An update on novel mechanisms of primary aldosteronism

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Abstract

Primary aldosteronism (PA) is the most common and curable form of secondary hypertension. It is caused in the majority of cases by either unilateral aldosterone overproduction due to an aldosterone-producing adenoma (APA) or by bilateral adrenal hyperplasia. Recent advances in genome technology have allowed researchers to unravel part of the genetic abnormalities underlying the development of APA and familial hyperaldosteronism. Recurrent somatic mutations in genes coding for ion channels (*KCNJ5* and *CACNA1D*) and ATPases (*ATP1A1* and *ATP2B3*) regulating intracellular ionic homeostasis and cell membrane potential have been identified in APA. Similar germline mutations of *KCNJ5* were identified in a severe familial form of PA, familial hyperaldosteronism type 3 (FH3), whereas *de novo* germline *CACNA1D* mutations were found in two cases of hyperaldosteronism associated with a complex neurological disorder. These results have allowed a pathophysiological model of APA development to be established. This model involves modifications in intracellular ionic homeostasis and membrane potential, accounting for ~50% of all tumors, associated with specific gender differences and severity of PA. In this review, we describe the different genetic abnormalities associated with PA and discuss the mechanisms whereby they lead to increased aldosterone production and cell proliferation. We also address some of the foreseeable consequences that genetic knowledge may contribute to improve diagnosis and patient care.

Key Words
- primary aldosteronism
- aldosterone producing adenoma
- bilateral adrenal hyperplasia
- potassium channels
- calcium channels
- ATPase
- phenotypic variability
- genotype-phenotype correlation
- genetic susceptibility
- somatic mutations
- endocrine tumor

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Introduction

Primary aldosteronism (PA) is the most common and curable form of secondary arterial hypertension, with an estimated prevalence of ~10% in referred patients and 4% in primary care (Hannemann & Wallaschofski 2012), but as high as 20% in patients with resistant hypertension (Calhoun *et al.* 2002, Douma *et al.* 2008). Increased aldosterone levels in PA are associated with increased cardiovascular risk compared with essential hypertension (Mulatero *et al.* 2013, Savard *et al.* 2013). Comparison of cardiovascular events in a large controlled cross-sectional study, involving 459 patients with PA and 1290 controls with essential hypertension, individually matched for sex, age, and office systolic blood pressure, showed an increased prevalence of left ventricular hypertrophy, coronary artery disease, nonfatal myocardial infarction, heart failure, and atrial fibrillation (Savard *et al.* 2013). The excess cardiovascular morbidity is related to blood pressure-independent cardiac remodeling (Rossi *et al.* 1997) and myocardial fibrosis (Freel *et al.* 2012), with left ventricular changes being reversible in the long-term with

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surgical or medical treatment of PA (Rossi *et al*. 2013). Vascular remodeling associated with aldosterone excess and duration of hypertension predict the outcome of adrenalectomy in patients with PA, emphasizing the importance of early diagnosis of unilateral forms of the disease (Rossi *et al*. 2008).

PA results from autonomous aldosterone production from the adrenal cortex. It is caused in the majority of cases by a unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia (BAH) and is often accompanied by hypokalemia. Aldosterone production from the zona glomerulosa (ZG) of the adrenal cortex is tightly controlled in an exquisite regulatory loop to maintain sodium and electrolyte homeostasis in the kidney. The main intracellular mechanism involved is calcium signaling, which is induced by both angiotensin II (AngII) and potassium, the major regulators of aldosterone biosynthesis (Spat & Hunyady 2004). Increased calcium signaling affects many of the steps involved in aldosterone biosynthesis, including increased cholesterol availability and influx into the mitochondria, as well as stimulation of the expression of *CYP11B2*, coding for the enzyme aldosterone synthase (AS), catalyzing the final steps of aldosterone biosynthesis (Guagliardo *et al*. 2012*a*). AngII acts through the AngII type 1 receptor (AT1R) to stimulate inositol trisphosphate-dependent Ca^{2+} release from the endoplasmic reticulum. In addition, both AngII and K^+ act by depolarizing the ZG cell membrane potential, leading to opening of voltage-gated calcium channels. Indeed, ZG cells have an intrinsic capacity to behave as electrical oscillators. In the ZG, the main ionic conductance is that of K^+, due to the expression of different types of K^+ channels. Thus, the cell membrane potential closely follows the equilibrium potential of K^+ over a large range of extracellular K^+ concentrations. Potassium channels thus play an important role in the pathogenesis of PA as they are central regulators of the ZG cell membrane potential, functioning as an extracellular potassium sensor. The concentration gradient of K^+ between the intracellular and extracellular space that is required for the establishment of the membrane potential is generated by the activity of the Na^+, K^+-ATPase. Elevation of extracellular K^+ concentration, decrease in intracellular K^+ concentration, inhibition of the Na^+, K^+-ATPase, or potassium channels all lead to cell membrane depolarization, allowing opening of voltage-dependent Ca^{2+} channels. The importance of the maintenance of the strongly negative resting membrane potential (−80 mV) and intracellular calcium concentrations of ZG cells for physiological aldosterone production has recently been highlighted by the discovery of genetic alterations affecting proteins involved in the regulation of ZG cell membrane potential and ionic homeostasis in familial and sporadic forms of PA.

Somatic mutations in APA

Availability of high throughput next-generation sequencing technologies has allowed for comparison of whole-exome genetic alterations between germline DNA and DNA extracted from APA (somatic DNA). Recurrent somatic mutations in genes coding for ion channels (*KCNJ5* (Choi *et al*. 2011) and *CACNA1D* (Azizan *et al*. 2013, Scholl *et al*. 2013)) and ATPases (*ATP1A1* and *ATP2B3* (Beuschlein *et al*. 2013)) regulating intracellular ionic homeostasis and cell membrane potential have been identified (Fig. 1). No germline mutations are found in patients harboring somatic mutations in APA (although a few germline mutations in *KCNJ5* were recently described in patients with BAH (Murthy *et al*. 2014), see below). *KCNJ5* encodes the G protein-activated inward rectifier potassium channel GIRK4 (alternative protein name KIR3.4). All *KCNJ5* mutations identified so far in APA, as well as different germline mutations identified in familial hyperaldosteronism type III (FH3) (see below), are located in exon 2; the most frequent of them are p.Gly151Arg and p.Leu168Arg, but less frequent ones located nearby have also been described (for an extensive list of identified mutations, see Gomez-Sanchez (2014) and Monticone *et al*. (2014)). All these mutations are located near or within the selectivity filter of GIRK4, which allows selective passage of potassium ions through the channel pore, and affect the ion selectivity of the channel. Increased sodium conductance leads to sodium influx into the cell with chronic cell membrane depolarization followed by opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production. Transfection of different mutant channels into adrenocortical cells leads to increased *CYP11B2* transcription and aldosterone production, as well as increased expression of two of the transcriptional regulators of *CYP11B2*, NURR1 (encoded by *NR4A2*) and NOR1 (encoded by *NR4A3*) (Monticone *et al*. 2012, Oki *et al*. 2012*a*). More recently, whole-exome sequencing in APA has identified recurrent somatic mutations in two members of the P-type ATPase gene family, namely *ATP1A1* encoding the α1 subunit of the Na^+, K^+-ATPase and *ATP2B3*, coding for the plasma membrane calcium-transporting ATPase 3 (PMCA3) (Beuschlein *et al*. 2013), as well as mutations in the *CACNA1D* gene, coding for

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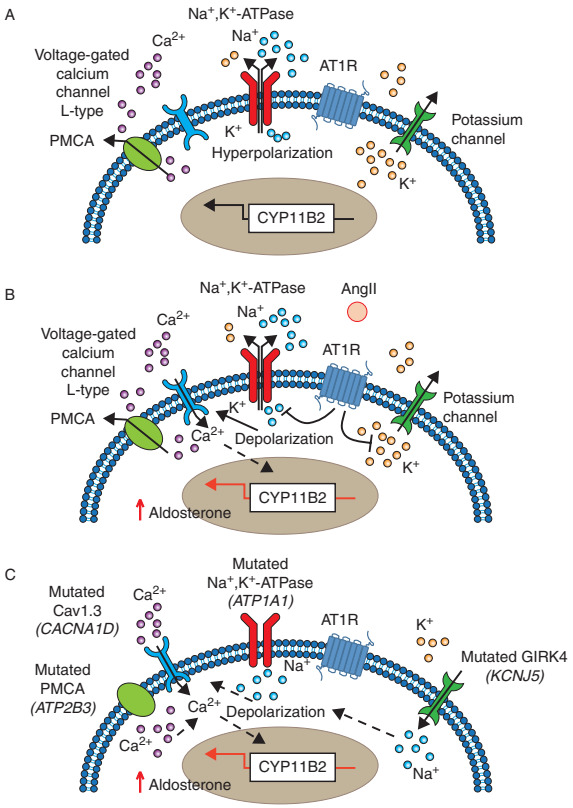

Figure 1

Aldosterone biosynthesis in normal and pathological conditions. (A) ZG cells have a very high conductance for K^+, which maintains a strongly negative membrane potential under resting conditions (−80 mV with normal plasma K^+ concentrations) due to the expression of a large number of potassium channels. The concentration gradient of K^+ between the intracellular and extracellular space that is required for the establishment of the membrane potential is generated by the activity of the Na^+, K^+-ATPase. Elevation of extracellular K^+ concentration; decrease in intracellular K^+ concentration; inhibition of the Na^+, K^+-ATPase, or potassium channels all lead to cell membrane depolarization, allowing opening of voltage-dependent Ca^{2+} channels. (B) AngII acts through the AngII type 1 receptor (AT1R) to stimulate inositol trisphosphate-dependent Ca^{2+} release from the endoplasmic reticulum. In addition, both AngII and K^+ act by depolarizing the ZG cell membrane potential, leading to opening of voltage-gated Ca^{2+} channels, which ultimately leads to an increase in intracellular Ca^{2+} concentration. AngII triggers cell membrane depolarization by inhibiting potassium channels and the sodium potassium ATPase (Na^+, K^+-ATPase). Increased intracellular Ca^{2+} concentration leads to the activation of the calcium signaling pathway, which triggers activation of specific transcription factors and positive regulation of *CYP11B2* transcription. (C) Genetic alterations leading to cell membrane depolarization and intracellular ionic modification. *KCNJ5* gain-of-function mutations affecting GIRK4 and *ATP1A1* mutations of the Na^+, K^+-ATPase affect intracellular Na^+ concentrations, leading to cell membrane depolarization triggering opening of voltage-gated Ca^{2+} channels. Mutations in *ATP2B3* coding for the plasma membrane Ca^{2+}-ATPase PMCA3 and mutations in *CACNA1D*, affecting the Cav1.3 subunit of the L-type voltage-gated calcium channel, have direct consequences on intracellular Ca^{2+} concentrations by affecting calcium recycling and influx. All genetic abnormalities ultimately lead to activation of Ca^{2+} signaling and increased aldosterone biosynthesis. Full arrow, direct activation; hatched arrows, indirect activation requiring intermediary steps.

the α1 subunit of the Cav1.3 calcium channel (calcium channel, voltage-dependent, L type, alpha-1d subunit) (Azizan *et al.* 2013, Scholl *et al.* 2013). Mutations in the α1 subunit of the Na^+, K^+-ATPase affect particular residues in transmembrane helices M1 and M4 of the protein, involved in the interaction with Na^+ and/or K^+, leading to a loss of pump activity and a strongly reduced affinity for K^+, as well as to an inward proton or sodium leak, which increases aldosterone production through cell membrane depolarization and increased calcium influx, similar to what is observed in the presence of *KCNJ5* mutations (Azizan *et al.* 2013, Beuschlein *et al.* 2013). *Ex vivo*, electrophysiological measurements of primary cultured adenoma cells with different *ATP1A1* mutations revealed inappropriate depolarization of cells with ATPase mutations (Beuschlein *et al.* 2013). In contrast, mutations of PMCA3 and Cav1.3 directly affect intracellular calcium homeostasis. PMCA3 mutations located in the highly conserved residues of transmembrane helix M4 are predicted to interact with Ca^{2+} and cause a major distortion of the calcium binding site, thus affecting intracellular calcium clearance. Somatic mutations in the Cav1.3 channel are located in particular segments known to form the channel activation gate, the voltage sensor, and the cytoplasmic domain coupling the voltage-sensing domain to the pore (Azizan *et al.* 2013, Scholl *et al.* 2013). Mutations result in gating changes suggesting gain-of-function mutations, in particular a shift of voltage-dependent channel activation to more negative voltages, or to reduced inactivation of the channel, depending on the mutation, leading, similarly to *ATP2B3* mutations, to alterations in intracellular calcium homeostasis.

Different studies have established the frequency of somatic mutations in cohorts with at least 100 patients with APA (Akerstrom *et al.* 2012, Boulkroun *et al.* 2012, Azizan *et al.* 2013, Beuschlein *et al.* 2013, Fernandes-Rosa *et al.* 2014, Kitamoto *et al.* 2014, Rossi *et al.* 2014). Among them, two subsequent multicenter studies, performed within the European Network for the Study of Adrenal Tumors (ENS@T, www.ensat.org), have investigated the genetic spectrum and clinical correlates of somatic mutations in APA (Boulkroun *et al.* 2012, Fernandes-Rosa *et al.* 2014). Analysis of 474 patients from seven centers has recently shown the presence of somatic mutations in 54% of APA, ranging from 27.2 to 56.8% across different centers. *KCNJ5* mutations represent the most prevalent genetic abnormality and were found in 38% of APA; *ATP1A1* and *ATP2B3* mutations were identified in 5.3 and 1.7% respectively (Fig. 2). Frequency of somatic mutations in *KCNJ5*, *ATP1A1*, and *ATP2B3* is consistent with those

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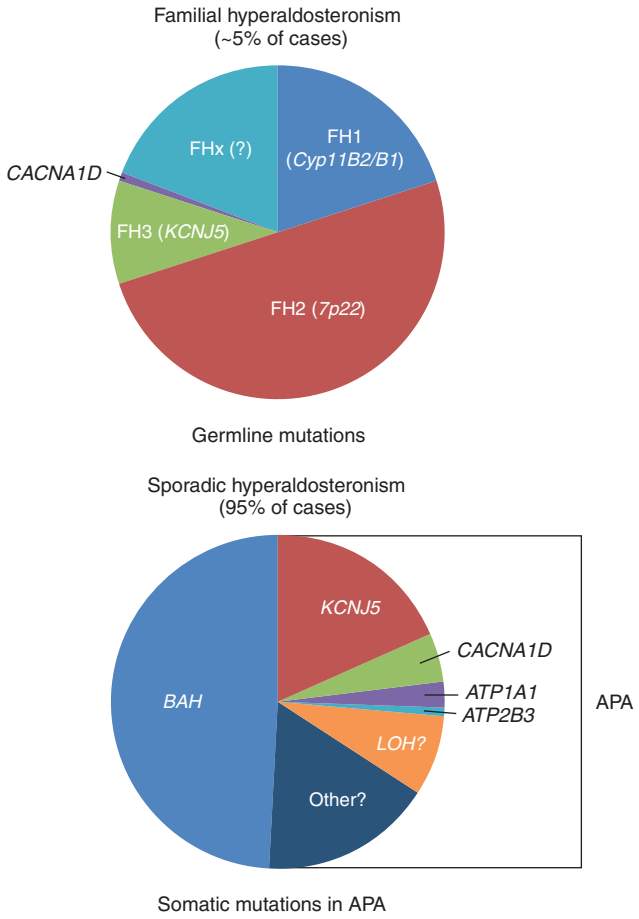

Figure 2
(Upper panel) Three familial forms of PA have been described. FH1 is associated with a chimeric *CYP11B1/11B2* gene, and FH3 with germline mutations in *KCNJ5*. FH2 is probably the most common form but without genetic abnormality identified so far. Germline *de novo CACNA1D* mutations have been identified in children with early-onset hypertension and hyperaldosteronism associated with a complex neurological disorder. Germline *KCNJ5* variants have also been identified in a few cases of sporadic PA. Other Mendelian forms of familial hyperaldosteronism (FHx) may also exist. (Lower panel) In sporadic PA, recurrent somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1*, and *ATP2B3* are found in >50% of APA. Other genes with point mutations or genomic rearrangements with loss of heterozygosity (LOH) have yet to be described. No mutation has been identified in BAH

previously reported in other cohorts (Azizan *et al*. 2013, Beuschlein *et al*. 2013, Williams *et al*. 2014), although *KCNJ5* mutations may be more or less frequent in certain populations (Kitamoto *et al*. 2014, Rossi *et al*. 2014). In particular, it has been shown that the frequency of *KCNJ5* mutations is higher in patients selected on more conservative criteria to define successful cannulation and lateralization during adrenal venous sampling than in centers adopting more permissive indices (Boulkroun *et al*. 2012). The frequency of *KCNJ5* mutations is as high as 70% in the series from Japan (Taguchi *et al*. 2012, Kitamoto *et al*. 2014). Mutations affecting *CACNA1D* were the second most prevalent genetic abnormalities being present in 9.3% of cases (Fernandes-Rosa *et al*. 2014), higher than the previously described prevalence of *CACNA1D* in 5 to 7.8% of APA (Azizan *et al*. 2013, Scholl *et al*. 2013). Fernandes-Rosa *et al*. also identified ten novel *CACNA1D* mutations, implying the necessity of a large genotyping of *CACNA1D* in APA. Correlations of *KCNJ5* and *CACNA1D* mutations with clinical and biochemical parameters (first analyzed in a subset of 199 patients from a single center and then replicated in two additional centers, to take into account between-center variability in the population structure) showed that patients with *KCNJ5* mutations were more frequently female and diagnosed younger compared with *CACNA1D* mutation carriers or noncarriers (Fernandes-Rosa *et al*. 2014). *CACNA1D* mutations were associated with smaller adenomas. There was no association between the mutation status and preoperative plasma aldosterone or renin levels, the aldosterone-to-renin ratio, or the number of medications taken before surgery. There was also no association with outcome of post-operative blood pressure as measured by blood pressure and treatment score at follow-up, cure, or improvement of hypertension. In another large multicenter study, cases with *ATP1A1* and *ATP2B3* mutations showed male dominance, increased plasma aldosterone concentration, and lower potassium levels (Beuschlein *et al*. 2013).

Familial forms of PA and germline mutations in BAH

While the majority of cases of PA are sporadic, 1–5% of cases are familial forms. Three different forms displaying Mendelian inheritance are described: familial hyperaldosteronism type I (FH1), type II (FH2), and type III (FH3). In addition, germline *KCNJ5* mutations and *de novo* *CACNA1D* germline mutations were also described in patients with sporadic PA and unexplained early-onset hypertension and PA respectively (Scholl *et al*. 2013, Murthy *et al*. 2014).

Familial hyperaldosteronism type I

FH1, also called glucocorticoid-remediable aldosteronism (GRA), is a disease with autosomal dominant mode of inheritance described by Sutherland *et al*. (1966). The authors reported the case of a father and a son presenting clinical and biochemical findings of PA, which has been

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relieved by treatment with dexamethasone. FH1 is characterized by early and severe hypertension, biochemical abnormalities of PA, significant production of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol, bilateral hyperplasia or in rare cases adrenal nodules (Sutherland *et al*. 1966, New 1980). The clinical and biochemical characteristics of the affected patients are largely variable, which suggests that FH1 is a heterogeneous disease with a wide spectrum of presentations even within the same family group (Aglony *et al*. 2011). This variability in clinical presentation may be related to other hereditary factors that regulate blood pressure or environmental factors such as variations in dietary sodium intake. Thus, the family history in FH1 does not invariably reveal a history of severe hypertension in first-degree relatives of affected subjects.

The molecular etiology of FH1 is a crossover of genetic material between the highly homologous genes coding for AS (*CYP11B2*) and 11β-hydroxylase (*CYP11B1*, responsible for the last steps of cortisol biosynthesis in the adrenal zona fasciculata (ZF)), creating a chimeric gene whereby the *CYP11B1* promoter and *CYP11B2*-specific coding sequences are juxtaposed leading to an ectopic expression of AS in the ZF (Lifton *et al*. 1992). In FH1, the aldosterone biosynthesis is regulated by the adrenocorticotropin (ACTH), rather than by AngII, resulting in a circadian pattern of aldosterone production, which parallels that of cortisol production (Stowasser & Gordon 2000).

In the hypertensive adult population, FH1 accounts for 0.5 to 1.0% of PA and occurs equally among women and men (Jackson *et al*. 2002, Pizzolo *et al*. 2005, Mulatero *et al*. 2011; Figure 2). However, in the pediatric population, a recent study has described a prevalence of 3% of the chimeric *CYP11B1*/*CYP11B2* gene in hypertensive children (Aglony *et al*. 2011). Different crossover patterns of the hybrid gene have been described across different pedigrees, suggesting that the mutations arose independently (Dluhy & Lifton 1995). More recently, an atypical gene segregation pattern has been described in four generations of a Chilean FH1 family. In this family, the chimeric *CYP11B1*/*CYP11B2* gene segregation differs from an autosomal disease, showing 100% of penetrance in generations II and III and 62.5% in generation IV. The authors suggested that this inheritance pattern was not due to random segregation but due to a preferential segregation of the chimeric *CYP11B1*/*CYP11B2* gene in the offspring (Carvajal *et al*. 2012). This may be explained by differential selection by viability of gametes, mitotic errors in germ cells, or differential survival of cells during development (Pardo-Manuel de Villena & Sapienza 2001*a*,*b*).

FH1 is a rare inherited disease with a variable degree of hypertension within and between pedigrees, sometimes misdiagnosed as essential hypertension. However, FH1 is associated with high morbidity and mortality at an early age. The diagnosis of FH1 must be considered in the presence of indicators of FH1: family history of hypertension and of cerebral hemorrhage before the age of 50 years (Litchfield *et al*. 1998), a history of hypertension from an early age (<20 years), hypertension that has proven difficult to control and hypokalemia (Gates *et al*. 2001, Funder *et al*. 2008). The measure of the urinary levels of 18-oxocortisol and 18-hydroxycortisol, and the dexamethasone suppression test may be misleading for the diagnosis of FH1 (Fardella *et al*. 2001). The genetic diagnosis of FH1 is usually made by Southern blotting or long PCR techniques, both tests with high sensitivity and specificity for the diagnosis (Funder *et al*. 2008).

The Endocrine Society Clinical Practice Guideline recommends that FH1 should be treated with a glucocorticoid, preferably a synthetic glucocorticoid longer acting than hydrocortisone (Funder *et al*. 2008). The administration of exogenous glucocorticoids suppresses ACTH secretion and, therefore, reduces aldosterone levels and reverses the state of mineralocorticoid excess. In order to avoid complete suppression of the circadian regulation of cortisol and the development of iatrogenic Cushing's syndrome, the lowest possible dose of glucocorticoids that normalize blood pressure and/or serum potassium should be used (Funder *et al*. 2008). It has been shown that only partial suppression of ACTH with low dose glucocorticoids (dexamethasone 0.125–0.250 mg/day or prednisone 2.5 or 5 mg/day) is required to correct hypertension for several years, as demonstrated by the maintenance of normal echocardiographic parameters (Stowasser *et al*. 2000). The addition of an MR antagonist (eplerenone, spironolactone) could be useful in cases where blood pressure control is unsatisfactory. Eplerenone should be used in children to avoid glucocorticoid effects on growth or antiandrogenic effects of spironolactone (Funder *et al*. 2008). In addition to their increased cardiovascular risk, it has been shown that the carriers of the chimeric *CYP11B1*/*CYP11B2* gene present increased left ventricular wall thicknesses and reduced diastolic function, well before the onset of hypertension (Stowasser *et al*. 2005). Also, subjects with FH1 present high serum levels of IL6, suggesting that inflammation plays a role in the blood pressure-independent cardiovascular damage occurring in the states of aldosterone excess (Staermose *et al*. 2009). These findings

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raise the question as to whether normotensive subjects found to have FH1 through familial genetic screening for FH1 would benefit from treatment with dexamethasone before the development of arterial hypertension (Staermose *et al*. 2009, Stowasser 2009).

Familial hyperaldosteronism type II

FH2 is a form of familial hyperaldosteronism first described by Gordon *et al*. (1991), not due to the presence of the chimeric *CYP11B1*/*CYP11B2* gene and not remediable by glucocorticoids. Transmission is consistent with an autosomal-dominant mode of inheritance (Stowasser & Gordon 2000, 2001). The prevalence of FH2 ranges from 1.2 to 6% in adult populations of PA (Stowasser & Gordon 2001, Medeau *et al*. 2005*a*, Mulatero *et al*. 2011, Pallauf *et al*. 2012). FH2 patients display a family history of PA and, within the same family, different subtypes of PA may be present (APA and BAH) with phenotypic variability (Stowasser & Gordon 2001, Stowasser 2009, Mulatero *et al*. 2011). FH2 has been reported to be clinically and biochemically indistinguishable from sporadic forms of PA and is diagnosed on the basis of two or more affected family members (Medeau *et al*. 2005*b*, Mulatero *et al*. 2011).

The genetic background of FH2 remains unknown, but a linkage between FH2 and the chromosomal region 7p22 has been established in some families; however, this linkage has not been observed in other FH2 families (Lafferty *et al*. 2000, So *et al*. 2005, Sukor *et al*. 2008). No mutations were found in different candidate genes located in this region, 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 (*PRKAR1B*) (Medeau *et al*. 2005*b*, So *et al*. 2006, Jeske *et al*. 2008). Other candidate genes including *CYP11B2*, *AGTR1*, and the p53 tumor-suppressor gene were also sequenced in FH2 patients and no mutations were identified (Ballantine *et al*. 1996*a*, *b*, Davies *et al*. 1997, Stowasser & Gordon 2001).

Recently, particular germline *KCNJ5* mutations have been found in patients with non-glucocorticoid-remediable PA, classified as FH2 (see below), due to a previously unknown phenotypic heterogeneity of FH3 (Mulatero *et al*. 2012). This implies that the genetic screening for *KCNJ5* mutations should be considered in patients with mild forms of familial PA classified as FH2. Also, somatic *KCNJ5* mutations have been reported in APA samples from patients classified as FH2 (Mulatero *et al*. 2012). Given the high frequency of PA among hypertensive subjects, these findings might be due to a causal association of sporadic PA in the same family. An alternative hypothesis could be a polygenic determinant of PA with a genetic background predisposing to familial PA that developed into an APA only in patients carrying somatic *KCNJ5* mutations.

Familial hyperaldosteronism type III

FH3 was first described in 2008 in a father and two daughters with early-onset severe arterial hypertension refractory to medical treatment (between ages 4 and 7 years) and hypokalemia (Geller *et al*. 2008). They exhibited marked hyperaldosteronism, very high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol, and aldosterone production was not suppressed by dexamethasone treatment. All three individuals presented massive BAH, needing bilateral adrenalectomy to control blood pressure. The pathology demonstrated massive hyperplasia of the adrenal cortex (Geller *et al*. 2008). Recently, the genetic background of FH3 has been elucidated and has been attributed to the mutation p.Thr158Ala in the *KCNJ5* gene (Choi *et al*. 2011; Fig. 2). Similar to the somatic mutations in APA, this mutation is located near the GYG motif that confers GIRK4 K^+ selectivity. Functional studies have demonstrated that GIRK4_Thr158Ala-transduced adrenocortical cells presented a loss in K^+ selectivity and greater influx of Na^+ into the cytoplasm resulting in the depolarization of the plasma membrane, thereby activating voltage-gated Ca^{2+} channels, leading to accumulation of intracellular Ca^{2+}. Increased intracellular Ca^{2+} activates the calcium signaling pathway resulting in the synthesis of steroidogenic enzymes and increased aldosterone production (Oki *et al*. 2012*b*).

Recent studies have described different germline *KCNJ5* mutations in families with FH3. The severity of PA depends on the type of *KCNJ5* mutation. Patients carrying the germline mutations p.Gly151Arg, identical to one of the recurrent mutations in APA, p.Thr158Ala and p.Ile157Ser, all presented a severe phenotype of PA and early-onset hypertension resistant to medical treatment (Charmandari *et al*. 2012, Scholl *et al*. 2012). On the other hand, affected members of three FH3 families carrying the *KCNJ5* p.Gly151Glu mutation and affected members from one family carrying the *KCNJ5* p.Tyr152Cys mutation exhibited a remarkably milder phenotype

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similar to FH2 (Mulatero *et al*. 2012, Scholl *et al*. 2012, Monticone *et al*. 2013). *In vitro* studies have demonstrated that, in a manner similar to other mutations, the mutation p.Gly151Glu alters GIRK4 selectively, resulting in Na^+ influx and membrane depolarization. Electrophysiological studies demonstrated that the mutation p.Gly151Glu leads to a much larger Na^+ conductance than other mutations, resulting in rapid Na^+-dependent cell lethality, which could limit adrenocortical cell proliferation and the severity of hyperaldosteronism *in vivo*. This finding may explain the milder phenotypes of families carrying p.Gly151Glu mutations and the absence of adrenal hyperplasia (Mulatero *et al*. 2012, Scholl *et al*. 2012). The notion of a strict genotype–phenotype correlation has however been challenged recently, with the description of a patient with sporadic hyperaldosteronism carrying a germline heterozygous p.Gly151Arg mutation, who had developed polyuria at 1.5 years of age and hypertension and hypokalemia by age 4 years. Thereafter, hyperaldosteronism was successfully treated for 7 years with spironolactone without visible adrenal enlargement (Adachi *et al*. 2014).

Germline *KCNJ5* and *CACNA1D* mutations in sporadic PA

Recently, the coding region of *KCNJ5* has been sequenced in peripheral blood DNA from 251 white subjects with apparently sporadic PA (Murthy *et al*. 2014). Three heterozygous missense mutations (p.Arg52His, p.Glu246Lys, and p.Gly247Arg) located outside the selectivity filter of GIRK4 were identified. In addition, 5% of PA patients carried a rare SNP (rs7102584, p.Glu282Gln), which presents a frequency of 2% in the 1000 genomes project. Functional studies demonstrated that, although outside the selectivity filter, the mutations p.Arg52His and p.Glu246Lys altered channel behavior in Xenopus oocytes and, in the H295R cells, depolarized the cell membrane and enhanced ATII-induced aldosterone synthesis and release (Murthy *et al*. 2014). In the same study, H259R cells transfected with GIRK4 channels carrying the SNP rs7102584 show reduced cell viability. In contrast, channels carrying the p.Gly247Arg mutation are functionally indistinguishable from the WT GIRK4. The authors suggest that germline variation in the *KCNJ5* gene could play a role in the common sporadic form of PA (Murthy *et al*. 2014).

CACNA1D germline mutations were described in two subjects with early-onset hypertension, aldosteronism, and cerebral palsy (Scholl *et al*. 2013; Fig. 2). The first subject exhibited hypertension at birth, biventricular hypertrophy, elevated plasma aldosterone, high ARR, and hypokalemia at the age of 1 month. Other features included a seizure disorder, apparent cerebral palsy, cortical blindness, and complex neuromuscular abnormalities. No family history of early-onset hypertension or seizures was present. Treatment with a calcium channel blocker, amlodipine, normalized blood pressure. Genetic analyses identified a germline mutation in exon 8B of *CACNA1D* introducing a missense mutation p.Gly403Asp. Somatic mutations of the same residue of *CACNA1D* (p.Gly403Arg) are found in APA (Azizan *et al*. 2013, Scholl *et al*. 2013, Fernandes-Rosa *et al*. 2014). Functional analysis of the p.Gly403Asp mutation demonstrated activation of Cav1.3 at less depolarizing potentials when compared with WT channel (Scholl *et al*. 2013). The second patient, diagnosed at birth with cerebral palsy and complex seizures, presented arterial hypertension, elevated plasma aldosterone, and suppressed PRA at the age of 5 years. Hypokalemia was noted at 8 years of age. Computed tomography (CT) scan showed no adrenal abnormality and there was no family history of early-onset hypertension or seizures. The patient carries a *de novo* germline mutation p.Ile770Met, which was also identified in APA samples. In HEK293T cells, p.Ile770Met Cav1.3 mutants showed maximum current amplitudes at less depolarized potentials and inactivation shifted to more hyperpolarized potentials (Scholl *et al*. 2013). The two germline mutations are predicted to increase intracellular Ca^{2+} concentration resulting in activation of the calcium signaling pathway and increased aldosterone production.

Genetic abnormalities, cellular phenotype, and the origin of APA

The current pathological diagnosis of APA is still difficult and is based on the description of yellow and well-circumscribed nodules and/or hyperplasia in the resected adrenal gland associated with normalization of aldosterone levels after surgery. During the last few years, the detection of aldosterone producing cells has become an important issue, yet rendered difficult by the absence of specific antibodies directed against AS. Use of specific cRNA probes and *in situ* hybridization allowed detection of *CYP11B2* expression in APA (Enberg *et al*. 2004, Lenzini *et al*. 2007, Boulkroun *et al*. 2010) and revealed that this was heterogeneous among tumor samples. Nishimoto *et al*. (2010) described for the first time the use of specific antibodies discriminating the AS and the 11β-hydroxylase in human cells (Nishimoto *et al*. 2010) and confirmed the

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heterogeneity of AS expression in APA: some APA were composed of AS-positive cells, whereas some were of a mixed population of AS and 11β-hydroxylase positive cells, and finally in some cases the expression of both enzymes was undetectable. More recently, generation of specific antibodies suitable for immunofluorescence has revealed that in APA a majority of cells express AS only, some cells express the 11β- and 17α-hydroxylases, but some cells are able to co-express the three enzymes (Nakamura *et al*. 2014). In 2013, two groups reported the use of specific antibodies against AS to improve the diagnosis of APA (Nanba *et al*. 2013, Volpe *et al*. 2013). However, similar to previous studies investigating *CYP11B2* mRNA expression, in some APA AS is not detected. Semi-quantitative analysis revealed that AS expression was inversely correlated with tumor size and tumor volume, indicating that smaller tumors have higher AS expression per area and cell (Nanba *et al*. 2013, Ono *et al*. 2014). These results may explain: i) why small APA below the detection limit of CT can result in clinical hyperaldosteronism and ii) why AS expression was not detectable in some large APA. Thus the identification of other specific markers of the ZG is an important issue to confirm the pathological diagnosis for patients who undergo adrenalectomy for PA. Interestingly, all investigated APA expressed the 3β-hydroxysteroid dehydrogenase, responsible for the conversion of pregnenolone into progesterone, independently of AS expression (Nishimoto *et al*. 2010, Ono *et al*. 2014). In the human adrenal, two 3β-hydroxysteroid dehydrogenase isoforms are expressed, namely *HSD3B1* and *HSD3B2* (Doi *et al*. 2010). Whereas peritumoral ZG express both isoforms, APA express almost exclusively *HSD3B2* (Doi *et al*. 2014); nevertheless, *HSD3B2* mRNA is also expressed in cortisol-producing adenoma and in nonfunctioning adenoma (Sakuma *et al*. 2013), making it unsuitable for diagnosis. Other specific markers of ZG have been described, such as disabled-2 (dab2), specifically expressed in the ZG (Romero *et al*. 2007, Boulkroun *et al*. 2010) and involved in aldosterone secretion (Romero *et al*. 2007) and GIRK4 (Boulkroun *et al*. 2013); both are expressed in APA (Boulkroun *et al*. 2010, 2013) making them the potential candidates to improve APA diagnosis.

While aldosterone is synthetized by ZG cells, paradoxically APA are essentially composed of cells resembling those of the ZF (so-called ZF-like cells) with high cytoplasm-to-nucleus ratio. Nevertheless, despite their morphological appearance, ZF-like cells still express markers of the ZG cells such as *CYP11B2*, *DAB2*, or *CD56*, suggesting that adenoma cells derive from ZG (Boulkroun *et al*. 2010, 2011). In different studies, mutational status of the tumor has been shown to be correlated with its cellular composition and size: *KCNJ5* mutations were associated with large ZF-like APA, while *ATP1A1* and *CACNA1D* mutations were associated with small ZG-like APA, suggesting that adenoma size is associated with cellular composition (Azizan *et al*. 2012, 2013, Dekkers *et al*. 2014). Nevertheless, these associations were not replicated in a recent study investigating a large cohort of patients (Fernandes-Rosa *et al*. 2014), probably due to differences in the investigated population with a large proportion of ZF-like APA in the latter cohort. Remarkably, APA harboring *KCNJ5* mutations are larger and those harboring *CACNA1D* mutations are smaller independent of their cellular composition (Fernandes-Rosa *et al*. 2014). The question on the origin of cells composing APA is still a matter of debate. Transcriptome analysis allowed researchers to identify a small set of differentially expressed genes between ZG- and ZF-like APA that in addition to *CYP17A1* and *CYP11B1* and the proportion of clear or compact cells (ZG-like APA are composed of at least 50% of compact cells) distinguish those tumors; it is interesting to note however that *CYP11B2* expression does not allow discrimination between ZG- and ZF-like APA (Azizan *et al*. 2012).

While the functional link between genetic alterations and aldosterone production has been clearly established, the natural history of the development of APA is still unknown. Our actual knowledge does not allow explanation of the mechanisms involved in APA formation and their histological variability. Different hypotheses have been formulated: i) if APA derive from ZG cells, the ZF-like phenotype would be due to greater metabolic activity or induction of lipid uptake (Boulkroun *et al*. 2010, 2011); ii) if APA derive from ZF cells, AS expression would be due to the presence of a somatic mutation in identified or not yet identified genes (Azizan *et al*. 2012, 2013); iii) APA derive from aldosterone-producing cell clusters (APCCs; Nishimoto *et al*. 2010). APCCs are AS-positive cell clusters of cuneiform or trapezoid shape located in the ZG and outer ZF that were reported recently in addition to the conventional adrenal cortex zonation (Nishimoto *et al*. 2010). They are formed of a mixed population of cells expressing *CYP11B2*, with cells located in the outer part of the structure co-expressing markers of the ZG, such as *DAB2*, whereas cells in the inner part express markers of the ZF, such as *CYP11B1* (Boulkroun *et al*. 2010, Nishimoto *et al*. 2010). This intermediate phenotype between ZG and ZF cells resembles what is observed in APA, suggesting that APA

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may originate from APCC (Boulkroun *et al*. 2010, Nishimoto *et al*. 2010). However, different gene expression profiles associated with different cellular compositions of APA and the large heterogeneity of APA at the transcriptome level rather support a model whereby APA originate from ZG cells that subsequently acquire some morphological characteristics of ZF.

Whereas adjacent adrenal cortex of cortisol-producing adenoma presents severe hypoplasia, we have shown that 'functional' ZG hyperplasia, increased nodulation, and decreased vascularization are the major features of adrenal cortex adjacent to APA (Boulkroun *et al*. 2010). This suggests that *KCNJ5* mutations could indeed occur within a proliferating cortex, leading to growth advantage, clonal expansion, and tumor formation; alternatively, they may represent isolated events leading to APA formation, with adrenal cortex hyperplasia being secondary to reduced vascularization and/or tissue hypoxia. We have shown that *KCNJ5* mutations are absent in somatic DNA from peritumoral cortices from adrenals carrying somatic *KCNJ5* mutations in the corresponding APA (Boulkroun *et al*. 2012). Also, no correlation is observed between *KCNJ5* mutations and parameters of adrenal cortex remodeling, suggesting that *KCNJ5* mutations do not arise in a proliferative microenvironment propitious to promote a specific mutational event leading to APA formation. Moreover, different studies have reported the presence of multiple functional nodules expressing *CYP11B2* in adrenals with APA (Enberg *et al*. 2004, Boulkroun *et al*. 2010, Dekkers *et al*. 2014). In multinodular adrenals, search for mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* genes is usually performed on the principal nodule, i.e. the largest one. Recently, Deckers *et al*. have assessed the genotypic characteristics of multinodular adrenals. All identified mutations were detected on AS-positive nodules; interestingly, one multinodular adrenal contained two nodules harboring different recurrent *KCNJ5* mutations (one p.Leu168Arg and the other p.Gly151Arg), suggesting independent genetic events leading to aldosterone overproduction.

To gain further insights into the potential mechanisms involved in the development of APA, different mouse models have been generated. Deletions of the *KCNK3* and *KCNK9* genes coding for the two-pore domain potassium channels task1 and task3 lead to the development of different forms of hyperaldosteronism or low renin hypertension (Davies *et al*. 2008, Heitzmann *et al*. 2008, Guagliardo *et al*. 2012*b*, Penton *et al*. 2012). Genetic invalidation of task1 leads to adrenocortical cell depolarization and ectopic expression of *CYP11B2* in ZF in female mice only, and to a glucocorticoid-remediable form of hyperaldosteronism resembling FH1 (Heitzmann *et al*. 2008). Deletion of *task3* in mice results in hyperaldosteronism in young animals and low renin hypertension in adults (Penton *et al*. 2012, Bandulik *et al*. 2013). Task1 and task3 double knock-out mice display a phenotype reminiscent of idiopathic primary hyperaldosteronism due to BAH with elevated levels of aldosterone and low circulating renin, and failure to suppress aldosterone production by dietary salt ingestion (Davies *et al*. 2008). 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. One potential mechanism could be the activation of the WNT/β-catenin pathway. Indeed, it has been shown that this pathway is activated in ~70% of APA (Boulkroun *et al*. 2011, Berthon *et al*. 2014), that in rare cases somatic activating mutations of *CTNN1B*, coding for β-catenin, have been described in APA (Azizan *et al*. 2013, Scholl *et al*. 2013) and that constitutive β-catenin activation in the adrenal cortex induces ectopic ZG differentiation and dedifferentiation of the orthotopic ZF, resulting in hyperaldosteronism in 10-month-old mice (Berthon *et al*. 2010). However some of these mice develop malignant characteristics, such as uncontrolled neovascularization and local invasion, a phenotype rarely observed in patients with PA (Berthon *et al*. 2010); it is possible that the levels of β-catenin dosage play a role in the development of specific types of tumors within the adrenal cortex (Berthon *et al*. 2014).

Study of K^+ channel expression showed that about 90 different potassium channels are expressed in the adrenal cortex (Choi *et al*. 2011), results confirmed by our own data retrieved from transcriptome analysis performed on 11 control adrenals (Boulkroun *et al*. 2012; Table 1). Among them, the most expressed are *KCNK3* and *KCNK5*, encoding respectively TASK1 and TASK2. In contrast to animal models, however (Heitzmann *et al*. 2008, Bandulik *et al*. 2010, 2013, Penton *et al*. 2012), to date no mutation in *KCNK3* has been reported in APA. Similarly, the low expression of *KCNK9*, coding for TASK3, in human adrenal cortex is not in favor of a major role of this channel in the development of APA and no somatic mutations have been reported. Recently Lenzini *et al*. (2014) reported reduced expression of TASK2, encoded by *KCNK5*, in APA compared with normal adrenal cortex. The overexpression of a dominant-negative mutant of TASK2 resulted in an increase in aldosterone production due to increase in *CYP11B2* and *STAR* expression in H295R cells.

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Table 1 Potassium channel expression in human adrenal cortex. Expression data are derived from a previously described transcriptome analysis (Boulkroun *et al*. 2012). Values represent median centered, log_2-transformed, and model-adjusted data. Expression is shown in log_2 scale

Gene	Expression	Gene	Expression	Gene	Expression
KCNK3	52.01	*KCND3*	0.96	*KCND1*	0.54
KCNMB4	28.38	*KCNK9*	0.95	*KCNT2*	0.53
KCNJ8	21.3	*KCNG4*	0.95	*KCNC1*	0.51
KCNJ5	12.21	*KCNJ6*	0.90	*KCNE4*	0.50
KCNJ9	11.89	*KCNK6*	0.89	*KCNAB1*	0.49
KCNQ4	9.96	*KCNA4*	0.87	*KCNJ16*	0.48
KCNC3	6.89	*KCNS3*	0.81	*KCNK17*	0.48
KCNK2	6.34	*KCND2*	0.81	*KCNA1*	0.48
KCNQ1	4.87	*KCNF1*	0.80	*KCNA3*	0.47
KCNK5	4.61	*KCNMB1*	0.80	*KCNS1*	0.46
KCNH2	4.16	*KCNV1*	0.75	*KCNH8*	0.45
KCNQ3	3.96	*KCNA5*	0.75	*KCNJ10*	0.44
KCNK12	3.39	*KCNE1*	0.74	*KCNG3*	0.44
KCNK1	3.17	*KCNN1*	0.72	*KCNE3*	0.44
KCNT1	2.99	*KCNJ15*	0.69	*KCNQ5*	0.43
KCNJ14	2.83	*KCNH7*	0.69	*KCNJ12*	0.41
KCNQ2	2.47	*KCNJ1*	0.68	*KCNC4*	0.41
KCNJ11	2.33	*KCNJ2*	0.64	*KCNMB3*	0.41
KCNK15	2.25	*KCNB2*	0.64	*KCNJ13*	0.40
KCNAB2	1.85	*KCNS2*	0.64	*KCNJ4*	0.38
KCNG2	1.85	*KCNMB2*	0.63	*KCNB1*	0.38
KCNH6	1.62	*KCNH5*	0.61	*KCNJ3*	0.38
KCNH1	1.34	*KCNK7*	0.60	*KCNH4*	0.37
KCNK13	1.29	*KCNK10*	0.59	*KCNA2*	0.35
KCNH3	1.25	*KCNE2*	0.58	*KCNK16*	0.35
KCNN4	1.23	*KCNV2*	0.58	*KCNA10*	0.33
KCNE1L	1.16	*KCNA6*	0.57	*KCNAB3*	0.32
KCNK4	1.11	*KCNN3*	0.56	*KCNK18*	0.31
KCNC2	1.01	*KCNMA1*	0.54	*KCNA7*	0.29

However, considering the absence of phenotype in heterozygous models of *task1* and *task3* inactivation, it is unclear how decreased expression of TASK2 alone may result in APA (Gomez-Sanchez 2014). *KCNJ5* is also found to be highly expressed in human adrenal cortex, explaining how mutations in this gene lead to APA formation. Interestingly, while GIRK4 is expressed in ZG and in APA, significantly lower levels were detected in APA harboring *KCNJ5* mutations compared with APA carrying other types of mutations or a new mutation (Boulkroun *et al*. 2013). Reduced GIRK4 expression may therefore counterbalance increased sodium-dependent cell mortality that has been observed *in vitro* in presence of mutated GIRK4 channels (Mulatero *et al*. 2012, Scholl *et al*. 2012). This may play a role in the development of APA harboring GIRK4 mutations, in addition to previously described mechanisms protecting the cells from calcium-induced apoptosis (Williams *et al*. 2012).

The expression of a large number of K^+ and Ca^{2+} channels in normal adrenal and the identification of recurrent mutations in *KCNJ5* and *CACNA1D* genes highlight the important role of the ionic homeostasis and intracellular calcium concentrations in physiological aldosterone production (Fig. 1). Evaluation of the expression of α subunits of L-, N-, and T-type calcium channels in normal adrenals revealed that at least two Ca^{2+} channel subunits, namely Cav1.3 (encoded by *CACNA1D*) and Cav3.2 (encoded by *CACNA1H*), were correlated with the mRNA levels of *CYP11B2* (Felizola *et al*. 2014). High expression of these two channels in the human adrenal cortex has been recently reported (Scholl *et al*. 2013). It might be expected that mutations in other K^+ and Ca^{2+} channels, as well as ion pumps will be identified in the future, although at a lower frequency. In favor of this hypothesis is the fact that genome-wide transcriptome analysis has not revealed major differences in the molecular phenotype of *KCNJ5*-mutated tumors compared with nonmutated APA. This result indicates that, with a few possible exceptions, genetic abnormalities in APA all result in increased intracellular calcium concentrations and altered calcium signaling triggered by different ionic abnormalities.

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Current clinical implications and future directions

Familial forms of PA altogether represent ~5% of cases, with genetic abnormalities identified only in FH1 (chimeric *CYP11B2/B1* gene) and FH3 (*KCNJ5*). Recurrent somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, and *CACNA1D* are found in more than half of APA. In addition, *de novo CACNA1D* germline mutations have been described in two subjects with a complex neurological disorder associated with PA, and functional germline *KCNJ5* variants have been identified in a few subjects with sporadic PA (Fig. 1). While genetic diagnosis of recurrent *KCNJ5*, *ATP1A1*, *ATP2B3* mutations is relatively simple and can be easily implemented in a hospital setting, the large number of different mutations in different regions of Cav1.3 implies the necessity of a large genetic screening of *CACNA1D* in APA.

Genotype–phenotype correlations have shown that patients with *KCNJ5* mutations are more frequently female, diagnosed younger and with higher minimal plasma potassium concentrations, while the presence of *CACNA1D* mutations is associated with smaller adenomas (Fernandes-Rosa *et al*. 2014). An important clinical issue is whether the mutation status influences the final diagnosis of PA, the treatment options, and/or the therapeutic outcome. *KCNJ5* mutations have been associated with a higher lateralization index at adrenal venous sampling, due to increased aldosterone production in the tumor side and more prominent contralateral suppression (Seccia *et al*. 2012). Thus, patients with *KCNJ5* mutations could be more likely to be identified at adrenal vein sampling and therefore to receive adrenalectomy. However, these results were not replicated in a subsequent study that did not find evidence for a clinically important impact of mutation status on steroid gradients during adrenal venous sampling (AVS) (Osswald *et al*. 2013).

Cardiovascular complications of aldosterone excess in APA have been correlated with the mutational status in two studies comprising >100 patients (Kitamoto *et al*. 2014, Rossi *et al*. 2014), with slightly different results. Although the frequency of *KCNJ5* mutations differed significantly between the studies (26% in Rossi *et al*. (2014) vs 69.4% in Kitamoto *et al*. (2014)), higher plasma aldosterone levels were associated with the presence of *KCNJ5* mutations. Rossi *et al*. identified more severe echocardiographic abnormalities (higher left ventricular mass index, LVMI) in *KCNJ5* mutation carriers compared with noncarriers, with a more pronounced fall after adrenalectomy resulting in a similar regression of left ventricular hypertrophy in both groups (Rossi *et al*. 2014). Although no differences in left ventricular hypertrophy before surgery were identified by Kitamoto *et al*. (2014) LVMI was significantly improved following surgery in carriers of *KCNJ5* mutations. In both studies, improvement in LVMI was independently associated with *KCNJ5* mutations. These results underscore the importance of identifying young patients carrying *KCNJ5* gene mutations, as such cases may be complicated by more prominent cardiovascular damage.

The knowledge of the pharmacology of mutant channels and pumps is useful for the interpretation of experimental and clinical data and might be relevant for the development of new therapies for APA. It has been demonstrated that somatic mutations of *KCNJ5* confer a pathological Na^+ permeability to the mutated GIRK4 (Choi *et al*. 2011), which leads to ZG cell membrane depolarization and increased aldosterone production through a calcium-dependent mechanism (Monticone *et al*. 2012, Oki *et al*. 2012*a*). Increased intracellular calcium concentrations are supposed to be due to opening of voltage-activated Ca^{2+} channels. Interestingly, in the adrenocortical NCI-H295R cell line, high intracellular Na^+ impaired Ca^{2+} export via Na^+/Ca^{2+} exchangers (NCX) and possibly allowed Ca^{2+} influx through NCX working in reverse transport mode (Tauber *et al*. 2014). Unlike WT GIRK4, which is inhibited by Tertiapin-Q (Jin *et al*. 1999), the mutant GIRK4 channels are only weakly inhibited by Tertiapin-Q (Tauber *et al*. 2014). Rather, *in vitro* studies have shown that the GIRK4 p.Leu168Arg mutant channel is inhibited by amiloride and, more potently, by the L-type Ca^{2+} channel blocker verapamil. The Gly151Arg and Thr158Ala mutants were also blocked by verapamil but less potently (Tauber *et al*. 2014). Verapamil may not only act on aldosterone secretion by directly blocking the mutated channel but also by inhibiting depolarization-activated voltage-gated Ca^{2+} channels. These findings point out the possible influence of the administration of verapamil on the diagnosis of PA, especially in patients with *KCNJ5* mutations. Similarly, calcium channel blockers are used in the treatment of patients with PA; they have been shown to decrease not only blood pressure but also plasma aldosterone levels (Tanaka *et al*. 2007, Aritomi *et al*. 2012). In one of the two carriers of germline *CACNA1D* mutations (see above), the use of calcium channel blocker amlodipine normalized blood pressure and resolved biventricular hypertrophy (Scholl *et al*. 2013). These data raise the possibility of specific treatment in patients with *KCNJ5* and *CACNA1D* mutations. It will be particularly relevant to identify

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surrogate biomarkers associated with the mutation status for stratifying patients for targeted treatment with verapamil or calcium channel blockers (alone or in association with amiloride) before surgery. In addition, it might be possible in the future to detect somatic mutations in circulating cell-free DNA derived from turnover of cells from APA. In particular for recurrent *KCNJ5* mutations, which represent the majority of cases, this would represent a sensitive and noninvasive screening test for APA.

Conclusion

Application of next generation sequencing technologies has allowed us to move an enormous step forward in our understanding of the genetic and mechanistic basis of sporadic and familial forms of PA. It can be expected that further studies in a larger number of samples will provide the missing elements allowing explanation of the remaining 50% of sporadic APA as well as the familial forms of PA without genetic defect identified so far. Other important questions that remain to be addressed by future research are whether there are susceptibility genes promoting the development of bilateral forms of PA and whether there are some common mechanisms involved in APA formation and BAH that are involved in adrenal cortex cell proliferation and/or aldosterone production.

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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