF according to further stimulation. 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-replete 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 hypothalamic–pituitary–adrenal activity would decrease the requirement for cortisol synthesis, and 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 adults. Whether the number of ZG cells, and consequently of ZP cells, changes, for example, in response to sodium intake, remains unclear, evidence from experimental models supports this contention. 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, and the width of the ZG and the number of CYP11B2-positive cells markedly increased in chronically sodium-depleted rats. However, adrenal analysis after manipulation of sodium intake is possible in experimental animals, not in humans, and 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 probably do not reflect normal physiological status.

### 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. 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 A). Using the antibodies generated by Ogishima et al, Nishimoto et al identified 3 cell types within the APA: (1) cells positive for CYP11B1 but negative for CYP11B2, (2) cells positive for CYP11B1 but negative for CYP11B2, and (3) cells negative for either CYP11B1 or CYP11B2 (Figure B). No cells showing double staining for CYP11B1 and CYP11B2 were found. In contrast, Nakamura et al reported that APAs contained not only a mix of CYP11B2 and CYP11B1-positive cells but also cells expressing both CYP11B1 enzymes. Immunoreactivity for CYP11B1 tended to be diffuse within the APA, whereas that for CYP11B2 was heterogeneous and spotted. CYP11B1 was strongly expressed outside the adenoma, whereas there was little expression of the CYP11B2 outside the adenoma. CYP11B1, but not CYP11B2, localized with 17β-hydroxylase/17,20-lyase in most APA cells. Because 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 and the tumor size. Using a simple semiquantitative system that assigned a score of 1 to dimly immunostained APAs, whereas a score 2 or 3 to well-markedly stained tumors, Nanba et al found that the score assessed for CYP11B2 inversely correlated with the tumor size calculated by assuming a spherical shape of the tumor. When adjusting the CYP11B2 score for the tumor volume, Nanba et al found a positive correlation between CYP11B2 staining score and plasma aldosterone concentration or the aldosterone/renin ratio. The score negatively correlated with serum K⁺ levels, thus leading support to the hypothesis that CYP11B2 score reflects aldosterone synthesis. 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, Nakamura et al observed that CYP11B2 H-score in the APA (44.0±5.1, mean±SE) was not significantly different from that calculated in the normal adrenal gland (58.6±9.5, mean±SE), but it was higher than that measured in the tissue adjacent to the APA (24.5±4.9, mean±SE). However, they found no relationship between intensity of CYP11B1 or CYP11B2 immunostaining and tumor size or age, in contrast to Nanba, thereby leaving unclear whether the intensity of immunostaining truly reflects the amount of steroidogenesis. Experimental evidence with cultured cells and animals suggests that it would. Because of the not perfectly circular shape of the tumors, the area of the adenoma was more precisely determined with a specific software by Ono et al. By analyzing a series of 40 APAs, they found that the CYP11B2 H-score was higher in APAs with an area smaller than the median value of 60 mm² compared with those with an area of ≥60 mm², suggesting that small APAs produce more aldosterone per cell than large tumors as previously reported by Nanba et al. However, when CYP11B2 H-score was multiplied by tumor area, the resulting value correlated positively with plasma aldosterone concentration. Using different CYP11B1 and 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 plasma aldosterone concentration and aldosterone/renin ratio. They also found that patients not expressing CYP11B1 had lower plasma potassium levels compared with patients expressing CYP11B1 in the adrenal nodule, but neither absolute aldosterone nor aldosterone/renin ratio levels differed between the groups.

The recent discovery of somatic mutations in the KCNJ5 gene opened a new scenario on the molecular mechanisms that control the autonomous aldosterone synthesis in APA. KCNJ5 encodes for the G-protein–activated inward rectifying potassium channel Kir 3.4, which allows the selective transport of K⁺ through the cell membrane to the extracellular space, keeping the membrane hyperpolarized. Choi et al 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 the loss of selectivity for K⁺, with ensuing leak of Na⁺ into the cell, membrane depolarization, increased [Ca²⁺], and finally CYP11B2 gene activation. KCNJ5-mutated APAs consisted mainly of ZF-like cells with high expression of CYP11B1. CYP11B2 immunostaining allowed identification of 1 case of 2 CYP11B2-positive nodules in the same APA exhibiting the same KCNJ5 mutation and 1 case reporting 2 nodules carrying 2 different KCNJ5 mutations (L168A
and G151A. 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\(^+\) channel Cav 1.3 gene CACNA1D. The loss-of-function mutations in ATPs and Ca\(^+\) channels resulted in increased intracellular [Ca\(^+\)]\(_{\text{load}}\). ATP1A1 and CACNA1D mutations were found in multinodular glands, whereas ATP2B3 mutation was found only in solitary adenomas. APAs with the ATP1A1, ATP2B3, and CACNA1D mutations most frequently had a ZG-like phenotype with high expression of CYP11B2. Nodules carrying an ATP2B3 mutation were smaller than those containing a KCNJ5 mutation, and in contrast to KCNJ5-mutated nodules, they did not contain atypical cells.

**Histology of the Adrenal Tissue Adjacent to the APA**

The adrenal tissue adjacent to an APA may show a pattern similar to that observed in the normal adrenal, consisting of diffuse CYP11B1 immunoreactivity in ZF and zona reticularis and sporadic expression of CYP11B2 in ZG, or a pattern resembling the APA, with APCCs expressing CYP11B2 and 3β-hydroxysteroid dehydrogenase but not CYP11B1 or 17β-hydroxylase/17,20-lyase. The peritumoral tissue often shows micronodules or macronodules, with some expressing CYP11B2, thereby suggesting active aldosterone production in the area surrounding APAs. These findings were consistent with those found from the in situ hybridization analysis, which showed mRNAs of steroidogenic enzymes necessary for aldosterone production (CYP11B1, CYP11B2, HSD3B2, and CYP21A2) in subcapsular micronodules adjacent to APAs. Collectively, these findings suggest that, in contrast to current opinion, a clear boundary between APA and adjacent tissue does not exist; moreover, coexisting subcapsular micronodules might be the initial foci for the development of APA.

**Clinical Implications of the Availability of the Antibodies Against CYP11B1 and CYP11B2**

Because these selective antibodies were made widely available a few years ago, several different studies were performed to analyze the CYP11B2/CYP11B1 immunohistochemical expression and clinical outcome of patients with APA. By analyzing 53 adrenals removed at surgery because of unilateral PA, Dekkers et al. observed that at follow-up, the patients with a solitary adenoma were cured more often than those with nodular hyperplasia. Volpe et al. retrospectively studied 120 consecutively unilaterally adrenalectomized patients selected on the basis of the AVS or NP59 scintigraphy. In 6 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. To date, studies searching for a relationship between immunohistochemistry pattern and clinical outcome have limitations in that they were retrospective, often spanning a long period of time, without uniform diagnostic workups for PA. The diagnostic strategies included computed tomographic scan or AVS or both, but it is well established that computed tomography can be misleading in identifying the side causing lateralized excess aldosterone production in ≤50% of the cases. Therefore, it could be that, in some cases, the removed adrenal lacked nodules with CYP11B2-positive cells simply because of side misclassification.

Because of these limitations, the clinical implications of the different cell type patterns, in terms of prediction of treatment outcome, remain to be determined.

**Conclusions**

The development of specific antibodies that selectively detect the enzymes that control the terminal reactions in the synthesis of aldosterone (CYP11B2) and cortisol (CYP11B1) allowed their precise localization in normal and pathological adrenals. Therefore, immunohistochemistry based on CYP11B2 and CYP11B1 antibodies offers great advancement when compared with the HE staining technique currently used in most laboratories, which does not allow recognition of ZG- and ZF-like cells that produce aldosterone and cortisol, respectively. The use of CYP11B2 and CYP11B1 antibodies is expected to markedly enhance the accuracy of the diagnosis.

If present, the nodule that produces aldosterone can be unequivocally revealed by the antibody against CYP11B2. However, the finding that the nodules were negative for CYP11B2 in some patients adrenalectomized for PA is puzzling. Excess aldosterone production was attributed to APCCs in these patients, but because APCCs are also present in normal adrenals, it is unclear how such cell clusters can be responsible for clinically relevant degrees of aldosterone excess. Moreover, the detection of unequivocally CYP11B2 negative nodules challenges the classic view of PA, considered as a single nodule that produces aldosterone. The content can be made that many different patterns sustain PA in patients with lateralized excess aldosterone production, but this should be tested in large data set of adrenalectomized patients with PA in whom the diagnosis was unequivocally confirmed by a well-defined set of criteria such as that of the 4 corners. If proven, this contention would support a continuum between APA and idiopathic hyperaldosteronism.

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**Disclosures**

None.

**References**


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Supplemental Figure 1

Figure S1. 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. The yellow highlighted letters are the sequences used by Ogishima and Gomez-Sanchez for synthesis of CYP11B1 peptides. The blue highlighted letters are the sequences used by Ogishima for synthesis of CYP11B2 peptides.
Figure S2: 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). This is a colour rendition of the Figure 1 presented in the text prepared to ease the identification of the different cell phenotypes. 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 phentotypes 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 steroidogenetic 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 exhibit 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.