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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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23

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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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.

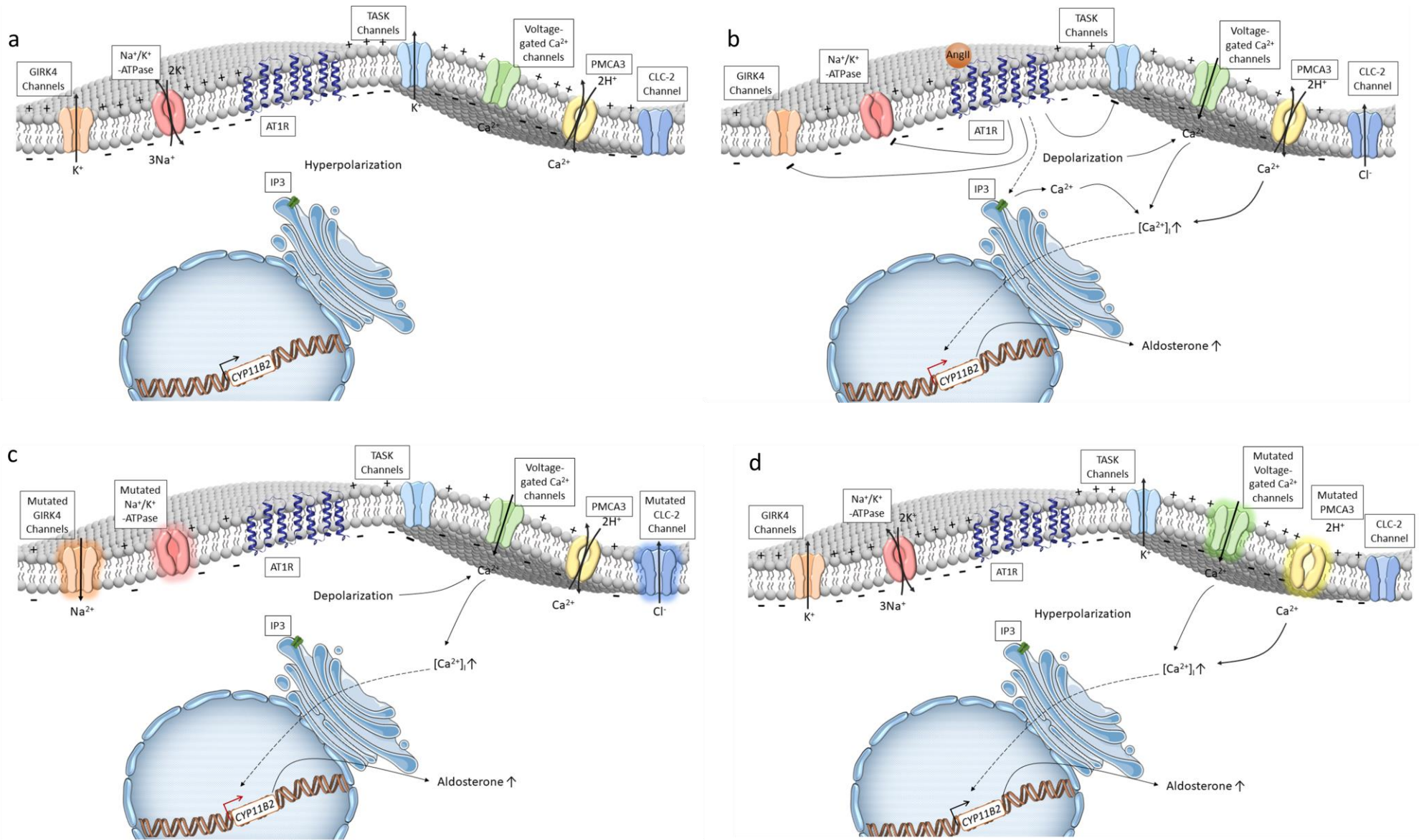


Figure 1
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