Genetic Causes of Functional Adrenocortical Adenomas

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ABSTRACT Aldosterone and cortisol, the main mineralocorticoid and glucocorticoid hormones in humans, are produced in the adrenal cortex, which is composed of three concentric zones with specific functional characteristics. Adrenocortical adenomas (ACAs) can lead to the autonomous secretion of aldosterone responsible for primary aldosteronism, the most frequent form of secondary arterial hypertension. In the case of cortisol production, ACAs lead to overt or subclinical Cushing syndrome. Genetic analysis driven by next-generation sequencing technology has enabled the discovery, during the past 7 years, of the genetic causes of a large subset of ACAs. In particular, somatic mutations in genes regulating intracellular ionic homeostasis and membrane potential have been identified in aldosterone-producing adenomas. These mutations all promote increased intracellular calcium concentrations, with activation of calcium signaling, the main trigger for aldosterone production. In cortisol-producing adenomas, recurrent somatic mutations in *PRKACA* (coding for the cyclic adenosine monophosphate-dependent protein kinase catalytic subunit α) affect cyclic adenosine monophosphate-dependent protein kinase A signaling, leading to activation of cortisol biosynthesis. In addition to these specific pathways, the Wnt/β-catenin pathway appears to play an important role in adrenal tumorigenesis, because β-catenin mutations have been identified in both aldosterone- and cortisol-producing adenomas. This, together with different intermediate states of aldosterone and cortisol cosecretion, raises the possibility that the two conditions share a certain degree of genetic susceptibility. Alternatively, different hits might be responsible for the diseases, with one hit leading to adrenocortical cell proliferation and nodule formation and the second specifying the hormonal secretory pattern. ***(Endocrine Reviews 38: 516 – 537, 2017)***

Adrenal masses are quite common, being found in ~3% of the general population, with the prevalence increasing with age (1). Functional adrenocortical adenomas are a group of benign tumors of the adrenal cortex autonomously secreting excess hormones. Aldosterone-producing adenomas (APAs) are responsible for primary aldosteronism (PA), the most frequent form of secondary hypertension. Cortisol-producing adenomas (CPAs) are responsible for overt Cushing syndrome (CS) or subclinical Cushing syndrome (sCS). In addition, both diseases can also result from different forms of adrenal hyperplasia: bilateral adrenal hyperplasia (BAH) in PA and primary bilateral macronodular adrenal hyperplasia (PBMAH) responsible for overt CS or sCS. Primary pigmented nodular adrenal hyperplasia is a rare condition responsible for overt CS. Unilateral adrenal hyperplasia leading to autonomous aldosterone or cortisol production has also been described. In between the two conditions are histological forms of multinodular adrenal glands in which hormone production can be localized to one large nodule or to multiple nodules. Sometimes cosecretion of aldosterone and cortisol is observed in one adrenal gland, either from one adenoma or from distinct adenomas.

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Aldosterone and Cortisol Biosynthesis in the Adrenal Cortex

Aldosterone and cortisol are produced in the adrenal cortex, which is composed, in humans, of three concentric functionally distinct zones. Steroid hormones are produced from cholesterol through sequential enzymatic steps involving different cytochrome P450 enzymes and hydroxysteroid dehydrogenases, and the functional properties of each zone results from

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ESSENTIAL POINTS

- During the past few years, extraordinary progress has been made in the understanding of the genetic bases of aldosterone- and cortisol-producing adrenocortical adenomas
- Somatic mutations in genes regulating intracellular ionic homeostasis and membrane potential, promoting increased intracellular calcium concentrations and activation of calcium signaling, have been identified in aldosterone-producing adenomas
- In cortisol-producing adenomas, recurrent somatic mutations in *PRKACA* (coding for the cyclic adenosine monophosphate-dependent protein kinase catalytic subunit α) affect cyclic adenosine monophosphate-dependent protein kinase A signaling, leading to activation of cortisol biosynthesis
- Recent data have suggested an overlap of genetic abnormalities converging to different endocrine abnormalities in functional adrenocortical adenomas; in particular, the Wnt/β-catenin pathway appears to play an important role in adrenal tumorigenesis for aldosterone producing adenomas and cortisol producing adenomas
- The presence of this overlap raises the question regarding whether the two conditions might share a certain degree of genetic susceptibility or whether different hits could be responsible for the diseases, with one hit leading to adrenocortical cell proliferation and nodule formation and the second specifying the hormonal secretory pattern
- Future research is necessary to address the sequence of events leading to adrenal cell proliferation, nodule formation, and specification of the hormonal secretory pattern

the expression of specific steroidogenic enzymes and the ability to respond to unique regulatory stimuli. In particular, the outer zona glomerulosa (ZG) produces aldosterone through the activity of aldosterone synthase (encoded by *CYP11B2*), which catalyzes the three terminal reactions of aldosterone biosynthesis: hydroxylation at position C11 of the steroid intermediate deoxycorticosterone, yielding corticosterone, followed by hydroxylation at position C18, yielding 18-hydroxy-corticosterone, and a subsequent oxidation of the hydroxyl group at C18, which results in the formation of aldosterone. Cortisol biosynthesis occurs in the zona fasciculata (ZF), where the expression of 17α hydroxylase mediates conversion of 17-deoxy-21-carbon steroids into 17-hydroxy-21-carbon steroids, precursors of cortisol. Because 17α hydroxylase is not expressed in rats and mice, the main glucocorticoid in these species is corticosterone (2). Cortisol is synthesized from 17OH-progesterone through 21-hydroxylation to form 11-deoxycortisol, followed by hydroxylation at position C11 by 11β-hydroxylase (encoded by *CYP11B1*). The two highly homologous genes *CYP11B2* and *CYP11B1* are located in tandem on human chromosome 8q21-22. Unequal crossing over of the two genes during meiosis results in a chimeric gene composed of the regulatory regions of *CYP11B1* fused to the coding sequence of *CYP11B2*, which is responsible for familial hyperaldosteronism (FH) type 1 (also known as glucocorticoid remediable aldosteronism) (3). Under these conditions, aldosterone synthase expression and aldosterone biosynthesis are regulated by adrenocorticotropic hormone (ACTH) in the ZF of the adrenal cortex, rather than by angiotensin II (AngII), with a circadian rhythmicity of aldosterone production following that of cortisol (4) and production of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol (5, 6).

The main regulators of aldosterone biosynthesis are the renin–angiotensin system, the concentration of extracellular potassium, and ACTH (7). The renin–angiotensin system translates changes in renal perfusion or sodium concentration sensed by the distal tubule into changes in aldosterone production from the adrenal cortex to maintain volume and electrolyte homeostasis. AngII acts via the AT1 receptor expressed in ZG cells, activating a Gαq-phospholipase C-mediated pathway, leading to increased inositol 1,4,5-triphosphhate (IP_3) and 1,2-diacylglycerol concentrations. IP_3 increases intracellular calcium concentrations by calcium release from intracellular stores.

AngII also inhibits the background potassium channel TASK (TWIK-related acid-sensitive potassium channel) and the Na^+,K^+-adenosine triphosphatase (ATPase), leading to cell membrane depolarization and activation of voltage-gated Ca^{2+} channels, again increasing intracellular calcium concentrations. Extracellular potassium is the other major regulator of aldosterone production, exerting a large amplitude of control over a wide range of potassium concentrations. This is possible because the main ionic conductance of ZG cells is that of K^+ and the cell membrane potential closely follows the equilibrium potential of K^+ over a large range of extracellular K^+ concentrations (8). Elevation of the extracellular K^+ concentration, a decrease in the intracellular K^+ concentration, inhibition of Na^+,K^+-ATPase, or closure of potassium channels all lead to cell membrane depolarization, opening of voltage-dependent Ca^{2+} channels, and an increase in intracellular calcium concentrations, resulting in activation of calcium signaling.

Increased calcium signaling affects aldosterone production in several methods. Calcium signaling

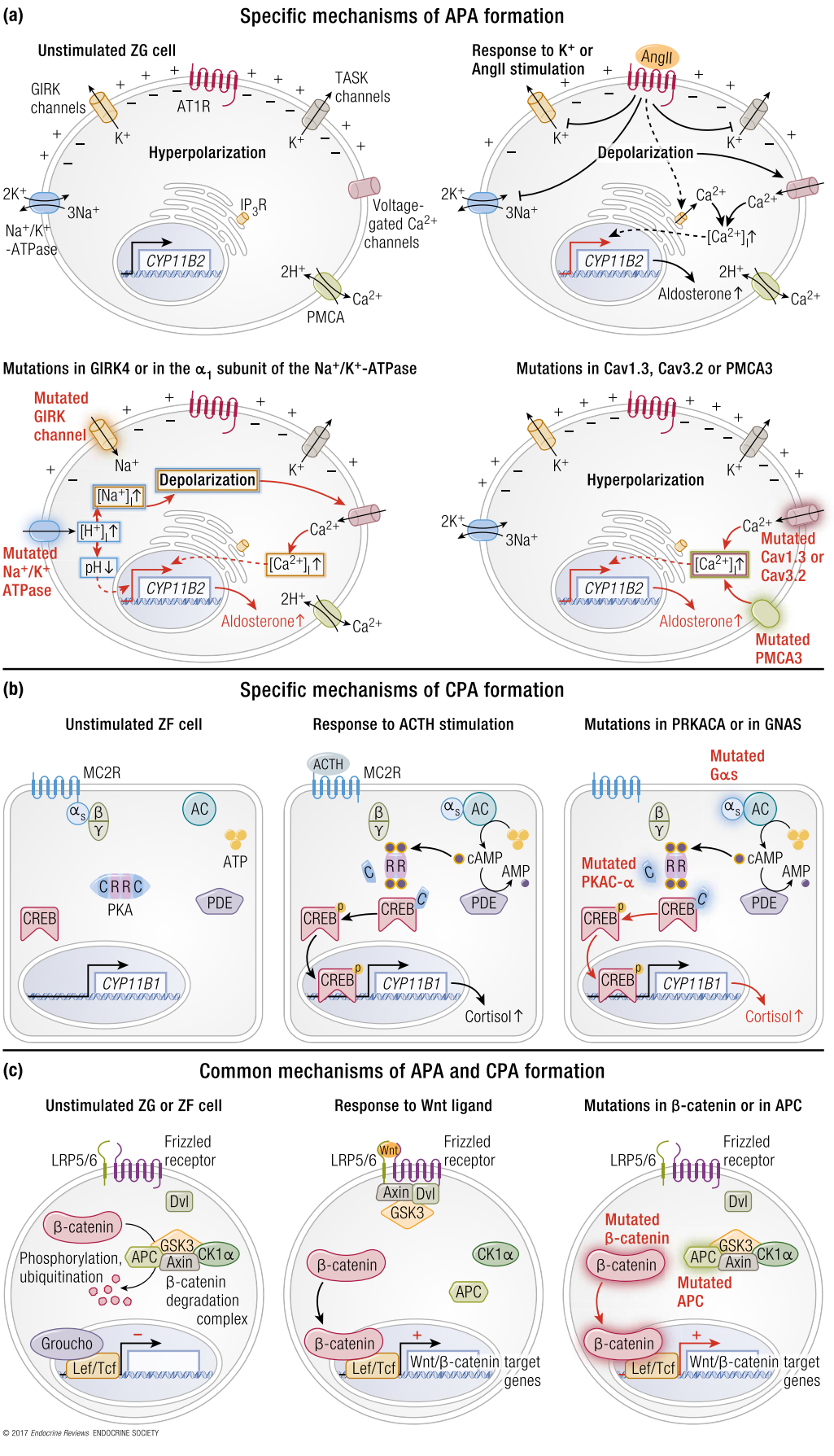


Figure 1. Specific and common mechanism of APA and CPA development. (A) Specific mechanisms of APA formation. In absence of stimulation (AngII or K^+), ZG cells are hyperpolarized owing to the presence of a large number of K^+ channels. In response to an increase of extracellular K^+ concentration or to the binding of AngII to its receptor (AT1R), inhibition of K^+ channels leads to cell membrane depolarization, opening of voltage gated Ca^{2+} channels, increased intracellular calcium concentrations, and activation of Ca^{2+} signaling, the major trigger for aldosterone biosynthesis. Moreover, binding of AngII to AT1R leads to inhibition of the Na^+/K^+-ATPase, increasing cell depolarization. In contrast, signaling through Gαq promotes the release of Ca^{2+} from the endoplasmatic reticulum through binding of IP3 to its receptor, again stimulating aldosterone biosynthesis. Mutations in *KCNJ5*, encoding the GIRK4 potassium channel, induce changes in channel selectivity, allowing Na^+ entry into the cell instead of K^+ efflux, resulting in cell depolarization, opening of voltage-gated Ca^{2+} channel, stimulation of Ca^{2+} signaling, and increased aldosterone production. Mutations in *ATP1A1*, coding for the α1 subunit of the Na^+/K^+-ATPase, lead to increased aldosterone production by increasing intracellular Na^+ and H^+ concentrations, resulting in cell membrane depolarization and decreases in intracellular pH. Mutations in *CACNA1D*, encoding the $Ca_v1.3$ calcium channel, and *ATP2B3*, coding for the Ca^{2+} pump PMCA3 directly affect intracellular Ca^{2+} concentrations and, thus, aldosterone biosynthesis. (B) Specific mechanisms of CPA formation. In the absence of stimulation, the catalytic and regulatory subunits of PKA form an inactive complex. Binding of ACTH to its receptor, the melanocortin 2 receptor (MC2R), leads to activation of adenylate cyclase, resulting in an increase in cAMP. cAMP binds to the regulatory subunits of the PKA complex, inducing conformational changes and release of the catalytic subunits from the complex. Active PKA phosphorylates specific target proteins such as the transcription factor cAMP-responsive element-binding protein (CREB) that binds to the promoter regions of specific target genes (*i.e.*, CYP11B1), leading to increased cortisol biosynthesis. In cells, cAMP is converted into AMP through the action of specific phosphodiesterase. Mutations in *PRKACA* or *GNAS* lead to the constitutive activation of PKA, phosphorylation of CREB, and increased expression of CYP11B1 and autonomous cortisol biosynthesis. (C) Common mechanisms of APA and CPA formation. In unstimulated cells, β-catenin is phosphorylated, ubiquitinated by a protein complex formed by APC, GSK3, axin, and CK1α and then degraded by the proteasome. Binding of Wnt ligand to the frizzled receptor and LRP5/6 coreceptor leads to the dissociation of the β-catenin degradation complex. β-Catenin can translocate into the nucleus, where it acts as a transcriptional coactivator for transcription factors of the TCF/LEF family, inducing the expression of specific target genes. In APAs and CPAs, *CTNNB1* mutations affecting specific residues of β-catenin lead to constitutive activation of the protein. In APAs, mutations in APC also lead to constitutive β-catenin activation. Color codes indicate the involved pathway.

increases activity of cholesterol ester hydrolase with subsequent release of deesterified cholesterol from cytoplasmic stores, promotes cholesterol delivery to the outer mitochondrial membrane and its transfer to the inner mitochondrial membrane by increasing expression of the steroid acute regulatory protein (StAR), and increases the synthesis of cofactors required for p450 cytochrome enzymes (9). Stimulation of *CYP11B2* transcription is mediated by calcium/calmodulin binding and activation of protein kinases regulating phosphorylation of several transcription factors, in particular, nuclear receptor subfamily 4 group A members 1 and 2 (NURR77 or NGF1B and NURR1), the cyclic adenosine monophosphate (cAMP)-dependent transcription factor ATF-1 and the cAMP-responsive element-binding protein (10) [Fig. 1(A)].

Cortisol production is regulated by the hypothalamus–pituitary–adrenal axis, whereby circadian input and stress stimuli induce release of cortisol via corticotrophin releasing hormone-dependent ACTH secretion, and cortisol signals back to exert negative feedback on the hypothalamus and the pituitary. In the ZF (and zona reticularis) of the adrenal cortex, ACTH binds to its receptor MC2R (melanocortin receptor 2), which signals through Gαs-mediated signaling to increase intracellular cAMP levels and activate protein kinase A (PKA) [Fig. 1(B)] (11). Acute stimulation by ACTH elicits effects on steroidogenesis by regulating cholesterol availability, mobilizing cholesterol from lipid stores, and increasing cholesterol transport into mitochondria and by stimulating transcription of steroidogenic genes. Rapid effects occurring within minutes mainly affect StAR activity through phosphorylation, and longer stimulation (days) by ACTH results in transcriptional regulation of StAR and steroidogenic enzymes. In particular, PKA signaling activates transcription factors regulating expression of steroidogenic genes, including steroidogenic factor 1, cAMP-responsive element-binding protein, cAMP responsive element modulator, CCAAT/enhancer-binding proteins, and activator protein 1 (11). ACTH also regulates aldosterone production acutely via cAMP-mediated pathways; however, chronically, ACTH also suppresses plasma aldosterone in both human and animal models and downregulates *CYP11B2*, inducing *CYP11B1* expression and promoting a ZF phenotype (7, 12). Long-term exposure to ACTH (weeks or months) stimulates adrenal growth and promotes adrenal cell hypertrophy and hyperplasia. In extreme cases of glucocorticoid deficiency or ACTH excess and in experimental models of corticotrophin releasing hormone or ACTH deficient mice, the trophic effects of the hormone lead to major hyperplasia or atrophy, respectively, of the inner zones of the adrenal cortex (12). In addition to acting through cAMP, ACTH stimulates calcium influx in glomerulosa cells by activation of L-type Ca^{2+} channels (9). Many other factors have been shown to modulate aldosterone and cortisol production, including neurotransmitters, cytokines, vascular products, and adipokines, as well as locally produced ACTH acting through a paracrine mechanism (12, 13).

Table 1. Clinical and Molecular Characteristics of Patients With APA

Total Patients Screened[a], n	*KCNJ5*, n (%)	*CACNA1D*, n (%)	*ATP1A1*, n (%)	*ATP2B3*, n (%)	*CTNNB1*, n (%)	Other	Correlations With Mutation Status	Reference
22	8 (36)	NA	NA	NA	NA	NA	7/8 patients with *KCNJ5* mutations were female	Choi *et al.* (24)
380	129 (34)	NA	NA	NA	NA	NA	*KCNJ5*: higher prevalence in females and younger patients; higher plasma aldosterone at diagnosis; no specific pangenomic transcriptomic profile; no association with preoperative blood pressure, serum potassium, adenoma size, or cure after adrenalectomy	Boulkroun *et al.* (42, 43)
							Lower relative GIRK4 protein expression in *KCNJ5*-mutated tumors	
348	180 (45)	NA	NA	NA	NA	NA	*KCNJ5*: higher prevalence in females, males younger at diagnosis and with larger tumors; no correlation with *KCNJ5* protein expression	Åkerström *et al.* (44)
23	15 (62.5)	NA	NA	NA	NA	NA	*KCNJ5*: higher *KCNJ5* mRNA expression compared with nonmutated tumors	Taguchi *et al.* (32)
73	30 (41)	NA	NA	NA	NA	NA	*KCNJ5*: larger APA, predominant ZF-like cells, younger patients, higher *CYP17A1* expression	Azizan *et al.* (45, 46)
91	29 (32)	NA	NA	NA	NA	NA	*KCNJ5*: higher aldosterone levels, lower plasma renin activity, higher ARR higher lateralization index; more frequent contralateral suppression	Seccia *et al.* (38)
							Higher *CYP11B2* mRNA expression	
308	118 (38.3)	NA	16 (5.2)	5 (1.6)	NA	NA	*ATP1A1* and *ATP2B3*: more frequent in males, higher preoperative aldosterone concentrations, lower serum potassium concentrations	Beuschlein *et al.* (27)
152	20 (13.2)	12 (7.9)	12 (7.9)	0/10 exomes	1/10 exomes	NA	*ATP1A1* and *CACNA1D*: more frequent in older males; smaller APAs with more compact, ZG-like cells	Azizan *et al.* (25)
							KCNJ5: more frequent ZF-like cells, higher *CYP11B1* mRNA expression	
							No difference in *ATP1A1* and *CACNA1D* expression among genotypes	
64	21 (32.8)	5 (7.8)	1 (1.6)	2 (3.1)	2 (3.1)	NA	*CACNA1D*: smaller APA, trend toward older age	Scholl *et al.* (26)
59	19 (32)	NA	5 (8.5)	3 (5)	NA	NA	No effect of mutation status on lateralization index and contralateral suppression index	Oßwald *et al.* (39)
474	180 (38)	44 (9.3)	25 (5.3)	8 (1.7)	NA	NA	*KCNJ5*: higher prevalence in females and younger patients; higher minimal plasma potassium at diagnosis	Fernandes-Rosa *et al.* (30)
							CACNA1D: smaller adenomas[b]	
							All mutations: no association with preoperative blood pressure, serum potassium, adenoma size, or cure after adrenalectomy; no correlation with cellular composition nor *CYP11B2* or *CYP11B2* expression; no correlation with *KCNJ5*, *CACNA1D*, and *ATP1A1* mRNA expression	
112[c]	44 (39.3)	NA	7 (6.3)	1 (0.9)	NA	NA	*ATP1A1*, *ATP2B3*: higher *CYP11B2* mRNA expression compared with *KCNJ5*-mutated APA	Williams *et al.* (47)
108	75 (69.4)	2 (1.9)	3 (2.8)		NA	NA	*KCNJ5*: younger patients, higher plasma and urine aldosterone levels; substantial improvement of left ventricular mass index after surgery; lower postoperative SBP and aldosterone levels	Kitamoto *et al.* (48)
195	48 (24.6)	NA	NA	NA	NA	NA	Not investigated	Kuppusamy *et al.* (49)
165	90 (54.5)	5 (3.0)	10 (6.1)	5 (3.0)	NA	NA	*KCNJ5*: higher prevalence in females and younger patients	Akerstrom *et al.* (40)

(*Continued*)

Table 1. *Continued*

Total Patients Screened[a], n	*KCNJ5*, n (%)	*CACNA1D*, n (%)	*ATP1A1*, n (%)	*ATP2B3*, n (%)	*CTNNB1*, n (%)	Other	Correlations With Mutation Status	Reference
							CACNA1D, *ATP1A1*, and *ATP2B3*: older at diagnosis, more often males with smaller tumors; higher *CYP11B2* and *NPNT* mRNA expression compared with APA with *KCNJ5* mutation	
							All mutations: no correlation with *ATP1A1*, *ATP2B3*, or *KCNJ5* mRNA expression	
69	26 (37.7)	NA	NA	NA	NA	NA	*KCNJ5*: lower relative GIRK4 protein expression in *KCNJ5*-mutated APA; higher prevalence in females and younger patients; larger tumors and lower plasma potassium	Cheng *et al.* (31)
168	129 (76.8)	1 (0.6)	4 (2.4)	1 (0.6)	NA	NA	*KCNJ5*: higher prevalence in females, larger tumors, higher aldosterone levels, lower minimal serum potassium, higher lateralization index, lower plasma renin activity, higher ARR	Zheng *et al.* (33)
71[d]	27 (38)	3 (4.2)	5 (7)	1 (1.4)	NA	NA	*KCNJ5*: more frequently ZF-like cells, high *CYP11B1* protein expression	Monticone *et al.* (50)
							ATP1A1, *ATP2B3*, and *CACNA1D*: more frequently ZG-like cells with high *CYP11B2* protein expression	
90	36 (37.1)	10 (10.3)	8 (8.2)	3 (3.1)	2 (2.1)	NA	*KCNJ5*: higher prevalence in females, larger tumors, lower precontrast Hounsfield units on CT; more frequently ZF-like cells	Scholl *et al.* (28)
							ATP1A1, *ATP2B3*, and *CACNA1D*: more frequent in males, heterogeneous cell composition	
148	88 (59.5)	0	2 (1.4)	1 (0.7)	NA	NA	Mutation carriers: younger, higher preoperative aldosterone and ARR, lower serum potassium, higher GFR, lower serum CRP levels, greater chance of recovery from hypertension after adrenalectomy	Wu *et al.* (35)
114	86 (75.4)	1 (0.09)	0	0	NA	NA	Mutation carriers: younger, lower preoperative serum potassium level, higher LV mass index, and substantial improvement after surgery; higher mRNA expression of *KCNJ5*, *CYP11B2*, and *ATP2B3*	Wang *et al.* (34)
							Male (but not female) mutation carriers: higher preoperative plasma aldosterone and blood pressure	
198	92 (46.5)	3 (1.5)	6 (3.0)	3 (1.5)	10 (5.1)	NA	*CTNNB1*: more frequent in females; larger tumors compared with APA without mutation; higher *CYP11B2* mRNA expression compared with APA with *KCNJ5* mutation	Åkerström *et al.* (29)
159	117 (73.6)	4 (2.5)	1 (0.6)	4 (2.5)	NA	NA	*ATPase*: tendency to smaller tumors, mainly ZG-like cells	Kitamoto *et al.* (36)
							CACNA1D: tendency to smaller tumors, predominant ZF-like cells	
							KCNJ5: not analyzed in their report	
66	47 (71.2)	0	0	0	NA	NA	*KCNJ5*: higher prevalence in females and younger patients, no association with preoperative blood pressure, plasma aldosterone, serum potassium, lateralization index, and adenoma size; better cure after adrenalectomy	Hong *et al.* (37)
219	116 (52.9)	0	3 (1.4)	1 (0.5)	8 (3.7)	NA	*CTNNB1*: more frequent in females, older patients, heterogeneous staining of β-catenin, and variable expression of gonadal receptors and both *CYP11B1* and *CYP11B2*	Wu *et al.* (41)

Abbreviations: CRP, C-reactive protein; CT, computed tomography; GFR, glomerular filtration rate; mRNA, messenger RNA; NA, not available.
[a]By WES and/or targeted sequencing.
[b]Frequency of mutations and associations dependent on center.
[c]Included 32 samples from the study by Beuschlein *et al.* (27).
[d]Patients included in the study by Fernandes-Rosa *et al.* (30).

Somatic Mutations in APA

PA is due to autonomous aldosterone production from the adrenal cortex and is diagnosed on the basis of hyperaldosteronism associated with suppressed renin and, often, hypokalemia (14). It is the most common form of secondary hypertension, with a prevalence of 5% to 10% in patients with arterial hypertension (15, 16) and ≤20% in patients with resistant hypertension (17). PA is associated with increased cardiovascular risk, in particular, coronary artery disease, nonfatal myocardial infarction, heart failure, atrial fibrillation, renal damage, and stroke (18–20), due to the blood pressure-independent effects of aldosterone on cardiac and vascular remodeling (21, 22).

Most cases of PA are due to APA or BAH (also called idiopathic hyperaldosteronism). Only in rare cases has unilateral adrenal hyperplasia been described, and aldosterone-producing adrenocortical cancers are extremely rare. APAs are generally small (<2 cm in diameter) and are diagnosed by adrenal computed tomography and adrenal venous sampling, which identifies lateralized aldosterone secretion in the presence or absence of a visible adrenal nodule on imaging (14, 23). Recent advances in genome technology have allowed for the identification, during the past 5 years, of several somatic mutations in APAs, providing a pathogenic model for PA development. Whole exome sequencing (WES) of paired tumor (somatic) and germline DNA performed by different groups on >100 APA samples identified recurrent somatic mutations in genes coding for ion channels [*KCNJ5* (24) and *CACNA1D* (25, 26)] and ATPases [*ATP1A1* and *ATP2B3* (25, 27)] regulating intracellular ionic homeostasis and cell membrane potential, thus defining APA as a channelopathy. These mutations all promote increased intracellular calcium concentrations, leading to activation of calcium signaling, the main trigger for aldosterone biosynthesis (Fig. 1). A small fraction of APAs also carries somatic mutations in *CTNNB1*, coding for β-catenin, similar to CPA (see subsequent sections) and adrenocortical cancer (28, 29) (Table 1). In the largest multicenter study reported, exploring the prevalence of somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* in 474 patients from seven centers belonging to the European Network for the Study of Adrenal Tumors, somatic mutations were detected in 54% of APAs, ranging from 27.2% to 56.8% across the different centers (30). In that study, *KCNJ5* mutations were the most prevalent genetic abnormality and were found in 38% of APAs. Mutations affecting *CACNA1D* were the second most prevalent genetic abnormalities, present in 9.3% of cases, and *ATP1A1* and *ATP2B3* mutations were identified in 5.3% and 1.7% of cases, respectively. No difference was found in cellular composition (percentage of cells resembling ZF or ZG) of APAs or in *CYP11B2*, *KCNJ5*, *CACNA1D*, or *ATP1A1* gene expression in APAs across genotypes. Patients with *KCNJ5* mutations were more frequently female and younger at diagnosis and had greater plasma potassium concentrations compared with *CACNA1D* mutation carriers or non-carriers. *CACNA1D* mutations were associated with smaller adenomas. No association was found between the mutation status and preoperative plasma aldosterone or renin levels, the aldosterone/renin ratio, or the number of medications taken before surgery. Also, no association was found with postoperative blood pressure outcomes, treatment scores during follow-up, or cure or improvement of hypertension. These associations were largely center-dependent, which might have resulted from the heterogeneity of APAs, which should also be considered when studying genotype–phenotype correlations (30).

Furthermore, less frequent mutations have been identified in a few genes in patients with peculiar phenotypes of PA, including early-onset PA and tumors cosecreting aldosterone and cortisol (50, 51) (Table 1). Recently, somatic mutations in *PRKACA* (coding for the cAMP-dependent protein kinase catalytic subunit α) have been reported in patients with APAs (51). Using WES and targeted sequencing in 122 patients, Rhayem *et al.* (51) identified two somatic heterozygous mutations in *PRKACA*, one of which was the p.Leu206Arg mutation previously reported in CPA (see subsequent sections). The carrier of this variant had biochemical CS and high expression of 11β-hydroxylase in the tumor. The second p.His88Asp variant was not associated with cortisol excess or with a gain of function of the PKA catalytic activity, leaving open the question of its role in the development of PA.

Pathogenic mechanisms of APA development

KCNJ5 codes for the G protein-activated inward rectifier potassium channel GIRK4 (alternative protein name Kir3.4), which is composed of two membrane-spanning domains (M1 and M2), one pore-forming region (H5), and cytoplasmic N- and C-termini that contribute to the pore structure. Recurrent somatic mutations identified in APAs cluster within or near the selectivity filter of the channel pore, between amino acids Thr149 and Gly153 (Table 2). They affect its ionic selectivity, rendering the channel permeable to sodium, with increased sodium influx into the cell leading to chronic cell membrane depolarization and opening of voltage-gated calcium channels (24). This, in turn, leads to increased intracellular calcium concentrations and activation of calcium signaling [Fig. 1(A)]. Transient transfection of *KCNJ5* mutants into adrenocortical cells increases aldosterone production and expression of *CYP11B2* in a calcium-dependent manner (58); in some cases, mutations lead to reduced membrane and total abundance of GIRK4 (31). Transiently transfected *KCNJ5* mutants did not lead to

Table 2. List of Different Mutations Identified in APA Driver Genes

Gene	Mutations	Reference (First Description)
KCNJ5	p.Gly151Arg, pLeu168Arg, pThr158Ala	Choi *et al.* (24)
	p.Gly151Glu	Mulatero *et al.* (52)
	p.Ile157del	Murthy *et al.* (54)
	p.Tyr152Cys	Monticone *et al.* (55)
	p.Trp126Arg	Williams *et al.* (47)
	p.Glu145Gln, p.Arg115Trp, p.Glu246Gly	Cheng *et al.* (31)
	p.Phe154Cys, p.Ile150_Gly151del_InsMet, p.Ser143_ile144del_InsAla	Scholl *et al.* (28)
	p.Ala139_Phe142dup	Hardege *et al.* (56)
ATP1A1	p.Leu104Arg, p.Val332Gly, pPhe100_Leu104del	Beuschlein *et al.* (27)
	p.Glu960_Ala963del	Azizan *et al.* (25)
	p.Gly99Arg	Williams *et al.* (47)
	p.Met102_Leu103del	Zheng *et al.* (33)
	p.Met102_Ile106del, p.Ieu103_Leu104del, p.Phe959_Glu961del, p.Glu960_Leu694del, p.Phe956_Glu961del	Åkerström *et al.* (40)
ATP2B3	p.Leu425_426del, p.Val426_427del	Beuschlein *et al.* (27)
	p.Val424_Leu425del	Fernandes-Rosa *et al.* (30)
	p.Ala428_Val429del	Dutta *et al.* (57)
	p.Val426_Val429del	Åkerström *et al.* (40)
	p.Val422_Val426del_InsSerThrLeu	Zheng *et al.* (33)
	p.Ala428_Leu433del_InsGlyGln	Scholl *et al.* (28)
CACNA1D	p.Val259Asp, p.Gly403Argp.Phe747Leu, p.Ile750Met, p.Arg990His	Azizan *et al.* (25)
	p.Gly403Arg, p.Ile750Met, p.Phe767Val, p.Val1373Met	Scholl *et al.* (26)
	p.Ser652Leu, p.Leu655Pro, p.Tyr741Cys, p.Val949Asp, p.Lys981Asnp.Ala998Ile, p.Ala998Valp.Val1151Phe, p.Ile1152Asnp.Val1338Met	Fernandes-Rosa *et al.* (30)
	p.Val401Leu	Åkerström *et al.* (40)
	p.Phe747Val	Nanba *et al.* (53)

enhanced cell proliferation *ex vivo* but, rather, to reduced cell viability or sodium-dependent cell death, which does not explain the cell proliferation and APA formation (58, 59). Whether these findings resulted from experimental artifacts caused by high expression of transfected channels, leading to excessively increased sodium influx into the cell, which does not reflect the *in vivo* situation, or whether additional hits are required for cell proliferation, remains a matter of debate.

CACNA1D codes for the α1 subunit of the L-type voltage-gated Cav1.3 calcium channel (calcium channel, voltage-dependent, L type, α-1d subunit), and *ATP1A1* codes for the α1 subunit of the Na^+,K^+-ATPase and *ATP2B3* for the plasma membrane calcium-transporting ATPase 3 (PMCA3). Na^+,K^+-ATPase and PMCA3 are members of the P-type family of ATPases and are composed of 10 transmembrane domains (M1 to M10) with intracellular N- and C-tails. *ATP1A1* mutations affect amino acids in transmembrane helices M1, M4, and M9 of the α1 subunit of the Na^+,K^+-ATPase (Table 2). They lead to a loss of pump activity and a reduced affinity for K^+ and to an inward proton or sodium leak, which has been proposed to increase aldosterone production through cell membrane depolarization and increased calcium influx, which has been observed in the presence of *KCNJ5* mutations (25, 27). However, transient transfections of Na^+,K^+-ATPase α1 subunits carrying the p.Leu104Arg, p.Val332Gly or p.Gly99Arg mutations in adrenocortical

NCI-H295R cells did not modify basal cytosolic calcium levels and hardly increased potassium-stimulated calcium concentrations, despite depolarizing adrenal cells and stimulating aldosterone secretion (60). Rather they induced increased intracellular acidification, which has been suggested to play a role in regulating aldosterone biosynthesis in this context [Fig. 1(A)] (60).

Mutations of PMCA3 are located in the transmembrane domain M4 and are all deletions of multiple amino acids in the region between Leu425 and Leu433 (Table 2). Electrophysiological and functional studies have shown that the p.Leu425_Val426del mutation leads to reduced Ca^{2+} export owing to loss of the physiological pump function and increased Ca^{2+} influx due to opening of depolarization-activated Ca^{2+} channels. A Ca^{2+} leak through the mutated pump has also been observed, inducing *CYP11B2* expression and aldosterone biosynthesis [Fig. 1(A)] (61).

Finally, *CACNA1D* mutations affect the function of the L-type Ca^{2+} channel α-subunit Cav1.3, which is composed of four repeat domains (I to IV), each consisting of six transmembrane segments S1 to S6, with a membrane-associated loop between S5 and S6 (Table 2) (62). Although the S4 segments are involved in voltage sensing, the S5 and S6 domains and the loop in between line the pore of the channel (63). Eighteen different *CACNA1D* mutations have been identified (25, 26, 30). They are gain-of-function mutations leading to a shift of voltage-dependent channel activation toward more negative voltages or to reduced inactivation of the channel, followed by increased intracellular calcium concentrations with induction of aldosterone biosynthesis [Fig. 1(A)] (25, 26).

Genotype–phenotype correlations in APA

KCNJ5 mutations are the most frequent type of somatic mutation within APAs (Table 1). Their reported frequency ranges from 13% to 77%, with greater frequency in studies from Japan, China, Korea, and Taiwan (32–37, 48). *CACNA1D* mutations are the second most frequent mutations ($\leq$10%), followed by *ATP1A1* and *ATP2B3*. Three studies have also explored *CTNNB1* mutations, which are found in 2.1% to 5.1% of cases (26, 28, 29).

The correlations of clinical and molecular parameters with the mutation status have been heterogeneous among studies (Table 1). In a recent meta-analysis, Lenzini *et al.* (64) analyzed the clinical correlates of *KCNJ5* mutations in 1636 patients from 13 studies. The overall prevalence of *KCNJ5* mutations was 43%, ranging from 12% to 80% among different studies, with a greater prevalence in studies from Japan and China compared with studies of populations from Europe, the United States, and Australia. The carriers of *KCNJ5* mutations were younger, were more often female, had larger tumors, and had higher plasma aldosterone levels compared with noncarriers. No association was observed between the mutation status and systolic or diastolic blood pressure and serum potassium levels overall, confirming previous results from the study by Fernandes-Rosa *et al.* (30). An association between the presence of *KCNJ5* mutations and the lateralization index at adrenal vein sampling was described in two studies (33, 38) but was not replicated in another study (39). In four studies, *KCNJ5* mutations were associated with tumors composed mainly of large, lipid-laden cells resembling ZF cells (Table 1). The other mutations were associated mainly with male sex and smaller tumors (25–28, 30, 36, 40), and the cellular phenotype of APA carrying *CACNA1D*, *ATP1A1*, or *ATP2B3* mutations was more heterogeneous than the cellular phenotype of *KCNJ5*-mutated tumors. Although few studies have investigated *CTNNB1* mutations in large cohorts, they seem to be more prevalent in females (26, 28, 29, 65).

Germline mutations in APA

In addition to somatic mutations, the occurrence of germline *CACNA1H* (coding for the voltage-dependent T-type calcium channel subunit α-1H) mutations, and, rarely, germline *KCNJ5* and *ARMC5* variants have been reported in PAs (66, 67, 68). Daniil *et al.* (66) performed WES in patients with different phenotypic presentations of PA, including 23 patients with APA, 10 patients with a family history of PA, and 1 patient with early-onset PA. *CACNA1H* mutations were identified in the patient with early-onset PA and the patients with a family history, and a germline *CACNA1H* p.Val1951Glu mutation was identified in one patient with an APA (66). *CACNA1H* encodes the pore-forming α1 subunit of the T-type voltage-dependent calcium channel Cav3.2, which has been implicated in membrane potential oscillations in ZG cells (69). The variant is located in the C-terminal cytoplasmic domain of Cav3.2, in a region possibly implicated in fast channel activation. Electrophysiological investigation revealed a gain-of-function phenotype, whereby p.Val1951Glu induced a faster recovery from inactivation and marked increase in voltage-dependent facilitation, two properties which could favor larger calcium entry during repetitive electrical activity. In H295R adrenocortical cells, transient transfection of the mutant channel led to an increase in aldosterone biosynthesis and expression of *CYP11B2* (66).

Genetic abnormalities in APA driver genes are present as germline mutations in some familial forms of PA, which account for 1% to 5% of cases and are transmitted as an autosomal dominant trait. Although the terminology for FH is still evolving, to date, four different forms of FH, FH-I to FH-IV, have been described. FH-I, resulting from the formation of a chimeric gene between *CYP11B2* and *CYP11B1* (see the preceding sections), is found in 0.5% to 1.0% of

cases of PA (70–72) but might represent ≤3% of cases in the pediatric hypertensive population (73). FH-I is associated with BAH; however, in rare cases, adrenocortical adenomas have been described. Heterozygous germline mutations in *KCNJ5* are found in FH-III, a severe form of PA presenting, in typical cases, in childhood with severe hypertension and profound hypokalemia, due to massive BAH, which requires bilateral adrenalectomy to control the hypertension (24). In other cases, the disease will be milder and with normal-appearing adrenals on imaging studies (74). Germline mutations in *CACNA1H* have recently been identified as the cause of a new form of early-onset PA associated or not with a developmental disorder (66, 75). In familial cases, the disease (termed FH-IV) is transmitted as an autosomal dominant trait. Similar mutations have also been found in patients with a diagnosis of FH-II, with or without an adrenal adenoma, and in those with APAs (66). Finally, *de novo* germline *CACNA1D* mutations have been described in a rare disease termed PASNA (primary aldosteronism, seizures, neurologic abnormalities), with no adrenal abnormality reported on imaging studies (26).

Pathological and Genetic Heterogeneity and the Origin of APA

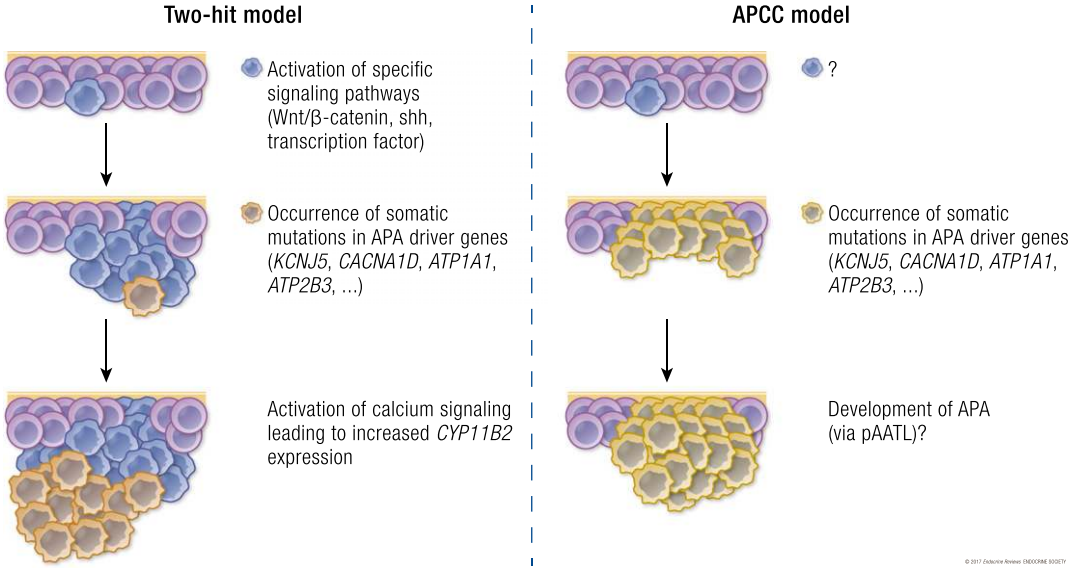
Although the functional link between mutations in driver genes in APA and aldosterone production has been clearly demonstrated, the origin of APA in terms of increased cell proliferation and nodule formation is not yet well understood. However, work from different groups has provided contradictory results on the sequence of events leading to aldosterone overproduction and adenoma formation. Familial forms of PA are due to germline mutations in driver genes, supporting a role for these mutations in adrenal cortex cell proliferation and nodulation. However, adrenal morphology is highly variable among these subjects. Patients with FH-III harboring germline *KCNJ5* mutations can exhibit adrenal phenotypes ranging from a normal adrenal gland to massive BAH or adrenal nodules (24, 52, 76). Similarly, patients carrying germline *CACNA1H* mutations show different adrenal phenotypes, including normal, hyperplastic, and adenomatous adrenal glands (66, 75). No abnormality in adrenal morphology was observed in patients with PASNA harboring germline *CACNA1D* mutations (26). *Ex vivo*, adrenocortical cells overexpressing mutant GIRK4 channels show increased intracellular Na^+ concentrations, leading, counterintuitively, to reduced cell proliferation and sodium-dependent apoptosis (58, 59).

It has been previously shown that adrenal glands with APA exhibit adrenal cortex remodeling, decreased vascularization, increased nodulation, and ZG hyperplasia (77, 78). These findings suggest that adrenal cortex remodeling might precede APA development and that the somatic mutations in driver genes are secondary events specifying the pattern of hormone secretion. The recent development of an aldosterone-synthase antibody (79) has allowed for assessment of the genetic and histological characteristics of adrenal glands with APA and reinforced the hypothesis of independent events leading to nodulation and aldosterone overproduction. Two studies in particular have identified different genetic abnormalities in different aldosterone-producing nodules from the same adrenal gland. In one study, Dekkers *et al.* (80) analyzed 28 multinodular adrenal glands and identified 5 adrenal glands with more than one nodule staining positively for aldosterone synthase. In one adrenal gland, two aldosterone-synthase positive nodules each harbored a different *KCNJ5* mutation (80). In the second study, assessment of the *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* mutation status in aldosterone synthase-positive nodules from 27 multinodular adrenals with APA identified seven adrenal glands harboring different mutations between the principal nodule and the secondary nodule within the same adrenal gland, including two adrenal glands with an APA harboring a *CACNA1D* mutation and, in one secondary nodule, a *KCNJ5* mutation (81). Further studies extended the heterogeneity of aldosterone synthase expression to the APA itself, with *KCNJ5* mutations only identified in APA regions positive for aldosterone synthase (53). Finally, in a young patient with PA, a germline *APC* mutation, and a multinodular adrenal gland with somatic biallelic inactivation of *APC*, we identified a somatic *KCNJ5* mutation in the nodule expressing aldosterone synthase only (see subsequent sections) (82). These data support a two-hit model for APA formation whereby a specific genetic or epigenetic event is responsible for adrenal cortex remodeling and nodule formation and somatic mutations in driver genes leading to excessive aldosterone secretion represent second hits specifying the pattern of hormonal secretion (Fig. 2).

A second model for the origin of APA has emerged from genetic studies performed on aldosterone-producing cell clusters (APCCs). APCCs, which are found in normal adult adrenal glands and adrenal glands with APAs, are composed by ZG-like cells expressing disabled homolog 2 (DAB2; a marker of ZG) in contact with the capsule and inner columnar DAB2-negative ZF-like cells, which do not express CYP11B1, forming cords along sinusoids (84, 85). APCC cells are positive for CYP11B2 and 3βHSD staining but not for CYP17, consistent with their capacity to synthesize aldosterone (84). Targeted next-generation sequencing was performed on the DNA of 23 APCCs micro-dissected from adrenal glands from kidney transplant donors. Somatic mutations in *CACNA1D*, *ATP1A1*, and *ATP2B3*, similar to those already described in APA, were identified in 35% of the

"New genetic studies of a larger number of normal and pathological adrenal glands with APCCs are necessary."

Figure 2. The two current models for APA development. (Left) Two hit model of APA development. In ZG cells, activation of specific signaling pathways, such as β-catenin, lead to abnormal cell proliferation. Abnormal cell proliferation creates a propitious environment for the occurrence of recurrent somatic mutations specifying the pattern of hormonal secretion. (Right) APCC hypothesis. In ZG cells, the occurrence of somatic mutations in a certain number of APA driver genes leads to the formation of APCCs with autonomous aldosterone production. In a certain number of cases, these structures would evolve toward an APA via a pAATL (83).



APCCs (86). The authors propose that APA may derive from APCC (Fig. 2) and suggest different hypotheses for this progression: single somatic mutations responsible for aldosterone production and cell proliferation or second-hit mutations within the APCCs leading to cell proliferation. In some cases, APCCs might represent terminal lesions without the capacity to progress toward an adenoma (86). No *KCNJ5* mutations, the most frequent genetic alteration observed in APAs, were identified in APCCs. The investigators suggested that this could be explained by a rapid progression of *KCNJ5*-mutated APCCs to APAs (86). Subsequently, *CACNA1D* mutations were identified in 17 of 26 APCCs from patients with lateralized PA negative for detectable masses on imaging, suggesting that these lesions might contribute to PA in this situation (87). Recently, a novel aldosterone-producing structure named a possible APCC-to-APA transitional lesion (pAATL) was described in multinodular adrenal glands from two patients with lateralized PA and was suggested to be an intermediate lesion between APCCs and APAs (83). Although the APCC-like portion consists of a subcapsular ZG-like region and an inner ZF-like region expressing CYP11B2 but not CYP11B1, the micro-APA–like portion is composed of a heterogeneous mixture of CYP11B1-positive and CYP11B2-positive cells. Sequencing of the known APA-associated mutations in three pAATLs identified a *KCNJ5* mutation and an *ATP1A1* mutation in the micro-APA–like portion of two pAATLs but not in their corresponding APCC-like portions. The investigators suggested that these data support the hypothesis that the micro-APAs are derived from the APCCs after the appearance of an APA driver mutation (83). However, another pAATL carried two novel *ATP1A1* mutations in both the micro-APA–like and APCC-like portions, supporting a clonal origin of the two portions (83). New genetic studies of a larger number of normal and pathological adrenal glands with APCCs are necessary to clarify the relationship between these aldosterone-producing lesions and the origin of APA.

Genetic Abnormalities in CPAs

The genetics of CPAs was unraveled in 2014 by the work of four independent groups identifying somatic mutations of *PRKACA* as a cause of CS. Beuschlein *et al.* (88) performed WES in 10 paired adrenal tumor and germline DNA samples from patients with CS, which revealed the presence of a recurrent somatic p.Leu206Arg mutation in the *PRKACA* gene in seven cases, with an additional p.Leu199_Cys200insTrp mutation in one case (88). Sequencing of 129 additional samples identified the p.Leu206Arg mutation in 22 of 59 CPAs (37%) from patients with overt CS but not in tumors from 40 patients with subclinical hypercortisolism (sCS), 20 APAs, 20 nonfunctioning adenomas, or 42 cases of adrenocortical carcinoma (ACC). Among the patients with different forms of BAH leading to CS, including bilateral micro- or macronodular hyperplasia, a germline copy-number gain (duplication) of the genomic region on chromosome 19 that includes

PRKACA was identified in five patients from four kindreds. In one family with bilateral macronodular hyperplasia, the genetic defect was transmitted in an autosomal dominant manner from the mother to her affected child. The p.Leu206Arg mutation is located in the highly conserved core of the interaction between the regulatory (RIIβ) and catalytic (Cα) subunits of PKA. *In vitro* studies demonstrated gain-of-function of the PKA catalytic subunit mutants with impaired inhibition by the PKA regulatory subunit and increased protein expression of the PKA catalytic subunit in cells from patients with germline chromosomal gains, with both mechanisms leading to increased PKA activity. Basal PKA activity in cells transfected with mutant Cα subunits was increased and was not reduced by excess nonmutant RIIβ subunit expression, nor was it responsive to cAMP analogs, indicating that the mutations made the catalytic subunit resistant to physiological suppression. The presence of *PRKACA* mutations was associated with a more severe phenotype in patients with overt CS and *PRKACA* mutation carriers had significantly increased expression of *MC2R*, *StAR*, *CYP21A1*, *HSD3B2*, and *CYP11A1* messenger RNA in adrenal tumors. These results were confirmed in three other studies. By performing WES in 25 tumor samples producing excess cortisol, including 22 CPAs and 3 ACC cases, Goh *et al.* (89) identified six cases carrying the recurrent p.Leu206Arg substitution. Six cases had gain-of-function mutations in *CTNNB1* and two cases in *GNAS* (coding for Gαs), with *PRKACA*, *GNAS*, and *CTNNB1* mutations being mutually exclusive. Additional targeted sequencing in 38 patients identified the p.Leu206Arg substitution collectively in 13 of 63 tumors, representing 24% of all ACAs and 35% of all ACAs associated with overt CS. In their study, adenomas carrying *PRKACA* or *GNAS* mutations were significantly smaller than those without these mutations, and the patients were younger at diagnosis of overt CS (89). WES also revealed a group of tumors harboring many somatic copy number variants, with frequent deletion of *CDC42* and *CDKN2A*, amplification of 5q31.2, and protein-altering mutations in *TP53* and *RB1*. This mutational spectrum is more similar to that of malignant tumors, indicating that they might have greater malignant potential or represent early steps toward the development of ACC.

A greater rate of somatic *PRKACA* mutations seems to occur in subjects with an Asian ancestry. Cao *et al.* (90) reported the occurrence of the p.Leu205Arg mutation (equivalent to the p.Leu206Arg mutation in the other reports) in 65.5% of a total of 87 CPAs from China screened by WES and targeted sequencing. In their study, no substantial correlation was found between the mutation status and clinical or biological parameters. The investigators also identified one somatic *PRKAR1A* mutation, one *CTNNB1* mutation, a truncating *APC* mutation, and two activating *GNAS* mutations in CPA (90). An additional *CTNNB1* mutation was found in an adrenal oncocytoma (a very rare form of a cortisol-producing adrenal tumor). The investigators also identified the recurrently mutated gene *DOT1L* coding for a histone H3 methyltransferase and one *HDAC9* mutation in ACTH-independent macronodular adrenocortical hyperplasia, suggesting involvement of chromatin deregulation, and *CLASP2* mutations in two of three adrenocortical oncocytomas. RNA-sequencing data comparing *PRKACA*-mutated and -nonmutated tumors identified 232 differentially expressed genes, with substantial enrichment of gene ontology terms "biosynthesis and metabolism of steroid and cholesterol" and "response to chemical stimulus," with increased expression of the genes *StAR*, *MC2R*, *GSTA1*, *CXCL2*, and *S100A8/9*.

Finally, in a Japanese cohort, 50% of cases (four of eight) with adrenocortical adenomas associated with corticotrophin-independent CS were carriers of the *PRKACA* p.Leu206Arg mutation (91). An additional patient carried a recurrent p.Arg201Cys *GNAS* mutation. Follow-up sequencing in an additional 57 cases identified the *PRKACA* p.Leu206Arg mutation in 30 cases and *GNAS* mutation in 10 cases, representing a total of 52.3% and 16.9% of mutational events and which were mutually exclusive. These mutations were found in 76% of patients with overt CS, and two patients with sCS were also carriers of a *PRKACA* and a *GNAS* mutation. In their study, not only carriers of the recurrent *PRKACA* p.Leu206Arg mutation, but also those with *GNAS* mutations had higher cortisol levels after an overnight dexamethasone suppression test and smaller adenoma size, suggesting greater cortisol output compared with that of nonmutated tumors.

After these seminal studies, a similar prevalence of *PRKACA* mutations was found in different cohorts of patients with CS from different countries (Table 3). The largest study published to date was performed within the European Network for the Study of Adrenal Tumors and included 149 samples from nine different European centers, among which were 64 CPAs from patients with overt CS, 36 CPA samples from patients with sCS, 32 nonsecreting adenomas, 4 androgen-producing tumors, 5 ACC cases, and 8 samples from PBMAH. Heterozygous somatic *PRKACA* mutations were found in 22 of 64 samples from patients with overt CS (34%); 18 patients carried the recurrent p.Leu206Arg mutation, and a p.Cys200_Gly201insVal mutation was identified in three patients and a p.Ser213Arg+p.Leu212_Lys214insIle-Ile-Leu-Arg mutation was identified in one patient. These newly identified mutations involved a region implicated in the interaction between the PKA regulatory and catalytic subunits. Just as in previous studies, patients with *PRKACA* mutations showed more severe cortisol abnormalities (greater levels of cortisol after an overnight dexamethasone suppression test) and smaller adenoma size compared

Table 3. Clinical and Genetic Characteristics of Patients With CPA

Total Patients Screened,[a] n	Clinical Phenotype	*PRKACA* (Phenotype), n	*GNAS* (Phenotype), n	*CTNNB1* (Phenotype), n	*APC* (Phenotype), n	Reference
139	59 CS, 40 sCS, 20 APA, 20 NSA	22 (CS)	0 in WES	0 in WES	0 in WES	Beuschlein *et al.* (88)
63 (55 CPA; 8 ACC)	36 CS, 27 sCS	13 (10 CS, 3 sCS)	3 CS	9 (3 CS, 6 sCS)	0 in WES	Goh *et al.* (89)
87	87 (CS)	57 (CS)	2 (CS)	1 (CS)	1 (CS)	Cao *et al.* (90)
64	55 CS, 9 sCS	34 (33 CS, 1 sCS)	11 (10 CS, 1 sCS)	0 in WES	0 in WES	Sato *et al.* (91)
144	64 CS, 36 sCS, 32 NSA, 4 androgen-producing tumors, 8 PBMAH	22 (CS)	ND	ND	ND	Di Dalmazi *et al.* (94)
60	36 CS, 22 sCS	12 (11 CS, 1 sCS)	4 (3 CS, 1 sCS[$])	13 (5 CS, 8 sCS[b])	ND	Thiel *et al.* (92)
57	15 CS, 9 sCS, 33 APA	4 CS	5 (2 CS, 1 sCS, 2 APA + sCS	ND	ND	Nakajima *et al.* (93); Okamura *et al.* (95)

Abbreviations: NSA, nonsecreting adenoma; ND, not determined.
[a]By WES and/or targeted sequencing, excluding ACC.
[b]Including one patient with a double *CTNNB1* plus *GNAS* mutation.

with noncarriers (88, 89). In a study exploring 60 patients with overt CS (n = 36) or sCS (n = 24), Thiel *et al.* (92) identified the recurrent somatic *PRKACA* p.Leu206Arg mutation in 23.1% of tumors, somatic *CTNNB1* mutations (p.Ser45Pro, p.Ser45Phe) in 23.1%, the recurrent *GNAS* p.Arg201Cys mutation in 5.8%, and a double mutation of *CTNNB1* and *GNAS* in one case. *PRKACA* and *GNAS* mutations were again mutually exclusive. In one patient with unilateral hyperplasia, a somatic *PRKACA* p.Leu206Arg mutation was identified, and patients with bilateral adenomas did not have known somatic mutations. The presence of *PRKACA* mutations was associated with younger age, overt CS, and greater cortisol levels after an overnight dexamethasone suppression test compared with non–*PRKACA*-mutant or *CTNNB1*-mutant lesions (92). Finally, targeted genetic screening for *GNAS*, *PRKACA*, and *KCNJ5* mutations in Japanese patients, 15 with CS, 9 with sCS, and 33 with an APA found mutations in the *PRKACA* gene in four patients (26%) with CS, and mutations in the *GNAS* gene were detected in two patients (13%). A mutation in the *GNAS* gene was detected in one patient with sCS (93, 96).

Altogether, >580 patients with a CPA or hyperplasia have been investigated. Somatic *PRKACA* mutations were found in 28%, *CTNNB1* mutations in 4%, and *GNAS* mutations in 4% of cases (Table 3). *PRKACA* mutations were associated with overt CS, more severe cortisol abnormalities, and smaller tumors. The question of whether other recurrent mutations or genetic abnormalities are found in CPAs has been addressed by Ronchi *et al.* (97), who performed WES in 99 patients with ACAs, including 74 CPAs and 25 nonfunctioning adenomas negative for *PRKACA* mutations. Different mutations affecting genes of the cAMP/PKA and Wnt/β-catenin signaling pathways were identified [Fig. 1(B) and 1(C)], the latter are more frequently associated with larger tumors and endocrine inactivity. Genetic variants were also identified in genes belonging to the calcium signaling pathway. To what extent these single variants are causative for cortisol production and/or adenoma formation remains to be established.

Genetics of Aldosterone and Cortisol Cosecretion

Cosecretion of aldosterone and cortisol has been described in several reports, and biochemical abnormalities of cortisol excess in PA have been associated with worse cardiovascular outcomes compared with APAs without cortisol secretion (98). Recent studies have suggested that cortisol excess might be more frequent in patients with APAs than previously thought. Histological examination of adrenal glands with APA has consistently shown coexpression of CYP11B2 and CYP11B1 in a number of cases (99).

A Japanese study explored somatic *GNAS*, *PRKACA*, and *KCNJ5* mutations in 15 patients with CS, 9 patients with sCS, and 33 patients with APAs (93, 96) (Table 4). From 33 APAs tested, 10 had autonomous cortisol secretion found from an overnight dexamethasone suppression test, increased midnight cortisol levels, and/or decreased plasma ACTH levels. Mutations in the *PRKACA* gene were found in four patients (26%) with CS, and mutations in the *GNAS* gene were detected in two (13%). Mutations in the

GNAS gene were also detected in one patient (11%) with sCS. Twenty-four patients with APA (72%) harbored *KCNJ5* mutations. Among the patients with APA with autonomous cortisol secretion, six carried a somatic *KCNJ5* mutation and two had a somatic *GNAS* mutation. The *KCNJ5* and *GNAS* mutations in APA were mutually exclusive. No *PRKACA* mutations were identified in patients with APA (96). In another study, the same investigators identified a *KCNJ5* mutation in two of three patients with APAs and sCS (100).

In a study of 60 patients with cortisol excess, Thiel *et al.* (92) identified four cases with aldosterone cosecretion, 2 with overt CS and 2 with sCS. Among those, two were carriers of mutations in *KCNJ5* (92). Finally, Rhayem *et al.* (51) identified somatic *PRKACA* mutations in two patients diagnosed with PA, a newly identified p.His88Asp variant and the recurrent p.Leu206Arg mutation. The patient with the recurrent p.Leu206Arg had biochemical CS, and the carrier of the p.His88Asp variant had normal cortisol levels.

A distinct histopathological entity is the adrenal harboring two nodules, each one secreting a different hormone. Fallo *et al.* (101) investigated the occurrence of concurrent PA and subclinical cortisol hypersecretion in a prospective series of 76 consecutive patients with PA. In 3 of the 76 patients, an overnight dexamethasone suppression test failed to appropriately suppress cortisol. One of these patients also had suppressed ACTH levels and mildly elevated urinary cortisol excretion. The resected adrenal gland showed a 4-cm nodule expressing *CYP11B1* by *in situ* hybridization. In contrast, *CYP11B2* expression was restricted to the peritumoral region composed of ZG-like cells, suggesting the coexistence of a CPA and aldosterone-producing hyperplasia in the same adrenal gland. Nanba *et al.* (102) described one patient with lateralized PA and CS, in whom examination of the adrenal gland revealed two distinct nodules, one expressing CYP11B1 and carrying a p.Leu206Arg *PRKACA* mutation and one expressing CYP11B2 and carrying a somatic *KCNJ5* mutation.

Altogether, these studies have raised a certain number of questions regarding the histological, cellular, and genetic nature of adrenal lesions producing aldosterone and cortisol. In particular, they emphasize the need to explore the common pathways leading to APA and CPA in terms of signals triggering cell proliferation and/or defining hormonal output.

Common Hits for APA and CPA

Role of the Wnt/β-catenin pathway in adrenal tumorigenesis

The role of the Wnt/β-catenin pathway has emerged as crucial in adrenal development and disease. β-Catenin and ARMC5 (armadillo repeat-containing protein 5; see the section "ARMC5 mutations in adrenocortical tumors") are both members of the Armadillo repeat containing (*ARMC*) gene family. Armadillo repeats are approximately 40 amino acid-long tandemly repeated domains that usually fold together to form a single, rigid protein domain termed the armadillo domain. β-Catenin is a key downstream component of the canonical Wnt signaling pathway. In the absence of Wnt ligand, β-catenin is in complex with AXIN1, AXIN2, APC, CSNK1A1, and GSK3B, which promotes its phosphorylation on N-terminal Ser and Thr residues, ubiquitination, and subsequent degradation by the proteasome. In the presence of Wnt ligand, the complex is disrupted, and β-catenin

Table 4. Clinical and Genetic Characteristics of Patients With Both APA and CPA

Characteristic	Nakajima *et al.* (93)	Yamada *et al.* (100)	Thiel *et al.* (92)	Rhayem *et al.* (51)	Vouillarmet *et al.* (82)
Total APA patients screened, n	33	Unknown	4	122	1
Patients with cortisol cosecretion (F/M)	10 (NR)	3 (3/0)	4	NR	1 (0/1)
Clinical phenotype	PA/sCS	PA/sCS	PA/2CS-2sCS	1 with PA/sCS, 1 with PA only	PA/sCS
KCNJ5 mutations	6	2	2	0	1 (somatic)
GNAS mutations	2	Not described	0	Not described	Not described
PRKACA mutations	0[a]	Not described	0	2	Not described
CTNNB1 mutations	Not described	Not described	0	Not described	0
APC mutations	Not described	Not described	Not described	Not described	1 (germline)

[a]In another report, the same investigators reported on the analysis of 33 APAs, 11 with sCS, with none carrying a PRKACA mutation (96); however, the overlap between these patients and those from Nakajima *et al.* (93) is unclear.

accumulates in the nucleus, where it acts as a coactivator for transcription factors of the TCF/LEF family, leading to transcriptional regulation of Wnt-responsive genes.

Modulation of different components of the Wnt/β-catenin pathway in mice leads to adrenal abnormalities. In the absence of Wnt4, one of the main ligands of the Wnt/β-catenin pathway, mice die immediately after birth of organ defects such as kidney dysfunction or poor pituitary gland development. Despite normal adrenal morphology, Wnt4 knockout animals produce less aldosterone owing to decreased expression of *cyp11b2* and show reduced expression of other markers of ZG cells, suggesting a defect in ZG development in the absence of Wnt4 (103). Just as observed in mice, loss of function mutations of WNT4 in humans leads to dysgenesis of the kidneys and to female and male sex reversal. Adrenal development and external genitalia are also affected in human fetuses (104). Along the same lines, the targeted disruption of β-catenin restricted to steroidogenic factor 1-expressing cells (adrenal cortex, somatic cells of the gonads, spleen, pituitary gonadotropes, and ventromedial hypothalamic nucleus (105) leads to complete adrenal aplasia when β-catenin is totally absent during development and to a defect in the maintenance of adrenocortical tissue due to increased apoptosis in adult mice when the level of expressed β-catenin is low (106). In H295R cells, a human adrenocortical cell line with activating *CTNNB1* mutations, a decrease in β-catenin expression induces cell cycle arrest, changes in cell morphology, modification of the expression of epithelial to mesenchymal transition markers, and modification of cell motility (107). Although these results highlight the central role of β-catenin in adrenal development, tight regulation of its activation is also crucial. Mice carrying a heterozygous mutation of APC, leading to the formation of a truncated protein and constitutive β-catenin activation, exhibit multiple intestinal tumors. In addition, these mice develop hyperaldosteronism, hypervolemia, and increased blood pressure; however, their adrenal phenotype has not been explored (108, 109). Mice expressing a constitutively active β-catenin in the adrenal cortex exhibit progressive adrenal hyperplasia and dysplasia, leading, with time, to the formation of nodules in both cortex and medulla. Abnormal vascularization was also observed and, in later stages in rare cases, the development of adrenal carcinoma (110). In humans, activation of β-catenin, demonstrated by its cytoplasmic and/or nuclear accumulation, was found in a large number of adrenocortical tumors, including adrenocortical adenomas (85, 111–113). Mutations in the *CTNNB1* gene, leading to constitutive activation of β-catenin, were found in both malignant and benign tumors [Fig. 1(C)] (111, 112). In benign tumors, the presence of β-catenin mutations was associated with larger adenomas and the absence of hormonal secretion (112). In hormonally active ACA, the prevalence of *CTNNB1* mutations has been estimated at 2% to 5% in APAs (28, 29, 41) and ~4% in CPA (see previous sections). The presence of *CTNNB1* mutations in APAs was not associated with a particular histological or morphological phenotype but seems to be more prevalent in females (26, 28, 29, 66). In addition, Teo *et al.* (66) reported the cases of three women with PA with *CTNNB1* mutations in their adenoma that were associated with aberrant expression of G protein-coupled receptors, two during pregnancy and one after menopause, suggesting a possible acceleration of the disease during pregnancy and menopause due to high gonadotropin levels acting on aberrantly expressed gonadotropin receptors. Because *CTNNB1* mutations were also reported in nonpregnant women (28, 29) and men, the relation between *CTNNB1* mutations and pregnancy and the possible interaction with aberrant gonadotropin receptors remain to be further clarified (114, 115).

The role of the Wnt/β-catenin pathway in the development of APA is further supported by the case of a young patient in whom severe arterial hypertension due to primary aldosteronism was diagnosed at age 26, followed by hemorrhagic stroke 4 years later owing to lateralized aldosterone secretion from an APA in the context of bilateral multinodular adrenal glands (82). The patient, in whom additional exploration identified asymptomatic familial adenomatous polyposis (FAP) associated with a heterozygous germline *APC* mutation, showed biallelic *APC* inactivation due to loss of heterozygosity in two adrenal nodules. The aldosterone-producing nodule carried an additional somatic *KCNJ5* mutation supporting a two-hit model for APA development (82). This patient also had incomplete cortisol suppression after an overnight dexamethasone suppression test but normal 24-hour urinary free cortisol, maintained circadian rhythm of cortisol secretion, and normal ACTH levels. *CYP11B1* expression was detected in all adrenal nodules, including the one carrying the *KCNJ5* mutation (data unpublished).

Remarkably, a link between the Wnt/β-catenin and the PKA pathway has recently been established, showing that activation of PKA prevents ZG differentiation through WNT4 repression and Wnt pathway inhibition, suggesting that PKA activation in the ZF is a key driver of Wnt inhibition and lineage conversion (116).

ARMC5 mutations in adrenocortical tumors

Evidence that ARMC5 is involved in adrenal tumorigenesis came from recent studies exploring PBMAH (also called ACTH-independent macronodular adrenal hyperplasia or ACTH-independent macronodular adrenocortical hyperplasia). PBMAH is a form of macronodular adrenal hyperplasia characterized by multiple, bilateral, nonpigmented, benign, adrenocortical nodules.

It results in excessive production of cortisol, leading to ACTH-independent overt CS or sCS (117).

Germline *ARMC5* mutations were identified in familial cases and are present in 50% of apparently sporadic cases. Somatic events occur independently in adrenal nodules, suggesting that *ARMC5* acts as a tumor suppressor gene (117).

A recent study has shown that PBMAH is not truly ACTH-independent because proopiomelanocortin expression was described in clusters of steroidogenic cells in PBMAH tissue, suggesting local ACTH production with paracrine regulation of cortisol biosynthesis (118). The existence of heterogeneous phenotypes (119), such as that observed in a Brazilian family with adrenal macronodular disease showing bilateral and unilateral adrenal nodules, has highlighted the complexity of the condition, leading some investigators to prefer the name primary macronodular adrenal hyperplasia (120). PBMAH is usually diagnosed after the investigation of an adrenal incidentaloma or by the presence of overt CS, although insidious or mild CS has been more frequent (121, 122). Aldosterone secretion was also described in patients with PBMAH (82, 123, 124). Previous studies have demonstrated that cortisol secretion in PBMAH might be regulated by various hormones via aberrant adrenal expression of several G protein-coupled receptors (121, 125, 126). Illegitimate G protein-coupled receptors expressed in PBMAH include the gastric inhibitory polypeptide receptor, vasopressin receptors, β-adrenergic receptors, luteinizing hormone/human chorionic gonadotropin receptors, and the serotonin 5-HT4 and 5-HT7 receptors (121).

Although sporadic forms appear to be more frequent, the description of familial forms and the occurrence of bilateral adrenal lesions support a genetic cause for PBMAH. Recently, combined single nucleotide polymorphism array and microsatellite marker analysis have shown a somatic 16p11 loss of heterozygosity (LOH) in 8 of 33 tumor samples from patients with PBMAH. In parallel, WES analysis, followed by direct sequencing of blood and tumor samples, has identified somatic mutations in *ARMC5*, the armadillo repeat-containing 5 gene mapped to 16p11.2, in 18 of 33 PBMAH patients (127). All patients carried a germline *ARMC5* mutation and a second somatic *ARMC5* event in the tumor DNA, including LOH, a second mutation, or microdeletion, suggesting a 2-hit model for the development of PBMAH (127). Distinct somatic *ARMC5* alterations have been observed in different nodules from the same adrenal gland (127), exemplified by an emblematic case of 16 different somatic *ARMC5* mutations in 20 adrenal nodules from the same patient (128).

The function of *ARMC5* is unknown, and no other diseases have been associated with mutations in this gene. *ARMC5* encodes a 935-amino acid protein of the armadillo repeat family containing an armadillo repeat domain in the N-terminal region and a BTB/POZ in the C-terminal region (129). Analysis of the expression of four *ARMC5* isoforms in 46 human tissues has shown that at least one *ARMC5* isoform is ubiquitously expressed throughout the body, but only seven tissues expressed all four isoforms, including the adrenal gland (130). *Armc5* knockout mice have defective development and immune responses, with adrenal hyperplasia in old animals (131). Transfection of wild-type *ARMC5* in H295R cells induces apoptosis, which is not observed when mutant *ARMC5* cells are transfected. Furthermore, silencing of *ARMC5* in H295R cells reduced the expression of the steroid enzymes *CYP17A1*, *CYP21A2*, *NR5A1*, and *MC2R* and cortisol production (127). Patients with PBMAH and *ARMC5* mutations experience a greater incidence of overt CS than patients with PBMAH without *ARMC5* mutations (127, 132). In a cohort of 98 unrelated subjects with PBMAH, *ARMC5* mutations were found in 24 patients (26%). Patients with *ARMC5* mutations again more frequently showed overt CS and had larger adrenal glands with a greater number of nodules than did patients without mutations (133). These results were confirmed in an Italian cohort of patients with PBMAH, in which patients with *ARMC5* mutations exhibited greater cortisol levels, more severe hypertension, diabetes, and larger adrenal lesions (134). *ARMC5* mutations have also been identified in familial forms of PBMAH (119, 135–137). In one Brazilian family with PBMAH, a germline *ARMC5* mutation was found that segregated with the disease, although the clinical phenotype and adrenal involvement (unilateral or bilateral) were variable among carriers (119). In five families with PBMAH, Gagliardi *et al.* (135) have found germline *ARMC5* mutations in four families with CS and no *ARMC5* mutation in one family with PA and sCS. In a large French-Canadian PBMAH family, *ARMC5* mutations were found in affected subjects, together with aberrant expression of β-adrenergic and V1-vasopressin receptors in adrenal nodules (137). Thus, the aberrant expression of G protein-coupled receptors might represent a secondary event in the development of PBMAH; however, the link between *ARMC5* mutations and this pattern of receptor expression has not yet been clarified.

Zilbermint *et al.* (68) sequenced *ARMC5* in germline DNA from 56 subjects with PA and identified 12 heterozygous variants, 9 missense variants, and 3 variants leading to alternative splicing. *In silico* analysis of the *ARMC5* variants has shown that six variants were predicted to be damaging, including the *ARMC5* variant p.R898W, which was previously described in one patient with PBMAH (132) and a novel missense mutation *ARMC5* p.P826H (68). All carriers of these variants were African American, two had bilateral macronodular hyperplasia, and tumor DNA sequencing showed neither additional somatic

"The functional consequences of these ARMC5 *variants are not completely understood."*

ARMC5 mutations nor *ARMC5* loss of heterozygosity. APAs with predicted damaging *ARMC5* variants exhibit lower ARMC5 expression than the adjacent adrenal cortex. The functional consequences of these *ARMC5* variants are not completely understood. Silencing of *ARMC5* in H295R cells leads to decreased *CYP11B2* expression and, therefore, a reduction of the aldosterone-secretory capacity. The investigators suggested that the greater aldosterone secretion in patients with PA carrying *ARMC5* variants might be explained by the increased adrenocortical mass, similar to that observed in patients with PBMAH and hypercortisolism (68). An association of *ARMC5* mutations with PA was not replicated in an Italian study assessing the presence of *ARMC5* mutations in 39 patients with PA and bilateral computed tomography-detected adrenal alterations, from which 8 had unilateral and 27 bilateral aldosterone secretion (138). Different *ARMC5* variants were identified in 18 subjects, including 11 common variants, 2 rare variants, and 2 previously unreported variants. However, *in silico* analysis did not identify possibly damaging *ARMC5* variants (138). These contradictory data do not allow confirmation of the role of *ARMC5* mutations in PA, and further studies are required to better understand the role of *ARMC5* in the development of adrenal nodules and aldosterone excess.

Germline mutations associated with ACA and macronodular hyperplasia

Genetic alterations leading to multiple tumor syndromes and mutations affecting genes of the cAMP/PKA signaling pathway have been associated with PBMAH [reviewed by Frageso *et al.* (120) and Drougat *et al.* (122)]. FAP is a disease characterized by multiple colonic polyps with an increased risk of colon carcinoma, owing to an inactivating germline mutation of *APC* and a somatic second hit (mutation or LOH), resulting in constitutive activation of the Wnt/β-catenin pathway (139). Adrenal masses are more frequently found in patients with FAP than in the general population (140), including nonfunctioning adrenal adenomas, adrenocortical cancers, and PBMAH (141, 142). Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant syndrome resulting from inactivating mutations in the *MEN1* gene, which is characterized by the development of multiple endocrine and nonendocrine tumors, most frequently hyperparathyroidism, pituitary adenoma, and pancreatic neuroendocrine tumors (143). MEN1 encodes the protein menin, which has a role in the regulation of cell cycle, cell growth, and proliferation (144). The analysis of adrenal involvement in 715 patients with MEN1 has shown adrenal tumors (>10 mm in size) in 10% of the cohort (145). In 19.4% of these cases, the tumors were bilateral, and in 12.5% of the cases, the tumors were >40 mm (145). A *MEN1* mutation was also identified in one patient with PBMAH and hyperparathyroidism, without a family history or other endocrine abnormalities (142). In addition, LOH at the MEN1 locus was also reported in APAs (146). Another hereditary disease associated with PBMAH is the hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC), a disease caused by germline mutations in the fumarate hydratase gene (147). Adrenal lesions are found in 7.8% of patients with HLRCC, including bilateral lesions associated with ACTH-independent hypercortisolism (148), and germline fumarate hydratase mutations were identified in patients with PBMAH associated with HLRCC (142, 147). Genetic alterations leading to activation of cAMP/PKA signaling were also described in patients with PBMAH. Postzygotic somatic mutations in the gene encoding the α subunit of stimulatory G protein (*GNAS*), leading to constitutive activation of adenylate cyclase, result in McCune-Albright syndrome, which is characterized by a triad constituted by polyostotic fibrous dysplasia, café au lait skin spots, and gonadotrophin-independent precocious puberty, associated with other endocrine and nonendocrine diseases, including CS (149). Somatic *GNAS* mutations were described in a few cases of PBMAH without other typical signs of McCune-Albright syndrome and might represent variants of this syndrome (142, 150). In isolated cases, variants in *PDE11A*, encoding the isoform 11A of the phosphodiesterase enzyme, and in *MC2R* were associated with PBMAH (151–153). Finally, duplication of a genomic region of chromosome 19, including *PRKACA*, was also identified in patients with PBMAH (88, 154).

Conclusion

During the past few years, extraordinary progress has been made in our understanding of the genetic abnormalities leading to APAs and CPAs. Although genetic testing in inherited forms of the diseases has been translated into clinical practice, and family screening is useful for the management of affected family members, the clinical utility of diagnosing somatic mutations is difficult to anticipate from available studies on genotype–phenotype correlations and outcome. Ideally, identification of mutations before surgery, through application of surrogate biomarkers or genetic testing on circulating cell-free DNA, could guide treatments to specifically target mutated proteins, such as was shown for APAs (155, 156). An overlap seems to exist of genetic abnormalities leading to different endocrine abnormalities in ACA. In particular, *CTNNB1* mutations are found at a relatively high frequency (at least comparable to that observed for *ATP1A1* or *ATP2B3* mutations in APA)

in CPA but also usually in APA. Furthermore, genetic variants affecting genes of the calcium signaling pathway are enriched in WES data from CPA, suggesting that calcium, in addition to PKA signaling, might lead to autonomous cortisol overproduction. This is not surprising, because a subset of APAs shows some cortisol cosecretion or biochemical glucocorticoid abnormalities (157). In some cases, those tumors harbor somatic *KCNJ5* mutations. In addition, overexpression of mutated GIRK4 channels in HAC15 cells not only increases *CYP11B2* expression but also increases *CYP11B1* (58). Future studies should address the sequence of events leading to adrenal cell proliferation, nodule formation, and specification of the hormonal secretory pattern. It will be of particular interest to explore whether common susceptibility alleles or rare variants might predispose to the development of APAs and CPAs.

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Abbreviations

ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; ACTH, adrenocorticotropic hormone; AngII, angiotensin II; APA, aldosterone producing adenoma; APCC, aldosterone-producing cell cluster; ARMC, armadillo repeat containing (gene); ARMC5, armadillo repeat-containing protein 5; ATPase, adenosine triphosphatase; BAH, bilateral adrenal hyperplasia; cAMP, cyclic adenosine monophosphate; CPA, cortisol producing adenoma; CS, Cushing syndrome; FAP, familial adenomatous polyposis; FH, familial hyperaldosteronism; HLRCC, hereditary leiomyomatosis and renal cell carcinoma syndrome; IP3, inositol 1,4,5-triphosphhate; LOH, loss of heterozygosity; MEN1, multiple endocrine neoplasia type 1; PA, primary aldosteronism; pAATL, possible APCC-to-APA transitional lesion; PBMAH, primary bilateral macronodular adrenal hyperplasia; PKA, protein kinase A; PMCA3, plasma membrane calcium-transporting ATPase 3; sCS, subclinical Cushing syndrome; StAR, steroid acute regulatory protein; WES, whole exome sequencing; ZF, zona fasciculata; ZG, zona glomerulosa.