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## **SOMATIC AND INHERITED MUTATIONS IN PRIMARY ALDOSTERONISM**

Fabio Luiz Fernandes-Rosa <sup>1,2,3\*</sup>, Sheerazed Boulkroun <sup>1,2\*</sup>, Maria-Christina Zennaro <sup>1,2,3</sup>

### **Affiliations:**

<sup>1</sup>INSERM, UMRS\_970, Paris Cardiovascular Research Center, Paris, France

<sup>2</sup>University Paris Descartes, Sorbonne Paris Cité, Paris, France

<sup>3</sup>Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Génétique, Paris, France

\* Equal contributions

Address correspondence to:

Fabio Luiz Fernandes-Rosa / Sheerazed Boulkroun

Institut National de la Santé et de la Recherche Médicale, Unité 970

Paris Cardiovascular Research Center – PARCC

56 rue Leblanc

75015 Paris, France

E-mail: [fabio.fernandes-rosa@inserm.fr](mailto:fabio.fernandes-rosa@inserm.fr) / [sheerazed.boulkroun@inserm.fr](mailto:sheerazed.boulkroun@inserm.fr)

**Running Title:** Genetic basis of primary aldosteronism

**Key words:** primary aldosteronism, aldosterone producing adenoma, Familial Hyperaldosteronism, somatic mutations, germline mutations, potassium channels, calcium channels, ATPase, wnt/ $\beta$ -catenin pathway

## **Abstract**

Primary aldosteronism (PA), the most common form of secondary hypertension, is caused in the majority of cases by unilateral aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia. Over the past few years, somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* have been proven to be associated with APA development, representing more than 50% of sporadic APA. The identification of these mutations has allowed the development of a model for APA involving modification on the intracellular ionic equilibrium and regulation of cell membrane potential, leading to autonomous aldosterone overproduction. Furthermore, somatic *CTNNB1* mutations have also been identified in APA, but the link between these mutations and APA development remains unknown. The sequence of events responsible for APA formation is not completely understood, in particular whether a single hit or a double hit are responsible for both aldosterone overproduction and cell proliferation. Germline mutations identified in patients with early onset PA have expanded the classification of familial forms (FH) of PA. The description of germline *KCNJ5* and *CACNA1H* mutations has identified FH-III and FH-IV based on genetic findings; germline *CACNA1D* mutations have been identified in patients with very early onset PA and severe neurological abnormalities. This review summarizes current knowledge on the genetic basis of PA, the association of driver gene mutations and clinical findings and in the contribution to patient care, plus the current understanding on the mechanisms of APA development.

## 1 **Background**

2 Arterial hypertension is a major cardiovascular risk factor with an estimated global age-  
3 standardized prevalence of 24.1% in adult males and 20.1% in adult females (NCD-Risc,  
4 2017). Detection of secondary hypertension is crucial for targeted management of the  
5 underlying disease and prevention of cardiovascular complications. Primary aldosteronism  
6 (PA) is the most common form of secondary hypertension with an estimated prevalence of 5  
7 to 10% in hypertensive patients (Douma, et al. 2008; Hannemann, et al. 2012; Hannemann  
8 and Wallaschofski 2012; Mulatero, et al. 2004; Plouin, et al. 2004; Rossi, et al. 2006). PA is  
9 due to autonomous aldosterone production from the adrenal gland, leading to hypertension  
10 with an increased aldosterone to renin ratio and on occasion hypokalemia (Funder, et al.  
11 2016).

12 Aldosterone is synthesized from cholesterol by a series of specific enzymatic reactions in  
13 the zona glomerulosa (ZG) of the adrenal cortex. The final steps are catalyzed by the  
14 aldosterone synthase (encoded by *CYP11B2*). Aldosterone biosynthesis is mainly regulated by  
15 the renin angiotensin system and extracellular potassium ( $K^+$ ) concentration. ACTH is also a  
16 regulator of *CYP11B2* expression and aldosterone secretion. Binding of ACTH to its receptor,  
17 the melanocortin 2 receptor, leads to the increase of cAMP and activation of PKA signaling  
18 pathway, however the complete mechanisms are not completely understood {Gallo-Payet,  
19 2016 #10097}{Ruggiero, 2016 #10098}{MacKenzie, 2017 #10099}. The stimulation by  
20 angiotensin II (AngII) or  $K^+$  results in depolarization of the ZG cell membrane and opening of  
21 voltage-dependent calcium ( $Ca^{2+}$ ) channels, leading to an increased intracellular  $Ca^{2+}$   
22 concentration. Through binding to AngII type 1 receptors, the hormone induces also  $Ca^{2+}$   
23 release from the endoplasmic reticulum (Spat and Hunyady 2004) (Figure 1). Thus  
24 aldosterone production is tightly controlled to maintain electrolyte and fluid homeostasis by  
25 the kidney. Aldosterone acts on epithelial cells, particularly in the renal collecting duct, where

26 it plays a major role in promoting sodium reabsorption and thus regulation of blood pressure  
27 (Bonvalet 1998). More recently aldosterone has emerged as a key hormone determining end  
28 organ damage. Given the major cardiovascular adverse effects of aldosterone excess, the early  
29 detection of PA has enormous impact on clinical outcome and survival (Rossi, et al. 2008).  
30 However, despite the publications of guidelines for the management of PA, critical issues  
31 related to diagnosis, subtype differentiation and treatment of non-surgically correctable forms  
32 still persist (Funder, et al. 2008; Funder et al. 2016). A better understanding of the pathogenic  
33 mechanisms underlying the development of aldosterone producing adenoma (APA) and  
34 bilateral adrenal hyperplasia (BAH), the most common forms of PA will improve diagnostic,  
35 prognostic and treatment for up to 10% of hypertensive subjects.

36 Over the past few years, major advances have been made in understanding the genetic  
37 basis of APA, with the identification of mutations in genes coding for ion channels (*KCNJ5*  
38 (Choi, et al. 2011), *CACNAID* (Azizan, et al. 2013; Scholl, et al. 2013) and *CACNAIH*  
39 (Daniil, et al. 2016)) and ATPases (*ATP1A1* (Azizan et al. 2013; Beuschlein, et al. 2013) and  
40 *ATP2B3* (Beuschlein et al. 2013)) in more than 50% of APA. All these mutations lead to the  
41 activation of calcium signaling, the major trigger for aldosterone production. In addition,  
42 recurrent activating mutations in *CTNNB1* gene coding for  $\beta$ -catenin have also been identified  
43 in APA (Akerstrom, et al. 2016; Tissier, et al. 2005).

44 In this review we will summarize the current knowledge of the genetic bases of PA  
45 acquired during the past few years using -omics approaches. These advances have highlighted  
46 the major role of the ionic equilibrium and regulation of cell membrane potential in  
47 autonomous aldosterone overproduction.

48

49 **Somatic mutations in APA**

50 Zona glomerulosa cells from the adrenal cortex are maintained in a hyperpolarized state,  
51 mainly due to the high expression of potassium channels. The study of mouse models in  
52 which the expression of specific potassium channels was invalidated has highlighted their  
53 crucial role in adrenal (Davies, et al. 2008; Guagliardo, et al. 2012; Heitzmann, et al. 2008;  
54 Penton, et al. 2012). Hence, the deletion of TWIK-related acid sensitive K<sup>+</sup> channel (Task) -1  
55 (Task1) and -3 (Task3) leads to alteration in the membrane properties of ZG cells, inducing  
56 autonomous aldosterone production with clinical features typical of glucocorticoid remediable  
57 aldosteronism (Heitzmann et al. 2008) or BAH (Davies et al. 2008). Task1<sup>-/-</sup> mice display  
58 severe hyperaldosteronism, hypokalemia and arterial low renin hypertension. Interestingly,  
59 this hyperaldosteronism was remediable by glucocorticoids due ectopic expression of AS in  
60 ZF before puberty in both male and female and then only in adult female (Heitzmann et al.  
61 2008). In contrast, the phenotype of Task3<sup>-/-</sup> mice recapitulates low renin and salt sensitive  
62 hypertension (Guagliardo et al. 2012; Penton et al. 2012). Surprisingly, invalidation of both  
63 Task1 and Task 3 in mice has no impact on cellular organization and zonation of the adrenal  
64 cortex, although aldosterone production by ZG cells is inappropriate for salt intake,  
65 recapitulating features of idiopathic primary aldosteronism (Davies et al. 2008). Invalidation  
66 of these different potassium channels leads to hyperaldosteronism due to abnormal  
67 depolarization of ZG cell membrane resulting in increased intracellular Ca<sup>2+</sup> concentration  
68 and stimulation of aldosterone biosynthesis; however formation of adrenal tumors has never  
69 been observed in these models, indicating that other mechanisms are required to promote  
70 increased cell proliferation in APA.

### 71 *Mutations affecting cell membrane potential*

72 The emergence of high throughput sequencing techniques has allowed major advances in  
73 the understanding of the genetic basis of many diseases. Thus in 2011, Choi and co-workers  
74 identified two different somatic mutations in the *KCNJ5* gene in 8 of 22 APA, as well as an

75 inherited mutation in the same gene in a Mendelian form of severe aldosteronism and massive  
76 bilateral adrenal hyperplasia (see below in familial forms of PA) (Choi et al. 2011). *KCNJ5*  
77 codes for the G protein-activated inward rectifier potassium channel 4 (GIRK4). The GIRK4  
78 channel belongs to a large family of inwardly rectifying K<sup>+</sup> channels. “Inward rectifiers” are a  
79 class of K<sup>+</sup> channels that conduct much larger inward currents at membrane voltages negative  
80 to the K<sup>+</sup> equilibrium potential. The GIRK channel family comprises four members (GIRK1  
81 to GIRK 4) that form hetero- or homotetramers. They are composed of two membrane-  
82 spanning domains (M1 and M2), one pore forming region (H5) and cytoplasmic N- and C-  
83 termini that participate in the pore structure. The first two mutations reported by Choi et al,  
84 p.Gly151Arg and p.Leu168Arg, are located near or within the selectivity filter of the channel  
85 (Choi et al. 2011). Subsequently many other studies have reported additional somatic or  
86 germline mutations in or surrounding the selectivity filter (from Thr149 to Gly153). All these  
87 mutations leads to a loss of K<sup>+</sup> selectivity and an increase of sodium (Na<sup>+</sup>) influx into the cells,  
88 producing a chronic depolarization of cell membrane, opening of voltage gated Ca<sup>2+</sup> channels,  
89 activation of calcium signaling, increase of aldosterone synthase expression and autonomous  
90 aldosterone production by the cells (Figure 2). Overexpression of the GIRK4 p.Thr158Ala  
91 mutant in HAC cells resulted in increased aldosterone, cortisol and 18-oxocortisol levels, as  
92 observed in patients harboring this mutation, associated with increased expression of  
93 *CYP11B2* and *CYP11B1* and decreased expression of *CYP17*, but a decrease of cell  
94 proliferation (Oki, et al. 2012). Scholl *et al* reported also a decrease of cell viability in 293T  
95 cells overexpressing p.Gly151Glu or p.Gly151Arg (Scholl, et al. 2012), due to increased Na<sup>+</sup>  
96 influx into the cells via the mutated channels. This additional effect of *KCNJ5* mutations may  
97 possibly be a mechanism limiting adrenocortical cell mass growth, but also strongly suggests  
98 that other mechanisms are involved in cell proliferation.

99 In addition to affecting  $K^+$  cell permeability, two *KCNJ5* mutations (p.Arg115Trp and  
100 p.Glu246Gly) decreased GIRK4 wt currents when co-expressed in HEK cells and altered the  
101 membrane abundance of the channels, suggesting that that reduced GIRK4 in cell surface may  
102 lead to inappropriate hypersecretion of aldosterone and formation of adrenal adenoma (Cheng,  
103 et al. 2015), although this is not the case for other mutations (Murthy, et al. 2012). Although  
104 *KCNJ5* mRNA expression is not affected by mutational status, at the protein level decreased  
105 GIRK4 expression in APA compared with peritumoral ZG was observed in APA with *KCNJ5*  
106 mutations compared to non-mutated APA or those with other mutations (Boukroun, et al.  
107 2013; Cheng et al. 2015; Fernandes-Rosa, et al. 2015a). *KCNJ5* mutations were associated  
108 neither with specific adrenal cortex remodeling nor specific transcriptional profiles  
109 (Boukroun, et al. 2012; Boukroun et al. 2013). Slight modifications of gene expression  
110 patterns have been reported, such as increased expression of *CYP11B2*, Visinin-like 1  
111 (*VSNL1*) or Acyl-CoA Synthetase Short-Chain Family Member 3 (*ACSS3*) in APA harboring  
112 *KCNJ5* expression (Monticone, et al. 2012; Williams, et al. 2012b; Zhou, et al. 2016). *VSNL*-  
113 1 is a calcium-sensor protein that may protect cells from apoptosis induced by the  
114 overexpression of *KCNJ5* mutants, counterbalancing to some extent the pro-apoptotic effect  
115 of overexpression of mutated GIRK4 channels in APA (Williams, et al. 2012a).

116 More recently, exome sequencing approaches allowed the identification of mutations in  
117 *ATP1A1*, encoding the  $\alpha 1$  subunit of the  $Na^+/K^+$ -ATPase in APA (Azizan et al. 2013;  
118 Beuschlein et al. 2013). The  $Na^+/K^+$ -ATPase is a member of the P-type ATPase family and is  
119 composed of two subunits,  $\alpha$  and  $\beta$ . By using energy derived from ATP hydrolysis, it  
120 generates electrochemical gradients for  $Na^+$  and  $K^+$  across the plasma membrane; hydrolysis  
121 of one ATP allows the exit of three  $Na^+$  and the entry of two  $K^+$ , generating an electrogenic  
122 gradient. The  $\alpha$  subunit is formed of ten transmembrane domains (M1 – M10) with  
123 intracellular N- and C-tails. Different somatic variants (p.Gly99Arg (Williams, et al. 2014),



124 p.Leu104Arg (Beuschlein et al. 2013), p.Val332Gly (Beuschlein et al. 2013)) or deletions  
125 (p.Phe100\_Leu104del (Azizan et al. 2013; Beuschlein et al. 2013), p. Met102\_Ile106del  
126 (Akerstrom, et al. 2015), p.Leu103\_Leu104del (Akerstrom et al. 2015), p.Phe956\_Glu961 del  
127 (Akerstrom et al. 2015), p.Phe959\_Glu961del (Akerstrom et al. 2015), p.Glu960\_Leu964del  
128 (Akerstrom et al. 2015), p.Glu960\_Ala963del (Azizan et al. 2013)) were identified in M1, M4  
129 and M9 domains. Residues mutated in M1 and M4 domains are essential for K<sup>+</sup> binding and  
130 gating of the binding pocket, their mutations leading to a major decrease in Na<sup>+</sup> and K<sup>+</sup>  
131 binding affinity and thus a loss of pump function (Figure 3) (Beuschlein et al. 2013)  
132 (Williams et al. 2014). Remarkably, in H295R\_S2 cells, whereas the expression of  
133 p.Leu104Arg or the p.Val332Gly mutants lead to cell membrane depolarization and impaired  
134 K<sup>+</sup> sensitivity, there is no increase in resting Ca<sup>2+</sup> activity but intracellular acidification of the  
135 cells due to H<sup>+</sup> leak. Although this acidification may contribute to the autonomous  
136 aldosterone production, specific mechanism remains to be established (Stindl, et al. 2015).  
137 Where residues are mutated in M9, these amino acids may be of importance for the Na<sup>+</sup>-  
138 specific site III, the Na<sup>+</sup> binding site involving specific residues located in M5 (Tyr778), M6  
139 (Gly813 and Thr814) and M9 (Glu 960 and Glu961) (Azizan et al. 2013; Li, et al. 2005).  
140 Until now, germline mutations in *ATPIA1* have never been reported, suggesting that loss of  
141 function of this protein would not be compatible with life.

#### 142 ***Mutations affecting directly intracellular calcium equilibrium***

143 The major trigger of aldosterone biosynthesis in response to increased plasma K<sup>+</sup> and  
144 angiotensin II is an increase of calcium concentration in ZG cells (see above). Whereas  
145 mutations in *KCNJ5* and *ATPIA1* affect cell membrane potential, leading to increased  
146 intracellular Ca<sup>2+</sup> concentration in case of *KCNJ5* mutations or disturbance of the pH, in the  
147 case of *ATPIA1* mutations, mutations identified in *ATP2B3*, *CACNAID* and *CACNAIH* (see

148 below, in familial forms of PA and germline mutations) are directly involved in the control of  
149 intracellular calcium equilibrium (Figure 4).

150 *ATP2B3* codes for the Plasma Membrane Calcium ATPase Isoform 3 (PMCA3), a P-type  
151 ATPase family, like the  $\text{Na}^+/\text{K}^+$ -ATPase. PMCA3 is essential to extrude  $\text{Ca}^{2+}$  from the  
152 cytoplasm and thereby has an essential role in regulating intracellular calcium homeostasis  
153 (Di Leva, et al. 2008). *ATP2B3* is located on chromosome X, and germline mutations have  
154 been shown to be responsible for the development of X-linked congenital cerebellar ataxia  
155 (Zanni, et al. 2012). Similar to the  $\alpha$  subunit of the  $\text{Na}^+/\text{K}^+$ -ATPase, PMCA3 is composed of  
156 10 transmembrane domains, a large cytoplasmic loop and cytoplasmic N- and C-tails. All the  
157 mutations identified in *ATP2B3* are located in the M4 domain, deletions of at least two amino  
158 acids in a specific region between amino acids 425 and 433 (Beuschlein et al. 2013);  
159 (Fernandes-Rosa, et al. 2014); (Dutta, et al. 2014) (Akerstrom et al. 2015); (Zheng, et al.  
160 2015) (Scholl, et al. 2015a), a region crucial for the binding of  $\text{Ca}^{2+}$  ions but also close to the  
161 catalytic core of the pump (Di Leva et al. 2008). Electrophysiological studies have shown  
162 that the *ATP2B3* p.Leu425\_Val426del mutation leads to increased intracellular  $\text{Ca}^{2+}$   
163 concentration in transfected human embryonic kidney (HEK-293) cells due to an increased  
164  $\text{Ca}^{2+}$  influx and reduced capacity to export  $\text{Ca}^{2+}$ , suggesting a loss of the physiological pump  
165 function (Tauber, et al. 2016). In addition to the alteration of  $\text{Ca}^{2+}$  equilibrium, *ATP2B3*  
166 mutations, like *KCNJ5* but to a less extent, lead to  $\text{Na}^+$  and possibly  $\text{Ca}^{2+}$  influx into the cells,  
167 contributing also to increased intracellular  $\text{Ca}^{2+}$  concentration through depolarization of the  
168 cell membrane and opening of voltage gated  $\text{Ca}^{2+}$  channel. These alterations are associated to  
169 increased aldosterone production and CYP11B2 expression in adrenocortical cells (Tauber et  
170 al. 2016)

171 *CACNA1D* encodes the L-type  $\text{Ca}^{2+}$  channel  $\alpha$ -subunit  $\text{Ca}_v1.3$  (Azizan et al. 2013; Scholl  
172 et al. 2013). The channel contains four repeat domains (I to IV), each composed of six

173 transmembrane segments (S1 to S6), plus a membrane-associated loop between S5 and S6  
174 (Catterall 2010). The S4 segment is involved in voltage sensing for activation, whereas S5, S6  
175 and the loop between these two segments form the pore lining of the channel (Dutta, et al.  
176 2016). Cav1.3 is one of the most abundant calcium channels in normal human adrenal and in  
177 APA, predominantly expressed in ZG (Daniil et al. 2016; Scholl et al. 2013). Whereas Cav3.2,  
178 encoded by *CACNA1H*, has been shown necessary for membrane potential oscillations in ZG  
179 cells, the role of Cav1.3 in adrenal is less clear (Hu, et al. 2012). In 2013, exome sequencing  
180 approaches identified somatic and germline mutations (see below) in *CACNAID* (Azizan et al.  
181 2013; Scholl et al. 2013). Nine different mutations were identified, all except two located  
182 specifically in S5, S6 or in the intracellular loop between the S5 and S6 segments.  
183 Electrophysiological studies showed different changes in channel properties depending on the  
184 mutations although all led to increased  $Ca^{2+}$  influx and activation of calcium signaling  
185 stimulating aldosterone production (Azizan et al. 2013; Scholl et al. 2013). By sequencing the  
186 entire gene, we identified nine additional mutations located in segments S4, S5 and S6,  
187 cytoplasmic segments or repeated region III (Fernandes-Rosa et al. 2014). Although the  
188 prevalence of the different mutations was not the same, the identification of all these new  
189 mutations suggests the necessity of sequencing the entire *CACNAID* gene when searching for  
190 causative mutations for APA (Fernandes-Rosa et al. 2014).

### 191 ***Mutations affecting $\beta$ -catenin activation***

192 Activation of Wnt/ $\beta$ -catenin pathway is one of the major features in APA, being found in  
193 at least two thirds of APA (Berthon, et al. 2012; Berthon and Stratakis 2014; Boulkroun, et al.  
194 2011). The Wnt/ $\beta$ -catenin pathway plays an important role in embryonic development, stem  
195 cell maintenance and differentiation in many tissues, especially normal adrenal development  
196 and tumorigenesis (El Wakil and Lalli 2011). In the absence of ligand,  $\beta$ -catenin is a part of  
197 the axin complex consisting of adenomatous polyposis coli (APC), axin, glycogen synthase

198 kinase-3 $\beta$  and casein kinase-1 $\beta$ . The phosphorylation of  $\beta$ -catenin in this complex results in  
199 its ubiquitination and degradation in the proteasome, preventing it from translocating to the  
200 nucleus and activating specific Wnt target genes. Wnt/ $\beta$ -catenin activation occurs via binding  
201 of ligand Wnt to its specific receptor frizzled resulting in inhibition of its phosphorylation and  
202 its subsequent dissociation from the axin complex, accumulation in the cytoplasm and  
203 translocation into the nucleus, where it serves as a transcriptional coactivator of transcription  
204 factors of the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family.

205 Different mouse models have highlighted the role of this pathway in adrenal development  
206 and in aldosterone production. *Wnt4*<sup>-/-</sup> mice display abnormal differentiation of the definitive  
207 zone of the adrenal cortex and decrease of the number of zona glomerulosa cells leading to a  
208 decrease of aldosterone production (Heikkila, et al. 2002; Jeays-Ward, et al. 2003). The  
209 constitutive activation of  $\beta$ -catenin in the adrenal cortex results in adrenocortical zonation  
210 defects by ectopic activation of ZG differentiation and inhibition of orthotopic ZF  
211 differentiation. With time, these mice develop hyperaldosteronism, similar to that observed in  
212 mice expressing a defective APC allele (Berthon, et al. 2010; Bhandaru, et al. 2009).

213 Until recently, although the activation of Wnt/ $\beta$ -catenin was common in APA, only rare  
214 mutations in *CTNNB1* gene, coding for  $\beta$ -catenin have been described (Tadjine, et al. 2008;  
215 Tissier et al. 2005). Different recent studies have reported known mutations in *CTNNB1* gene  
216 in specific residues involved in the stability of the protein (p.Ser45Phe, p.Ser45Pro,  
217 p.Thr41Ala) (Akerstrom et al. 2016; Wu, et al. 2017), leading to constitutive activation of the  
218 Wnt/ $\beta$ -catenin pathway. A novel frameshift mutation has also been reported (A39Efs\*3)  
219 located close to previously described mutation (Scholl et al. 2015a), resulting in an early  
220 termination of the protein.

221 Teo and coworkers reported the case of three women with PA due to an APA with somatic  
222 *CTNNB1* mutations (p.Ser33Cys, pSer45Phe and p.Gly34Arg), two diagnosed during

223 pregnancy and the third after menopause (Teo, et al. 2015). Their APA expressed high levels  
224 of luteinizing hormone–chorionic gonadotropin receptor (LHCGR) and gonadotropin-  
225 releasing hormone receptor (GNRHR), suggesting that pregnancy may reveal underlying PA  
226 (Teo et al. 2015). Although these data provide a substantial addition to our understanding of  
227 the mechanisms linking *CTNNB1* mutation and APA development, the pathophysiology of  
228 APA harbouring *CTNNB1* mutations seems more complex. Previous studies have reported  
229 somatic *CTNNB1* mutations in APA from male patients or non-pregnant females (Akerstrom  
230 et al. 2016; Scholl et al. 2015a; Wu et al. 2017). Moreover, while GNRHR and LHCGR  
231 overexpression is found in more than 40% of APA (Nakamura, et al. 2014; Nicolini, et al.  
232 2014), *CTNNB1* mutations are identified only in 5% (Akerstrom et al. 2016), with  
233 immunohistochemical evidence for  $\beta$ -catenin activation observed in about two thirds of APA  
234 (Berthon, et al. 2014). Further studies are necessary to establish the complex mechanisms  
235 affected by *CTNNB1* mutations in APA.

### 236 ***Prevalence and clinical correlates of somatic mutations in APA***

237 Since the identification of *KCNJ5* mutations in 2011, many studies have reported the  
238 prevalence and the clinical correlates of somatic mutations in APA (Akerstrom, et al. 2012;  
239 Azizan et al. 2013; Boulkroun et al. 2012; Fernandes-Rosa et al. 2014; Kitamoto, et al. 2014;  
240 Tauber, et al. 2014). A first multicenter European study performed on 380 APA showed a  
241 prevalence of 34% of somatic *KCNJ5* mutations (Boulkroun et al. 2012). More recently, a  
242 second European study performed on 474 APA identified somatic mutations in 54.3% of  
243 tumors: *KCNJ5* mutations were found in 38% of APA, *CACNA1D* in 9.3%, *ATP1A1* in 5.3%,  
244 and *ATP2B3* in 1.7% (Fernandes-Rosa et al. 2014); a similar prevalence was reported in the  
245 American population (Scholl et al. 2015a). The prevalence of somatic *CTNNB1* mutations in  
246 APA ranged from 2.1% to 5.1 % (Akerstrom et al. 2016; Scholl et al. 2015a; Wu et al. 2017).  
247 Differences were observed in the distribution of mutations across the different centers

248 participating in the multicentre European study: the prevalence of *CACNA1D* mutations was  
249 highest in the Paris cohort, whereas *ATP2B3* mutation rate was highest in the Munich cohort  
250 (Fernandes-Rosa et al. 2014). In Asian populations *KCNJ5* mutations appear to have a higher  
251 prevalence, ranging from 59% to 76% (Hong, et al. 2016; Taguchi, et al. 2012; Wang, et al.  
252 2015; Wu, et al. 2015; Zheng et al. 2015). A meta-analysis of somatic *KCNJ5* mutations,  
253 comprising 1636 patients with APA, reported that the overall prevalence of somatic *KCNJ5*  
254 mutations was 43%, ranging from 63% in Asia to 35% in Europe + the United States +  
255 Australia (Lenzini, et al. 2015). This contrast in the prevalence could be explained by ethnic  
256 background, environmental differences or different selection criteria.

257         Various studies have tried to establish genotype-phenotype correlations or to find  
258 biomarkers to determine the mutational status of APA before surgery. Genotype-phenotype  
259 analysis performed in 199 patients from a cohort from Paris have shown that patients with  
260 *KCNJ5* mutations are more frequently female, diagnosed younger and with higher minimal  
261 plasma potassium concentrations, and the presence of *CACNA1D* mutations associated with  
262 smaller adenomas (Fernandes-Rosa et al. 2014). No association was found between the  
263 mutation status and preoperative plasma aldosterone, renin levels, aldosterone to renin ratio or  
264 number of medications taken before surgery. Replication analyses, performed on APA from  
265 two other European cohorts, confirmed the association of *KCNJ5* mutations with female sex  
266 and younger age of diagnosis (Fernandes-Rosa et al. 2014). The association of *KCNJ5*  
267 mutations with female sex and younger age was also observed in a meta-analysis study  
268 comprising 1636 APA patients from 13 studies (Lenzini et al. 2015), as were higher plasma  
269 levels of aldosterone and larger tumors (Lenzini et al. 2015). Cardiovascular complications of  
270 aldosterone excess in APA in relation to the mutational status were evaluated in two studies  
271 (Kitamoto et al. 2014; Rossi, et al. 2014). Left ventricular mass index (LVMI) was found to  
272 be higher in patients carrying *KCNJ5* mutations (Rossi et al. 2014), and in both studies LVMI

273 was independently associated with *KCNJ5* mutations (Kitamoto et al. 2014; Rossi et al. 2014).  
274 Finally, steroid profiles performed on adrenal and peripheral venous plasma samples of 79  
275 APA patients by liquid chromatography–tandem mass spectrometry (LC–MS/MS) established  
276 that specific somatic mutations in APAs can be defined by distinct steroid profiles in adrenal  
277 vein plasma. This approach may translate to identifying specific steroid signatures in  
278 peripheral venous plasma, and thus potentially useful in determining the underlying APA  
279 genotype (Williams, et al. 2016).

280 An unanswered question concerns the influence of somatic mutations on the  
281 management of PA. Higher lateralization indices on adrenal venous sampling (AVS) were  
282 found in patients carrying somatic *KCNJ5* mutations (Seccia, et al. 2012). More recently,  
283 Osswald et al observed no difference in steroid gradients during AVS as a function of the  
284 mutation status (Osswald, et al. 2013). Another difficulty in establishing genotype-phenotype  
285 correlations or surrogate biomarkers of the mutational status is APA heterogeneity. Different  
286 somatic mutations have been identified in distinct nodules within the same adrenal, including  
287 mutations in different genes (Dekkers, et al. 2014; Fernandes-Rosa, et al. 2015b). Moreover,  
288 APA mutations are reported to occur only in aldosterone synthase (AS) positive regions with  
289 two different mutations identified in distinct AS positive regions within the same nodule  
290 (Nanba, et al. 2016a), pointing to the complexity of genetic determinants of APA.

291

## 292 **Familial forms of PA and germline mutations**

293 Familial forms are rare causes of PA that account for 1–5% of cases. Different forms  
294 displaying Mendelian inheritance have been described: familial hyperaldosteronism type I  
295 (FH-I) or glucocorticoid-remediable aldosteronism (GRA), familial hyperaldosteronism type  
296 II (FH-II), familial hyperaldosteronism type III (FH-III) with *KCNJ5* mutations, and familial  
297 hyperaldosteronism type IV (FH-IV) due to *CACNA1H* mutations. Germline *KCNJ5*,

298 *CACNAID* and *CACNAIH* mutations are also associated with sporadic PA and/or early-onset  
299 hypertension due to PA (Daniil et al. 2016; Murthy, et al. 2014; Scholl et al. 2013).  
300 Furthermore, germline *ARMC5* variants have been identified in African American patients  
301 with PA (Zilbermint, et al. 2015).

### 302 ***Familial hyperaldosteronism type I***

303 FH-I is characterized by clinical and biochemical findings of PA relieved by  
304 dexamethasone. The clinical characteristics comprise early and severe hypertension,  
305 biochemical abnormalities of PA, increased production of 18-hydroxycortisol and 18-  
306 oxocortisol, bilateral hyperplasia or adrenal nodules (New 1980; Sutherland, et al. 1966). FH-  
307 I has an autosomal dominant mode of inheritance, accounting for 0.5% to 1.0% of PA in adult  
308 patients (Jackson, et al. 2002; Mulatero, et al. 2011a; Pizzolo, et al. 2005) and for 3% in  
309 hypertensive children (Aglony, et al. 2011). The genetic etiology is a crossover of the  
310 homologous genes coding for AS (*CYP11B2*) and 11 $\beta$ -hydroxylase (*CYP11B1*), creating a  
311 chimeric gene with the *CYP11B1* promoter and *CYP11B2* coding sequences juxtaposed. This  
312 crossing over leads to an ectopic expression of AS throughout the ZF, with aldosterone being  
313 produced not only in isolated cells from ZG (Lifton, et al. 1992; Stowasser and Gordon 2000).  
314 The description of different crossover patterns of the hybrid gene suggests that mutations  
315 arise independently (Dluhy and Lifton 1995). The genetic diagnosis is usually made by  
316 Southern blot or long PCR, both techniques with high sensitivity and specificity (Funder et al.  
317 2016).

318 The Endocrine Society Clinical Practice Guideline suggests that in patients with an onset  
319 of confirmed PA earlier than 20 years of age and in those who have a family history of PA or  
320 stroke at a young age (<40 years), genetic testing for FH-I should be performed (Funder et al.  
321 2016). FH-I patients should be treated with glucocorticoid hormones, which suppress ACTH  
322 secretion leading to the reduction of aldosterone levels. Low dose glucocorticoids are



323 sufficient, as only partial suppression of adrenocorticotropin is required to correct  
324 hypertension over several years (Stowasser, et al. 2000).

### 325 ***Familial hyperaldosteronism type II***

326 FH-II is a non-glucocorticoid remediable form of familial hyperaldosteronism first  
327 described by Gordon et al. in 1991 (Gordon, et al. 1991), with transmission consistent with an  
328 autosomal dominant mode of inheritance (Stowasser and Gordon 2000, 2001) and his  
329 prevalence ranging from 1.2% to 6% in adult patients with PA (Medeau, et al. 2005;  
330 Mulatero, et al. 2011b; Pallauf, et al. 2012; Stowasser and Gordon 2001). FH-II patients  
331 display a familial history of PA and different subtypes may be present within the same family  
332 (Mulatero et al. 2011b; Stowasser 2009; Stowasser and Gordon 2001).

333 The genetic bases of FH-II are not completely understood. No mutations in candidate  
334 genes including *CYP11B2*, *AGTR1* and the p53 tumor-suppressor gene have been identified in  
335 FH-II patients (Ballantine, et al. 1996a, b; Davies, et al. 1997; Stowasser and Gordon 2001).  
336 On the other hand, some studies have established evidence for an association of a genetic  
337 locus on the chromosomal region 7p22 with FH-II in some affected families (Lafferty, et al.  
338 2000; So, et al. 2005; Sukor, et al. 2008). Candidate genes located in this region were  
339 sequenced in FH-II patients, including retinoblastoma-associated Kruppel-associated box  
340 gene (*RBaK*), postmeiotic segregation increased 2 (*PMS2*), guanine nucleotide-binding  
341 protein  $\alpha$ -12 (*GNAI2*), replication protein A3 (*RPA3*), zinc finger protein 12 (*ZNF12*),  
342 glucocorticoid-induced transcript 1 (*GLCC1*), fascin 1 (*FSCN1*), and the cAMP-dependent  
343 protein kinase type I  $\beta$ -regulatory subunit (*PRKAR1B*), but no mutations were identified  
344 (Jeske, et al. 2008; Medeau et al. 2005; So, et al. 2006).

345 FH-II is clinically and biochemically indistinguishable from sporadic forms of PA. With  
346 the discovery in the last years of the genetic basis of FH-III and FH-IV (see below), the  
347 diagnosis of FH-II is made on the basis of two or more affected family members, without

348 *KCNJ5* and *CACNA1H* mutations. Advances in genetic techniques, with whole exome and  
349 whole genome sequencing studies, should allow discovery of genes associated with familial  
350 PA and thus change the current definition of FH-II.

351 ***Familial hyperaldosteronism type III and germline KCNJ5 mutations***

352 FH-III is a non-glucocorticoid remediable form of FH first described in a father and two  
353 daughters with early-onset severe arterial hypertension and hypokalemia associated with  
354 marked hyperaldosteronism and very high levels of the hybrid steroids 18-oxocortisol and 18-  
355 hydroxycortisol (Geller, et al. 2008). The patients showed massive BAH with bilateral  
356 adrenalectomy required to control blood pressure (Geller et al. 2008). The genetic background  
357 of FH-III is a mutation in the *KCNJ5* gene (p.Thr158Ala) (Choi et al. 2011). This mutation is  
358 located near the region responsible for GIRK4 K<sup>+</sup> selectivity (GYG motif), leading to a loss in  
359 K<sup>+</sup> selectivity and increased influx of Na<sup>+</sup> into the cytoplasm, resulting in the depolarization  
360 of the plasma membrane and opening of voltage-gated Ca<sup>2+</sup> channels. The calcium signalling  
361 pathway is activated by increased intracellular Ca<sup>2+</sup>, resulting in an increase of steroidogenic  
362 enzyme expression and aldosterone production (Oki et al. 2012).

363 Different studies have identified germline *KCNJ5* mutations in FH-III families with a  
364 phenotypic variability, ranging from spironolactone-responsive hyperaldosteronism to  
365 massive adrenal hyperplasia with drug-resistant hypertension (Adachi, et al. 2014;  
366 Charmandari, et al. 2012; Choi et al. 2011; Monticone, et al. 2015; Monticone, et al. 2013;  
367 Mulatero, et al. 2012; Mussa, et al. 2012; Scholl et al. 2012; Tong, et al. 2016). This  
368 variability was attributed to the type of *KCNJ5* mutation, with a severe phenotype associated  
369 with GIRK4 p.Gly151Arg, p.Thr158Ala, p.Ile157Ser, and p.Tyr152Cys mutations, and a  
370 milder phenotype associated with *KCNJ5* p.Gly151Glu mutations. *In vitro* studies showed  
371 that the milder phenotype in patients carrying the *KCNJ5* p.Gly151Glu mutation may be  
372 explained by rapid cell lethality due to much higher sodium conductance of the channel

373 preventing adrenal hyperplasia (Scholl et al. 2012). A recent study, however, described a  
374 patient with sporadic spironolactone-responsive PA with a germline *KCNJ5* mutation  
375 previously associated with a severe phenotype (p.Gly151Arg) (Adachi et al. 2014). In the  
376 same way, patients carrying the same germline *KCNJ5* mutation may display differences in  
377 adrenal morphology, even within the same family (Adachi et al. 2014). These differences  
378 suggest that the clinical severity in FH-III cannot be defined only on the basis of the type of  
379 *KCNJ5* mutation.

380 The complexity of the FH-III phenotype associated with *KCNJ5* mutations has been  
381 highlighted by two recent studies. Combined aldosterone and cortisol oversecretion associated  
382 with massive bilateral adrenal hyperplasia was described in a patient carrying a germline  
383 *KCNJ5* mutation (p.Glu145Gln) (Tong et al. 2016). In primary cell culture of the resected  
384 hyperplastic adrenal, calcium channel blockers inhibited the secretion of both aldosterone and  
385 cortisol and the mRNA expression of steroidogenic enzymes. The authors suggest that the  
386 hypersecretion of cortisol might be ascribed to overly large size of the hyperplastic adrenal  
387 (with low *CYP11B1* expression) and, like aldosterone secretion, may be mediated by voltage-  
388 gated  $\text{Ca}^{2+}$  channels (Tong et al. 2016). A second study reported that adrenals from patients  
389 carrying a germline *KCNJ5* mutation (p. p.Thr158Ala) were markedly enlarged with loss of  
390 zonation and cells expressing aldosterone synthase and/or  $11\beta$ -hydroxylase, with and without  
391  $17\alpha$ -hydroxylase, and some expressing all three enzymes (Gomez-Sanchez, et al. 2017).  
392 *GIRK4* was abundantly expressed in almost all cells, suggesting that the mutation not only  
393 altered the potassium filter selectivity of the channel, but also a signal that designated  
394 specificity for *CYP11B2*-expressing cells, or the presence of a second mutation that abolishes  
395 selectivity of steroidogenesis on the basis of adrenal zonation (Gomez-Sanchez et al. 2017).

396 Germline *KCNJ5* mutations have also been suggested to play a role in the common  
397 sporadic form of PA, although the functional consequences of these germline mutations are

398 not completely established. Sequencing germline DNA from 251 subjects with sporadic PA,  
399 Murthy et al identified three *KCNJ5* missense mutations (p.Arg52His, p.Glu246Lys, and  
400 p.Gly247Arg), located outside the selectivity filter of GIRK4, and a rare SNP (rs7102584,  
401 p.Glu282Gln) in 5% of patients (Murthy et al. 2014). The mutations p.Arg52His and  
402 p.Glu246Lys altered channel properties and the cells expressing GIRK4 channels carrying the  
403 SNP rs7102584 showed reduced viability, whereas channels carrying the p.Gly247Arg  
404 mutation were functionally indistinguishable from the wild type GIRK4 (Murthy et al. 2014).

405 The role of common *KCNJ5* SNPs was also assessed in cohorts of Asian patients with PA  
406 and essential hypertension (Li, et al. 2013; Wang, et al. 2017). Comparing 235 subjects with  
407 sporadic PA to 913 subjects with essential hypertension, Li and coworkers showed that in  
408 Chinese population the minor allele of the *KCNJ5* SNP rs2604204, located in the *KCNJ5* 3'-  
409 UTR, was more frequent in males with PA (43.4%) than in males with essential hypertension  
410 (33.3%,  $p=0.001$ ) (Li et al. 2013), suggesting that *KCNJ5* may be involved in the  
411 pathogenesis of sporadic PA in Chinese patients. The same SNP was studied in 229 Chinese  
412 patients with newly diagnosed essential hypertension (Wang et al. 2017). *KCNJ5* rs2604204  
413 was related to increased plasma aldosterone, plasma renin, AngI and AngII levels in patients  
414 with essential hypertension (Wang et al. 2017). The mechanism underlying the association  
415 between a *KCNJ5* 3'-UTR polymorphism and activation of the renin-angiotensin-aldosterone  
416 system remains unknown.

#### 417 ***Germline CACNAID mutations in sporadic PA***

418 *De novo* germline *CACNAID* mutations have been described in two children with PASNA  
419 (Primary aldosteronism, seizures, and neurological abnormalities), a syndrome of PA and  
420 neuromuscular abnormalities (Scholl et al. 2013). The first subject, without family history of  
421 early-onset hypertension or seizures, showed hypertension with biventricular hypertrophy as a  
422 newborn, with the diagnostic of aldosteronism (high plasma aldosterone, suppressed renin and

423 hypokalemia) at age 1 month. Neurological abnormalities (cerebral palsy, cortical blindness  
424 and complex neuromuscular abnormalities) were observed, with seizures starting at age 7  
425 months. Treatment with a calcium channel blocker normalized blood pressure, with cure of  
426 hypertension and PA on follow up (Korah and Scholl 2015). A germline mutation was  
427 identified in exon 8B of *CACNAID* (p.Gly403Asp), in a residue also mutated in APA  
428 (p.Gly403Arg) (Azizan et al. 2013; Scholl et al. 2013). The second patient, also without  
429 family history of early-onset hypertension or seizures, was diagnosed at birth with cerebral  
430 palsy and complex seizures, arterial hypertension at the age of 5 and hypokalemia at the age  
431 of 8 due to hyperaldosteronism. No adrenal abnormality was observed on CT scan. The  
432 patient carried a germline *CACNAID* mutation (p.Ile770Met) also found in somatic DNA  
433 from APA patients.

#### 434 ***Familial Hyperaldosteronism type-IV (FH-IV) and germline CACNAIH mutations***

435 To identify new genetic abnormalities associated to the development of early onset  
436 hypertension due to PA, Scholl et al performed whole exome sequencing analysis of 40  
437 unrelated patients. A recurrent germline mutation in *CACNAIH* (encoding the pore-forming  
438  $\alpha 1$  subunit of the T-type voltage-dependent calcium channel Cav3.2) (p.Met1549Val) was  
439 identified in 5 children with PA before age 10 (Scholl, et al. 2015b). Familial analysis showed  
440 autosomal dominant transmission of the mutation. Differences in clinical presentation  
441 (incomplete penetrance) were observed within family members, attributed to age-dependent  
442 variability, or to genetic or environmental modifiers. Despite the previously described  
443 association between epilepsy and autism with *CACNAIH* mutations (Chen, et al. 2003; Heron,  
444 et al. 2007; Splawski, et al. 2006), and the presence of severe neurological abnormalities  
445 observed in patients carrying *CACNAID* mutations (Scholl et al. 2013), no neurological  
446 abnormalities or seizures were described in the patients carrying the *CACNAIH*  
447 p.Met1549Val mutation. However, developmental delay or attention deficit were reported in

448 two mutation carriers (Scholl et al. 2015b). Functional studies demonstrated increased  
449 aldosterone production and increased *CYP11B2* mRNA expression in cells transfected with  
450 Cav3.2 1549Val compared with cells transfected with WT Cav 3.2. In addition, the T-type  
451 calcium channel blocker Mibefradil reduced the increased aldosterone production and  
452 *CYP11B2* expression caused by transfection of WT Cav3.2 and Cav3.2 1549Val in untreated  
453 cells, suggesting that T-type calcium channel inhibitors may be an useful treatment option for  
454 patients with *CACNA1H* gain-of-function mutations (Reimer, et al. 2016).

455 Using a similar approach, we identified four germline *CACNA1H* mutations in PA patients  
456 with different phenotypic presentations. A *de novo* germline *CACNA1H* mutation  
457 (p.Met1549Ile) was identified in a patient with early onset hypertension and  
458 hyperaldosteronism, associated with multiplex development disorders (Daniil et al. 2016).  
459 This mutation modifies the same amino acid as the *CACNA1H* p.Met1549Val variant reported  
460 in FH-IV (Scholl et al. 2015b). The germline *CACNA1H* variation p.Ser196Leu was identified  
461 in a patient with family history of HT and PA, diagnosed with HT and PA at 36 (HT) and 51  
462 years of age (PA), and in her sister (HT and PA diagnosed at 35 years of age). A third  
463 *CACNA1H* variant (p.Pro2083Leu) was identified in a PA subject with familial history of PA  
464 and his brother. This variant is located in the C-terminal cytoplasmic domain of Cav3.2.  
465 Finally, in one patient with APA, a germline *CACNA1H* missense variant (p.Val1951Glu)  
466 was found, located in the C-terminal cytoplasmic domain of Cav3.2, in a region possibly  
467 implicated in fast channel activation. Electrophysiological analysis of these four mutant  
468 channels demonstrated that the Ca<sup>2+</sup> current properties were affected, suggesting a gain of  
469 function phenotype. Additionally, results from functional studies in adrenocortical H295R-S2  
470 cells directly linked electrophysiological abnormalities to increased aldosterone production  
471 and/or expression of genes coding for steroidogenic enzymes (Daniil et al. 2016).  
472 Identification of new *CACNA1H* mutations associated with early onset PA and multiplex

473 developmental disorder, or with familial forms diagnosed as FH-II or APA, broadens the  
474 phenotypic spectrum of PA associated with *CACNA1H* mutations.

#### 475 ***ARMC5* variants**

476 Germline and somatic mutations in the armadillo repeat containing 5 (*ARMC5*) gene  
477 causes hypercortisolism in primary bilateral macronodular adrenal hyperplasia (PBMAH)  
478 (Assie, et al. 2013). *ARMC5* belongs to the large family of armadillo (ARM)-repeat-  
479 containing proteins, of which the best-known member is  $\beta$ -catenin, and is likely to be a  
480 tumor-suppressor gene (Berthon and Stratakis 2014). *ARMC5* inactivation appears to affect  
481 steroidogenesis. Upon sequencing the *ARMC5* gene in germline DNA from 56 PA patients,  
482 12 different *ARMC5* variants were identified in 20 unrelated and two related individuals  
483 (39%) (Zilbermint et al. 2015); *in silico* analysis revealed that six of these variants were  
484 predicted to be damaging. Interestingly, all carriers of these variants were African-American.  
485 Tumor DNA sequencing confirmed the presence of the germline mutations but failed to  
486 identify additional somatic mutations in *ARMC5*. Furthermore, the silencing of *ARMC5* in  
487 H295R cells decreased *CYP11B2* expression (Zilbermint et al. 2015). The authors suggested  
488 that the increased adrenocortical mass leads to higher aldosterone secretion, despite a  
489 reduction of aldosterone-secretory capacity of individual zona glomerulosa cells, as seen with  
490 hypercortisolism in patients with PBMAH (Zilbermint et al. 2015). Recently, Mulatero and  
491 coworkers reported 18 *ARMC5* variants, including 5 rare (allele frequency >1%) and 2 new  
492 variants (Mulatero, et al. 2016). *In silico* analysis of these variants, however, did not detect  
493 sequence variations that were predicted to alter protein function (Mulatero et al. 2016). The  
494 link between *ARMC5* mutations and PA has still to be determined.

#### 495 **Molecular and histopathological heterogeneity of APA**

496 As APA may be composed of cells exhibiting characteristics of different zones of the  
497 adrenal cortex (Azizan, et al. 2012; Neville and O'Hare 1985), the association between APA

498 cellular composition and molecular markers of adrenal cortex with the mutation status was  
499 proposed, although conflictual results were observed among different studies. Some have  
500 described that APA harboring *KCNJ5* mutations are mainly composed by cells resembling  
501 cortisol-secreting cells of the adrenal zona fasciculata (ZF-like) and APA harboring  
502 *CACNA1D* and *ATP1A1* mutations are mainly composed by cells resembling the aldosterone-  
503 secreting cells of the adrenal zona glomerulosa (ZG-like) (Azizan et al. 2012; Azizan et al.  
504 2013). Accordingly to these results, *CYP17A1* expression was found to be higher in APA  
505 harboring *KCNJ5* mutations, suggesting that these adenomas arise from the ZF (Azizan et al.  
506 2012; Azizan et al. 2013). However, these results were not replicated in a large cohort of  
507 APA with known somatic mutational status. Analyzing histological characteristics of 78  
508 APA, we found that 72% of APA were composed of a majority of ZF-like cells and no  
509 difference neither in the cellular composition of APA nor in *CYP11B2* expression according  
510 to the mutation status was observed (Fernandes-Rosa et al. 2014). Furthermore, we have  
511 shown that APA, independently of the cellular composition phenotype, were composed of  
512 cells expressing markers of ZG (*CYP11B2*, *Dab2* and *CD56*), and that both APA and adjacent  
513 ZG exhibit histological characteristics of stem/progenitor cells, which may underlie excessive  
514 proliferation and APA formation (Boukroun et al. 2011). We have also shown that *Dab2* is  
515 coexpressed with *CYP11B2* in APA, suggesting that APA is formed by ZG cells acquiring  
516 morphological characteristics of ZF cells due to the excess of steroid production (Boukroun,  
517 et al. 2010).

518 Although the direct link between recurrent somatic mutations and aldosterone production  
519 is well established, the effects of these mutations on nodulation and cell proliferation remain  
520 to be clarified. *KCNJ5* germline mutations originally described in FH-III were associated with  
521 massive bilateral hyperplasia, suggesting that the *KCNJ5* mutation was responsible for  
522 increased cell proliferation and autonomous aldosterone production (Choi et al. 2011). On the



523 other hand, patients with PASNA and germline *CACNA1D* mutations did not exhibit enlarged  
524 adrenals or adrenal nodules (Scholl et al. 2013), as also observed for some patients with  
525 germline *KCNJ5* mutations (Adachi et al. 2014; Mulatero et al. 2012). Furthermore, *in vitro*  
526 studies demonstrated that cells overexpressing a mutant *GIRK4* exhibited lower proliferation,  
527 or even apoptosis, due to increased intracellular Na<sup>+</sup> concentration (Oki et al. 2012; Scholl et  
528 al. 2012). Although adrenal nodules can be observed in normal adrenals associated with older  
529 age and severity of hypertension (Wolkersdorfer and Bornstein 1998), we found an increase  
530 in nodulation of the cortex adjacent to APA compared with control adrenals (Boukroun et al.  
531 2010). This raises the possibility that remodeling of the adrenal cortex precedes the  
532 development of an APA, and thus, that somatic mutations could be a secondary event in APA  
533 development.

534 Different studies have provided strong arguments in favor of the hypothesis of independent  
535 events leading to nodulation and aldosterone overproduction. Recently, we and others have  
536 reported different somatic mutations in different *CYP11B2* positive nodules within the same  
537 multinodular adrenal (Dekkers et al. 2014; Fernandes-Rosa et al. 2015b). Furthermore, a  
538 number of APA exhibited heterogeneity of expression of *CYP11B2*; among these APAs,  
539 recurrent somatic mutations were identified in 3 nodules of the same adrenal, localized only  
540 in positive *CYP11B2* regions. Interestingly, in one APA two different mutations were  
541 identified in two distinct *CYP11B2* positive regions within the same APA (Nanba et al.  
542 2016a). Moreover, the co-existence of two different adrenocortical adenomas within the same  
543 adrenal causing the concurrent diagnosis of PA and Cushing syndrome was recently reported  
544 (Nanba, et al. 2016b). Taken together, all these results suggest that somatic mutations  
545 responsible for aldosterone biosynthesis could be a secondary event in APA development. In  
546 agreement with this possibility, we have described the concurrence of different germline and  
547 somatic mutations leading to APA development (Vouillarmet, et al. 2016). A young patient

548 with severe PA aged 26 years, bilateral macronodular adrenal hyperplasia seen on CT, and  
549 lateralized aldosterone secretion, showed clinical improvement of PA after right  
550 adrenalectomy. The resected adrenal contained three nodules, one expressing aldosterone  
551 synthase, with Weiss index of 3, and a somatic *KCNJ5* mutation. A FDG-PET performed  
552 after surgery revealed abnormal rectal activity despite the absence of clinical symptoms, and  
553 gastrointestinal exploration revealed familial adenomatous polyposis. We identified the  
554 presence of a germline heterozygous *APC* gene mutation associated with nodular biallelic  
555 *APC* inactivation due to loss of heterozygosity (Vouillarmet et al. 2016). This case supports a  
556 two-hit model for APA formation whereby the first hit (*APC* germline mutation) drives the  
557 nodule development, whereas the second hit (*KCNJ5* somatic mutation) specifies the pattern  
558 of hormonal secretion.

559 On the other hand, some authors suggest that APA could be derived from aldosterone-  
560 producing cell clusters (APCCs) (Nishimoto, et al. 2010). APCCs are composed of  
561 morphological ZG cells in contact with the capsule and inner columnar ZF-like cells forming  
562 cords along sinusoids, staining positive for both CYP11B2 and CYP11B1 and found in both  
563 normal and PA adrenal tissue (Boulkroun et al. 2011; Nishimoto et al. 2010). The sequencing  
564 of DNA from 23 APCCs from normal adrenals showed known aldosterone driver mutations  
565 in *CACNAID*, *ATP1A1* and *ATP2B3* in 35% of the APCCs; however no *KCNJ5* mutations,  
566 the most frequent alteration observed in APA, were found (Nishimoto, et al. 2015). The  
567 authors suggest that as APCCs share the same *ATP1A1*, *ATP2B3* and *CACNAID* mutations as  
568 APA, they may represent a precursor population of cells that lead to APA. The lack of *KCNJ5*  
569 mutations in APCC may reflect rapid progress of *KCNJ5*-mutated APCCs to APA, so  
570 that *KCNJ5* mutations may only be rarely detected in APCCs (Nishimoto et al. 2015).

571 **Conclusion**

572 In the past five years, since the original article from Choi and coworkers (Choi et al.  
573 2011), large advances have been done in the field of genetic causes of PA. Somatic mutations  
574 in five genes (*KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3* and *CTNNB1*) are proven to be  
575 associated with APA development, explaining more than 50% of sporadic cases. Germline  
576 mutations identified in *KCNJ5* and *CACNA1H* have expanded the classification of familial  
577 forms of PA, now based on genetic findings. Clinical studies to establish genotype-phenotype  
578 correlations and biomarkers associated with recurrent somatic mutations are yet largely  
579 unsuccessful, *in vitro* studies have found pharmacological characteristics of mutant channels  
580 which may provide potential treatment options.

581 In future studies larger numbers of samples should progressively explain the genetic basis  
582 of the remaining 50% of sporadic APA, as well as the familial forms of PA without genetic  
583 defects identified to date. The development of biomarkers or techniques allowing the  
584 identification of somatic mutations before surgery may open new options in PA diagnosis and  
585 treatment. Whether somatic mutations in APA are responsible for both cellular proliferation  
586 and aldosterone production or whether they are independent events leading to aldosterone  
587 production occurring in a previously altered adrenal cortex is yet to be verified. Finally, the  
588 existence of possible genetic determinants of BAH, as well as the presence of common  
589 mechanisms involved in APA and BAH development, is still unknown and constitutes a new  
590 field for PA research in future studies.

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601

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603 The authors have nothing to disclose.

604

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1002

1003 **Figure legends**

1004 **Figure 1: Regulation of aldosterone biosynthesis in zona glomerulosa under**  
1005 **physiological conditions. (A)** In basal condition, zona glomerulosa cells are hyperpolarized  
1006 due to the presence of a high number of potassium channels at the cell surface. The gradient  
1007 of  $K^+$  concentration between the intracellular and extracellular spaces required to maintain  
1008 this membrane potential is generated by the  $Na^+/K^+$ -ATPase pump activity. **(B)** Extracellular  
1009 increase or intracellular decrease of  $K^+$  concentration as well as inhibition of the  $Na^+/K^+$ -  
1010 ATPase pump activity or of  $K^+$  channels lead to the depolarization of cell membrane, opening  
1011 of voltage gated  $Ca^{2+}$  channel, increased intracellular  $Ca^{2+}$  concentration and activation of  
1012  $Ca^{2+}$  signal pathway, the major trigger for aldosterone biosynthesis. The binding of  
1013 angiotensin II (AngII) to the AngII type 1 receptor (AT1R) leads, through triphosphate  
1014 inositol (IP3) increase, to the release of stored  $Ca^{2+}$  by the endoplasmic reticulum. AngII and  
1015  $K^+$  induced, both, cell membrane depolarization leading to opening of voltage gated  $Ca^{2+}$   
1016 channels and increased intracellular  $Ca^{2+}$  concentration. In addition to this effect, AngII also  
1017 inhibit potassium channels and  $Na^+/K^+$ -ATPase pump activity, leading again to cell  
1018 membrane depolarization. The following rise in intracellular  $Ca^{2+}$  concentration leads to  
1019 activation of the  $Ca^{2+}$  signaling pathway, inducing activation of specific transcription factors  
1020 (Nurr1, NGFIB) and thus increase of *CYP11B2* mRNA expression and aldosterone  
1021 biosynthesis.

1022

1023 **Figure 2: Aldosterone biosynthesis in APA in presence of *KCNJ5* mutations.** Mutations  
1024 affecting the GIRK4 potassium channel, encoded by *KCNJ5*, lead to increase of intracellular  
1025  $Na^+$  concentration, thus resulting in cell membrane depolarization and opening of voltage  
1026 gated  $Ca^{2+}$  channel.

1027

1028 **Figure 3: Aldosterone biosynthesis in APA in presence of *ATP1A1* mutations.** Mutations  
1029 affecting the Na<sup>+</sup>/K<sup>+</sup>-ATPase pump, encoded by *ATP1A1*, lead to increase of intracellular Na<sup>+</sup>  
1030 and H<sup>+</sup> concentration, thus resulting in cell membrane depolarization and decrease of  
1031 intracellular pH.

1032

1033 **Figure 4: Aldosterone biosynthesis in APA in presence of mutations affecting**  
1034 **intracellular calcium balance.** Mutations in *ATP2B3* coding for PMCA3, *CACNA1D*,  
1035 encoding the  $\alpha$  subunit of the L-type Ca<sup>2+</sup> channel Cav1.3, and *CACNA1H*, encoding the  $\alpha$   
1036 subunit of the T-type Ca<sup>2+</sup> channel Cav3.2 affect directly intracellular Ca<sup>2+</sup> concentrations.

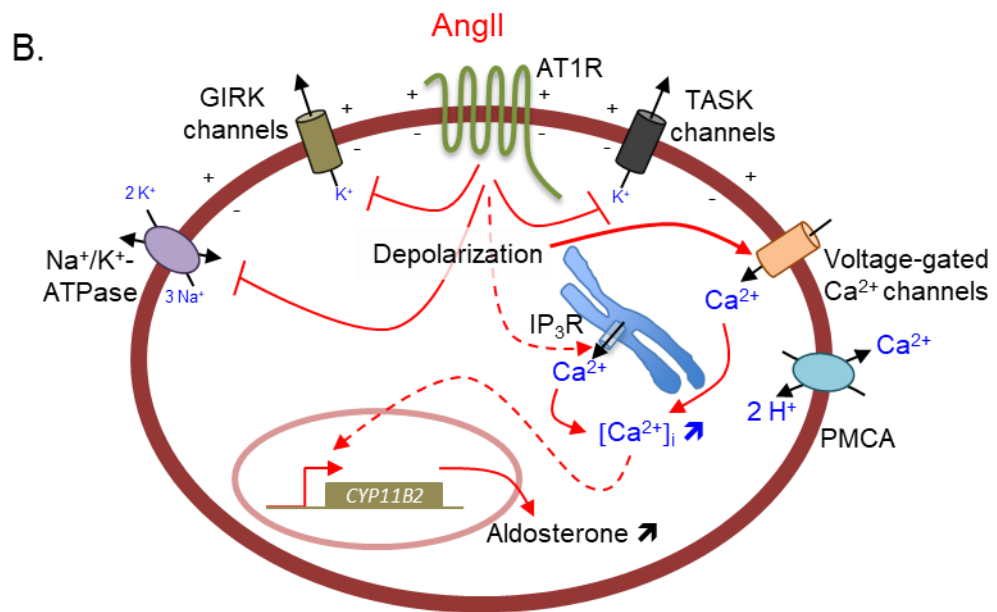
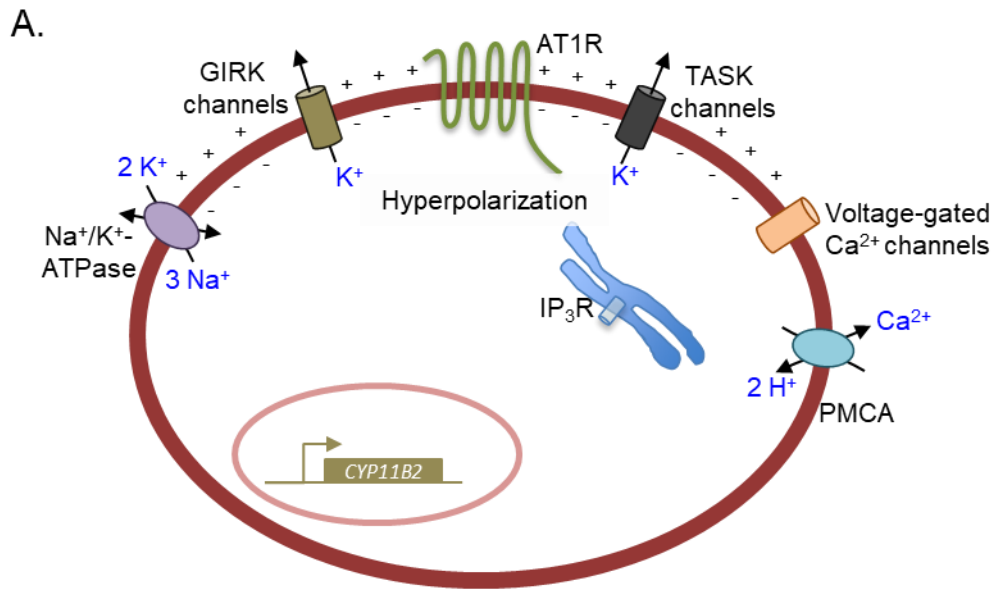


Figure 1



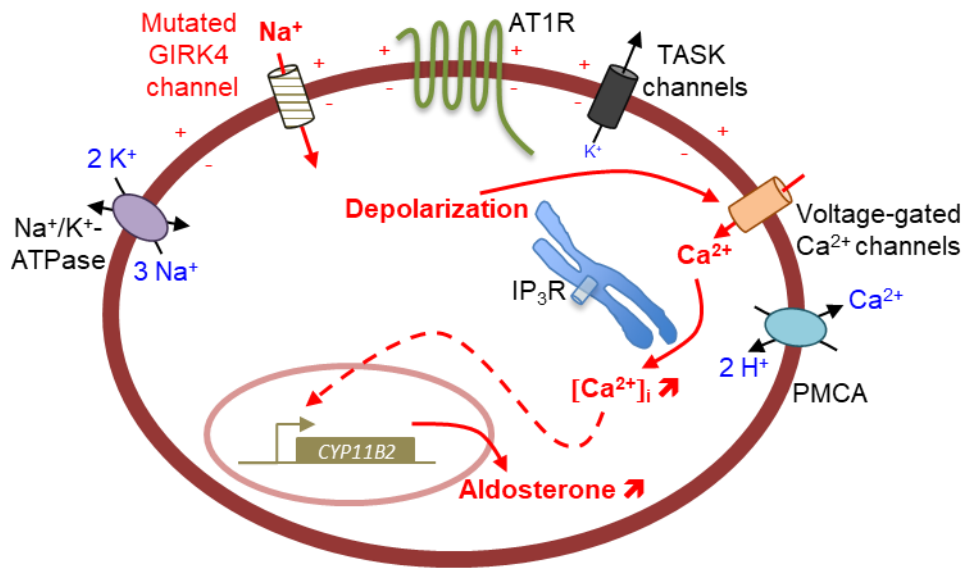


Figure 2

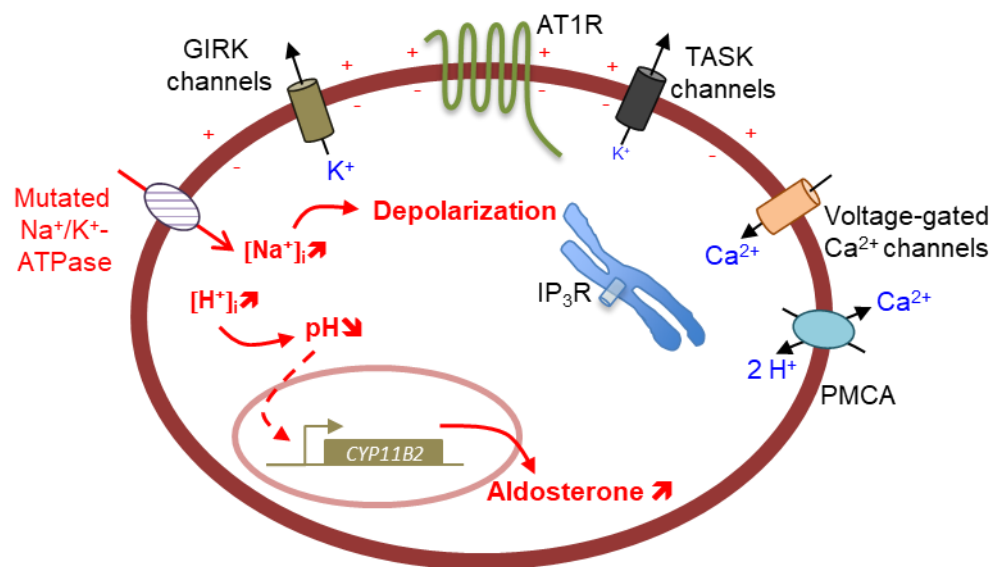


Figure 3

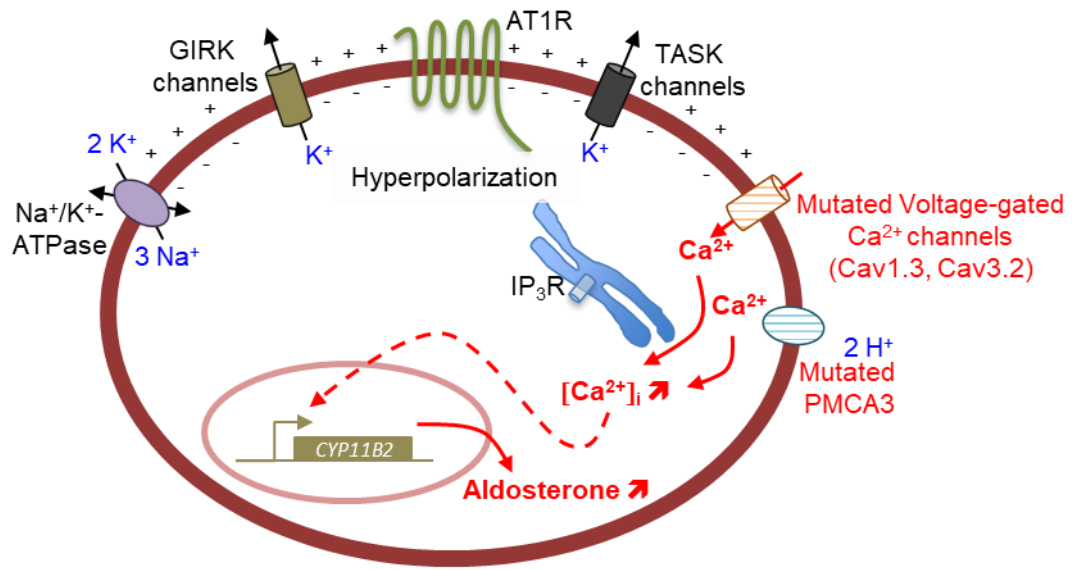


Figure 4