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Old and new genes in primary aldosteronism

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Abstract

Primary aldosteronism (PA) is the most common form of secondary hypertension affecting 5% to 10% of patients with arterial hypertension. In PA, high blood pressure is associated with high aldosterone and low renin levels, and often hypokalemia. In a majority of cases, autonomous aldosterone production by the adrenal gland is caused by an aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia (BAH). During the last ten years, a better knowledge of the pathophysiology of PA came from the discovery of somatic and germline mutations in different genes in both sporadic and familial forms of the disease. Those genes code for ion channel and pumps, as well as proteins involved in adrenal cortex development and function. Targeted next generation sequencing following immunohistochemistry guided detection of aldosterone synthase expression allows detection of somatic mutations in up to 90% of APA, while whole exome sequencing has discovered the genetic causes of four different familial forms of PA. The identification, in BAH, of somatic mutations in aldosterone producing cell clusters open new perspectives in our understanding of the bilateral form of the disease and the development of new therapeutic approaches.

1 **Introduction**

2

3 Aldosterone is synthesized by the zona glomerulosa of the adrenal cortex and plays a major
4 role in regulating blood pressure, mainly by regulating sodium and water reabsorption in the
5 kidney [1]. Effects of aldosterone on target tissues are mediated through its binding to the
6 mineralocorticoid receptor (MR), a member of the nuclear receptor superfamily. The MR is a
7 hormone dependent transcription factor, which binds to specific hormone responsive elements
8 on regulatory regions of target genes. In the kidney, the MR is expressed in the aldosterone
9 sensitive distal tubule, where aldosterone regulates sodium reabsorption and potassium
10 excretion. In addition, MR is expressed also in other epithelial tissues such as the colon, sweat
11 and salivary glands, where it regulates water and electrolyte homeostasis, and in non-
12 epithelial tissues, such as the heart, vessels, and macrophages, where it mediates major
13 deleterious effects of aldosterone excess on the cardiovascular system [2].

14

15 *Aldosterone biosynthesis*

16 The adrenal cortex is composed of three distinct zones with different functions. The Zona
17 Glomerulosa (ZG) forms the outer part of the cortex underneath the capsule, and produces
18 mineralocorticoids, mainly aldosterone but also 11-deoxycorticosterone (DOC). Whereas
19 aldosterone is produced only in ZG, due to specific expression of aldosterone synthase in this
20 zone, DOC is produced in both ZG and ZF. The Zona Fasciculata (ZF), the thickest zone of
21 the cortex, is involved in biosynthesis of the glucocorticoid hormone cortisol, which plays
22 important roles in metabolism, stress response and inflammation. Finally, the Zona Reticularis
23 (ZR), in contact with the medulla, produces sex steroids.

24 Aldosterone is synthesized from cholesterol, whose availability constitutes the limiting step in
25 aldosterone biosynthesis. The translocation of cholesterol into the mitochondria, where it is

26 converted into pregnenolone by the P450 side-chain cleavage enzyme (*CYP11A1*), is
27 mediated by the steroidogenic acute regulatory protein (StAR). A cascade of enzymatic
28 reactions then leads to the biosynthesis of aldosterone (Figure 1). Briefly, in ZG, the 3 β -
29 hydroxysteroid dehydrogenase converts pregnenolone into progesterone and the 21-
30 hydroxylase catalyzes the 21-hydroxylation of progesterone to form 11-deoxycorticosterone.
31 These two enzymes are expressed throughout the adrenal cortex. The peculiar functional
32 zonation of the adrenal cortex is due to zone-specific expression of different steroidogenic
33 enzymes. In particular, the ZG expresses aldosterone synthase (encoded by *CYP11B2*), but
34 not 17 α -hydroxylase (encoded by the *CYP17A1* gene) converting pregnenolone and
35 progesterone to 17-OH-pregnenolone and 17-OH-progesterone); the ZF in contrast expresses
36 both 17 α -hydroxylase and 11 β -hydroxylase (encoded by *CYP11B1*) converting 11-
37 deoxycortisol to cortisol (Figure 1). Thus in the ZG, aldosterone synthase catalyzes the three
38 final enzymatic reactions leading to aldosterone biosynthesis: the 11 β -hydroxylation of 11-
39 deoxycorticosterone, the 18-hydroxylation of corticosterone and finally the 18
40 methyloxidation of 18-OH-corticosterone to produce aldosterone [3].

41

42 *Physiological regulation of aldosterone biosynthesis*

43 Aldosterone biosynthesis is under the control of many factors, the two most important being
44 angiotensin II (AngII) and extracellular potassium concentration (K⁺). Its biosynthesis is
45 tightly controlled in order to maintain electrolyte homeostasis by the kidney. In absence of
46 stimulation, ZG cells are hyperpolarized with a resting potential around -80mV. This is due to
47 a dominant K⁺ conductance due to the presence of a large number of potassium channels [4]
48 and the Na⁺/K⁺-ATPase that maintain the K⁺ gradient concentration between the plasma and
49 the cytosol. Stimulation of ZG cells by AngII or increased plasma K⁺ concentration lead to
50 cell membrane depolarization, opening of voltage-gated Ca²⁺ channels and increase of the

51 intracellular Ca^{2+} concentration. In parallel, binding of AngII to the angiotensin II Type 1
52 receptor (AT1R) stimulates inositol triphosphate-dependent Ca^{2+} release from the
53 endoplasmic reticulum. Increase of intracellular Ca^{2+} concentration leads to activation of a
54 phosphorylation cascade involving calmodulin and calcium/calmodulin-dependent protein
55 kinases (CaMKI and/or CaMKIV) and results in the activation of Nurr1 and NGF1B, two
56 transcription factors that positively regulate the transcription of *CYP11B2* [5, 6].
57 In addition to these two major regulators, a number of other factors are known to induce or
58 inhibit aldosterone biosynthesis. Although ACTH is known to be important in glucocorticoid
59 biosynthesis, this hormone also contributes to the regulation of aldosterone biosynthesis [7].
60 Whereas acutely ACTH stimulates aldosterone biosynthesis via cAMP and activation of PKA
61 signaling, chronically it inhibits aldosterone biosynthesis. One potential explanation for this
62 phenomenon is the conversion by ACTH of ZG cells into ZF cells [8, 9]. Serotonin (5HT)
63 produced by mast cells present in the adrenal cortex has also been shown to stimulate
64 aldosterone biosynthesis through binding to 5HT4R receptors and activation of the PKA
65 signaling pathway [10]. *In vitro* data have shown that other factors can positively
66 (Interleukine 6 [11], Epidermal Growth Factor [12]) or negatively (Transforming Growth
67 Factor-beta 1 [13], dopamine [14]) regulate aldosterone biosynthesis (for review, see [15].
68 Recently, the adipocyte derived factor leptin has been shown also to be able to stimulate
69 aldosterone biosynthesis in both cell and mouse models [16].

70

71 *Primary aldosteronism*

72 Primary aldosteronism (PA) is the most common form of secondary hypertension. Its
73 prevalence was estimated between 5% to 10% of patients with arterial hypertension [17, 18]
74 and up to 20% of patients with resistant hypertension [19]. PA is described as a group of
75 disorders in which aldosterone is inappropriately high considering salt status, relatively

76 autonomous from the renin-angiotensin-system and non-suppressible by salt loading [20].
77 High blood pressure is associated to high aldosterone level and suppressed renin and often
78 hypokalemia. The two major causes of PA are aldosterone producing adenoma (APA), first
79 described by Jerome Conn in 1954 [21, 22], and bilateral adrenal hyperplasia (BAH),
80 accounting together for more than 95% of cases. In rare cases, unilateral adrenal hyperplasia
81 and adrenal carcinoma may be responsible for PA.

82 Patients with PA are more likely to develop cardiovascular complications than patients with
83 primary hypertension matched for sex, age and blood pressure [23]. They have an increased
84 rate of cardiovascular events such as stroke, myocardial infarction and atrial fibrillation [24].
85 They are also more likely to present left ventricular hypertrophy, diastolic dysfunction,
86 alterations in left ventricular filling, nonfatal myocardial infarction, coronary artery disease,
87 heart failure and atrial fibrillation [25-27]. This is due to the well-known deleterious effects of
88 aldosterone on the heart and blood vessels [2]. Despite publication of guidelines for the
89 diagnosis, subtype identification and management of PA in 2008, and their update in 2016
90 [20, 28], the condition is still underdiagnosed or diagnosed years after the discovery of
91 hypertension. This delay has a negative impact on the therapeutic outcome of adrenalectomy
92 in patients with APA.

93 Over the last 10 years our knowledge on the genetic basis of PA has improved spectacularly
94 and different new Mendelian forms of PA have been described. In parallel, somatic mutations
95 in different genes have been discovered in APA as well as other aldosterone producing
96 structures of the adrenal gland.

97

98 **Familial forms of primary aldosteronism**

99

100 Although the majority of cases of PA are sporadic, approximately 5% are found in a familial
101 context and transmitted as an autosomal dominant trait [29]. Four different Mendelian forms
102 of PA have been identified: Familial Hyperaldosteronism (FH) Type I to Type IV (Figure 2).
103 However, while somatic mutations were identified in about 90% of APA, the genetic causes
104 of an important number of familial forms are still unknown.

105

106 *Familial Hyperaldosteronism Type I (FH-I)*

107 In 1966, Sutherland and colleagues reported the case of a father and his son with moderate
108 hypertension, hypokalemia, increased aldosteronemia and suppressed plasma renin activity
109 suggesting a Conn's syndrome [30]. Remarkably, dexamethasone treatment normalized their
110 blood pressure, aldosterone and potassium levels and plasma renin activity [30]; more
111 surprisingly, aldosterone biosynthesis was found to be sensitive to ACTH stimulation [31].
112 These patients presented also high levels of 18-hydroxycortisol and 18-oxocortisol, their
113 secretion being again under the control of ACTH and suppressible by glucocorticoids. FH-I is
114 more often associated with bilateral adrenal hyperplasia rather than adrenocortical adenoma
115 [32, 33].

116 In 1992, the molecular basis of this autosomal dominant form of PA was elucidated by Lifton
117 and coll [34, 35]. FH-I is due to unequal crossing-over between *CYP11B1* and *CYP11B2*,
118 coding for 11 β -hydroxylase and aldosterone synthase, both localized on tandem in the same
119 region of chromosome 8 and sharing 95% of sequence homology. This crossing-over leads to
120 the fusion of the regulatory sequences of *CYP11B1* to the coding sequence of *CYP11B2*,
121 resulting in ectopic expression of aldosterone synthase in the ZF and regulation of its
122 expression by ACTH (Figure 2A).

123 The prevalence of FH-I is estimated between 0.5% and 1% of cases of PA diagnosed in adult
124 patients [29]; however the screening of a hypertensive pediatric population revealed that the
125 prevalence of FH-I could represent 3% of cases [36].

126

127 *Familial Hyperaldosteronism Type II (FH-II)*

128 For many years FH-II was referring to familial forms of PA with an autosomal dominant
129 mode of inheritance and for which FH-I had been excluded [37, 38]. FH-II is clinically and
130 biochemically indistinguishable from sporadic forms of PA and within the same family APA
131 and bilateral adrenal hyperplasia may be present with variable phenotypes [29, 39]. Diagnosis
132 of FH-II is done on the basis of at least two affected members in the same family [40]. In
133 some families a linkage was established with the chromosomal region 7p22 [41], however no
134 mutations were found in different candidate genes located in this region [40, 42]. The
135 sequencing of other candidate genes including *CYP11B2*, *AGTR1* coding for the AngII
136 Receptor Type 1 and *p53*, a tumor-suppressor gene, was also negative [32, 43, 44].

137 Recently, whole exome sequencing performed in a large family first described by Michael
138 Stowasser and colleagues [45] allowed the identification of a causal germline mutation in the
139 *CLCN2* gene (p.Arg172Gln). The same mutation was also found in three other families and
140 four additional mutations (p.Tyr26Asn, p.Lys362del, p.Met22Lys, p.Ser865Arg) were
141 identified in unrelated patients [46]. At the same time, Fernandes-Rosa and colleagues
142 reported the identification of a *de novo* p.Gly24Asp mutation in *CLCN2* in a 9-year-old girl
143 presenting severe hypertension, profound hypokalemia, elevated plasma aldosterone and
144 suppressed renin activity [47] (Figure 2B).

145 *CLCN2* encodes the ubiquitously expressed inwardly rectifying chloride channel CLC-2, that
146 opens upon hyperpolarization, hypoosmotic cell swelling and acidic extracellular pH [48]. In
147 humans, loss of function mutations in *CLCN2* are responsible for leukodystrophy [49] that

148 may also be associated with azoospermia [50]. Similarly in mice, invalidation of *Clcn2* leads
149 to testicular degeneration, leukodystrophy as well as retinal degeneration [51, 52].
150 Interestingly, mutations identified in PA result in a gain of function of the channel, leading to
151 increased chloride currents. In particular, the p.Gly24Asp mutation is located in a well-
152 conserved inactivation domain of the protein and abolishes the voltage- and time-dependent
153 gating of the channel, strongly increasing chloride conductance at resting potentials [47].
154 Increased efflux of Cl⁻ ions leads to cell membrane depolarization, opening of voltage gated
155 Ca²⁺ channel and ultimately increased aldosterone biosynthesis [46, 47]. Different mutations
156 have slightly different effects on channel function. In particular, the p.Arg172Gln mutation
157 seems to be milder than other mutations when tested in two-electrode voltage clamp studies in
158 *Xenopus* oocytes [53], explaining the incomplete penetrance and phenotypic heterogeneity
159 observed in affected family members [46]. Similar to the human disease, mice expressing a
160 constitutively open CLC-2 chloride channel, recapitulating gain of function mutations found
161 in FH-II, display marked hypertension, high aldosterone levels, low renin activity and
162 hypokalemia, the principal features of human PA [53]. This is due to increased chloride
163 conductance of zona glomerulosa cells, leading to a strong cell membrane depolarization and
164 increased cytoplasmic calcium concentration, followed by increased expression of aldosterone
165 synthase, thus confirming the role of *CLC2* mutations in the pathogenesis of PA. A similar
166 phenotype is observed in mice carrying a mutation at the position homologous to
167 p.Arg172Gln. Heterozygous *Clcn2*^{R180Q/+} mice show increased aldosterone levels and
168 *Cyp11b2* expression; in addition, male mice have increased aldosterone to renin ratio and
169 slightly increase blood pressure [54] In contrast, *Clcn2* knockout mice show normal
170 aldosterone levels and *Cyp11b2* expression, but increased plasma renin concentration,
171 suggesting that activation of the renin angiotensin system maintains normal aldosterone levels

172 in these animals and that CLC-2 plays a role in regulating physiological aldosterone
173 biosynthesis [54].

174

175 *Familial Hyperaldosteronism Type III (FH-III)*

176 In 2008, Geller and coll reported a new familial form of primary aldosteronism in a father and
177 his two daughters [55]. At 5 years of age, the father presented severe hypertension, high
178 aldosterone, profound hypokalemia and evidence of left ventricular hypertrophy associated
179 with adrenal hyperplasia [56]. He underwent bilateral adrenalectomy four years later. His two
180 daughters presented at 7 and 4 years of age severe hypertension associated with profound
181 hypokalemia, elevated plasma aldosterone despite suppressed renin activity. Only the older
182 girl presented also concentric left ventricular hypertrophy with preserved ejection fraction.
183 These three patients presented also high levels of the hybrid steroids 18-oxocortisol and 18-
184 hydroxycortisol. However, lack of suppression of aldosterone by dexamethasone excluded
185 FH-I. Despite multiple therapies, both patients had to undergo bilateral adrenalectomy to
186 achieve normalization of BP and kalemia. Their adrenals presented ZF hyperplasia and
187 atrophy of the ZG, with CYP11B2 expression throughout the adrenal cortex and coexpression
188 of CYP11B2, CYP11B1 and CYP17A1, suggesting that aldosterone and hybrid steroid
189 production was due to ectopic expression of aldosterone synthase in the ZF [55, 57].

190 The molecular abnormality responsible for this new form of familial PA is the mutation
191 p.Thr158Ala in the *KCNJ5* gene, encoding the G protein-activated inward rectifier potassium
192 channel GIRK4 [58] (Figure 2C). The mutation is located in the selectivity filter of GIRK4
193 and modifies its ion selectivity, with the loss of K⁺ selectivity in favor of sodium and increase
194 in Na⁺ influx into the cell. Different germline mutations in *KCNJ5* were identified in familial
195 cases of PA, with a correlation between the type of mutation and the severity of the disease.
196 Whereas a severe phenotype with early-onset hypertension resistant to treatment was

197 associated with the mutations p.Gly151Arg, p.Thr158Ala and p.Ile157Ser [58-60], a milder
198 phenotype was observed in patients carrying the mutations p.Gly151Glu [59, 61] and
199 p.Tyr152Cys [62]. These observations were also supported by *in vitro* data showing that the
200 p.Gly151Glu affects the GIRK4 channel selectivity similarly to other mutations; however the
201 resulting Na⁺ conductance was larger for this mutation than for the others, leading to rapid
202 cell lethality probably limiting cell proliferation [59]. However, this phenotype-genotype
203 association between *KCNJ5* mutations and the severity of the disease was questioned by the
204 description of a sporadic patient with FH-III harboring the p.Gly151Arg mutation that was
205 associated to a severe phenotype. Against all odds, this patient responded well to
206 spironolactone and no adrenal enlargement was observed after seven years of follow-up [63].
207 FH-III is a rare disease, its prevalence is estimated to be less than 0.5% of cases of PA
208 diagnosed in adult patients and around 8% among familial forms [61, 64, 65].

209

210 *Familial Hyperaldosteronism Type IV (FH-IV)*

211 Exome sequencing performed on 40 unrelated patients with early onset hypertension due to
212 PA before age 10 years allowed the identification of a germline mutation in the voltage gated
213 T-type calcium channel Cav3.2 (p.Met1549Val), encoded by *CACNA1H*, in five patients [66].
214 A similar approach led to the identification of four different mutations (p.Met1549Ile,
215 p.Ser196Leu, p.Pro2083Leu and p.Val1951Glu) in the same gene in patients with early onset
216 and familial PA [67] (Figure 2D). These mutations are inherited in an autosomal dominant
217 manner with incomplete penetrance of the disease, including asymptomatic patients. Different
218 *CACNA1H* variants have been associated with epileptic disorders [68-71], autism spectrum
219 disorders [72] and chronic pain [73]. Accordingly, one patient carrying a p.Met1549Ile
220 mutation was diagnosed with multiplex developmental disorder at age 10 years [67] and two
221 patients carrying the p.Met1549Val mutation presented developmental delay or attention

222 deficit [66]. Phenotypic variability was observed among patients carrying different
223 *CACNA1H* mutation, some patients being diagnosed with FH-II and some others with
224 sporadic PA or APA. This was also observed among member of the same family carrying the
225 same *CACNA1H* mutation and was attributed to age-dependent variability or to genetic or
226 environmental modifiers [66]. All the mutations identified in Cav3.2 affect significantly the
227 electrophysiological properties of the channel, modifying their activation and/or inactivation
228 properties, their steady-state inactivation and/or their voltage-dependent facilitation [66, 67].
229 These functional modifications lead to shift of the voltage dependence of the channel towards
230 more negative potentials, increasing calcium influx into the cell, with consequent activation of
231 calcium signaling and aldosterone production [66, 67, 74].

232

233 *Other genetic forms of Hyperaldosteronism*

234 *De novo* germline mutations in *CACNA1D* (p.Gly403Asp and p.Ile770Met), encoding the $\alpha 1$
235 subunit of the L-type voltage gated calcium channel Cav1.3, were identified in two children
236 with early-onset PA with no adrenal abnormality [75]. These mutations induce a gain of
237 function, allowing the channel to open at lower voltages and leading to increased intracellular
238 calcium concentration. In these two young patients, PA was associated with seizures and
239 neurologic abnormalities (PASNA for Primary Aldosteronism associated with Seizures and
240 Neurologic Abnormalities).

241

242 *Genetic testing in Familial Hyperaldosteronism*

243 Genetic testing for Familial Hyperaldosteronism has been proposed in different categories of
244 patients [20, 76, 77]. Genetic testing for FH-I is recommended in patients with onset of PA
245 before 20 years of age and/or a family history of PA or strokes before 40 years of age.
246 Familial screening should also be performed in relatives of patients with confirmed FH-I, as

247 adverse cardiovascular damage by aldosterone may precede the onset of high blood pressure
248 [76, 78]. Genetic screening for FH-III is recommended in young patients with PA [20, 76,
249 77]. Genetic screening for FH-IV may be performed in patients with early onset hypertension
250 and PA before the age of 10 years, while mutations in *CACNAID* have to be searched for in
251 patients with early onset hypertension and PA associated with complex neurologic syndrome
252 with seizures [20, 76, 77]. The recent discovery of *CLCN2* mutations in FH-II strongly
253 supports the addition of this gene among those tested, if the criteria for a genetic form are
254 strong and other inherited forms were excluded. Practically speaking, it is difficult to clearly
255 separate the different familial forms of PA based on clinical and biochemical data, as there is
256 important clinical heterogeneity and overlap between them. Therefore, the main criteria for
257 performing genetic testing are a young age of onset and/or a family history of PA. Genetic
258 screening may allow identifying patients for targeted treatment and exclude patients from
259 undergoing AVS and possibly unilateral adrenalectomy in case of asymmetric aldosterone
260 production. It also allows better management of affected families and prevention of
261 cardiovascular complications.

262

263 **Somatic mutations in Aldosterone Producing Adenoma**

264

265 Whole exome sequencing studies performed on DNA from APA and paired germline DNA
266 led to the identification of recurrent somatic mutations in genes coding for ion channels
267 (*KCNJ5* and *CACNAID*) and ATPases (*ATP1A1* and *ATP2B3*) in a majority of APA [58, 75,
268 79, 80]. Similarly to familial forms, these mutations increase aldosterone biosynthesis by
269 affecting cell membrane potential and/or intracellular ion homeostasis in zona glomerulosa
270 cells. This ultimately increases intracellular calcium concentration and activates calcium
271 signaling, increasing *CYP11B2* expression and aldosterone production.

272 In 2011, Choi and co-workers performed whole exome sequencing in 22 APA, identifying
273 two different somatic mutations (p.Gly151Arg and p.Leu168Arg) affecting the *KCNJ5* gene
274 in 8 APA [58]. Using a similar strategy, somatic mutations were identified in *ATP1A1*, coding
275 for the α subunit of the Na^+, K^+ -ATPase [79, 80], *ATP2B3* coding for the plasma membrane
276 calcium ATPase 3 [80] and *CACNA1D*, coding for the voltage gated calcium channel Cav1.3
277 [75, 79]. The *KCNJ5* mutations affect GIRK4 ion selectivity and lead to sodium influx into
278 the cell. *ATP1A1* mutations lead to loss of pump activity and inward proton and sodium leak
279 and intracellular acidification [81]. The resulting chronic cell membrane depolarization
280 induces opening of voltage-dependant calcium channels with activation of calcium signalling
281 and stimulation of aldosterone production. Mutations in *ATP2B3* lead to a reduced calcium
282 export from the cell as well as increased calcium influx due to different mechanisms [82],
283 while mutations in *CACNA1D* lead to channel opening at less depolarized membrane
284 potentials. Again, these mutations increase intracellular calcium signalling.

285 The prevalence of somatic mutations in APA was investigated in different studies by Sanger
286 sequencing on DNA extracted from nodules identified macroscopically [83-88]. The largest
287 study, performing target sequencing of hot spot regions of APA driver genes in 474 APA,
288 identified somatic mutations in 54% of samples [84]. *KCNJ5* mutations were found in 38% of
289 APA, *CACNA1D* in 9.3%, *ATP1A1* in 5.3%, and *ATP2B3* in 1.7% of the samples (Table 1).

290 Recently, a different sequencing approach using full-gene-based next generation sequencing
291 (NGS) performed on DNA extracted from aldosterone synthase positive regions on formalin-
292 fixed paraffin-embedded (FFPE) tissues completely changed the prevalence of somatic
293 mutations in APA. This approach has allowed identifying somatic mutations in up to 88% of
294 APA, showing that in some cases APA were satellite small nodules neighboring large
295 adenomas without CYP11B2 expression [89-91] (Table 1).

296 In the majority of studies, somatic *KCNJ5* mutations are the most prevalent, with an overall
297 prevalence of 43% in a meta-analysis of somatic *KCNJ5* mutations comprising 1636 patients
298 with APA [92]. Ethnic differences were observed among cohorts. The frequency of somatic
299 *KCNJ5* mutations ranges from 40% in European and white American cohorts to up to 76% in
300 Asian cohorts [87, 93-95]. In the meta-analysis, the frequency of somatic *KCNJ5* mutations
301 was 63% in patients from East Asia and 35% in patients from Europe + the United States +
302 Australia [92]. Moreover, somatic mutations in *CACNA1D* are most frequent in APA from
303 African-American patients (42%) while the frequency of somatic *KCNJ5* mutations in this
304 population was 34% [90]. The CYP11B2 immunohistochemistry-guided next-generation
305 sequencing (IHC-guided NGS) approach has allowed the identification of a larger number of
306 somatic *CACNA1D* and *ATP1A1* mutations in APA. In one cohort of white Americans, the
307 prevalence of *ATP1A1* and *CACNA1D* mutations was 21% and 17%, respectively [89], much
308 higher than in previous studies performing Sanger sequencing [84-86]. The frequency of
309 *ATP2B3* mutations ranges from 1.7% to 4% in different cohorts [84-86, 89, 90]. In addition to
310 these well characterized mutations, a small proportion of APA also carries mutations in
311 *CTNNB1*, coding for β -catenin. The frequency of *CTNNB1* mutations ranges from 2.1% to 5.1
312 % [85, 89, 90, 96, 97], but may be higher in women with PA during pregnancy or after
313 menopause [98]. Finally, a somatic *CLCN2* mutation was recently described in one APA [99],
314 while somatic *PRKACA* (coding for the catalytic subunit of protein kinase A) mutations were
315 observed in two patients, one with biochemical findings of aldosterone and cortisol co-
316 secretion [100].

317 Several studies aimed at identifying clinical and biochemical surrogate markers of the somatic
318 mutation status, allowing to predict mutations of APA before surgery. Analyzing the clinical
319 correlates of *KCNJ5* mutations in 1636 patients from 13 studies, Lenzini et al [92] showed
320 that carriers of *KCNJ5* mutations were younger and more often females, associations largely

321 observed in other studies, including studies using CYP11B2 IHC-guided NGS [83-86, 89, 90,
322 101, 102]. *KCNJ5* mutations were also associated with higher preoperative aldosterone levels
323 and larger tumor size [92], although these correlations were not observed in all cohorts [84].
324 The mutation status may influence the diagnosis of PA, the therapeutic options and the
325 outcome, including cardiovascular complications. Association of *KCNJ5* mutations with
326 higher lateralization index at adrenal venous sampling (AVS) was described, suggesting
327 increased aldosterone production on the APA side and suppression on the contralateral
328 adrenal [103]. This association was not replicated in a subsequent study [104]. Concerning the
329 identification of somatic mutations before surgery and a potential impact for the selection of
330 PA patients undergoing AVS, Williams et al described a steroid fingerprint in peripheral vein
331 plasma able to correctly classify the somatic genotype of 92% of APA [105]. In addition,
332 based on the PASO criteria for APA outcome [106], *KCNJ5* mutations were shown to be an
333 independent predictor of hypertension cure after adrenalectomy [107]. Finally, *KCNJ5*
334 mutations were correlated to more prominent cardiovascular damage and higher left
335 ventricular mass in an Italian study [108], and significant postoperative improvements in left
336 ventricular mass in Japanese subjects [109]. The discovery of somatic mutations in APA
337 driver genes also opens new perspectives in PA care, particularly in therapeutic options
338 targeting mutant ion channels. It has been shown that high therapeutic doses of calcium
339 channel blockers such as verapamil are able to block a *KCNJ5* mutant channel *in vitro* [110].
340 In addition, recent studies revealed that macrolides may also inhibit mutant *KCNJ5* channels,
341 decreasing *CYP11B2* expression and aldosterone production both in the adrenocortical
342 HAC15 cell line [111] and in primary culture of APA harboring *KCNJ5* mutations [112].
343 Currently, study of the potential use of macrolides for the preoperative identification of
344 *KCNJ5* positive APA and for PA subtype diagnosis is ongoing [113].

345

346 **Somatic mutations in other aldosterone producing structures in the adrenal gland**

347

348 CYP11B2 expression is not only found in a limited number of ZG cells, but also in specific
349 morphological structures with a defined boundary from the neighboring areas, but without an
350 apparent fibrous capsule, called Aldosterone Producing Cell Clusters (APCC) [114, 115].
351 These specific structure express CYP11B2 and 3 β -HSD2, but not Disabled-2, a marker of ZG
352 cells, or CYP11B1, a marker of ZF cells, suggesting that APCC may constitute an
353 intermediate structure between ZG and ZF cells [114, 115]. APCC were described in normal
354 adrenal cortex as well as in adrenal cortex adjacent to an APA; they have been proposed to
355 constitute renin-independent aldosterone producing structures.

356 RNA-sequencing revealed that APCC have a molecular profile very similar to the ZG, but
357 different from ZF samples [116]. CYP11B2 immunohistochemistry guided NGS of FFPE
358 adrenal tissue allowed to sequence APCC from normal adrenal glands and to identify somatic
359 mutations in *CACNA1D*, *ATP1A1* and *ATP2B3* [116]. Based on this finding, it was
360 hypothesised that APA derive from APCC. Interestingly, this hypothesis was reinforced by
361 the identification of specific lesions called APCC-to-APA translational lesions (pAATL);
362 these structures are composed of a sub-capsular APCC-like structure and an inner micro-
363 APA-like (mAPA-like) structure [117]. Mutations in APA driver genes were identified in
364 different parts of pAATL; in one case a somatic *KCNJ5* mutation was identified only in the
365 mAPA-like structure, whereas in another case a somatic *ATP1A1* mutation was identified in
366 the entire structure [117]. Taken together, these results suggest that APA with *ATP1A1*,
367 *ATP2B3* and *CACNA1D* mutations may derive from APCC, however the sequence of events
368 leading to APA development remains unknown.

369 Recently, the presence of APCC was also described in a cohort of 15 patients with bilateral
370 adrenal hyperplasia who underwent unilateral adrenalectomy [118]. All adrenals harbored at

371 least one APCC; sequencing of 99 APCC from 14 out of 15 of these adrenals revealed the
372 presence of somatic mutations in at least one APCC per adrenal. In contrast to the prevalence
373 of mutations observed in APA, mutations are almost exclusively found in *CACNA1D* [118].
374 Interestingly, the number of APCC was significantly higher in adrenals with BAH than in
375 adrenals from normotensive controls, suggesting that accumulation of APCC harboring
376 somatic mutations in APA driver genes may contribute to BAH. Despite a small number of
377 samples, and the need for replication in other cohorts of patients, this study for the first time
378 identified a potential role for APCC in the development of BAH.

379

380 **Conclusions**

381

382 During the last ten years, whole exome sequencing allowed to decipher the genetic basis of
383 familial and sporadic forms of PA. Until recently, the frequency of somatic mutations in APA
384 was considered to be $\approx 50\%$. However, the recent application of CYP11B2-
385 immunohistochemistry guided NGS revealed the presence of somatic mutations in known
386 APA driver genes in up to 90% of APA, suggesting that a majority of APA is due to
387 mutations in known genes. Our understanding of inherited forms of PA has been significantly
388 improved with the identification of germline mutations in *KCNJ5*, *CACNA1H* and *CLCN2*
389 and the definition of four familial forms of hyperaldosteronism, FH-I to FH-IV. Nevertheless,
390 the genetic defects underlying many familial cases remain still unknown. Considering the
391 high prevalence of PA in hypertensive patients and the current criteria for defining familial
392 forms, a certain number of familial cases may however be due to co-occurrence of sporadic
393 PA within the same family. Further studies will need to address whether other genes are
394 involved in APA and familial hyperaldosteronism and replicate genetic findings observed in
395 bilateral forms of the disease.

396

397 **Summary**

398

399 Understanding the genetic basis of primary aldosteronism is important to improve diagnosis
400 and therapeutic approaches of the disease. During the last ten years, whole exome sequencing
401 has been successful applied to identify the genetic determinants of PA. Mutations in genes
402 coding for ion channels and pumps have been identified in APA as well as in familial
403 hyperaldosteronism, all affecting intracellular ion homeostasis. Moreover, the recent
404 identification of mutations in APA driver genes in APCC constitutes a new path to understand
405 the mechanisms responsible for bilateral adrenal hyperplasia and opens new perspectives for
406 therapeutic approaches in PA.

407

408 **Practice points**

409

- 410 • Somatic mutations in genes regulating intracellular ion homeostasis are present in at
411 least 90% of APA.
- 412 • APCC harboring somatic mutations in APA driver genes may contribute to APA
413 development and to bilateral adrenal hyperplasia
- 414 • Genetic discoveries in PA have the potential to improve diagnosis and treatment of PA
415 in the near future.

416

417 **Research agenda**

418

- 419 • Whole genome sequencing and characterization of the epigenome to further decipher
420 the genetic basis of PA, particularly for BAH.

421 • Development of new methods allowing preoperative detection of somatic mutations to
422 improve the diagnosis and treatment of patients with PA.

423 • Need of functional studies to decipher the sequence of events leading to APA
424 formation.

425

426 **Disclosure statement**

427 The authors declare that none have either any financial interest or conflicts of interest.

428

429 **References**

430

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801

802

803 **Figure legends**

804

805 **Figure 1: Steroidogenic pathway for aldosterone and cortisol production in the adrenal**
806 **cortex.** P450scc: cytochrome P450 side chain cleavage; 3 β -HSD2: 3 β -hydroxysteroid
807 dehydrogenase type II.

808

809 **Figure 2: Mechanisms responsible for Familial Hyperaldosteronism.** (A) FH-I is due to
810 unequal crossing-over between *CYP11B1* and *CYP11B2*, leading to the fusion of the
811 regulatory sequences of *CYP11B1* to the coding sequence of *CYP11B1*, resulting in ectopic
812 expression of aldosterone synthase in the ZF and regulation by ACTH. (B) FH-II is due to
813 mutations in the chloride channel CLC-2. (C) FH-III is due to mutations in the potassium
814 channel GIRK4. (D) FH-IV is due to mutations in the T-type calcium channel Cav3.2.
815 Mutations in CLC-2, GIRK4 and Cav3.2 lead, directly or indirectly, to increase in
816 intracellular calcium concentration, triggering increased expression of CYP11B2 and
817 increased aldosterone biosynthesis.

818

819

1 Table 1. Frequency of somatic mutations in Aldosterone Producing Adenomas

2

Mutation	Sanger Sequencing	References	IHC-guided NGS	References
<i>KCNJ5</i>	13.2% - 76.8%	[58, 75, 79, 80, 83-87, 93-96, 101-104, 109, 119-126]	43% - 44%	[89, 91]
<i>CACNA1D</i>	0% - 10.3%	[75, 79, 84-87, 94-97, 109, 122, 124-126]	13% - 21%	[89, 91]
<i>ATP1A1</i>	0% - 8.5%	[75, 79, 80, 84-87, 94-97, 104, 109, 120, 122, 124-126]	13% - 17%	[89, 91]
<i>ATP2B3</i>	0% - 3.1%	[75, 79, 80, 84-87, 94-97, 104, 120, 122, 124-126]	4% - 10%	[89, 91]
<i>CTNNB1</i>	3.7% - 5.1%	[75, 79, 85, 96, 97]	3%	[89]
<i>CLCN2</i>	one case	[99]	NA	

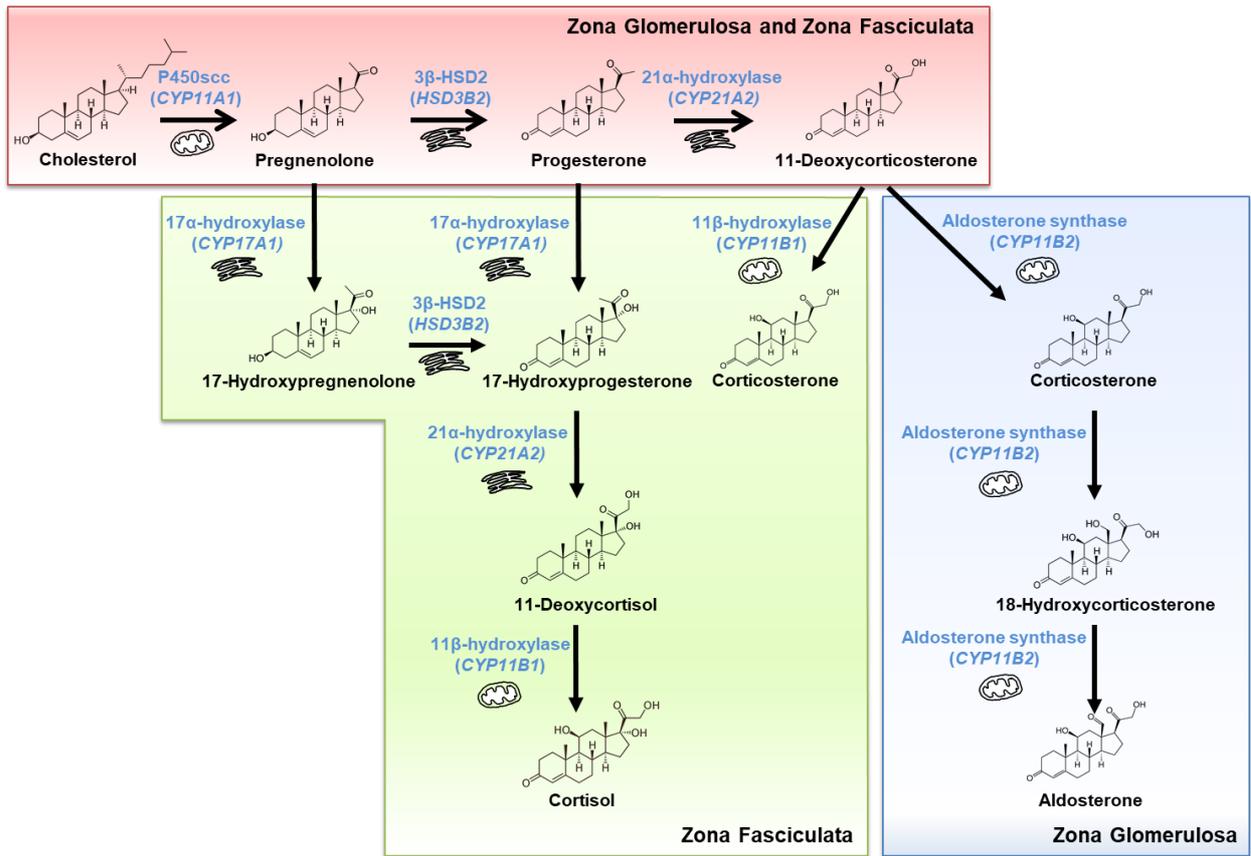
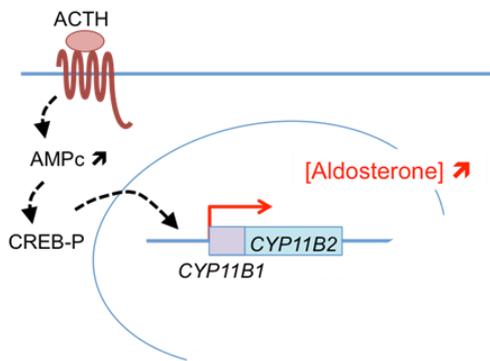
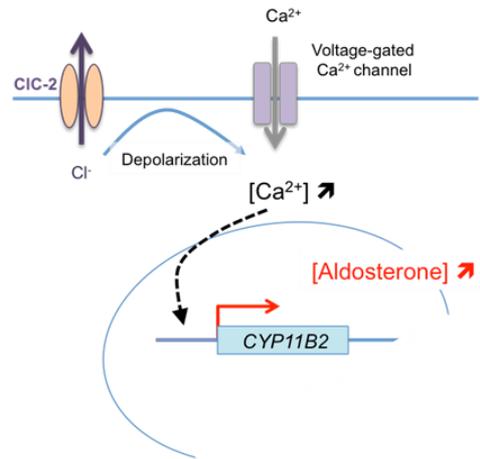


Figure 1

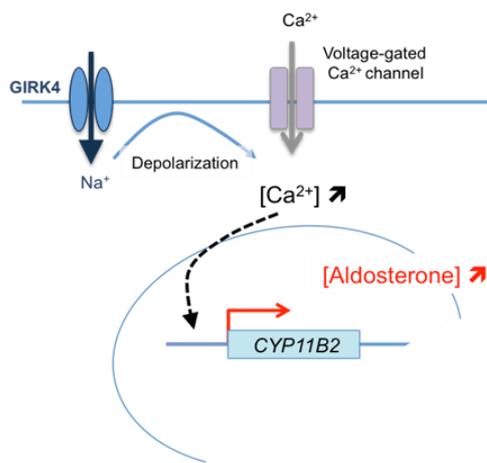
A. **FH-I: CYP11B1-CYP11B2 chimeric gene**



B. **FH-II: Mutation in CLCN2**



C. **FH-III: Mutation in KCNJ5**



D. **FH-IV: Mutation in CACNA1H**

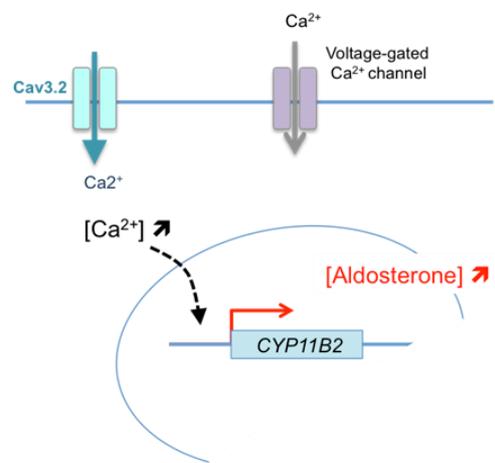


Figure 2