Genetics of primary hyperaldosteronism

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Abstract

Hypertension is a common medical condition and affects approximately 20% of the population in developed countries. Primary aldosteronism is the most common form of secondary hypertension and affects 8–13% of patients with hypertension. The two most common causes of primary aldosteronism are aldosterone-producing adenoma and bilateral adrenal hyperplasia. Familial hyperaldosteronism types I, II and III are the known genetic syndromes, in which both adrenal glands produce excessive amounts of aldosterone. However, only a minority of patients with primary aldosteronism have one of these syndromes. Several novel susceptibility genes have been found to be mutated in aldosterone-producing adenomas: *KCNJ5*, *ATP1A1*, *ATP2B3*, *CTNNB1*, *CACNA1D*, *CACNA1H* and *ARMC5*. This review describes the genes currently known to be responsible for primary aldosteronism, discusses the origin of aldosterone-producing adenomas and considers the future clinical implications based on these novel insights.

Key Words

- ▶ primary aldosteronism
- ▶ genetics
- ▶ familial
- ▶ sporadic
- ▶ calcium channel

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Introduction

Hypertension, which is characterized by the presence of high blood pressure, is a serious medical condition. According to the World Health Organization (WHO), approximately 40% of adults aged 25 and older have hypertension. It is divided into two forms, primary or essential hypertension that accounts for 90–95% of all hypertension cases and secondary hypertension. Concerning secondary hypertension, primary aldosteronism (PA) accounts for 8–13% of the patients with unselective hypertension (Funder 2012). The two most common causes of primary aldosteronism are an aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH); both are characterized by a constitutive overproduction of aldosterone.

APAs are also known as Conn adenomas (named after the American endocrinologist Jerome W Conn)

and are characterized by the presence of tumors in the adrenal cortex (Conn 1966). Unilateral APA is most commonly found in patients; bilateral APAs are rarely reported. Patients with APAs are often identified due to hypertension that is difficult to control with medication. To screen patients, the guidelines recommend the measurement of the aldosterone-to-renin ratio (ARR), which may be accompanied by hypokalemia (Funder et al. 2008, Nishikawa et al. 2011). If the ARR is >200, confirmative tests such as the captopril challenge test, the upright furosemide-loading test or the saline-loading test should be performed. Unilateral adrenalectomy often normalizes or markedly improves the blood pressure in patients with APAs (Funder et al. 2008, Nishikawa et al. 2011). The majority of APAs are only 1–3 cm in size (Choi et al. 2011, Mulatero et al. 2012, Taguchi et al. 2012,

Azizan *et al.* 2012a, Azizan *et al.* 2013, Beuschlein *et al.* 2013, Dutta *et al.* 2014, Zilbermint *et al.* 2015, Scholl *et al.* 2015b). APAs may occur in patients of all ages, but a high prevalence is seen in patients 40–50 years of age.

Bilateral adrenal hyperplasia (BAH) is another form of PA, which accounts for 60–70% of patients with PA (Zennaro *et al.* 2015). It is diagnosed if adrenal venous sampling shows hyperaldosterone secretion from both adrenals. Standard treatment involves antagonists of the mineralocorticoid receptor, e.g., spironolactone. BAH is also associated with genetic syndromes such as familial hyperaldosteronism types I, II or III, in which both adrenal glands produce excessive amounts of aldosterone.

Aldosterone is a mineralocorticoid that under physiological conditions is produced by the zona glomerulosa of the adrenal cortex and regulated by the renin–angiotensin system (Morgan *et al.* 1996, Tanabe *et al.* 1998). The proteolytic enzyme, renin, converts angiotensinogen to angiotensin I, which is subsequently cleaved by angiotensinogen-converting enzyme (ACE) to angiotensin II (Fig. 1). Next, angiotensin II binds to angiotensin receptors on the surface of adrenal cortex cells, which leads to the production of aldosterone. Moreover, aldosterone binds to the mineralocorticoid receptor (MR) in different segments of the renal tubule. It activates the expression of the epithelial Na⁺ channel (ENaC), Na⁺-K⁺-ATPase and NaCl cotransporter, which leads to the reabsorption of sodium and chloride ions (Garty & Palmer 1997, Rossier *et al.* 2013, Czogalla *et al.* 2016). In addition, aldosterone production and secretion

by the adrenals and the antidiuretic hormone (ADH) from the pituitary gland further contribute to the increase in blood pressure by regulating the reabsorption of water in the collecting ducts of the kidney (Share & Crofton 1982). Aldosterone thus plays a central role in the regulation of blood pressure mainly by acting on the distal tubules and collecting ducts of the nephron. To a lesser extent, the adrenocorticotrophic hormone (ACTH) also stimulates the secretion of aldosterone from adrenals (Gallo-Payet 2016). In healthy adults, the adrenal cortex produces 20–200 µg of aldosterone per day.

The two most important physiological stimuli of aldosterone production are angiotensin II and the quantity of serum potassium (Fig. 1) (Tanabe *et al.* 1998). Decreased blood volume activates the renin–angiotensin system, in which angiotensin II signals via the angiotensin receptor (Fig. 1). The difference in K⁺ concentration across the membrane sets the resting membrane potential; both hyperkalemia and hypokalemia cause depolarization of the membrane and generate an action potential to open a voltage-gated Ca²⁺ channel (Spat 2004). An enhanced intracellular Ca²⁺ concentration provides the normal signal for aldosterone production. In PA, autonomous production of aldosterone is found independent of angiotensin II (Funder 2012).

Research of PA and familial cases of hyperaldosteronism has accelerated the mechanistic insights of how different genes are linked to these diseases. Next-generation sequencing of APA samples has uncovered mutations in several novel genes: *KCNJ5*, *ATP1A1*,

Figure 1

The renin–angiotensin system and aldosterone production under normal physiologic conditions. The liver continuously produces angiotensinogen that is released into the plasma. When the blood pressure and volume decreases, the kidney granular cells start to produce renin, which cleaves angiotensinogen into angiotensin I. Angiotensin I is subsequently cleaved into angiotensin II, which binds to angiotensin II binding receptors (AT_{1A}, AT_{1B} and AT₂) on zona glomerulosa cells of the adrenal cortex. This triggers the production of aldosterone. Aldosterone binds to the mineralocorticoid receptor in the kidney. ACE, angiotensinogen.

ATP2B3, *CTNNB1*, *CACNA1D*, *CACNA1H* and *ARMCS* (Choi et al. 2011, Mulatero et al. 2012, Azizan et al. 2012a, 2013, Taguchi et al. 2012, Beuschlein et al. 2013, Dutta et al. 2014, Zilbermint et al. 2015, Scholl et al. 2015b). So far, these identified genes are mutated in approximately 60% of patients with APAs. Still, the genetic factors that contribute to APA in the remaining 40% of patients are unknown. Here, we review the current knowledge regarding the genetics that contribute to PA.

Genes associated with hereditary hyperaldosteronism

Familial hyperaldosteronism type I

Sutherland and coworkers described the first familial hyperaldosteronism type I (FH-I) or glucocorticoid-remedial aldosteronism (GRA) in their case report of a father and son suffering from hypertension due to hyperaldosteronism in 1966 (Sutherland et al. 1966). FH-I has been found in 0.5–1% of the adult population with PA as opposed to children with PA, where FH-I is found in 1–3% of cases (Aglony et al. 2011, Carvajal et al. 2012). Among patients or within a family, FH-I is characterized by the presence of bilateral adrenal hyperplasia or a rare adrenal nodule exhibiting variable clinical and biochemical features (Fallo et al. 2004, Aglony et al. 2011). Patients with FH-I come to clinical attention early in life (Table 1). However, some patients exhibit a mild clinical phenotype, and even normotensive patients have been reported (Fallo et al. 2004). A high rate of cerebrovascular complications has been found in patients with GRA (Litchfield et al. 1998). There is no gender bias found among patients. Sutherland and coworkers found that

treatment with dexamethasone, a suppressor of the ACTH, suppressed hypertension and decreased the aldosterone level (Sutherland et al. 1966).

The molecular etiology of FH-I was first elucidated by Lifton and coworkers in 1992. This study reported that patients with FH-I inherit a chimeric *CYP11B1* and *CYP11B2* hybrid gene. This hybrid gene includes the promoter region of *CYP11B1* and the majority of the coding sequence of *CYP11B2* (Lifton et al. 1992a). Consequently, aldosterone production in these patients is regulated by ACTH instead of angiotensin II. As a result, the aldosterone production follows a circadian secretion pattern with cortisol. Of note, the hybrid gene was found to be expressed in all cell layers of the adrenal cortex. In subsequent studies, Lifton and coworkers and Pascoe and coworkers showed that the crossing-over breakpoints of *CYP11B1/B2* differ among cases, which suggest that the mutations arose independently in each individual (Pascoe et al. 1992, Lifton et al. 1992b). Patients with FH-I display increased levels of the secreted hybrid steroids, 18-hydroxycortisol and 18-oxocortisol. Ion channels and transporters are not reported to be involved in this type of familial hyperaldosteronism.

Familial hyperaldosteronism type II

In 1992, Stowasser and coworkers first described a second form of hereditary hyperaldosteronism, which is a nonglucocorticoid remedial form of PA (Stowasser et al. 1992). Patients are diagnosed with familial hyperaldosteronism type II (FH-II) when at least two first-degree members of the same family have confirmed primary aldosteronism (either APA or BAH) and when FH-I and familial hyperaldosteronism type III (FH-III) have been

Table 1 Clinical and biochemical data of patients with familial hyperaldosteronism (FH).

	FH-I	FH-II	FH-III
Gene mutation	Hybrid <i>CYP11B1/CYP11B2</i>	unknown	<i>KCNJ5</i>
Mode of inheritance	Autosomal dominant	Autosomal dominant	Autosomal dominant
Gender predominance (male:female)	1:1	1:1	1:1
Age at diagnosis	Variable, mostly young	Variable or like-APA	Mostly in childhood
Hypertension	Normotensive to severely hypertensive	Normotensive to severely hypertensive	Severely hypertensive
Aldosterone levels	Normal to markedly increased	Normal to markedly increased	Extremely high
Potassium level	Normal to low	Normal to low	Hypokalemic
Hybrid steroid	Higher	Normal or mildly elevated	Extremely high
Adrenal morphology	Normal or occasional evidence of hyperplasia	APA/BAH	Normal or BAH
Adrenal lateralization	Bilateral	Unilateral or bilateral	Bilateral
Treatment	Dexamethasone or MRA	Unilateral adrenalectomy or MRA	Bilateral adrenalectomy

APA, aldosterone-producing adenoma; BAH, bilateral adrenal hyperplasia; MRA, mineralocorticoid receptor antagonist.

excluded. In FH-II, the clinical and biochemical features as well as the severity of the disease vary among patients and family members (So *et al.* 2005). The phenotype of FH-II may be uni- or bilateral hyperplasia, which is very similar to sporadic PA (Table 1). Besides age, aldosterone levels, potassium levels and hybrid steroids (18-hydroxycortisol and 18-oxocortisol) levels are in the range of those observed in patients with sporadic APA (Funder *et al.* 2008, Mulatero *et al.* 2011). Depending on the severity and phenotype, FH-II is treated with either adrenalectomy or mineralocorticoid receptor antagonists. The prevalence of FH-II is higher (approximately 6%) when compared with other familial forms of PA, and unlike FH-I, the age of FH-II patients varies from 14 to 78 years (Mulatero *et al.* 2011).

The molecular etiology of FH-II is still unknown; however, the mode of inheritance found among FH-II families is autosomal dominant (So *et al.* 2005, Sukor *et al.* 2008). Genome-wide linkage analysis in one family showed that chromosome 7p22 is associated with FH-II (Lafferty *et al.* 2000). In a subsequent study, Sukor and coworkers obtained similar results using samples from five different families. However, sequencing of candidate genes in this chromosomal region has failed to identify causative mutations. Recently, Mulatero and coworkers identified germline mutations in *KCNJ5* in patients considered to have FH-II but who actually suffered from FH-III instead (Mulatero *et al.* 2012).

Familial hyperaldosteronism type III

In 2008, Geller and coworkers first described the third form of familial hyperaldosteronism in their case study of a father and two daughters (Geller *et al.* 2008). The affected persons had severe hypertension in childhood with distinctive clinical and biochemical features (Table 1). They also exhibited high levels of hybrid steroids (18-hydrocortisol and 18-oxocortisol) accompanied with hypokalemia. Dexamethasone suppression tests unexpectedly increased both serum aldosterone and blood pressure levels. The adrenal size was unexpectedly large, and histopathology demonstrated an atrophic zona glomerulosa and diffuse hyperplasia in the zona fasciculata. Bilateral adrenalectomies normalized the patients' blood pressure and aldosterone levels. FH-III is characterized by the early onset of hypertension and the presence of bilateral adrenal hyperplasia exhibiting resistant hypertension with hypokalemia (Geller *et al.* 2008). Very severe clinical phenotypes have been found among patients with FH-III (Geller *et al.* 2008, Monticone *et al.* 2015a). However, one case reported by Monticone

and coworkers described patients with a mild phenotype (Monticone *et al.* 2013).

In 2011, Choi and coworkers identified the genetic etiology behind FH-III by whole exome sequencing (Choi *et al.* 2011). A germline *KCNJ5* mutation was identified in one affected person at p.T158A and a somatic mutation in sporadic APAs (Choi *et al.* 2011). Expression of mutant *KCNJ5* leads to higher membrane depolarization, which results in voltage-gated calcium channels opening. In 2012, Scholl and coworkers identified *KCNJ5* germline mutations in four families with FH-III (Scholl *et al.* 2012). The mutations targeted the same codon, p.G151E and p.G151R, which is part of a highly conserved selective filter region of the potassium channel.

Genes associated with aldosterone-producing adenomas (APAs)

KCNJ5

KCNJ5 encodes the potassium ion channel Kir3.4, is located on chromosome 11q24.3 and consists of 3 exons. Further, this gene encodes a membrane protein of 419 amino acids. Kir3.4 forms the ion channel as homo- or heterotetramers with Kir3.1 (Corey & Clapham 1998). This channel is expressed in different tissues including the heart, central and peripheral neurons, various endocrine tissues as well as nonexcitable structures such as blood platelets (Marionneau *et al.* 2005, Mintert *et al.* 2007, Choi *et al.* 2011, Monticone *et al.* 2012). Immunohistochemical studies of the human adrenal cortex have established that Kir3.4 is localized mainly in the zona glomerulosa and the outer part of zona fasciculata. Inwardly rectifying potassium channels are a type of G protein-gated ion channels (Corey & Clapham 1998). Inward rectifier potassium ion channels are activated (opened) via a signal transduction cascade starting with ligand-stimulated G protein-coupled receptors (GPCRs). GPCRs, in turn, release activated G protein $\beta\gamma$ -subunits ($G\beta\gamma$) from inactive heterotrimeric G protein complexes ($G\alpha\beta\gamma$). Finally, the $G\beta\gamma$ dimeric protein interacts with G protein-regulated inwardly rectifying K^+ (GIRK) channels to open them, so that they become permeable to K^+ ions, resulting in the hyperpolarization of the cell membrane. Potassium channels share a highly conserved stretch of eight amino acids, the K^+ channel signature sequence, that forms the selective filter region for K^+ ions (Heginbotham *et al.* 1994). The Kir3.4 channel is an inward rectifier channel that is very selective for monovalent ions and remarkably discriminate between

Figure 2

Showing one subunit of the Kir3.4 channel. Red circles indicate the most frequently found somatic and germline mutations in aldosterone-producing adenomas.

K⁺ and Na⁺ ions (Roux 2005). Loss-of-function germline mutations in *KCNJ5* have been reported in congenital long QT syndrome (Yang et al. 2010).

Choi and coworkers identified germline and somatic mutations in *KCNJ5* by whole exome sequencing of samples from patients with either the familial or sporadic form of APA (Choi et al. 2011). Two hot spot somatic mutations were identified at p.G151R and p.L168R of the Kir3.4 channel (Fig. 2). The mutations p.G151R and p.L168R are situated on the highly conserved glycine–tyrosine–glycine (GYG) motif of the selective filter and the second transmembrane (TM) domain of *KCNJ5*, respectively. The mutations abolish the K⁺ selectivity of the channel. Electrophysiological measurements of cells expressing both mutants show that the selectivity for the K⁺ ion is lost, which leads to higher Na⁺ conductance and depolarization of the cell. Further, this depolarization results in the opening of the voltage-gated Ca²⁺ channels, which results in an influx of Ca²⁺ and increased aldosterone production.

Since the discovery of the *KCNJ5* mutations in APAs by Choi and coworkers, there have been several confirmative reports from different research groups worldwide (Boukroun et al. 2012, Taguchi et al. 2012, Dutta et al. 2014, Fernandes-Rosa et al. 2014, Murthy et al. 2014, Williams et al. 2014). Fernandes-Rosa and coworkers sequenced the hot spot region in 474 APAs collected from the European Network for the Study of Adrenal Tumor (ENSAT). *KCNJ5* mutations were identified in 180 of the 474 samples (38%), in which p.G151R (62.7%) and p.L168R (36.1%) were highly prevalent (Fernandes-Rosa et al. 2014). Recently, Murthy and coworkers identified one somatic mutation (p.E246K) and three polymorphisms (p.R52H, rs144062083; p.G247R, rs200170681; and p.E282Q, rs7102584) of *KCNJ5* in sporadic cases (Murthy et al. 2014). All variants affected an amino acid that is highly conserved among species. When the polymorphism was expressed by

Xenopus oocytes, p.R52H and p.E282Q showed loss of the selectivity filter for the K⁺ ion. The polymorphisms were inherited by the offspring (<30 years), but no adenomas were found in their adrenals using a computed tomography scan.

Dekkers and coworkers sequenced multinodular adrenals for known genes associated with APAs (Dekkers et al. 2014) and, of interest, found one case having two different nodules with two different *KCNJ5* mutations. In a similar study, Scholl and coworkers observed that APA with *KCNJ5* mutations had larger clear cells with numerous lipid droplets and abundant microvascular cytoplasm (zona fasciculata-like cells) (Scholl et al. 2015a). Additionally, they identified that *KCNJ5* mutations are more common in patients with uninodular rather than multinodular adrenal disease.

Several groups performed genome-wide expression array analysis to explore the expression pattern in sporadic APAs with and without *KCNJ5* mutations (Boukroun et al. 2012, Azizan et al. 2012b, Fernandes-Rosa et al. 2014, Monticone et al. 2015b). The mRNA expression of *CYP17A1* (marker of zona fasciculata) was enhanced in *KCNJ5*-mutated tumors (Azizan et al. 2012b). Adenomas harboring *KCNJ5* mutations showed a higher percentage of zona fasciculata-like cells, whereas wild-type *KCNJ5* adenomas had a higher percentage of zona glomerulosa-like cells. However, Fernandes-Rosa and coworkers could not confirm any correlation between the *KCNJ5* mutation status and the cellular morphology. They showed that 72% of APAs in their cohort were composed of >50% of zona fasciculata-like cells. This divergent result is probably due to a different selection of the APA at this center. No obvious gene expression clustering pattern could be identified that differentiated APAs with and without *KCNJ5* mutations (Boukroun et al. 2012).

KCNJ5 mutations are present in approximately 30–65% of APA patients, with a higher prevalence in the Asian population and among females (~70%) (Table 2) (Taguchi et al. 2012, Williams et al. 2015). Among *KCNJ5* mutations, G151R and L168R are found in approximately 90–99% of cases, whereas other mutations are rare (Choi et al. 2011, Fernandes-Rosa et al. 2014). A summary of the reported *KCNJ5* mutations is shown in Table 3.

ATPases

ATP1A1

ATP1A1 encodes for the $\alpha 1$ subunit of Na⁺/K⁺ ATPase, which is a membrane-bound ion transporter of P-type ATPase.

Table 2 Clinical and biochemical data of patients with APAs.

Clinical parameter	Gene mutated (frequency)				
	<i>KCNJ5</i> 35–50%	<i>ATP1A1</i> 5.2–8.2%	<i>ATP2B3</i> 0.9–1.7%	<i>CACNA1D</i> 11%	<i>CTNNB1</i> (unknown)
Gender predominance (frequency)	Females (60–74%)	Males (75–89%)	Females (60–70%)	Males (67%)	Majority female
Age at diagnosis (years)	35–50	34–71	33–60	35–48	24–65
Preoperative plasma aldosterone (pmol/L)	411–1200	373–6963	300–9567	520–1012	672–2590
Preoperative plasma renin concentration (ng/L)	1–4.2	1.3–5.2	1.0–8.7	1.2–4.7	<2.0
Adenoma size (mm)	12–25	10–25	12–25	8–12	9–30
Preoperative SBP (mmHg)	140–170	138–180	150–167	138–164	130–150
Preoperative DBP (mmHg)	93–110	84–110	90–111	85–95	85–95
K ⁺ concentration (mmol/L)	2.1–3.6	2.5–3.5	1.7–3.3	2.8–3.3	2.9–4.1

APA, aldosterone-producing adenoma; DBP, diastolic blood pressure; SBP, systolic blood pressure.

It is located on chromosome 1p13.1. It has 23 exons and 11 splice variants. In general, Na⁺/K⁺ ATPases consist of α - and β -subunits. The α 1 subunit is the most abundant and the major form found in the kidney and epithelial cells. Na⁺/K⁺ ATPases are expressed throughout the adrenal cortex; the highest mRNA expression is in the zona glomerulosa. The Na⁺/K⁺ and ATP binding sites are located in the α -subunit, whereas the β -subunits are responsible for

directing the α -subunit to the plasma membrane (Einholm et al. 2007). Na⁺/K⁺ ATPases transport three Na⁺ ions in exchange for two K⁺ ions, and this process is driven by the hydrolysis of ATP. The exchange of ions generates an electrochemical gradient across the membrane that facilitates the cellular uptake of ions. There are at least four isoforms of the α -subunit identified. The α 1 subunit is a 110kDa protein with more than 1000 amino acid residues. It has 10 transmembrane (TM) domains and a large cytoplasmic domain; K⁺ ion binding sites are present between the helices of TM4, TM5 and TM6 (Morth et al. 2007). L104 and V332 interact with E334 and cooperate in K⁺ binding and gating of the K⁺ sites (Einholm et al. 2007).

By exome sequencing, Beuschlein and coworkers identified two somatic substitutions at p.L104R and p.V332G and one deletion at p.F100_L104 in *ATP1A1* in 6.8% of the patients with APAs (16/328) (Fig. 3) (Beuschlein et al. 2013). The reported mutations are at highly conserved amino acid residues present in all ATPases. Mutations at L104 and V332 may abolish E334's ability to regulate K⁺ ion influx. The mutant protein displayed severely impaired ATPase activity, which indicates decreased Na⁺ and K⁺ binding. The mutant also showed considerably higher levels of membrane depolarization when expressed by an adrenal cell line, H295R. Membrane depolarization leads to opening of a voltage-gated ion channel, increased intracellular calcium, and subsequently enhanced production of aldosterone (Beuschlein et al. 2013).

Azizan and coworkers sequenced DNA from 10 APAs, which consisted of zone glomerulosa-like cells (Azizan et al. 2013). They identified mutations at p.L104R and p.F100_L104del, which supports the critical function

Table 3 Somatic and germline *KCNJ5* mutations in patients with APAs.

Coding alteration	Altered amino acid	Mutation type
c.155G>A	p.R52H	Germline (rare polymorphism)
c.376T>C	p.W126R	Somatic
c.414-425dupCGCTTCTCTGTT	p.A139_F142dup	Somatic
c.433G>C	p.E145Q	Somatic/germline
c.433_434CCATTG	p.I144_E144insAl	Somatic
c.451G>A/C	p.G151R	Somatic/germline
c.451insATG	p.I150_G151insM	Somatic
c.452G>A	p.G151E	Germline
c.455A>G	p.Y152C	Germline
c.461T>G	p.F154C	Somatic
c.469_471delATC	p.I157del	Somatic
c.470T>G	p.I157S	Germline
c.470_471delinsAA	p.I157K	Somatic
c.472A>G	p.T158A	Somatic/germline
c.503T>G	p.L168R	Somatic
c.730G>A	p.E246K	Somatic
c.739G>A	p.G247R	Germline (rare polymorphism)
c.844C>G	p.E282Q	Germline (polymorphism, 2%)

Accession number: NM_000890.3; APA, aldosterone-producing adenoma.

Figure 3
Illustrating the structure of Na⁺/K⁺ ATPase subunit α1. Red circles indicate the mutations found in *ATP1A1* in sporadic aldosterone-producing adenomas. All mutations are located in the second and fourth transmembrane domain of *ATP1A1*.

of L104. In addition, they found an in-frame deletion of residues 960–963 and a substitution of 963 by a serine (p.EETA963S). This mutation includes E961 (TM9) that has been identified in forming a third Na⁺-specific binding site (Morth *et al.* 2007). When mutant p.EETA963S was expressed in *Xenopus* oocytes, the mutant ATPase still transports K⁺ ions. However, mutant cells showed an enhanced inward current under normal physiological conditions. Williams and coworkers found a novel mutation at p.G99R that was associated with a severe phenotype with hypokalemia (Williams *et al.* 2014). G99 is in the transmembrane domain (TM1) and interacts with I292 and E334 in K⁺ binding site I. Substitution of glycine for the large positive charge of arginine resulted in significantly increased expression of *CYP11B2* and its transcription factor, *NR4A2/Nur77* (Williams *et al.* 2014).

In a recent study, Stindl and coworkers expressed the mutants G99R, L104R and V332G in NCI H295R and looked at the effect on aldosterone production (Stindl *et al.* 2015). Among all the mutants, L104R showed the highest depolarization of the cell membrane in NCI H295R cells. However, expression of any of the mutants leads to an increase in intracellular Na⁺ and to a decrease in K⁺ ions. Interestingly, L104R and V332G mutants resulted in leakage of H⁺ and acidification of adrenal cells. Of note,

an increase in extracellular K⁺ resulted in an increase in pH in the mutant-expressing cells. In a subsequent study, acidification of control cells with acetate was found to increase *CYP11B2* mRNA and aldosterone levels.

The clinical and biochemical data of patients with *ATP1A1* mutations is shown in Table 2 and the *ATP1A1* mutations are summarized in Table 4. Most patients have the following mutations: p.L104R, p.V332G, deletion at p.F100_L104 and p. F959_E961. Mutations are present in 5–8% of patients with APAs and are more prevalent in males than in females.

ATP2B3

ATP2B3 encodes the plasma membrane calcium transporter, ATPase 3 (PMCA3), which belongs to the superfamily of P-type transporters. It is highly expressed in the adrenal cortex. *ATP2B3* is a large gene of 20 exons located on chromosome Xq28. The Ca²⁺ATPases are conserved and have a single polypeptide chain organized into 10 transmembrane (TM) domains and 4 cytoplasmic loops. The cytoplasmic domains contain an actuator domain A, a phosphorylation domain P and a nucleotide binding domain N. The loop between TM4 and TM5 contains the phosphorylation and ATP binding sites. There are two calcium ion binding sites

Table 4 Somatic mutations in genes coding for ATPases in patients with APAs.

Gene	Coding alteration	Altered amino acid	Mutation type
<i>ATP1A1</i> ^a	c.295G>A	p.G99R	Somatic
	c.299_313del TCTCAATGTTACTGT	p.F100L	Somatic
	c.311T>G	p.L104R	Somatic
	c.995T>G	p.V332G	Somatic
	c. 2879AAGAGACAGdel	p.960-963del	Somatic
	&c.2887G>T	p.A963S	Somatic
<i>ATP2B3</i> ^b	c.1270_1275delGTGCTG/or	p.V424_L425	Somatic
	c.1269_1274delTGTGCT		
	c.1272_1277delGCTGGT or	p.L425_V426del	Somatic
	c.1273_1278delCTGGTC		
	c.1277_1282delTCGTGG	p.V426_V427	Somatic

Accession numbers: ^aNM_001160233.1; ^bNM_001001344.2; APA, aldosterone-producing adenoma.

Figure 4

Illustrating the structure of the Ca^{2+} ATPase 3 subunit. Red circles indicate the mutations found in *ATP2B3* in sporadic aldosterone-producing adenomas. All mutations are located in the fourth transmembrane domain of *ATP2B3*. This is the Ca^{2+} binding site.

(I and II). Site I is located in the space between TM4 and TM5; TM8 also contributes to this binding. Site II is formed on TM4. Ca^{2+} ATPases adopt an autoinhibitory state under normal physiological cytoplasmic Ca^{2+} concentrations, but ion transportation may be activated by binding of calmodulin to the calmodulin binding site. Ca^{2+} ATPases are characterized by the formation of a high-energy phosphorylated intermediate during the exchange of one Ca^{2+} ion for one H^{+} ion. This exchange is at the expense of one ATP. Germline mutations in *ATP2B3* have previously been reported in X-linked congenital cerebellar ataxia and Laminin syndromes (Baig et al. 2011, Cali et al. 2015).

Deletion mutations of *ATP2B3* were reported together with *ATP1A1* (Beuschlein et al. 2013) at p.L425_V426del and p.V426_V427del, which are located in TM4 (Fig. 4). The amino acids L425, V426 and V427 interact with glutamic acid at 462 and are crucial for calcium binding. The mutations potentially lead to the distortion of both Ca^{2+} binding regions. Functional *ex vivo* studies have shown substantial depolarization in the mutated samples, indicating a selective advantage for these tumor cells.

To date, all mutations identified are in-frame deletions and located between the amino acids L424 and V429 (Table 4). *ATP2B3* mutations have a low frequency (about 1–1.5%) in APAs, are more prevalent in females, and are associated with a more severe form of APA (Table 2).

Calcium channels

CACNA1D

CACNA1D encodes the α_1 subunit of L-type voltage calcium channel, $\text{Ca}_v1.3$, and is located on chromosome 3p14.3. *CACNA1D* is a large gene with 49 exons and belongs to the Ca_v1 family of proteins, which are activated by

high-voltage depolarization and slow inactivation (Lipscombe et al. 2004). These L-type channels are expressed by endocrine tumors and are sensitive to selective inhibition by dihydropyridine (Catterall 2011). Ca_v1 channels are formed by five subunits, α_1 , α_2 , β , γ and δ . The α_1 subunit is the principle transmembrane and pore-forming subunit of the $\text{Ca}_v1.3$ channel (Catterall 2011). *CACNA1D* encodes a protein of more than 2100 amino acids that are arranged in four homologous repeats (I–IV); each repeat contains six transmembrane segments (S1–S6) and a membrane-associated loop between the transmembrane segments S5 and S6. The S4 segment of each homologous domain serves as the voltage sensor for activation. It moves outward and rotates under the influence of an electric field, which initiates a conformational change that opens the pore. The S5 and S6 segments and the membrane-associated pore loop between them form the pore lining of the channel. The narrow external end of the pore is lined by the pore loop, which contains a pair of glutamate (E) residues in each domain. These glutamate residues are required for Ca^{2+} selectivity that is unique to a Ca^{2+} channel (Heinemann et al. 1992).

Exome sequencing of APAs identified somatic and *de novo* germline mutations in the *CACNA1D* gene (Scholl et al. 2013). The identified somatic mutations were at p.G403R and p.I770M, which are located on the S5 and S6 segments of domain I and II, respectively (Fig. 5 and Table 5). The identified mutations are at conserved sites in orthologs that range from invertebrates to humans. Segments S5 and S6 are important for pore formation and gating of the channel. Electrophysiological experiments revealed that the mutant channels open early (lower potential) and have a sustained activation. Early activation at a lower depolarization leads to an increase in Ca^{2+} entry, which is associated with enhanced aldosterone production (Scholl et al. 2013).

Scholl and coworkers further sequenced the *CACNA1D* S6 segment of domain I and II in 100 patients

Figure 5

Showing the structure of the alpha subunit of $\text{Ca}_v1.3$ channel. Red and black circles indicate the mutations found in *CACNA1D* and *CACNA1H*, respectively, in sporadic aldosterone-producing adenomas. *CACNA1D* mutations are distributed throughout the protein.

that displayed an early onset of PA. The identified germline mutations were *de novo* at position p.G403R and p.I770M, and these patients had no family history of hypertension or seizure (Scholl et al. 2013). One case had a mutation at p.G403R and suffered from high blood pressure since birth. Further studies of this individual identified a significantly higher aldosterone-to-renin ratio and this patient also had hypokalemia. In addition, this patient had biventricular hypertrophy, a ventricular septal defect, pulmonary hypertension and second-degree heart blockage. Treatment with a calcium blocker normalized the hypertension as well as the biventricular hypertrophy. The second case carried a p.I770M mutation and was diagnosed with hypertension at age 5. At birth, the patient was diagnosed with cerebral palsy, spastic quadriplegia, mild athetosis and seizures. Treatment with clonidine and spironolactone normalized the patient's blood pressure. So far, no other group has reported germline mutations in *CACNA1D*.

At the same time, Azizan and coworkers also reported somatic mutations in *CACNA1D* in zona glomerulosa-like

APAs (Azizan et al. 2013). These mutations are scattered throughout the entire gene, although they always affect conserved amino acids or regions. Functional studies also showed that the channels were activated at lower membrane potentials. *CACNA1D* insertional germline mutations at p.Gly403_404 have previously been reported for sinoatrial node dysfunction and deafness syndrome (Baig et al. 2011, Pinggera et al. 2015). Recently, mutations in *CACNA1D* were also reported in patients with sporadic autism and intellectual disability at p.G403R (8A) and p.A749G (Baig et al. 2011, Pinggera et al. 2015). Notably, the reported mutation, p.G403, is also frequent in APAs. The mutation is located in the transmembrane S6 segment of repeat I and II, which highlights the importance of this region for proper channel function (Baig et al. 2011, Scholl et al. 2013).

During the last few years, the concept of APCCs (aldosterone-producing cell clusters) has given hope for finding the precursor cells of APAs. The development of specific antibodies against aldosterone synthase (AS; *CYP11B2*) and 11β -hydroxylase (*CYP11B1*) has allowed for the detection of cells that do and do not produce AS in APAs. Thus, *CYP11B2*- and *CYP11B1*-expressing cells can be determined. Studies with these antibodies reveal that the normal adrenal cortex consists of aldosterone-producing cell clusters (APCCs) (Nishimoto et al. 2010). In subsequent studies, Nishimoto and coworkers sequenced the captured APCC for known genes and identified mutations in *ATP1A1* and *CACNA1D* (Nishimoto et al. 2015). When they compared the mRNA expression of *CYP11B2* in APCCs with different layers of the adrenal cortex, the mRNA expression in ZG (zona glomerulosa) cells was similar to that in APCCs. However, upon histological examination, the majority of samples showed a mixture of ZG- and ZF (zona fasciculata)-like cells.

More recently, Fernandes-Rosa and coworkers screened for mutations in functional secondary nodules of 27 patients with multinodular adrenal disease. They found two cases with somatic *CACNA1D* mutations in one nodule that also contained the somatic *KCNJ5* mutation p.G151R in the secondary nodule (Fernandes-Rosa et al. 2015). In addition, a mutation in *KCNJ5* was identified

Table 5 Germline and somatic *CACNA1D* and *CACNA1H* mutations in patients with APAs.

Gene	Coding alteration	Altered amino acid	Mutation type
<i>CACNA1D</i>	c.1201G>T	p.V401I ^a	Somatic
	c.1207G>C	p.G403R	Somatic/ <i>de novo</i> germline
	c.1955C>T	p.S652L	Somatic
	c.1964T>C	p.L655P	Somatic
	c.2222A>G	p.Y741C	Somatic
	c.2239T>C	p.F747L	Somatic/ <i>de novo</i> germline
	c.2936T>A	p.V979N	Somatic
	c.2992_2993GC>AT	p.A998I	Somatic
	c.2993C>T	p.A998V	Somatic
	c.3451G>T	p.V1151F	Somatic
	c.3455T>A	p.I1152N	Somatic
	c.4012G>A	p.V1338M	Somatic
	c.4057G>A	p.V1353M ^a	Somatic
	<i>CACNA1H</i> ^b c.4645A>G	p.M1549V	Germline/ <i>de novo</i> germline

Accession numbers: NM_000720.3, ^aNM_001128840.2, ^bNM_021098; APA, aldosterone-producing adenoma.

in one of the secondary nodules at p.G151R without any other mutation in the known APA susceptibility genes.

CACNA1D mutations are predominantly found among males and are present in 3–11% of patients with APAs (Table 2). Unlike *KCNJ5* mutants, *CACNA1D* mutants contain a mixture of zona glomerulosa and zona fasciculata cells in the adrenal adenomas (Akerstrom et al. 2015, Scholl et al. 2015a).

CACNA1H

CACNA1H encodes the α_1 subunit of the T-type voltage calcium channel, $\text{Ca}_v3.2$. The gene is situated on chromosome 16p13.3 and contains 35 exons that encode 2300 amino acids. $\text{Ca}_v3.2$ channels are activated at higher negative membrane potentials and display voltage-dependent inactivation. The α_1 subunit is the principle transmembrane portion and the pore-forming subunit of $\text{Ca}_v3.2$ channels. Expression of T-type channels in endocrine cells has been reported previously (Catterall 2011). The structure of *CACNA1H* is similar to *CACNA1D*, which is described in the *CACNA1D* section (Fig. 5).

Scholl and coworkers identified mutations in *CACNA1H* by exome sequencing of samples from 40 unrelated early-onset cases of hypertension (Scholl et al. 2015b). One hot spot mutation was identified at p.M1549V in the *CACNA1H* gene in five cases (Table 5). In three cases, the variant was inherited from a parent, whereas one case had a *de novo* germline mutation. In the last case, the mother had a *de novo* germline mutation, which was inherited by her daughter. The author further sequenced *CACNA1H* from an additional 1632 samples obtained from patients diagnosed with PA after the age of 10. They could not identify any mutation, which suggests that this mutation is specific for the early onset of hypertension (Table 2).

Methionine at 1549 is in a methionine–phenylalanine–valine (MFV) motif, which is conserved in orthologs from invertebrates to humans. In a previous study, substitution of methionine was found to delay the inactivation of the channel (Marksteiner et al. 2001). When the mutant was expressed in HEK293T cells, whole-cell patch clamp recording showed slow activation and a 10-fold delayed inactivation. Slower inactivation leads to prolonged opening of the channel, which results in higher Ca^{2+} influx.

It is currently unknown whether germline mutations in *CACNA1D* and *CACNA1H* represent a new type of familial hyperaldosteronism. In particular, mutations in *CACNA1H* are specific for early onset of hypertension.

Armadillo (ARM)-repeat-containing proteins

CTNNB1 (β -catenin)

Wnt signaling is a well-known signal transduction pathway that regulates aspects of embryonic development, solid cancers and stem cells (Anastas & Moon 2013). Wnt signaling has been divided into two pathways: a β -catenin-dependent (canonical) and a β -catenin-independent (noncanonical) pathway. The best understood Wnt signaling transduction cascade is the Wnt/ β -catenin pathway. This is a receptor-mediated signaling pathway where the Frizzled/LRP6 receptor is the target of 19 highly conserved Wnt glycoprotein ligands (Morin et al. 1997, Angers & Moon 2009). Until now, the Frizzled family of G protein-coupled receptors (GPCRs), as well as the receptor tyrosine kinases (RTKs), ROR1 and ROR2, and the RTK-like protein RYK were identified as receptors involved in Wnt signaling (Angers & Moon 2009). Upon binding of a Wnt ligand, the Frizzled/LRP6 receptor activates Dishevelled cytoplasmic phosphoproteins, which inhibits phosphorylation of β -catenin (Fig. 6). β -Catenin then accumulates in the

Figure 6

The Wnt/ β -catenin signaling pathway is involved in the formation of sporadic APA. Wnt ligands bind to frizzled (Fz) and its coreceptor, LRP5/6, to inhibit the degradation of β -catenin. The stabilized β -catenin translocates into the nucleus, which results in enhanced expression of the transcription factor TCF/LEF1 and ultimately leads to the expression of target genes. In aldosterone-producing adenomas, TCF/LEF1 binds to NURR1/NUR77, which is a transcription factor of *CYP11B2*.

Figure 7

Structure of β -catenin. Glycogen synthase kinase 3 (GSK3) phosphorylate the serines (colored) in the GSK3 β binding domain of β -catenin. The mutations are localized to the phosphorylation sites and indicated with red circles.

nucleus, binds to lymphoid enhancer-binding factor (LEF) and T cell factor (TCF) proteins and acts as a transcriptional coactivator to modulate the expression of target genes. In the absence of Wnt ligands, β -catenin becomes phosphorylated by a degradation complex in the cytoplasm, which leads to its ubiquitin-dependent degradation (Fig. 6).

CTNNB1 consists of 15 exons, encodes for β -catenin, and is located on chromosome 3p22.1. Gain of function mutations in *CTNNB1* have been identified in several different forms of cancer (e.g., colorectal cancer and ovarian cancer) (Kim et al. 2008). β -catenin signaling is essential for the development of the adrenal cortex, especially for adrenal glomerulosa. *CTNNB1* is also frequently mutated in benign and malignant adrenocortical tumors that are not related to PA (Tissier et al. 2005).

CTNNB1 mutations (Fig. 7 and Table 6) have also been found in APAs (Azizan et al. 2013, Scholl et al. 2013, Shaikh et al. 2015), although the frequency of *CTNNB1* mutations in APAs is very low (Table 2). Of note, the

majority of *CTNNB1* mutations are reported in female patients (Tissier et al. 2005, Azizan et al. 2013, Scholl et al. 2013, Teo et al. 2015, Scholl et al. 2015a). The mutations result in the loss of phosphorylation sites, which prevents ubiquitination by the E3 ligase, TrCP1 and further proteasomal degradation of β -catenin. Expression of β -catenin is found in the nuclear or cytoplasmic compartments in 70% of APAs (Berthon et al. 2014). It appears to regulate the transcription of *NURR1* and *NUR77* (via its binding partners, LEF/TCF), which, in turn, is involved in the regulation of the aldosterone synthesis rate-limiting enzyme, *CYP11B2* (Bassett et al. 2004, Berthon et al. 2014).

Berthon and coworkers observed that the Wnt/ β -catenin pathway is active in the adrenal cortex of transgenic mice, in which exon 3 of *CTNNB1* was deleted (Berthon et al. 2010). Hyperproliferation in the zona glomerulosa was observed in 10-month-old mice, which resulted in primary hyperaldosteronism. β -Catenin activation resulted in the malignant characteristics of

Table 6 Mutations in genes coding for armadillo (ARM)-repeat-containing proteins in patients with APAs.

Gene	Coding alteration	Altered amino acid	Mutation type
<i>CTNNB1</i> ^a	c.98C>G	p.S33C	Somatic
	c.100C>A	p.G34R	Somatic
	c.134C>T	p.S45F	Somatic
	C1276_1298insGACA_DelTCGTGGCTGTCCCAGAGGGCCT	p.V426G_V427Q_A428_L433del	Somatic
	c.1641G>A	p.A547	Germline
	c.133T>C	p.S45P	Somatic
<i>ARMC5</i> ^b	c.41T>A	p.F14Y	Germline
	c.466C>T	p.L156F	Germline
	c.438G>A	p.R146	Germline
	c.508A>G	p.I170V	Germline
	c.968G>C	p.G323A	Germline
	c.1505G>A	p.R502H	Germline
	c.1520C>T	p.P507L	Germline
	c.1928C>T	p.T643M	Germline
	c.2476C>A	p.P826H	Germline
	c.2692C>T	p.R898W	Germline

Splice site mutation are not included in the table. Accession numbers: ^aNM_001904.3, ^bNM_024742.2; APA, aldosterone-producing adenoma.

these transgenic mice over a 17-month period of time. Of note, female mice showed an aggressive progression in tumorigenesis (Berthon et al. 2010).

Recently, three different *CTNNB1* mutations were identified in case studies of two pregnant and one postmenopausal woman (Teo et al. 2015). The identified mutations were at the target phosphorylation sites, p.S33C, p.S45F and p.G34C (Teo et al. 2015). Increased expression of luteinizing hormone-chorionic gonadotropin receptor (*LH-CGR*) and gonadotropin-releasing hormone receptor (*GNRHR*) was identified in the adenomas. When the mutant *CTNNB1* was expressed in an adrenal cell line, H295R, the expression of *GATA4*, a critical transcription factor during embryogenesis, was increased, which suggests that mutant *CTNNB1* activates precursor cells in adrenal cortex.

ARMC5

The gene encoding for armadillo-repeat-containing 5 (*ARMC5*) is located on chromosome 16p11.2 and consists of 8 exons encoding for a protein of 1030 amino acids. *ARMC5* belongs to the large family of armadillo (ARM)-repeat-containing proteins (ACPs). ACPs contain a common tandem repeat of 42 amino acids (Berthon & Stratakis 2014). The three-dimensional structure of these proteins is highly conserved among ACPs; however, the structure of *ARMC5* has yet to be determined. The best-known member of this family is β -catenin. Unlike β -catenin, the function of *ARMC5* is still unknown, but the presence of tandem ARM-repeats suggests involvement in protein–protein interactions. Germline and somatic *ARMC5* mutations have been reported earlier in adrenals from patients with Cushing syndrome (Assie et al. 2013).

Zilbermint and coworkers identified 12 different germline mutations in *ARMC5* using samples from patients with APA (Fig. 8 and Table 6) (Zilbermint et al. 2015). The mutations were present in 39.3% (22/56) of the patients and distributed throughout the gene. All cases with predicted damaging mutations were of African-American origin. Nine mutations were germline missense mutations and three of them were germline splice site mutations. *ARMC5* mutations were complementary to the *KCNJ5* mutation,

indicating a driving gene rather than a passenger gene. The expression of *ARMC5* was downregulated in the mutated cells when compared with nonmutated APAs. Developmental abnormalities of the adrenal were found in mice and zebrafish harboring a loss-of-function mutation in *ARMC5*, which supports *ARMC5*'s role as a tumor-suppressor gene (Assie et al. 2013, Faucz et al. 2014a,b). Furthermore, overexpression of mutant *ARMC5* was found to downregulate *CYP11B2* expression. Recently, Mulatero and coworkers also reported *ARMC5* mutations in Caucasian patients (Mulatero et al. 2016). They identified 18 variants of *ARMC5*. However, the observed mutations were not associated with an altered protein function by *in silico* analysis. More studies are needed to clarify the role of *ARMC5* in PA. It also appears that germline mutations of *ARMC5* represent a new type of familial hyperaldosteronism.

SLC26A2

SLC26A2, located on chromosome 5q32, has at least three exons that encode the solute carrier 26 A2 transporter, a membrane protein of 739 amino acids that acts as a $\text{SO}_4^{2-}/\text{Cl}^-$ exchanger (Ohana et al. 2009). The SO_4^{2-} ions transported by *SLC26A2* are important for the development of cartilage, and mutations in this gene have been associated with diastrophic dysplasia, achondrogenesis 1B, atelosteogenesis 2 (AO2) and multiple epiphyseal dysplasia 4 (EDM4) diseases.

Spyroglou and coworkers performed a genome-wide association study using a large population of patients with PA and controls (Spyroglou et al. 2014). It revealed an association with chromosome 5q32. The relevant gene present in this region was *SLC26A2*, which is downregulated in sporadic APAs. However, no mutation has been found in *SLC26A2*. Still, treatment with angiotensin II and induction of hypokalemia were found to downregulate *SLC26A2* expression in mice. However, no morphological adrenal changes or adrenal tumors were observed in *SLC26A2* knockin mice, but more aldosterone was produced. This study also addresses the possibility of epigenetic regulation, which could subsequently lead to the formation of sporadic APAs as well as increased aldosterone production.

Figure 8

Structure of *ARMC5*. The red circles indicate mutations found in *ARMC5* of patients with APA. Mutations are distributed throughout the protein.

The origin of APAs

Mutations found in ion channel and transporter-related genes have clearly explained the underpinning cause of ectopic production of aldosterone in the majority of patients with PA (Fig. 9). However, tumor formation in the adrenal cortex is still unexplained and the cellular origin of sporadic APAs is controversial. Recent findings showed that mutational target genes determine the cellular composition of APAs. Upon histological examination, tumors harboring *KCNJ5* mutations have a ZF-like cellular morphology (Azizan et al. 2012b). However, these tumors also express the biomarker of zona glomerulosa cells, disabled-2 (Dab-2). *CACNA1D* and ATPase mutants phenotypically resemble zona glomerulosa cells (Azizan et al. 2012b, Akerstrom et al. 2015). In contrast to previous results, Fernandes-Rosa and coworkers found that the majority of APAs were composed of heterogeneous cells and had a high percentage of ZF-like cells (Fernandes-Rosa et al. 2014). In a similar study, Scholl and coworkers observed that tumors with *KCNJ5* mutations phenotypically resemble ZF, whereas other mutations result in tumors with a more heterogeneous phenotype (Scholl et al. 2015a).

It has been debated whether adrenal cortex remodeling itself can lead to the development of APAs and whether somatic mutations in different genes are secondary, independent events. In recent studies, Dekkers and coworkers as well as Fernandes-Rosa and coworkers reported two different mutations in two

different functional nodules of the same adrenal gland, which supports the concept of remodeling (Dekkers et al. 2014, Fernandes-Rosa et al. 2015). However, the underlying genetic mechanisms that lead to tumor formation are still unknown. Potential mechanisms could include the sonic hedgehog and/or the Wnt/ β -catenin signaling pathways (Boukroun et al. 2011). Expression of both pathways has been found in a subset of APAs. Further, it is possible that β -catenin plays a role in gender-specific tumor formation in the adrenal cortex because the majority of somatic mutations in *CTNNB1* are found in females with sporadic APAs (Tissier et al. 2005, Azizan et al. 2013, Teo et al. 2015, Scholl et al. 2015a, Akerstrom et al. 2016).

Several mouse models have been developed to study factors that contribute to the gender differences of APAs. Female *TASK1*^{-/-} mice have a more severe phenotype than the corresponding males (Heitzmann et al. 2008). Female *TASK1*^{-/-} mice also have a compromised adrenal cortex zonation after puberty, and a higher expression of *CYP11B2* was observed in the zone fasciculata. When female *TASK1*^{-/-} mice were treated with the male sex hormone, testosterone, a regression to normal aldosterone synthase expression was observed. Estrogen did not affect aldosterone production in either female or male knock-out mice. Furthermore, female *SLC26A2* knockin mice have been found to produce higher levels of aldosterone (Spyroglou et al. 2014). Nevertheless, adrenal tumors have never been found in any of the mouse models.

Figure 9

Pathways and channels involved in the biosynthesis of aldosterone. Under physiological conditions, binding of angiotensin II on angiotensin receptor type I (AT1R) activates the voltage-gated Ca^{2+} channels, which results into influx of Ca^{2+} ions. AT1R also elicit inositol 1,4,5-trisphosphate receptors (IP3Rs) to release Ca^{2+} release from the endoplasmic reticulum. Under pathological conditions, genes coding for Na^+/K^+ -ATPase, the potassium channels *KCNJ5* or the Ca^{2+} ATPase and Ca^{2+} channel *CACNA1D* are mutated, leading to membrane depolarization and influx of calcium through voltage-gated calcium channel. Mutations in *ATP2B3* affect the recycling of Ca^{2+} . However, mutations in L-type Ca^{2+} channel *CACNA1D* lead to early opening at lower potential and a sustained activation of the channel, which increases the calcium influx. An increase in the intracellular calcium concentration is found to trigger the production of aldosterone.

While critically analyzing data from previous publications concerning the age of patients and size of tumors between males and females, irrespective of mutational status (Choi *et al.* 2011, Dutta *et al.* 2014, Akerstrom *et al.* 2015, Scholl *et al.* 2015a), it became obvious that the majority of females were younger and had not entered menopause (<50 years ($N=56$) vs >50 years ($N=26$), $P=0.0001$). Although there was no size difference in the tumors before or after menopause, the aldosterone-to-renin ratio was higher before initiation of menopause (289.5 ± 44.2 ($N=56$) vs 204.1 ± 42.7 ($N=26$), $P=0.26$ (mean $\pm$ s.e.m)). On the contrary, males with APAs were older (>50 years ($N=70$) vs <50 years ($N=40$)). In addition, the size of the tumors was significantly smaller in males than in females (1.55 ± 0.098 cm ($N=110$) vs 1.95 ± 0.12 cm ($N=82$), $P=0.0079$). It is possible that sex hormones influence the development of adrenal tumors. Previous studies have shown that the androgen and estrogen receptors regulate the L-type voltage calcium channel, $Ca_v1.3$, which is mutated in cases of sporadic APAs (Chen *et al.* 2014, Hao *et al.* 2015). We hypothesize that there is a gender-specific origin of APAs for several reasons. First, there is a gender bias among several of the mutations, and the majority of β -catenin mutations are found in females. Further, gender-specific differences are also observed in level of β -catenin activation and several mouse models. In females, APAs originate from the zona fasciculata, due to the activity of β -catenin, estrogen or unidentified pathways/genes. Moreover, mutations in ion channels and transporters activate aldosterone synthase. This results in morphological changes and zona glomerulosa-like phenotypes due to more metabolic activity. Another interesting observation is that β -catenin is frequently mutated in females with Cushing syndrome (Tissier *et al.* 2005, Goh *et al.* 2014). It is possible that tumors originate from zona fasciculate cells, and subsequent mutations in different genes cause the overproduction of different steroids. In male patients, the tumor originates from zona glomerulosa cells. There is not enough data to perform statistical analyses, but the available reports showed that *ATP1A1* and *CACNA1D* mutations were primarily found in males with smaller adenomas and higher aldosterone levels.

In summary, the cellular origin of sporadic APAs is still unclear. However, we propose a theory that the origin and regulation of sporadic APAs is gender specific. Clinical data, β -catenin expression and activation, tumor cell morphology and the expression of zona glomerulosa and zona fasciculata cell markers should all be examined in future studies. A comparison of males and females

using large cohorts will increase our understanding of the cellular origin of APAs.

Clinical consequences

What is the clinical implication of all this knowledge? One clinical consequence might be more obvious in those cases arising from germline mutations. Here, family members harboring one of the mutations can be screened for the development of hypertension, leading to earlier diagnosis and treatment. Future research will tell us more about the prevalence of hypertension due to an APA in the presence of a specific germline mutation.

One important clinical issue is determining the subtype of PA in order to administer the appropriate treatment. Until now, AVS has been used as the gold standard for subtype's differentiation (APA/BAH). It requires a skilled radiologist. Recently, genotype-specific steroid profiles were found in the plasma of APA patients (Williams *et al.* 2016). This finding may be used in clinical practice to select PA patients that need to undergo AVS. The development of noninvasive imaging techniques could further improve the evaluation of patients with PA.

The question is whether specific nonsurgical treatments that take the underlying mutation into consideration can be developed. In the case of activating mutations, drugs blocking this activity are theoretically possible. In the case of inactivating mutations, the development of specific drugs may be more difficult. Because the mutations affect membrane-bound proteins, the development of specific antibodies would theoretically be possible. In order to create a definitive treatment, these antibodies could be conjugated to a radioactive substance that would kill cells recognized by the antibodies. Obviously, these antibodies would have to be very specific. Severe side effects of these potential nonsurgical treatments would not be acceptable, because the side effects of surgery are generally low and the underlying disease is benign.

In hereditary cases, the underlying mutation can be screened for by analyzing constitutive DNA. In sporadic cases, however, it is much more difficult to identify the mutation status. As shown in Table 2, it seems unlikely that one can predict the genotype by looking at the phenotype, even though some mutations seem to be associated with tumor size and a patient's age. Until we find the specific marker for each genotype, one has to get some tumor tissue from each adrenal nodule in order to be able to identify the specific mutation present in

that nodule. In theory, imaging techniques specific for a certain mutation would also be imaginable, but these techniques currently do not exist.

In summary, our knowledge of the underlying genetic mechanism leading to APAs is increasing. The development of specific therapies awaits further research.

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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