Molecular basis of Primary Aldosteronism and adrenal Cushing

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Abstract

This review reports the main molecular alterations leading to development of benign cortisol and/or aldosterone secreting adrenal tumors. Causes of adrenal Cushing can be divided in two groups: multiple bilateral tumors or adenomas secreting cortisol. Bilateral causes are mainly Primary Pigmented Nodular Adrenocortical Disease (PPNAD), most of the time due to PRKAR1A germline inactivating mutations, and Primary Bilateral Macronodular Adrenal Hyperplasia (PBMAH), that can be caused in some rare syndromic cases by germline inactivating mutations of MEN1, APC and FH and of ARMC5 in isolated forms. PRKACA somatic activating mutations are the main alterations in unilateral cortisol producing adenomas (CPA). In primary hyperaldosteronism (PA), familial forms were identified in 1-5% of cases: familial hyperaldosteronism type I (FH-I) due to a chimeric CYP11B1/CYP11B2 hybrid gene, FH-II due to CLCN-2 germline mutations, FH-III due to KCNJ5 germline mutations, FH-IV due to CACNA1H germline mutations and PA, seizures and neurological abnormalities (PASNA) syndrome due to CACNA1D germline mutations. Several somatic mutations have been found in aldosterone producing adenomas (APAs) in KCNJ5, ATP1A1, ATP2B3, CACNA1D and CTNNB1 genes.

Key words: Cushing syndrome, primary aldosteronism, cAMP, CTNNB1

In addition to these genetic alterations, genome wide approaches identified several new alterations, in transcriptome, methylome and miRnome studies, highlighting new pathways involved in steroids dysregulation.
Introduction

Adrenocortical tumors are quite frequent, most of them are incidentally discovered (incidentalomas) ranging from 1 to 7% in general population, their frequency increasing with age. Unilateral benign adrenocortical tumors have various patterns of steroids secretion, from clinically inactive to overt Cushing or aldosterone secretion. New mass-spectrometry techniques revealed that most of the tumors considered as clinically inactive, are in fact producing excess of some steroids. In adrenal incidentalomas, cortisol excess, leading to Cushing syndrome, is seen in about 15% of cases and aldosterone excess, from Conn adenomas, is seen in around 3% of cases. Bilateral adrenocortical tumors are less common. Cortisol secreting tumors can be roughly divided in two entities, depending on the size of the nodules (micro- or macro-nodular adrenocortical hyperplasia).

Different mechanisms of tumorigenesis are involved in the growth of these adrenal tumors, with alterations in various signaling pathways. There are several points of interest in understanding their alterations:

- understanding the mechanism of steroid secretion
- exploring mechanisms of tumorigenesis, non-only in these benign tumors but also in malignant tumors, using the same metabolic pathways
- public health importance as these tumors are common and often associated with cardiovascular morbidity
- better patient care, as classifying tumors with similar pathogenesis leads to an easier choice for medical or surgical treatment and adequate genetic counselling
- easier diagnosis, thanks to specific markers detected by non-invasive techniques as miRNAs or circulation tumor DNA

Adrenal Cushing

In normal adrenocortical cells, the pituitary hormone adrenocorticotropic hormone (ACTH) binds to its seven-transmembrane G protein-coupled receptor MC2R, resulting in Gs protein activation, then adenyl cyclase activation and finally cAMP production. The binding of 4 cAMP molecules to PKA (protein kinase A) regulatory subunits dimer, allows the release and subsequent activation of the 2 catalytic subunits. These catalytic subunits will further phosphorylate several nuclear and cytoplasmic targets, including the transcription factor CREB (cAMP Response Element Binding protein), responsible for the stimulation of cAMP-dependent genes transcription. The negative
regulators of this pathway are the phosphodiesterases, responsible for cAMP degradation. Constitutive activation of the cAMP/PKA pathway can lead to tumorigenesis and Cushing syndrome, as ACTH stimulates both adrenocortical cell growth and cortisol synthesis (Figure 1).

**Bilateral nodular hyperplasia**

Bilateral adrenocortical tumors cover a spectrum of several entities. Micronodular adrenal hyperplasia (MiAH) is defined by multiples nodules, less than 1 cm diameter, in each adrenal. The most common form of MiAH is the Primary Pigmented Nodular Adrenal Disease (PPNAD). Macronodular hyperplasia is defined by nodules more than 1 cm diameter, the most common cause being the Primary Bilateral Macronodular Adrenal Hyperplasia. The bilateral nature of these tumors suggests a possible genetic predisposition, several mutations have been identified in these tumors, through candidate genes approaches. Initially, study of families with segregation of several bilateral adrenocortical tumors identified the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway as the first altered signaling pathway leading to tumorigenesis. Since these studies, other pathways have been identified as responsible of adrenal tumors (Figure 2).

**Primary Pigmented Nodular Adrenal Disease (PPNAD)**

PPNAD is a rare cause of ACTH-independent Cushing syndrome, most often diagnosed in children and young adults and characterized by the presence of pigmented micro nodules (diameter less than 1 cm by definition, but that in fact often less than 3 mm) widespread in the cortex of both adrenals. PPNAD is the most frequent endocrine manifestations of the Carney complex (CNC), a tumor predisposition syndrome, reported in 26 to 60% of patients but can also be isolated without tumors suggestive of CNC in 12% of the patients. The clinical presentation is variable, with more or less marked hypercortisolism, sudden, insidious or cyclic onset.

CNC is familial in 70% of cases, with an autosomal dominant transmission. In the majority of cases, the disease is due to an inactivating mutation of the PRKAR1A gene, coding for the type I alpha regulatory subunit of PKA, located in 17q22-24. These mutations are found in 37% of cases with sporadic CNC, and more than 70% of cases with familial CNC, with almost complete penetrance.

Most of the mutations of PRKAR1A identified first were frameshift, leading to a premature stop codon. The mutant mRNA is therefore unstable and degraded by non-sense mediated mRNA decay (NMD). Often within the tumor DNA a loss of heterozygosity (LOH) is observed leading to loss of the wild type allele in CNC tumors. The PKA regulatory subunit R1A protein is then lacking in tumor cells, resulting in constitutive activation of cAMP/PKA pathway. PRKAR1A is considered as a tumor suppressor gene. No clear hotspot mutations were identified, and mutations are distributed all along the coding sequence and even sometimes in non-coding sequence (intronic mutations affecting splicing). However, two mutations are found with a higher prevalence: c.709-7del6 (intron 7) and c.491-492del1G (exon 5). The first one was mainly seen in isolated PPNAD and the second was significantly associated with cardiac myxroma, lentigines, and thyroid tumors. The mutations
escaping NMD (20%), can give rise to the expression of an altered protein and have been suggested to be responsible for a more aggressive form of CNC.

For some cases of CNC, linkage studies revealed a second potential locus in 2p16 responsible for CNC. This region including Proopiomelanocortin (POMC) gene and DNA-mismatch repair gene MSH2, but these 2 genes were further excluded. The anomalies found at this locus were generally losses of heterozygosity and gains in the number of copies, suggesting the potential presence of an oncogene, not determined to date.

In addition, anomalies in the regulation of the catalytic subunits of PKA were reported. Triplication of 1p31.1 chromosome region, including PRKACB gene, were observed in a patient with CNC, having abnormal skin pigmentation, myxomas and acromegaly, but no PPNAD. Duplication of 19p region, including PRKACA gene, was identified in patients with bilateral adrenal hyperplasia (micro nodular and macro nodular forms) and Cushing syndrome of various degrees.

Other actors of cAMP/PKA pathway has been described as altered in patients with PPNAD. Three germline deleterious variants of PDE11A were identified in patients with MiAH/PPNAD without PRKAR1A mutation, suggesting a possible causative role of this gene. Moreover, single nucleotide variants (SNV) of PDE11A, with decreased enzymatic activity in vitro, were found more often in CNC patients due to PRKAR1A mutations that present with PPNAD than without PPNAD. So PDE11A could also act as a modifier gene in patients with CNC. The single base substitution c.914A>C was identified in another phosphodiesterase gene, PDE8A, in a patient with PPNAD and early onset of Cushing’s syndrome. Alterations of PDE11A and PDE8B were not specific of PPNAD and were further described in other types of adrenocortical tumors: PBMAH, adrenocortical adenomas (ACA), non-secreting adrenocortical adenoma and adrenocortical carcinomas (ACC).

In addition to cAMP/PKA pathway, involvement of Wnt/ beta catenin pathway was also reported in a study including 13 patients with PPNAD or sporadic cortisol secreting adenomas (ACA) with PRKAR1A somatic mutation. Beta catenin accumulation was found in all the tumors studied and somatic mutations in CTNNB1, gene coding for beta catenin, were identified in some tumors. This result was confirmed in another study which reported 2 CTNNB1 somatic mutations in 18 patients with PPNAD.

Primary Bilateral Macronodular Adrenal Hyperplasia (PBMAH)

PBMAH is usually defined by the presence of bilateral adrenal macronodules and cortisol autonomous secretion with low circulating ACTH, even if unilateral nodules with enlargement of the contralateral gland have been reported. The median age of diagnosis is around 50 years old. The degree of cortisol autonomous secretion ranges from overt Cushing syndrome to almost hormonally inactive forms with minimal or subtle hormonal alterations.

Historically this disease was called “ACTH-Independent Macronodular Adrenal Hyperplasia” (AIMAH), but the name changed when was found, in some forms of the disease, intra-adrenal ACTH synthesis responsible for local stimulation of cortisol production. In case of the rare syndromic forms of PBMAH (genetic tumors predisposition syndromes), multiple genetic alterations are known. Mutations of MEN 1 gene lead to type 1 multiple endocrine neoplasia, mutations of APC gene lead to familial adenomatous polyposis and (APC) and alterations...
of the *FH* gene leads to hereditary leiomyomatosis \(^{32-35}\). These genetic causes are however rarely found, as isolated PBMAH is by far the most common form of the disease.

As in MiAH/PPNAD, genetic alterations of actors of cAMP/PKA pathway were reported in PBMAH. *PDE11A* variants were found with a high prevalence in PBMAH patients (24-28%) \(^{27,36}\), some of them being associated with decreased enzymatic activity confirmed in vitro \(^{36}\). Activating mutations of the ACTH receptor (*MC2R*) gene is very rare but has been reported \(^{37}\). Post-zygotic activating mutations of the *GNAS 1* gene, resulting in constitutive activation of the cAMP/PKA pathway and autonomous cortisol secretion in the context of McCune Albright syndrome \(^{38,39}\) in very young children.

In cases of isolated bilateral macronodular adrenal hyperplasia, the bilateral nature of the adrenal involvement and the existence of familial cases suggested the existence of a genetic cause for PBMAH. In the study of blood and tumor DNA samples of 33 patients with PBMAH, who had undergone adrenal surgery, LOH was detected at 16p locus by SNP array in 24% of the cases \(^{40}\).

Whole genome sequencing of 5 paired tumor and leucocyte DNA samples identified *ARMCS* gene alterations, mapping to 16p11.2, as responsible of PBMAH. A first inactivating alteration was observed in the patients' leucocyte DNA, and a second event in somatic DNA extracted from adrenal nodules, suggesting a tumor suppressor role of the *ARMCS* gene. Inactivation of the second allele was due to LOH or point mutations, different from one nodule to another in the same patient. Other studies also confirmed the involvement of *ARMCS* in the pathophysiology of HMB5, in cohorts with sporadic or family cases \(^{41-44}\). The prevalence of *ARMCS* damaging germline mutations in PBMAH with a sporadic presentation was further estimated from 21% to 26% in following studies \(^{41,45}\).

*ARMCS* mutated index case PBMAH patients present higher cortisol secretion levels compared to patients without *ARMCS* mutation, with larger adrenals and a higher number of adrenocortical nodules \(^{45,46}\).

The precise function of *ARMCS* is yet unknown. It is a cytosolic protein, containing 7 armadillo domains, as beta-catenin, and a BTB domain \(^{47}\). Functional studies showed that inactivation of *ARMCS* decreases expression of steroidogenesis enzymes and cortisol secretion in adrenal cells in vitro, but at the same time expression of *ARMCS* mutant reduces apoptosis by comparison with the wild type *ARMCS* protein \(^{40,45}\). This suggests that the increased number of adrenocortical cells explains the excess in cortisol secretion in PBMAH patients, despite the reduced capacity of each cell \(^{40}\). It has been more recently shown that wild type ARMCS protein interacts with culin 3 (*CUL3*). Missense variants of *ARMCS* losing the ability to interact with *CUL3* are not ubiquitinated and further degraded by the proteasome and this could take part in cell cycle dysregulation \(^{48}\).

Whole Exome Sequencing (WES) studies in patients with PBMAH reported alterations of other potential causal genes as *DOT1L* (coding for a histone H3 lysine methyl-transferase), *HDAC9* (coding for a histone deacetylase) and Endothelin Receptor type A (*EDNRA*) gene \(^{49,50}\).

Finally, PBMAH might be due to illegitimate membrane receptors on adrenocortical cells, cortisol secretion being secondary to non-physiological stimuli. The stimulating ligands, binding to G-protein coupled receptors are various: Glucose-dependent insulinitropic peptide receptor (GIPR) responsible for food-dependent Cushing syndrome \(^{51,52}\), LH/HCG receptor responsible for Cushing syndrome during pregnancy and after menopause \(^{53}\), vasopressin, catecholamine, serotonin 5-HT, angiotensin II and glucagon receptors \(^{54-60}\). The prevalence of illegitimate membrane receptors in PBMAH is high, varying from 77% to 87% among studies \(^{59,60}\). In vitro and animals studies for the
GIPR showed the role of this ectopic expression on adrenal tumorigenesis and excessive cortisol secretion secondary to cAMP/PKA pathway activation \textsuperscript{61,62}. At present in PBMAH no genetic alteration have been given to explain the mechanism of illegitimate membrane receptors despite whole genome approaches \textsuperscript{63}. However in unilateral adenoma with GIPR ectopic expression gene rearrangement have been recently found at the GIPR locus \textsuperscript{64}.

**Unilateral adenomas**

As in bilateral cortisol secreting tumors, alterations of the cAMP/PKA pathway were described in unilateral cortisol secreting adenomas. In 2014, were identified, by four independent teams, activating somatic mutations of the PKA catalytic alpha-subunit (PRKACA) \textsuperscript{23,49,65,66}. Around 40\% of cortisol producing adenomas (CPA) harbor PRKACA mutations, most of them presenting the hotspot mutation L206R \textsuperscript{67}. In patient with CPA carrying a somatic PRKACA mutation, the clinical phenotype was more severe than wild-type ones and in fact the mutations are only found in CPA responsible for overt Cushing. An activating somatic mutation of the catalytic sub-unit Beta of PKA gene (PRKACB) has also been reported in CPA responsible for overt Cushing but this alteration is apparently rare \textsuperscript{68}. Somatic alterations of PRKAR1A were also described in CPA, with LOH in PRKAR1A locus 17q found in 7 of the 29 studied adenomas and somatic inactivating mutations in 3 CPA responsible for overt Cushing \textsuperscript{69}. A PRKAR1A somatic mutation was also more recently described in a WES study, including 39 CPA \textsuperscript{49}. Somatic activating mutations of GNAS 1, the gene coding for Gs protein alpha-subunit, were also reported in a few rare cases of CPA \textsuperscript{65,70,71}.

Activation of cAMP/PKA signaling leads to different pathway alterations in CPA. In GNAS 1 mutated tumors an overexpression of extracellular matrix receptor interaction and focal adhesion pathways was observed, while in PRKAR1A mutated tumors genes related to Wnt signaling pathway are overexpressed \textsuperscript{72}. Activation of the Wnt/beta catenin pathway was also reported in around 40\% of adrenocortical adenomas without any somatic mutation of PRKAR1A, most of them being explained by the occurrence of a somatic activating mutation of the beta-catenin gene (CTNNB1) \textsuperscript{73}. Activating mutations CTNNB1 are more frequent in non-secreting adrenal adenomas \textsuperscript{74,75}. This suggest that the consequences Wnt/beta-catenin activation on cortisol dysregulation differs from the one of cAMP/PKA signaling activation.

**Omic alterations in adrenal Cushing**

In chromosome alteration studies comparing adrenocortical adenomas (ACA) to adrenocortical carcinomas, the first group shows a smaller proportion of copy number alteration and loss of heterozygosity \textsuperscript{76,77}. The 9q34 region, including the steroidogenic factor 1 locus, is commonly gained in ACA \textsuperscript{77}.

Transcriptome studies showed various genes differentially expressed between ACA and ACC, with up regulation of steroidogenic genes in ACA, compared to ACC \textsuperscript{78} and overexpression of IGF2 and IGF2
related genes in ACC. A distinct gene expression profile in patients with overt Cushing versus subclinical Cushing and non-functional tumors, confirming the activation of the cAMP/PKA pathway and over expression of steroidogenic genes. Lampron et al. showed that 723 differentially expressed genes are identified in GIP-dependent tumors, including perilipin, overt expression of 13G protein-coupled receptors and potential involvement of Rho-GTPases.

Transcriptome, as well as methylome and miRNA studies are limited at present in ACA, but show interesting results in aldosterone producing adenomas.

Benign adrenal tumors associated with primary hyperaldosteronism (PA)

The two most common causes of PA are aldosterone-producing adenomas (APA), also called Conn adenomas, or bilateral adrenal hyperplasia (BAH). APA are usually unilateral, small in size (1-2 cm) and diagnosed in patients 40-50 years of age. BAH accounts for 60-70% of patients with PA. Aldosterone synthesis is tightly regulated by the renin-angiotensin system and extracellular potassium concentration. Angiotensin II (Ang II), binding to its Ang II type 1 receptors, stimulate inositol triphosphate signaling pathway, inducing a Ca\(^{2+}\) release from endoplasmic reticulum. Stimulation by potassium and Ang II also result in zona glomerulosa cell membrane depolarization, responsible for opening of voltage-dependent Ca\(^{2+}\)-channels. Both signals contribute to increase intracellular Ca\(^{2+}\) concentration, triggering a phosphorylation cascade, leading to increased transcription of enzymes responsible for aldosterone synthesis (Figure 3). While the majority of cases of PA are sporadic, 1-5% of cases are inherited familial forms, transmitted as autosomal dominant traits. Four different forms have been described, based on the underling genetic defects. At the same time, recurrent somatic mutations in several genes have been identified in 88% of APA (Figure 2).

Genes associated with familial hyperaldosteronism

Familial hyperaldosteronism type I (FH-I)

FH-I, also reported as Glucorticoid Remediable Aldosteronism (GRA), is an autosomal dominant disease, described for the time in 1966 in a father and his son, suffering from hypertension due to PA. The particularity of their phenotype relied on aldosterone suppression and therefore hypertension resolution with dexamethasone treatment. FH-I is usually due to bilateral hyperplasia or in rare cases adrenal nodules, and associates a significant production of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol. Clinical and biochemical characteristics are variable, even within the same family. In the adult hypertensive population, FH-I accounts for 0.5-1% of primary hyperaldosteronism, occurring in the same proportion in men and women. The molecular origin of FH-I was elucidated by Lifton and al. in 1992, who identified a chimeric CYP11B1/CYP11B2 hybrid gene in FH-I patients. These two genes are highly homologous and localized in tandem on the chromosome 8q21-8q22, CYP11B1 coding for 11-beta-hydroxylase, the enzyme responsible for the
last steps of cortisol synthesis and CYP11B2 coding for aldosterone synthase, the enzyme responsible for the last steps of aldosterone synthesis. The hybrid gene includes the promoter region of CYP11B1 and a large part of the CYP11B2 coding sequence, making aldosterone production dependent on ACTH regulation. Patients with FH-I are at increased cardiovascular risk, even for normotensive patients\textsuperscript{95}, with increased number of cerebrovascular events at young age\textsuperscript{96}.

**Familial hyperaldosteronism type II (FH-II):**

FH-II, was first described by Gordon et al., as a second form of autosomal dominant form of PA, not remediable with glucocorticoids and not due to the presence of the chimeric CYP11B1/CYP11B2 gene\textsuperscript{97}. The prevalence of FH-II ranges from 1.2% to 6% in adult population of primary hyperaldosteronism\textsuperscript{88}. APA and BAH have been reported, with a high phenotypic variability, even within the same family\textsuperscript{98–101}. FH-II is clinically and biochemically indistinguishable from sporadic forms of primary hyperaldosteronism and is only diagnosed on the basis of two or more affected family members.

Linkage analysis found an association between FH-II and the chromosomal region 7p22 but no mutations were found in different candidate genes located in this region\textsuperscript{102}. In 2018, two independent teams identified several gain of function mutations in the CLCN2 gene, coding for the CLC2 chloride channel, in patients with FH-II and early onset of primary hyperaldosteronism\textsuperscript{89,90}. Mutations were located in different domains of the protein and can therefore explain the phenotypic heterogeneity. It is the first time that implication of a chloride channel is shown in regulation of aldosterone production. A recent study also found one somatic mutation in CLCN2, after screening 80 apparently sporadic APAs, in a male patient with primary hyperaldosteronism, which was cured after surgery of its small adenoma\textsuperscript{103}.

**Familial hyperaldosteronism type III (FH-III)**

FH-III was first described in 2008 by Geller and al. in a father and two daughters with early-onset severe resistant arterial hypertension and hypokalemia\textsuperscript{104}. Their phenotype was particularly severe, associating marked hyperaldosteronism, very high levels of the hybrid steroids 18-oxocortisol and 18-hydroxycortisol but no suppression of aldosterone production by dexamethasone treatment and massive BAH. Bilateral adrenalectomy was necessary to control blood pressure. Recently, WES identified a germline mutation in the KCNJ5 gene (p.Thr158Ala) in an FH-III patient, unraveling the genetic origin of this syndrome\textsuperscript{105}. KCNJ5 is located on chromosome 11q24.3, and coding for the G protein-activated inward rectifier potassium channel GIRK4. This mutation affects channel ion selectivity for K\textsuperscript{+}, leading to Na\textsuperscript{+} influx into zona glomerulosa cell, cell membrane depolarization, activation of voltage-dependent Ca\textsuperscript{2+}-channels, increase in intracellular Ca\textsuperscript{2+} concentration and stimulation of steroidogenesis enzymes synthesis resulting in aldosterone synthesis. Different germline KCNJ5 mutations were further reported in families with FH-III. Patients carrying the germline mutations p.Gly151Arg, p.Thr158Ala and p.Ile157Ser all presented a severe phenotype of primary hyperaldosteronism whereas affected members from three FH-III families carrying p.Gly151Glu mutation and from one family carrying the p.Tyr152Cys mutation exhibited a milder phenotype similar to FH-II\textsuperscript{88}. However, like for the other forms of familial hyperaldosteronism, a strict genotype-phenotype correlation is difficult to establish and a certain degree of phenotypic variability exists.
Familial hyperaldosteronism type IV (FH-IV)

T-type voltage calcium channel (Cav 3) are activated at higher negative membrane potential and display voltage-dependent inactivation. The alpha 1 subunit is the main transmembrane and pore-forming portion of Cav3.2 channels, and is encoded by CACNA1H gene, located on chromosome 16p13.3. WES in blood samples from 40 patients with very early-onset hypertension identified a hotspot de novo mutation in CACNA1H p.M1549V in 5 cases. Four germline mutation of the same gene were identified later in patients with diverse phenotypic presentation of primary hyperaldosteronism. Whole patch clamp study in HEK293T cells transfected with the mutant CACNA1H showed slower inactivation and longer opening in the channel, resulting in higher Ca\textsuperscript{2+} influx.

Primary aldosteronism, seizures and neurological abnormalities (PASNA) syndrome

CACNA1D mutations have been described in two children with PASNA. This gene, located on chromosome 3p14.3, encodes the alpha 1 subunit of L-type voltage Ca\textsuperscript{2+}-channels (Cav1). These mutations induce a gain of function, with channel opening at lower voltage leading to excessive aldosterone production, by increasing intracellular calcium levels. Exome sequencing in 100 patients with early-onset of primary hyperaldosteronism identified two germline de novo mutations in CACNA1D p.G403R and p.I770M. The phenotype was very severe in both patients. The first one, carrying p.G403R mutation, presented hypertension since birth resulting in biventricular hypertrophy, associated with ventricular septal defect and pulmonary hypertension. The second case, harboring p.I770M mutation was diagnosed with hypertension at the age of 5 but presented central nervous system attempt since birth, neurological symptoms including spastic quadriplegia and seizures.

Somatic mutations in APAs

KCNJ5

The first gene whose somatic alteration was reported in APA is KCNJ5, identified by WES of samples from patients with sporadic form of APA. Two hot-spot mutations were reported, p.G151R and p.L168R, respectively localized in the highly conserved glycine-tyrosine-glycine (GYG) motif of the selective filter and in the second transmembrane domain of KCNJ5. They both abolish K\textsuperscript{+}-selectivity of the channel. The consequences of this alteration was further confirmed in other studies. KCNJ5 mutations were thus identified in 180 of 474 (38%) APA samples collected from the European Network for the Study of Adrenal Tumor (ENSAT), the two hotspot mutations being largely prevalent with respective prevalence of 63% for p.G151R and 36% for p.L168R. KCNJ5 mutations prevalence in APA was found to be higher in some specific populations, particularly in Asian population, confirmed by a meta-analysis on 1636 patients, showing a prevalence of KCNJ5 mutations of 43%, and up to 77% in populations from East Asia. APAs harboring mutations in KCNJ5
were more frequent in women, young patients and were associated with larger tumors and higher plasma aldosterone concentration \(^{11}\).

**ATPases**

ATPases somatic mutations have also been reported in APAs. Na\(^+\)/K\(^+\)-ATPases are composed of alpha- and beta-subunits, alpha-subunit including the Na\(^+\)/K\(^+\) and ATP-binding sites and beta-subunit directing alpha-subunit to plasma membrane. Na\(^+\)/K\(^+\)-ATPases transport 3 Na\(^+\) ions in exchange of 2 K\(^+\) ions, using the driving force of ATP hydrolysis and generating an electrochemical gradient across the membrane that facilitates ions cellular uptake. At least four alpha-subunit isoforms have been described. ATP1A1 codes for the alpha 1 subunit of Na\(^+\)/K\(^+\)-ATPase, located on chromosome 1p13.1 \(^{106}\). Three different somatic alterations of ATP1A1 (2 substitutions p.L104R and p.V332G and one deletion p.F100_L104) were identified in 16 of 328 APAs (6.8%) by Beuschlein and al. \(^{113}\). Expression in an adrenal cell line showed that ATPase activity of mutant proteins was severely impaired, with decreased Na\(^+\) and K\(^+\) binding, inducing high level membrane depolarization, opening of voltage-gated calcium channel and enhanced aldosterone production \(^{113}\). Thirteen different mutations were reported so far, with a global prevalence of 5-8% in patients with APAs among studies \(^{85,106}\).

**Calcium channels**

WES of APAs identified somatic alterations in CACNA1D. These mutations were the same than those identified at the germline level, p.G403R and p.I770M \(^{110}\). Other somatic mutations along the coding region of CACNA1D were further identified \(^{85}\), with a global prevalence of 3-11% in patients with APAs among studies \(^{106}\). CACNA1D mutations were the most frequent ones in APAs from patients with African ancestry, up to 42% \(^{114}\).

**Beta-catenin**

Like in cortisol-producing adrenal tumors, beta-catenin accumulation in both nuclear and cytoplasmic compartments is very common in APAs with a prevalence of about 70% \(^{115}\). Nuclear beta-catenin can stimulate the expression of the transcription factor TCF/LEF, which further activates the transcription of transcription factors NURR1 and NURR7, finally activating the transcription of CYP11B2 \(^{115,116}\). Interestingly, in transgenic mice harboring beta catenin inactivation specifically targeted in the adrenal cortex, hyperproliferation of zona glomerulosa cells and primary hyperaldosteronism were observed in 10-months-old mice \(^{117}\). However, CTNNB1 mutations are rare, found in around 3% of sporadic APAs, suggesting Wnt/beta catenin pathway activation through
other mechanisms. Somatic mutations of \(\text{CTNNB1}\) have been associated to female gender and relatively large adenomas.

**Aldosterone-producing cell clusters (APCCs)**

In normal human adrenal gland, Aldosterone-producing cell clusters (APCCs) has been recently observed and it has been suggested that they autonomously produce aldosterone. APCCs are increased in patients with primary hyperaldosteronism and negative CT. APCC are characterized by a uniform expression of CYP11B2, in cell clusters and non CYP11B1 expression, and are composed of subcapsular zona glomerulosa-like cells and inner large zona-fasciculata-like cells.

35% of APCC harbor mutations observed in APA, causing aldosterone over production: \(\text{CACNA1D}\) mainly and \(\text{ATP1A1}\), but no mutation in \(\text{KCNJ5}\).

APCCs and APAs are different by their size, cellular arrangements and enzyme expression profile, as APA is composed by heterogeneous cell types expressing either CYP11B1 or CYP11B2. Recently, were described some transitional structures, consisting of a subcapsular APCC-like structure and an inner micro-APA-like structure without well-defined histological border, called pAATL (possible APCC-to-APA transitional lesions), characterized by the presence of \(\text{KCNJ5}\) and \(\text{ATP1A1}\) mutations. Thus, some APA could derive from APCC with \(\text{CACNA1D}\) and \(\text{ATP1A1}\) mutations or from pAATL, but the precise mechanism of these transition has not been found yet.

**Molecular basis of bilateral hyperaldosteronism**

Contrary to APAs, for which molecular determinants are well established, the molecular basis of bilateral hyperaldosteronism and bilateral adrenal cell proliferation are unknown. In 2011, it has been shown that \(\text{KCNJ5}\) mutations were not only involved in driving aldosterone secretion, but also in promoting cell proliferation. Thus, a study carried on 251 patients affected by sporadic bilateral hyperaldosteronism revealed three heterozygous missenses germline mutations in \(\text{KCNJ5}\), two of them resulting in membrane depolarization. \(\text{ARMCS}\) germline variants, first described in PBMAH, were also reported in patients with primary hyperaldosteronism. In a cohort of 56 patients with PA, some of them presenting bilateral adrenal hyperplasia, almost 40% carried a genetic variant in the \(\text{ARMCS}\) gene, among which about one quarter were predicted to be deleterious in \textit{in silico} analysis. However, all concerned patients were African-American and this association was not confirmed in a caucasian cohort of 39 patients presenting primary aldosteronism and bilateral adrenal hyperplasia. Eleven common variants, 5 rare variants and 2 unknown variants were indeed identified in this cohort but none of them was predicted to alter protein function, so the role of \(\text{ARMCS}\) in PA needs further confirmation.
Omic alterations in APA

A recent study performed deep quantitative proteomic profiling on APA and adjacent non tumoral adrenal tissue and showed 11 significantly upregulated proteins out of 5555. This upregulation concerned steroidogenic enzymes as HSD3B2, CYP21A2, CYP11B2 and proteins involved in cholesterol uptake as LSR (lipolysis stimulated lipoprotein receptor)\(^2\). Higher levels of proteins involved in N-glycosylation and enzymes involved in GABA degradation are seen in APAs. N-glycosylation affects the activity of steroidogenesis regulators as MC2R (adrenocorticotropic hormone receptor) and AT1R (angiotensin II receptor), lipoprotein receptors and ion channels. GABAergic signaling mediates steroidogenesis decrease in vivo, in rat adrenal cortex\(^2\). Moreover, the activity of the mTOR pathway, involved in cell proliferation, steroidogenesis and immortalized adrenocortical cells, is increased in APA\(^2\). Inhibiting this pathway could then be a new perspective of treating patients with APA.

In APAs, Bassett et al. revealed up regulation of genes encoding transcription factors NURR1 and NGF1B, that regulate CYP11B2, as well as SF-1 and DAX1, that play a role in adrenal development and steroidogenesis\(^2\).\(^3\)\(^4\).

There are also some specific transcriptomic markers of APCCs. In addition to CYP11B2, genes like SLC35F1 (role in glucose transport), MC2R (role in aldosterone production) and PPP4R4 (role in phosphorylation) have higher transcript expression in APCCs compared to zona glomerulosa or fasciculata\(^2\). Thus, aldosterone synthesis in APCC, known to be independent of renin and angiotensin II stimulation, could be regulated, at least partially, by ACTH\(^2\). Based on differential gene expression, we are now able to distinguish APAs with KCNJ5 somatic mutations from ATP1A1, ATP2B3 and wild type tumors\(^2\).\(^3\)\(^4\).

Several microRNAs (small size non coding RNAs that promote translational repression) as miR-24, miR-23b, miR-34a, miR-203 and miR-375, were identified in APAs and CPAs, as involved in modulating steroid biosynthesis and secretion by different mechanism, such as modulation of CYP11B1 and CYP11B2 expression\(^2\).\(^3\)\(^4\).\(^5\)\(^6\).

APAs are characterized by a global hypomethylation, compared to normal adrenal and non-functioning adenomas, correlating to upregulation of CYP11B2 and genes involved in tumorigenesis\(^2\)\(^7\)\(^8\), as well as higher demethylation of G protein-coupled receptors (GPCRs) and GPCR-related genes\(^2\).

Concomitant aldosterone and cortisol production

Several reports described conditions related to glucocorticoid excess, as insulin resistance type 2 diabetes mellitus, osteoporosis, depressions and anxiety in patients with primary aldosteronism\(^2\).\(^9\). However current guidelines for diagnosis of PA do not indicate hypercortisolism assessment\(^2\).\(^9\).

In 2015, a study compared steroid hormone production in patients with aldosterone producing adenomas to patients with bilateral hyperplasia and showed that this last group had higher urinary, peripheral, and adrenal venous concentrations of the hybrid steroids, 18-oxocortisol and 18-hydroxycortisol\(^2\).\(^9\). Two years later a spectrometry-based analysis of 24-hour urine steroid...
metabolome showed that patients with PA had significantly increased cortisol and glucocorticoid metabolite excretion when compared with healthy controls, inactive adrenal adenomas and mild subclinical adrenal cortisol excess, correlated with several parameters indicative of adverse metabolic risk. This lead to the speculation that, at least in a subset of PA patients, mineralocorticoid receptor antagonist therapy might not be sufficient and that additional glucocorticoid-targeted treatment would be important to reduce the cardiovascular damages. In patients with PA cortisol co-secretion could have an additional impact on cardiac remodeling, with more severe left ventricular hypertrophy, compared to aldosterone secretion only and adrenalectomy had a better efficiency on left ventricular mass index than mineralocorticoid receptor therapy.

In the study by Arlt et al. the glucocorticoid excretion was significantly associated with intratumoral CYP11B1 expression, required for the synthesis of glucocorticoids and 11beta-hydroxyandrostenedione, explaining also the normal or increased androgen output of these tumors.

Moreover, it has been shown that a distinct steroid signature can predict APA genotype in adrenal venous and peripheral plasma; for example a 7-steroid fingerprint in peripheral plasma correctly classified 92% of the APA according to the genotype. High plasma levels of 18-hydroxy cortisol and 18-oxocortisol have been identified in patients with APA and were predictive of KCNJ5 mutations. Furthermore, based on matrix-assisted laser desorption/ionization (MALDI) technology, it was shown that 137 metabolites were significantly different in KCNJ5- and CACNA1D-mutated patients. An increased intratumoral content of 18-oxocortisol and 18-hydroxy cortisol was found in KCNJ5 mutated APAs. The 18-oxocortisol tumoral content and CYP11B1 expression levels (inversely correlated) are associated with outcome (higher probability of complete clinical success after surgery), independent of clinical parameters. Concordantly, Arnesen et al. showed that KCNJ5 somatic mutations were associated with a better surgical outcome in 28 APAs, which was confirmed recently by Vilela et al. on 100 patients with unilateral primary hyperaldosteronism, where somatic KCNJ5 mutation was an independent predictor of hypertension remission after adrenalectomy. ATP1A1-mutated APAs had higher CYP11B2 staining intensities.

**Conclusion**

Molecular basis of cortisol secreting adenoma is nowadays well defined, even if genetic explanation is still missing for some cases. Alterations of cAMP /PKA pathway can explain the occurrence of a significant proportion of the tumor responsible for overt-Cushing. Other pathways, as the Wnt/beta-catenin or the still to be explained ARMCS signaling, can also play a role in cortisol secreting tumors that are often less active in term of cortisol-secretion. Major advances in determination of molecular mechanism leading to sporadic and familial primary aldosteronism have been made in the last years. Novel familial forms have been characterized. Somatic mutations driving aldosterone overproduction have been found in around 60% of sporadic APAs. Recent studies showed that some adenomas were producing concomitantly aldosterone and cortisol in excess.
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Figures Legends

Figure 1: Signaling pathways and molecular alterations in adrenal Cushing

Adrenocorticotropic hormone (ACTH) binds to a G protein-coupled receptor, the Melanocortin receptor (MC2R), resulting in Gs protein activation. This in turn activates adenylate cyclase (AC) leading to cAMP production. Four cAMP molecules bind to the protein kinase A (PKA) regulatory subunits dimer, which allows the releasing and activation of the 2 catalytic subunits of PKA. The free catalytic subunits will phosphorylate the transcription factor CREB (cAMP response element-binding protein), stimulating the transcription of several cAMP-dependant genes. Phosphodiesterases (PDE) involved in cAMP degradation, are negative regulators of this pathway.

Different alterations of various component of the cAMP/PKA signalling pathway are observed in adrenal Cushing.

In primary bilateral macronodular adrenal hyperplasia (PBMAH)

1. Activating mutation of MC2R
2. Activating mutation of GNAS1 in Mac Cune Albright syndrome
3. Illegitimate G-protein-coupled receptors expression
4. Phosphodiesterase (PDE11A) inactivating mutations

In bilateral adrenal hyperplasia

5. PRKACA duplication

In primary pigmented nodular adrenal dysplasia (PPNAD)

4. Phosphodiesterases’ (PDE11A and PDE8B) inactivating mutations
6. PRKAR1A inactivating mutations or deletion (germline mutation and somatic second hit)

In unilateral cortisol secreting adenomas

2. Activating mutation of GNAS1, apart from Mac Cune Albright syndrome
6. PRKAR1A inactivating mutations
7. Activating somatic mutation of PRKACA

Apart from cAMP/PKA pathway:

8. ARMCS5 inactivation (germline mutation and somatic second hit) reducing steroidogenesis and adrenocortical cells apoptosis
Figure 2: Genetic alterations in adrenal Cushing and primary aldosteronism
Type of genetic alterations: \( ^{\text{a}} \) loss of function; \( ^{\text{b}} \) gain of function; \( ^{\text{c}} \) duplication; \( ^{\text{d}} \) chimeric fusion gene
Gene function: bold black = certain causal gene; grey = causal gene to be confirmed; (brackets black) = causal/modifier gene

Figure 3: Signaling pathway and molecular alterations in Primary Aldosteronism
Glomerulosa cells stimuli, as angiotensin II (AT-II) and hyperkalaemia, lead to membrane depolarization, then opening of voltage-gated calcium channels, signal for aldosterone synthase expression and aldosterone production.

Different generic alterations lead to multiple forms of primary aldosteronism

1. Expression of the hybrid variant of CYP11B1/CYP11B2 (germline mutation in FH-I) dependent of pituitary ACTH increases aldosterone production
2. Depolarization of glomerulosa cells by increased chloride efflux due to CLCN-2 variants (germline mutation in FH-II)
3. Depolarization of cell membrane by change of ion selectivity of the K+ channel with increased sodium influx due to KCNJ5 variants (germline mutation in FH-III and somatic mutations in APAs)
4. Increased calcium permeability due to CACNA1H mutations (germline mutation in FH-IV) and CACNA1D (germline mutation in PASNA syndrome and somatic mutations in APAs)
5. Depolarization of cell membrane by increased permeability for Na+ or H+ due to ATP1A1 and ATP2B3 variants (somatic mutations in APAs)
6. Stimulation of aldosterone production by CTNNB1 variants (somatic mutations in APAs)

MC2R: Melanocortin receptor; AT1R: Angiotensin receptor; ACTH: Adrenocorticotropic hormone
Figure 1

[Diagram showing the interaction between ACTH and illegitimate receptors, with pathways involving GS, AC, PDE, PKA, CREB, and ARMC5, leading to apoptosis and steroidogenesis.]
Figure 2

**Adrenal Cushing**

| Bilateral | Post-zygotic alterations | Somatic alterations |
|-----------|--------------------------|--------------------|
| PPNAD     | PRKARIA<sup>*</sup>      | PRKARIA<sup>*</sup> |
|           | (PDE11A)<sup>*</sup>     | PRKACA<sup>*</sup>  |
| Hyperplasia | PRKACA<sup>*</sup>      | GNAS1<sup>*</sup>  |
| PBMAH     | MEN1<sup>*</sup>         | CTNNB1<sup>*</sup>  |
|           | APC<sup>*</sup>          |                    |
|           | FH<sup>*</sup>           |                    |
|           | ARMCS<sup>*</sup>        |                    |
|           | DOT1L<sup>*</sup>        |                    |
|           | HDAC9<sup>*</sup>        |                    |
|           | EDNR<sup>*</sup>         |                    |
|           | (PDE11A)<sup>*</sup>     |                    |
|           | (MC2R)<sup>*</sup>       |                    |

**Primary aldosteronism**

| Familial | Unilateral | Bilateral |
|----------|------------|-----------|
| FH I     | CYP11B1/CYP11B2<sup>§</sup> | KCNJ5<sup>*</sup> |
| FH II    | CLCN2<sup>*</sup>          | ATP1A1<sup>*</sup> |
| FH III   | KCNJ5<sup>*</sup>          | ATP2B3<sup>*</sup> |
| FH IV    | CACNA1H<sup>*</sup>        | CACNA1D<sup>*</sup> |
| PASNA    | CACNA1D<sup>*</sup>        | CTNNB1<sup>*</sup> |

**Unilateral**

GNAS1<sup>*</sup>
