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## Genetic causes of functional adrenocortical adenomas

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**Abbreviations:** PA, primary aldosteronism; CS, Cushing syndrome; sCS, subclinical Cushing syndrome; APA, aldosterone producing adenomas; CPA, cortisol producing adenomas; A/CPA, aldosterone and cortisol producing adenoma; ACA, adrenocortical adenoma; BAH: bilateral adrenal hyperplasia; PBMAH, primary bilateral macronodular adrenal hyperplasia; FH, familial hyperaldosteronism

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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 zone with specific functional characteristics. Adrenocortical adenomas (ACA) may 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, ACA lead to overt or subclinical Cushing syndrome. Genetic analysis driven by next generation sequencing technology has enabled the discovery, during the last seven years, of the genetic causes of a large subset of ACA. In particular, somatic mutations in genes regulating intracellular ionic homeostasis and membrane potential have been identified in aldosterone producing adenoma. 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 cAMP-dependent protein kinase catalytic subunit alpha) affect cyclic AMP-dependent protein kinase A signaling, leading to activation of cortisol biosynthesis. In addition to these specific pathways, the Wnt/ $\beta$ -catenin pathway appears to play an important role in adrenal tumorigenesis, as  $\beta$ -catenin mutations have been identified in both aldosterone as well as cortisol producing adenomas. This, together with different intermediate states of aldosterone and cortisol co-secretion, raises the possibility that the two conditions share a certain degree of genetic susceptibility. Alternatively, different hits may be responsible for the diseases, with one hit leading to adrenocortical cell proliferation and nodule formation, with the second one specifying the hormonal secretory pattern.

## 1 **1. Introduction**

2 Adrenal masses are quite common being found in  $\approx 3\%$  of the general population with the  
3 prevalence increasing with age (1). Functional adrenocortical adenomas are a group of benign  
4 tumors of the adrenal cortex autonomously secreting excess hormones. Aldosterone producing  
5 adenomas (APA) are responsible for primary aldosteronism (PA), the most frequent form of  
6 secondary hypertension. Cortisol producing adenomas (CPA) are responsible for overt or  
7 subclinical Cushing syndrome (CS). In addition, both diseases may also result from different  
8 forms of adrenal hyperplasia: bilateral adrenal hyperplasia (BAH) in PA and primary bilateral  
9 macronodular adrenal hyperplasia (PBMAH) responsible for overt or subclinical CS. Primary  
10 pigmented nodular adrenal hyperplasia is a rare condition responsible for overt CS. Unilateral  
11 adrenal hyperplasia leading to autonomous aldosterone or cortisol production has also been  
12 described. Importantly, in between the two conditions are histological forms of multinodular  
13 adrenals where hormone production may be localized to one large nodule or to multiple  
14 nodules; sometimes co-secretion of aldosterone and cortisol is observed in one adrenal, either  
15 from one adenoma or from distinct adenomas.

16

## 17 **2. Aldosterone and cortisol biosynthesis in the adrenal cortex**

18 Aldosterone and cortisol are produced in the adrenal cortex, which is composed in humans of  
19 three concentric functionally distinct zones. Steroid hormones are produced from cholesterol  
20 through sequential enzymatic steps involving different cytochrome P450 enzymes and  
21 hydroxysteroid dehydrogenases and the functional properties of each zone results from the  
22 expression of specific steroidogenic enzymes and the ability to respond to unique regulatory  
23 stimuli. In particular, the outer zona glomerulosa produces aldosterone through the activity of  
24 aldosterone synthase (encoded by *CYP11B2*), which catalyzes the three terminal reactions of  
25 aldosterone biosynthesis: hydroxylation at position C11 of the steroid intermediate  
26 deoxycorticosterone (DOC) yielding corticosterone, followed by a hydroxylation at position

27 C18 yielding 18-hydroxy-corticosterone and a subsequent oxidation of the hydroxyl group at  
28 C18, which results in the formation of aldosterone. Cortisol biosynthesis occurs in the zona  
29 fasciculata, where the expression of 17 $\alpha$  hydroxylase mediates conversion of 17-deoxy-21-  
30 carbon steroids into 17-hydroxy-21-carbon steroids, precursors of cortisol. As 17 $\alpha$   
31 hydroxylase is not expressed in rats and mice, the main glucocorticoid in these species is  
32 corticosterone (2). Cortisol is synthesized from 17OH-progesterone through 21-hydroxylation  
33 to form 11-deoxycortisol, followed by hydroxylation at position C11 by 11 $\beta$ -hydroxylase  
34 (encoded by *CYP11B1*). The two highly homologous genes *CYP11B2* and *CYP11B1* are  
35 located in tandem on human chromosome 8q21-22. Unequal crossing over of the two genes  
36 during meiosis results in a chimeric gene composed of the regulatory regions of *CYP11B1*  
37 fused to the coding sequence of *CYP11B2*, which is responsible for familial  
38 hyperaldosteronism (FH) type 1 (also known as glucocorticoid remediable aldosteronism) (3).  
39 Under these conditions, aldosterone synthase expression and aldosterone biosynthesis are  
40 regulated by ACTH in the zona fasciculata of the adrenal cortex, rather than by angiotensin II  
41 (AngII), with a circadian rhythmicity of aldosterone production following that of cortisol (4)  
42 and production of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol (5,6).  
43 The main regulators of aldosterone biosynthesis are the renin-angiotensin system, the  
44 concentration of extracellular potassium and ACTH (7). The renin-angiotensin system  
45 translates changes in renal perfusion or sodium concentration sensed by the distal tubule into  
46 changes in aldosterone production from the adrenal cortex, in order to maintain volume and  
47 electrolyte homeostasis. AngII acts via the AT1 receptor expressed in zona glomerulosa cells,  
48 activating a G $\alpha$ q-phospholipase C mediated pathway leading to increased inositol 1,4,5-  
49 triphosphate (IP3) and 1,2-diacylglycerol concentrations. IP3 increases intracellular calcium  
50 concentrations by calcium release from intracellular stores.

51 AngII also inhibits the background potassium channel TASK (TWIK-related acid-sensitive  
52 potassium channel) and the Na<sup>+</sup>,K<sup>+</sup>-ATPase, leading to cell membrane depolarisation and  
53 activation of voltage-gated Ca<sup>2+</sup> channels, again increasing intracellular calcium  
54 concentrations. Extracellular potassium is the other major regulator of aldosterone production,  
55 exerting a large amplitude of control over a wide range of potassium concentrations. This is  
56 possible because the main ionic conductance of zona glomerulosa cells is that of K<sup>+</sup> and the  
57 cell membrane potential closely follows the equilibrium potential of K<sup>+</sup> over a large range of  
58 extracellular K<sup>+</sup> concentrations (8). Elevation of the extracellular K<sup>+</sup> concentration, a decrease  
59 in the intracellular K<sup>+</sup> concentration, inhibition of Na<sup>+</sup>, K<sup>+</sup>-ATPase or closure of potassium  
60 channels all lead to cell membrane depolarization, opening of voltage-dependent Ca<sup>2+</sup>  
61 channels and an increase in intracellular calcium concentrations resulting in activation of  
62 calcium signaling.

63 Increased calcium signalling affects aldosterone production in several ways. Calcium  
64 signalling increases activity of cholesterol ester hydrolase with subsequent release of  
65 deesterified cholesterol from cytoplasmic stores, promotes cholesterol delivery to the outer  
66 mitochondrial membrane and its transfer to the inner mitochondrial membrane by increasing  
67 expression of the steroid acute regulatory protein (StAR), and increases the synthesis of  
68 cofactors required for p450 cytochrome enzymes (9). Stimulation of *CYP11B2* transcription is  
69 mediated by calcium/calmodulin binding and activation of protein kinases regulating  
70 phosphorylation of several transcription factors, in particular nuclear receptor subfamily 4  
71 group A (NR4A) members 1 and 2 (NURR77 or NGF1B and NURR1), the cyclic AMP-  
72 dependent transcription factor ATF-1 and the cyclic AMP-responsive element-binding protein  
73 (CREB) (10) (Figure 1A).

74 Cortisol production is regulated by the hypothalamus-pituitary-adrenal (HPA) axis, whereby  
75 circadian input and stress stimuli induce release of cortisol via corticotrophin releasing

76 hormone (CRH) -dependent ACTH secretion and cortisol signals back to exert negative  
77 feedback on the hypothalamus and the pituitary. In the zona fasciculata (and zona reticularis)  
78 of the adrenal cortex, ACTH binds to its receptor MC2R (Melanocortin receptor 2), which  
79 signals through a Gas mediated signaling to increase intracellular cAMP levels and to activate  
80 protein kinase A (PKA) (Figure 1B) (11). Acute stimulation by ACTH elicits effects on  
81 steroidogenesis by regulating cholesterol availability, mobilizing cholesterol from lipid stores  
82 and increasing cholesterol transport into mitochondria and by stimulating transcription of  
83 steroidogenic genes. Rapid effects occurring within minutes mainly affect StAR activity  
84 through phosphorylation, while longer stimulation (days) by ACTH results in transcriptional  
85 regulation of StAR as well as steroidogenic enzymes. In particular, PKA signaling activates  
86 transcription factors regulating expression of steroidogenic genes, including steroidogenic  
87 factor 1 (SF-1), CREB, CRE modulator (CREM), CCAAT/enhancer-binding proteins  
88 (C/EBPs), and activator protein 1 (AP-1) (11). ACTH also regulates aldosterone production  
89 acutely via cAMP mediated pathways, whereas chronically, ACTH suppresses plasma  
90 aldosterone both in human and animal models and downregulates *CYP11B2*, inducing  
91 *CYP11B1* expression and promoting a zona fasciculata phenotype (7,12). Long-term exposure  
92 to ACTH (weeks or months) stimulates adrenal growth and promotes adrenal cell hypertrophy  
93 and hyperplasia. In extreme cases of glucocorticoid deficiency or ACTH excess, as well as in  
94 experimental models of CRH or ACTH deficient mice, the trophic effects of the hormone lead  
95 to major hyperplasia or atrophy respectively of the inner zones of the adrenal cortex (12). In  
96 addition to acting through cAMP, ACTH also stimulates calcium influx in glomerulosa cells  
97 by activation of L-type Ca<sup>2+</sup> channels (9). Many other factors have been shown to modulate  
98 aldosterone and cortisol production, including neurotransmitters, cytokines, vascular products,  
99 and adipokines, as well as locally produced ACTH acting through a paracrine mechanism  
100 (12,13).

101

### 102 **3. Somatic mutations in APA**

103 PA is due to autonomous aldosterone production from the adrenal cortex and is diagnosed on  
104 the basis of hyperaldosteronism associated with suppressed renin and often hypokalemia (14).

105 It is the most common form of secondary hypertension (HT), with a prevalence of 5-10% in  
106 patients with arterial hypertension (15,16) and up to 20% in patients with resistant HT (17).

107 PA is associated with increased cardiovascular risk, in particular coronary artery disease,  
108 nonfatal myocardial infarction, heart failure, atrial fibrillation, renal damage and stroke (18-  
109 20), due to blood-pressure independent effects of aldosterone on cardiac and vascular  
110 remodeling (21,22),

111 The majority of cases of PA are due to an APA or to bilateral adrenal hyperplasia (BAH, also  
112 called idiopathic hyperaldosteronism). Only in rare cases has unilateral adrenal hyperplasia  
113 been described, and aldosterone producing adrenocortical cancers are extremely rare. APA are  
114 generally small (< 2 cm in diameter) and are diagnosed by adrenal CT and adrenal venous  
115 sampling, which identifies lateralized aldosterone secretion in the presence or absence of a  
116 visible adrenal nodule at imaging (14,23). Recent advances in genome technology have  
117 allowed identification, during the last five years, of several somatic mutations in APA,  
118 providing a pathogenic model for PA development. Whole exome sequencing of paired  
119 tumour (somatic) and germline DNA performed by different groups on a total of more than  
120 100 APA samples has identified recurrent somatic mutations in genes coding for ion channels  
121 (*KCNJ5* (24) and *CACNA1D* (25,26)) and ATPases (*ATP1A1* and *ATP2B3*, (25,27))

122 regulating intracellular ionic homeostasis and cell membrane potential, thus defining APA as  
123 a channelopathy. These mutations all promote increased intracellular calcium concentrations,  
124 leading to activation of calcium signaling, the main trigger for aldosterone biosynthesis  
125 (Figure 1). A small fraction of APA also carries somatic mutations in *CTNNB1*, coding for  $\beta$ -



126 catenin, similar to CPA (see below) and adrenocortical cancer (28,29) (Table 1). In the largest  
127 multicenter study published, exploring the prevalence of somatic mutations in *KCNJ5*,  
128 *CACNAID*, *ATP1A1* and *ATP2B3* in 474 patients from seven centers belonging to the  
129 ENS@T network (European Network for the Study of Adrenal Tumors), somatic mutations  
130 were detected in 54% of APA, ranging from 27.2% to 56.8% across different centers (30). In  
131 this study, *KCNJ5* mutations were the most prevalent genetic abnormality and were found in  
132 38% of APA. Mutations affecting *CACNAID* were the second most prevalent genetic  
133 abnormalities being present in 9.3% of cases, while *ATP1A1* and *ATP2B3* mutations were  
134 identified in 5.3% and 1.7% of cases respectively. There was no difference in cellular  
135 composition (percentage of cells resembling to zona fasciculata (ZF) or zona glomerulosa  
136 (ZG)) of APA or in *CYP11B2*, *KCNJ5*, *CACNAID* or *ATP1A1* gene expression in APA across  
137 genotypes. Patients with *KCNJ5* mutations were more frequently female, diagnosed younger  
138 and with higher plasma potassium concentrations when compared to *CACNAID* mutation  
139 carriers or non-carriers. *CACNAID* mutations were associated with smaller adenomas. There  
140 was no association between the mutation status and preoperative plasma aldosterone or renin  
141 levels, the aldosterone to renin ratio or the number of medications taken before surgery. There  
142 was also no association with post-operative blood pressure outcomes, treatment scores at  
143 follow-up, cure or improvement of hypertension. These associations were largely center-  
144 dependent, which might be due to the heterogeneity of APA that has to be taken into account  
145 when considering genotype-phenotype correlations (30).

146 Furthermore, less frequent mutations have been identified in a few genes in patients with  
147 peculiar phenotypes of PA, including early-onset PA and tumors cosecreting aldosterone and  
148 cortisol (31,32) (Table 1). Recently, somatic mutations in *PRKACA* (coding for the cAMP-  
149 dependent protein kinase catalytic subunit alpha) have been reported in patients with APA  
150 (32). Using whole exome and targeted sequencing in 122 patients, Rhayem et al identified

151 two somatic heterozygous mutations in *PRKACA*, one of them being the p.Leu206Arg  
152 mutation previously reported in CPA (see below). The carrier of this variant had biochemical  
153 Cushing syndrome and high expression of 11 $\beta$ -hydroxylase in the tumor. The second  
154 p.His88Asp variant was not associated with cortisol excess, nor with a gain of function of the  
155 PKA catalytic activity, leaving open the question of its role in the development of PA.

156

### 157 **3.1. Pathogenic mechanisms of APA development**

158 *KCNJ5* codes for the G protein-activated inward rectifier potassium channel GIRK4  
159 (alternative protein name Kir3.4), which is composed of two membrane-spanning domains  
160 (M1 and M2), one pore forming region (H5) and cytoplasmic N- and C-termini that contribute  
161 to the pore structure. Recurrent somatic mutations identified in APA cluster within or near the  
162 selectivity filter of the channel pore, between amino acids Thr149 to Gly153 (Table 2). They  
163 affect its ionic selectivity, rendering the channel permeable to sodium, with increased sodium  
164 influx into the cell leading to chronic cell membrane depolarization and opening of voltage-  
165 gated calcium channels (24). This in turn, leads to increased intracellular calcium  
166 concentrations and activation of calcium signaling (Figure 1A). Transient transfection of  
167 *KCNJ5* mutants into adrenocortical cells increase aldosterone production and expression of  
168 *CYP11B2* in a calcium-dependent manner (33); in some cases, mutations lead to reduced  
169 membrane and total abundance of GIRK4 (34). Surprisingly, transiently transfected *KCNJ5*  
170 mutants did not lead to enhanced cell proliferation *ex vivo*, but rather to reduced cell viability  
171 or sodium dependent cell death, which does not explain the cell proliferation and APA  
172 formation (33,35). Whether these results are due to experimental artefacts, due to high  
173 expression of transfected channels leading to excessively increased sodium influx into the  
174 cell, which does not reflect the *in vivo* situation, or whether additional hits are required for  
175 cell proliferation, remains a matter of debate.

176 *CACNA1D* codes for the  $\alpha 1$  subunit of the L-type voltage gated Cav1.3 calcium channel  
177 (calcium channel, voltage-dependent, L type, alpha-1d subunit), whereas *ATP1A1* codes for  
178 the  $\alpha 1$  subunit of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and *ATP2B3* for the plasma membrane calcium-  
179 transporting ATPase 3 (PMCA3).  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and PMCA3 are members of the P-type  
180 family of ATPases and are composed of ten transmembrane domains (M1 – M10) with  
181 intracellular N- and C-tails. *ATP1A1* mutations affect amino acids in transmembrane helices  
182 M1, M4 and M9 of the  $\alpha 1$  subunit of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (Table 2). They lead to a loss of  
183 pump activity and a reduced affinity for  $\text{K}^+$ , as well as to an inward proton or sodium leak,  
184 which is proposed to increase aldosterone production through cell membrane depolarization  
185 and increased calcium influx, as is observed in the presence of *KCNJ5* mutations (25,27).  
186 However, transient transfections of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase  $\alpha 1$  subunits carrying the p.Leu104Arg,  
187 p.Val332Gly or p.Gly99Arg mutations in adrenocortical NCI-H295R cells did not modify  
188 basal cytosolic calcium levels and hardly increased potassium-stimulated calcium  
189 concentrations, despite depolarizing adrenal cells and stimulating aldosterone secretion (36).  
190 Rather they induced increased intracellular acidification which is suggested to play a role in  
191 regulating aldosterone biosynthesis in this context (Figure 1A) (36).  
192 Mutations of PMCA3 are located in the transmembrane domain M4 and are all deletions of  
193 multiple amino acids in the region between Leu425 and Leu433 (Table 2).  
194 Electrophysiological and functional studies have shown that the p.Leu425\_Val426del  
195 mutation leads to reduced  $\text{Ca}^{2+}$  export due to loss of the physiological pump function and  
196 increased  $\text{Ca}^{2+}$  influx due to opening of depolarization-activated  $\text{Ca}^{2+}$  channels. A  $\text{Ca}^{2+}$  leak  
197 through the mutated pump has also been observed, inducing *CYP11B2* expression and  
198 aldosterone biosynthesis (Figure 1A) (37).  
199 Finally, *CACNA1D* mutations affect the function of the L-type  $\text{Ca}^{2+}$  channel  $\alpha$ -subunit  $\text{Ca}_v1.3$ ,  
200 which is composed of four repeat domains (I to IV), each one consisting of six

201 transmembrane segments S1-S6 and a membrane associated loop between S5 and S6 (Table  
202 2) (38). Whereas the S4 segments are involved in voltage sensing, the S5 and S6 domains as  
203 well as the loop in between line the pore of the channel (39). 18 different *CACNAID*  
204 mutations have been identified (25,26,30); they are gain-of-function mutations leading to a  
205 shift of voltage-dependent channel activation towards more negative voltages, or to reduced  
206 inactivation of the channel, followed by increased intracellular calcium concentrations with  
207 induction of aldosterone biosynthesis (Figure 1A) (25,26).

208

### 209 **3.2. Genotype-phenotype correlations in APA**

210 *KCNJ5* mutations are the most frequent type of somatic mutation within APA (Table 1). Their  
211 reported frequency ranges from 13-77%, with higher frequency in studies from Japan, China,  
212 Korea and Taiwan (40-46). *CACNAID* mutations are the second most frequent mutations (up  
213 to 10%), followed by *ATP1A1* and *ATP2B3*. Three studies have also explored *CTNNB1*  
214 mutations, which are found in 2.1 – 5.1% of cases (26,28,29).

215 The correlations of clinical and molecular parameters with the mutation status are  
216 heterogeneous among studies (Table 1). In a recent meta-analysis, Lenzini et al analyzed the  
217 clinical correlates of *KCNJ5* mutations in 1636 patients from 13 studies (47). The overall  
218 prevalence of *KCNJ5* mutations was 43%, ranging from 12-80% among different studies,  
219 with a higher prevalence in studies from Japan and China compared to studies on populations  
220 from Europe, US and Australia. Carriers of *KCNJ5* mutations were younger, more often  
221 females, had larger tumors and had higher plasma aldosterone levels when compared to non-  
222 carriers. No association was observed between the mutation status and systolic or diastolic  
223 blood pressure and serum potassium levels overall, confirming previous results from the study  
224 by Fernandes-Rosa et al (30). An association between the presence of *KCNJ5* mutations and  
225 the lateralization index at adrenal vein sampling was described in two studies ((42,48)

226 , but not replicated in another study (49). In four studies, *KCNJ5* mutations were associated  
227 with tumors composed mainly of large, lipid-laden cells resembling zona fasciculata cells  
228 (Table 1). The other mutations were associated mainly with male sex and smaller tumors (25-  
229 28,30,45,50), and the cellular phenotype of APA carrying *CACNAID*, *ATPIA1* or *ATP2B*  
230 mutations was more heterogeneous than *KCNJ5* mutated tumors . Although few studies have  
231 investigated *CTNNT1* mutations in large cohorts, they seem to be more prevalent in females  
232 (26,28,29,51).

233

### 234 **3.3. Germline mutations in APA**

235 In addition to somatic mutations, the occurrence of germline *CACNAIH* (coding for the  
236 voltage-dependent T-type calcium channel subunit alpha-1H) mutations, and rarely, germline  
237 *KCNJ5* and *ARMC5* variants has been reported in PA (31,52,53). Daniil et al performed  
238 whole exome sequencing in patients with different phenotypic presentations of PA, including  
239 23 subjects with APA, 10 patients with a family history of PA and one subject with early-  
240 onset PA. *CACNAIH* mutations were identified in early onset PA and patients with a family  
241 history, and a germline *CACNAIH* p.Val1951Glu mutation was identified in one patient with  
242 an APA (31). *CACNAIH* encodes the pore-forming  $\alpha 1$  subunit of the T-type voltage-  
243 dependent calcium channel Cav3.2, which has been implicated in membrane potential  
244 oscillations in ZG cells (54). The variant is located in the C-terminal cytoplasmic domain of  
245 Cav3.2, in a region possibly implicated in fast channel activation. Electrophysiological  
246 investigation revealed a gain-of-function phenotype, whereby p.Val1951Glu induced a faster  
247 recovery from inactivation and marked increase in voltage-dependent facilitation, two  
248 properties which could favor larger calcium entry during repetitive electrical activity. In  
249 H295R adrenocortical cells, transient transfection of the mutant channel led to an increase in  
250 aldosterone biosynthesis and expression of *CYP11B2* (31).

251 Genetic abnormalities in APA driver genes are present as germline mutations in some familial  
252 forms of PA, which account for 1 to 5% of cases and are transmitted as an autosomal  
253 dominant trait. Although the terminology for FH is still evolving, to date four different forms  
254 of familial hyperaldosteronism, FH-I to FH-IV, have been described. FH-I, resulting from the  
255 formation of a chimeric gene between *CYP11B2* and *CYP11B1* (see above), is found in 0.5%  
256 to 1.0% of cases of PA (55-57), but may represent up to 3% of cases in the paediatric  
257 hypertensive population (58). FH-I is associated with BAH, but in rare cases adrenocortical  
258 adenomas have been described. Heterozygous germline mutations in *KCNJ5* are found in FH-  
259 III, a severe form of PA presenting in typical cases in childhood with severe hypertension and  
260 profound hypokalemia, due to massive bilateral adrenal hyperplasia which requires bilateral  
261 adrenalectomy to control the hypertension (24). In other cases, the disease is milder and with  
262 normal appearing adrenals on imaging studies (59). Germline mutations in *CACNA1H* have  
263 recently been identified as the cause of a new form of early onset PA associated or not with a  
264 developmental disorder (31,60). In familial cases, the disease (named FH-IV) is transmitted as  
265 an autosomal dominant trait. Similar mutations are also found in patients diagnosed as FH-II  
266 with or without an adrenal adenoma as well as in APA (31). Finally, *de novo* germline  
267 *CACNA1D* mutations have been described in a rare disease called PASNA (Primary  
268 aldosteronism, seizures, neurological abnormalities), with no adrenal abnormality reported on  
269 imaging (26).

270

#### 271 **4. Pathological and genetic heterogeneity and the origin of APA**

272 While the functional link between mutations in driver genes in APA and aldosterone  
273 production has been clearly demonstrated, the origin of APA in terms of increased cell  
274 proliferation and nodule formation is not yet well understood, and work from different groups  
275 has provided contradictory results on the sequence of events leading to aldosterone

276 overproduction and adenoma formation. Although the fact that familial forms of PA are due  
277 to germline mutations in driver genes supports a role for these mutations in adrenal cortex cell  
278 proliferation and nodulation, adrenal morphology is highly variable among these subjects.  
279 Patients with FH-III harboring germline *KCNJ5* mutations may exhibit adrenal phenotypes  
280 ranging from a normal adrenal to massive bilateral adrenal hyperplasia or adrenal nodules  
281 (24,61,62). Similarly, patients carrying germline *CACNA1H* mutations show different adrenal  
282 phenotypes including normal, hyperplastic and adenomatous adrenals  
283 (31,60). No abnormality in adrenal morphology was observed in patients with PASNA  
284 harboring germline *CACNA1D* mutations (26). *Ex vivo*, adrenocortical cells overexpressing  
285 mutant *GIRK4* channels show increased intracellular Na<sup>+</sup> concentration leading,  
286 counterintuitively, to reduced cell proliferation and sodium-dependent apoptosis (33,35).  
287 It has been previously shown that adrenals with APA exhibit adrenal cortex remodeling,  
288 decreased vascularization, increased nodulation and ZG hyperplasia (63,64). These findings  
289 suggest that adrenal cortex remodeling may precede APA development and that somatic  
290 mutations in driver genes are secondary events specifying the pattern of hormone secretion. The  
291 recent development of an aldosterone-synthase antibody (65) has allowed assessment of the  
292 genetic and histological characteristics of adrenals with APA and has reinforced the hypothesis  
293 of independent events leading to nodulation and aldosterone overproduction. Two studies in  
294 particular have identified different genetic abnormalities in different aldosterone producing  
295 nodules from the same adrenal. In one study, Dekkers et al analyzed 28 multinodular adrenals  
296 and identified five adrenals with more than one nodule staining positively for aldosterone  
297 synthase; in one adrenal two aldosterone-synthase positive nodules each harbored a different  
298 *KCNJ5* mutation (66). In the second study, assessment of the *KCNJ5*, *ATP1A1*, *ATP2B3* and  
299 *CACNA1D* mutation status in aldosterone synthase positive nodules from 27 multinodular  
300 adrenals with APA identified 7 adrenals harboring different mutations between the principal

301 nodule and the secondary nodule within the same adrenal, including two adrenals with an APA  
302 harboring a *CACNA1D* mutation and in one secondary nodule, a *KCNJ5* mutation (67). Further  
303 studies extended the heterogeneity of aldosterone synthase expression to the APA itself, with  
304 *KCNJ5* mutations only identified in APA regions positive for aldosterone synthase (68).  
305 Finally, in a young PA patient with a germline *APC* mutation and a multinodular adrenal with  
306 somatic biallelic inactivation of *APC*, we identified a somatic *KCNJ5* mutation in the nodule  
307 expressing aldosterone synthase only (see below) (69). These data support a two-hit model for  
308 APA formation whereby a specific genetic or epigenetic event is responsible for adrenal cortex  
309 remodeling and nodule formation and somatic mutations in driver genes leading to excessive  
310 aldosterone secretion represent second hits specifying the pattern of hormonal secretion (Figure  
311 2).

312 A second model for the origin of APA has emerged from genetic studies performed on  
313 aldosterone-producing cell clusters (APCC). APCC, which are found in normal adult adrenals  
314 and in adrenals with APA, are composed by ZG-like cells expressing disabled 2 (*Dab2*, a  
315 marker of ZG) in contact with the capsule and inner columnar *Dab2*-negative ZF-like cells,  
316 which do not express *CYP11B1*, forming cords along sinusoids (70,71). APCC cells are  
317 positive for *CYP11B2* and  $3\beta$ HSD staining, but not for *CYP17*, consistent with their capacity  
318 to synthesize aldosterone (70). Targeted next generation sequencing was performed on the  
319 DNA of 23 APCC microdissected from adrenals from kidney transplant donors; somatic  
320 mutations in *CACNA1D*, *ATP1A1* and *ATP2B3*, similar to those already described in APA, were  
321 identified in 35% of APCC (72). The authors proposed that these findings are consistent with  
322 the development of APA from APCC (Figure 2) and suggest different hypotheses for this  
323 progression: single somatic mutations responsible for aldosterone production and cell  
324 proliferation or second-hit mutations within the APCC leading to cell proliferation. In some  
325 cases APCCs may represent terminal lesions not having the capacity to progress towards an



326 adenoma (72). Interestingly, no *KCNJ5* mutations, the most frequent genetic alteration  
327 observed in APA, were identified in APCC. The authors suggest that this could be explained  
328 by a rapid progression of *KCNJ5* mutated APCC to APA (72). Subsequently, *CACNA1D*  
329 mutations were identified in 17 of 26 APCC from patients with lateralized PA negative for  
330 detectable masses at imaging, suggesting that these lesions may contribute to PA in this  
331 situation (73). Recently, a novel aldosterone-producing structure named a possible APCC-to-  
332 APA transitional lesion (pAATL) was described in multinodular adrenals from two patients  
333 with lateralized PA, which was suggested to be an intermediate lesion between APCC and APA  
334 (74). While the APCC-like portion consists of a subcapsular ZG-like region and an inner ZF-  
335 like region expressing CYP11B2 but not CYP11B1, the micro-APA-like portion is composed  
336 of a heterogeneous mixture of CYP11B1-positive and CYP11B2-positive cells. Sequencing of  
337 the known APA-associated mutations in three pAATL identified a *KCNJ5* mutation and an  
338 *ATP1A1* mutation in the micro-APA-like portion of two pAATL, but not in their corresponding  
339 APCC-like portions. The authors suggest that these data support the hypothesis that the micro-  
340 APA derived from the APCC following the appearance of an APA driver mutation (74).  
341 However, another pAATL carried two novel *ATP1A1* mutations in both the mAPA-like and  
342 APCC-like portions, in this case supporting a clonal origin of the two portions (74). New  
343 genetic studies in a larger number of normal and pathological adrenals with APCC are necessary  
344 to clarify the relationship between these aldosterone-producing lesions and the origin of APA.

345

## 346 **5. Genetic abnormalities in Cortisol Producing Adenomas**

347 The genetics of CPA was unraveled in 2014 by the work of four independent groups  
348 identifying somatic mutations of *PRKACA* as a cause of CS. Beuschlein and coworkers  
349 performed WES in 10 paired adrenal tumor and germline DNA samples from patients with  
350 CS, which revealed the presence of a recurrent somatic p.Leu206Arg mutation in the

351 *PRKACA* gene in 7 cases, with an additional p.Leu199\_Cys200insTrp mutation in one case  
352 (75). Sequencing of 129 additional samples identified the p.Leu206Arg mutation in 22 of 59  
353 CPA (37%) from patients with overt CS, but not in tumors from 40 patients with subclinical  
354 hypercortisolism (sCS), 20 APA, 20 non-functioning adenomas or 42 adrenocortical  
355 carcinomas. Interestingly, among patients with different forms of bilateral adrenal hyperplasia  
356 leading to CS, including bilateral micro- or macronodular hyperplasia, a germline copy-  
357 number gain (duplication) of the genomic region on chromosome 19 that includes *PRKACA*  
358 was identified in 5 patients from four kindreds. In one family with bilateral macronodular  
359 hyperplasia, the genetic defect was transmitted in an autosomal dominant manner from the  
360 mother to her affected child. The p.Leu206Arg mutation is located in the highly conserved  
361 core of the interaction between the regulatory (RII $\beta$ ) and catalytic (C $\alpha$ ) subunits of PKA. *In*  
362 *vitro* studies demonstrated gain-of-function of the PKA catalytic subunit mutants with  
363 impaired inhibition by the PKA regulatory subunit, and increased protein expression of the  
364 PKA catalytic subunit in cells from patients with germline chromosomal gains, with both  
365 mechanisms leading to increased PKA activity. Basal PKA activity in cells transfected with  
366 mutant C $\alpha$  subunits was increased and was not reduced by excess nonmutant RII $\beta$  subunit  
367 expression, nor was it responsive to cAMP analogues, indicating that the mutations made the  
368 catalytic subunit resistant to the physiological suppression. The presence of *PRKACA*  
369 mutations was associated with a more severe phenotype in patients with overt CS and  
370 *PRKACA* mutation carriers had significantly increased expression of *MC2R*, *StAR*, *CYP21A1*,  
371 *HSD3B2* and *CYP11A1* mRNA in adrenal tumors. These results were confirmed in the three  
372 other studies. By performing WES in 25 tumor samples producing excess cortisol, including  
373 22 CPA and 3 ACC, Goh et al identified six cases carrying the recurrent p.Leu206Arg  
374 substitution (76). Six cases had gain-of-function mutations in *CTNNB1* and two cases in  
375 *GNAS* (coding for G $\alpha$ s), with *PRKACA*, *GNAS* and *CTNNB1* mutations being mutually

376 exclusive. Additional targeted sequencing in 38 patients identified the p.Leu206Arg  
377 substitution collectively in 13 out of 63 tumors representing 24% of all ACA and 35% of all  
378 ACA associated with overt CS. In this study, adenomas carrying *PRKACA* or *GNAS*  
379 mutations were significantly smaller than those without these mutations, and the patients were  
380 of younger age at diagnosis with overt CS. WES also revealed a group of tumors harboring  
381 many somatic copy number variants with frequent deletion of *CDC42* and *CDKN2A*,  
382 amplification of 5q31.2 and protein-altering mutations in *TP53* and *RBI*. This mutational  
383 spectrum is more similar to that of malignant tumors, indicating that they may have greater  
384 malignant potential or represent early steps towards the development of ACC.

385 A higher rate of somatic *PRKACA* mutations seems to occur in subjects with an Asian  
386 ancestry. Cao et al (77) reported the occurrence of the p.Leu205Arg mutation (equivalent to  
387 the p.Leu206Arg mutation in the other reports) in 65.5% of a total of 87 CPA from China  
388 screened by WES and targeted sequencing. In this study, there was no significant correlation  
389 between the mutation status and clinical or biological parameters. The authors also identified  
390 one somatic *PRKARIA* mutation, one *CTNNB1* mutation, a truncating *APC* mutation and two  
391 activating *GNAS* mutations in CPA; an additional *CTNNB1* mutation was found in an adrenal  
392 oncocytoma (a very rare form of cortisol producing adrenal tumor). The authors also  
393 identified the recurrently mutated gene *DOTIL* coding for a histone H3 methyltransferase as  
394 well as one *HDAC9* mutation in ACTH-independent macronodular adrenocortical hyperplasia  
395 (AIMAH), suggesting involvement of chromatin deregulation, and *CLASP2* mutations in 2 of  
396 three adrenocortical oncocytomas. RNA-sequencing data comparing *PRKACA* mutated and  
397 non-mutated tumors identified 232 differentially expressed genes, with significant enrichment  
398 of gene ontology terms “biosynthesis and metabolism of steroid and cholesterol” and  
399 “response to chemical stimulus”, with increased expression of the genes *StAR*, *MC2R*,  
400 *GSTAI*, *CXCL2*, and *S100A8/9*.

401 Finally, in a Japanese cohort, 50% of cases (4/8) with adrenocortical adenomas associated  
402 with corticotropin-independent Cushing's syndrome were carriers of the *PRKACA*  
403 p.Leu206Arg mutation (78). An additional patient carried a recurrent p.Arg201Cys *GNAS*  
404 mutation. Follow-up sequencing in an additional 57 cases identified the *PRKACA*  
405 p.Leu206Arg mutation in 30 cases and *GNAS* mutation in 10 cases, representing a total of  
406 52.3% and 16.9% of mutational events and which were mutually exclusive. These mutations  
407 were found in 76% of patients with overt CS; interestingly, two patients with sCS were also  
408 carriers of a *PRKACA* and a *GNAS* mutation. In this study, carriers of the recurrent *PRKACA*  
409 p.Leu206Arg mutation, but also those with *GNAS* mutations had higher cortisol levels after an  
410 overnight dexamethasone suppression test and smaller adenoma size, suggesting a higher  
411 cortisol output compared to non-mutated tumors.

412 After these seminal works, a similar prevalence of *PRKACA* mutations was found in different  
413 cohorts of CS patients from different countries (Table 3). The largest study published to date  
414 was performed within the ENS@T and included 149 samples from nine different European  
415 centers, among which were 64 CPA from patients with overt CS, 36 CPA samples from  
416 patients with sCS, 32 nonsecreting adenomas, 4 androgen producing tumors, 5 ACC and 8  
417 samples from PBMAH. Heterozygous somatic *PRKACA* mutations were found in 22/64  
418 samples from patients with overt CS (34%). 18 patients carried the recurrent p.Leu206Arg  
419 mutation, while a new mutation p.Cys200\_Gly201insVal was identified in three patients and a  
420 p.Ser213Arg+p.Leu212\_Lys214insIle-Ile-Leu-Arg mutation was identified in one patient.

421 These newly identified mutations involved a region implicated in the interaction between the  
422 PKA regulatory and catalytic subunits. As in previous studies, patients with *PRKACA*  
423 mutations showed more severe cortisol abnormalities (higher levels of cortisol after an  
424 overnight dexamethasone suppression test) and smaller adenoma size compared with non-  
425 carriers (75,76). In a study exploring 60 patients with overt (n=36) or subclinical (n=24) CS,

426 Thiel et al (79) identified the recurrent somatic *PRKACA* p.Leu206Arg mutation in 23.1% of  
427 tumors, somatic *CTNNB1* mutations (p.Ser45Pro, p.Ser45Phe) in 23.1%, the recurrent *GNAS*  
428 p.Arg201Cys mutation in 5.8%, and a double mutation of *CTNNB1* and *GNAS* in one case.  
429 *PRKACA* and *GNAS* mutations were again mutually exclusive. In one patient with unilateral  
430 hyperplasia, a somatic *PRKACA* p.Leu206Arg mutation was identified, while patients with  
431 bilateral adenomas did not have known somatic mutations. The presence of *PRKACA*  
432 mutations was associated with younger age, overt Cushing's syndrome, and higher cortisol  
433 levels after an overnight dexamethasone suppression test compared with non-*PRKACA*-  
434 mutant or *CTNNB1*-mutant lesions (79). Lastly, targeted genetic screening for *GNAS*,  
435 *PRKACA*, and *KCNJ5* mutations in Japanese patients, 15 with CS, 9 with sCS, and 33 with  
436 an APA, found mutations in the *PRKACA* gene in 4 (26%) patients with CS, while mutations  
437 in the *GNAS* gene were detected in 2 (13%). A mutation in the *GNAS* gene was detected in 1  
438 patient with sCS (80,81).

439 Altogether, more than 580 patients with CPA or hyperplasia have been investigated. Somatic  
440 *PRKACA* mutations were found in 28%, *CTNNB1* mutations in 4%, and *GNAS* mutations in  
441 4% of cases (Table 3). *PRKACA* mutations were associated with overt CS, more severe  
442 cortisol abnormalities and smaller tumors. The question as to whether other recurrent  
443 mutations or genetic abnormalities are found in CPA has been addressed by Ronchi et al, who  
444 performed WES in 99 patients with ACA, including 74 CPA and 25 non-functioning  
445 adenomas negative for *PRKACA* mutations (82). Different mutations affecting genes of the  
446 cAMP/PKA and Wnt/ $\beta$ -catenin signalling pathway were identified (Figure 1B and 1C), the  
447 latter are more frequently associated with larger tumors and endocrine inactivity. Genetic  
448 variants were also identified in genes belonging to the calcium signalling pathway. To what  
449 extent these single variants are causative for cortisol production and/or adenoma formation  
450 remains to be established.

451

## 452 **6. Genetics of aldosterone and cortisol co-secretion**

453 Co-secretion of aldosterone and cortisol has been described in several reports and biochemical  
454 abnormalities of cortisol excess in PA have been associated with worse cardiovascular  
455 outcomes compared to APA without cortisol secretion (83). Recent studies suggest that  
456 cortisol excess may be more frequent in patients with APA than previously thought.  
457 Histological examination of adrenals with APA has consistently shown co-expression of  
458 CYP11B2 and CYP11B1 in a number of cases (84).

459 A Japanese study explored somatic *GNAS*, *PRKACA*, and *KCNJ5* mutations in 15 patients  
460 with CS, 9 patients with sCS, and 33 patients with APA (80,81) (Table 4). From 33 APA  
461 tested, 10 had autonomous cortisol secretion based on an overnight dexamethasone  
462 suppression test, increased midnight cortisol and/or decreased plasma ACTH levels.

463 Mutations in the *PRKACA* gene were found in 4 (26%) patients with CS, while mutations in  
464 the *GNAS* gene were detected in 2 (13%). Mutations in the *GNAS* gene were also detected in 1  
465 patient (11%) with sCS. Twenty-four patients with APA (72%) harboured *KCNJ5* mutations.

466 Among APA patients with autonomous cortisol secretion, six carried a somatic *KCNJ5*  
467 mutation, while two had a somatic *GNAS* mutation. *KCNJ5* and *GNAS* mutations in APA  
468 were mutually exclusive. No *PRKACA* mutations were identified in patients with APA (80).

469 In another study, the same authors identified a *KCNJ5* mutation in 2 out of 3 patients with  
470 APA and sCS (85).

471 In a study exploring 60 patients with cortisol excess, Thiel et al identified 4 cases with  
472 aldosterone co-secretion, 2 with overt and 2 with subclinical CS. Among those, 2 were  
473 carriers of mutations in *KCNJ5* (79). Finally, Rhayem et al identified somatic *PRKACA*  
474 mutations in two patients diagnosed with PA, a newly identified p.His88Asp variant and the

475 recurrent p.Leu206Arg mutation (32). The patient with the recurrent p.Leu206Arg had  
476 biochemical CS, while the carrier of the p.His88Asp variant had normal cortisol levels.  
477 A distinct histopathological entity is the adrenal harboring two nodules each one secreting a  
478 different hormone. Fallo and coworkers have investigated the occurrence of concurrent PA  
479 and subclinical cortisol hypersecretion in a prospective series of 76 consecutive patients with  
480 PA (86). Three out of 76 patients failed to appropriately suppress cortisol after an overnight  
481 dexamethasone suppression test; one of them also showed suppressed ACTH levels and  
482 mildly elevated urinary cortisol excretion. The resected adrenal showed a 4-cm nodule  
483 expressing *CYP11B1* by *in-situ* hybridization, whereas *CYP11B2* expression was restricted to  
484 the peritumoral region composed of zona glomerulosa-like cells, suggesting the co-existence  
485 of a cortisol-producing adenoma and an aldosterone-producing hyperplasia in the same  
486 adrenal. Namba et al described a patient with lateralized PA and CS, in whom examination of  
487 the adrenal revealed two distinct nodules, one expressing CYP11B1 and carrying a  
488 p.Leu206Arg *PRKACA* mutation, while the other nodule expressed CYP11B2 and carried a  
489 somatic *KCNJ5* mutation (87).

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

494

## 495 **7. Common hits for APA and CPA**

### 496 **7.1. The role of the Wnt/ $\beta$ -catenin pathway in adrenal tumorigenesis**

497 The role of the Wnt/ $\beta$ -catenin pathway has emerged as crucial in adrenal development and  
498 disease.  $\beta$ -catenin, as well as ARMC5 (Armadillo repeat-containing protein 5, see below), are  
499 both members of the Armadillo repeat containing (ARMC) gene family. Armadillo repeats are

500 approximately 40 amino acid long tandemly repeated domains which usually fold together to  
501 form a single, rigid protein domain called the armadillo (ARM) domain.  $\beta$ -catenin is a key  
502 downstream component of the canonical Wnt signaling pathway. In the absence of Wnt  
503 ligand,  $\beta$ -catenin is in complex with AXIN1, AXIN2, APC, CSNK1A1 and GSK3B, which  
504 promotes its phosphorylation on N-terminal Ser and Thr residues, ubiquitination and  
505 subsequent degradation by the proteasome. In the presence of Wnt ligand, the complex is  
506 disrupted and  $\beta$ -catenin accumulates in the nucleus, where it acts as a coactivator for  
507 transcription factors of the TCF/LEF family, leading to transcriptional regulation of Wnt  
508 responsive genes.

509 Modulation of different components of the Wnt/ $\beta$ -catenin pathway in mice leads to adrenal  
510 abnormalities. In the absence of Wnt4, one of the main ligands of the Wnt/ $\beta$ -catenin pathway,  
511 mice die immediately after birth due to organ defects such as kidney dysfunction or poor  
512 pituitary gland development. Despite normal adrenal morphology, Wnt4 knockout animals  
513 produce less aldosterone due to decreased expression of *cyp11b2* and show reduced  
514 expression of other markers of ZG cells, suggesting a defect in ZG development in the  
515 absence of Wnt4 (88). As observed in mice, loss of function mutations of WNT4 in humans  
516 lead to dysgenesis of the kidneys and to female and male sex reversal; adrenal development  
517 and external genitalia are also affected in human fetuses (89). Along the same lines, the  
518 targeted disruption of  $\beta$ -catenin restricted to sf-1 expressing cells (adrenal cortex, somatic  
519 cells of the gonads, spleen, pituitary gonadotropes and ventromedial hypothalamic nucleus  
520 (90) leads to complete adrenal aplasia when  $\beta$ -catenin is totally absent during development  
521 and to a defect in the maintenance of adrenocortical tissue due to increased apoptosis in adult  
522 mice when the level of expressed  $\beta$ -catenin is low (91). In H295R cells, a human  
523 adrenocortical cell line with activating *CTNNB1* mutations, a decrease in  $\beta$ -catenin expression  
524 induces cell cycle arrest, changes in cell morphology, modification of the expression of



525 epithelial to mesenchymal transition markers and a modification of cell motility (92).

526 Whereas these results highlight the central role of  $\beta$ -catenin in adrenal development, tight  
527 regulation of its activation is also crucial. Mice carrying a heterozygous mutation of APC,  
528 leading to the formation of a truncated protein and constitutive  $\beta$ -catenin activation, exhibit  
529 multiple intestinal tumors. In addition these mice also develop hyperaldosteronism,  
530 hypervolemia and increased blood pressure, but their adrenal phenotype has not been explored  
531 (93,94). Mice expressing a constitutively active  $\beta$ -catenin in the adrenal cortex exhibit  
532 progressive adrenal hyperplasia and dysplasia leading, with time, to the formation of nodules  
533 in both cortex and medulla; abnormal vascularization was also observed, and in later stages in  
534 rare cases, development of adrenal carcinoma (95). In humans, activation of  $\beta$ -catenin,  
535 demonstrated by its cytoplasmic and/or nuclear accumulation, was found in a large number of  
536 adrenocortical tumors, including adrenocortical adenomas (71,96-98). Mutations in the  
537 *CTNNB1* gene, leading to constitutive activation of  $\beta$ -catenin were found in both malignant  
538 and benign tumors (Figure 1C) (96,97). In benign tumors, the presence of  $\beta$ -catenin mutations  
539 was found to be associated with larger adenomas and absence of hormonal secretion (97). In  
540 hormonally active ACA, the prevalence of *CTNNB1* mutations is estimated between 2 to 5%  
541 in APA (28,29,99) and  $\approx$ 4% in CPA (see previous sections). The presence of *CTNNB1*  
542 mutations in APA was not associated with a particular histological or morphological  
543 phenotype, but seems to be more prevalent in females (26,28,29,51). In addition, Teo and co-  
544 workers reported three women with PA, harboring *CTNNB1* mutations in their adenoma that  
545 were associated with aberrant expression of G-protein coupled receptors (GPCR), two during  
546 pregnancy and one after menopause (51), suggesting a possible acceleration of the disease  
547 during pregnancy and menopause due to high gonadotropin levels acting on aberrantly  
548 expressed gonadotropin receptors. Since *CTNNB1* mutations were also reported in non-  
549 pregnant women (28,29) and men, the relation between *CTNNB1* mutations and pregnancy

550 and the possible interaction with aberrant gonadotropin receptors remain to be further  
551 clarified (100,101).

552 The role of the Wnt  $\beta$ -catenin pathway in the development of APA is further supported by the  
553 case of a young patient who was diagnosed with severe arterial hypertension due to primary  
554 aldosteronism at age 26, followed by hemorrhagic stroke four years later, due to lateralized  
555 aldosterone secretion from an APA in the context of bilateral multinodular adrenal glands  
556 (69). The patient, in whom additional exploration identified asymptomatic Familial  
557 Adenomatous Polyposis (FAP) associated with a heterozygous germline *APC* mutation,  
558 showed biallelic *APC* inactivation due to loss of heterozygosity in two adrenal nodules. The  
559 aldosterone producing nodule carried an additional somatic *KCNJ5* mutation supporting a  
560 two-hit model for APA development (69). This patient also had incomplete cortisol  
561 suppression after an overnight dexamethasone suppression test, but normal 24-h urinary free  
562 cortisol, maintained circadian rhythm of cortisol secretion and normal ACTH levels;  
563 *CYP11B1* expression was detected in all adrenal nodules, including the one carrying the  
564 *KCNJ5* mutation (unpublished).

565 Remarkably, a link between the Wnt/ $\beta$ -catenin and the PKA pathway has recently been  
566 established, showing that activation of PKA prevents zona glomerulosa differentiation  
567 through WNT4 repression and Wnt pathway inhibition, suggesting that PKA activation in the  
568 zona fasciculata is a key driver of Wnt inhibition and lineage conversion. (102).

569

## 570 **7.2. ARMC5 mutations in adrenocortical tumors**

571 Evidence that ARMC5 is involved in adrenal tumorigenesis came from recent studies  
572 exploring PBMAH (also called ACTH-independent macronodular adrenal hyperplasia or  
573 AIMAH). PBMAH is a form of macronodular adrenal hyperplasia characterized by multiple,

574 bilateral, non-pigmented, benign, adrenocortical nodules. It results in excessive production of  
575 cortisol leading to ACTH-independent overt or subclinical Cushing syndrome (103).  
576 Germline *ARMC5* mutations were identified in familial cases and are present in 50% of  
577 apparently sporadic cases. Somatic events occur independently in adrenal nodules, suggesting  
578 that *ARMC5* acts as a tumor suppressor gene (103).

579 A recent study has shown that PBMAH is not truly ACTH-independent since  
580 proopiomelanocortin expression was described in clusters of steroidogenic cells in PBMAH  
581 tissue, suggesting local ACTH production with paracrine regulation of cortisol biosynthesis  
582 (104). The existence of heterogeneous phenotypes (105), such as that observed in a Brazilian  
583 family with adrenal macronodular disease showing bilateral and unilateral adrenal nodules, has  
584 highlighted the complexity of the condition, leading some authors to prefer the name primary  
585 macronodular adrenal hyperplasia (PMAH) (106). PBMAH is usually diagnosed after the  
586 investigation of an adrenal incidentaloma or by the presence of overt CS, although insidious or  
587 mild CS is more frequent (107,108). Aldosterone secretion was also described in patients with  
588 PBMAH (69,109,110). Previous studies have demonstrated that cortisol secretion in PBMAH  
589 may be regulated by various hormones via aberrant adrenal expression of several G-protein-  
590 coupled receptors (107,111,112). Illegitimate G-protein-coupled receptors expressed in  
591 PBMAH include the gastric inhibitory polypeptide (GIP) receptor, vasopressin receptors,  $\beta$ -  
592 adrenergic receptors, LH/hCG receptors, and the serotonin 5-HT<sub>4</sub> and 5-HT<sub>7</sub> receptors ((107)  
593 and references therein).

594 Although sporadic forms appear to be more frequent, the description of familial forms and the  
595 occurrence of bilateral adrenal lesions support a genetic cause for PBMAH. Recently, combined  
596 SNP array and microsatellite marker analysis have shown a somatic 16p11 loss of  
597 heterozygosity (LOH) in 8 of 33 tumor samples from patients with PBMAH. In parallel, WES  
598 analysis followed by direct sequencing of blood and tumor samples has identified somatic

599 mutations in *ARMC5*, the armadillo repeat containing 5 gene mapped to 16p11.2, in 18 of 33  
600 PBMAH patients (113). All patients carried a germline *ARMC5* mutation and a second somatic  
601 *ARMC5* event in the tumor DNA, including LOH, a second mutation or microdeletion,  
602 suggesting a 2-hit model for the development of PBMAH (113). Interestingly, distinct somatic  
603 *ARMC5* alterations are observed in different nodules from the same adrenal (113), exemplified  
604 by an emblematic case of 16 different somatic *ARMC5* mutations in 20 adrenal nodules from  
605 the same patient (114).

606 The function of *ARMC5* is unknown and no other diseases have been associated with mutations  
607 in this gene. *ARMC5* encodes a 935 amino acid protein of the armadillo repeat family containing  
608 an armadillo repeat domain in the N-terminal region and a BTB/POZ in the C-terminal region  
609 (115). Analysis of the expression of four *ARMC5* isoforms in 46 human tissues has shown that  
610 at least one *ARMC5* isoform is ubiquitously expressed throughout the body, but only seven  
611 tissues expressed all four isoforms, including the adrenal gland (116). *Armc5* knockout mice  
612 have defective development and immune responses, with adrenal hyperplasia in old animals  
613 (117). Transfection of wild-type *ARMC5* in H295R cells induces apoptosis, which is not  
614 observed when mutant *ARMC5* are transfected. Furthermore, silencing of *ARMC5* in H295R  
615 cells reduced the expression of the steroid enzymes *CYP17A1* and *CYP21A2*; *NR5A1* and  
616 *MC2R*, as well as cortisol production (113). PBMAH patients with *ARMC5* mutations show  
617 more overt CS than patients without *ARMC5* mutations (113,118). In a cohort of 98 unrelated  
618 PBMAH subjects, *ARMC5* mutations were found in 24 patients (26%). Patients with *ARMC5*  
619 mutations again showed more frequently overt CS and had larger adrenals with a higher number  
620 of nodules than non-mutated patients (119). These results were confirmed in an Italian cohort  
621 of PBMAH patients, in which *ARMC5* mutated patients exhibited higher cortisol levels, more  
622 severe hypertension and diabetes, and larger adrenal lesions (120). *ARMC5* mutations have also  
623 been identified in familial forms of PBMAH (105,121-123). In one Brazilian family with

624 PBMAH, a germline *ARMC5* mutation was found which segregated with the disease, although  
625 the clinical phenotype and adrenal involvement (unilateral or bilateral) were variable among  
626 carriers (105). In five families with PBMAH, Gagliardi et al have found germline *ARMC5*  
627 mutations in four families with CS and no *ARMC5* mutation in one family with PA and sCS  
628 (121). Interestingly, in a large French-Canadian PBMAH family, *ARMC5* mutations were found  
629 in affected subjects together with aberrant expression of  $\beta$ -adrenergic and V1-vasopressin  
630 receptors in adrenal nodules (123). In this case, the aberrant expression of GPCR may represent  
631 a secondary event in the development of PBMAH, but the link between *ARMC5* mutations and  
632 this pattern of receptor expression is not yet clarified.

633 Zilbermint et al have sequenced *ARMC5* in germline DNA from 56 subjects with PA and have  
634 identified 12 heterozygous variants, 9 missense variants and three variants leading to alternative  
635 splicing (124). *In silico* analysis of the *ARMC5* variants has shown that six variants were  
636 predicted to be damaging, including the *ARMC5* variant p.R898W, which was previously  
637 described in one patient with PBMAH (118), and a novel missense mutation *ARMC5* p.P826H  
638 (124). All carriers of these variants were African-American, two had bilateral macronodular  
639 hyperplasia, and tumor DNA sequencing showed neither additional somatic *ARMC5* mutations  
640 nor *ARMC5* loss of heterozygosity. APA with predicted damaging *ARMC5* variants exhibit  
641 lower *ARMC5* expression than the adjacent adrenal cortex. The functional consequences of  
642 these *ARMC5* variants are not completely understood. Silencing of *ARMC5* in H295R cells  
643 leads to decreased *CYP11B2* expression, and therefore a reduction of the aldosterone-secretory  
644 capacity. The authors suggest that the higher aldosterone secretion in PA patients carrying  
645 *ARMC5* variants may be explained by the increased adrenocortical mass, similar to what was  
646 observed in patients with PBMAH and hypercortisolism (124). An association of *ARMC5*  
647 mutations with PA was not replicated in an Italian study assessing the presence of *ARMC5*  
648 mutations in 39 PA patients with bilateral computed tomography-detectable adrenal alterations,

649 from which 8 had unilateral and 27 bilateral aldosterone secretion (125). Different *ARMC5*  
650 variants were identified in 18 subjects, among them 11 common variants, two rare ones and two  
651 previously unreported variants were found, but *in silico* analysis did not identify possibly  
652 damaging *ARMC5* variants (125). These contradictory data do not allow confirmation of the  
653 role of *ARMC5* mutations in PA and further studies are required to better understand the role of  
654 *ARMC5* in the development of adrenal nodules and aldosterone excess.

655

### 656 **7.3. Germline mutations associated with ACA and macronodular hyperplasia**

657 Genetic alterations leading to multiple tumor syndromes and mutations affecting genes of the  
658 cAMP/PKA signaling pathway have been associated with PBMAH (review in (106,108)).  
659 Familial adenomatous polyposis (FAP) is a disease characterized by multiple colonic polyps  
660 with an increased risk of colon carcinomas, due to an inactivating germline mutation of *APC*  
661 and a somatic second hit (mutation or LOH), resulting in constitutive activation of the Wnt/ $\beta$ -  
662 catenin pathway (126). Adrenal masses are more frequently found in patients with FAP than in  
663 the general population (127), including non-functioning adrenal adenomas, adrenocortical  
664 cancers, and PBMAH (128,129). Multiple endocrine neoplasia type 1 (MEN1) is an autosomal  
665 dominant syndrome resulting from inactivating mutations in the *MEN1* gene, which is  
666 characterized by the development of multiple endocrine and non-endocrine tumors, most  
667 frequently hyperparathyroidism, pituitary adenomas, and pancreatic neuroendocrine tumors  
668 (130). MEN1 encodes the protein menin which has a role in the regulation of cell cycle, cell  
669 growth and proliferation (131). The analysis of adrenal involvement in 715 patients with MEN1  
670 has shown adrenal tumors (>10mm in size) in 10% of the cohort; in 19.4% of these cases the  
671 tumors were bilateral, and in 12.5% of the cases the tumors were >40mm in size (132). A *MEN1*  
672 mutation was also identified in one patient with PBMAH and hyperparathyroidism, without a  
673 family history or other endocrine abnormalities (129). In addition, LOH at the MEN1 locus was

674 also reported in APA (133). Another hereditary disease associated with PBMAH is the  
675 hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC), a disease caused by  
676 germline mutations in the fumarate hydratase (FH) gene (134). Adrenal lesions are found in  
677 7.8% of patients with HLRCC, including bilateral lesions associated with ACTH-independent  
678 hypercortisolism (135), and germline FH mutations were identified in patients with PBMAH  
679 associated with HLRCC (129,134). Genetic alterations leading to activation of cAMP/PKA  
680 signaling were also described in patients with PBMAH. Post-zygotic somatic mutations in the  
681 gene encoding the alpha subunit of stimulatory G protein (*GNAS*), leading to constitutive  
682 activation of adenylate cyclase, result in McCune Albright syndrome, which is characterized  
683 by a triad constituted by polyostotic fibrous dysplasia, cafe' au lait skin spots, and gonadotropin-  
684 independent precocious puberty, associated with other endocrine and non-endocrine diseases,  
685 including CS (136). Somatic *GNAS* mutations were described in a few cases of PBMAH  
686 without other typical signs of McCune Albright syndrome and might represent variants of this  
687 syndrome (129,137). In isolated cases, variants in *PDE11A*, encoding the isoform 11A of the  
688 phosphodiesterase enzyme, and in *MC2R* were associated with PBMAH (138-140). Finally,  
689 duplication of a genomic region of chromosome 19 including *PRKACA* was also identified in  
690 patients with PBMAH (75,141).

691

## 692 **8. Concluding remarks**

693 Over the past few years, extraordinary progress has been made in our understanding of the  
694 genetic abnormalities leading to APA and CPA. While genetic testing in inherited forms of  
695 the diseases has been translated into clinical practice, and family screening is useful for  
696 management of affected family members, the clinical utility of diagnosing somatic mutations  
697 is difficult to anticipate from available studies on genotype-phenotype correlations and  
698 outcome. Ideally, identification of mutations prior to surgery, through the application of

699 surrogate biomarkers or genetic testing on circulating cell-free DNA, could guide treatments  
700 to specifically target mutated proteins, as shown for APA (142,143). There seems to be an  
701 overlap of genetic abnormalities leading to different endocrine abnormalities in ACA. In  
702 particular, *CTNNB1* mutations are found at relatively high frequency (at least comparable to  
703 what observed for *ATP1A1* or *ATP2B3* mutations in APA) in CPA but also usually in APA.  
704 Furthermore, genetic variants affecting genes of the calcium signaling pathway are enriched  
705 in WES data from CPA, suggesting that calcium, in addition to PKA signaling, may lead to  
706 autonomous cortisol overproduction. This is not surprising, as a subset of APA show some  
707 cortisol co-secretion, or biochemical glucocorticoid abnormalities (144), and in some cases  
708 those tumors harbor somatic *KCNJ5* mutations. In addition, overexpression of mutated  
709 *GIRK4* channels in HAC15 cells not only increases *CYP11B2* expression but also *CYP11B1*  
710 (33). Future studies need to address the sequence of events leading to adrenal cell  
711 proliferation, nodule formation and specification of the hormonal secretory pattern. It will be  
712 of particular interest to explore whether common susceptibility alleles or rare variant may  
713 predispose to the development of APA and CPA.

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717 **References**

718

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1326 **Figure legends**

1327

1328 **Figure 1. Specific and common mechanism of aldosterone and cortisol producing**

1329 **adenoma development. A. Specific mechanisms of APA formation.** In absence of

1330 stimulation (AngII or K<sup>+</sup>), ZG cells are hyperpolarized due to the presence of a large number

1331 of K<sup>+</sup> channels. In response to an increase of extracellular K<sup>+</sup> concentration or to the binding

1332 of AngII to its receptor (AT1R), inhibition of K<sup>+</sup> channels leads to cell membrane

1333 depolarization, opening of voltage gated Ca<sup>2+</sup> channels, increased intracellular calcium

1334 concentrations and activation of Ca<sup>2+</sup> signaling, the major trigger for aldosterone biosynthesis.

1335 Moreover, binding of AngII to AT1R leads to the inhibition of the Na<sup>+</sup>/K<sup>+</sup>-ATPase, increasing

1336 cell depolarization, whereas signaling through Gαq promotes the release of Ca<sup>2+</sup> from the

1337 endoplasmatic reticulum through binding of IP3 to its receptor, again stimulating aldosterone

1338 biosynthesis. Mutations in *KCNJ5*, encoding the GIRK4 potassium channel, induce changes

1339 in channel selectivity, allowing Na<sup>+</sup> entry into the cell instead of K<sup>+</sup> efflux, resulting in cell

1340 depolarization, opening of voltage gated Ca<sup>2+</sup> channel, stimulation of Ca<sup>2+</sup> signaling and

1341 increase in aldosterone production. Mutations in *ATP1A1*, coding for the α1 subunit of the

1342 Na<sup>+</sup>/K<sup>+</sup>-ATPase, lead to increased aldosterone production by increasing intracellular Na<sup>+</sup> and

1343 H<sup>+</sup> concentration, resulting in cell membrane depolarization and decrease of intracellular pH.

1344 Mutations in *CACNA1D*, encoding the Cav1.3 calcium channel, and *ATP2B3*, coding for the

1345 Ca<sup>2+</sup> pump PMCA3 directly affect intracellular Ca<sup>2+</sup> concentrations and thus aldosterone

1346 biosynthesis. **B. Specific mechanisms of CPA formation.** In absence of stimulation, the

1347 catalytic and regulatory subunits of the PKA form an inactive complex. Binding of ACTH to

1348 its receptor, the melanocortin 2 receptor (MC2R), leads to activation of adenylate cyclase

1349 resulting in an increase of cAMP. cAMP binds to the regulatory subunits of the PKA

1350 complex, inducing conformational changes and release of the catalytic subunits from the

1351 complex. Active PKA phosphorylates specific target proteins such as the transcription factor

1352 CREB that binds to the promoter regions of specific target genes, ie CYP11B1 leading to  
1353 increased cortisol biosynthesis. In cells, the cAMP is converted into AMP through the action  
1354 of specific PDE. Mutations in *PRKACA* or in *GNAS* lead to the constitutive activation of  
1355 PKA, phosphorylation of CREB and increased expression of CYP11B1 and autonomous  
1356 cortisol biosynthesis. **C. Common mechanisms of APA and CPA formation.** In  
1357 unstimulated cells,  $\beta$ -catenin is phosphorylated, ubiquitinated by a protein complex formed by  
1358 APC, GSK3, Axin and CK1 $\alpha$  and then degraded by the proteasome. Binding of Wnt ligand to  
1359 Frizzled receptor and LRP5/6 co-receptor leads to the dissociation of the  $\beta$ -catenin  
1360 degradation complex.  $\beta$ -catenin can translocate into the nucleus where it acts as a  
1361 transcriptional coactivator for transcription factors of the TCF/LEF family inducing the  
1362 expression of specific target genes. In APA as well as in CPA, *CTNNB1* mutations affecting  
1363 specific residues of  $\beta$ -catenin lead to constitutive activation of the protein. In APA, mutations  
1364 in APC also lead to constitutive  $\beta$ -catenin activation.

1365

1366 **Figure 2. The two current models for APA development. Left panel: Two hit model of**  
1367 **APA development.** In ZG cells, activation of specific signaling pathways, such as  $\beta$ -catenin,  
1368 lead to abnormal cell proliferation. Abnormal cell proliferation creates a propitious  
1369 environment for occurrence of recurrent somatic mutations specifying the pattern of hormonal  
1370 secretion. **Right panel: APCC hypothesis.** In ZG cells, occurrence of somatic mutations in a  
1371 certain number of APA driver genes, lead to the formation of aldosterone producing cell  
1372 clusters (APCC) with autonomous aldosterone production. In a certain number of cases these  
1373 structures would evolve towards an aldosterone producing adenoma a possible APCC-to-APA  
1374 transitional lesion (pAATL).

1375

1376

**Table 1. Clinical and molecular characteristics of patients with APA.**

<b>Total n of patients screened*</b>	<b><i>KCNJ5</i> N (%)</b>	<b><i>CACNA1D</i> N (%)</b>	<b><i>ATP1A1</i> N (%)</b>	<b><i>ATP2B3</i> N (%)</b>	<b><i>CTNNB1</i> N (%)</b>	<b>Other</b>	<b>Correlations of mutation status</b>	<b>Reference</b>
22	8 (36%)	NA	NA	NA	NA	NA	7/8 patients with <i>KCNJ5</i> mutations were female	(24)
380	129 (34%)	NA	NA	NA	NA	NA	<i>KCNJ5</i> : Higher prevalence in females and younger patients; higher plasma aldosterone at diagnosis; no specific pangenomic transcriptomic profile; no association with pre-operative blood pressure, serum potassium and adenoma size or cure after adrenalectomy  Lower relative GIRK4 protein expression in <i>KCNJ5</i> mutated tumors	(145,146)
348	180 (45%)	NA	NA	NA	NA	NA	<i>KCNJ5</i> : Higher prevalence in females, males younger at diagnosis and with larger tumors; no correlation with <i>KCNJ5</i> protein expression	(147)
23	15 (62.5%)	NA	NA	NA	NA	NA	<i>KCNJ5</i> : higher <i>KCNJ5</i> mRNA expression compared to non-mutated tumors	(40)
73	30 (41%)	NA	NA	NA	NA	NA	<i>KCNJ5</i> : larger APA, predominant ZF-like cells, younger patients, higher <i>CYP17A1</i> expression;	(148,149)
<b>91</b>	29 (32%)	NA	NA	NA	NA	NA	<i>KCNJ5</i> : Higher aldosterone levels, lower plasma renin activity, higher ARR higher lateralization index; more frequent contralateral suppression Higher <i>CYP11B2</i> mRNA expression	(48)

308	118 (38.3)	NA	16 (5.2%)	5 (1.6%)	NA	NA	<i>ATP1A1</i> and <i>ATP2B3</i> : more frequent in males, higher preoperative aldosterone concentrations, lower serum potassium concentrations	(27)
152	20 (13.2%)	12 (7.9%)	12 (7.9%)	0/10 exomes	1/10 exomes	NA	<i>ATP1A1</i> and <i>CACNAID</i> : more frequent in older males; smaller APAs with more compact, zona glomerulosa-like cells;  <i>KCNJ5</i> : more frequent ZF-like cells, higher <i>CYP11B1</i> mRNA expression:  No difference in <i>ATP1A1</i> and <i>CACNAID</i> expression among genotypes	(25)
64	21 (32.8%)	5 (7.8%)	1 (1.6%)	2 (3.1%)	2 (3.1%)	NA	<i>CACNAID</i> : smaller APA, trend towards older age	(26)
<b>59</b>	19 (32%)	NA	5 (8.5%)	3 (5%)	NA	NA	No impact of mutation status on lateralization index and contralateral suppression index	(49)
474	180 (38%)	44 (9.3%)	25 (5.3%)	8 (1.7%)	NA	NA	<i>KCNJ5</i> : Higher prevalence in females and younger patients; higher minimal plasma potassium at diagnosis;  <i>CACNAID</i> : smaller adenomas**  All mutations: no association with pre-operative blood pressure, serum potassium and adenoma size or cure after adrenalectomy; no correlation with cellular composition nor <i>CYP11B2</i> or <i>CYP11B2</i> expression; no correlation with <i>KCNJ5</i> , <i>CACNAID</i> , and <i>ATP1A1</i> mRNA expression	(30)
112****	44 (39.3%)	NA	7 (6.3%)	1 (0.9%)	NA	NA	<i>ATP1A1</i> , <i>ATP2B3</i> : higher <i>CYP11B2</i> mRNA expression compared to <i>KCNJ5</i> mutated APA	(150)

108	75 (69.4%)	2 (1.9)	3 (2.8)	NA	NA	<i>KCNJ5</i> : younger patients, higher plasma and urine aldosterone levels; significant improvement of left ventricular mass index after surgery; lower post-op SBP and aldosterone levels	
195	48 (24.6%)	NA	NA	NA	NA	<i>Not investigated</i>	(151)
165	90 (54.5%)	5 (3.0%)	10 (6.1%)	5 (3.0%)	NA	<i>KCNJ5</i> : Higher prevalence in females and younger patients; <i>CACNA1D</i> , <i>ATP1A1</i> and <i>ATP2B3</i> : older at diagnosis, more often males with smaller tumors; higher <i>CYP11B2</i> and <i>NPNT</i> mRNA expression compared to APA with <i>KCNJ5</i> mutation; All mutations: No correlation with <i>ATP1A1</i> , <i>ATP2B3</i> or <i>KCNJ5</i> mRNA expression	(50)
69	26 (37.7%)	NA	NA	NA	NA	<i>KCNJ5</i> : Lower relative GIRK4 protein expression in <i>KCNJ5</i> mutated APA; Higher prevalence in females and younger patients; larger tumors and lower plasma potassium	(34)
168	129 (76.8%)	1 (0.6%)	4 (2.4%)	1 (0.6%)	NA	<i>KCNJ5</i> : Higher prevalence in females, larger tumors, higher aldosterone levels and lower minimal serum potassium, higher lateralization index, lower plasma renin activity, higher ARR	(42)
71***	27 (38%)	3 (4.2%)	5 (7%)	1 (1.4%)	NA	<i>KCNJ5</i> : more frequently zona fasciculata-like cells, high <i>CYP11B1</i> protein expression; <i>ATP1A1</i> , <i>ATP2B3</i> and <i>CACNA1D</i> : more frequently ZG-like cells with high <i>CYP11B2</i> protein expression	(152)

90	36 (37.1%)	10 (10.3%)	8 (8.2%)	3 (3.1%)	2 (2.1%)	NA	<i>KCNJ5</i> : Higher prevalence in females, larger tumors, lower pre-contrast Hounsfield units on CT; more frequently ZF-like cells  <i>ATP1A1</i> , <i>ATP2B3</i> and <i>CACNA1D</i> : more frequent in males, heterogenous cell composition	(28)
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 glomerular filtration rate, lower serum CRP levels, greater chance of recovery from hypertension post-adrenalectomy	(44)
114	86 (75.4%)	1 (0.09%)	0	0	NA	NA	Mutation carriers: younger, lower preoperative serum potassium level, higher LV mass index and significant improvement after surgery; higher mRNA expression of <i>KCNJ5</i> , <i>CYP11B2</i> , and <i>ATP2B3</i>  male (but not female) mutation carriers: higher preoperative plasma aldosterone and blood pressure;	(43)
198	92 (46.5%)	3 (1.5%)	6 (3.0%)	3 (1.5%)	10 (5.1%)	NA	<i>CTNNB1</i> : more frequent in females; larger tumors compared to APA without mutation; Higher <i>CYP11B2</i> mRNA expression compared to APA with <i>KCNJ5</i> mutation;	(29)
159	117 (73.6%)	4 (2.5%)	1 (0.6%)	4 (2.5%)	NA	NA	<i>ATPase</i> : tendency to smaller tumors, mainly ZG-like cells  <i>CACNA1D</i> : tendency to smaller tumors, predominant ZF-like cells  <i>KCNJ5</i> : not analyzed in this report	(45)

66	47 (71.2%)	0	0	0	NA	NA	<i>KCNJ5</i> : Higher prevalence in females and younger patients, no association with pre-operative blood pressure, plasma aldosterone, serum potassium, lateralization index, and adenoma size; better cure following adrenalectomy	(46)
219	116 (52.9%)	0	3 (1.4%)	1 (0.5%)	8 (3.7%)		<i>CTNNB1</i> : more frequent in females, older patients, heterogeneous staining of $\beta$ -catenin and variable expression of gonadal receptors and both <i>CYP11B1</i> and <i>CYP11B2</i>	(99)

\*by WES and/or targeted sequencing; \*\*frequency of mutations and associations dependent on center; \*\*\*patients included in (30) ;\*\*\*\* included 32 samples from (27). ZF, zona fasciculata; ZG, zona glomerulosa.



**Table 2. List of the different mutations identified in APA driver genes.**

<b>Gene</b>	<b>Mutations</b>	<b>References first description</b>
<i>KCNJ5</i>	p.Gly151Arg, p.Leu168Arg, p.Thr158Ala	(24)
	p.Gly151Glu	(62)
	p.Ile157del	(153)
	p.Tyr152Cys	(154)
	p.Trp126Arg	(150)
	p.Glu145Gln, p.Arg115Trp, p.Glu246Gly	(34)
	p.Phe154Cys, p.Ile150_Gly151del_InsMet,	(28)
	p.Ser143_ile144del_InsAla	
	p.Ala139_Phe142dup	(155)
<i>ATPIA1</i>	p.Leu104Arg, p.Val332Gly, p.Phe100_Leu104del	(27)
	p.Glu960_Alala963del	(25)
	p.Gly99Arg	(150)
	p.Met102_Leu103del	(42)

	p.Met102_Ile106del, p.leu103_Leu104del, p.Phe959_Glu961del,	(50)
	p.Glu960_Leu694del, p.Phe956_Glu961del	
<b>ATP2B3</b>	p.Leu425_426del, p.Val426_427del	(27)
	p.Val424_Leu425del	(30)
	p.Ala428_Val429del	(156)
	p.Val426_Val429del	(50)
	p.Val422_Val426del_InsSerThrLeu	(42)
	p.Ala428_Leu433del_InsGlyGln	(28)
<b>CACNAID</b>	p.Val259Asp, p.Gly403Argp.Phe747Leu, p.Ile750Met,	(25)
	p.Arg990His	
	p.Gly403Arg, p.Ile750Met, p.Phe767Val, p.Val1373Met	(26)
	p.Ser652Leu, p.Leu655Pro, p.Tyr741Cys, p.Val949Asp,	(30)
	p.Lys981Asnp.Ala998Ile, p.Ala998Valp.Val1151Phe,	
	p.Ile1152Asnp.Val1338Met	
	p.Val401Leu	(50)
	p.Phe747Val	(68)

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**Table 3. Clinical and genetic characteristics of patients with CPA.**

<b>Total n of patients screened*</b>	<b>Clinical phenotype</b>	<b><i>PRKACA</i> N (phenotype)</b>	<b><i>GNAS</i> N (phenotype)</b>	<b><i>CTNNB1</i> N (phenotype)</b>	<b><i>APC</i> N (phenotype)</b>	<b>Reference</b>
139	59 CS, 40 sCS, 20 APA, 20 NSA	22 (CS)	0 in WES	0 in WES	0 in WES	(75)
63 (55 CPA + 8 ACC)	36 CS, 27 sCS	13 (10 CS, 3 sCS)	3 CS	9 (3 CS, 6 sCS)	0 in WES	(76)
87	87 (CS)	57 (CS)	2 (CS)	1 (CS)	1 (CS)	(77)
64	55 CS, 9 sCS	34 (33 CS, 1 sCS)	11 (10 CS, 1 sCS)	0 in WES	0 in WES	(78)
144	64 CS, 36 sCS, 32 NSA, 4 androgen producing tumors, 8 PBMAH	22 (CS)	ND	ND	ND	(157)
60	36 CS, 22 sCS	12 (11 CS, 1 sCS)	4 (3 CS, 1 sCS <sup>§</sup> )	13 (5 CS, 8 sCS <sup>§</sup> )	ND	(79)
57	15 CS, 9 sCS, 33 APA	4 CS	5 (2 CS, 1 sCS, 2 APA+sCS)	ND	ND	(81,158)

\*by whole exome sequencing and/or targeted sequencing, excluding ACC; CS, overt Cushing syndrome; sCS, subclinical Cushing syndrome; APA, aldosterone producing adenoma; NSA, non-secreting adenoma; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; <sup>§</sup>including one patient with a double *CTNNB1* + *GNAS* mutation; ND, not determined.

**Table 4. Clinical and genetic characteristics of patients with A/CPA**

<b>Characteristics</b>	<b>(81)</b>	<b>(85)</b>	<b>(79)</b>	<b>(32)</b>	<b>(69)</b>
<b>Total n of APA patients screened</b>	33	unknown	4	122	1
<b>N of patients with cortisol co-secretion (F:M)</b>	10 (x:x)	3 (3:0)	4	?	1 (0:1)
<b>Clinical phenotype</b>	PA/sCS	PA/sCS	PA/2CS-2sCS	1 case with PA/sCS, 1 with PA only	PA/sCS
<b><i>KCNJ5</i> mutations</b>	6	2	2	0	1 (somatic)
<b><i>GNAS</i> mutations</b>	2	Not described	0	Not described	Not described
<b><i>PRKACA</i> mutations</b>	0*	Not described	0	2	Not described
<b><i>CTNNB1</i> mutations</b>	Not described	Not described	0	Not described	0
<b><i>APC</i> mutations</b>	Not described	Not described	Not described	Not described	1 (germline)

\*in another paper, the same authors report about the analysis of 33 APA, 11 of them with sCS, with none of them carrying a PRKACA mutation (80). It is unclear, what the overlap is between these patients and those of this reference.

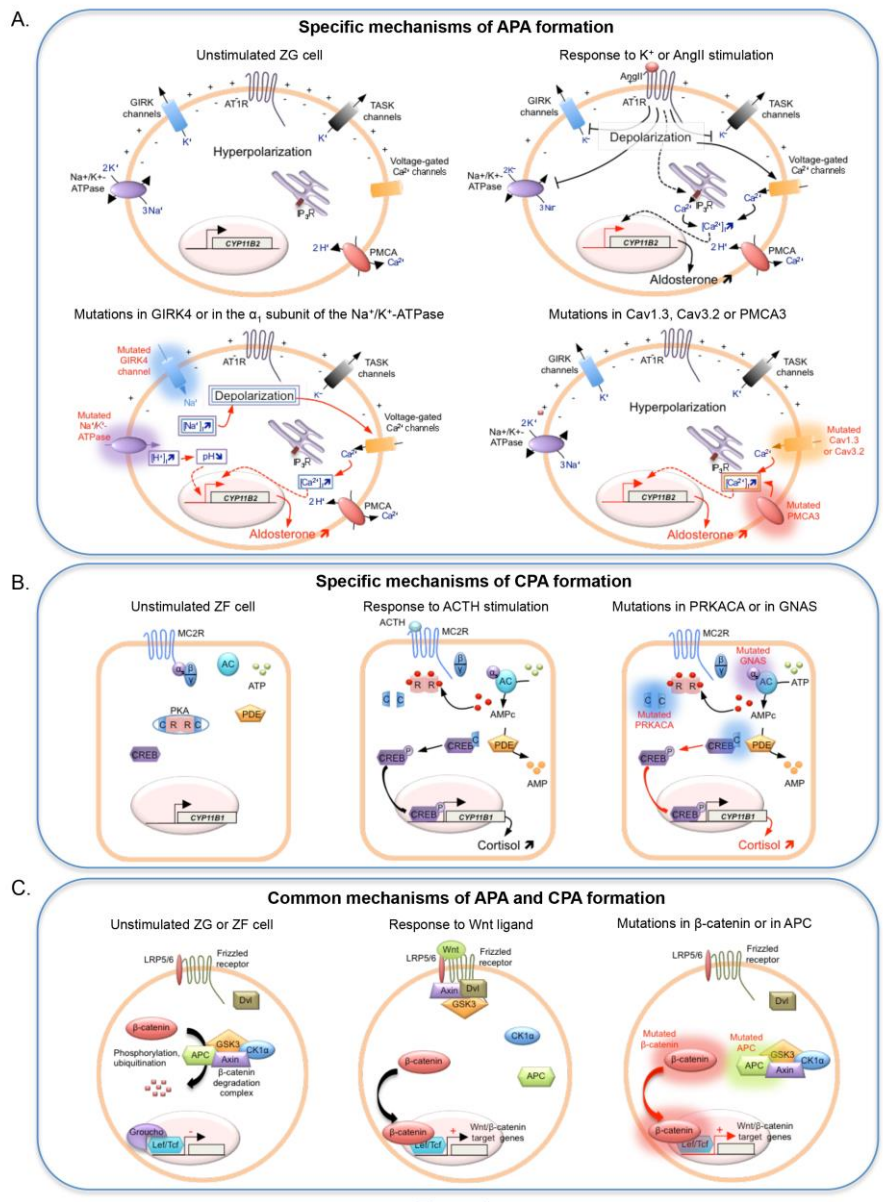


Figure 1

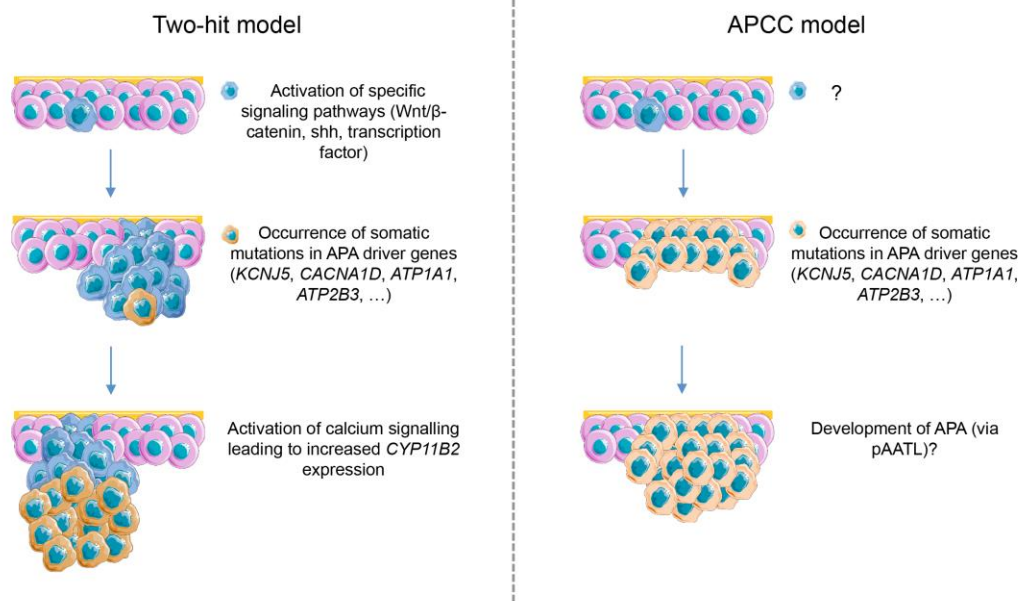


Figure 2