REVIEW

Molecular mechanisms in primary aldosteronism

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Abstract

Primary aldosteronism (PA) is the most common form and an under-diagnosed cause of secondary arterial hypertension, accounting for up to 10% of hypertensive cases and associated to increased cardiovascular risk. PA is caused by autonomous overproduction of aldosterone by the adrenal cortex. It is mainly caused by a unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia. Excess aldosterone leads to arterial hypertension with suppressed renin, frequently associated to hypokalemia. Mutations in genes coding for ion channels and ATPases have been identified in APA, explaining the pathophysiology of increased aldosterone production. Different inherited genetic abnormalities led to the distinction of four forms of familial hyperaldosteronism (type I to IV) and other genetic defects very likely remain to be identified. Somatic mutations are identified in APA, but also in aldosterone-producing cell clusters (APCCs) in normal adrenals, in image-negative unilateral hyperplasia, in transitional lesions and in APCC from adrenals with bilateral adrenal hyperplasia (BAH). Whether these structures are precursors of APA or whether somatic mutations occur in a proliferative adrenal cortex, is still a matter of debate. This review will summarize our knowledge on the molecular mechanisms responsible for PA and the recent discovery of new genes related to early-onset and familial forms of the disease. We will also address new issues concerning genomic and proteomic changes in adrenals with APA and discuss adrenal pathophysiology in relation to aldosterone-producing structures in the adrenal cortex.

Introduction

Primary aldosteronism (PA) is due to excessive and autonomous aldosterone production by the adrenal cortex. It is the most frequent form of secondary hypertension with an estimated prevalence up to 10% in referred patients, 4% in primary care (Hannemann & Wallaschfski 2012, Monticone et al. 2017) and 20% in patients with resistant hypertension (Calhou et al. 2002, Douma et al. 2008). PA is characterized by an increased aldosterone-to-renin ratio, which is the principal feature used for screening, often hypokalemia and metabolic alkalosis and an increased risk of cardiovascular complications. The prevalence of left ventricular hypertrophy is higher in patients with PA even after adjustment for hypertension duration (Rossi et al. 1996). Moreover, PA patients have a significantly higher prevalence of coronary artery disease, nonfatal myocardial infarction, heart failure and atrial fibrillation (Savard et al. 2013). The two major causes of PA are APA and BAH, also called idiopathic hyperaldosteronism (IHA). Although guidelines for the management of PA have been published and are
widely used in reference centers (Funder et al. 2016), improvements for better diagnosis, subtype identification and treatment for non-operable patients are urgently required to allow for improved care and prevention of cardiovascular complications.

Recently, whole exome sequencing had allowed identifying several somatic genetic abnormalities in APA, providing a pathogenic model for PA development. Recurrent somatic mutations were identified 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, Azizan et al. 2013, Beuschlein et al. 2013) regulating intracellular ionic homeostasis and cell membrane potential. Germline mutations were identified in familial forms of PA, allowing to distinguish four different forms of familial hyperaldosteronism (FH), based on the underlying genetic defect (Fernandes-Rosa et al. 2018, Scholl et al. 2018, Zennaro 2019). These mutations increase intracellular calcium concentrations, leading to the activation of the calcium signaling pathway and to increased aldosterone production by increasing the expression of CYP11B2, coding for aldosterone synthase. In addition, somatic mutations were also identified in CTNNB1 coding for b-catenin in a small proportion of APA (Teo et al. 2015, Scholl et al. 2015a, Akerstrom et al. 2016) and mutations in PRKACA (encoding protein kinase cAMP-activated catalytic subunit α) have been described in two cases (Rhayem et al. 2016).

Even though the consequences of these mutations on aldosterone production are well demonstrated, their impact on cell proliferation and APA formation is not well understood. Different pathogenic mechanisms have been proposed, including different hits being responsible for adrenocortical cell proliferation and hormonal secretion or the development of APA from aldosterone-producing cell clusters (APCC) of the adrenal cortex.

**Steroid biosynthesis in the adrenal cortex**

The adrenal cortex is composed of three separate layers, the zona glomerulosa, the zona fasciculata and the zona reticularis. This zonation is functional, each zone producing specific steroid hormones due to the zone-specific expression of two enzymes, the aldosterone synthase and the 11β-hydroxylase. Cholesterol is the common precursor in steroid hormone biosynthesis through a process that implicates multiple enzymatic steps, the major effectors being enzymes belonging to the cytochrome P450 family. In zona glomerulosa, where aldosterone is produced, cholesterol is converted into 11-deoxycorticosterone through the successive actions of the cytochrome P450 side-chain cleavage (CYP11A1), the 3β-hydroxysteroid dehydrogenase type II (HSD3B2) and the 21α-hydroxylase (CYP21A2). Aldosterone synthase (encoded by the CYP11B2 gene) catalyzes the three final steps in aldosterone biosynthesis. The first step consists in the hydroxylation at the C11 position of the intermediate steroid deoxycorticosterone into corticosterone, followed by a hydroxylation at the C18 position and the formation of 18-hydroxy-corticosterone. The last step is the oxidation of the hydroxyl group on C18, finally resulting in the production of aldosterone (Connell & Davies 2005).

Cortisol biosynthesis occurs in the zona fasciculata, where 17α-hydroxylase is expressed. This enzyme is responsible for the conversion of pregnenolone and progesterone to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, precursors of cortisol, whereas 21-hydroxylase induces a hydroxylation in the C21 position of the 17α-hydroxyprogesterone producing 11-deoxycortisol. The final step of cortisol biosynthesis is the hydroxylation in the C21 position of 11-deoxycortisol by 11β-hydroxylase, encoded by CYP11B1 and specifically expressed in the zona fasciculata and reticularis (Connell & Davies 2005).

Aldosterone production is mainly regulated by the renin angiotensin system and potassium concentrations and, to a lesser extent, by the adrenocorticotropic hormone (ACTH) (Connell & Davies 2005). The renin angiotensin system plays a major role in sodium homeostasis. In response to a decrease in blood volume, dehydration or hyponatremia, renin is secreted by the juxta-glomerulus apparatus of the kidney and cleaves the angiotensinogen produced in the liver to produce angiotensin I. In the lungs, angiotensin I in converted by the angiotensin-converting enzyme (ACE) to angiotensin II (Ang II). Ang II acts via its AT1 receptor expressed in zona glomerulosa cells, activating a Gαq-phospholipase C-mediated pathway, inducing the production of inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol. IP3 binding to its receptor leads to calcium release from the endoplasmic reticulum, thus increasing intracellular calcium concentrations. In parallel, Ang II also inhibits the potassium channels TASK (TWIK-related acid-sensitive potassium channel) and GIRK4 (G-protein-activated inward rectifier potassium channel) and the Na+/K+ ATPase, therefore inducing cell membrane depolarization, and the opening of voltage-dependent membrane calcium channels, leading to calcium influx into the cell and activation of calcium signaling (Spat & Hunyady 2004, Oki et al. 2012a) (Fig. 1A and B).
Activation of calcium signaling increases the availability of cholesterol in the cell, by stimulating the activity of the enzyme cholesterol ester hydrolase, but also by increasing the expression of StAR (steroid acute regulatory protein), which is responsible for cholesterol transport into the mitochondria, and of other steroidogenic enzymes (Spat & Hunyady 2004). Calcium/calmodulin-dependent protein kinases also activate transcription factors such as nuclear receptor subfamily 4 group A members 1 and 2 (NUR77/NGF1B and NURR1 encoded by NR4A1 and NR4A2 respectively), which increase transcription of the CYP11B2 gene (Bassett et al. 2004b).

Cortisol production is regulated by the hypothalamic-pituitary-adrenal axis via ACTH. ACTH binds to the melanocortin receptor 2 (MC2R), inducing an increase in intracellular cAMP levels via the activation of a Gαs protein. cAMP activates PKA signaling following its binding to the regulatory subunits of PKA (Ruggiero & Lalli 2016). The duration of ACTH stimulation affects its role in the biosynthesis of steroids. Short-term stimulation increases the availability of intracellular cholesterol and its transfer to the internal membrane of the mitochondria, primarily through the phosphorylation of StAR at the Ser195 position; it also induces the expression of steroidogenic enzymes. However, long-term stimulation by ACTH induces transcriptional regulation of StAR and steroidogenic enzymes. Particularly, PKA signaling activates transcription factors such as steroidogenic

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**Figure 1**

Regulation of aldosterone biosynthesis in normal conditions and in PA. (A) In unstimulated conditions, zona glomerulosa cells are hyperpolarized. This state is maintained by the activity of potassium channels such as TASK and GIRK4 and the Na+/K+ ATPase pump. (B) Increases in extracellular potassium levels inhibit K+ currents of TASK channels and lead to cell membrane depolarization. Activation of AngII signaling through its receptor (AT1R) on the surface of ZG cells inhibits K+ channels and the Na+/K+ ATPase, which leads to cell membrane depolarization, opening of voltage-gated Ca2+ channels and increased intracellular calcium concentrations. Intracellular calcium activates signaling pathways leading to CYP11B2 expression and thus increasing aldosterone biosynthesis. Activation of AT1R also activates a Gαq-phospholipase C-mediated pathway, inducing an increase in the concentration of inositol 1,4,5-triphosphate (IP3), which binds to its receptor expressed on the endoplasmic reticulum, inducing the release of calcium from intracellular stores. (C) Mutations in KCNJ5 encoding the potassium channel GIRK4 are responsible for a loss of selectivity of the channel and lead to sodium influx into the cell; mutations in ATP1A1 encoding the α1 subunit of the Na+/K+ ATPase increase intracellular Na+ and H+ concentrations; mutations in CLCN2, encoding the chloride channel ClC-2, increase chloride conductance at resting potentials; all these abnormalities lead to membrane depolarization of glomerulosa cells. Cell membrane depolarization induces opening of voltage-gated calcium channels, increased intracellular calcium concentrations and activation of calcium signaling, triggering increased expression of CYP11B2 and aldosterone production. (D) Mutations in CACNA1H and CACNA1D encoding the voltage-gated calcium channels Cav3.2 and Cav1.3 as well as mutations in ATP2B3, encoding the Ca2+ pump PMCA3, directly increase intracellular calcium concentrations and lead to increased aldosterone biosynthesis through activation of calcium signaling.
factor I (SF-1), cAMP-responsive element-binding protein (CREB), cAMP-responsive element modulator and CCAAT/enhancer-binding protein and activator protein 1, which regulate the expression of steriodogenic genes (Ruggiero & Lalli 2016).

ACTH stimulation also induces aldosterone production by similar mechanisms. Moreover, ACTH stimulates calcium flow into zona glomerulosa cells by activating L-type calcium channels. However, in the case of a chronic ACTH stimulation, CYP11B2 expression is inhibited, which causes a reduction in aldosterone production, as opposed to the expression of CYP11B1 which is responsible of cortisol production (Vinson 2016).

There exist other factors that regulate aldosterone production in a paracrine fashion. Among them, serotonin (5-HT) produced by mastocytes stimulates steroid production (El Ghorayeb et al. 2016) through mechanisms involving the 5-HT receptor type 4 (HTR4) (Lefebvre et al. 1993, Lefebvre et al. 2001) which is expressed in adrenocortical cells. Activation of HTR4 activates adenylyl cyclase and induces calcium influx into the cell through T-type channels, thus regulating cortisol and aldosterone production (Louiset et al. 2017). Remarkably, activation of 5-HT4 by agonists such as cisapride or zacopride induces aldosterone production in healthy volunteers (Lefebvre et al. 2000).

Genetic alterations in PA

The mechanisms that regulate aldosterone production are severely altered in PA. PA is the most common form of secondary arterial hypertension, with an estimated prevalence of 10% in referred patients in specialized centers and up to 5% in the general hypertensive population (Rossi et al. 2006, Hannemann & Wallaschofski 2012, Monticone et al. 2017). Although the majority of cases are sporadic, PA may be transmitted as a Mendelian trait in familial forms of the disease. Familial forms of PA account for 1–5% of cases and are transmitted as autosomal dominant traits. Four different forms have been described, based on the underlying genetic defect. Similarly, recurrent somatic mutations in different genes have been identified in up to 88% of APA (Nanba et al. 2018).

Germline mutations in FH

FH type I

FH-I or glucocorticoid-remediable aldosteronism (GRA) is an autosomal dominant disease that accounts for 0.5–1% of PA in adult patients, but up to 3% in pediatric cohorts (Medeau et al. 2005, Aglony et al. 2011, Mulatero et al. 2011, Pallauf et al. 2012). It is characterized by early and severe hypertension, often associated to hypokalemia. Peculiar features of FH-I are the presence of hybrid steroids 18-hydroxycortisol and 18-oxocortisol in the urines and the suppression of aldosterone by dexamethasone (Sutherland et al. 1966). These features are a consequence of the underlying genetic defect, which consists in the formation of a chimeric gene between the regulatory regions of CYP11B1 and the coding regions of CYP11B2 due to unequal crossing over during meiosis. This results in ectopic expression of CYP11B2 in the zona fasciculata of the adrenal cortex, and ACTH-regulated expression of aldosterone synthase (Lifton et al. 1992, Stowasser et al. 2000). Those patients can be efficiently treated with low-dose glucocorticoids (Stowasser et al. 2000); however, cardiovascular consequences of high aldosterone excess may manifest even in young normotensive patients (Stowasser et al. 2005) and patients with FH-I are at increased cardiovascular risk, with increased number of cerebrovascular events at a young age (Litchfield et al. 1998).

FH type II

FH-II is a non-glucocorticoid-remediable form of familial hyperaldosteronism, transmitted in an autosomal dominant fashion, found in 1.2–6% of adult patients with PA. Patients display a familial history of PA, and different subtypes of the disease (APA or BAH) may be present within the same family (Stowasser & Gordon 2001, Medeau et al. 2005, Mulatero et al. 2011, Pallauf et al. 2012). FH-II is not distinguishable from other sporadic forms of PA, the diagnosis is based on the identification of two or more affected family members. While a genetic locus was associated with FH-II on 7p22 (Lafferty et al. 2000), no pathogenic mutations were identified in several candidate genes located in this chromosomal region. It is only very recently that use of whole exome sequencing allowed the discovery of a new gene associated to FH-II. Two groups have indeed identified several gain-of-function mutations in the CLCN2 gene coding for the CLC-2 chloride channel in patients with FH-II and early-onset PA and described for the first time the implication of a chloride channel in the regulation of aldosterone production (Fig. 1C).

In one study, a de novo heterozygous p.Gly24Asp mutation in the CLCN2 gene was identified in a patient diagnosed at 9 years of age with hypertension due to PA, associated to profound hypokalemia (Fernandes-Rosa et al. 2018). This gain-of-function mutation affects a highly
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conserved region in the ClC-2 chloride channel, in which mutations lead to constitutive activation of the channel (Grunder et al. 1992). The channel is highly expressed in the human and mouse adrenal zona glomerulosa and patch clamp analyses of zona glomerulosa cells from mouse adrenal gland slices show bona fide chloride currents, which are abolished in Clcn2 knockout mice, indicating that ClC-2 is the foremost chloride channel in the adrenal zona glomerulosa (Fernandes-Rosa et al. 2018). Expression of the mutated ClCN2 in adrenocortical cells leads to increased chloride currents and depolarization of the zona glomerulosa cell membrane, opening voltage-gated calcium channels and triggering autonomous aldosterone production (Fig. 1C). This work indicates for the first time the implication of an anion channel in the regulation of aldosterone biosynthesis, PA and hypertension (Fernandes-Rosa et al. 2018).

In the other study, whole exome sequencing of three members of a large Australian family with FH-II previously described by Stowasser et al. (Stowasser et al. 1992, Lafferty et al. 2000) allowed the identification of a germline mutation in the CLCN2 gene, p.Arg172Gln, in affected family members. The same mutation was identified in three additional cases with early-onset PA, one within a familial context, another occurring de novo. Furthermore, the authors identified four additional germline ClC-2 variants, p.Met22Lys, p.Tyr26Asn, p.Lys362del and p.Ser865Arg in four unrelated patients with early-onset PA (Scholl et al. 2018). Immunohistochemistry confirmed the expression of ClC-2 in human adrenal zona glomerulosa. Mutant channels showed gain of function, with higher open probabilities at the glomerulosa resting potential.

Altogether, these results indicate that CLCN2 should be included in the panel of genes that are screened in patients who develop early-onset PA and in families with a diagnosis of FH-II. Mutations may be located in different domains of ClC-2 and therefore affect channel function at different degrees, which explains phenotypic heterogeneity and the presence of relatively mild cases.

FH type III and germline KCNJ5 mutations

FH-III is a non-glucocorticoid-remediable form of FH, showing early-onset severe hypertension, hyperaldosteronism, profound hypokalemia and high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol (Geller et al. 2008). Patients require bilateral adrenalectomy to control blood pressure, which shows massive bilateral hyperplasia of the adrenal cortex, although mild forms have also been described.

Recurrent germline mutations in the KCNJ5 gene (coding for the potassium channel GIRK4) have been identified in FH-III (Choi et al. 2011). KCNJ5 codes for the G protein-activated inward rectifier potassium channel 4 (GIRK4), which is composed of two membrane-spanning domains (M1 and M2), one pore-forming region (H5) and cytoplasmic N- and C-termini that participate in the formation of pore structure. The mutations are located in a highly conserved domain within or near the channel selectivity filter. They induce a gain of function, with a loss of potassium selectivity in favor of an increased unspecific sodium influx into the cytoplasm. This results in cell membrane depolarization, increased intracellular calcium concentrations, activation of Ca2+ signaling and autonomous aldosterone biosynthesis (Choi et al. 2011, Oki et al. 2012b) (Fig. 1C). Different KCNJ5 mutations have been described in the literature, which not all are associated with severe PA (Mulatero et al. 2013). Indeed, KCNJ5 mutations were also identified in patients diagnosed with mild PA in a context of FH-II, with no BAH at imaging and blood pressure controlled by conventional medication. The genetic diagnosis allowed reclassifying those patients into mild forms of FH-III. Remarkably, mutations associated with milder phenotypes have more pronounced functional effects in vitro. In particular, the channel’s selectivity was less affected by the p.Thr158Ala mutation compared to the channels carrying the other mutations (p.Leu168Arg and p.Gly151Arg) (Choi et al. 2011). The p.Gly151Glu mutation, identified in mild cases diagnosed as FH-II, generates significantly larger Na+ currents than the p.Gly151Arg mutation and leads to sodium-dependent cell death (Scholl et al. 2012), with strong depolarization of adrenocortical cells expressing the mutated channel (Mulatero et al. 2012).

FH type IV and germline CACNA1H mutations

FH-IV is a non-glucocorticoid-remediable form of FH, which has been attributed to germline CACNA1H mutations. Familial analysis showed that despite autosomal dominant transmission of the mutation, differences could be observed within family members on a clinical level, indicating incomplete penetrance of the disease (Scholl et al. 2015b).

CACNA1H codes for the α1 subunit of the voltage-gated T-type calcium channel Cav3.2. The structure of this subunit consists of four homologous domains (named from I to IV) where each domain contains six transmembrane helices (S1–S6). A recurrent p.Met1549Val mutation in CACNA1H was first identified in five patients...
with hypertension due to PA by age 10 years and their relatives. The mutation affects the electrophysiological properties of the channel, inducing specifically a shift in the voltage sensitivity of the channel to a more negative current and a modification of its inactivating properties, which triggers an opening of the channel at more negative membrane potentials, allowing activation of calcium signaling in the absence of stimulation (Scholl et al. 2015b) (Fig. 1D). Scholl et al. demonstrated in their study that cells transfected with the Cav3.2 1549Val had more aldosterone biosynthesis as well as higher CYP11B2 expression when compared to cells transfected with the WT Cav3.2 channel (Reimer et al. 2016).

In a similar study, Daniil et al. identified four germline mutations in the CACNA1H gene in PA patients with different phenotypic presentations. Two of the variants (p.Val1951Glu and p.Pro2083Leu) in this study are located in the cytoplasmic C terminus of the channel. A p.Ser196Leu variant was located in the S4 segment of domain I of the channel, and one mutation, p.Met1549Ile, is located in the S6 segment of domain III. The p.Met1549Ile mutation, affecting the same amino acid described by Scholl et al. in patients with FH-IV, was identified in a patient with early-onset PA and multiplex developmental disorder. Cav3.2 p.Ser196Leu and p.Pro2083Leu were found in two patients with FH-II, and p.Val1951Glu was identified in one patient with APA. Electrophysiological analysis revealed significant changes in the Ca\(^{2+}\) current properties for all mutants, suggesting a gain-of-function phenotype. Moreover, transfection of the mutant Cav3.2 in the adrenocortical cell line H295R-S2 led to increased aldosterone production and increases the expression of steroidogenic enzymes following potassium stimulation (Daniil et al. 2016).

**Germline CACNA1D mutations in PA**

CACNA1D mutations have been described in two children with PASNA (primary aldosteronism, seizures and neurological abnormalities) (Scholl et al. 2013). CACNA1D codes for the α1 subunit of the voltage-gated L-type calcium channel Cav1.3. Again, these mutations induce a gain of function, with the channel opening at lower voltages. This leads to excessive aldosterone production by increasing Ca\(^{2+}\)-mediated intracellular acidification which causes an increased intracellular Ca\(^{2+}\) concentrations, but showed intracellular acidification, which caused increased aldosterone production (Stindl et al. 2015).

The first case was identified in a 3-year-old female who suffered severe hypertension, biventricular hypertrophy, neurological abnormalities and aldosteronism as a newborn, associated with high aldosterone levels, a high aldosterone-to-renin ratio and hypokalemia. Genetic analyses identified a germline mutation in CACNA1D (p.Gly403Asp), a variant that had also been identified in APA (Scholl et al. 2013, 2015a). The second subject exhibited cerebral palsy and complex seizures just after birth, with HT and hyperaldosteronism with hyperkalemia at age 5 years. Genetic analysis leads to the identification of a CACNA1D mutation (p.Ile770Met) also found as recurrent mutation in APA (Scholl et al. 2013, Korah & Scholl 2015) (see below).

**Somatic mutations in APA**

Numerous recurrent somatic mutations have been identified in APA. These affect genes coding for ion channels ( KCNJ5 Choi et al. 2011 and CACNA1D Azizan et al. 2013, Scholl et al. 2013) also involved in inherited forms. In addition, recurrent mutations have been identified in genes coding for ATPases ( ATP1A1 which codes for the α1 subunit of the Na\(^{+}\)/K\(^{+}\)-ATPase Azizan et al. 2013, Beuschlein et al. 2013 and ATP2B3 encoding the plasma membrane calcium-transporting ATPase 3 (PMCA3) Beuschlein et al. 2013) that regulate the intracellular ionic homeostasis and membrane potential. Na\(^{+}\)/K\(^{+}\)-ATPase and PMCA3 are members of the P-type family of ATPases and are composed of ten transmembrane domains (M1–M10) with intracellular N- and C-tails. ATP1A1 mutations affect amino acids in the transmembrane helices M1, M4, and M9 of the α1 subunit. They lead to a loss of pump activity and a reduction in its affinity for potassium as well as an inward leakage of sodium, inducing membrane depolarization (Azizan et al. 2013, Beuschlein et al. 2013). In addition, mutant pumps may induce a proton leak (Azizan et al. 2013), leading to intracellular acidification which contributes to aldosterone production. Indeed, expression of mutant α1 subunits of the Na\(^{+}\)/K\(^{+}\)-ATPase carrying the L104R, V332G and G99R mutations in adrenocortical NCI-H295R cells failed to increase intracellular Ca\(^{2+}\) concentrations, but showed intracellular acidification, which caused increased aldosterone production (Stindl et al. 2015). Under physiological conditions, the PMCA3 extrudes one cytosolic Ca\(^{2+}\) in exchange for two H\(^{+}\). PMCA3 mutations occur in the transmembrane domain M4 and induce the deletion of a stretch of amino acids between Leu425 and Leu433 (Beuschlein et al. 2013, Fernandes-Rosa et al. 2014). Cells expressing the mutant PMCA3 have a reduced capacity to export Ca\(^{2+}\), suggesting a loss
of the physiological pump function. In addition, they induce an increased Ca\textsuperscript{2+} influx possibly due to opening of depolarization-activated Ca\textsuperscript{2+} channels or to Ca\textsuperscript{2+} leak through the mutated pump (Tauber et al. 2016). All these mutations induce an increase in intracellular calcium levels, followed by an activation of calcium signaling and therefore increase the biosynthesis of aldosterone.

A study on 474 subjects presenting an APA, performed within the European Network for the Study of Adrenal Tumors (ENS@T), found mutations in 54.2% of APA, where 38% of these were found in the \textit{KCNJ5} gene, 9.3% in \textit{CACNA1D}, 5.3% in \textit{ATP1A1} and 1.7% in \textit{ATP2B3} (Fernandes-Rosa et al. 2014). Patients presenting mutations in \textit{KCNJ5} were mostly women and younger patients, while APA with \textit{CACNA1D} mutations were smaller than the other mutational groups (Fernandes-Rosa et al. 2014).

A recent meta-analysis on 1636 patients from 13 studies showed a prevalence of \textit{KCNJ5} mutations of 43%, with much higher frequencies (up to 77%) in populations from East Asia in comparison to studies performed on European populations. APA harboring \textit{KCNJ5} mutations were more frequent in women and associated with larger tumors and a higher plasma aldosterone concentration when compared with mutation-negative APA. APA where \textit{KCNJ5} mutations are present are also associated with APA composed mainly of ZF-like cells. Other mutations were associated mainly with male gender and smaller tumors (Lenzini et al. 2015).

In a recent study, Nanba et al. performed CYP11B2 immunohistochemistry on 75 adrenal samples from white American patients, in order to identify APA and other aldosterone-producing regions prior to genetic analysis. DNA was then extracted directly from formalin-fixed paraffin-embedded (FFPE) tissue, positive for CYP11B2 expression. Complete next-generation sequencing of all genes involved in PA identified somatic mutations in 88% of APA (Nanba et al. 2018). Again, \textit{KCNJ5} mutations were the most frequent mutational events (43%), followed by \textit{CACNA1D} (21%), \textit{ATP1A1} (17%), \textit{ATP2B3} (4%) and \textit{CTNNB1} (3%) mutations. Also, \textit{KCNJ5} mutations were more frequent in women (70 vs 24% in men). This study shows that CYP11B2 immunohistochemistry-guided next-generation sequencing allows the identification of a larger portion of somatic mutations in APA. Similar results were obtained by analyzing adrenocortical adenomas from 79 black patients with PA. Somatic mutations in aldosterone driver genes were found in 89% of APAs. Interestingly, \textit{CACNA1D} mutations were the most frequent (42%) ones in APA from patients with African ancestry, followed by \textit{KCNJ5} (34%), \textit{ATP1A1} (8%) and \textit{ATP2B3} mutations (4%) (Nanba et al. 2019).

Other less frequent mutations that play an important role in APA were identified in \textit{CTNNB1} and \textit{PRKACA}. \textit{CTNNB1} codes for \(\beta\)-catenin. This protein is a central element of the Wnt/\(\beta\)-catenin signaling pathway, which has an essential role in the development of the adrenal cortex, in the differentiation of the zona glomerulosa and steroid hormone biosynthesis (El Wakil & Lalli 2011). In unstimulated conditions, \(\beta\)-catenin is present in the cytoplasm and is associated to the degradation complex with adaptor proteins such as axin, APC (adenomatous polyposis coli), two serine/threonine kinases, caseine kinase 1 (CK1\(\alpha\)) and glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)). This complex phosphorylates \(\beta\)-catenin, which is then recognized by the ubiquitin ligase E3 that results in its destruction by the proteasome, thus preventing its translocation into the nucleus and the activation of different Wnt target genes. Activation of the Wnt/\(\beta\)-catenin signaling pathway by binding of Wnt to its receptor frizzled inhibits the phosphorylation of \(\beta\)-catenin which dissociates from the axin complex and translocates to the nucleus where it induces the expression of Wnt target genes, by binding to the transcription factors TCF (T-cell factor) and LEF (lymphocyte enhancer factor) as a transcriptional co-activator (MacDonald et al. 2009). It has been shown that \(\beta\)-catenin indirectly regulates \textit{CYP11B2} expression via transcriptional induction of the nuclear receptors NURR1 and NUR77 (Berthon et al. 2014). The Wnt/\(\beta\)-catenin signaling pathway has been shown to be constitutively active in 70% of APA (Berthon et al. 2014). Mutations in \textit{CTNNB1} have been found in 2–5% of APA and are more frequent in women (Teo et al. 2015, Scholl et al. 2015a, Akerstrom et al. 2016). Mutations specifically occur in exon 3, which codes for the serine/threonine residue which when phosphorylated results in the degradation of \(\beta\)-catenin (Berthon et al. 2010). To a much lesser extent, somatic mutations in \textit{PRKACA}, which codes for the cAMP-dependent protein kinase catalytic subunit \(\alpha\), have been described in APA (Rhayem et al. 2016). A study performed on a cohort of 12 patients showed a prevalence of 1.6% of this mutation. One of the described mutations in this study was new (p.His88Asp) (Rhayem et al. 2016), whereas the other mutation, p.Leu206Arg, had been previously described as a recurrent somatic mutation in patients with cortisol-producing adenoma and Cushings’s syndrome (Goh et al. 2014). Mutations in both \textit{PRKACA} and \textit{CTNNB1} have been mainly associated with cortisol-producing adenomas and/or adrenocortical...
cancer. Therefore, the mechanisms explaining how the same mutations result in two different hormonal phenotypes remain to be established.

### Genomic and proteomic alterations in APA

Despite the major advances on the genetics of PA, many questions remain open concerning the pathophysiology of the disease, in particular, the mechanisms and dynamics of adenoma formation. Several studies have explored genomic and proteomic alterations in APA, to better understand the mechanisms involved in its development. A recent extremely original work performed deep quantitative proteomic and phosphoproteomic profiling on APA and adjacent non-tumoral adrenal cortex from six patients with PA. The authors identified, out of 5555 proteins common to all samples, 18 which were significantly downregulated and 11 significantly upregulated in all APA. Proteome data confirmed that upregulation of the steroidogenic enzymes HSD3B2, CYP21A2, CYP11B2 and of proteins involved in cholesterol uptake (lipolysis-stimulated lipoprotein receptor (LSR)) is involved in the increase of steroidogenesis in APA (Swierczynska et al. 2019). This study also showed that steroidogenic enzymes are regulated by phosphorylation; in particular, HSD3B2 is phosphorylated at Ser95 or 96 and CYP21A2 has a novel phosphorylation site at Ser489. An altered composition of the extracellular matrix (ECM) was also revealed in APA, with reduction in proteins related to collagen and collagen fibril assembly, enzymes involved in extracellular matrix turnover and structural components of the extracellular matrix. The deregulation of kinases affecting cytoskeleton remodeling and axonal guidance-related pathways suggests that APA development may be accompanied or be related to changes in cytoskeleton rearrangements and possibly innervation. Remarkably, the GTPase RHOC controlling actin organization, which was upregulated in APA, significantly increased the expression of CYP11B2 at the transcriptional level in transduced adrenocortical H295R cells.

APA also showed higher levels of proteins involved in N-glycosylation and enzymes involved in GABA degradation. Remarkably, the activity of the main regulators of steroidogenesis, MC2R (receptor for ACTH) and AT1R (receptor for Ang II) is affected by N-glycosylation and the level and the activity of N-glycoproteins like lipoprotein receptors (LDLR and SR-B1) and many ion channels (TASK1, TASK3, TREK1, Kv1.4, CACNA1D) are affected by N-glycosylation. The inhibition of TASK1, TASK3 and Kv1.4 glycosylation decreases the number of cell-surface channels, leading to a reduced current flow, protein instability and intracellular channel retention (Watanabe et al. 2004, Mant et al. 2013, Swierczynska et al. 2019). Reduced GABAergic signaling in APA may also modulate aldosterone production. Indeed, in physiological conditions, GABAergic signaling mediates a decrease of steroidogenesis in vivo in the rat adrenal cortex (Mishunina & Kononenko 2002). Finally, one of the most dysregulated pathways in APAs appeared to be the mTORC1 signaling pathway. Its activity was increased in APA; this pathway is involved in cell proliferation and steroidogenesis in APA cells and immortalized adrenocortical cells. Thus, the increase of its activation may lead to an increased steroidogenic output and adenoma development. This opens new perspectives on the benefits of inhibiting mTORC1 in patients with APA (Su et al. 2013, Swierczynska et al. 2019).

Similarly, gene expression profiling of APA allowed identifying genes and intracellular signaling pathways involved in the pathogenesis and pathophysiology of this tumor. As expected, microarray studies and SAGE (serial analysis of gene expression) showed higher expression of CYP11B2 in APA compared to normal adrenals and adjacent cortical tissue (Assie et al. 2005, Williams et al. 2010), although other studies report a subgroup of APA with reduced expression of aldosterone synthase (Lenzini et al. 2007). However, these data require to be reevaluated in the context of recent work showing that in a certain number of multinodular adrenals some of the larger nodules do not express CYP11B2, which is localized to smaller nodules or APCC (Fernandes-Rosa et al. 2015, Nanba et al. 2018); this finding may have led to some sample biases in earlier studies. Despite this, APA show a genotype–phenotype correlation indicating that transcriptional signatures may be influenced by APA genotype, although different studies report slightly different results. Indeed, APA with different gene mutations show distinct expression profiles (Azizan et al. 2012). While some studies reported a higher CYP11B2 expression in APA with KCNJ5 mutations compared to APA without KCNJ5 mutation (Monticone et al. 2012, Seccia et al. 2012), a higher expression of CYP11B2 was reported in APA with ATP1A1 and ATP2B3 mutations, compared to KCNJ5-mutated tumors (Akerstrom et al. 2015). In a large transcriptome analysis performed on 102 samples, KCNJ5-mutated APA did not show differential clustering compared to tumors not harboring KCNJ5 mutations (Boulkroun et al. 2012, Zennaro 2019).

Several studies revealed an upregulation of genes encoding transcription factors (NURR1 and NGF1B)
that regulate the transcription of CYP11B2 (Bassett et al. 2004a), genes encoding the transcription factors SF-1 (NR5A1) and DAX1 (NROB1) that play an essential role in adrenal development and steroidogenesis (Bassett et al. 2005) and a target gene of SF-1, VSNL1, which increases aldosterone production in the NCI H295R cells (Williams et al. 2012). Furthermore, Azizan et al. reported higher CYP17A1 expression in APA composed of cells resembling to the zona fasciculata carrying KCNJ5 mutations (Azizan et al. 2012). This result was also confirmed in KCNJ5-mutated tumors compared with ATP1A1/CACNA1D mutant adenomas by immunohistochemistry (Tan et al. 2017).

Finally, a recent methylose analysis revealed hypomethylation of CYP11B2 in APA and a higher demethylation of G protein-coupled receptors (GPCRs) and GPCR-related genes (Itcho et al. 2018). Thus, this hypomethylation may stimulate aldosterone production in APA through receptors that would regulate gene transcription like ACTH, glucagon, somatostatin, parathyroid hormone and glutamate metabotropic receptors. Remarkably, HTR4, MC2R and PTGER1 (prostaglandin E receptor 1), which all regulate aldosterone biosynthesis, showed hypomethylation and upregulation of their mRNA in APA.

APCC and the development of APA

Different studies showed the existence of APCC in the subcapsular portion of the normal human adrenal gland. These APCC, which are suggested to autonomously produce aldosterone, are characterized by the presence of subcapsular zona glomerulosa-like cells and inner zona fasciculata-like cells (Nishimoto et al. 2010) and a uniform CYP11B2, but absent CYP11B1 expression (Nishimoto et al. 2016). Therefore, APCC and APA are distinctive in their sizes, cellular arrangements and enzyme expression profiles, as APA may consist of heterogeneous cell types expressing either CYP11B1 or CYP11B2. In the human adrenal gland, aldosterone synthase staining is continuous in the zona glomerulosa at young age between 0 and 11 years, whereas with increasing age, the adrenal cortex loses the continuous staining of aldosterone synthase and acquires a variegated zonation with increased number of CYP11B2 expressing APCC (Omata et al. 2015). The discontinuous CYP11B2 expression is suggested to be the consequence of a negative feedback produced by the autonomous aldosterone production by APCC.

Even though there is no sex difference in APA prevalence, a higher APCC score was described in female adrenals (Nishimoto et al. 2015). Transcriptome analyses identified markers for APCC in addition to CYP11B2, which showed significantly higher transcript expressions in APCC compared to zona glomerulosa or fasciculata. These genes code for proteins playing a role in glucose transport (SLC35F1), aldosterone production (MC2R) and regulation of intracellular phosphorylation and dephosphorylation (PPP4R4) (Nishimoto et al. 2015).

Different somatic mutations leading to autonomous aldosterone production were identified in APCC from normal subjects, supporting a pathological behavior of APA (Nishimoto et al. 2015). Next-generation sequencing of 23 APCC samples from FFPE and frozen tissues from normal adrenals showed that 35% of APCC harbored mutations observed in APA and known to cause aldosterone overproduction in CACNA1D (26%) and ATP1A1 (6%). No mutations in KCNJ5 have been identified in APCC so far. Different explanations are given for this observation, including that APCC with KCNJ5 mutations may rapidly progress into APA (Nishimoto et al. 2015).

Omata et al. demonstrated that the accumulation of APCC is a cellular and molecular cause of IHA. Examination of a cohort of 15 adrenals with IHA with CYP11B2 immunohistochemistry and next-generation sequencing showed that the adrenal cortex of all IHA adrenals harbored at least one CYP11B2-positive APCC or aldosterone-producing microadenomas. IHA adrenals had a significantly larger number of APCC than normotensive controls. Somatic mutations were identified in CACNA1D in 58% of cases and only in one case in KCNJ5. These findings suggest that not only hyperplasia but also the enlargement of APCC with somatic aldosterone driver gene mutations (most likely CACNA1D) are responsible for IHA (Omata et al. 2018).

Finally, transitional structures were described showing a subcapsular APCC-like portion and inner APA-like portion (Nishimoto et al. 2016). These structures are called pAATL, possible APCC-to-APA transitional lesions. These pAATL are characterized by the presence of mutations in APA driver genes, in particular KCNJ5 and ATP1A1, with KCNJ5 mutations identified exclusively in the APA-like portion of the structure (Nishimoto et al. 2016).

Conclusion

Although much progress has been made over the last ten years in our understanding of the molecular mechanisms leading to PA, there still remain a number of open
questions which need to be addressed. In particular, in a certain number of familial cases, diagnosed mainly as FH-II, no genetic abnormality has been identified in known genes. This is also the case for a small proportion of APA, in which even CYP11B2-guided next-generation sequencing of all coding exons of all APA driver genes fails identifying somatic mutations. This suggests that additional genes may be involved in the disease, which remain to be discovered. Another question relates to the mechanisms leading to APA development. Indeed, germline mutations identified in familial forms lead to adrenal cortex hyperplasia, but not to APA. Whether nodule formation requires an additional genetic hit favoring increased proliferation, or whether APA derive from APCC carrying somatic mutations remains to be clarified. A related question is how do APCC develop, in particular how and when do somatic mutations occur and whether there is any difference between APCC with and without somatic mutations. Future work addressing these issues will allow progress in our knowledge on the molecular mechanisms involved in normal aldosterone physiology and development of PA and possibly provide new tools for the development of better diagnostic and therapeutic approaches.

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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