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.

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).

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 adrenocorticotropin hormone (ACTH) also stimulates the secretion of aldosterone from adrenals (Gallo-Payet 2016). In healthy adults, the adrenal cortex produces 20–200µg 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,
Genetics of primary hyperaldosteronism

R K Dutta et al.

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, Agloný 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 of FH-I usually consists of 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 excluded.

Genes associated with hereditary hyperaldosteronism

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

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

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

In a subsequent study, Sukor 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 βγ-subunits (Gβγ) from inactive heterotrimERIC G protein complexes (Gαi). Finally, the Gβγ 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⁺ selectivity filter region of the potassium channel.

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 by hypokalemia. Dexamethasone suppression tests unexpectedly increased both serum aldosterone and potassium levels and hybrid steroids (18-hydroxycortisol and 18-oxocortisol). 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, Multero 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

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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 (Boulkroun 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 adrenal glands 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 uniodal 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 (Boulkroun 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 (Boulkroun 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 α1 subunit of Na⁺/K⁺-ATPase, which is a membrane-bound ion transporter of P-type ATPase.
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 α1 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 α1 subunit is a 110 kDa 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 (16/238) (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 of Na+/K+ ATPases in aldosterone production.
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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coding alteration</th>
<th>Altered amino acid</th>
<th>Mutation type</th>
</tr>
</thead>
</table>

Accession numbers: *NM_001160233.1;* *NM_001001344.2; APA, aldosterone-producing adenoma."
(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\textsuperscript{2+}-ATPases adopt an autoinhibitory state under normal physiological cytoplasmic Ca\textsuperscript{2+} concentrations, but ion transportation may be activated by binding of calmodulin to the calmodulin binding site. Ca\textsuperscript{2+}-ATPases are characterized by the formation of a high-energy phosphorylated intermediate during the exchange of one Ca\textsuperscript{2+} ion for one H\textsuperscript{+} 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\textsuperscript{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 α1 subunit of L-type voltage calcium channel, Ca\textsubscript{α1.3}, and is located on chromosome 3p14.3. CACNA1D is a large gene with 49 exons and belongs to the Ca\textsubscript{α1} 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\textsubscript{α1} channels are formed by five subunits, α1, α2, β, γ and δ. The α1 subunit is the principle transmembrane and pore-forming subunit of the Ca\textsubscript{α1.3} 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\textsuperscript{2+} selectivity that is unique to a Ca\textsuperscript{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.1770M, 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\textsuperscript{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
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 insertion germline mutations at p.G403 (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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coding alteration</th>
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</tr>
</thead>
<tbody>
<tr>
<td>CACNA1D</td>
<td>c.1201G&gt;T</td>
<td>p.V401I</td>
<td>Somatic</td>
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<td>c.2222A&gt;G</td>
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<td></td>
<td>c.4057G&gt;A</td>
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<td>CACNA1H</td>
<td>c.4645A&gt;G</td>
<td>p.M1549V</td>
<td>Germline/de novo</td>
</tr>
</tbody>
</table>

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 KCNJS 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 α1 subunit of the T-type voltage calcium channel, Ca.3.2. The gene is situated on chromosome 16p13.3 and contains 35 exons that encode 2300 amino acids. Ca.3.2 channels are activated at higher negative membrane potentials and display voltage-dependent inactivation. The α1 subunit is the principle transmembrane portion and the pore-forming subunit of Ca.3.2 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 in 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 Disheveled cytoplasmic phosphoproteins, which inhibits phosphorylation of β-catenin (Fig. 6). β-Catenin then accumulates in the nucleus 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 CYP1B2.

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, LRPS/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 CYP1B2.
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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coding alteration</th>
<th>Altered amino acid</th>
<th>Mutation type</th>
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<td>p.G34R</td>
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<td>c.134C&gt;T</td>
<td>p.V426G_V427Q_A428_L433del</td>
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<td></td>
<td>c.1641G&gt;A</td>
<td>p.A547</td>
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<td>c.133T&gt;C</td>
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<tr>
<td></td>
<td>c.2692C&gt;T</td>
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</tr>
</tbody>
</table>

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, Fauz 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 SO$_4^{2−}$/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 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 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 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).

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 (Heitmann 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 knockout 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 Ca2+ channels, which results into influx of Ca2+ ions. AT1R also elicit inositol 1,4,5-trisphosphate receptors (IP3Rs) to release Ca2+ release from the endoplasmic reticulum. Under pathological conditions, genes coding for Na+/K+ ATPase, the potassium channels KCNJ5 or the Ca2+ ATPase and Ca2+ channel CACNA1D are mutated, leading to membrane depolarization and influx of calcium through voltage-gated calcium channel. Mutations in ATP2B3 affect the recycling of Ca2+. However, mutations in L-type Ca2+ 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 ± 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\textsubscript{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 \(\beta\)-catenin mutations are found in females. Further, gender-specific differences are also observed in level of \(\beta\)-catenin activation and several mouse models. In females, APAs originate from the zona fasciculata, due to the activity of \(\beta\)-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 \(\beta\)-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, \(\beta\)-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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Review

R K Dutta et al.

23:10

Genetics of primary hyperaldosteronism

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