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Molecular genetics of Conn adenomas in the era of exome analysis

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Abstract

Aldosterone producing adenomas (APA) are a major cause of primary aldosteronism (PA), the most common form of secondary hypertension. Exome analysis of APA has allowed the identification of recurrent somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* in more than 50% of sporadic cases. These gain of function mutations in ion channels and pumps lead to increased and autonomous aldosterone production. In addition, somatic *CTNNB1* mutations have also been identified in APA. The *CTNNB1* mutations were also identified in cortisol producing adenomas and adrenal cancer, but their role in APA development and the mechanisms specifying the hormonal production or the malignant phenotype remain unknown. The role of the somatic mutations in the regulation of aldosterone production is well understood, while the impact of these mutations on cell proliferation remains to be established. Furthermore, the sequence of events leading to APA formation is currently the focus of many studies. There is evidence for a two-hit model where the somatic mutations are second hits occurring in a previously remodeled adrenal cortex. On the other hand, the APA-driver mutations were also identified in aldosterone-producing cell clusters (APCC) in normal adrenals, suggesting that these structure may represent precursors for APA development. As PA due to APA can be cured by surgical removal of the affected adrenal gland, the identification of the underlying genetic abnormality by novel biomarkers could improve diagnostic and therapeutic approaches of the disease. In this context, recent data on steroid profiling in peripheral venous samples of APA patients and on new drugs capable of inhibiting mutated potassium channels provide promising preliminary data with potential for translation into clinical care.

1 **Introduction**

2 Arterial hypertension (HT) is a worldwide health problem which affects ~25% of the
3 global population [1], resulting in an estimated 9.4 million deaths or approximately 12.8% of
4 all deaths (Global Health Observatory data, WHO). A vast and diverse array of drugs exists
5 for the treatment of HT, such as diuretics, antagonists of the renin-angiotensin-aldosterone
6 system, notably angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor
7 blockers, calcium channel blockers, vasodilators, β adrenergic blocking agents. Optimal blood
8 pressure control, however, is still far from being achieved in up to two thirds of the
9 hypertensive population. In a certain proportion of cases, HT can arise from a specific
10 disease; endocrine hypertension, a frequent form of secondary arterial hypertension, emerges
11 following a dysregulation of one or more hormones that are involved in blood pressure
12 regulation. Primary aldosteronism (PA), also known as Conn's syndrome, is the most frequent
13 form of secondary hypertension with estimates of up to 10% of cases in referred patients, 4%
14 in primary care [2] and 20% in patients with resistant hypertension [3,4]. PA is mainly due to
15 aldosterone producing adenoma (APA) and bilateral adrenal hyperplasias (BAH, or idiopathic
16 hyperaldosteronism, IHA). The clinical picture of patients with PA consists of HT, a high
17 aldosterone to renin ratio, which has become one of the major diagnostic tools for PA
18 alongside different confirmation tests and adrenal venous sampling for subtype diagnosis, and
19 variable hypokalemia and metabolic alkalosis [5]. PA is associated to an increased risk of
20 cardiovascular complications, which occur beyond the effect of hypertension, such as
21 coronary artery disease, heart failure, myocardial infarction, atrial fibrillation and renal
22 damage. Different studies insist on the importance of screening most hypertensive patients for
23 PA to either confirm or exclude the diagnosis [6,7]. Indeed, early PA diagnosis can improve
24 prognosis and prevent the development of target organ damage.

25 Although the management of PA in hypertensive patients has come a long way and the
26 treatment is much better established, the prognosis of unilateral PA depends on different
27 criteria such as age, sex, BMI, age upon diagnosis and the duration of hypertension [7,8].
28 Indeed, younger patients and female patients show a better clinical outcome after
29 adrenalectomy in comparison to older or male patients [7].

30

31 **Aldosterone biosynthesis in the adrenal cortex**

32 The human adrenal cortex is composed of three distinct zones that are characterized by
33 their respective functions. Steroid hormones are synthesized following the sequential
34 enzymatic breakdown of cholesterol by different cytochrome P450 enzymes as well as
35 hydroxysteroid dehydrogenases, the particularity of each zone lies in the expression of
36 specific steroidogenic enzymes and the ability to have each its own regulators. The most outer
37 zone of the adrenal cortex, the zona glomerulosa (ZG), expresses aldosterone synthase
38 (encoded by *CYP11B2*), which catalyzes the hydroxylation at the C11 position of the 11-
39 deoxycorticosterone into corticosterone, furthermore, its hydroxylation at C18 into
40 18(OH)corticosterone followed by an oxidation of C18's hydroxyl group giving as an end
41 result aldosterone. The main trigger for aldosterone biosynthesis is the activation of
42 intracellular calcium signaling in the zona glomerulosa which is induced by either angiotensin
43 II (Ang II) from the renin-angiotensin system or by extracellular potassium levels. The zona
44 fasciculata (ZF) of the adrenal cortex mainly produces cortisol. In this process, the conversion
45 of progesterone into 17-hydroxyprogesterone is catalyzed by the activity of 17 α -hydroxylase
46 which is not expressed in ZG cells. 17-hydroxyprogesterone undergoes a hydroxylation at
47 C21 by the 21-hydroxylase enzyme, and finally a hydroxylation at position C11 by 11 β -
48 hydroxylase (encoded by *CYP11B1*) forming cortisol. The main regulator of cortisol

49 production is the hypothalamus-pituitary-adrenal (HPA) axis primarily through the
50 adrenocorticotrophic hormone (ACTH).

51 Ang II is one of the major regulators of aldosterone secretion by ZG cells. The binding of
52 AngII to its receptor (AT1) will lead to the activation of the Gαq-phospholipase C-mediated
53 pathway, increasing inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol concentrations.
54 Ultimately, IP3 is responsible for increased intracellular calcium concentration due to calcium
55 release from intracellular stores. AngII also inhibits the background TWIK-related acid-
56 sensitive potassium channel (TASK), as well as GIRK4 and the Na⁺,K⁺ -ATPase, leading to
57 a cell membrane depolarization [9]. This will lead to opening of voltage-gated Ca²⁺ channels,
58 also increasing intracellular calcium concentrations.

59 The other main stimulator for aldosterone biosynthesis is the increase in extracellular
60 potassium levels. In the normal ZG cell at a resting state, the cell membrane potential is
61 hyperpolarized, the reason is that the membrane potential follows closely the equilibrium
62 potential of potassium in these cells which largely express potassium channels. Small
63 increases in extracellular potassium levels cause ZG cell membrane depolarization. The
64 depolarization of the ZG cell membrane leads to the opening of voltage gated Ca²⁺ channels
65 and an increase in intracellular calcium levels resulting in the activation of calcium signaling.

66 Calcium signaling acts by increasing the release of deesterified cholesterol from
67 cytoplasmic stores, as well as cholesterol delivery to the outer mitochondrial membrane and
68 then to the inner mitochondrial membrane by increasing the expression of the steroid acute
69 regulatory protein (StAR). Calcium signaling also increases the expression of cofactors
70 required for p450 cytochrome enzymes. Calcium/Calmodulin binding in the cytosol of the ZG
71 cell induces the activation of protein kinases that regulate phosphorylation of transcription
72 factors involved in *CYP11B2* transcriptional induction, mainly nuclear receptor subfamily 4

73 group A 1 and 2 (*NR4A1* and *NR4A2* coding for NUR77/NGF1B and NURR1 respectively)
74 and the cyclic AMP-responsive element-binding protein (CREB).

75

76 **Genetic abnormalities in APA**

77 PA is due to inappropriate aldosterone production by the adrenal cortex in spite of the
78 suppression of the renin-angiotensin system. In the last years, whole exome sequencing
79 (WES) performed on DNA from APA led to the identification of recurrent somatic mutations
80 in genes coding for ion channels (*KCNJ5* and *CACNA1D*) and ATPases (*ATP1A1* and
81 *ATP2B3*). These genes are essential for regulating intracellular ion homeostasis and cell
82 membrane potential. All these mutations promote an increased intracellular calcium signaling
83 through cell membrane depolarization and opening of voltage-dependent calcium channels, or
84 impaired intracellular calcium recycling, therefore leading to high aldosterone levels by
85 constitutive expression of *CYP11B2*.

86 In a large multicenter study from the European Network for the Study of Adrenal Tumors
87 (ENS@T) that analyzed somatic mutations in APA from 474 patients [10], hot spot regions
88 for mutations in *KCNJ5*, *CACNA1D*, *ATP1A1* and *ATP2B3* were sequenced. Somatic
89 mutations were identified in 54.2% of APA, with *KCNJ5* being the most prevalent at 38 %,
90 *CACNA1D* at 9.3%, *ATP1A1* at 5.3% and *ATP2B3* 1.7% of these mutations. However,
91 *KCNJ5* mutations are more prevalent in Asian populations, with up to 76% of prevalence [11-
92 15]. These observations were corroborated by a meta-analysis of clinical and genetic data
93 from 1636 patients with APA showing an overall prevalence of *KCNJ5* mutations of 43%,
94 with higher prevalence in patients from Asia [16]. Some aldosterone producing adenomas
95 also carry somatic mutations in the gene that codes for β -catenin (*CTNNB1*), less common
96 mutations have also been identified in *PRKACA* (encoding Protein Kinase cAMP-Activated
97 Catalytic Subunit α) [17-19].

98 *KCNJ5* codes for an inwardly rectifying K⁺ channel, which is the G-protein-activated
99 inward rectifier potassium channel GIRK4 (also known as Kir3.4). It is mainly expressed in
100 the ZG of the adrenal cortex. *KCNJ5* mutations were found to be more frequent in female and
101 younger patients, and the expression of GIRK4 in APA was found to be correlated to the
102 mutation status [20]. GIRK4 is composed of 2 membrane spanning helices with one pore-
103 forming region in between and N- and C- termini that contribute to the pore structure [21].
104 Choi et al identified two somatic *KCNJ5* mutations mapping to the selectivity filter of GIRK4
105 (p.Gly151Arg and p.Leu168Arg). In addition to these two mutations (the most prevalent
106 mutations in APA), the majority of the *KCNJ5* mutations described are located within or near
107 the selectivity filter, rendering the channel permeable to sodium, which leads to chronic cell
108 membrane depolarization [22]. Transient transfection of *KCNJ5* mutants in HAC-15 resulted
109 in a calcium-dependent increase in *CYP11B2* expression and aldosterone biosynthesis in the
110 cells; the mutant GIRK4, however, did not induce any increase in proliferation but rather a
111 reduced cell viability or sodium-induced cell death [23,24]. This leaves the question of the
112 role of *KCNJ5* mutations on the cell proliferation and APA formation unanswered in tumors
113 where these mutations occur. *KCNJ5* mRNA expression is not affected by *KCNJ5* mutations,
114 but APA harboring *KCNJ5* mutations show decreased GIRK4 protein expression when
115 compared with adjacent ZG, allowing the differentiation from APA harboring other mutations
116 or without mutations identified [20,25].

117 More than 20 mutations have been identified in *CACNA1D* (encoding the voltage-
118 dependent L-type calcium channel subunit alpha-1D, Cav1.3) [26]. The Cav1.3 calcium
119 channel consists of 4 repeat domains, each one consisting of six transmembrane segments,
120 with a membrane-associated loop between S5 and S6 [27-29]. Mutations occurring in
121 *CACNA1D* are gain of function mutations that lead to a decrease in the threshold of the
122 voltage-dependent activation or impaired channel inactivation, which is followed by increased

123 intracellular calcium concentrations and thereby an induction of aldosterone biosynthesis
124 [28,29].

125 *ATP1A1* and *ATP2B3* are members of the P-type family of ATPases and are composed of
126 10 transmembrane domains (M1 - M10) with intracellular N- and C- termini. *ATP1A1* codes
127 for the Na(+)/K(+) ATPase alpha-1 subunit. Mutations in this pump lead to a loss of its
128 activity and affinity to K⁺ and to an inward proton or sodium leak, which has been proposed
129 to induce aldosterone production through cell membrane depolarization and increased calcium
130 influx [28,30]. Nevertheless, transient transfection of 2 of the described *ATP1A1* mutations in
131 the adrenocortical cell line H295R did not result in modifications of basal cytosolic calcium
132 levels, and barely increased potassium-stimulated calcium concentrations, in spite of
133 depolarizing the cells and stimulating aldosterone biosynthesis. In these cells, Stindl et al
134 found that there was an increased intracellular acidification, which was suggested to regulate
135 *CYP11B2* biosynthesis [31].

136 *ATP2B3* codes for the plasma membrane calcium-transporting ATPase 3 (PMCA3).
137 Mutations of PMCA3 are found in the transmembrane domain M4 and result in the deletion
138 of different amino acids in the region between Leu422 and Leu433. One mutation in
139 particular, p.Leu425_Val426del, leads to reduced calcium export which is due to the loss of
140 the physiological pump functions, and an increased intracellular calcium signaling due to the
141 depolarization-activated Ca²⁺ channels [32]. Recently, a second mechanism explaining
142 aldosterone production due to *ATP2B3* mutations was suggested. *ATP2B3* mutations induce
143 an increase in calcium influx by the opening of depolarization-activated calcium channels and
144 by a possible calcium leak through the mutated PMCA3 [32].

145 *CACNA1H* encoding the pore-forming $\alpha 1$ subunit of the T-type voltage-dependent calcium
146 channel Cav3.2 has been recently shown to be involved in familial forms of PA in some cases
147 associated with developmental disorders [33,34]. In addition, it was also described as

148 germline mutation in a patient with APA [34]. This channel consists of a single polypeptide
149 chain of four homologous domains (I-IV), each one containing six transmembrane spans (S1-
150 S6) and cytoplasmic C- and N- Termini. Mutant Cav3.2 channels show significant changes in
151 their electrophysiological properties, specifically a shift in activation towards more negative
152 voltages and modifications of their inactivation properties. Consequently, the channels are
153 activated at less depolarized voltages leading to activation of calcium signaling and
154 autonomous aldosterone production [33,34]. A germline *CACNA1H* variant was identified in
155 one patient with APA without somatic mutations and improvement after adrenalectomy
156 [33,34]. This case suggest that *CACNA1H* might be a susceptibility gene for different types of
157 PA, including APA.

158 The Wnt/ β -Catenin signaling pathway has been shown to play an important role in the
159 development of the adrenal cortex and in aldosterone biosynthesis [35]. This signaling
160 pathway is constitutively active in ~70% of APA [36]. In unstimulated conditions, β -catenin
161 is located in the cytosol, and is part of the axin complex along with adenomatous poliposis
162 coli protein (APC), axin, Glycogen Synthase Kinase-3 β (GSK-3 β) and Caseine Kinase-1 β .
163 Eventually, β -catenin in this complex will be phosphorylated resulting in its degradation by
164 the proteasome, and preventing its translocation to the nucleus and the activation of different
165 Wnt target-genes. The activation of the pathway occurs through binding of Wnt ligand to its
166 receptor Frizzled resulting in the inhibition of the phosphorylation of β -catenin, which
167 dissociates from the axin complex and translocates to the nucleus where it induces the
168 expression of Wnt target genes, most notably the transcription factors T-cell factor (*TCF*) and
169 lymphocyte enhancer factor (*LEF*), through its actions as a transcriptional coactivator [35].
170 Mutations in the *CTNNB1* gene, encoding β -Catenin, have been described in 2-5% of APA
171 [17,19]. The description of somatic *CTNNIB* mutations associated with higher expression of
172 luteinizing hormone–chorionic gonadotropin receptor (LHCGR) and gonadotropin-releasing

173 hormone receptor (GNRHR) in APA diagnosed during pregnancy or menopause suggested
174 that pregnancy may reveal an underlying PA [37]. Other studies, however, showed high
175 expression of GNRHR and LHCGR in more than 40% of APA [38,39], and the presence of
176 *CTNNB1* mutations both in females and males [17,19]. Further studies are necessary to
177 establish the mechanism of *CTNNB1* mutations in the development of APA.

178 To a much lesser extent, somatic *PRKACA* (encoding the catalytic α subunit of Protein
179 Kinase A) mutations have been described in APA [18]. Rhayem et al identified somatic
180 mutations of the *PRKACA* gene in two patients with APA by whole exome sequencing. The
181 mutation p.Leu206Arg, previously identified in CPA [40-42], was found in one patient with
182 PA and Cushing syndrome. The second mutation (p.His88Asp) was identified in a patient
183 without cortisol hypersecretion [18]. This particular mutation was not associated with a gain
184 of function, the mechanism underlying increased PKA signaling and tumorigenesis in cortisol
185 producing adenoma. The role of these mutations on aldosterone secretion and their frequency
186 in APA remains to be established.

187 *CTNNB1* mutations and *PRKACA* mutations are also identified in cortisol producing
188 adenomas (CPA). Other evidences for an overlap of genetic determinants of aldosterone and
189 cortisol excess have been described, including the cortisol co-secretion observed in a subset of
190 APA, notably those harboring *KCNJ5* mutations [43]. The complex mechanisms that would
191 explain how the same mutations could end up in two different hormonal phenotypes remain to
192 be discovered.

193

194 **Clinical correlates of somatic mutations**

195 The discovery of clinical or biochemical surrogate markers of somatic mutations in
196 APA could be of benefit for the management of the disease. Different studies described the
197 higher prevalence of somatic *KCNJ5* mutations in women and in young patients with APA

198 [10,16,44]. *KCNJ5* mutations were also associated to higher levels of plasma aldosterone and
199 larger tumors [16], and with higher left ventricular mass index [45]. *CACNA1D* mutations
200 were associated with smaller APA [10]. More recently, *KCNJ5* mutations were described as a
201 predictor of better outcome in young patients with APA [46]. Promising data for the
202 identification of the underlying APA genotype came from a study that analyzed steroid
203 profiles in adrenal and peripheral venous plasma samples from APA patients by liquid
204 chromatography–tandem mass spectrometry [47]. The authors identified a 7-steroid
205 fingerprint in peripheral venous samples allowing to correct classify 92% of the APA
206 accordingly to genotype. Additionally, specific steroid profiles were associated with *KCNJ5*
207 mutations, in particular the presence of significantly higher hybrid steroids 18-
208 hydroxycortisol and 18-oxocortisol. This approach may be translated into clinical care,
209 allowing to identify the APA genotype from peripheral venous plasma samples before surgery.
210 This could be useful for the selection of patients for adrenal vein sampling.

211

212 **Heterogeneity of APA**

213 In spite of the fact that the relation between aldosterone production and the presence of
214 somatic mutations is well established, the impact of these mutations on nodule/APA
215 formation and cell proliferation is still far from fully understood. APA present a highly
216 pronounced molecular heterogeneity not only on a mutational status but also in terms of
217 aldosterone synthase expression within the same APA. Recent studies showed the presence of
218 different somatic mutations in different aldosterone-producing nodules from the same adrenal
219 [48,49], suggesting that somatic mutations are independent events occurring in a previously
220 remodeled adrenal cortex. In the same context, Nanba et al described one case of a patient that
221 was diagnosed with PA and Cushing syndrome with double adrenocortical adenomas
222 harboring each a *KCNJ5* and a *PRKACA* somatic mutation [50]. Furthermore, in APA

223 exhibiting heterogeneity of aldosterone synthase expression, APA driver mutations were
224 identified only in positive aldosterone synthase regions [51]. Interestingly, two different
225 mutations were identified in the same APA, lying in two distinct positive aldosterone synthase
226 regions [51]. These findings suggest that somatic mutations are second hits in APA
227 development that emerge from specific mechanisms that remain to be elucidated. Supporting
228 this hypothesis, our group described the occurrence of a germline *APC* mutation and a
229 somatic *KCNJ5* mutation leading to the development of an APA in a young patient with
230 severe unilateral PA, bilateral macronodular adrenal hyperplasia and Gardner syndrome [52],
231 suggesting a two-hit model for APA development with the *APC* mutation driving nodule
232 formation and the *KCNJ5* mutation being responsible for aldosterone hypersecretion.

233 Another theory was suggested by Nishimoto et al, in a study that describes aldosterone
234 producing cell clusters (APCC) in being the origin behind APA development [53]. APCCs are
235 structures of outer morphological ZG cells in contact with the capsule and inner ZF-like cells,
236 staining positive for both CYP11B2 and CYP11B1 [53,54]. They are found in normal adrenal
237 tissue and in adrenals with APA. APCC express important amounts of aldosterone synthase in
238 normal and pathological conditions. In a later study, Nishimoto et al sequenced DNA from
239 APCCs that were collected from normal adrenal glands and identified mutations in APA
240 driver genes in up to 35% of the collected samples; specifically, mutations in *CACNA1D*,
241 *ATP1A1* and *ATP2B3*. Interestingly, no mutations in *KCNJ5* were reported, which is the most
242 frequently mutated gene in APA [55]. The authors suggest that APCCs could represent
243 cellular precursors that could lead to APA with their specific mutations through unknown
244 mechanisms. On the other hand, they propose that APCCs with *KCNJ5* could be rarer, or that
245 APCCs that develop *KCNJ5* mutations tend to become APAs quite quickly and are hard to be
246 witnessed before the APA development [55]. It was suggested that the sequence of events
247 leading to APA development from an APCC occurs through the development of structures

248 called possible APCC-to-APA translational lesions (pAATL) [56]. pAATL are composed by
249 an outer APCC-like portion and inner micro-APA (mAPA)-like portion. The genetic
250 characterization of pAATL is complex. In one adrenal, a *KCNJ5* mutation was identified only
251 in the mAPA-like portion of a pAATL, not observed in the APCC-like portion and was
252 different from the mutation identified in an APA within the same adrenal. This suggests that
253 the APA and the pAATL do not share the same origin and that the *KCNJ5* mutation leads to
254 differentiation of the mAPA portion from the APCC. In a second adrenal, both portions of the
255 pAATL carried an *ATP1A1* mutation indicating its clonal origin. Although the model
256 whereby APA arise from APCC through pAATL and mAPA is intriguing, further studies are
257 required to better clarify the suite of genomic events involved in this transition.

258

259 **Conclusion**

260 The role of each mutation in the regulation of aldosterone production is well studied, while
261 the impact of these mutations on cell proliferation remains to be established. In the future it
262 would be of relevance to distinguish additional biomarkers or the development of techniques
263 that are able to identify somatic mutations in APA. This could be of interest since PA is the
264 most frequent form of secondary hypertension and is curable by the surgical removal of the
265 APA carrying adrenal if recognized early enough. An additional benefit is the possibility of
266 developing new diagnostic and therapeutic approaches. This is particularly the case for the
267 use of macrolides in the detection and treatment of APA with *KCNJ5* mutations [57]. A recent
268 work has shown that macrolide antibiotics, including roxithromycin, are potent inhibitors of
269 *KCNJ5* channels carrying the most frequent mutations p.Gly151Arg and p.Leu168Arg. Use of
270 clarithromycin in primary cultures from APA showed a significant inhibition of *CYP11B2*
271 gene expression and aldosterone production [58]. These compounds could therefore be used

272 to identify patients carrier of APA with KCNJ5 mutations and as targeted treatments in
273 patients who are not candidates for surgery.

274

275

276

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284

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286 The authors have nothing to disclose.

287

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476 **Figure legend**

477 **Figure 1. Regulation of aldosterone biosynthesis in zona glomerulosa cells.** (A) In
478 basal conditions, zona glomerulosa cells are in a hyperpolarized state due to the activity of
479 potassium channels at the cell membrane. (B) The binding of AngII to its receptor AT1R or
480 the increase of extracellular K^+ concentration lead to inhibition of K^+ currents through TASK
481 and GIRK4 channels, followed by cell membrane depolarization; AngII also inhibits the
482 activity of the Na^+/K^+ -ATPase pump (*ATP1A1*) activity. This depolarization leads to the
483 opening of voltage gated calcium channels on the cell membrane increasing Ca^{2+}
484 concentrations in the cytosol. AngII also induces, through inositol triphosphate (IP3), the
485 release of Ca^{2+} from the sarco/endoplasmic reticulum. The increased intracellular Ca^{2+}
486 concentration leads to the activation of the calcium signaling pathway, the major trigger for
487 aldosterone biosynthesis. (C) In pathological conditions, mutations affecting specific ion
488 channels (*CACNA1D*, *CACNA1H*, *KCNJ5*) and ATPases (*ATP1A1*, *ATP2B3*) lead to
489 constitutively depolarized ZG cell membrane or directly to increased intracellular Ca^{2+}
490 concentrations, constitutively activating Ca^{2+} signaling. The net result is an increased
491 expression of *CYP11B2* and an autonomous aldosterone biosynthesis.

