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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 $\beta$ -  
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 $\alpha$ -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 $\alpha$ -hydroxylase and 11 $\beta$ -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 $\beta$ -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<sup>+</sup>). 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<sup>+</sup> conductance due to the presence of a large number of potassium channels [4]  
48 and the Na<sup>+</sup>/K<sup>+</sup>-ATPase that maintain the K<sup>+</sup> gradient concentration between the plasma and  
49 the cytosol. Stimulation of ZG cells by AngII or increased plasma K<sup>+</sup> concentration lead to  
50 cell membrane depolarization, opening of voltage-gated Ca<sup>2+</sup> channels and increase of the

51 intracellular  $\text{Ca}^{2+}$  concentration. In parallel, binding of AngII to the angiotensin II Type 1  
52 receptor (AT1R) stimulates inositol triphosphate-dependent  $\text{Ca}^{2+}$  release from the  
53 endoplasmic reticulum. Increase of intracellular  $\text{Ca}^{2+}$  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 $\beta$ -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<sup>-</sup> ions leads to cell membrane depolarization, opening of voltage gated  
155 Ca<sup>2+</sup> 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*<sup>R180Q/+</sup> 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<sup>+</sup> selectivity in favor of sodium and increase  
194 in Na<sup>+</sup> 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<sup>+</sup> 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  $\alpha$  subunit of the  $\text{Na}^+, \text{K}^+$ -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  $\beta$ -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 $\beta$ -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

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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.** P450<sub>scc</sub>: cytochrome P450 side chain cleavage; 3 $\beta$ -HSD2: 3 $\beta$ -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

<b>Mutation</b>	<b>Sanger Sequencing</b>	<b>References</b>	<b>IHC-guided NGS</b>	<b>References</b>
<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	

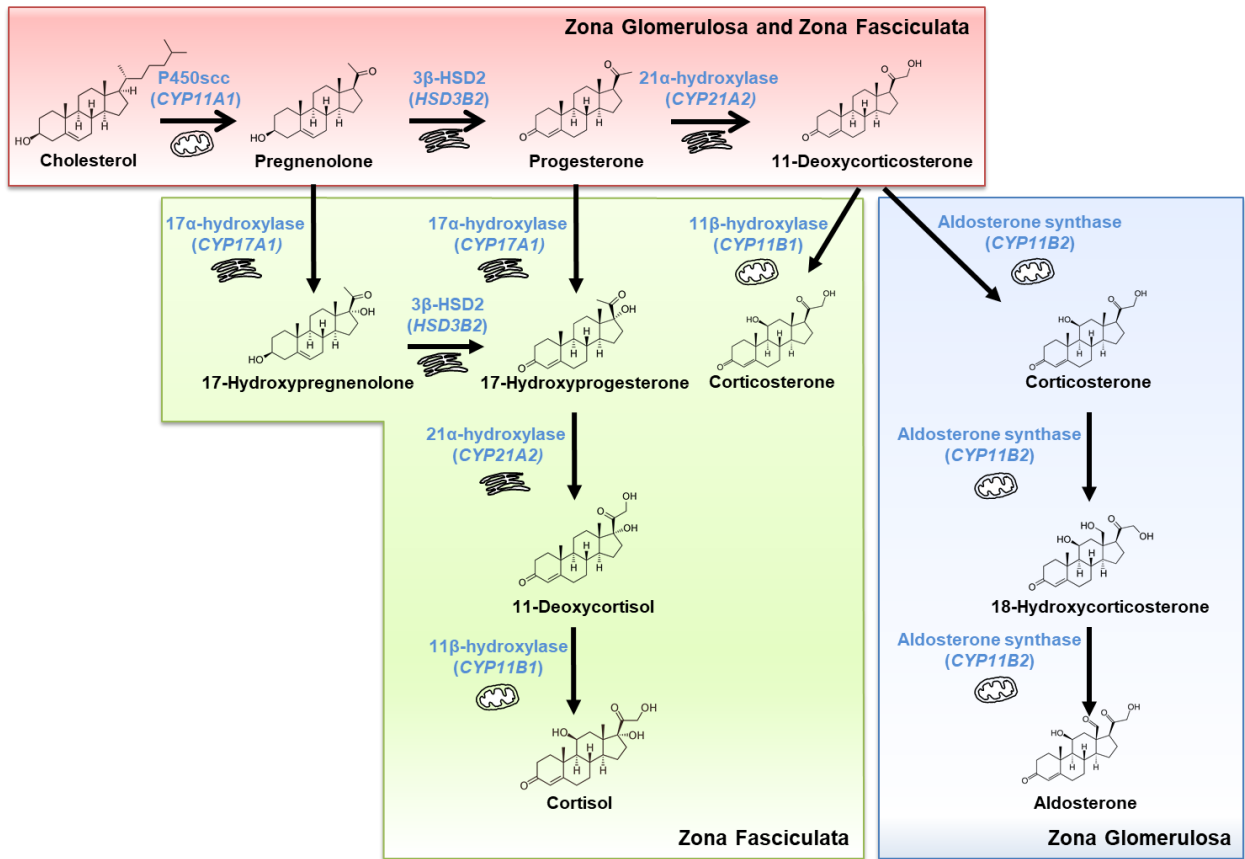
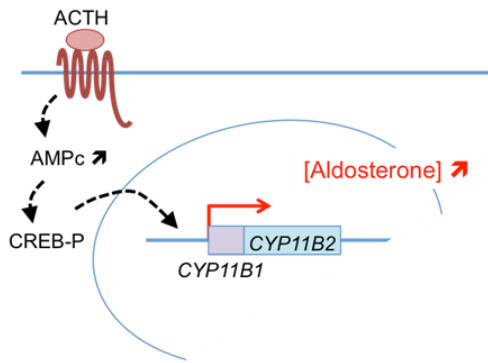


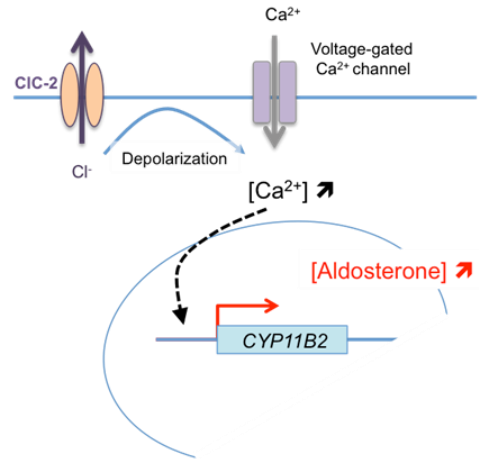
Figure 1



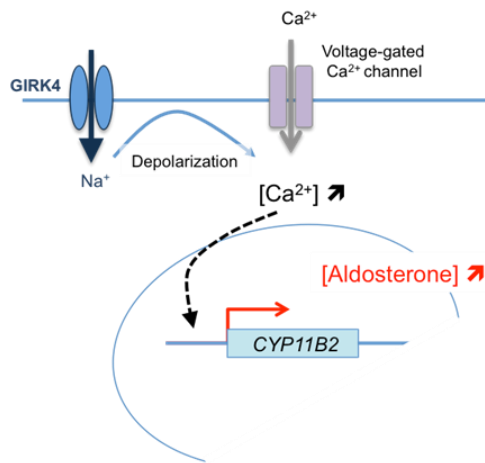
A. **FH-I: CYP11B1-CYP11B2 chimeric gene**



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



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



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

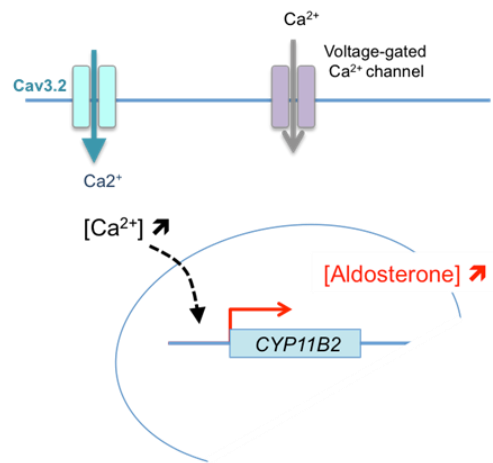


Figure 2