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Molecular mechanisms in primary aldosteronism

Kelly De Sousa^{1,2}, Alaa B Abdellatif^{1,2}, Rami M El Zein^{1,2}, Maria-Christina Zennaro^{1,2,3},

Affiliations:

¹INSERM, UMRS_970, Paris Cardiovascular Research Center, Paris, France

²Université Paris Descartes, Sorbonne Paris Cité, Paris, France

³Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Service de Génétique, Paris, France

Address correspondence to:

Maria-Christina Zennaro, MD, PhD

INSERM, U970

Paris Cardiovascular Research Center – PARCC

56, rue Leblanc,

75015 Paris – France

Tel : +33 (0)1 53 98 80 42

Fax : + 33 (0)1 53 98 79 52

e-mail: maria-christina.zennaro@inserm.fr

1 **Abstract**

2 Primary aldosteronism (PA) is the most common form and an under-diagnosed cause of
3 secondary arterial hypertension, accounting for up to 10% of hypertensive cases and associated
4 to increased cardiovascular risk. PA is caused by autonomous overproduction of aldosterone by
5 the adrenal cortex. It is mainly caused by a unilateral aldosterone-producing adenoma (APA)
6 or by bilateral adrenal hyperplasia. Excess aldosterone leads to arterial hypertension with
7 suppressed renin, frequently associated to hypokalemia. Mutations in genes coding for ion
8 channels and ATPases have been identified in APA, explaining the pathophysiology of
9 increased aldosterone production. Different inherited genetic abnormalities led to the
10 distinction of four forms of familial hyperaldosteronism (Type I to IV) and other genetic defects
11 very likely remain to be identified. Somatic mutations are identified in APA, but also in
12 aldosterone producing cell clusters (APCC) in normal adrenals, in image-negative unilateral
13 hyperplasia, in transitional lesions and in APCC from adrenals with BAH. Whether these
14 structures are precursors of APA or whether somatic mutations occur in a proliferative adrenal
15 cortex, is still a matter of debate. This review will summarize our knowledge on the molecular
16 mechanisms responsible for PA and the recent discovery of new genes related to early onset
17 and familial forms of the disease. We will also address new issues concerning genomic and
18 proteomic changes in adrenals with APA and discuss adrenal pathophysiology in relation to
19 aldosterone producing structures in the adrenal cortex.

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21
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Keywords: hypertension, adrenal, primary aldosteronism; ion channels; calcium

Introduction

24 Primary aldosteronism is due to excessive and autonomous aldosterone production by the
25 adrenal cortex. It is the most frequent form of secondary hypertension with an estimated
26 prevalence up to 10% in referred patients, 4% in primary care (Hannemann and Wallaschofski
27 2012; Monticone, et al. 2017) and 20% in patient with resistant hypertension (Calhoun, et al.
28 2002; Douma, et al. 2008). PA is characterized by an increased aldosterone to renin ratio, which
29 is the principal feature used for screening, often hypokalemia and metabolic alkalosis and an
30 increased risk of cardiovascular complications. The prevalence of left ventricular hypertrophy
31 is higher in patients with PA even after adjustment for hypertension duration (Rossi, et al.
32 1996). Moreover, PA patients have a significantly higher prevalence of coronary artery disease,
33 nonfatal myocardial infarction, heart failure, and atrial fibrillation (Savard, et al. 2013). The
34 two major causes of PA are aldosterone producing adenomas (APA) and bilateral adrenal
35 hyperplasia (BAH), also called idiopathic hyperaldosteronism (IHA). Although guidelines for
36 the management of PA have been published and are widely used in reference centers (Funder,
37 et al. 2016), improvements for better diagnosis, subtype identification and treatment for non-
38 operable patients are urgently required to allow for improved care and prevention of
39 cardiovascular complications.

40 Recently, whole exome sequencing had allowed identifying several somatic genetic
41 abnormalities in APA, providing a pathogenic model for PA development. Recurrent somatic
42 mutations were identified in genes coding for ion channels [*KCNJ5* (Choi, et al. 2011) and
43 *CACNA1D* (Azizan, et al. 2013; Scholl, et al. 2013)] and ATPases [*ATP1A1* and *ATP2B3*,
44 (Azizan et al. 2013; Beuschlein, et al. 2013)] regulating intracellular ionic homeostasis and cell
45 membrane potential. Germline mutations were identified in familial forms of PA, allowing to
46 distinguish four different forms of familial hyperaldosteronism (FH), based on the underlying
47 genetic defect (Fernandes-Rosa, et al. 2018; Scholl, et al. 2018; Zennaro 2019). These mutations
48 increase intracellular calcium concentrations, leading to activation of the calcium signaling
49 pathway and to increased aldosterone production by increasing the expression of *CYP11B2*,
50 coding for aldosterone synthase. In addition, somatic mutations were also identified in *CTNNB1*
51 coding for b-catenin in a small proportion of APA (Akerstrom, et al. 2016; Scholl, et al. 2015a;
52 Teo, et al. 2015) and mutations in *PRKACA* (encoding Protein kinase cAMP-Activated
53 Catalytic Subunit α) have been described in two cases (Rhayem, et al. 2016).

54 Even though the consequences of these mutations on aldosterone production are well
55 demonstrated, their impact on cell proliferation and APA formation is not well understood.

56 Different pathogenic mechanisms have been proposed, including different hits being
57 responsible for adrenocortical cell proliferation and hormonal secretion, or the development of
58 APA from aldosterone producing cell clusters (APCC) of the adrenal cortex.

59

60 **Steroid biosynthesis in the adrenal cortex**

61 The adrenal cortex is composed of three separate layers, the zona glomerulosa, the zona
62 fasciculata and the zona reticularis. This zonation is functional, each zone producing specific
63 steroid hormones due to the zone-specific expression of two enzymes, the aldosterone synthase
64 and the 11 β -hydroxylase. Cholesterol is the common precursor in steroid hormone biosynthesis
65 through a process that implicates multiple enzymatic steps, the major effectors being enzymes
66 belonging to the cytochrome P450 family. In zona glomerulosa, where aldosterone is produced,
67 cholesterol is converted into 11-deoxycorticosterone through the successive actions of the
68 cytochrome P450 side-chain cleavage (*CYP11A1*), the 3 β -hydroxysteroid dehydrogenase type
69 II (*HSD3B2*) and the 21 α -hydroxylase (*CYP21A2*). Aldosterone synthase (encoded by the
70 *CYP11B2* gene) catalyses the three final steps in aldosterone biosynthesis. The first step consists
71 in the hydroxylation at the C11 position of the intermediate steroid deoxycorticosterone into
72 corticosterone, followed by a hydroxylation at the C18 position and the formation of 18-
73 hydroxy-corticosterone. The last step is the oxidation of the hydroxyl group on C18, finally
74 resulting in the production of aldosterone (Connell and Davies 2005).

75 Cortisol biosynthesis occurs in the zona fasciculata, where 17 α -hydroxylase is expressed. This
76 enzyme is responsible for the conversion of pregnenolone and progesterone to 17-OH-
77 pregnenolone and 17-OH-progesterone, precursors of cortisol, whereas 21-hydroxylase induces
78 a hydroxylation in the C11 position of the 17OH-progesterone producing 11-deoxycortisol. The
79 final step of cortisol biosynthesis is the hydroxylation in the C11 position of 11-deoxycortisol
80 by 11 β -hydroxylase, encoded by *CYP11B1* and specifically expressed in the zona fasciculata
81 and reticularis (Connell and Davies 2005).

82 Aldosterone production is mainly regulated by the renin angiotensin system and potassium
83 concentrations and, to a lesser extent, by the adrenocorticotrophic hormone (ACTH) (Connell
84 and Davies 2005). The renin angiotensin system plays a major role in sodium homeostasis. In
85 response to a decrease in blood volume, dehydration or hyponatremia, renin is secreted by the
86 juxta-glomerulus apparatus of the kidney, and cleaves the angiotensinogen produced in the liver
87 to produce angiotensin I. In the lungs, angiotensin I is converted by the angiotensin converting

88 enzyme (ACE) to angiotensin II (Ang II). Ang II acts via its AT1 receptor expressed in zona
89 glomerulosa cells, activating a Gαq-phospholipase C-mediated pathway, inducing the
90 production of inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol. IP3 binding to its
91 receptor leads to calcium release from the endoplasmic reticulum, thus increasing intracellular
92 calcium concentrations. In parallel, Ang II also inhibits the potassium channels TASK (TWIK-
93 related acid-sensitive potassium channel) and GIRK4 (G-protein-activated inward rectifier
94 potassium channel) and the Na⁺/K⁺ ATPase, therefore inducing cell membrane depolarization,
95 and the opening of voltage-dependent membrane calcium channels, leading to calcium influx
96 into the cell and activation of calcium signaling (Oki, et al. 2012a; Spat and Hunyady 2004)
97 (Figure 1A and B). Activation of calcium signaling increases the availability of cholesterol in
98 the cell, by stimulating the activity of the enzyme cholesterol ester hydrolase, but also by
99 increasing the expression of StAR (steroid acute regulatory protein), which is responsible for
100 cholesterol transport into the mitochondria, and of other steroidogenic enzymes (Spat and
101 Hunyady 2004). Calcium/calmodulin dependent protein kinases also activate transcription
102 factors such as nuclear receptor subfamily 4 group A members 1 and 2 (NUR77/NGF1B and
103 NURR1 encoded by NR4A1 and NR4A2 respectively), which increase transcription of the
104 *CYP11B2* gene (Bassett, et al. 2004b).

105 Cortisol production is regulated by the hypothalamic-pituitary-adrenal axis via ACTH. ACTH
106 binds to the melanocortin receptor 2 (MC2R), inducing an increase in intracellular cAMP levels
107 via the activation of a Gαs protein. cAMP activates PKA signaling following its binding to the
108 regulatory subunits of PKA (Ruggiero and Lalli 2016). The duration of ACTH stimulation
109 affects its role in the biosynthesis of steroids. Short-term stimulation increases the availability
110 of intracellular cholesterol and its transfer to the internal membrane of the mitochondria,
111 primarily through the phosphorylation of StAR at the Ser195 position; it also induces the
112 expression of steroidogenic enzymes. However, long-term stimulation by ACTH induces
113 transcriptional regulation of StAR and steroidogenic enzymes. Particularly, PKA signaling
114 activates transcription factors such as steroidogenic factor 1 (SF-1), cAMP-responsive element
115 binding protein (CREB), cAMP responsive element modulator, CCAAT/enhancer-binding
116 protein and activator protein 1, which regulate the expression of steroidogenic genes (Ruggiero
117 and Lalli 2016).

118 ACTH stimulation also induces aldosterone production by similar mechanisms. Moreover,
119 ACTH stimulates calcium flow into zona glomerulosa cells by activating L-type calcium
120 channels. However, in the case of a chronic ACTH stimulation, *CYP11B2* expression is

121 inhibited, which causes a reduction in aldosterone production, as opposed to the expression of
122 *CYP11B1* which is responsible of cortisol production (Vinson 2016).

123 There exist other factors that regulate aldosterone production in a paracrine fashion. Among
124 them, serotonin (5-HT) produced by mastocytes stimulates steroid production (El Ghorayeb, et
125 al. 2016) through mechanisms involving the 5-HT receptor type 4 (HTR4) (Lefebvre, et al.
126 2001; Lefebvre, et al. 1993) which is expressed in adrenocortical cells. Activation of HTR4
127 activates adenylyl cyclase and induces calcium influx into the cell through T-type channels,
128 thus regulating cortisol and aldosterone production (Louiset, et al. 2017). Remarkably,
129 activation of 5-HT4 by agonists such as cisapride or zacopride induces aldosterone production
130 in healthy volunteers (Lefebvre, et al. 2000).

131

132 **Genetic alterations in PA**

133 The mechanisms that regulate aldosterone production are severely altered in PA. PA is the most
134 common form of secondary arterial hypertension, with an estimated prevalence of 10% in
135 referred patients in specialized centers and up to 5% in the general hypertensive population
136 (Hannemann and Wallaschofski 2012; Monticone et al. 2017; Rossi, et al. 2006). Although the
137 majority of cases are sporadic, PA may be transmitted as a Mendelian trait in familial forms of
138 the disease. Familial forms of PA account for 1-5% of cases and are transmitted as autosomal
139 dominant traits. Four different forms have been described, based on the underlying genetic
140 defect. Similarly, recurrent somatic mutations in different genes have been identified in up to
141 88% of APA (Nanba, et al. 2018).

142

143 **Germline mutations in familial hyperaldosteronism**

144 **Familial hyperaldosteronism Type I**

145 FH-I or glucocorticoid-remediable aldosteronism (GRA) is an autosomal dominant disease
146 that accounts for 0.5 to 1% of PA in adult patients, but up to 3% in pediatric cohorts (Aglony,
147 et al. 2011; Medeau, et al. 2005; Mulatero, et al. 2011; Pallauf, et al. 2012). It is characterized
148 by early and severe hypertension, often associated to hypokalemia. Peculiar features of FH-I
149 are the presence of hybrid steroids 18-hydroxycortisol and 18-oxocortisol in the urines and the
150 suppression of aldosterone by dexamethasone (Sutherland, et al. 1966). These features are a
151 consequence of the underlying genetic defect, which consists in the formation of a chimeric

152 gene between the regulatory regions of *CYP11B1* and the coding regions of *CYP11B2* due to
153 unequal crossing over during meiosis. This results in ectopic expression of *CYP11B2* in the
154 zona fasciculata of the adrenal cortex, and ACTH-regulated expression of aldosterone synthase
155 (Lifton, et al. 1992; Stowasser, et al. 2000). Those patients can be efficiently treated with low
156 dose glucocorticoids (Stowasser et al. 2000); however, cardiovascular consequences of high
157 aldosterone excess may manifest even in young normotensive patients (Stowasser, et al. 2005)
158 and patients with FH-I are at increased cardiovascular risk, with increased number of
159 cerebrovascular events at young age (Litchfield, et al. 1998).

160 Familial hyperaldosteronism Type II

161 FH-II is a non-glucocorticoid remediable form of familial hyperaldosteronism, transmitted
162 in an autosomal dominant fashion, found in 1.2 to 6% of adult patients with PA. Patients display
163 a familial history of PA, and different subtypes of the disease (APA or BAH) may be present
164 within the same family (Medeau et al. 2005; Mulatero et al. 2011; Pallauf et al. 2012; Stowasser
165 and Gordon 2001). FH-II is not distinguishable from other sporadic forms of PA, the diagnosis
166 is based on the identification of two or more affected family members. While a genetic locus
167 was associated with FH-II on 7p22 (1996) (Lafferty, et al. 2000), no pathogenic mutations were
168 identified in several candidate genes located in this chromosomal region. It is only very recently
169 that use of whole exome sequencing allowed the discovery of a new gene associated to FH-II.
170 Two groups have indeed identified several gain-of-function mutations in the *CLCN2* gene
171 coding for the CLC-2 chloride channel in patients with FH-II and early onset PA and described
172 for the first time the implication of a chloride channel in the regulation of aldosterone
173 production (Figure 1C).

174 In one study, a *de novo* heterozygous p.Gly24Asp mutation in the *CLCN2* gene was
175 identified in a patient diagnosed at 9 years of age with hypertension due to PA, associated to
176 profound hypokalemia (Fernandes-Rosa et al. 2018). This gain-of-function mutation affects a
177 highly conserved region in the CLC-2 chloride channel, in which mutations lead to constitutive
178 activation of the channel (Grunder, et al. 1992). The channel is highly expressed in the human
179 and mouse adrenal zona glomerulosa and patch clamp analyses of zona glomerulosa cells from
180 mouse adrenal gland slices show *bona fide* chloride currents, which are abolished in *Clcn2*
181 knock-out mice, indicating that CLC-2 is the foremost chloride channel in the adrenal zona
182 glomerulosa (Fernandes-Rosa et al. 2018). Expression of the mutated CLC-2 in adrenocortical
183 cells leads to increased chloride currents and depolarization of the zona glomerulosa cell
184 membrane, opening voltage-gated calcium channels and triggering autonomous aldosterone

185 production (Figure 1C). This work indicates for the first time the implication of an anion
186 channel in the regulation of aldosterone biosynthesis, primary aldosteronism and hypertension
187 (Fernandes-Rosa et al. 2018).

188 In the other study, whole exome sequencing of three members of a large Australian family
189 with FH-II previously described by Stowasser et al (Lafferty et al. 2000; Stowasser, et al. 1992)
190 allowed the identification of a germline mutation in the *CLCN2* gene, p.Arg172Gln, in affected
191 family members. The same mutation was identified in three additional cases with early onset
192 PA, one within a familial context, another occurring *de novo*. Furthermore, the authors
193 identified 4 additional germline *CLCN2* variants, p.Met22Lys, p.Tyr26Asn, p.Lys362del et
194 p.Ser865Arg in 4 unrelated patients with early onset PA (Scholl et al. 2018).
195 Immunohistochemistry confirmed the expression of ClC-2 in human adrenal zona glomerulosa.
196 Mutant channels showed gain of function, with higher open probabilities at the glomerulosa
197 resting potential.

198 Altogether, these results indicate that *CLCN2* should be included in the panel of genes
199 that are screened in patients who develop early-onset primary aldosteronism and in families
200 with a diagnosis of FH-II. Mutations may be located in different domains of ClC-2 and therefore
201 affect channel function at different degrees, which explains phenotypic heterogeneity and the
202 presence of relatively mild cases.

203 Familial hyperaldosteronism Type III and germline *KCNJ5* mutations

204 FH-III is a non-glucocorticoid remediable form of FH, showing early-onset severe
205 hypertension, hyperaldosteronism, profound hypokalemia and high levels of the hybrid steroids
206 18-oxocortisol and 18-hydroxycortisol (Geller, et al. 2008). Patients require bilateral
207 adrenalectomy to control blood pressure, which shows massive bilateral hyperplasia of the
208 adrenal cortex, although mild forms have also been described. Recurrent germline mutations in
209 the *KCNJ5* gene (coding for the potassium channel GIRK4) have been identified in FH-III
210 (Choi et al., 2011). *KCNJ5* codes for the G protein-activated inward rectifier potassium channel
211 4 (GIRK4), which is composed of two membrane-spanning domains (M1 and M2), one pore
212 forming region (H5) and cytoplasmic N- and C-termini that participate to the pore structure.
213 The mutations are located in a highly conserved domain within or near the channel selectivity
214 filter. They induce a gain-of-function, with a loss of potassium selectivity in favor of an
215 increased unspecific sodium influx into the cytoplasm. This results in cell membrane
216 depolarization, increased intracellular calcium concentrations, activation of Ca²⁺ signaling and

217 autonomous aldosterone biosynthesis (Choi et al. 2011; Oki, et al. 2012b) (Figure 1C). Different
218 *KCNJ5* mutations have been described in the literature, which not all are associated with severe
219 PA (Mulatero, et al. 2013). Indeed, *KCNJ5* mutations were also identified in patients diagnosed
220 with mild PA in a context of FH-II, with no bilateral adrenal hyperplasia at imaging and blood
221 pressure controlled by conventional medication. The genetic diagnosis allowed reclassifying
222 those patients into mild forms of FH-III. Remarkably, mutations associated with milder
223 phenotypes have more pronounced functional effects *in vitro*. In particular, the channel's
224 selectivity was less affected by the p.Thr158Ala mutation compared to the channels carrying
225 the other mutations (p.Leu168Arg and p.Gly151Arg) (Choi et al. 2011). The p.Gly151Glu
226 mutation, identified in mild cases diagnosed as FH-II, generates significantly larger Na⁺
227 currents than the p.Gly151Arg mutation and leads to sodium-dependent cell death (Scholl, et
228 al. 2012), with strong depolarization of adrenocortical cells expressing the mutated channel
229 (Mulatero, et al. 2012).

230 Familial hyperaldosteronism Type IV and germline CACNA1H mutations

231 FH-IV is a non-glucocorticoid remediable form of FH, which has been attributed to germline
232 *CACNA1H* mutations. Familial analysis showed that despite autosomal dominant transmission
233 of the mutation, differences could be observed within family members on a clinical level,
234 indicating incomplete penetrance of the disease (Scholl, et al. 2015b).

235 *CACNA1H* codes for the $\alpha 1$ subunit of the voltage-gated T-type calcium channel Cav3.2.
236 The structure of this subunit consists of 4 homologous domains (named from I-IV) where each
237 domain contains 6 transmembrane helices (S1-S6). A recurrent p.Met1549Val mutation in
238 *CACNA1H* was first identified in 5 patients with hypertension due to PA by age 10 and their
239 relatives. The mutation affects the electrophysiological properties of the channel, inducing
240 specifically a shift in the voltage sensitivity of the channel to a more negative current and a
241 modification of its inactivating properties, which triggers an opening of the channel at less
242 negative membrane potentials, allowing activation of calcium signaling in the absence of
243 stimulation (Scholl et al. 2015b) (Figure 1D). Scholl et al demonstrated in their study that cells
244 transfected with the Cav3.2 1549Val had more aldosterone biosynthesis as well as higher
245 *CYP11B2* expression when compared to cells transfected with the wild type Cav3.2 channel.

246 In a similar study, Daniil et al identified four germline mutations in the *CACNA1H* gene in
247 PA patients with different phenotypic presentations. Two of the variants (p.Val1951Glu and
248 p.Pro2083Leu) in this study are located in the cytoplasmic C terminus of the channel. A

249 p.Ser196Leu variant was located in the S4 segment of domain I of the channel, and one
250 mutation, p.Met1549Ile, is located in the S6 segment of domain III. The p.Met1549Ile mutation,
251 affecting the same amino acid described by Scholl et al in patients with FH-IV, was identified
252 in a patient with early onset PA and multiplex developmental disorder. Cav3.2 p.Ser196Leu
253 and p.Pro2083Leu were found in two patients with FH-II, and p.Val1951Glu was identified in
254 one patient with APA. Electrophysiological analysis revealed significant changes in the Ca²⁺
255 current properties for all mutants, suggesting a gain of function phenotype. Moreover,
256 transfection of the mutant Cav3.2 in the adrenocortical cell line H295R-S2 led to increased
257 aldosterone production and increases the expression of steroidogenic enzymes following
258 potassium stimulation (Daniil, et al. 2016)

259 Germline CACNAID mutations in PA

260 *CACNAID* mutations have been described in two children with PASNA (primary
261 aldosteronism, seizures and neurological abnormalities) (Scholl et al. 2013). *CACNAID* codes
262 for the $\alpha 1$ subunit of the voltage-gated L-type calcium channel Cav1.3. Again, these mutations
263 induce a gain-of-function, with the channel opening at lower voltages. This leads to excessive
264 aldosterone production by increasing Ca²⁺ entry that causes an increased intracellular Ca²⁺-
265 mediated signaling with induction of aldosterone biosynthesis (Azizan et al. 2013; Scholl et al.
266 2013).

267 The first case was identified in a 3-year old female who suffered severe hypertension,
268 biventricular hypertrophy, neurological abnormalities and aldosteronism as a newborn,
269 associated with high aldosterone levels, a high aldosterone to renin ratio and hypokalemia.
270 Genetic analyses identified a germline mutation in *CACNAID* (p.Gly403Asp), a variant that
271 had also been identified in APA (Scholl et al. 2013; Scholl et al. 2015a). The second subject
272 exhibited cerebral palsy and complex seizures just after birth, HT and hyperaldosteronism with
273 hyperkalemia. Genetic analysis lead to the identification of a *CACNAID* mutation
274 (p.Ile770Met) also found as recurrent mutation in APA (Korah and Scholl 2015; Scholl et al.
275 2013) (see below).

276

277 **Somatic mutations in APA**

278 Numerous recurrent somatic mutations have been identified in APA. These affect genes
279 coding for ion channels (*KCNJ5* (Choi et al. 2011) and *CACNAID* (Azizan et al. 2013; Scholl

280 et al. 2013)) also involved in inherited forms. In addition, recurrent mutations have been
281 identified in genes coding for ATPases [*ATP1A1* which codes for the $\alpha 1$ subunit of the Na^+/K^+ -
282 ATPase (Azizan et al. 2013; Beuschlein et al. 2013) and *ATP2B3* encoding the plasma
283 membrane calcium-transporting ATPase 3 (PMCA3) (Beuschlein et al. 2013)] that regulate the
284 intracellular ionic homeostasis and membrane potential. Na^+/K^+ -ATPase and PMCA3 are
285 members of the P-type family of ATPases and are composed of ten transmembrane domains
286 (M1 – M10) with intracellular N- and C-tails. *ATP1A1* mutations affect amino acids in the
287 transmembrane helices M1, M4, and M9 of the $\alpha 1$ subunit. They lead to a loss of pump activity
288 and a reduction in its affinity for potassium as well as an inward leakage of sodium, inducing
289 membrane depolarization (Azizan et al. 2013; Beuschlein et al. 2013). In addition, mutant
290 pumps may induce a proton leak (Azizan et al. 2013), leading to intracellular acidification
291 which contributes to aldosterone production. Indeed, expression of mutant $\alpha 1$ subunits of the
292 Na^+/K^+ ATPase carrying the L104R, V332G, G99R mutations in adrenocortical NCI-H295R
293 cells failed to increase intracellular Ca^{2+} concentrations, but showed intracellular acidification,
294 which caused increased aldosterone production (Stindl, et al. 2015). Under physiological
295 conditions, the PMCA3 extrudes one cytosolic Ca^{2+} in exchange for two H^+ . PMCA3 mutations
296 occur in the transmembrane domain M4 and induce the deletion of a stretch of amino acids
297 between Leu425 and Leu433 (Beuschlein et al. 2013; Fernandes-Rosa, et al. 2014). Cells
298 expressing the mutant PMCA3 have a reduced capacity to export Ca^{2+} , suggesting a loss of the
299 physiological pump function. In addition, they induce an increased Ca^{2+} influx possibly due to
300 opening of depolarization-activated Ca^{2+} channels or to Ca^{2+} leak through the mutated pump
301 (Tauber, et al. 2016). All these mutations induce an increase in intracellular calcium levels,
302 followed by an activation of calcium signaling and therefore increase the biosynthesis of
303 aldosterone.

304 A study on 474 subjects presenting an APA, performed within the European Network for the
305 Study of Adrenal Tumors (ENS@T), found mutations in 54.2% of APA, where 38% of these
306 were found in the *KCNJ5* gene, 9.3% in *CACNAID*, 5.3% in *ATP1A1* and 1.7% in *ATP2B3*
307 (Fernandes-Rosa et al. 2014). Patients presenting mutations in *KCNJ5* were mostly women and
308 younger patients, while APA with *CACNAID* mutations were smaller than the other mutational
309 groups (Fernandes-Rosa et al. 2014). A recent meta-analysis on 1636 patients from 13 studies
310 showed a prevalence of *KCNJ5* mutations of 43%, with much higher frequencies (up to 77%)
311 in populations from East Asia in comparison to studies performed on European populations.
312 APA harboring *KCNJ5* mutations were more frequent in women and associated with larger

313 tumors and a higher plasma aldosterone concentration when compared with mutation-negative
314 APA. APA where *KCNJ5* mutations are present are also associated with APA composed mainly
315 of ZF-like cells. Other mutations were associated mainly with male gender and smaller tumors
316 (Lenzini, et al. 2015).

317 In a recent study, Nanba et al performed CYP11B2 immunohistochemistry on 75 adrenal
318 samples from white American patients, in order to identify APA and other aldosterone
319 producing regions prior to genetic analysis. DNA was then extracted directly from formalin
320 fixed paraffin embedded (FFPE) tissue, positive for CYP11B2 expression. Complete next
321 generation sequencing of all genes involved in PA identified somatic mutations in 88% of APA
322 (Nanba et al. 2018). Again, *KCNJ5* mutations were the most frequent mutational events (43%),
323 followed by *CACNAID* (21%), *ATPIA1* (17%), *ATP2B3* (4%) and *CTNNB1* (3%) mutations.
324 Also, *KCNJ5* mutations were more frequent in women (70% vs 24% in men). This study shows
325 that CYP11B2 immunohistochemistry-guided next generation sequencing allows the
326 identification of a larger portion of somatic mutations in APA. Similar results were obtained by
327 analyzing adrenocortical adenomas from 79 black patients with PA. Somatic mutations in
328 aldosterone-driver genes were found in 89% of APAs. Interestingly, *CACNAID* mutations were
329 the most frequent (42%) ones in APA from patients with African ancestry, followed by *KCNJ5*
330 (34%), *ATPIA1* (8%), and *ATP2B3* mutations (4%) (Nanba, et al. 2019).

331 Other less frequent mutations that play an important role in APA were identified in *CTNNB1*
332 and *PRKACA*. *CTNNB1* codes for β -Catenin. This protein is a central element of the Wnt/ β -
333 Catenin signaling pathway, which has an essential role in the development of the adrenal cortex,
334 in the differentiation of the zona glomerulosa and steroid hormone biosynthesis (El Wakil and
335 Lalli 2011). In unstimulated conditions, β -catenin is present in the cytoplasm, and is associated
336 to the degradation complex with adaptor proteins such as Axin, APC (adenomatous polyposis
337 coli), two serine/threonine kinases, casein kinase 1 (CK1 α) and glycogen synthase kinase 3 β
338 (GSK3 β). This complex phosphorylates β -Catenin, which is then recognized by the ubiquitin
339 ligase E3 that results in its destruction by the proteasome, thus preventing its translocation into
340 the nucleus and the activation of different Wnt target-genes. Activation of the Wnt/ β -Catenin
341 signaling pathway by binding of Wnt to its receptor frizzled inhibits the phosphorylation of β -
342 Catenin which dissociates from the axin complex, and translocates to the nucleus where it
343 induces the expression of Wnt target genes, by binding to the transcription factors TCF (T-cell
344 factor) and LEF (lymphocyte enhancer factor) as a transcriptional co-activator (MacDonald, et
345 al. 2009). It has been shown that β -Catenin indirectly regulates *CYP11B2* expression via

346 transcriptional induction of the nuclear receptors NURR1 and NUR77 (Berthon, et al. 2014).
347 The Wnt/ β -Catenin signaling pathway has been shown to be constitutively active in 70% of
348 APA (Berthon et al. 2014). Mutations in *CTNNB1* have been found in 2-5% of APA and are
349 more frequent in women (Akerstrom et al. 2016; Scholl et al. 2015a; Teo et al. 2015). Mutations
350 specifically occur in exon 3, which codes for the serine/threonine residue which when
351 phosphorylated results in the degradation of β -Catenin (Berthon, et al. 2010). To a much lesser
352 extent, somatic mutations in *PRKACA*, which codes for the cAMP-dependent protein kinase
353 catalytic subunit α , have been described in APA (Rhayem et al. 2016). A study performed on a
354 cohort of 12 patients showed a prevalence of 1.6% of this mutation. One of the described
355 mutations in this study was new (p.His88Asp) (Rhayem et al. 2016), whereas the other
356 mutation, p.Leu206Arg, had been previously described as a recurrent somatic mutation in
357 patients with cortisol producing adenoma and Cushing's syndrome (Goh, et al. 2014).
358 Mutations in both *PRKACA* and *CTNNB1* have been mainly associated with cortisol producing
359 adenomas and/or adrenocortical cancer. Therefore, the mechanisms explaining how the same
360 mutations result in two different hormonal phenotypes remain to be established.

361

362 **Genomic and proteomic alterations in APA**

363 Despite the major advances on the genetics of PA, many questions remain open concerning the
364 pathophysiology of the disease, in particular the mechanisms and dynamics of adenoma
365 formation. Several studies have explored genomic and proteomic alterations in APA, to better
366 understand the mechanisms involved in its development. A recent extremely original work
367 performed deep quantitative proteomic and phosphoproteomic profiling on APA and adjacent
368 non-tumoral adrenal cortex from 6 patients with PA. The authors identified, out of 5555 proteins
369 common to all samples, 18 which were significantly down-regulated and 11 significantly up-
370 regulated in all APA. Proteome data confirmed that upregulation of the steroidogenic enzymes
371 HSD3B2, CYP21A2, CYP11B2 and of proteins involved in cholesterol uptake (lipolysis
372 stimulated lipoprotein receptor, LSR) is involved in the increase of steroidogenesis in APA
373 (Swierczynska, et al. 2019). This study also showed that steroidogenic enzymes are regulated
374 by phosphorylation; in particular, HSD3B2 is phosphorylated at Ser95 or 96 and CYP21A2 has
375 a novel phosphorylation site at Ser489. An altered composition of the extracellular matrix
376 (ECM) was also revealed in APA, with reduction in proteins related to collagen and collagen
377 fibril assembly, enzymes involved in extracellular matrix turnover and structural components
378 of the extracellular matrix. The deregulation of kinases affecting cytoskeleton remodeling and

379 axonal guidance related pathways suggests that APA development may be accompanied or be
380 related to changes in cytoskeleton rearrangements and possibly innervation. Remarkably, the
381 GTPase RHOC controlling actin organization, which was upregulated in APA, significantly
382 increased expression of *CYP11B2* at the transcriptional level in transfected adrenocortical
383 H295R cells.

384 APA also showed higher levels of proteins involved in N-glycosylation and enzymes involved
385 in GABA degradation. Remarkably, the activity of the main regulators of steroidogenesis,
386 MC2R (receptor for ACTH) and AT1R (receptor for Ang II) is affected by N-glycosylation and
387 the level and the activity of N-glycoproteins like lipoprotein receptors (LDLR and SR-B1) and
388 many ion channels (TASK1, TASK3, TREK1, Kv1.4, CACNA1D) are affected by N-
389 glycosylation. The inhibition of TASK1, TASK3 and KV1.4 glycosylation decreases the
390 number of cell surface channels, leading to a reduced current flow, protein instability and
391 intracellular channel retention (Mant, et al. 2013; Swierczynska et al. 2019; Watanabe, et al.
392 2004). Reduced GABAergic signaling in APA may also modulate aldosterone production.
393 Indeed, in physiological conditions, GABAergic signaling mediates a decrease of
394 steroidogenesis in vivo in the rat adrenal cortex (Mishunina and Kononenko 2002). Finally, one
395 of the most dysregulated pathways in APAs appeared to be the mTORC1 signaling pathway.
396 Its activity was increased in APA; this pathway is involved in cell proliferation and
397 steroidogenesis in APA cells and immortalized adrenocortical cells. Thus, the increase of its
398 activation may lead to an increased steroidogenic output and adenoma development. This opens
399 new perspectives on the benefits of inhibiting mTORC1 in patients with APA (Su, et al. 2013;
400 Swierczynska et al. 2019).

401 Similarly, gene expression profiling of APA allowed identifying genes and intracellular
402 signaling pathways involved in the pathogenesis and pathophysiology of this tumor. As
403 expected, microarray studies and SAGE (serial analysis of gene expression) showed higher
404 expression of *CYP11B2* in APA compared to normal adrenals and adjacent cortical tissue
405 (Assie, et al. 2005; Williams, et al. 2010), although other studies report a subgroup of APA with
406 reduced expression of aldosterone synthase (Lenzini, et al. 2007). However, these data require
407 to be reevaluated in the context of recent work showing that in a certain number of multinodular
408 adrenals some of the larger nodules do not express *CYP11B2*, which is localized to smaller
409 nodules or APCC (Fernandes-Rosa, et al. 2015; Nanba et al. 2018); this finding may have led
410 to some sample biases in earlier studies. Despite this, APA show a genotype-phenotype
411 correlation indicating that transcriptional signatures may be influenced by APA genotype,

412 although different studies report slightly different results. Indeed, APA with different gene
413 mutations show distinct expression profiles (Azizan, et al. 2012). While some studies reported
414 a higher *CYP11B2* expression in APA with *KCNJ5* mutations compared to APA without
415 *KCNJ5* mutation (Monticone, et al. 2012; Seccia, et al. 2012), a higher expression of *CYP11B2*
416 was reported in APA with *ATP1A1* and *ATP2B3* mutations, compared to *KCNJ5* mutated
417 tumors (Akerstrom, et al. 2015). In a large transcriptome analysis performed on 102 samples,
418 *KCNJ5*-mutated APA did not show differential clustering compared to tumors not harboring
419 *KCNJ5* mutations (Boulkroun, et al. 2012; Zennaro 2019).

420 Several studies revealed an upregulation of genes encoding transcription factors (*NURR1* and
421 *NGF1B*) that regulate the transcription of *CYP11B2* (Bassett, et al. 2004a), genes encoding the
422 transcription factors SF-1 (*NR5A1*) and DAX1 (*NR0B1*) that play an essential role in adrenal
423 development and steroidogenesis (Bassett, et al. 2005), and a target gene of SF-1, *VSNLI*, which
424 increases aldosterone production in the NCI H295R cells (Williams, et al. 2012). Furthermore,
425 Azizan et al reported higher *CYP17A1* expression in APA composed of cells resembling to the
426 zona fasciculata carrying *KCNJ5* mutations (Azizan et al. 2012). This result was also confirmed
427 in *KCNJ5*-mutated tumors compared with *ATP1A1/CACNAID* mutant adenomas by
428 immunohistochemistry (Tan, et al. 2017).

429 Finally, a recent methylome analysis revealed hypomethylation of *CYP11B2* in APA and a
430 higher demethylation of G protein-coupled receptors (GPCR) and GPCR-related genes (Itcho,
431 et al. 2018). Thus, this hypomethylation may stimulate aldosterone production in APA through
432 receptors that would regulate gene transcription like ACTH, glucagon, somatostatin,
433 parathyroid hormone and glutamate metabotropic receptors. Remarkably, HTR4, MC2R, and
434 PTGER1 (prostaglandin E receptor 1), which all regulate aldosterone biosynthesis, showed
435 hypo-methylation and up-regulation of their mRNA in APA.

436

437 **Aldosterone producing cell clusters and the development of APA**

438 Different studies showed the existence of APCC in the subcapsular portion of the normal human
439 adrenal gland. These APCC, which are suggested to autonomously produce aldosterone, are
440 characterized by the presence of subcapsular zona glomerulosa-like cells and inner zona
441 fasciculata-like cells (Nishimoto, et al. 2010) and a uniform *CYP11B2*, but absent *CYP11B1*
442 expression (Nishimoto, et al. 2016). Therefore, APCC and APA are distinctive in their sizes,
443 cellular arrangements, and enzyme expression profiles, as APA may consist of heterogeneous

444 cell types expressing either CYP11B1 or CYP11B2. In the human adrenal gland, aldosterone
445 synthase staining is continuous in the zona glomerulosa at young age between 0 and 11 years,
446 whereas with increasing age the adrenal cortex loses the continuous staining of aldosterone
447 synthase and acquires a variegated zonation with increased number of CYP11B2 expressing
448 APCC (Omata, et al. 2017). The discontinuous CYP11B2 expression is suggested to be the
449 consequence of a negative feedback produced by the autonomous aldosterone production by
450 APCC.

451 Even though there is no sex difference in APA prevalence, a higher APCC score was described
452 in female adrenals (Nishimoto, et al. 2015). Transcriptome analyses identified markers for
453 APCC in addition to CYP11B2, which showed significantly higher transcript expressions in
454 APCC compared to zona glomerulosa or fasciculata. These genes code for proteins playing a
455 role in glucose transport (SLC35F1), aldosterone production (MC2R) and regulation of
456 intracellular phosphorylation and dephosphorylation (PPP4R4) (Nishimoto et al. 2015).

457 Different somatic mutations leading to autonomous aldosterone production were identified in
458 APCC from normal subjects, supporting a pathological behavior of APCC (Nishimoto et al.
459 2015). Next generation sequencing of 23 APCC samples from FFPE and frozen tissues from
460 normal adrenals showed that 35% of APCC harbored mutations observed in APA and known
461 to cause aldosterone overproduction in *CACNAID* (26%), and *ATP1A1* (6%). No mutations in
462 *KCNJ5* have been identified in APCC so far. Different explanations are given for this
463 observation, including that APCC with *KCNJ5* mutations may rapidly progress into APA
464 (Nishimoto et al. 2015).

465 Omata et al demonstrated that the accumulation of APCC is a cellular and molecular cause of
466 IHA. Examination of a cohort of 15 adrenals with IHA with CYP11B2 immunohistochemistry
467 and next-generation sequencing showed that the adrenal cortex of all IHA adrenals harbored at
468 least 1 CYP11B2-positive APCC or aldosterone-producing microadenomas. IHA adrenals had
469 a significantly larger number of APCC than normotensive controls. Somatic mutations were
470 identified in *CACNAID* in 58% of cases and only in 1 case in *KCNJ5*. These findings suggest
471 that not only hyperplasia but also the enlargement of APCC with somatic aldosterone driver
472 gene mutations (most likely *CACNAID*) are responsible for IHA (Omata et al. 2017).

473 Finally, transitional structures were described showing a subcapsular APCC-like portion and
474 inner APA-like portion (Nishimoto et al. 2016). These structures are called pAATL, possible
475 APCC-to-APA transitional lesions. These pAATL are characterized by the presence of

476 mutations in APA driver genes, in particular *KCNJ5* and *ATP1A1*, with *KCNJ5* mutations
477 identified exclusively in the APA-like portion of the structure (Nishimoto et al. 2016).

478

479 **Conclusion**

480 Although much progress has been made over the last ten years in our understanding of the
481 molecular mechanisms leading to PA, there still remain a number of open questions which
482 need to be addressed. In particular, in a certain number of familial cases, diagnosed mainly as
483 FH-II, no genetic abnormality has been identified in known genes. This is also the case for a
484 small proportion of APA, in which even CYP11B2-guided next generation sequencing of all
485 coding exons of all APA driver genes fails identifying somatic mutations. This suggests that
486 additional genes may be involved in the disease, which remain to be discovered. Another
487 question relates to the mechanisms leading to APA development. Indeed, germline mutations
488 identified in familial forms lead to adrenal cortex hyperplasia, but not to APA. Whether
489 nodule formation requires an additional genetic hit favoring increased proliferation or whether
490 APA derive from APCC carrying somatic mutations remains to be clarified. A related
491 question is how do APCC develop, in particular how and when do somatic mutations occur
492 and whether there is any difference between APCC with and without somatic mutations.
493 Future work addressing these issues will allow progressing in our knowledge on the molecular
494 mechanisms involved in normal aldosterone physiology and development of PA and possibly
495 provide new tools for the development of better diagnostic and therapeutic approaches.

496

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The authors have nothing to disclose.

497

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505 **References**

506

- 507 1996 Effectiveness of spironolactone added to an angiotensin-converting enzyme inhibitor
508 and a loop diuretic for severe chronic congestive heart failure (the Randomized Aldactone
509 Evaluation Study [RALES]). *Am J Cardiol* **78** 902-907.
- 510 Aglony M, Martinez-Aguayo A, Carvajal CA, Campino C, Garcia H, Bancalari R, Bolte L,
511 Avalos C, Loureiro C, Trejo P, et al. 2011 Frequency of familial hyperaldosteronism type 1 in
512 a hypertensive pediatric population: clinical and biochemical presentation. *Hypertension* **57**
513 1117-1121.
- 514 Akerstrom T, Maharjan R, Sven Willenberg H, Cupisti K, Ip J, Moser A, Stalberg P,
515 Robinson B, Alexander Iwen K, Dralle H, et al. 2016 Activating mutations in CTNNB1 in
516 aldosterone producing adenomas. *Sci Rep* **6** 19546.
- 517 Akerstrom T, Willenberg HS, Cupisti K, Ip J, Backman S, Moser A, Maharjan R, Robinson
518 B, Iwen KA, Dralle H, et al. 2015 Novel somatic mutations and distinct molecular signature
519 in aldosterone-producing adenomas. *Endocr Relat Cancer* **22** 735-744.
- 520 Assie G, Auzan C, Gasc JM, Baviera E, Balaton A, Elalouf JM, Jeunemaitre X, Plouin PF,
521 Corvol P & Clauser E 2005 Steroidogenesis in aldosterone-producing adenoma revisited by
522 transcriptome analysis. *J Clin Endocrinol Metab* **90** 6638-6649.
- 523 Azizan EA, Lam BY, Newhouse SJ, Zhou J, Kuc RE, Clarke J, Happerfield L, Marker A,
524 Hoffman GJ & Brown MJ 2012 Microarray, qPCR, and KCNJ5 Sequencing of Aldosterone-
525 Producing Adenomas Reveal Differences in Genotype and Phenotype between Zona
526 Glomerulosa- and Zona Fasciculata-Like Tumors. *J Clin Endocrinol Metab* **97** E819-829.
- 527 Azizan EA, Poulsen H, Tuluc P, Zhou J, Clausen MV, Lieb A, Maniero C, Garg S,
528 Bochukova EG, Zhao W, et al. 2013 Somatic mutations in ATP1A1 and CACNA1D underlie
529 a common subtype of adrenal hypertension. *Nat Genet* **45** 1055-1060.
- 530 Bassett MH, Mayhew B, Rehman K, White PC, Mantero F, Arnaldi G, Stewart PM, Bujalska
531 I & Rainey WE 2005 Expression profiles for steroidogenic enzymes in adrenocortical disease.
532 *J Clin Endocrinol Metab* **90** 5446-5455.
- 533 Bassett MH, Suzuki T, Sasano H, White PC & Rainey WE 2004a The orphan nuclear
534 receptors NURR1 and NGFIB regulate adrenal aldosterone production. *Mol Endocrinol* **18**
535 279-290.
- 536 Bassett MH, White PC & Rainey WE 2004b The regulation of aldosterone synthase
537 expression. *Mol Cell Endocrinol* **217** 67-74.
- 538 Berthon A, Drelon C, Ragazzon B, Boulkroun S, Tissier F, Amar L, Samson-Couterie B,
539 Zennaro MC, Plouin PF, Skah S, et al. 2014 WNT/beta-catenin signalling is activated in
540 aldosterone-producing adenomas and controls aldosterone production. *Hum Mol Genet* **23**
541 889-905.
- 542 Berthon A, Sahut-Barnola I, Lambert-Langlais S, de Joussineau C, Damon-Soubeyrand C,
543 Louiset E, Taketo MM, Tissier F, Bertherat J, Lefrancois-Martinez AM, et al. 2010
544 Constitutive beta-catenin activation induces adrenal hyperplasia and promotes adrenal cancer
545 development. *Hum Mol Genet* **19** 1561-1576.
- 546 Beuschlein F, Boulkroun S, Osswald A, Wieland T, Nielsen HN, Lichtenauer UD, Penton D,
547 Schack VR, Amar L, Fischer E, et al. 2013 Somatic mutations in ATP1A1 and ATP2B3 lead
548 to aldosterone-producing adenomas and secondary hypertension. *Nat Genet* **45** 440-444.
- 549 Boulkroun S, Beuschlein F, Rossi GP, Golib-Dzib JF, Fischer E, Amar L, Mulatero P,
550 Samson-Couterie B, Hahner S, Quinkler M, et al. 2012 Prevalence, Clinical, and Molecular
551 Correlates of KCNJ5 Mutations in Primary Aldosteronism. *Hypertension* **59** 592-598.

552 Calhoun DA, Nishizaka MK, Zaman MA, Thakkar RB & Weissmann P 2002
553 Hyperaldosteronism among black and white subjects with resistant hypertension.
554 *Hypertension* **40** 892-896.

555 Choi M, Scholl UI, Yue P, Bjorklund P, Zhao B, Nelson-Williams C, Ji W, Cho Y, Patel A,
556 Men CJ, et al. 2011 K⁺ channel mutations in adrenal aldosterone-producing adenomas and
557 hereditary hypertension. *Science* **331** 768-772.

558 Connell JM & Davies E 2005 The new biology of aldosterone. *J Endocrinol* **186** 1-20.

559 Daniil G, Fernandes-Rosa FL, Chemin J, Blesneac I, Beltrand J, Polak M, Jeunemaitre X,
560 Boulkroun S, Amar L, Strom TM, et al. 2016 CACNA1H Mutations Are Associated With
561 Different Forms of Primary Aldosteronism. *EBioMedicine* **13** 225-236.

562 Douma S, Petidis K, Doumas M, Papaefthimiou P, Triantafyllou A, Kartali N, Papadopoulos
563 N, Vogiatzis K & Zamboulis C 2008 Prevalence of primary hyperaldosteronism in resistant
564 hypertension: a retrospective observational study. *Lancet* **371** 1921-1926.

565 El Ghorayeb N, Bourdeau I & Lacroix A 2016 Role of ACTH and Other Hormones in the
566 Regulation of Aldosterone Production in Primary Aldosteronism. *Front Endocrinol*
567 *(Lausanne)* **7** 72.

568 El Wakil A & Lalli E 2011 The Wnt/beta-catenin pathway in adrenocortical development and
569 cancer. *Mol Cell Endocrinol* **332** 32-37.

570 Fernandes-Rosa FL, Daniil G, Orozco IJ, Goppner C, El Zein R, Jain V, Boulkroun S,
571 Jeunemaitre X, Amar L, Lefebvre H, et al. 2018 A gain-of-function mutation in the CLCN2
572 chloride channel gene causes primary aldosteronism. *Nat Genet* **50** 355-361.

573 Fernandes-Rosa FL, Giscos-Douriez I, Amar L, Gomez-Sanchez CE, Meatchi T, Boulkroun S
574 & Zennaro MC 2015 Different Somatic Mutations in Multinodular Adrenals With
575 Aldosterone-Producing Adenoma. *Hypertension* **66** 1014-1022.

576 Fernandes-Rosa FL, Williams TA, Riester A, Steichen O, Beuschlein F, Boulkroun S, Strom
577 TM, Monticone S, Amar L, Meatchi T, et al. 2014 Genetic spectrum and clinical correlates of
578 somatic mutations in aldosterone-producing adenoma. *Hypertension* **64** 354-361.

579 Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, Stowasser M &
580 Young WF, Jr. 2016 The Management of Primary Aldosteronism: Case Detection, Diagnosis,
581 and Treatment: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*
582 **101** 1889-1916.

583 Geller DS, Zhang J, Wisgerhof MV, Shackleton C, Kashgarian M & Lifton RP 2008 A novel
584 form of human mendelian hypertension featuring nonglucocorticoid-remediable
585 aldosteronism. *J Clin Endocrinol Metab* **93** 3117-3123.

586 Goh G, Scholl UI, Healy JM, Choi M, Prasad ML, Nelson-Williams C, Kunstman JW, Korah
587 R, Suttrop AC, Dietrich D, et al. 2014 Recurrent activating mutation in PRKACA in cortisol-
588 producing adrenal tumors. *Nat Genet* **46** 613-617.

589 Grunder S, Thiemann A, Pusch M & Jentsch TJ 1992 Regions involved in the opening of
590 CIC-2 chloride channel by voltage and cell volume. *Nature* **360** 759-762.

591 Hannemann A & Wallaschofski H 2012 Prevalence of primary aldosteronism in patient's
592 cohorts and in population-based studies--a review of the current literature. *Horm Metab Res*
593 **44** 157-162.

594 Itcho K, Oki K, Kobuke K, Yoshii Y, Ohno H, Yoneda M & Hattori N 2018 Aberrant G
595 protein-receptor expression is associated with DNA methylation in aldosterone-producing
596 adenoma. *Mol Cell Endocrinol* **461** 100-104.

597 Korah HE & Scholl UI 2015 An Update on Familial Hyperaldosteronism. *Horm Metab Res*
598 **47** 941-946.

599 Lafferty AR, Torpy DJ, Stowasser M, Taymans SE, Lin JP, Huggard P, Gordon RD &
600 Stratakis CA 2000 A novel genetic locus for low renin hypertension: familial
601 hyperaldosteronism type II maps to chromosome 7 (7p22). *J Med Genet* **37** 831-835.

602 Lefebvre H, Cartier D, Duparc C, Contesse V, Lihrmann I, Delarue C, Vaudry H,
603 Fischmeister R & Kuhn JM 2000 Effect of serotonin₄ (5-HT₄) receptor agonists on
604 aldosterone secretion in idiopathic hyperaldosteronism. *Endocr Res* **26** 583-587.
605 Lefebvre H, Compagnon P, Contesse V, Delarue C, Thuillez C, Vaudry H & Kuhn JM 2001
606 Production and metabolism of serotonin (5-HT) by the human adrenal cortex: paracrine
607 stimulation of aldosterone secretion by 5-HT. *J Clin Endocrinol Metab* **86** 5001-5007.
608 Lefebvre H, Contesse V, Delarue C, Soubrane C, Legrand A, Kuhn JM, Wolf LM & Vaudry
609 H 1993 Effect of the serotonin-4 receptor agonist zacopride on aldosterone secretion from the
610 human adrenal cortex: in vivo and in vitro studies. *J Clin Endocrinol Metab* **77** 1662-1666.
611 Lenzini L, Rossitto G, Maiolino G, Letizia C, Funder JW & Rossi GP 2015 A Meta-Analysis
612 of Somatic KCNJ5 K(+) Channel Mutations In 1636 Patients With an Aldosterone-Producing
613 Adenoma. *J Clin Endocrinol Metab* **100** E1089-1095.
614 Lenzini L, Seccia TM, Aldighieri E, Belloni AS, Bernante P, Giuliani L, Nussdorfer GG,
615 Pessina AC & Rossi GP 2007 Heterogeneity of aldosterone-producing adenomas revealed by
616 a whole transcriptome analysis. *Hypertension* **50** 1106-1113.
617 Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S & Lalouel JM 1992 A chimaeric
618 11beta-hydroxylase aldosterone synthase gene causes glucocorticoid-remediable
619 aldosteronism and human hypertension. *Nature* **355** 262-265.
620 Litchfield WR, Anderson BF, Weiss RJ, Lifton RP & Dluhy RG 1998 Intracranial aneurysm
621 and hemorrhagic stroke in glucocorticoid-remediable aldosteronism. *Hypertension* **31** 445-
622 450.
623 Louiset E, Duparc C, Lenglet S, Gomez-Sanchez CE & Lefebvre H 2017 Role of cAMP/PKA
624 pathway and T-type calcium channels in the mechanism of action of serotonin in human
625 adrenocortical cells. *Mol Cell Endocrinol* **441** 99-107.
626 MacDonald BT, Tamai K & He X 2009 Wnt/beta-catenin signaling: components,
627 mechanisms, and diseases. *Dev Cell* **17** 9-26.
628 Mant A, Williams S, Roncoroni L, Lowry E, Johnson D & O'Kelly I 2013 N-glycosylation-
629 dependent control of functional expression of background potassium channels K2P3.1 and
630 K2P9.1. *J Biol Chem* **288** 3251-3264.
631 Medeau V, Assie G, Zennaro MC, Clauser E, Plouin PF & Jeunemaitre X 2005 [Familial
632 aspect of primary hyperaldosteronism: analysis of families compatible with primary
633 hyperaldosteronism type 2]. *Ann Endocrinol (Paris)* **66** 240-246.
634 Mishunina TM & Kononenko VY 2002 Specific GABA binding in the adrenals and blood
635 corticosteroid levels in stress in intact rats and rats with changes in the functional activity of
636 the hypothalamo-pituitary-adrenal system. *Neurosci Behav Physiol* **32** 109-112.
637 Monticone S, Burrello J, Tizzani D, Bertello C, Viola A, Buffolo F, Gabetti L, Mengozzi G,
638 Williams TA, Rabbia F, et al. 2017 Prevalence and Clinical Manifestations of Primary
639 Aldosteronism Encountered in Primary Care Practice. *J Am Coll Cardiol* **69** 1811-1820.
640 Monticone S, Hattangady NG, Nishimoto K, Mantero F, Rubin B, Cicala MV, Pezzani R,
641 Auchus RJ, Ghayee HK, Shibata H, et al. 2012 Effect of KCNJ5 Mutations on Gene
642 Expression in Aldosterone-Producing Adenomas and Adrenocortical Cells. *J Clin Endocrinol*
643 *Metab* **97** E1567-1572.
644 Mulatero P, Monticone S, Rainey WE, Veglio F & Williams TA 2013 Role of KCNJ5 in
645 familial and sporadic primary aldosteronism. *Nat Rev Endocrinol* **9** 104-112.
646 Mulatero P, Tauber P, Zennaro MC, Monticone S, Lang K, Beuschlein F, Fischer E, Tizzani
647 D, Pallauf A, Viola A, et al. 2012 KCNJ5 mutations in European families with
648 nonglucocorticoid remediable familial hyperaldosteronism. *Hypertension* **59** 235-240.
649 Mulatero P, Tizzani D, Viola A, Bertello C, Monticone S, Mengozzi G, Schiavone D,
650 Williams TA, Einaudi S, La Grotta A, et al. 2011 Prevalence and characteristics of familial

651 hyperaldosteronism: the PATOGEN study (Primary Aldosteronism in TORino-GENetic
652 forms). *Hypertension* **58** 797-803.

653 Nanba K, Omata K, Else T, Beck PCC, Nanba AT, Turcu AF, Miller BS, Giordano TJ,
654 Tomlins SA & Rainey WE 2018 Targeted Molecular Characterization of Aldosterone-
655 Producing Adenomas in White Americans. *J Clin Endocrinol Metab* **103** 3869-3876.

656 Nanba K, Omata K, Gomez-Sanchez CE, Stratakis CA, Demidowich AP, Suzuki M,
657 Thompson LDR, Cohen DL, Luther JM, Gellert L, et al. 2019 Genetic Characteristics of
658 Aldosterone-Producing Adenomas in Blacks. *Hypertension* **73** 885-892.

659 Nishimoto K, Nakagawa K, Li D, Kosaka T, Oya M, Mikami S, Shibata H, Itoh H, Mitani F,
660 Yamazaki T, et al. 2010 Adrenocortical zonation in humans under normal and pathological
661 conditions. *J Clin Endocrinol Metab* **95** 2296-2305.

662 Nishimoto K, Seki T, Kurihara I, Yokota K, Omura M, Nishikawa T, Shibata H, Kosaka T,
663 Oya M, Suematsu M, et al. 2016 Case Report: Nodule Development From Subcapsular
664 Aldosterone-Producing Cell Clusters Causes Hyperaldosteronism. *J Clin Endocrinol Metab*
665 **101** 6-9.

666 Nishimoto K, Tomlins SA, Kuick R, Cani AK, Giordano TJ, Hovelson DH, Liu CJ,
667 Sanjanwala AR, Edwards MA, Gomez-Sanchez CE, et al. 2015 Aldosterone-stimulating
668 somatic gene mutations are common in normal adrenal glands. *Proc Natl Acad Sci U S A* **112**
669 E4591-4599.

670 Oki K, Plonczynski M, Lam M, Gomez-Sanchez E & Gomez-Sanchez C 2012a The
671 potassium channel, Kir3.4 participates in angiotensin II-stimulated aldosterone production by
672 a human adrenocortical cell line. *Endocrinology* **153** 4328-4335.

673 Oki K, Plonczynski MW, Luis Lam M, Gomez-Sanchez EP & Gomez-Sanchez CE 2012b
674 Potassium Channel Mutant KCNJ5 T158A Expression in HAC-15 Cells Increases
675 Aldosterone Synthesis. *Endocrinology* **153** 1774-1782.

676 Omata K, Anand SK, Hovelson DH, Liu CJ, Yamazaki Y, Nakamura Y, Ito S, Satoh F,
677 Sasano H, Rainey WE, et al. 2017 Aldosterone-Producing Cell Clusters Frequently Harbor
678 Somatic Mutations and Accumulate With Age in Normal Adrenals. *J Endocr Soc* **1** 787-799.

679 Pallauf A, Schirpenbach C, Zwermann O, Fischer E, Morak M, Holinski-Feder E, Hofbauer
680 L, Beuschlein F & Reincke M 2012 The prevalence of familial hyperaldosteronism in
681 apparently sporadic primary aldosteronism in Germany: a single center experience. *Horm*
682 *Metab Res* **44** 215-220.

683 Rhayem Y, Perez-Rivas LG, Dietz A, Bathon K, Gebhard C, Riester A, Mauracher B,
684 Gomez-Sanchez C, Eisenhofer G, Schwarzmayr T, et al. 2016 PRKACA Somatic Mutations
685 Are Rare Findings in Aldosterone-Producing Adenomas. *J Clin Endocrinol Metab* **101** 3010-
686 3017.

687 Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, Ferri C, Ganzaroli C, Giacchetti G,
688 Letizia C, Maccario M, et al. 2006 A prospective study of the prevalence of primary
689 aldosteronism in 1,125 hypertensive patients. *J Am Coll Cardiol* **48** 2293-2300.

690 Rossi GP, Sacchetto A, Visentin P, Canali C, Graniero GR, Palatini P & Pessina AC 1996
691 Changes in left ventricular anatomy and function in hypertension and primary aldosteronism.
692 *Hypertension* **27** 1039-1045.

693 Ruggiero C & Lalli E 2016 Impact of ACTH Signaling on Transcriptional Regulation of
694 Steroidogenic Genes. *Front Endocrinol (Lausanne)* **7** 24.

695 Savard S, Amar L, Plouin PF & Steichen O 2013 Cardiovascular complications associated
696 with primary aldosteronism: a controlled cross-sectional study. *Hypertension* **62** 331-336.

697 Scholl UI, Goh G, Stolting G, de Oliveira RC, Choi M, Overton JD, Fonseca AL, Korah R,
698 Starker LF, Kunstman JW, et al. 2013 Somatic and germline CACNA1D calcium channel
699 mutations in aldosterone-producing adenomas and primary aldosteronism. *Nat Genet* **45**
700 1050-1054.

701 Scholl UI, Healy JM, Thiel A, Fonseca AL, Brown TC, Kunstman JW, Horne MJ, Dietrich D,
702 Riemer J, Kucukkoylu S, et al. 2015a Novel Somatic Mutations in Primary
703 Hyperaldosteronism are related to the Clinical, Radiological and Pathological Phenotype. *Clin*
704 *Endocrinol (Oxf)*.

705 Scholl UI, Nelson-Williams C, Yue P, Grekin R, Wyatt RJ, Dillon MJ, Couch R, Hammer
706 LK, Harley FL, Farhi A, et al. 2012 Hypertension with or without adrenal hyperplasia due to
707 different inherited mutations in the potassium channel KCNJ5. *Proc Natl Acad Sci U S A* **109**
708 2533-2538.

709 Scholl UI, Stolting G, Nelson-Williams C, Vichot AA, Choi M, Loring E, Prasad ML, Goh G,
710 Carling T, Juhlin CC, et al. 2015b Recurrent gain of function mutation in calcium channel
711 CACNA1H causes early-onset hypertension with primary aldosteronism. *Elife* **4** e06315.

712 Scholl UI, Stolting G, Schewe J, Thiel A, Tan H, Nelson-Williams C, Vichot AA, Jin SC,
713 Loring E, Untiet V, et al. 2018 CLCN2 chloride channel mutations in familial
714 hyperaldosteronism type II. *Nat Genet* **50** 349-354.

715 Seccia TM, Mantero F, Letizia C, Kuppusamy M, Caroccia B, Barisa M, Cicala MV, Miotto
716 D & Rossi GP 2012 Somatic mutations in the KCNJ5 gene raise the lateralization index:
717 implications for the diagnosis of primary aldosteronism by adrenal vein sampling. *J Clin*
718 *Endocrinol Metab* **97** E2307-2313.

719 Spat A & Hunyady L 2004 Control of aldosterone secretion: a model for convergence in
720 cellular signaling pathways. *Physiol Rev* **84** 489-539.

721 Stindl J, Tauber P, Sterner C, Tegtmeier I, Warth R & Bandulik S 2015 Pathogenesis of
722 Adrenal Aldosterone-Producing Adenomas Carrying Mutations of the Na(+)/K(+)-ATPase.
723 *Endocrinology* **156** 4582-4591.

724 Stowasser M, Bachmann AW, Huggard PR, Rossetti TR & Gordon RD 2000 Treatment of
725 familial hyperaldosteronism type I: only partial suppression of adrenocorticotropin required to
726 correct hypertension. *J Clin Endocrinol Metab* **85** 3313-3318.

727 Stowasser M & Gordon RD 2001 Familial hyperaldosteronism. *J Steroid Biochem Mol Biol*
728 **78** 215-229.

729 Stowasser M, Gordon RD, Tunny TJ, Klemm SA, Finn WL & Krek AL 1992 Familial
730 hyperaldosteronism type II: five families with a new variety of primary aldosteronism. *Clin*
731 *Exp Pharmacol Physiol* **19** 319-322.

732 Stowasser M, Sharman J, Leano R, Gordon RD, Ward G, Cowley D & Marwick TH 2005
733 Evidence for abnormal left ventricular structure and function in normotensive individuals with
734 familial hyperaldosteronism type I. *J Clin Endocrinol Metab* **90** 5070-5076.

735 Su H, Gu Y, Li F, Wang Q, Huang B, Jin X, Ning G & Sun F 2013 The PI3K/AKT/mTOR
736 signaling pathway is overactivated in primary aldosteronism. *PLoS One* **8** e62399.

737 Sutherland DJ, Ruse JL & Laidlaw JC 1966 Hypertension, increased aldosterone secretion
738 and low plasma renin activity relieved by dexamethasone. *Can Med Assoc J* **95** 1109-1119.

739 Swierczynska MM, Betz MJ, Colombi M, Dazert E, Jenö P, Moes S, Pfaff C, Glatz K,
740 Reincke M, Beuschlein F, et al. 2019 Proteomic Landscape of Aldosterone-Producing
741 Adenoma. *Hypertension* **73** 469-480.

742 Tan GC, Negro G, Pinggera A, Tizen Laim NMS, Mohamed Rose I, Ceral J, Ryska A, Chin
743 LK, Kamaruddin NA, Mohd Mokhtar N, et al. 2017 Aldosterone-Producing Adenomas:
744 Histopathology-Genotype Correlation and Identification of a Novel CACNA1D Mutation.
745 *Hypertension* **70** 129-136.

746 Tauber P, Aichinger B, Christ C, Stindl J, Rhayem Y, Beuschlein F, Warth R & Bandulik S
747 2016 Cellular Pathophysiology of an Adrenal Adenoma-Associated Mutant of the Plasma
748 Membrane Ca(2+)-ATPase ATP2B3. *Endocrinology* **157** 2489-2499.

749 Teo AE, Garg S, Shaikh LH, Zhou J, Karet Frankl FE, Gurnell M, Happerfield L, Marker A,
750 Bienz M, Azizan EA, et al. 2015 Pregnancy, Primary Aldosteronism, and Adrenal CTNNB1
751 Mutations. *N Engl J Med* **373** 1429-1436.
752 Vinson GP 2016 Functional Zonation of the Adult Mammalian Adrenal Cortex. *Front*
753 *Neurosci* **10** 238.
754 Watanabe I, Zhu J, Recio-Pinto E & Thornhill WB 2004 Glycosylation affects the protein
755 stability and cell surface expression of Kv1.4 but Not Kv1.1 potassium channels. A pore
756 region determinant dictates the effect of glycosylation on trafficking. *J Biol Chem* **279** 8879-
757 8885.
758 Williams TA, Monticone S, Crudo V, Warth R, Veglio F & Mulatero P 2012 Visinin-like 1 is
759 upregulated in aldosterone-producing adenomas with KCNJ5 mutations and protects from
760 calcium-induced apoptosis. *Hypertension* **59** 833-839.
761 Williams TA, Monticone S, Morello F, Liew CC, Mengozzi G, Pilon C, Asioli S, Sapino A,
762 Veglio F & Mulatero P 2010 Teratocarcinoma-derived growth factor-1 is upregulated in
763 aldosterone-producing adenomas and increases aldosterone secretion and inhibits apoptosis in
764 vitro. *Hypertension* **55** 1468-1475.
765 Zennaro MC 2019 From Transcripts to Proteins. *Hypertension* **73** 284-285.

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767

768 **Figure legends.**

769

770 **Figure 1. Regulation of aldosterone biosynthesis in normal conditions and in PA. A.** In
771 unstimulated conditions, zona glomerulosa cells are hyperpolarized. This state is maintained by
772 the activity of potassium channels such as TASK and GIRK4 and the Na⁺/K⁺ ATPase pump.
773 **B.** Increases in extracellular potassium levels inhibit K⁺ currents of TASK channels and lead to
774 cell membrane depolarization. Activation of AngII signaling through its receptor (AT1R) on
775 the surface of ZG cells inhibits K⁺ channels and the Na⁺/K⁺ ATPase, which leads to cell
776 membrane depolarization, opening of voltage gated Ca²⁺ channels, increased intracellular
777 calcium concentrations. Intracellular calcium activates signaling pathways leading to *CYP11B2*
778 expression and thus increasing aldosterone biosynthesis. Activation of AT1R also activates a
779 Gαq-phospholipase C-mediated pathway, inducing an increase in the concentration of inositol
780 1,4,5-triphosphate (IP3) which binds to its receptor expressed on the endoplasmic reticulum,
781 inducing the release of calcium from intracellular stores. **C.** Mutations in *KCNJ5* encoding the
782 potassium channel GIRK4 are responsible for a loss of selectivity of the channel and lead to
783 sodium influx into the cell; mutations in *ATP1A1* encoding the α1 subunit of the Na⁺/K⁺ ATPase
784 increase intracellular Na⁺ and H⁺ concentrations; mutations in *CLCN2*, encoding the chloride
785 channel ClC-2, increase chloride conductance at resting potentials; all these abnormalities lead
786 to membrane depolarization of glomerulosa cells. Cell membrane depolarization induces
787 opening of voltage gated calcium channels, increased intracellular calcium concentrations and
788 activation of calcium signaling, triggering increased expression of *CYP11B2* and aldosterone
789 production. **D.** Mutations in *CACNA1H* and *CACNA1D* encoding the voltage-gated calcium
790 channels Cav3.2 and Cav1.3 as well as mutations in *ATP2B3*, encoding the Ca²⁺ pump PMCA3,
791 directly increase intracellular calcium concentrations and lead to increased aldosterone
792 biosynthesis through activation of calcium signalling.

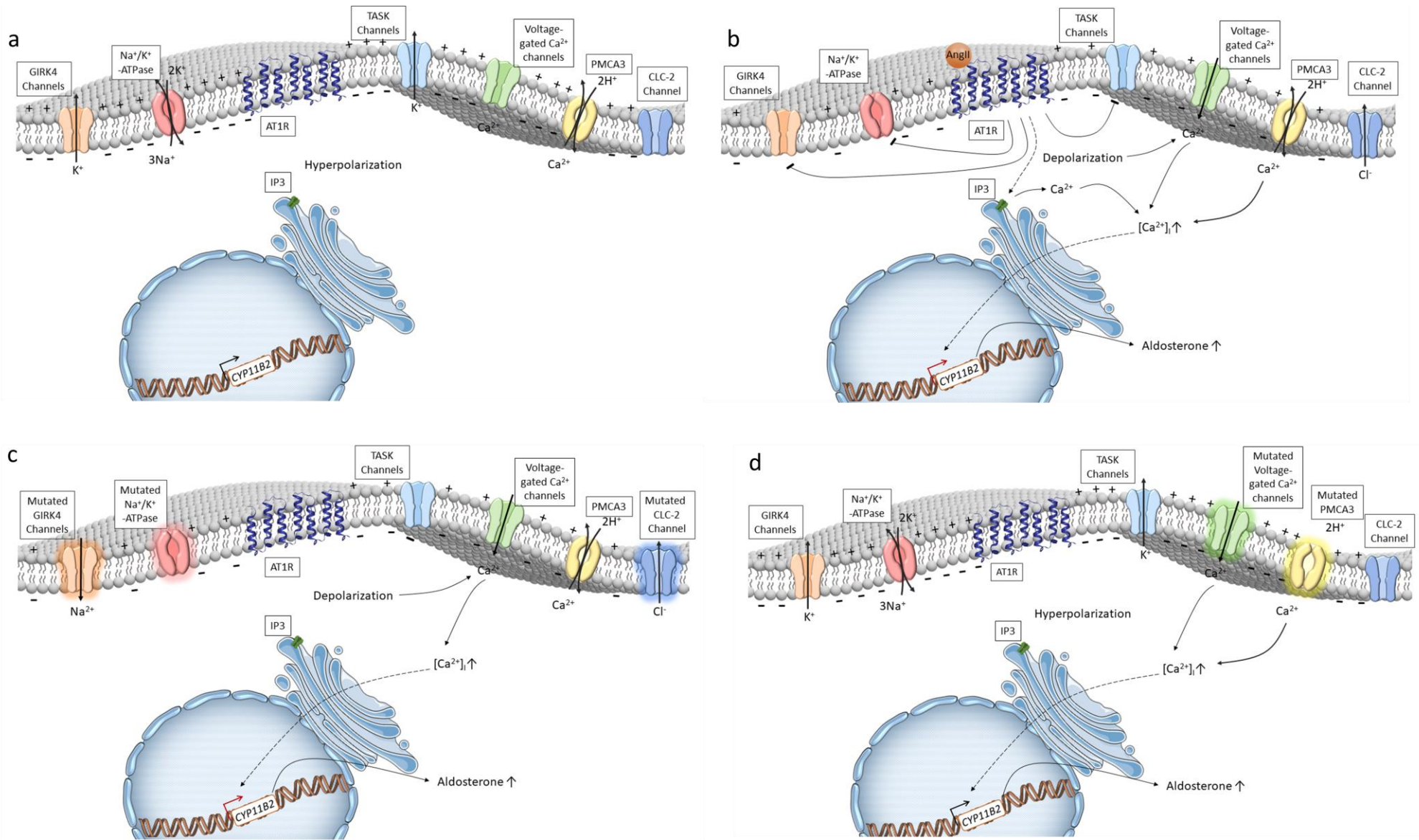


Figure 1
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