Genetic and Genomic Mechanisms of Primary Aldosteronism

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Abstract

Aldosterone producing adenomas (APA) and bilateral adrenal hyperplasia are the main cause of primary aldosteronism (PA), the most frequent form of secondary hypertension. Mutations in ion channels and ATPases have been identified in APA and inherited forms of PA, highlighting the central role of calcium signalling in PA development. Different somatic mutations are also found in aldosterone producing cell clusters in adrenals from normal subjects and from patients with unilateral and bilateral PA, suggesting additional pathogenic mechanisms. Recent mouse models have also contributed to a better understanding of PA. Application of genetic screening in familial PA, development of surrogate biomarkers for somatic mutations in APA and use of targeted treatment directed at mutated proteins may allow improved management of patients.

Primary aldosteronism (PA) is the most frequent form of secondary arterial hypertension with a prevalence of 5% of hypertensive patients in primary care and until 10% of hypertensive subjects in reference centers [1, 2]. PA is due to autonomous aldosterone production by the adrenal gland, the two major causes being the presence of an aldosterone producing adenoma (APA) or bilateral adrenal hyperplasia (BAH). In addition to sporadic forms, four different familial forms were described, accounting for approximately 5% of PA cases. PA patients exhibit hypertension associated in many cases with hypokalemia. The biochemical diagnosis is based on high levels of plasma aldosterone and low plasma renin, with an increased aldosterone to renin ratio (ARR). The confirmation of PA diagnosis is performed by one of different tests, including oral salt loading or captopril challenge tests, followed by adrenal image and adrenal vein sampling (AVS) to subtype PA as unilateral or bilateral forms [3]. PA subtyping guides the optimal treatment, unilateral adrenalectomy for lateralized APA or long-life drug therapy with mineralocorticoid receptor antagonists in the presence of bilateral forms. Due to deleterious effects of aldosterone on blood vessels and heart, patients with PA have an increased cardiovascular risk when compared with age, sex and blood pressure matched patients with primary hypertension [4, 5].

Aldosterone is synthetized from cholesterol in the cells of the zona glomerulosa (ZG) of the adrenal cortex by a cascade of enzymatic reactions. The ZG specifically expresses the enzyme aldosterone synthase (encoded by *CYP11B2*), responsible for the three final enzymatic reactions leading to aldosterone biosynthesis [6]. Aldosterone biosynthesis is tightly regulated to maintain salt and water homeostasis, the two most important factors being angiotensin II (AngII) and extracellular potassium concentration (K^+) (Box 1). In basal conditions ZG cells are maintained in a hyperpolarized state due to the presence of a large number of potassium channels [7] and the Na^+/K^+-ATPase. ZG cell membrane depolarization is induced by AngII or increase in plasma K^+ concentration, causing the opening of voltage-gated Ca^{2+} channels and the increase of the intracellular Ca^{2+} concentration. AngII also stimulates Ca^{2+} release from the endoplasmic reticulum by binding the angiotensin II Type 1 receptor (AT1R) and stimulating the inositol triphosphate pathway. The increase in cytosolic Ca^{2+} activates calcium signaling (via calmodulin and calcium/calmodulin-dependent protein kinases), the main trigger for *CYP11B2* expression and aldosterone production [8, 9]. Other regulators of aldosterone biosynthesis include ACTH, serotonin, interleukine 6, epidermal growth factor and leptin [10-13].

In the last decade, following the discovery of *KCNJ5* mutations associated with PA, different studies performing whole exome sequencing identified mutations in genes coding for ion channels and ATPases as responsible for APA and familial PA and highlighted the central role of calcium signaling on PA pathogenesis. These studies allowed the identification of somatic mutations in more than 85% of APA and completely changed the classification of familial forms (Box 2. Clinician's Corner).

Genetics of primary aldosteronism

The first genetic defect associated with PA was described almost 30 years ago. A chimeric gene, where the regulatory regions of *CYP11B1* are juxtaposed with the coding sequence of *CYP11B2*, was identified as responsible of familial hyperaldosteronism Type 1 (FH-I) [14, 15] (Table 1). FH-I is an autosomal dominant disease presenting as early-onset hypertension associated with biochemical abnormalities of PA and increased production of 18-hydroxycortisol and 18-oxocortisol, which improves with glucocorticoid treatment [16, 17]. The chimeric *CYP11B1/CYP11B2* gene leads to ectopic expression of *CYP11B2* throughout the adrenal cortex, with aldosterone being produced not only in cells from ZG, and with an inappropriate ACTH-dependent regulation of aldosterone biosynthesis following the circadian rhythm of cortisol (Figure 1) [18, 19].

During the last decade, whole exome sequencing (WES) performed on DNA from APA has allowed the identification of somatic mutations in genes coding for ion channels (*KCNJ5* encoding for the potassium channel GIRK4, *CACNA1D* coding for the α1 subunit of the L-type Ca^{2+} channel Cav1.3) and ATPases (*ATP1A1*, coding for the α1 subunit of the Na^{+},K^{+}-ATPase, and *ATP2B3*, coding for the plasma membrane calcium-transporting ATPase 3 (PMCA3) [20-23]. The frequency of somatic mutations was reported to be approximately 50% in studies performing Sanger sequencing of hot spot regions of APA-driver genes on DNA extracted from frozen pieces of tumor [24, 25]. The recent development of aldosterone synthase (CYP11B2) immunohistochemistry (IHC)–guided next-generation sequencing (NGS) allowed the identification of mutations in APA-driver genes in 88% to 93% of APA [26-28].

In parallel, the discovery of germline mutations in genes coding for the same ion channels identified in APA and also in *CACNA1H* (coding for the α1 subunit of the T-type Ca2+ channel Cav3.2) and *CLCN2* (coding for the chloride channel ClC-2) have complexified the

classification of familial forms of PA [20, 22, 29-32], with four different forms now classified in FH-I to FH-IV, based on the underlying genetic defect.

All the different somatic and germline mutations increase *CYP11B2* expression and aldosterone biosynthesis by affecting cell membrane potential and/or intracellular ionic homeostasis, resulting in increased intracellular calcium concentration and activation of calcium signaling (Figure 1).

KCNJ5 mutations

Somatic and germline *KCNJ5* mutations were identified in APA and in patients with a familial form of PA (FH-III) [20]. The most prevalent *KCNJ5* mutations (p. Gly151Arg, p. Thr158Ala, p. Leu168Arg) are localized near or within the selectivity filter of the channel. These mutations cause loss of K^+ selectivity and increase sodium permeability, resulting in cell membrane depolarization followed by opening of voltage-gated Ca^{2+} channels and stimulation of Ca^{2+} signaling [20, 33, 34] (Figure 1). A small number of *KCNJ5* mutations are localized in regions far from the selectivity filter and could be responsible for decreased abundance of the channel at the plasma membrane [35]. While the impact of GIRK4 downregulation on aldosterone production was not observed in cell models [33], a recent study suggested that cell proliferation and apoptosis may be regulated by the level of GIRK4 expression [36].

Recurrent somatic *KCNJ5* mutations are the most frequent genetic abnormality observed in APA, being identified in approximately 40% of adenomas [24, 37, 38]. Ethnic differences were observed among cohorts, with a higher prevalence of somatic *KCNJ5* mutations in patients from East Asian cohorts [35, 39-41]. Different studies have shown that *KCNJ5* mutations are more frequent in female and younger patients [24-28, 35, 38, 42, 43], and may also be associated with higher aldosterone levels and larger tumor size in some studies [37, 38] but not in others [28]. *KCNJ5* mutations were also suggested to be a marker of better surgical outcome after adrenalectomy [44]. Compared with other mutational groups, APA carrying *KCNJ5* mutations present lower percentage of cells expressing CYP11B2 and higher percentage of cells expressing CYP11B1 [28]. A recent study, however, reported no difference in overall aldosterone synthase expression as a function of mutational status [45]. In addition, *KCNJ5*-mutated APA exhibit decreased GIRK4 expression in comparison with the adjacent ZG [28, 36, 46, 47].

Germline *KCNJ5* mutations are also responsible for FH-III [20] (Table 1). FH-III was first described in 2008 as severe early-onset hypertension associated with hypokalemia, high urinary concentrations of hybrid steroids 18-oxocortisol and 18-hydroxycortisol, and massive bilateral

adrenal hyperplasia [48]. Germline *KCNJ5* mutations were found in different FH-III families presenting variability in the severity of hyperaldosteronism and in the subtype of PA, sometimes within the same family [49-53].

In vitro studies revealed that mutated GIRK4 may be blocked by high therapeutic doses of the calcium channel blocker verapamil, or by macrolides, decreasing *CYP11B2* expression and aldosterone biosynthesis [54-56]. Clinical studies are currently ongoing, in hypertensive patients with PA, to evaluate the potential clinical use of macrolides on PA subtyping or the identification of somatic *KCNJ5* mutations before surgery [57].

CACNA1D and CACNA1H mutations

CACNA1D mutations affect different properties of Cav1.3 channel, delaying the voltage-dependent inactivation or inducing opening of the channel at less depolarized potentials. This results in increased Ca^{2+} influx leading to the activation of Ca^{2+} signaling and aldosterone overproduction (Figure 1) [21, 22]. Using CYP11B2 IHC-guided NGS, the prevalence of somatic *CACNA1D* mutations was 21% in APA from white American patients, 37% in a French cohort, and as high as 42% in APA from African-American patients, being the most frequent genetic abnormality in this population [26-28]. While *CACNA1D* mutations were associated with smaller tumors in studies using Sanger sequencing to genotype APA [24], this association was not described in a recent study using CYP11B2 IHC-guided NGS [28]. In addition to somatic mutations, d*e novo* germline mutations in *CACNA1D* were identified in two children exhibiting a severe form of hyperaldosteronism in the first year of life associated with a complex neurologic disorder (Primary Aldosteronism, Seizures and Neurologic Abnormalities (PASNA) [22] (Table 1).

Germline *CACNA1H* mutations are responsible for FH-IV. Mutations modify the Cav3.2 channel properties inducing a gain of function, resulting in increased Ca^{2+} influx followed by activation of calcium signaling and overproduction of aldosterone (Figure 1) [29, 30]. The recurrent germline *CACNA1H* mutation p.Met1549Val was described in five children with onset of PA before age 10 years [29]. Micronodular adrenal hyperplasia was observed in the histological analysis of one case [29]. A *de novo* germline *CACNA1H* mutation also affecting codon 1549 (p.Met1549Ile) was described in one children with PA associated with multiplex developmental disorder [30]. Germline *CACNA1H* mutations were also identified in adult patients with a familial inheritance of PA and in one patient with APA, suggesting that *CACNA1H* mutations may predispose to PA with different phenotypic presentation [30]. Recently, somatic *CACNA1H* mutations were also observed in a rare subset of APA [58].

ATP1A1 and ATP2B3 mutations

ATP1A1 mutations were identified in 8% to 17% of APA by CYP11B2 IHC-guided NGS [26-28]. The α subunit of the Na^+,K^+-ATPase, encoded by *ATP1A1*, is composed of 10 transmembrane domains (M1 to M10) and intracellular N- and C- termini. Somatic *ATP1A1* mutations are localized in M1, M4 and M9 domains [21, 23, 25, 40, 59]. While mutations in the M1 and M4 domains affect the K^+ binding pocket decreasing the pump affinity for K^+, mutations in the M9 domain affect a supposed sodium-specific site of the pump, all mutations decreasing the pump activity [60, 61]. The reduced affinity for K^+ and the loss of pump activity result in cell membrane depolarization and cell acidification, without overt increase of intracellular Ca^{2+} (Figure 1) [60]. However, the role of cytosolic acidification on autonomous aldosterone production is not established.

ATP2B3 mutations were identified in 1.7% to 10% of APA, with higher prevalence in studies using CYP11B2 IHC-guided NGS [24-28, 43]. PMCA3, encoded by *ATP2B3* is composed of 10 transmembrane domains (M1 to M10) and intracellular N- and C- termini. All APA-driver *ATP2B3* mutations are in frame deletions affecting a "PEGL" motif in the M4 domain, a region involved in calcium binding and ion gating [23-25, 43, 62]. Mutations in *ATP2B3* decrease pump activity, impairing calcium export and resulting in increased cytosolic calcium concentration and activation of calcium signaling (Figure 1). A second mechanism for autonomous aldosterone biosynthesis was suggested; *ATP2B3* mutations may induce a sodium leak resulting in cell membrane depolarization and opening of voltage-dependent calcium channels, but may also induce a calcium leak through mutated pumps [63].

CLCN2 mutations

Germline *CLCN2* mutations are responsible for FH-II (Table 1). FH-II was used to group all familial forms of PA without an established genetic cause, with a diagnosis based on the presence of two or more affected family members [64, 65]. Whole-exome sequencing performed in a large Australian FH-II family identified a germline mutation in *CLCN2* segregating with the disease in 8 subjects [32]. Three other PA pedigrees carried the same mutation and, additionally, four different germline *CLCN2* mutations were identified in unrelated patients with early-onset PA [32]. A *de novo* germline *CLCN2* mutation was identified, concomitantly, in a 9 years-old PA patient exhibiting severe hypertension and profound hypokalemia [31]. This mutation changes the properties of ClC-2 leading to increased chloride conductance at resting potentials and chloride efflux followed by cell membrane

depolarization, activation of calcium signaling and stimulation of *CYP11B2* expression and autonomous aldosterone production (Figure 1) [31]. In addition to germline mutations and FH-II, a somatic *CLCN2* mutation was reported in one APA [66].

CTNNB1, PRKACA and ARMC5 mutations

Somatic mutations in *CTNNB1*, coding for β-catenin, were identified in 2.1% to 5.1% of APA [43, 67]. *CTNNB1* mutations are localized in exon 3 and were previously described in cortisol-producing adenomas and adrenocortical cancer. *CTNNB1* mutations are more prevalent in women and an association with PA during pregnancy due to aberrant expression of gonadal receptors in APA was suggested [68]. However, it has been shown that the regulation of aldosterone production by gonadal hormones may also occur in APA without somatic *CTNNB1* mutations [69]. Further studies are necessary to establish the role of *CTNNB1* mutations on APA development.

Somatic *PRKACA* (encoding the cAMP-dependent protein kinase catalytic subunit alpha) mutations were identified in two patients with APA, one presenting aldosterone and cortisol co-secretion [70]. *PRKACA* mutations were also described in cortisol producing adenomas and their roles in autonomous aldosterone production and APA development are unknown.

Finally, germline *ARMC5* (armadillo repeat containing 5) variants, predicted to be damaging, were observed in African-American patients with PA [71]. Germline *ARMC5* variants were also observed in a European cohort of patients with PA, although they were not predicted to be damaging using bioinformatics tools [72]. *ARMC5* mutations were previously identified in primary bilateral macronodular adrenal hyperplasia and associated with hypercortisolism [73]. The link between *ARMC5* mutations and PA remains to be further determined.

Animal models of primary aldosteronism

Genetic studies have greatly contributed to a better understanding of the mechanisms responsible for PA development, highlighting the role of ion channels and pumps, but also of the Wnt/β-catenin pathway (Table 2). Remarkably, potassium channels and the Wnt/β-catenin pathway had been shown previously to play crucial roles in the regulation of aldosterone biosynthesis and homeostasis of the adrenal cortex by specific mouse models.

Invalidation of *kcnk3* or/and *kcnk9*, coding for the two-pore domain potassium channels Task1 and Task3, resulted in different forms of hyperaldosteronism or low renin hypertension

[74-78]. Simultaneous invalidation of *kcnk3* and *kcnk9* leads to ZG cell depolarization and PA resistant to salt suppression, without affecting cellular morphology and adrenal zonation [75]. ZG-specific invalidation of both potassium channels resulted in a milder phenotype consisting of a moderate increase of plasma aldosterone that was not associated with a reduction of plasma renin or increased ARR; nevertheless, it was sufficient to significantly increase blood pressure [78]. Different phenotypes were observed when *Kcnk3* or *kcnk9* were invalidated separately. Task3 channel deletion causes salt-sensitive hypertension with low renin [76, 77], associated with ZG cell depolarization, modification of Ca^{2+} signaling and alteration of the physiological regulation of aldosterone, without changes in adrenal morphology. In neonatal mice, Task3 deletion led to major adrenal dysfunction, associated with increased aldosterone, corticosterone and progesterone levels [79]. Deletion of Task1 resulted in severe hyperaldosteronism remediable by glucocorticoids associated with abnormal expression of aldosterone synthase in the zona fasciculata (ZF), despite normal morphological zonation of the adrenal cortex [74]. Whereas this phenotype was observed in female mice all along their life, it was only observed in pre-pubertal male mice, suggesting an androgen-driven upregulation of compensatory mechanisms [74]. In these mice, a subset of genes was closely associated with hyperaldosteronism, including *Dkk3* coding for Dickkopf3, a member of the Wnt signaling pathway [80]. Interestingly, invalidation of *dkk3* in *kcnk3*$^{-/-}$ mice resulted in hyperaldosteronism also in male mice, suggesting a role for *dkk3* in the development of PA [80].

After the identification of *CLCN2* mutations in patients with FH-II, two different knock-in mouse models provided important insight regarding the role of chloride channels in PA development [81, 82]. Deletion of eight N-terminal residues constituting the inactivation domain of the Clc-2 chloride channel, where the p.Met22Lys, p.Gly24Asp and p.Tyr26Asn mutations are located [31, 32], resulted in hypertension and hypokalemia in mice, with high aldosterone and low renin levels, recapitulating the major features of PA [81]. Mice harboring the heterozygous p.Arg180Gln mutation, mimicking the human p.Arg172Gln mutation found in FH-II [32], present a mild form of PA with increased aldosterone associated with a mild elevation of blood pressure, but absence of morphological adrenal abnormalities [82].

Through different mechanisms, the mutations identified in APA converge upon increased calcium signaling to increase aldosterone production. The constitutive expression in mice of a gain-of-function mutant of the AII receptor type 1A (AT1R) led to the development of low-renin hypertension and relative hyperaldosteronism [83]. More recently, specific activation of Gq signaling in adrenal cortex, using a Designer Receptors Exclusively Activated by Designers

Drugs (DREADD) activated by clozapine N-oxide (CNO), was used to establish a mouse model of PA [84]. CNO treatment results in increase of Cyp11b2 expression and aldosterone levels, decreased plasma renin concentration and abnormal zonation of Cyp11b2, with ZF cells expressing both Cyp11b1 and Cyp11b2. In addition, high blood pressure was observed after CNO treatment in mice submitted to high salt diet.

Whereas these mouse models develop hyperaldosteronism, the presence of tumors was never observed, suggesting that modification of intracellular ion balance is not sufficient to induce both increase of aldosterone production and adenoma development. Activation of the Wnt/β-catenin pathway, playing an important role in adrenal development, has been involved in adrenal carcinoma and adenoma [85] and was found in two-thirds of APA [86]. In mice, constitutive activation of β-catenin results in adrenal hyperplasia and dysplasia as well as increased cell proliferation. More interestingly, β-catenin activation also led to increased aldosterone production and hyperaldosteronism [87]. However, the development of tumors was observed only in rare cases with features of carcinoma rather than adenoma [87].

Recently, retinoic acid receptor α (RARα) signaling has been identified as involved in nodule formation by transcriptome analysis performed in APA. Some years ago, proteomic analyses have suggested a role of retinoic acid receptor signaling in the early stage of adrenal development [88]. Invalidation of rarα expression in mice results in structural disorganization of the adrenal cortex characterized by the loss of the radial organization of the ZF and abnormalities in vessel architecture and extracellular matrix, in both male and female mice, with no major modifications of aldosterone production [89]. Beyond the role of rarα in the maintenance of the normal adrenal cortex structure and cell proliferation, dysregulation of this signaling may contribute to abnormal cell proliferation in PA [89].

APCC and their role in aldosterone biosynthesis and PA

Until the description of aldosterone producing cell clusters (APCC), expression of *CYP11B2* and aldosterone biosynthesis was thought to occur exclusively in ZG cells under the tight control of different factors, the two majors being AngII and K^+ (see above). APCC have been described in 2010 [90]. They are located under the capsule and composed of ZG-like cells in the outer part and of columnar ZF-like cells in the inner part. Despite their morphological properties and the fact that Disabled-2, a marker of ZG, is expressed only in the outer part, the entire structure expresses CYP11B2 but not CYP11B1, suggesting that APCC may constitute

intermediate structure between ZG and ZF [86, 91]. However, transcriptome profiling of APCC revealed a greater homology to ZG cells than to ZF cells [92, 93]. APCC are found in normal adrenals and their number and size are correlated with age, but not with gender [45]. APCC are also found in adrenal cortex adjacent to an APA and in adrenals from patients with BAH [90, 91, 94].

Genetic analysis of APCC, using CYP11B2 IHC-guided guided NGS on FFPE tissue, identified somatic mutations in APA driver genes, including *CACNA1D*, *ATP1A1* and *ATP2B3*, but not *KCNJ5*, in 35% of APCC from normal adrenals [92]. More recently, the description of APCC-to-APA translational lesions (pAATL), composed of a subcapsular APCC-like structure and a micro-APA-like structure and carrying somatic mutations suggested the possibility for APCC to constitute the starting point for the development of APA (Figure 2) [93]. Other evidences, however, support the occurrence of two independent events leading to APA development [95]. It has been shown that the adrenal cortex adjacent to an APA may exhibit ZG hyperplasia, increased nodulation and reduced vascularization [86]. Distinct CYP11B2 positive nodules may carry different mutations within the same adrenal [28, 96] and different APCC located in the cortex adjacent to an APA may also carry different somatic mutations, including *KCNJ5* mutations [28]. Finally, a patient with lateralized PA and familial adenomatous polyposis further supports a two-hit model for APA development. The patients carried a germline APC mutation with loss of heterozygosity in adrenals (first hit), responsible for a macronodular hyperplasia. In one CYP11B2 positive nodule, a somatic *KCNJ5* mutation was identified, being responsible for autonomous aldosterone production (second hit) (Figure 2) [97].

APCC appear also to play a role in the pathogenesis of bilateral forms of PA. In a study investigating adrenals from 15 patients with BAH who underwent adrenalectomy, the number of APCC was significantly higher compared to adrenals from normotensive controls, suggesting that APCC, through autonomous aldosterone production, may contribute to the development of PA. Interestingly, sequencing of 99 APCC from the 15 adrenals identified somatic mutations in at least one APCC in all but one case; all mutations affected the *CACNA1D* gene [94]. These results suggest for the first time that the presence of APCC in BAH may be, in part, responsible for PA development; however, they need to be confirmed in a larger cohort of patients.

Concluding remarks

Over the last years, CYP11B2 guided NGS has allowed the identification of somatic mutations in APA-driver genes in more than 85% of APA. In addition, the description of germline mutations in genes coding for different ion channels has allowed refining the classification of familial forms of PA and the description of *de novo* early-onset PA. In mechanistic terms, these mutations highlight the central role of plasma membrane potential, intracellular calcium concentrations and activation of calcium signaling in the pathogenesis of PA. However, the sequence of events leading to APA formation as well as the mechanisms responsible for bilateral PA remains unknown. Somatic mutations in APA-driver genes were also identified in APCC from normal adrenals and from adrenals with APA. While this discovery suggests a possible role of APCC in PA, the identification of different mutations among APA, APCC and micronodules within the same adrenal implies the contribution of additional mechanisms (see Outstanding Questions). Finally, the investigation of different mouse models has largely contributed to our understanding of some aspects of the development of hyperaldosteronism. However, none of these models leads to APA development, suggesting that there might be the requirement for additional events to trigger nodulation, which remain to be established. New studies identifying surrogate markers of APA mutational status before surgery, associated with the recent description of drugs specifically blocking mutated KCNJ5 channels, could open new and exciting perspectives for a better diagnosis and targeted treatment of PA.

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Box 1. Regulation of aldosterone biosynthesis

Aldosterone is produced in the zona glomerulosa of the adrenal cortex to maintain blood volume as well as sodium and potassium homeostasis in the body. Aldosterone is synthetized from cholesterol through sequential enzymatic steps catalyzed by different cytochrome P450 enzymes and hydroxysteroid dehydrogenases. In the zona glomerulosa, the enzyme aldosterone synthase (encoded by *CYP11B2*) catalyzes the three terminal steps of aldosterone biosynthesis: hydroxylation at position C11 of deoxycorticosterone (DOC) resulting in corticosterone, hydroxylation at position C18 of corticosterone yielding 18-hydroxy-corticosterone and the oxidation of the hydroxyl group at C18, producing aldosterone.

Aldosterone biosynthesis is mainly regulated by the renin angiotensin system and plasma potassium concentrations. Under basal condition zona glomerulosa cells are hyperpolarized, due to the presence of a large number of K^+ channels. Both increased extracellular potassium concentrations and angiotensin II inhibit K^+ channels, leading to cell membrane depolarization, opening of voltage gated Ca^{2+} channels, increasing intracellular Ca^{2+} concentrations. Angiotensin II also signals through the Angiotensin II Type 1 receptor via Gαq proteins leading to activation of phospholipase C-β, increase of inositol triphosphate, and mobilization of intracellular Ca^{2+} stores from the endoplasmatic reticulum, increasing intracellular calcium levels. This leads to activation of Ca^{2+} signaling, which increases aldosterone biosynthesis by activating transcription factors, in particular the nuclear receptor subfamily 4 group A (NR4A) members 1 and 2 (NURR77 or NGF1B and NURR1), the cyclic AMP-dependent transcription factor ATF-1 and the cyclic AMP-responsive element-binding protein (CREB), which increase the expression of *CYP11B2*, coding for aldosterone synthase. Increased Ca^{2+} signaling also enhances early and intermediary steps of adrenal steroidogenesis, including release of cholesterol from cytoplasmic stores, activation of the expression of the steroid acute regulatory protein (StAR), which promotes the transfer of cholesterol to the inner mitochondrial membrane, and the synthesis of cofactors for p450 cytochrome enzyme.

Box 2. Clinician's Corner

Primary aldosteronism (PA) is the most frequent form of secondary arterial hypertension with a prevalence of up to 10% of hypertensive patients. PA patients have a higher cardiovascular risk compared to patients with essential hypertension. The two main causes of PA are unilateral aldosterone producing adenomas (APA) and bilateral adrenal hyperplasia. The diagnosis of PA includes screening, confirmation tests and subtype identification. Treatment is

tailored to the underlying cause: adrenalectomy for unilateral disease and drug therapy for bilateral forms.

Familial forms of PA (FH) account for 5% of the cases. They are classified according to the underlying genetic defect. FH-I is caused by a chimeric gene composed of the promoter regions of *CYP11B1* and the coding region from *CYP11B2*. FH-II is caused by germline mutations in the gene *CLCN2*, coding for the chloride channel ClC-2. Germline mutations in *KCNJ5*, coding for the potassium channel GIRK4 are responsible FH-III. Finally, germline mutations in *CACNA1H*, coding for the voltage dependent T-type calcium channel Cav 3.2 are responsible by FH-IV. Germline *CACNA1D* mutations were associated with a rare form of early-onset hyperaldosteronism associated with neurological abnormalities.

Somatic mutations in genes coding for ion channels (*KCNJ5*, *CACNA1D*) and ATPases (*ATP1A1* and *ATP2B3*) were identified in the majority of APA. Their frequency may vary in different populations, with *KCNJ5* being more frequent in cohorts from Asia and *CACNA1D* mutations in cohorts from African ancestry. *KCNJ5* mutations are more frequent in women and young patients. Mutations in *CTNNB1*, coding for β-catenin, were identified in 5% of APA, most frequently in women and possibly associated to pregnancy.

Somatic mutations are also found in aldosterone producing cell clusters (APCC), adrenal structures responsible for autonomous aldosterone production, in normal adrenals. Somatic mutations in APCC are also found in the adrenal cortex adjacent to APA and adrenals from bilateral adrenal hyperplasia. APCC may play a role in the development of PA, as well as in the physiology of aldosterone biosynthesis in normal adrenals.

The discovery of the genetic basis of PA opens new perspectives for the development of new and better diagnostic tools and for drug therapy targeting mutated proteins. Also, it allows for genetic screening and counseling in affected families and improved management of mutation carriers.

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595

Figure legends

Figure 1. Cellular mechanisms of autonomous aldosterone biosynthesis. In unstimulated conditions, zona glomerulosa cells are in a hyperpolarized state, due to the presence of a large number of potassium channels. Cell membrane depolarization, following stimulation of aldosterone biosynthesis, leads to opening of voltage-gated calcium channels and activation of calcium signaling, the main trigger for aldosterone biosynthesis. Mutations in different channels and pumps have been identified in different forms of familial hyperaldosteronism and APA. Mutations in GIRK4 potassium channels in APA and FH-III lead to sodium entry into the cells instead of extrusion of potassium. Mutations in CLC-2 chloride channels in FH-II and APA increase chloride efflux, and mutations in the α1 subunit of the Na^+,K^+-ATPase, found in APA, are responsible for loss of pump activity, but also lead to proton entry into the cells. These mutations lead to cell membrane depolarization and opening of voltage gated calcium channels. Mutations in the α1 subunit of the L-type Ca^{2+} channel Cav1.3, found in APA and PASNA, and in α1 subunit of the T-type Ca^{2+} channel Cav3.2 in FH-IV and APA, result in changes in their functional properties, particularly allowing calcium entry at lower membrane potential, whereas mutations in PMCA3 are responsible for loss of pump activity and accumulation of calcium in the cells. All these mutations induce an increase of intracellular calcium concentration and activation of calcium signaling, followed by stimulation of CYP11B2 transcription and increase in aldosterone biosynthesis. In FH-I, a chimeric gene in which the coding region of *CYP11B2* is fused to the regulatory region of *CYP11B1* leads to ectopic expression of *CYP11B2* throughout the adrenal cortex and control of aldosterone biosynthesis mainly by ACTH.

Figure 2: Pathogenic model of aldosterone producing adenoma development. Two different models may explain APA development in the adrenal cortex. (Left panel) "APCC model": occurrence of somatic mutations in different APA driver genes (*KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3, KCNJ5*) lead to the development of aldosterone producing cell clusters (APCC). APCC may develop into APA through the formation of possible APCC-to-APA translational lesions (pAATL). (Right panel) "Two-hit model": Abnormal cell proliferation and nodulation in the zona glomerulosa, due to genetic or environmental factors, may create a propitious environment for the occurrence of somatic mutations in APA driver genes (*KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3, CACNA1H, CLCN2*). In both hypothesis, activation of calcium signaling, due to the presence of somatic mutations, leads to increased *CYP11B2* expression and autonomous aldosterone production.

629 **Table 1. Familial and inherited forms of primary aldosteronism.**

Disease	Age of onset	Specific features	Gene	Transmission	Treatment
Familial hyperaldosteronism type I	Variable Often before 20 ys	Cerebrovascular events at young age (<30 ys)	Chimeric *CYP11B1/B2*	AD	Glucocorticoids, MRA
Familial hyperaldosteronism type II	Variable Young onset in patients with *CLCN2* mutations	none	*CLCN2*	AD	MRA
Familial hyperaldosteronism type III	Before 20 ys Variable in mild forms	Massive bilateral adrenal hyperplasia in severe cases	*KCNJ5*	AD	MRA Bilateral adrenalectomy in severe cases
Familial hyperaldosteronism type IV	Variable Most frequent before 20 ys	Developmental disorder in some cases	*CACNA1H*	AD	MRA
Primary Aldosteronism, Seizures and Neurologic Abnormalities (PASNA)	Childhood	Seizures and neurological abnormalities	*CACNA1D*	? (de novo)	Calcium channel blocker

AD: autosomal dominant; MRA: Mineralocorticoid Receptor antagonist

Table 2. Recent mouse models of hyperaldosteronism.

Model	Phenotype	Adrenal Phenotype	Mechanism	Ref
TASK1 inactivation	• Hyperaldosteronism remediable by glucocorticoids • Both sexes in young mice • Restricted to females in adult mice	• Cyp11B2 absent in the ZG • Ectopic Cyp11B2 expression in the ZF restricted to females in adults.	• More depolarized membrane voltage at resting conditions	[74]
TASK1 and TASK3 inactivation	• Overproduction of aldosterone resistant to salt suppression • Normal/low renin	• Cellular morphology and zonation preserved in adult mice (only males)	• Marked depolarization of ZG cells.	[75]
TASK3 inactivation	• Salt-sensitive, low renin, arterial hypertension • Abnormalities in the regulation of aldosterone • Severe hyperaldosteronism in neonatal knockout mice	• No changes in adrenal morphology	• Depolarization of adrenocortical cells. • Abnormal Ca^{2+} signaling.	[76, 79]
TASK3 inactivation	• Salt-sensitive, low renin, arterial hypertension • Mild hyperaldosteronism resistant to salt suppression	• No changes in adrenal morphology	• More depolarized membrane voltage at resting conditions	[77]
TASK1 and DKK3 inactivation	• Hyperaldosteronism remediable by glucocorticoids in both sexes	• Absence of altered functional zonation in male mice	• More depolarized membrane voltage at resting conditions	[80]
Constitutive activation of β-catenin	• Increased aldosterone production independent of renin	• Ectopic expression of Dab2, Beta-catenin and Cyp11b2 in ZF	• Beta-catenin dependent activation of *at1r* and *cyp21* • Increased expression of Nurr1 and Nur77 leading to *Cyp11b2* activation	[87]
ZG Specific inactivation of TASK1 and TASK3	• Arterial hypertension • Mild hyperaldosteronism • Mild aldosterone autonomy	NA	NA	[78]

Constitutively open ClC-2	• Marked hypertension • High serum aldosterone • Low renin activity • Hypokalemia	• No changes in adrenal morphology, increased expression of Cyp11b2 in the ZG	• Increased chloride conductance in the ZG • Cell membrane depolarization • Increased cytoplasmic Ca^{2+} concentration • Increased *Cyp11b2* expression	[81]
Knock-in *Clcn2* model (homologous to human *CLCN2* mutation in FH-II)	• Mildly elevated blood pressure • Mildly elevated aldosterone levels •	• No changes in adrenal morphology • Incomplete suppression of Cyp11b2 expression upon high-salt challenge	• Increased calcium oscillatory activity in adrenal cells • Increased *Cyp11b2* expression	[82]
Specific and inducible adrenal activation of Gq signaling	• Hypertension • Increased aldosterone levels • Decreased renin	• Disorganization of adrenal functional zonation	• Gq signaling dependent *Cyp11b2* expression in ZF cells	[84]

ZG: zona glomerulosa; ZF: zona fasciculata; NA: not available

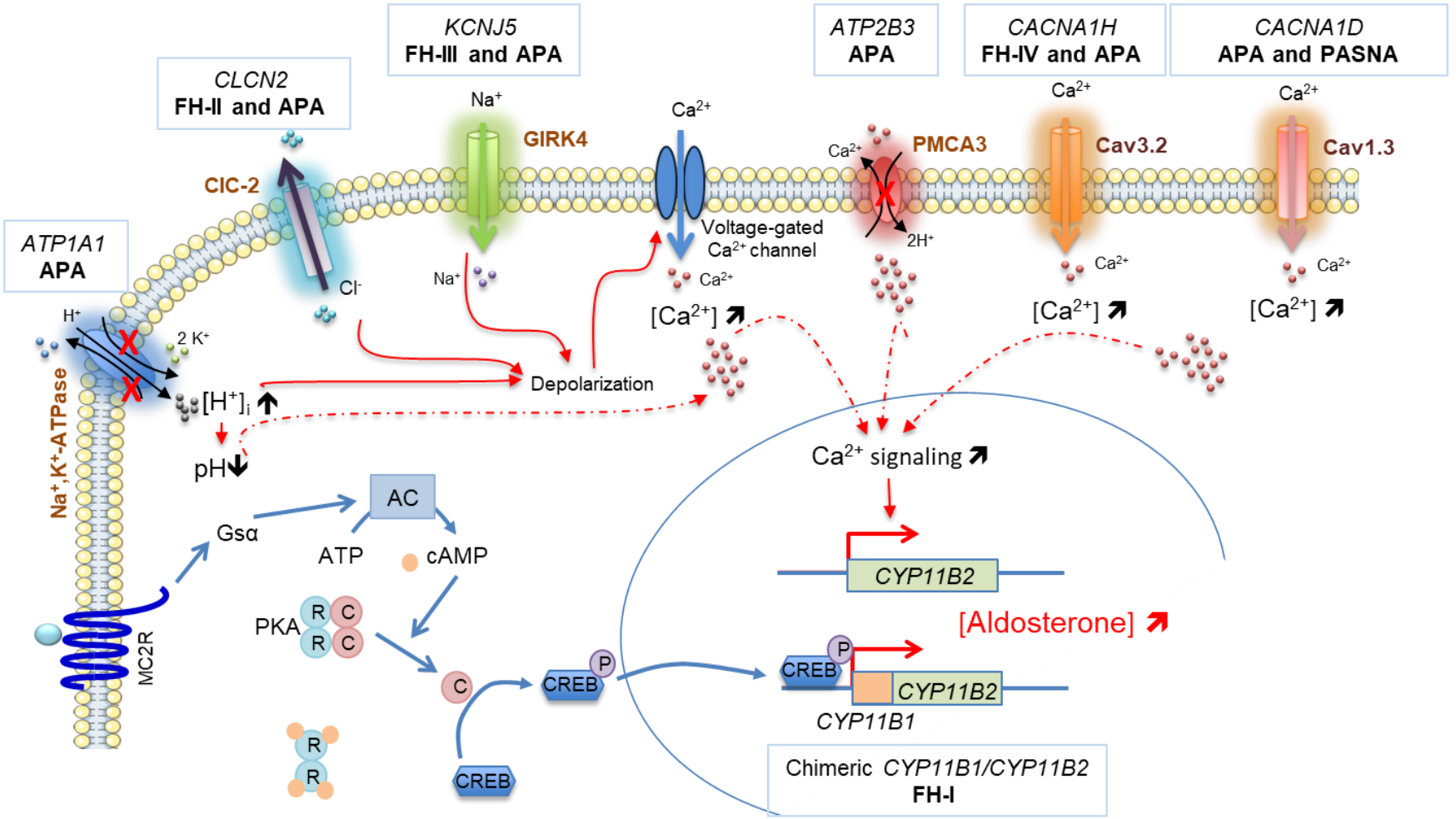
CLCN2
FH-II and APA
KCNJ5
FH-III and APA
ATP2B3
APA
CACNA1H
FH-IV and APA
CACNA1D
APA and PASNA
ATP1A1
APA
CIC-2
GIRK4
Voltage-gated
Ca2+ channel
PMCA3
Cav3.2
Cav1.3
Na+
Ca2+
Cl-
H+
2 K+
2H+
Na+,K+-ATPase
[H+]i
pH
Depolarization
[Ca2+]
Ca2+ signaling
AC
Gsα
ATP
cAMP
PKA
R
C
CREB
P
MC2R
CYP11B2
[Aldosterone]
CYP11B1
Chimeric CYP11B1/CYP11B2
FH-I

Figure 1

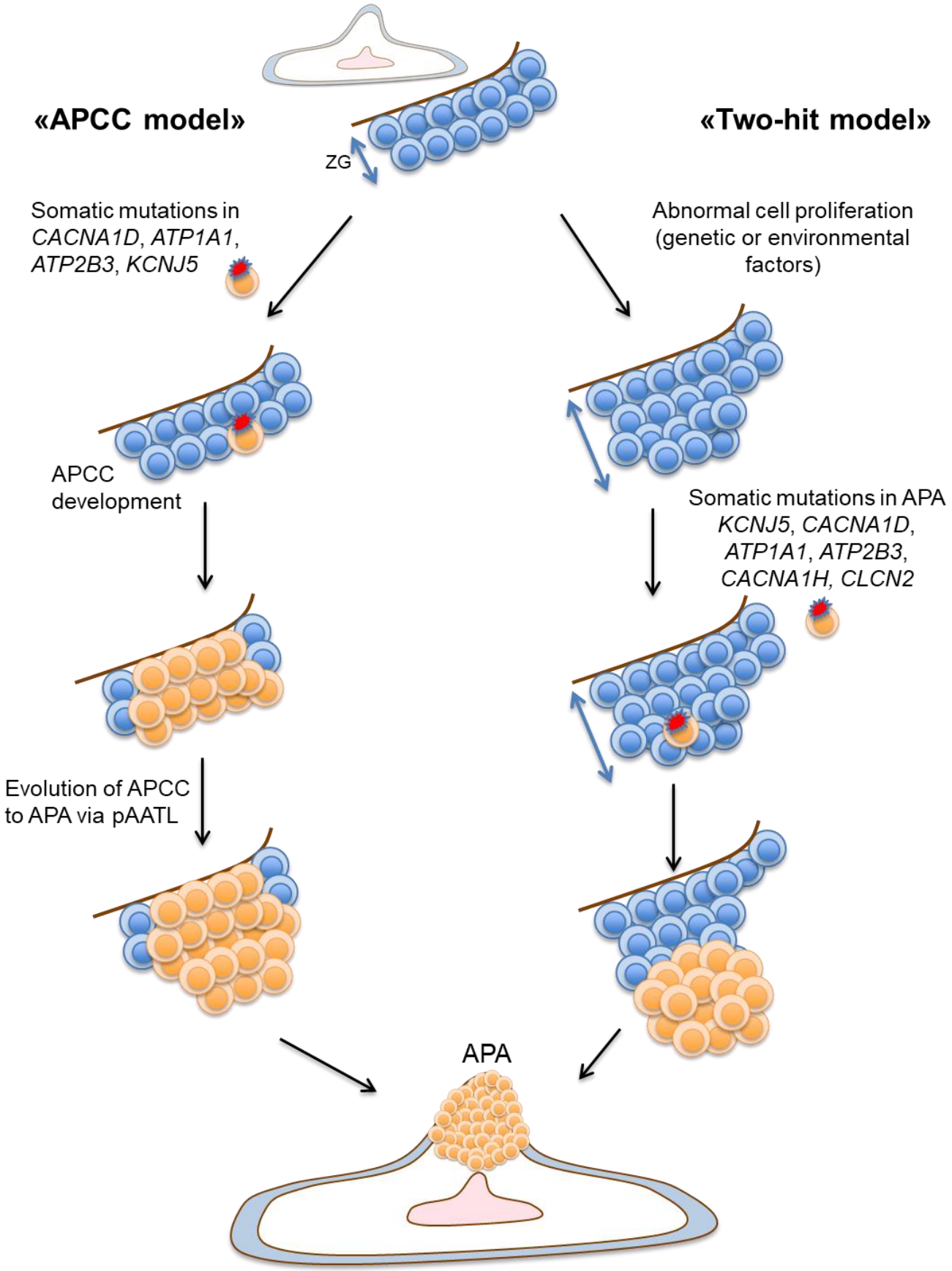
«APCC model»
«Two-hit model»
ZG
Somatic mutations in
CACNA1D, ATP1A1,
ATP2B3, KCNJ5
Abnormal cell proliferation
(genetic or environmental
factors)
APCC
development
Somatic mutations in APA
KCNJ5, CACNA1D,
ATP1A1, ATP2B3,
CACNA1H, CLCN2
Evolution of APCC
to APA via pAATL
APA

Figure 2