Somatic and inherited mutations in primary aldosteronism

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Abstract

Primary aldosteronism (PA), the most common form of secondary hypertension, is caused in the majority of cases by unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia. Over the past few years, somatic mutations in KCNJ5, CACNA1D, ATP1A1 and ATP2B3 have been proven to be associated with APA development, representing more than 50% of sporadic APA. The identification of these mutations has allowed the development of a model for APA involving modification on the intracellular ionic equilibrium and regulation of cell membrane potential, leading to autonomous aldosterone overproduction. Furthermore, somatic CTNNB1 mutations have also been identified in APA, but the link between these mutations and APA development remains unknown. The sequence of events responsible for APA formation is not completely understood, in particular, whether a single hit or a double hit is responsible for both aldosterone overproduction and cell proliferation. Germline mutations identified in patients with early-onset PA have expanded the classification of familial forms (FH) of PA. The description of germline KCNJ5 and CACNA1H mutations has identified FH-III and FH-IV based on genetic findings; germline CACNA1D mutations have been identified in patients with very early-onset PA and severe neurological abnormalities. This review summarizes current knowledge on the genetic basis of PA, the association of driver gene mutations and clinical findings and in the contribution to patient care, plus the current understanding on the mechanisms of APA development.

Background

Arterial hypertension is a major cardiovascular risk factor with an estimated global age-standardized prevalence of 24.1% in adult males and 20.1% in adult females (NCD-RisC 2017). Detection of secondary hypertension is crucial for targeted management of the underlying disease and prevention of cardiovascular complications. Primary aldosteronism (PA) is the most common form of secondary hypertension with an estimated prevalence of 5–10% in hypertensive patients (Mulatero et al. 2004, Plouin et al. 2004, Rossi et al. 2006, Douma et al. 2008, Hannemann & Wallascofski 2012, Hannemann et al. 2012). PA is due to autonomous aldosterone production from the adrenal gland, leading to hypertension with an increased aldosterone-to-renin ratio and on occasion hypokalemia (Funder et al. 2016).
Aldosterone is synthetized from cholesterol by a series of specific enzymatic reactions in the zona glomerulosa (ZG) of the adrenal cortex. The final steps are catalyzed by the aldosterone synthase (encoded by CYP11B2). Aldosterone biosynthesis is mainly regulated by the renin-angiotensin system and extracellular potassium (K⁺) concentration. ACTH is also a regulator of CYP11B2 expression and aldosterone secretion. Binding of ACTH to its receptor, the melanocortin 2 receptor, leads to the increase of cAMP and activation of PKA signaling pathway; however, the complete mechanisms are not completely understood (Gallo-Payet 2016, Ruggiero & Lalli 2016, MacKenzie et al. 2017). The stimulation by angiotensin II (AngII) or K⁺ results in depolarization of the ZG cell membrane and opening of voltage-dependent calcium (Ca²⁺) channels, leading to an increased intracellular Ca²⁺ concentration. Through binding to AngII type 1 receptors, the hormone also induces Ca²⁺ release from the endoplasmic reticulum (Spat & Hunyady 2004) (Fig. 1). Thus, aldosterone production is tightly controlled to maintain electrolyte and fluid homeostasis by the kidney. Aldosterone acts on epithelial cells, particularly in the renal collecting duct, where it plays a major role in promoting sodium reabsorption and thus regulation of blood pressure (Bonvalet 1998). More recently, aldosterone has emerged as a key hormone determining end-organ damage. Given the major cardiovascular adverse effects of aldosterone excess, the early detection of PA has enormous impact on clinical outcome and survival (Rossi et al. 2008). However, despite the publications of guidelines for the management of PA, critical issues related to diagnosis, subtype differentiation and treatment of non-surgically correctable forms still persist (Funder et al. 2008, 2016). A better understanding of the pathogenic mechanisms underlying the development of aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH), the most common forms of PA will improve diagnosis, prognosis and treatment for up to 10% of hypertensive subjects.

Over the past few years, major advances have been made in understanding the genetic basis of APA, with the identification of mutations in genes coding for ion channels (KCNJ5 (Choi et al. 2011), CACNA1D (Azizan et al. 2013, Scholl et al. 2013) and CACNA1H (Danill et al. 2016)) and ATPases (ATP1A1 (Azizan et al. 2013, Beuschlein et al. 2013) and ATP2B3 (Beuschlein et al. 2013)) in more than 50% of APA. All these mutations lead to the activation of calcium signaling, the major trigger for aldosterone production. In addition, recurrent activating mutations in CTNN1B gene coding for β-catenin have also been identified in APA (Tissier et al. 2005, Akerstrom et al. 2016).

In this review, we will summarize the current knowledge of the genetic bases of PA acquired during the past few years using-omics approaches. These advances

![Figure 1](image_url)

**Figure 1**
Regulation of aldosterone biosynthesis in zona glomerulosa under physiological conditions. (A) In basal condition, zona glomerulosa cells are hyperpolarized due to the presence of a high number of potassium channels at the cell surface. The gradient of K⁺ concentration between the intracellular and extracellular spaces required to maintain this membrane potential is generated by the Na⁺/K⁺-ATPase pump activity. (B) Extracellular increase or intracellular decrease of K⁺ concentration as well as inhibition of the Na⁺/K⁺-ATPase pump activity or of K⁺ channels lead to the depolarization of cell membrane, opening of voltage gated Ca²⁺ channel, increased intracellular Ca²⁺ concentration and activation of Ca²⁺ signal pathway, the major trigger for aldosterone biosynthesis. The binding of angiotensin II (AngII) to the AngII type 1 receptor (AT1R) leads, through triphosphate inositol (IP3) increase, to the release of stored Ca²⁺ by the endoplasmic reticulum. AngII and K⁺ induced, both, cell membrane depolarization leading to opening of voltage gated Ca²⁺ channels and increased intracellular Ca²⁺ concentration. In addition to this effect, AngII also inhibit potassium channels and Na⁺/K⁺-ATPase pump activity, leading again to cell membrane depolarization. The following rise in intracellular Ca²⁺ concentration leads to activation of the Ca²⁺ signaling pathway, inducing activation of specific transcription factors (NurrI, NGFIB) and thus increase of CYP11B2 mRNA expression and aldosterone biosynthesis.
have highlighted the major role of the ionic equilibrium and regulation of cell membrane potential in autonomous aldosterone overproduction.

**Somatic mutations in APA**

Zona glomerulosa cells from the adrenal cortex are maintained in a hyperpolarized state, mainly due to the high expression of potassium channels. The study of mouse models in which the expression of specific potassium channels was invalidated has highlighted their crucial role in adrenal (Davies et al. 2008, Heitzmann et al. 2008, Guagliardo et al. 2012, Penton et al. 2012). Hence, the deletion of TWIK-related acid-sensitive K+ channel (Task)-1 (Task 1) and -3 (Task 3) leads to alteration in the membrane properties of ZG cells, inducing autonomous aldosterone production with clinical features typical of glucocorticoid-remediable aldosteronism (Heitzmann et al. 2008) or BAH (Davies et al. 2008). Task1−/− mice display severe hyperaldosteronism, hypokalemia and arterial low renin hypertension. Interestingly, this hyperaldosteronism was remediable by glucocorticoids due to ectopic expression of AS in ZF before puberty in both male and female and then only in adult female (Heitzmann et al. 2008). In contrast, the phenotype of Task3−/− mice recapitulates low renin and salt-sensitive hypertension (Guagliardo et al. 2012, Penton et al. 2012). Surprisingly, invalidation of both Task 1 and Task 3 in mice has no impact on cellular organization and zonation of the adrenal cortex, although aldosterone production by ZG cells is inappropriate for salt intake, recapitulating features of idiopathic primary aldosteronism (Davies et al. 2008). Invalidation of these different potassium channels leads to hyperaldosteronism due to abnormal depolarization of ZG cell membrane resulting in increased intracellular Ca2+ concentration and stimulation of aldosterone biosynthesis; however, formation of adrenal tumors has never been observed in these models, indicating that other mechanisms are required to promote increased cell proliferation in APA.

**Mutations affecting cell membrane potential**

The emergence of high throughput sequencing techniques has allowed major advances in the understanding of the genetic basis of many diseases. Thus in 2011, Choi and coworkers identified two different somatic mutations in the KCNJ5 gene in 8 of 22 APA, as well as an inherited mutation in the same gene in a Mendelian form of severe aldosteronism and massive bilateral adrenal hyperplasia (see below in the Familial forms of PA section) (Choi et al. 2011). KCNJ5 codes for the G protein-activated inward rectifier potassium channel 4 (GIRK4). The GIRK4 channel belongs to a large family of inwardly rectifying K+ channels. ‘Inward rectifiers’ are a class of K+ channels that conduct much larger inward currents at membrane voltages negative to the K+ equilibrium potential. The GIRK channel family comprises four members (GIRK1 to GIRK 4) that form heterotetramers or homotetramers. They are composed of two membrane-spanning domains (M1 and M2), one pore-forming region (H5) and cytoplasmic N- and C-termini that participate in the pore structure. The first two mutations reported by Choi and coworkers, p.Gly151Arg and p.Leu168Arg, are located near or within the selectivity filter of the channel (Choi et al. 2011). Subsequently, many other studies have reported additional somatic or germline mutations in or surrounding the selectivity filter (from Thr149 to Gly153). All these mutations leads to a loss of K+ selectivity and an increase of sodium (Na+) influx into the cells, producing a chronic depolarization of cell membrane, opening of voltage-gated Ca2+ channels, activation of calcium signaling, increase of aldosterone synthase expression and autonomous aldosterone production by the cells (Fig. 2). Overexpression of the GIRK4 p.Thr158Ala mutant in HAC cells resulted in increased aldosterone, cortisol and 18-oxocortisol levels, as observed in patients harboring this mutation, associated with increased expression of CYP11B2 and CYP11B1 and decreased expression of CYP17, but a decrease of cell proliferation (Oki et al. 2012). Scholl and coworkers also reported a decrease of cell viability in 293T cells overexpressing p.Gly151Glu or p.Gly151Arg (Scholl et al. 2012), due to increased Na+ influx into the cells via the mutated channels.

![Figure 2](image_url)

**Figure 2**

Aldosterone biosynthesis in APA in presence of KCNJ5 mutations. Mutations affecting the GIRK4 potassium channel, encoded by KCNJ5, lead to increase of intracellular Na+ concentration, thus resulting in cell membrane depolarization and opening of voltage gated Ca2+ channel.
This additional effect of KCNJ5 mutations may possibly be a mechanism limiting adrenocortical cell mass growth, but it also strongly suggests that other mechanisms are involved in cell proliferation.

In addition to affecting K+ cell permeability, two KCNJ5 mutations (p.Arg115Trp and p.Glu246Gly) decreased GIRK4 wt currents when coexpressed in HEK cells and altered the membrane abundance of the channels, suggesting that reduced GIRK4 in cell surface may lead to inappropriate hypersecretion of aldosterone and formation of adrenal adenoma (Cheng et al. 2015), although this is not the case for other mutations (Murthy et al. 2012). Although KCNJ5 mRNA expression is not affected by mutational status, at the protein level, decreased GIRK4 expression in APA compared with peritumoral ZG was observed in APA with KCNJ5 mutations compared to non-mutated APA or those with other mutations (Boulkroun et al. 2013, Cheng et al. 2015, Fernandes-Rosa et al. 2015a). KCNJ5 mutations were associated neither with specific adrenal cortex remodeling nor specific transcriptional profiles (Boulkroun et al. 2012, 2013). Sligt modifications of gene expression patterns have been reported, such as increased expression of CYP11B2, Visinin-like 1 (VSNL1) or acyl-coA synthetase short-chain family member 3 (ACS3) in APA harboring KCNJ5 expression (Monticone et al. 2012, Williams et al. 2012b, Zhou et al. 2016). VSNL-1 is a calcium-sensor protein that may protect cells from apoptosis induced by the overexpression of KCNJ5 mutants, counterbalancing to some extent the pro-apoptotic effect of overexpression of mutated GIRK4 channels in APA (Williams et al. 2012a).

More recently, exome sequencing approaches allowed the identification of mutations in ATP1A1, encoding the α1 subunit of the Na+/K+-ATPase pump, by Beuschlein et al. (2013). The Na+/K+-ATPase is a member of the P-type ATPase family and is composed of two subunits, α and β. The energy derived from ATP hydrolysis, it generates electrochemical gradients for Na+ and K+ across the plasma membrane; hydrolysis of one ATP allows the exit of three Na+ and the entry of two K+, generating an electric gradient. The α-subunit is formed of ten transmembrane domains (M1–M10) with intracellular N- and C-tails. Different somatic variants (p.Gly99Arg (Williams et al. 2014), p.Leu104Arg (Beuschlein et al. 2013), p.Val323Gly (Beuschlein et al. 2013)) or deletions (p.Phe100_Leu104del (Azizan et al. 2013, Beuschlein et al. 2013), p. Met102_Leu106del (Akerstrom et al. 2015), p. Leu103_Leu104del (Akerstrom et al. 2015), p.Phe956_Glu961del (Akerstrom et al. 2015), p.Phe959_Glu961del (Akerstrom et al. 2015), p.Glu960_Leu964del (Akerstrom et al. 2015)) in ATP1A1 (Azizan et al. 2013) were identified in M1, M4 and M9 domains. Residues mutated in M1 and M4 domains are essential for K+ binding and gating of the binding pocket, their mutations leading to a major decrease in Na+ and K+ binding affinity and thus a loss of pump function (Fig. 3) (Beuschlein et al. 2013, Williams et al. 2014). Remarkably, in H295R S2 cells, although the expression of p.Leu104Arg or the p.Val323Gly mutations leads to cell membrane depolarization and impaired K+ sensitivity, there is no increase in resting Ca2+ activity but intracellular acidification of the cells due to H+ leak. Although this acidification may contribute to the autonomous aldosterone production, specific mechanism remains to be established (Stindl et al. 2015). Where residues are mutated in M9, these amino acids may be of importance for the Na+-specific site III, the Na+-binding site involving specific residues located in M5 (Tyr778), M6 (Gly813 and Thr814) and M9 (Glu 960 and Glu961) (Li et al. 2005, Azizan et al. 2013). Until now, germline mutations in ATP1A1 have never been reported, suggesting that loss of function of this protein would not be compatible with life.

Mutations affecting directly intracellular calcium equilibrium

The major trigger of aldosterone biosynthesis in response to increased plasma K+ and angiotensin II is an increase of calcium concentration in ZG cells (see above). Although mutations in KCNJ5 and ATP1A1 affect cell membrane potential, leading to increased intracellular Ca2+ concentration in case of KCNJ5 mutations or disturbance of the pH, in the case of ATP1A1 mutations, mutations
are associated to increased aldosterone production and CYP11B2 expression in adrenocortical cells (Tauber et al. 2016).

CACNA1D encodes the L-type Ca\(^{2+}\) channel α-subunit Ca\(_{\alpha1.3}\) (Azizan et al. 2013, Scholl et al. 2013). The channel contains four repeat domains (I-IV), each composed of six transmembrane segments (S1–S6), plus a membrane-associated loop between S5 and S6 (Catterall 2010). The S4 segment is involved in voltage sensing for activation, whereas S5, S6 and the loop between these two segments form the pore lining of the channel (Dutta et al. 2016). Cav1.3 is one of the most abundant calcium channels in normal human adrenal and in APA, predominantly expressed in ZG (Scholl et al. 2013, Daniil et al. 2016). Although Cav3.2, encoded by CACNA1H, has been shown necessary for membrane potential oscillations in ZG cells, the role of Cav1.3 in adrenal is less clear (Hu et al. 2012). In 2013, exome sequencing approaches identified somatic and germline mutations (see below) in CACNA1D (Azizan et al. 2013, Scholl et al. 2013). Nine different mutations were identified, all except two located specifically in S5, S6 or in the intracellular loop between the S5 and S6 segments. Electrophysiological studies showed different changes in channel properties depending on the mutations although all led to increased Ca\(^{2+}\) influx and activation of calcium signaling stimulating aldosterone production (Azizan et al. 2013, Scholl et al. 2013). By sequencing the entire gene, we identified nine additional mutations located in segments S4, S5 and S6, cytoplasmic segments or repeated region III (Fernandes-Rosa et al. 2014). Although the prevalence of the different mutations was not the same, the identification of all these new mutations suggests the necessity of sequencing the entire CACNA1D gene when searching for causative mutations for APA (Fernandes-Rosa et al. 2014).

**Mutations affecting β-catenin activation**

Activation of Wnt/β-catenin pathway is one of the major features in APA, being found in at least two-thirds of APA (Boulkroun et al. 2011, Berthon et al. 2012, Berthon & Stratakis 2014). The Wnt/β-catenin pathway plays an important role in embryonic development, stem cell maintenance and differentiation in various tissues, especially normal adrenal development and tumorigenesis (El Wakil & Lalli 2011). In the absence of ligand, β-catenin is a part of the axin complex consisting of adenomatous polyposis coli (APC), axin, glycogen synthase kinase-3β and casein kinase-1β. The phosphorylation of β-catenin in this complex results in its ubiquitination and degradation

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Figure 4

Aldosterone biosynthesis in APA in presence of mutations affecting intracellular calcium balance. Mutations in ATP2B3 coding for PMCA3, CACNA1D, encoding the α subunit of the L-type Ca\(^{2+}\) channel Cav1.3, and CACNA1H, encoding the α subunit of the T-type Ca\(^{2+}\) channel Cav3.2 affect directly intracellular Ca\(^{2+}\) concentrations.
in the proteasome, preventing it from translocating to the nucleus and activating specific Wnt target genes. Wnt/β-catenin activation occurs via binding of ligand Wnt to its specific receptor frizzled resulting in the inhibition of its phosphorylation and its subsequent dissociation from the axin complex, accumulation in the cytoplasm and translocation into the nucleus, where it serves as a transcriptional coactivator of transcription factors of the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family.

Different mouse models have highlighted the role of this pathway in adrenal development and in aldosterone production. Wnt4−/− mice display abnormal differentiation of the definitive zone of the adrenal cortex and decrease of the number of zona glomerulosa cells leading to a decrease of aldosterone production (Heikkila et al. 2002, Jeays-Ward et al. 2003). The constitutive activation of β-catenin in the adrenal cortex results in adrenocortical zonation defects by ectopic activation of ZG differentiation and inhibition of orthotopic ZF differentiation. With time, these mice develop hyperaldosteronism, similar to that observed in mice expressing a defective APC allele (Bhandaru et al. 2009, Berthon et al. 2010).

Until recently, although the activation of Wnt/β-catenin was common in APA, only rare mutations in CTNN1B gene, coding for β-catenin have been described (Tissier et al. 2005, Tadjine et al. 2008). Different recent studies have reported known mutations in CTNNB1 gene in specific residues involved in the stability of the protein (p.Ser45Phe, p.Ser45Pro and p.Thr41Ala) (Akerstrom et al. 2016, Wu et al. 2017), leading to constitutive activation of the Wnt/β-catenin pathway. A novel frame-shift mutation has also been reported (A39Efs*3) located close to previously described mutation (Scholl et al. 2015a), resulting in an early termination of the protein.

Teo and coworkers reported the case of three women with PA due to an APA with somatic CTNN1B mutations (p.Ser33Cys, p.Ser45Phe and p.Gly34Arg), two diagnosed during pregnancy and the third after menopause (Teo et al. 2015). Their APA expressed high levels of luteinizing hormone–chorionic gonadotropin receptor (LHCGR) and gonadotropin-releasing hormone receptor (GNRHR), suggesting that pregnancy may reveal underlying PA (Teo et al. 2015). Although these data provide a substantial addition to our understanding of the mechanisms linking CTNNB1 mutation and APA development, the pathophysiology of APA harboring CTNNB1 mutations seems more complex. Previous studies have reported somatic CTNNB1 mutations in APA from male patients or non-pregnant females (Scholl et al. 2015a, Akerstrom et al. 2016, Wu et al. 2017). Moreover, although GNRHR and LHCGR overexpression is found in more than 40% of APA (Nakamura et al. 2014, Nicolini et al. 2014), CTNNB1 mutations are identified only in 5% (Akerstrom et al. 2016), with immunohistochemical evidence for β-catenin activation observed in about two-thirds of APA (Berthon et al. 2014). Further studies are necessary to establish the complex mechanisms affected by CTNNB1 mutations in APA.

Prevalence and clinical correlates of somatic mutations in APA

Since the identification of KCNJ5 mutations in 2011, many studies have reported the prevalence and the clinical correlates of somatic mutations in APA (Akerstrom et al. 2012, Boulkroun et al. 2012, Azizan et al. 2013, Fernandes-Rosa et al. 2014, Kitamoto et al. 2014, Tauber et al. 2014). A first multicenter European study performed on 380 APA showed a prevalence of 34% of somatic KCNJ5 mutations (Boulkroun et al. 2012). More recently, a second European study performed on 474 APA identified somatic mutations in 54.3% of tumors: KCNJ5 mutations were found in 38% of APA, CACNA1D in 9.3%, ATP1A1 in 5.3% and ATP2B3 in 1.7% (Fernandes-Rosa et al. 2014); a similar prevalence was reported in the American population (Scholl et al. 2015a). The prevalence of somatic CTNNB1 mutations in APA ranged from 2.1% to 5.1% (Scholl et al. 2015a, Akerstrom et al. 2016, Wu et al. 2017). Differences were observed in the distribution of mutations across the different centers participating in the multicenter European study: the prevalence of CACNA1D mutations was highest in the Paris cohort, whereas ATP2B3 mutation rate was highest in the Munich cohort (Fernandes-Rosa et al. 2014). In Asian populations, KCNJ5 mutations appear to have a higher prevalence, ranging from 59% to 76% (Taguchi et al. 2012, Wang et al. 2015, Wu et al. 2015, Zheng et al. 2015, Hong et al. 2016). A meta-analysis of somatic KCNJ5 mutations, comprising 1636 patients with APA, reported that the overall prevalence of somatic KCNJ5 mutations was 43%, ranging from 63% in Asia to 35% in Europe+the United States+Australia (Lenzini et al. 2015). This contrast in the prevalence could be explained by ethnic background, environmental differences or different selection criteria.

Various studies have tried to establish genotype–phenotype correlations or to find biomarkers to determine the mutational status of APA before surgery. Genotype–phenotype analysis performed in 199 patients from a cohort from Paris have shown that patients with
KCNJ5 mutations are more frequently female, diagnosed younger and with higher minimal plasma potassium concentrations, and the presence of CACNA1D mutations associated with smaller adenomas (Fernandes-Rosa et al. 2014). No association was found between the mutation status and preoperative plasma aldosterone, renin levels, aldosterone-to-renin ratio or number of medications taken before surgery. Replication analyses, performed on APA from two other European cohorts, confirmed the association of KCNJ5 mutations with female sex and younger age of diagnosis (Fernandes-Rosa et al. 2014). The association of KCNJ5 mutations with female sex and younger age was also observed in a meta-analysis study comprising 1636 APA patients from 13 studies (Lenzini et al. 2015), as were higher plasma levels of aldosterone and larger tumors (Lenzini et al. 2015). Cardiovascular complications of aldosterone excess in APA in relation to the mutational status were evaluated in two studies (Kitamoto et al. 2014, Rossi et al. 2014). Left ventricular mass index (LVMI) was found to be higher in patients carrying KCNJ5 mutations (Rossi et al. 2014), and in both studies, LVMI was independently associated with KCNJ5 mutations (Kitamoto et al. 2014, Rossi et al. 2014). Finally, steroid profiles performed on adrenal and peripheral venous plasma samples of 79 APA patients by liquid chromatography–tandem mass spectrometry (LC–MS/MS) established that specific somatic mutations in APA can be defined by distinct steroid profiles in adrenal vein plasma. This approach may translate to identifying specific steroid signatures in peripheral venous plasma, and be potentially useful in determining the underlying APA genotype (Williams et al. 2016).

An unanswered question concerns the influence of somatic mutations on the management of PA. Higher lateralization indices on adrenal venous sampling (AVS) were found in patients carrying somatic KCNJ5 mutations (Seccia et al. 2012). More recently, Osswald and coworkers observed no difference in steroid gradients during AVS as a function of the mutation status (Osswald et al. 2013). Another difficulty in establishing genotype–phenotype correlations or surrogate biomarkers of the mutational status is APA heterogeneity. Different somatic mutations have been identified in distinct nodules within the same adrenal, including mutations in different genes (Dekkers et al. 2014, Fernandes-Rosa et al. 2015b). Moreover, APA mutations are reported to occur only in aldosterone synthase (AS)-positive regions with two different mutations identified in distinct AS-positive regions within the same nodule (Nanba et al. 2016a), pointing to the complexity of genetic determinants of APA.

Familial forms of PA and germline mutations

Familial forms are rare causes of PA that account for 1–5% of cases. Different forms displaying Mendelian inheritance have been described: familial hyperaldosteronism type I (FH-I) or glucocorticoid-remediable aldosteronism (GRA), familial hyperaldosteronism type II (FH-II), familial hyperaldosteronism type III (FH-III) with KCNJ5 mutations and familial hyperaldosteronism type IV (FH-IV) due to CACNA1H mutations. Germline KCNJ5, CACNA1D and CACNA1H mutations are also associated with sporadic PA and/or early-onset hypertension due to PA (Scholl et al. 2013, Murthy et al. 2014, Daniil et al. 2016). Furthermore, germline ARMC5 variants have been identified in African American patients with PA (Zilbermint et al. 2015).

Familial hyperaldosteronism type I

FH-I is characterized by clinical and biochemical findings of PA relieved by dexamethasone. The clinical characteristics comprise early and severe hypertension, biochemical abnormalities of PA, increased production of 18-hydroxy cortisol and 18-oxocortisol, bilateral adrenal hyperplasia or adrenal nodules (Sutherland et al. 1966, New 1980). FH-I has an autosomal dominant mode of inheritance, accounting for 0.5–1.0% of PA in adult patients (Jackson et al. 2002, Pizzolo et al. 2005, Mulatero et al. 2011a) and for 3% in hypertensive children (Aglony et al. 2011). The genetic etiology is a crossover of the homologous genes coding for AS (CYP11B2) and 11β-hydroxylase (CYP11B1), creating a chimeric gene with the CYP11B1 promoter and CYP11B2 coding sequences juxtaposed. This crossing over leads to an ectopic expression of AS throughout the ZF, with aldosterone being produced not only in isolated cells from ZG (Lifton et al. 1992, Stowasser & Gordon 2000). The description of different crossover patterns of the hybrid gene suggests that mutations arise independently (Dluhy & Lifton 1995). The genetic diagnosis is usually made by Southern blot or long PCR, both techniques with high sensitivity and specificity (Funder et al. 2016).

The Endocrine Society Clinical Practice Guideline suggests that in patients with an onset of confirmed PA earlier than 20 years of age and in those who have a family history of PA or stroke at a young age (<40 years), genetic testing for FH-I should be performed.
(Funder et al. 2016). FH-I patients should be treated with glucocorticoid hormones, which suppress ACTH secretion leading to the reduction of aldosterone levels. Low-dose glucocorticoids are sufficient, as only partial suppression of adrenocorticotropic hormone is required to correct hypertension over several years (Stowasser et al. 2000).

**Familial hyperaldosteronism type II**

FH-II is a non-glucocorticoid-remediable form of familial hyperaldosteronism first described by Gordon and coworkers in 1991 (Gordon et al. 1991), with transmission consistent with an autosomal dominant mode of inheritance (Stowasser & Gordon 2000, 2001), and his prevalence ranging from 1.2% to 6% in adult patients with PA (Stowasser & Gordon 2001, Medeau et al. 2005, Mulatero et al. 2011b, Pallauf et al. 2012). FH-II patients display a familial history of PA and different subtypes may be present within the same family (Stowasser & Gordon 2001, Stowasser 2009, Mulatero et al. 2011b).

The genetic bases of FH-II are not completely understood. No mutations in candidate genes including CYP11B2, AGTR1 and the p53 tumor-suppressor gene have been identified in FH-II patients (Ballantine et al. 1996a,b, Davies et al. 1997, Stowasser & Gordon 2001). On the other hand, some studies have established evidence for an association of a genetic locus on the chromosomal region 7p22 with FH-II in some affected families (Lafferty et al. 2000, So et al. 2005, Sukor et al. 2008). Candidate genes located in this region were sequenced in FH-II patients, including retinoblastoma-associated Kruppel-associated box gene (RBaK), postmeiotic segregation increased 2 (PMS2), guanine nucleotide-binding protein α-12 (GNA12), replication protein A3 (RPA3), zinc finger protein 12 (ZNF12), glucocorticoid-induced transcript 1 (GLCCI1), fascin 1 (FSCN1) and the cAMP-dependent protein kinase type I β-regulatory subunit (PRKARIB), but no mutations were identified (Medeau et al. 2005, So et al. 2006, Jeske et al. 2008).

FH-II is clinically and biochemically indistinguishable from sporadic forms of PA. With the discovery in the last years of the genetic basis of FH-III and FH-IV (see below), the diagnosis of FH-II is made on the basis of two or more affected family members, without KCNJ5 and CACNA1H mutations. Advances in genetic techniques, with whole-exome and whole-genome sequencing studies, should allow the discovery of genes associated with familial PA and thus change the current definition of FH-II.

**Familial hyperaldosteronism type III and germline KCNJ5 mutations**

FH-III is a non-glucocorticoid-remediable form of FH first described in a father and two daughters with early-onset severe arterial hypertension and hypokalemia associated with marked hyperaldosteronism and very high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol (Geller et al. 2008). The patients showed massive BAH with bilateral adrenalectomy required to control blood pressure (Geller et al. 2008). The genetic background of FH-III is a mutation in the KCNJ5 gene (p.Thr158Ala) (Choi et al. 2011). This mutation is located near the region responsible for GIRK4 K⁺ selectivity (GYG motif), leading to a loss in K⁺ selectivity and increased influx of Na⁺ into the cytoplasm, resulting in the depolarization of the plasma membrane and opening of voltage-gated Ca²⁺ channels. The calcium signaling pathway is activated by increased intracellular Ca²⁺, resulting in an increase of steroidogenic enzyme expression and aldosterone production (Oki et al. 2012).

Different studies have identified germline KCNJ5 mutations in FH-III families with a phenotypic variability, ranging from spironolactone-responsive hyperaldosteronism to massive adrenal hyperplasia with drug-resistant hypertension (Choi et al. 2011, Charmandari et al. 2012, Mulatero et al. 2012, Mussa et al. 2012, Scholl et al. 2012, Monticone et al. 2013, 2015, Adachi et al. 2014, Tong et al. 2016). This variability was attributed to the type of KCNJ5 mutation, with a severe phenotype associated with GIRK4 p.Gly151Arg, p.Thr158Ala, p.Ile157Ser and p.Tyr152Cys mutations, and a milder phenotype associated with KCNJ5 p.Gly151Glu mutations. In vitro studies showed that the milder phenotype in patients carrying the KCNJ5 p.Gly151Glu mutation may be explained by rapid cell lethality due to much higher sodium conductance of the channel preventing adrenal hyperplasia (Scholl et al. 2012). A recent study, however, described a patient with sporadic spironolactone-responsive PA with a germline KCNJ5 mutation previously associated with a severe phenotype (p.Gly151Arg) (Adachi et al. 2014). In the same way, patients carrying the same germline KCNJ5 mutation may display differences in adrenal morphology, even within the same family (Adachi et al. 2014). These differences suggest that the clinical severity in FH-III cannot be defined only on the basis of the type of KCNJ5 mutation.

The complexity of the FH-III phenotype associated with KCNJ5 mutations has been highlighted by two recent
studies. Combined aldosterone and cortisol oversecretion associated with massive bilateral adrenal hyperplasia was described in a patient carrying a germline KCNJ5 mutation (p.Glu145Gln) (Tong et al. 2016). In primary cell culture of the resected hyperplastic adrenal, calcium channel blockers inhibited the secretion of both aldosterone and cortisol and the mRNA expression of steroidogenic enzymes. The authors suggest that the hypersecretion of cortisol might be ascribed to overly large size of the hyperplastic adrenal (with low CYP11B1 expression) and, like aldosterone secretion, may be mediated by voltage-gated Ca\textsuperscript{2+} channels (Tong et al. 2016). A second study reported that adrenals from patients carrying a germline KCNJ5 mutation (p. pThr158AIA) were markedly enlarged with loss of zonation and cells expressing aldosterone synthase and/or 11β-hydroxylase, with and without 17α-hydroxylase, and some expressing all three enzymes (Gomez-Sanchez et al. 2017). GIRK4 was abundantly expressed in almost all cells, suggesting that the mutation not only altered the potassium filter selectivity of the channel, but also a signal that designated specificity for CYP11B2-expressing cells or the presence of a second mutation that abolishes selectivity of steroidogenesis on the basis of adrenal zonation (Gomez-Sanchez et al. 2017).

Germline KCNJ5 mutations have also been suggested to play a role in the common sporadic form of PA, although the functional consequences of these germline mutations are not completely established. Sequencing germline DNA from 251 subjects with sporadic PA, Murthy and coworkers identified three KCNJ5 missense mutations (p.Arg52His, p.Glu246Lys and p.Gly247Arg), located outside the selectivity filter of GIRK4, and a rare SNP (rs7102584, p.Glu246Lys and p.Gly247Arg), located outside the KCNJ5 3′-UTR (Gomez-Sanchez et al. 2017). The mechanism underlying the association between a KCNJ5 3′-UTR polymorphism and activation of the renin–angiotensin–aldosterone system remains unknown.

### Germline CACNA1D mutations in sporadic PA

**Denovo** germline CACNA1D mutations have been described in two children with PASNA (primary aldosteronism, seizures and neurological abnormalities), a syndrome of PA and neuromuscular abnormalities (Scholl et al. 2013). The first subject, without family history of early-onset hypertension or seizures, showed hypertension with biventricular hypertrophy, new born, with the diagnostic of aldosteronism (high plasma aldosterone, suppressed renin and hypokalemia) at age 1 month. Neurological abnormalities (cerebral palsy, cortical blindness and complex neuromuscular abnormalities) were observed, with seizures starting at age 7 months. Treatment with a calcium channel blocker normalized blood pressure, with cure of hypertension and PA on follow-up (Korah & Scholl 2015). A germline mutation was identified in exon 8B of CACNA1D (p.Gly403Asp), in a residue also mutated in APA (p.Gly403Arg) (Azizan et al. 2013, Scholl et al. 2013). The second patient, also without family history of early-onset hypertension or seizures, was diagnosed at birth with cerebral palsy and complex seizures, arterial hypertension at the age of 5 years and hypokalemia at the age of 8 years due to hyperaldosteronism. No adrenal abnormality was observed on CT scan. The patient carried a germline CACNA1D mutation (p.1le770Met) also found in somatic DNA from APA patients.

### Familial hyperaldosteronism type IV (FH-IV) and germline CACNA1H mutations

To identify new genetic abnormalities associated to the development of early-onset hypertension due to PA, Scholl and coworkers performed whole-exome sequencing analysis of 40 unrelated patients. A recurrent germline mutation in CACNA1H (encoding the pore-forming α1 subunit of the T-type voltage-dependent calcium channel Cav3.2) (p.Met1549Val) was identified in 5 children with PA before age 10 years (Scholl et al. 2015b).

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showed autosomal dominant transmission of the mutation. Differences in clinical presentation (incomplete penetrance) were observed within family members, attributed to age-dependent variability or to genetic or environmental modifiers. Despite the previously described association between epilepsy and autism with CACNA1H mutations (Chen et al. 2003, Splawski et al. 2006, Heron et al. 2007), and the presence of severe neurological abnormalities observed in patients carrying CACNA1D mutations (Scholl et al. 2013), no neurological abnormalities or seizures were described in the patients carrying the CACNA1H p.Met1549Val mutation. However, developmental delay or deficit were reported in two mutation carriers (Scholl et al. 2015b). Functional studies demonstrated increased aldosterone production and increased CYP11B2 mRNA expression in cells transfected with Cav3.2 1549Val compared with cells transfected with WT Cav 3.2. In addition, the T-type calcium channel blocker Mibefradil reduced the increased aldosterone production and CYP11B2 expression caused by transfection of WT Cav3.2 and Cav3.2 1549Val in untreated cells, suggesting that T-type calcium channel inhibitors may be an useful treatment option for patients with CACNA1H gain-of-function mutations (Reimer et al. 2016).

Using a similar approach, we identified four germline CACNA1H mutations in PA patients with different phenotypic presentations. A de novo germline CACNA1H mutation (p.Met1549Val) was identified in a patient with early-onset hypertension and hyperaldosteronism, associated with multiplex development disorders (Daniil et al. 2016). This mutation modifies the same amino acid as the CACNA1H p.Met1549Val variant reported in FH-IV (Scholl et al. 2015b). The germline CACNA1H variation p.Ser196Leu was identified in a patient with family history of HT and PA, diagnosed with HT and PA at 36 (HT) and 51 years of age (PA), and in her sister (HT and PA diagnosed at 35 years of age). A third CACNA1H variant (p.Pro2083Leu) was identified in a PA subject with familial history of PA and his brother. This variant is located in the C-terminal cytoplasmic domain of Cav3.2. Finally, in one patient with APA, a germline CACNA1H missense variant (p.Val1951Glu) was found, located in the C-terminal cytoplasmic domain of Cav3.2, in a region possibly implicated in fast channel activation. Electrophysiological analysis of these four mutant channels demonstrated that the Ca²⁺ current properties were affected, suggesting a gain-of-function phenotype. Additionally, results from functional studies in adrenocortical H295R-S2 cells directly linked electrophysiological abnormalities to increased aldosterone production and/or expression of genes coding for steroidogenic enzymes (Daniil et al. 2016). Identification of new CACNA1H mutations associated with early-onset PA and multiplex developmental disorder or with familial forms diagnosed as FH-II or APA, broadens the phenotypic spectrum of PA associated with CACNA1H mutations.

**ARMC5 variants**

Germline and somatic mutations in the armadillo repeat containing 5 (ARMC5) gene causes hypercortisolism in primary bilateral macronodular adrenal hyperplasia (PBMAH) (Assie et al. 2013). ARMC5 belongs to the large family of armadillo (ARM)-repeat-containing proteins, of which the best-known member is β-catenin and is likely to be a tumor-suppressor gene (Berthon & Stratakis 2014). ARMC5 inactivation appears to affect steroidogenesis. Upon sequencing the ARMC5 gene in germline DNA from 56 PA patients, 12 different ARMC5 variants were identified in 20 unrelated and two related individuals (39%) (Zilbermint et al. 2015); in silico analysis revealed that six of these variants were predicted to be damaging. Interestingly, all carriers of these variants were African American. Tumor DNA sequencing confirmed the presence of the germline mutations but failed to identify additional somatic mutations in ARMC5. Furthermore, the silencing of ARMC5 in H295R cells decreased CYP11B2 expression (Zilbermint et al. 2015). The authors suggested that the increased adrenocortical mass leads to higher aldosterone secretion, despite a reduction of aldosterone-secretory capacity of individual zona glomerulosa cells, as seen with hypercortisolism in patients with PBMAH (Zilbermint et al. 2015). Recently, Mulatero and coworkers reported 18 ARMC5 variants, including 5 rare (allele frequency >1%) and 2 new variants (Mulatero et al. 2016). In silico analysis of these variants, however, did not detect sequence variations that were predicted to alter protein function (Mulatero et al. 2016). The link between ARMC5 mutations and PA is still to be determined.

**Molecular and histopathological heterogeneity of APA**

As APA may be composed of cells exhibiting characteristics of different zones of the adrenal cortex (Neville & O’Hare 1985, Azizan et al. 2012), the association between APA cellular composition and molecular markers of adrenal
cortex with the mutation status was proposed, although conflictual results were observed among different studies. Some have described that APA harboring KCNJ5 mutations are mainly composed by cells resembling cortisol-secreting cells of the adrenal zona fasciculate (ZF-like) and APA harboring CACNA1D and ATP1A1 mutations are mainly composed by cells resembling the aldosterone-secreting cells of the adrenal zona glomerulosa (ZG-like) (Azizan et al. 2012, 2013). Accordingly to these results, CYP17A1 expression was found to be higher in APA harboring KCNJ5 mutations, suggesting that these adenomas arise from the ZF (Azizan et al. 2012, 2013). However, these results were not replicated in a large cohort of APA with known somatic mutational status. Analyzing histological characteristics of 78 APA, we found that 72% of APA were composed of a majority of ZF-like cells and no difference neither in the cellular composition of APA nor in CYP11B2 expression according to the mutation status was observed (Fernandes-Rosa et al. 2014). Furthermore, we have shown that APA, independently of the cellular composition phenotype, were composed of cells expressing markers of ZG (CYP11B2, Dab2 and CD56), and that both APA and adjacent ZG exhibit histological characteristics of stem/progenitor cells, which may underlie excessive proliferation and APA formation (Boulkroun et al. 2011). We have also shown that Dab2 is coexpressed with CYP11B2 in APA, suggesting that APA is formed by ZG cells acquiring morphological characteristics of ZF cells due to the excess of steroid production (Boulkroun et al. 2010).

Although the direct link between recurrent somatic mutations and aldosterone production is well established, the effects of these mutations on nodulation and cell proliferation remain to be clarified. KCNJ5 germline mutations originally described in FH-III were associated with massive bilateral hyperplasia, suggesting that the KCNJ5 mutation was responsible for increased cell proliferation and autonomous aldosterone production (Choi et al. 2011). On the other hand, patients with PASNA and germline CACNA1D mutations did not exhibit enlarged adrenals or adrenal nodules (Scholl et al. 2013), as also observed for some patients with germline KCNJ5 mutations (Mulatero et al. 2012, Adachi et al. 2014). Furthermore, in vitro studies demonstrated that cells overexpressing a mutant GIRK4 exhibited lower proliferation, or even apoptosis, due to increased intracellular Na+ concentration (Oki et al. 2012, Scholl et al. 2012). Although adrenal nodules can be observed in normal adrenals associated with older age and severity of hypertension (Wolkersdorfer & Bornstein 1998), we found an increase in nodulation of the cortex adjacent to APA compared with control adrenals (Boulkroun et al. 2010). This raises the possibility that remodeling of the adrenal cortex precedes the development of an APA, and thus, that somatic mutations could be a secondary event in the APA development.

Different studies have provided strong arguments in favor of the hypothesis of independent events leading to nodulation and aldosterone overproduction. Recently, we and others have reported different somatic mutations in different CYP11B2-positive nodules within the same multinodular adrenal (Dekkers et al. 2014, Fernandes-Rosa et al. 2015b). Furthermore, a number of APA exhibited heterogeneity of expression of CYP11B2; among these APAs, recurrent somatic mutations were identified in 3 nodules of the same adrenal, localized only in positive CYP11B2 regions. Interestingly, in one APA, two different mutations were identified in two distinct CYP11B2-positive regions within the same APA (Nanba et al. 2016a). Moreover, the co-existence of two different adrenocortical adenomas within the same adrenal causing the concurrent diagnosis of PA and Cushing syndrome was recently reported (Nanba et al. 2016b). Taken together, all these results suggest that somatic mutations responsible for aldosterone biosynthesis could be a secondary event in APA development. In agreement with this possibility, we have described the concurrence of different germline and somatic mutations leading to APA development (Vouillarmet et al. 2016). A young patient with severe PA aged 26 years, bilateral macronodular adrenal hyperplasia seen on CT, and lateralized aldosterone secretion, showed clinical improvement of PA after right adrenalectomy. The resected adrenal contained three nodules, one expressing aldosterone synthase, with Weiss index of 3, and a somatic KCNJ5 mutation. A FDG-PET performed after surgery revealed abnormal rectal activity despite the absence of clinical symptoms, and gastrointestinal exploration revealed familial adenomatous polyposis. We identified the presence of a germline heterozygous APC gene mutation associated with nodular biallelic APC inactivation due to loss of heterozygosity (Vouillarmet et al. 2016). This case supports a two-hit model for APA formation whereby the first hit (APC germline mutation) drives the nodule development, whereas the second hit (KCNJ5 somatic mutation) specifies the pattern of hormonal secretion.

On the other hand, some authors suggest that APA could be derived from aldosterone-producing cell clusters (APCCs) (Nishimoto et al. 2010). APCCs are composed
of morphological ZG cells in contact with the capsule and inner columnar ZF-like cells forming cords along sinusoids, staining positive for both CYP11B2 and CYP11B1 and found in both normal and PA adrenal tissue (Nishimoto et al. 2010, Boulkroun et al. 2011). The sequencing of DNA from 23 APCCs from normal adrenals showed known aldosterone driver mutations in CACNA1D, ATP1A1 and ATP2B3 in 35% of the APCCs; however, no KCNJ5 mutations, the most frequent alteration observed in APA, were found (Nishimoto et al. 2015). The authors suggest that as APCCs share the same ATP1A1, ATP2B3 and CACNA1D mutations as APA, they may represent a precursor population of cells that lead to APA. The lack of KCNJ5 mutations in APCC may reflect rapid progress of KCNJ5-mutated APCCs to APA, so that KCNJ5 mutations may only be rarely detected in APCCs (Nishimoto et al. 2015).

Conclusion

In the past five years, since the original article from Choi and coworkers (Choi et al. 2011), large advances have been done in the field of genetic causes of PA. Somatic mutations in five genes (KCNJ5, CACNA1D, ATP1A1, ATP2B3 and CTNNB1) are proven to be associated with APA development, explaining more than 50% of sporadic cases. Germline mutations identified in KCNJ5 and CACNA1H have expanded the classification of familial forms of PA, now based on genetic findings. Clinical studies to establish genotype–phenotype correlations and biomarkers associated with recurrent somatic mutations are yet largely unsuccessful; in vitro studies have found pharmacological characteristics of mutant channels, which may provide potential treatment options.

In future studies, larger numbers of samples should progressively explain the genetic basis of the remaining 50% of sporadic APA, as well as the familial forms of PA without genetic defects identified to date. The development of biomarkers or techniques allowing the identification of somatic mutations before surgery may open new options in PA diagnosis and treatment. Whether somatic mutations in APA are responsible for both cellular proliferation and aldosterone production or whether they are independent events leading to aldosterone production occurring in a previously altered adrenal cortex is yet to be verified. Finally, the existence of possible genetic determinants of BAH, as well as the presence of common mechanisms involved in APA and BAH development, is still unknown and constitutes a new field for PA research in future studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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