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Understanding primary aldosteronism: impact of next generation sequencing and expression profiling

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Abstract

Primary aldosteronism (PA) encompasses a broad, heterogeneous group of disorders including both sporadic and familial forms (familial hyperaldosteronism type I, II and III). PA is the most common form of secondary hypertension and associated with a higher rate of cardiovascular complications, compared to essential hypertension. Despite significant progress in the diagnosis and management of PA, until recently the molecular mechanisms leading to inappropriate aldosterone production were largely unknown. The introduction of next-generation sequencing has had a profound impact on the field of human genetics and has given new insight in the molecular determinants that lead to both sporadic and familial forms of PA. Here we review the recent progress towards understanding of the genetic and molecular mechanisms leading to autonomous aldosterone production in PA.

Keywords

primary aldosteronism; exome sequencing; KCNJ5; ATP1A1; ATP2B3; CACNA1D

1. Introduction

Primary aldosteronism (PA) is a heterogeneous group of disorders, characterized by inappropriate aldosterone secretion and concomitant suppression of its main physiological

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regulator, the renin-angiotensin system. Since its first description by Jerome Conn in 1955 (Conn, 1955) as a secondary form of hypertension associated with hypokalemia and metabolic alkalosis, significant progress has been made in the management of PA patients and in our understanding of the molecular determinants leading to inappropriate aldosterone production. Classically, PA was thought to be a rare and relatively benign form of endocrine hypertension, accounting for less than 2% of all hypertension (Ganguly, 1998). However, over the last 20 years, the widespread use of the aldosterone/plasma renin activity ratio (ARR) as a screening test has led to a 5–15 fold increase in the identification of patients affected by PA (Mulatero et al., 2004a), which is now widely recognized as the most common form of secondary hypertension.

Similarly, a wealth of studies have extensively demonstrated the detrimental role of aldosterone in the cardiovascular system (Mulatero et al., 2006), resulting in a higher rate of cardio- and cerebrovascular events, target organ damage, and metabolic syndrome in PA patients compared to essential hypertension with similar blood pressure and risk profiles (Fallo et al., 2006; Milliez et al., 2005; Mulatero et al., 2013a; Savard et al., 2013). In light of these considerations, early detection of PA is of particular importance because it allows for targeted therapy, which has been proven to reverse the excess of organ damage and cardio- and cerebrovascular events (Catena et al., 2008). As recommended by the Endocrine Society Guidelines (Funder et al., 2008), diagnostic work-up for PA is a three-step process that consists of case-finding, confirmation/exclusion and subtype differentiation, based on imaging techniques and selective adrenal vein sampling, to distinguish between unilateral and bilateral forms.

PA is either regarded to be sporadic or hereditary. Approximately 70% of PA patients are affected by bilateral disease, that in the great majority of cases can be attributed to idiopathic hyperaldosteronism (IHA) while the remaining 30% present with a unilateral form are mainly due to aldosterone producing adenomas (APA) (Mulatero et al., 2004a). Rarer subtypes are unilateral adrenal hyperplasia and adrenal carcinoma (Else et al., 2014). Current estimates are that up to 5% of PA are caused by Familial Hyperaldosteronism type I, type II and type III (FH-I to FH-III) (Mulatero et al., 2011; Mulatero et al., 2013b).

Until recently, the only subtype of PA whose underlying genetic and molecular basis was clearly understood was FH-I (or Glucocorticoid remediable aldosteronism, GRA). GRA is a form of monogenic hypertension transmitted as an autosomal dominant disease. Inappropriate aldosterone production is caused by a hybrid gene, resulting from unequal crossing over between *CYP11B1* and *CYP11B2*, which encode 11 β -hydroxylase and aldosterone synthase, respectively (Lifton et al., 1992). As a result, *CYP11B2* is controlled by the ACTH-responsive promoter of *CYP11B1*. The main clinical features are ACTH-dependent aldosterone secretion, renin suppression and high levels of the hybrid steroids 18-hydroxycortisol and 18-oxocortisol (Mulatero et al., 2004b). Based on these observations, genetic changes in *CYP11B1/B2* were further analyzed in APAs (targeted gene approach). However, despite a moderate increase in hybrid steroid levels and some degree of aldosterone responsiveness to ACTH in APA patients, a chimeric *CYP11B1/B2* gene was not found in sporadic adenomas (Carroll et al., 1996). Similarly, the chimeric *CYP11B1/B2* gene was not present in a large population of IHA patients (Mulatero et al., 1998).

FH-II is a familial form of unknown genetic basis that has been shown to be in linkage with chromosomal region 7p22 in families from different continents (Sukor et al., 2008); however, this linkage has not been demonstrated in other FH-II families (So et al., 2005). FH-III is a severe form of hyperaldosteronism due to mutations in *KCNJ5*, which will be discussed in more detail below in this manuscript.

Until recently, studies of sporadic PA have mainly focused on genetic variants that potentially increase the susceptibility to develop PA or affect the clinical phenotype. Three main polymorphisms of *CYP11B2* have been identified: (i) a c.-344C>T substitution in the promoter region; (ii) an intron 2 gene conversion in which part of intron 2 of *CYP11B2* is substituted with the corresponding region of *CYP11B1* and (iii) a single nucleotide substitution in codon 173 (c.518A>G) leading to a substitution of the arginine with a lysine (Mulatero et al., 2004b). The c.-344C>T polymorphism is located in the putative binding site for the steroidogenic factor 1 (SF1, NR5A1). The biological effect of this variant is unclear: despite SF1 binding being increased in the presence of the c.-344C allele, no effect on *CYP11B2* transcription has been shown by *in vitro* studies (Bassett et al., 2002; Clyne et al., 1997). Interestingly, the c.-344C>T polymorphism has been shown recently to be in tight linkage disequilibrium with another T/C polymorphism at position -1651 (c.-1651T>C) at the binding site of the multifunctional protein DNA (apurinic/apyrimidinic site) lyase called APEX1 (McManus et al., 2012). The substitution affects APEX1 binding and results in different repressor effects on *CYP11B2* transcription; intriguingly, this polymorphism associates also with lower excretion rates of aldosterone metabolites in human subjects (McManus et al., 2012). The gene conversion in intron 2 and the p.Arg173Lys substitution have both been shown to have a strong linkage disequilibrium with the c.-344C>T polymorphism. However, the p.Arg173Lys does not affect aldosterone synthase activity *in vitro* (Portrat-Doyen et al., 1998). Overall, there are several reports that consistently link the *CYP11B2* locus to hypertension and PA not entirely explained by the known polymorphisms (Davies and Kenyon, 2003; Mulatero et al., 2000). Recently, polymorphisms in other genes were found to associate with PA. Polymorphisms in *KCNJ5* and *HSD3 β* associate with PA in the Chinese populations (Li et al., 2013; Wu et al., 2013). Finally, polymorphisms in α -adducin (p.Gly460Trp) and bradykinin B2 receptor (p.Cys58Thr) affect blood pressure in PA patients presumably by effects on renal sodium handling (Mulatero et al., 2002).

Over the last few years, the advent of affordable large scale methods of analyses, particularly of gene expression (e.g. transcriptome profiling, cDNA arrays) and gene sequencing (e.g. next generation sequencing, NGS) has dramatically changed the approach to basic, applied and clinical genetic analysis and had a profound impact on genomic variant/mutation discovery. Taking advantage of these next-generation technologies, recent efforts have been directed towards a better understanding of the pathogenic mechanism of the disease. In this review we summarize the impact of these methods on our understanding of the molecular determinants of PA in both sporadic and familial PA.

2. Gene-expression studies

Gene expression profiling is based on hybridization of cDNA, which is generated through reverse transcription from mRNA of the cells or tissue of interest, to short oligomers of DNA. This method was first introduced in the 1990s and has over the last decade become widely available at fairly low cost on different platforms (e.g. Affymetrix, Illumina). In part DNA chip-based technologies have been replaced by direct sequencing of cDNA generated from cellular mRNA (RNAseq). However, it is still a matter of debate whether this NGS based system will show comparable results to the older hybridization based assays. RNAseq has the advantage of defining fusion genes and additional sequence data beyond that found with simple expression data. This method has not been employed for PA samples.

Over the last decade several studies investigated the gene-expression profile of APAs compared to normal adrenals or adjacent adrenal cortex with the aim of identifying transcriptional modulators of aldosterone overproduction. Under physiological conditions, aldosterone is synthesized in adrenal zona glomerulosa cells through the successive action of four different enzymes, cholesterol side-chain cleavage (encoded by *CYP11A1*), type 2 3 β -hydroxysteroid dehydrogenase (encoded by *HSD3B2*), 21-hydroxylase (encoded by *CYP21A1*) and finally aldosterone synthase (encoded by *CYP11B2*) (Hattangady et al., 2012). The two main physiological regulators of aldosterone production are angiotensin II (AngII) and serum potassium levels, that exert their effects through increasing cytoplasmic calcium concentration and activating the calcium/calmodulin-dependent protein kinase I/II (CaMKI/II) (Hattangady et al., 2012). This results in the activation of a number of transcription factors such as nuclear receptor related 1 (NURR1)/nerve growth factor 1b (NGF1B), cAMP response element-binding protein (CREB) and activating transcription factor (ATF), which in turn increase the transcription of *CYP11B2* and stimulate aldosterone production.

Results regarding *CYP11B2* expression in APAs have been conflicting with most studies reporting a significant up-regulation of *CYP11B2* in comparison to non-adenomatous adrenal tissue (Assiè et al., 2005; Boulkroun et al., 2010; Fallo et al., 2002; Wang et al., 2011; Williams et al., 2010). However, this finding was not confirmed by other authors (Lenzini et al., 2007; Enberg et al., 2004), who reported subgroups of APAs whose *CYP11B2* expression was unchanged or even reduced as compared to control adrenal tissue. Possible explanations for the heterogeneity of *CYP11B2* expression are i) true differences in patient populations and disease entities, ii) differences in technical approaches employed (real-time PCR or *in situ* hybridization) iii) the variations in *CYP11B2* expression in different control tissues (e.g. normal adrenals removed from nephroadrenalectomized patients vs. adjacent adrenal cortex), iv) heterogeneity of *CYP11B2* within APA or v) the potential for misdiagnosis of benign tumors as APA (Zennaro et al., 2012).

Analysis of other genes encoding steroidogenic enzymes, found decreased *CYP11B1* mRNA (Fallo et al., 2002; Assiè et al., 2005) and increased *CYP21A1* levels (Bassett et al., 2005; Assiè et al., 2005). *CYP11A1*, *CYP17A* and *HSD3B2* expression levels showed a relative heterogeneity amongst studies, being either increased, unchanged or decreased (Zennaro et al., 2012). In addition, several genes involved in calcium signaling or in endoplasmic

reticulum calcium storage displayed differential expression in APAs compared to normal adrenals (Assiè et al., 2005), including calmodulin 2 (*CALM2*), calreticulin (*CALR*) and *CAMK-1* (Lenzini et al., 2007).

Aberrant or ectopic hormone receptor expression has been reported to play a central role in ACTH-independent macronodular adrenal hyperplasia and in some unilateral cortisol producing adenomas leading to cortisol hypersecretion regulated by hormones other than ACTH (Lacroix et al., 2004). Similarly, several G-protein-coupled hormone receptors are expressed in APAs, including receptors for GnRH (Ye et al., 2007), LH (Saner-Amigh et al., 2006), vasopressin (Perraudin et al., 2006), serotonin (Cartier et al. 2005; Lefebvre et al., 2002; Williams et al., 2010; Ye et al., 2007), G protein-coupled receptor 37 (endothelin receptor type B-like, GPR37) and glutamate receptor metabotropic 3 (GRM3) (Ye et al., 2007); a role for these receptors in aldosterone secretion has been demonstrated in vivo in PA patients (Zwermann et al., 2009). In particular, LH receptor (*LHR*) mRNA levels have been found to be over-expressed in 50% of APAs (n=18) when compared to normal adrenals. Functional studies in H295R adrenocortical cells demonstrated that LH treatment increases the transcriptional activity of *CYP11B2* in a dose-dependent manner (Saner-Amigh et al., 2006). Interestingly, the aberrant expression of LHR and/or gonadotropin-releasing hormone receptors (GnRHRs) in aldosterone-producing adenomas is associated with stimulation of aldosterone secretion during pregnancy (Albiger et al., 2011).

The effects of GnRHR in aldosterone production have been recently explored by Nakamura et al. (2014). In H295R cells with doxycycline inducible *GnRHR*, chronic stimulation with the agonist (GnRH) resulted in a significant increase in both *CYP11B2* mRNA (over 100-fold) and aldosterone production (50-fold). These effects clearly involved Ca^{2+} signaling since they were specifically blocked by the Ca^{2+} signaling inhibitors calmidazolium and KN93.

In 2010 Williams et al. identified a series of differentially expressed genes in APAs compared to normal adrenals using an oligonucleotide microarray. Among the overexpressed genes, teratocarcinoma-derived growth factor-1 (*TDGF1*) and visinin-like 1 (*VSNL1*) were studied in detail (Williams et al., 2010; Williams et al., 2012). *TDGF1* is part of the epidermal growth factor–Cripto–FRL1–Cryptic (CFC) protein family and plays a key role in early vertebrate development and in carcinoinogenesis. Overexpression of *TDGF1* in H295R cells resulted in increased aldosterone secretion and protection from apoptosis through the activation of the PI3K/AKT signaling pathway, thus indicating a potential role of TDGF1 in the pathogenesis of APA (Williams et al., 2010). Consistently, the overactivation of the PI3K/AKT/mTOR signaling pathway has been observed in APA compared to normal adrenals (Su et al., 2013). A recent study showed that *VSNL1* is a target of the nuclear receptor SF1 in the H295R human adrenocortical carcinoma cell line (Ferraz-de-Souza et al., 2011). SF1 plays a central role in the regulation of steroidogenesis and adrenal development and therefore, this study raises the possibility that *VSNL1* is involved in the regulation of these functions.

The WNT/ β -catenin pathway has been extensively demonstrated to play a key role in both normal development (regulating stem cell maintenance and differentiation in several tissues)

and tumorigenesis, being deregulated in a wide variety of human cancers (El Wakil and Lalli, 2011). In mice, β -catenin is involved in the development and maintenance of the subcapsular zone of adrenal cortex (Kim et al., 2009) and *Wnt4* knockout mice display alteration of the differentiation of the zona glomerulosa with reduced *CYP11B2* expression and reduced aldosterone production (Heikkilä et al., 2002). On the contrary, in mice, constitutive activation of β -catenin in the adrenal cortex resulted in adrenal hyperplasia and a phenotype of PA, as well as dysplasia of the cortex with 30% of transgenic mice developing malignant adrenal tumors (Berthon et al., 2010).

The involvement of Wnt/ β -catenin in the pathogenesis of adrenocortical adenomas is now well documented (Berthon et al., 2012; Berthon et al., 2014) and activation of β -catenin (without discernable *CTNNB1* mutation) has been reported in 66% of APAs (Boukroun et al., 2011). The observation of the transcriptome profiles of the Wnt/ β -catenin pathway genes showed different clustering between APAs and control adrenals; furthermore, APAs with nuclear (active) catenin localization had different patterns of gene expression compared to those with nonactive-catenin (Boukroun et al., 2011).

A major advance in our understanding of the mechanisms involved in inappropriate aldosterone secretion has recently come from the development of the TWIK related acid sensitive 1 (*Task1*) knock out and *Task1/Task3* double knock-out mice (Davies et al., 2008; Heitzmann et al., 2008). TASK1 and TASK3 (encoded by *KCNK3* and *KCNK9* respectively) belong to the 2-pore domain K^+ channels family and are known to be expressed in mouse central nervous system (Linden et al., 2008) and adrenal cortex, in particular *KCNK3* mRNA localizes in the zona glomerulosa and zona fasciculata, while *KCNK9* expression is limited to the zona glomerulosa (Davies et al., 2008).

TASK1 and TASK3 through a high resting outward K^+ current, are the main determinants of the membrane potential of adrenal zona glomerulosa cells in rodents. Inhibition of these channels by AngII results in cell membrane depolarization and activates the intracellular cascade that finally leads to aldosterone production (Bandulik et al., 2010). In *Task1*^{-/-} mice the PA phenotype is restricted to female animals and the sex-dependent effect is completely reversed by administration of testosterone to females, which might be explained by an androgen-dependent compensatory overexpression of *Task3* (Heitzmann et al., 2008). To further elucidate the role of TASK channels in regulating aldosterone secretion the double knock out *Task1*^{-/-} *Task3*^{-/-} animal model has been generated (Davies et al., 2008). Genetic deletion of both Task1 and Task3 resulted, in male mice, in marked cell membrane depolarization in adrenal zona glomerulosa and autonomous aldosterone production which was not suppressed by dietary Na^+ loading and not fully normalized by AngII receptor blockade (Davies et al., 2008).

Using gene expression profiling, El Wakil et al. identified *Dkk3* (encoding a Wnt signaling modulator) as a differentially expressed gene in the adrenal glands of male and female *Task1*^{-/-} mice under basal conditions or after hormonal treatment (El Wakil et al., 2012). Inactivation of the *Dkk3* gene in *Task1*^{-/-} mice resulted in the extension of the PA phenotype to male animals, without inducing abnormal zonation of the adrenal cortex (El Wakil et al., 2012).

This has led several authors to hypothesize that alterations in the *KCNK* family genes might be causative of PA also in man. Interestingly, microarray analyses revealed that in normal adrenals *KCNK3* (encoding for TASK1) expression was significantly higher compared to other genes in its family, in particular, it was about 15-fold higher than *KCNK2*, and over 40-fold higher than the other members. However, in this report, no differences could be identified in *KCNK* family genes expression between APAs and normal adrenals (Nogueira et al., 2010). On the contrary, Lenzini et al. (2013) observed a lower expression of *KCNK5* (*TASK2*) in APAs compared to normal adrenal cortex at both the transcript and protein level, moreover they found that *KCNK9* was barely detectable in both normal adrenals and APAs. To explore the effect of *KCNK5* inactivation on aldosterone secretion H295R cell were transfected with a dominant negative mutant with subsequent increase in *CYP11B2* gene expression and aldosterone production.

3. Next-generation massive parallel sequencing, whole exome-sequencing, whole genome sequencing

Next generation sequencing has truly advanced the methodology to find disease causing somatic and germline mutations. Traditionally disease and phenotype causing genes had been cloned through a targeted candidate gene approach. This was based on the detailed knowledge of the underlying phenotype and biochemical changes. Based on the available clinical and biochemical data, a prediction on genes potentially involved in the pathogenesis of the disease was made and subsequently the candidate genes were cloned and sequenced. Interestingly, the approach of defining the underlying pathophysiology is still extremely valuable, when interpreting the plethora of potential changes uncovered by NGS. For example, even the most recently discovered genes involved in APA pathogenesis fall into categories one might have suspected to impact signaling leading to increased aldosterone production. Until recently, the only unbiased approach to identify disease-linked genes was positional cloning. In short this method is based on analyses of germline DNA from affected and unaffected family members, aiming to find stretches of the same haplotype in affected individuals. However, the success of this method is strongly limited by the requirement of large, informative and well characterized families. Similarly, the use of automated Sanger sequencing (which is perfectly suitable for a candidate gene approach as described above) in whole genomes sequencing is hampered by a number of limitations related to cost and throughput.

NGS satisfies the growing demand for low-cost sequencing producing massive parallel nucleotide sequencing to enable the production of an enormous volume of sequence data at relatively low cost (Metzker, 2010). Although there are differences in NGS methodologies, they share main important features (Metzker, 2010). Briefly, the first step consists of the generation of a library of randomly sheared small genomic DNA fragments, usually less than 1 Kb. These are ligated to platform-specific adapter oligonucleotides and amplified on a glass slide or microbead solid surface by a polymerase-mediated reaction. The amplification process is required in order to allow the subsequent reaction sequences to produce a signal sufficiently strong to be detected by an optical system. Unfortunately, this amplification may introduce a number of errors that are maintained through the downstream

processes (Mardis, 2011). Single-molecule template DNA sequencing systems have been developed to overcome this issue (Eid et al., 2009). The sequence of these fragments is then determined by either ligation or synthesis-based assays, which are different between each platform.

The particular challenge with NGS generated sequencing is the further bioinformatic analysis. In short, the DNA reads are aligned to a reference genome and analyzed for differences. In case of defining somatic mutations the sequences are aligned to the germline sequences of the same patient in order to enrich for somatic mutations. One shortcoming of NGS methods, however, is that they are very good in detecting single base pair mutations, very small and large deletions. Medium sized deletions, such as exome deletions may be difficult to detect, unless areas of specific interest are reviewed with specific attention.

4. Genes responsible for PA identified by NGS methods

The use of NGS has resulted in the identification of several genes underlying hereditary forms of PA and/or involved APA-associated PA, such as *KCNJ5*, *ATP1A1*, *ATB2B3* and *CACNAD1*.

4.1. *KCNJ5*

KCNJ5 encodes an inward-rectifying K^+ channel, also known as GIRK4, a member of the G-protein activated K^+ channel subfamily. The potassium channel is localized in the plasma membrane and forms a homotetramer or a heterotetrameric complex with GIRK1 (encoded by *KCNJ3*) (Krapivinsky et al., 1995).

The primary structure of the channel comprises two membrane-spanning domains, an extracellular pore forming loop (H5), cytosolic NH_2 and $COOH$ terminal domains. The H5 pore forming domain contains the ion selectivity filter of the channel, which is characterized by the signature sequence Gly-Tyr-Gly (Hibino et al., 2010). Under physiological conditions, in mice, *Kcnj5* is expressed in different tissues, including central nervous system (Wickman et al., 2000) and heart (Marionneau et al., 2005), where it is activated by acetylcholine and its conductance controls parasympathetic slowing of the heart rate and repolarization of atrial action potential (Kurachi et al., 1992), as demonstrated also by the phenotype of *Kcnj5* knockout animals (Wickman et al., 1998). In humans, *KCNJ5* is highly expressed in both adult and fetal adrenal glands and localizes to both the outer part of the zona fasciculata and the glomerulosa, (Azizan et al., 2012a; Choi et al., 2011; Monticone et al., 2012). In the HAC15 human adrenocortical carcinoma cell line, *KCNJ5* is involved in the modulation of the Ang-II stimulation of aldosterone secretion (Oki et al., 2012a), however its functional relevance in adrenal cell function in vivo remains controversial.

Through a whole exome capture approach, Choi et al. identified two *KCNJ5* somatic point mutations (p.Leu168Arg and p.Gly151Arg) in 8/22 sporadic APAs (Choi et al., 2011). The mutations, located near or within the selectivity filter of the channel, are responsible for loss of ion selectivity, facilitating Na^+ entry, chronic cell membrane depolarization, opening of the voltage-gated calcium channels, increase in intracellular calcium and constitutive aldosterone production (Choi et al., 2011) (Figure 2, panel A). Interestingly, the same

authors, identified an inherited *KCNJ5* mutation (p.Thr158Ala) with similar electrophysiological effects in a family affected by FH-III.

FH-III is a rare form of inherited hyperaldosteronism, initially described in a father and his two daughters affected by an early-onset and particularly severe form of PA, with profound hypokalemia and marked bilateral adrenal hyperplasia (Geller et al., 2008; Therien et al., 1959). All three family members displayed severe hypertension, resistant to multidrug therapy (including spironolactone and amiloride) and required bilateral adrenalectomy to obtain blood pressure reduction. Other interesting features of this family were the extremely high levels of hybrid steroids 18OH-cortisol and 18oxo-cortisol and the paradoxical increases in blood pressure and aldosterone levels after dexamethasone administration (Geller et al., 2008; Therien et al., 1959).

Over the last two years, an additional seven families and four different germline *KCNJ5* mutations have been reported (Table 1) (Mulatero et al., 2013b; Monticone et al., 2013). In most cases, with the exception of the three families with the p.Gly151Glu mutation and the index case carrying the p.Tyr152Cys substitution, affected members displayed marked bilateral adrenal hyperplasia and a severe clinical and biochemical phenotype requiring bilateral adrenalectomy. Interestingly, the two mutations (p.Gly151Glu and p.Tyr152Cys) responsible for a mild clinical and biochemical phenotype, displayed different electrophysiological effects in human embryonic kidney HEK293T cells with the p.Gly151Glu mutation having a severe impact on the channel function and the p.Tyr152Cys substitution showing a small effect on Na⁺ conductance (Mulatero et al., 2012; Scholl et al., 2012; Monticone et al., 2013).

Following the original finding of two recurrent *KCNJ5* mutations in sporadic APAs, several centers have investigated the prevalence of *KCNJ5* mutations in adrenal tumors. In addition to the originally identified p.Gly151Arg and p.Leu168Arg substitutions, three additional somatic *KCNJ5* mutations have been reported, resulting in p.Glu145Gln (Akerstrom et al., 2012), p.Glu145Lys (that was found concurrent with p.Leu168Arg *KCNJ5* mutation in a Czech patient) (Azizan et al., 2013) and p.Trp126Arg amino acid change (Williams et al., 2014), one deletion of isoleucine at residue 157 (Azizan et al., 2012b; Murthy et al., 2012) and one threonine insertion at position 149 (Kuppusamy et al., 2014) (Table 1). Furthermore, the p.Thr158Ala substitution, that has also been described as a germline mutation (Choi et al., 2011), was identified as a somatic mutation in an Italian patient (Mulatero et al., 2012). So far no APAs carrying mutations in more than one gene among *KCNJ5*, *ATP1A1*, *ATP2B3* or *CACNA1D* have been identified.

Recently, sequencing of the flanking and coding region of *KCNJ5* in peripheral blood DNA from 251 PA patients led to the identification of three heterozygous missense mutation (p.Arg52His, p.Glu246Lys and p.Gly247Arg); in addition, 12 patients resulted to be carriers of the rare SNP rs7102584, resulting in the p.Glu282Gln substitution. Functional in vitro studies demonstrated that the p.Arg52His, p.Glu246Lys and p.Glu282Gln mutations can induce cell membrane depolarization, while the p.Gly247Arg mutant was undistinguishable from the wild-type channel. Taken together this data indicate that also germline mutations in *KCNJ5* might also play a role in the pathogenesis of sporadic PA (Murthy et al., 2014).

Overall, the prevalence of *KCNJ5* somatic mutations in APAs is ~40%, varying by different centers and ethnic backgrounds (Mulatero et al., 2013b; Fernandes-Rosa et al., 2014). In particular they appear to be more frequent in Japanese PA patients compared to other populations, which might at least in part explain the high prevalence of PA in Japanese individuals (Monticone et al., 2012; Taguchi et al., 2012). In a large series of 380 APAs from France, Germany and Italy, Boulkroun et al. observed a lower prevalence of *KCNJ5* mutations in centers adopting more permissive adrenal vein sampling criteria compared to those centers using strict criteria to define successful cannulation and lateralization of aldosterone production (Boulkroun et al., 2012).

Of note, the comparison of clinical and biochemical parameters between APA carrying mutations in *KCNJ5* and noncarriers, revealed that *KCNJ5* mutations were more prevalent in female than in male patients. This sex association is reminiscent of the features seen in the *Task1* deficient mouse model of primary aldosteronism in which the phenotype is restricted to female animals as discussed above (Heitzmann et al., 2008).

KCNJ5 mutations have been associated with a young age at diagnosis (Boulkroun et al., 2012), increased preoperative aldosterone levels (Boulkroun et al., 2012) and reduced potassium levels (Monticone et al., 2012) nonetheless, this apparently more severe form of hyperaldosteronism was not associated with higher systolic and diastolic blood pressure levels. Furthermore, *KCNJ5*-mutated APAs have been reported to be larger than those in patients with wild-type *KCNJ5* (Akerstrom et al., 2012; Azizian et al., 2012a; Choi et al., 2011), and associated with a zona fasciculata-like histological phenotype (Azizian et al., 2012a).

In order to better define the molecular mechanism responsible for inappropriate aldosterone production and adrenal cell proliferation, several groups compared the transcriptome profile of APAs with and without *KCNJ5* mutations. In the largest of these studies, including 100 APAs from a cohort of French patients, hierarchical clustering showed that APAs carrying p.Gly151Arg and p.Leu168Arg substitutions were not associated with a unique molecular phenotype in comparison to tumors without *KCNJ5* mutations (Boulkroun et al., 2012).

Recently, Williams et al. (2012) reported that *VSNL1* was highly overexpressed in APAs with *KCNJ5* mutations compared to APAs without *KCNJ5* mutations. *VSNL1* is a calcium sensor protein belonging to the visinin-like protein subfamily that includes 4 other members, *VSNL2*, *VSNL3*, hippocalcin and neurocalcin- δ . In H295R cells *VSNL1* overexpression increases basal and AngII-stimulated *CYP11B2* gene expression, whereas silencing *VSNL1* negatively modulates *CYP11B2* transcription and reduces aldosterone secretion. Moreover, *VSNL1* had been shown to promote cell survival from the calcium toxicity induced by *KCNJ5* mutations (Williams et al., 2012).

To better define the influence of *KCNJ5* mutations on *CYP11B2* expression, we overexpressed wild type or mutant *KCNJ5* in the HAC15 adrenal cell culture model (Monticone et al., 2012). Both p.Gly151Arg and p.Leu168Arg mutations increased *CYP11B2* transcript levels compared to wild type *KCNJ5*, supporting a mechanistic role for these mutations in the activation of aldosterone production through increased *CYP11B2*

expression. Importantly, we also observed a large increase in two of the transcriptional regulators of CYP11B2, NURR1 (encoded by *NR4A2*) and NOR-1 (encoded by *NR4A3*). NURR-1 and NOR-1 belong to the NGFI-B family of orphan nuclear receptors and represent the final effectors of the multiple signaling pathways activated by Ang-II and K⁺ in adrenal zona glomerulosa cells (Bassett et al., 2004).

Moreover, Oki et al. demonstrated that the expression of the p.Thr158Ala mutation in HAC15 cells was responsible for a significant increase in aldosterone production and this effect was dependent on calcium influx, as it was specifically inhibited by the calcium channel blocker nifedipine and the calmodulin antagonist W-7 (Oki et al., 2012b). While the causal relationship between *KCNJ5* mutations and autonomous aldosterone secretion is now well established, it is still unknown whether *KCNJ5* mutations stimulate cell proliferation or promote APA formation. *KCNJ5* mutations have in fact been associated with a slight inhibitory effect on cell proliferation (Oki et al., 2012b) and to some degree of cell lethality induced by osmotic shock (Scholl et al., 2012). Boulkroun et al. (2013) have recently explored the relationship between adrenal cortex remodeling and *KCNJ5* mutations without finding any correlation between the nodulation score in the peritumoral tissue, vascularization and zona glomerulosa hyperplasia in the peritumoral cortex and *KCNJ5* status, indicating that *KCNJ5* mutations are not likely to be responsible for a proliferative microenvironment leading to APA formation. The observation that most APA are associated with a different degree of zona glomerulosa hyperplasia and to other micronodules outside the adenoma lead to the hypothesis that APA formation requires two subsequent alteration facilitating proliferation and deregulation of aldosterone production (Gomez-Sanchez C.E. and Gomez-Sanchez E.P., 2012). Although unilateral adrenalectomy is the preferred treatment for APA patients, the development of highly selective inhibitors of mutant *KCNJ5*, able to suppress autonomous aldosterone overproduction and perhaps stop adrenal cell proliferation would be highly attractive since more than one third of patients with APA carry mutations in *KCNJ5* (Al-Salameh et al., 2014; Fischer et al., 2014).

To summarize, *KCNJ5* mutations have been implicated in the pathogenesis of both a consistent proportion of sporadic APAs and familial hyperaldosteronism type III. The mutations, located near or within the selectivity filter of the channel result in cell membrane depolarization and autonomous aldosterone production via a pathological Na⁺ permeability through the mutated channel. The mutations are significantly more prevalent in female patients than in males, an observation that is still to be accounted for.

4.2. ATP1A1 and ATP2B3

Following the seminal discovery of the role of *KCNJ5* in sporadic and familial PA (Choi et al., 2011), Beuschlein et al. performed exome sequencing of nine *KCNJ5* mutation negative APAs from male PA patients (Beuschlein et al., 2013). Two different somatic mutations, affecting *ATP1A1* (encoding the α -subunit of the Na⁺/K⁺ ATPase) and two different in-frame deletions of *ATP2B3* (encoding the Ca²⁺ ATPase) were identified in three out of nine and two out of nine APAs, respectively (Table 1). The affected amino acids lie in positions highly conserved across species and between different members of the P-type ATPase family. The main role of the Na⁺/K⁺ ATPase, which is located at the plasma membrane, is

to maintain the resting membrane potential and cellular excitability, by exchanging three cytoplasmic sodium ions for two extracellular potassium ions against their concentration gradient (Figure 1). A direct connection between Na^+/K^+ ATPase and aldosterone secretion has been demonstrated by Yingst et al. (1999): inhibition of Na^+/K^+ ATPase by ouabain resulted in increased aldosterone production in rat adrenal glomerulosa cells, through a mechanism involving cell membrane depolarization and opening of voltage-gated Ca^{2+} channels. Moreover, *ATP1A1* haploinsufficient mice displayed 2-fold higher aldosterone levels compared to wild-type animals (Moseley et al., 2005). *ATP2B3* also belongs to the ATPase gene family and encodes the plasma membrane Ca^{2+} -ATPase 3, which removes calcium ions from the cytoplasm of eukaryotic cells (Figure 1), thus playing a key role in calcium homeostasis. In normal adrenals, Na^+/K^+ ATPase mainly localizes to the zona glomerulosa and less to the zona fasciculata whereas Ca^{2+} -ATPase 3 expression is similar throughout all the adrenal cortex (Beuschlein et al., 2013).

In vitro studies demonstrated that the two point *ATP1A1* mutations, leading to the p.Leu104Arg and p.Val332Gly substitutions, resulted in a disturbed gating mechanism, reduced affinity for potassium compared to wild-type Na^+/K^+ -ATPase and induced cell membrane depolarization (Figure 2, panel B). In fact, both leucine 104 and valine 322 have been shown to interact with glutamic acid 334, which is crucial for gating of the binding pocket and potassium ion binding. Similarly, the two *ATP2B3* deletions (c.1272_1277delGCTGGT and c.1273_1278delCTGGTC, both resulting in p.Leu425_Val426del) (Table 1), affect the same region in which lies the glutamate homologous to glutamic acid 334 in Na^+/K^+ -ATPase and are predicted to affect the calcium binding ion site (Beuschlein et al., 2013) (Figure 2, panel C). The additional sequencing of 299 APAs led to the identification of a novel *ATP1A1* in-frame deletion of five amino acids (c.299_313delTCTCAATGTTACTGT; p.Phe100_Leu104del) and a novel *ATP2B3* in-frame deletion of two amino acids (c.1277_1282delTCGTGG; p.Val426_Val427del), with an overall prevalence of *ATP1A1* and *ATP2B3* mutations of 5.2% and 1.6% respectively. Clinical and biochemical correlates of the *ATP1A1* and *ATP2B3* somatic mutations indicate predominance in males, increased plasma aldosterone concentrations and lower potassium concentrations compared with mutation-negative cases (Beuschlein et al., 2013).

Similarly Azizan et al. performed exome sequencing in ten APAs with the zona glomerulosa-like phenotype. Four out of ten adenomas harbored somatic mutations in the gene *ATP1A1* (including a newly described substitution of residues 960–963 by a serine residue; p.GluGluThrAla963Ser) (Azizan et al., 2013). The analysis of 152 APAs (including the ten samples used for exome sequencing) from UK, Dutch and Czech cohorts revealed a prevalence of *ATP1A1* somatic mutations of 11% (7/63), 2% (1/50) and 10% (4/39) respectively (overall 7.9%, 12/152). Compared to *KCNJ5* mutant APAs, tumors carrying mutations in *ATP1A1* in this report, seemed to be associated with older age at diagnosis, male sex and zona glomerulosa-like phenotype.

The prevalence of *ATP1A1* and *ATP2B3* somatic mutations has been recently explored in two large cohorts of 112 and 474 APAs collected in Italian referral hypertension centers (Williams et al., 2014) and through the European Network for the Study of Adrenal Tumors (ENS@T) respectively (Fernandes-Rosa et al., 2014). Sequencing analysis revealed a

prevalence of *ATP1A1* and *ATP2B3* mutations of 6.3% and 0.9% by Williams et al. and of 5.3% and 1.7% by Fernandes-Rosa et al. A novel *ATP1A1* somatic (c.295G>A) mutation (p.Gly99Arg) was identified; in vitro studies revealed that the p.Gly99Arg mutant displayed severely impaired ATPase and decreased cation binding, resulting in cell membrane depolarization (Williams et al., 2014).

To summarize, somatic mutations in ATPases have been found in around 7% of sporadic APAs and no clear clinical and biochemical correlates associated to the mutational status have been identified so far.

4.3. CACNA1D

CACNA1D encodes for $\text{Ca}_v1.3$, the $\alpha_1\text{D}$ (pore-forming) subunit of L-type voltage-gated calcium channels, which are composed of alpha-1, beta, alpha-2/delta, and gamma subunits. Voltage-gated calcium channels mediate the entry of calcium ions into excitable cells and are involved in a variety of calcium dependent processes, including muscle contraction and hormone release.

Azizan et al. identified 7 different somatic mutations (p.Val259Asp, p.Gly403Arg, p.Phe747Leu, p.Ile750Met, p.Arg990His, p.Pro1336Arg, p.Met1354Ile), while Scholl et al. identified 4 mutations (p.Gly403Arg, p.Phe767Val, p.Ile770Met, and p.Val1373Met) in sporadic APAs without *KCNJ5* mutations (Azizan et al., 2013; Scholl et al., 2013) (Table 1). Expression of *CACNA1D* mutations in the human embryonic kidney cell line resulted in a shift of the voltage-dependent activation to more negative potentials, suppression of the inactivation or increase in the currents, suggestive of increased Ca^{2+} entry in APAs harboring mutations in *CACNA1D* (Azizan et al., 2013) (Figure 2, panel D). Interestingly, two *de-novo* *CACNA1D* germline mutations (p.Gly403Asp and p.Ile770Met) (Table 1) were detected in two subjects with early onset PA associated with seizure and neuromuscular disease (Scholl et al., 2013). Notably, treatment with the calcium channel blocker amlodipine normalized blood pressure in one of the two affected subjects, raising the possibility of specific treatment for PA patients affected by APAs carrying mutations in *CACNA1D*. Overall, *CACNA1D* mutations have been identified in 7/64 (11%) APAs and in 12/152 (7.9%) in these two sample sets (Azizan et al., 2013; Scholl et al., 2013).

In a large series of 474 APA samples collected through the ENS@T, *CACNA1D* mutations were identified in 44 tumors (9.3%); in addition to previously reported somatic mutations, 10 novel substitutions were identified (Table 1) (Fernandes-Rosa et al., 2014). Interestingly, patients with *CACNA1D* mutations had smaller adenomas compared to those with *KCNJ5* mutations or no mutations (Fernandes-Rosa et al., 2014), as previously reported (Azizan et al., 2013; Scholl et al., 2013), while the association with other clinical and biochemical parameters was mainly dependent on the population structures of different centers (Fernandes-Rosa et al., 2014).

In conclusion *CACNA1D* mutation are an infrequent cause of APA and are responsible for a novel genetic form of primary aldosteronism associated with neurological developmental disorders.

5. Conclusions

Large scale analysis of APAs and FH, particularly NGS-based methods have significantly increased our understanding of the genetics and pathophysiology of PA. Most strikingly, all to date identified disease causing genes fall in well-defined pathways involved in regulation of aldosterone production, such as potassium sensing, membrane potential regulation and steroidogenesis. Therefore these findings do not only define new disease causing genes, but also corroborate our understanding of the pathophysiology of the regulation of aldosterone production. This also opens the door to identifying new candidate genes and candidate pathways for the remaining genetic causes for APA and for hereditary contributions to PA and ultimately will identify new approaches to diagnosis and therapy.

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Highlights

- NGS-based methods increased our knowledge of genetics and pathophysiology of PA.
- Somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* are present in 50% of APAs.
- Germline mutations in *KCNJ5* are causative of familial hyperaldosteronism type III.

Figure 1. Regulation of aldosterone production

Increase of intracellular calcium concentration is the convergence point of the main physiological stimuli of aldosterone secretion (angiotensin II – AngII-, increased plasma K^+ concentration, and adrenocorticotrophic hormone). Binding of AngII to its receptor (AT1R) increases intracellular calcium concentration by inhibiting the two-P-domain K^+ channels (TASK), which in turn leads to cell membrane depolarization and opening of voltage-gated calcium channels. Intracellular Ca^{2+} binds to calmodulin and increases the transcription of *CYP11B2* (encoding aldosterone synthase, the last rate-limiting enzyme for aldosterone production) through the activation of its main transcriptional factors (NURR-1, ATF, CREB). GIRK4, Na^+/K^+ ATPase, Ca^{2+} ATPase3 and Cav1.3 are shown in their physiological role and effects on intracellular ion homeostasis. NURR-1, nuclear receptor related 1; ATF, activating transcription factor; CREB, cAMP response element-binding protein; GIRK4, G-protein-activated inwardly rectifying K^+ channel.

Figure 2. Proposed molecular mechanism responsible for autonomous aldosterone production in adrenal cells carrying mutations in *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D*

A. GIRK4 physiologically keeps the cell in a hyperpolarized state. The mutation, located near or within the selectivity filter of the channel, cause a loss of ion selectivity, Na⁺ entry, cell membrane depolarization and activation of membrane calcium channels. **B.** Na⁺/K⁺ ATPase sustains a transmembrane concentration gradient for potassium and sodium. Mutations in the *ATP1A1* gene affecting the ion binding capacity disrupt this function leading to cell membrane depolarization. **C.** Ca²⁺ ATPase3 physiologically pumps calcium out of the cell. Loss of this function leads to an increase in intracellular calcium. **D.** Cav1.3 is the $\alpha 1$ (pore-forming) subunit of an L-type (long-lasting) voltage-gated calcium channel. Mutations in *CACNA1D* causes activation of the channel at a less depolarized state, suppresses the channel's inactivation or directly increases the currents flux of calcium.

Table 1

Somatic and germline *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* mutations in sporadic and familial primary aldosteronism

Gene	Location	Protein	Somatic mutations	Germline mutations
KCNJ5	11q24	G protein-activated inward rectifier potassium channel 4, GIRK4 Inward rectifier K ⁺ channel 3.4, KIR3.4	p.Trp126Arg p.Glu145Gln [#] p.Glu145Lys [#] p.insThr149 p.Gly151Arg p.Ile157del p.Thr158Ala p.Leu168Arg	p.Gly151Arg p.Gly151Glu p.Tyr152Cys p.Ile157Ser p.Thr158Ala
ATP1A1	1p21	sodium/potassium-transporting ATPase subunit alpha-1	p.Gly99Arg p.Phe100_Leu104del p.Leu104Arg p.Val332Gly p.GluGluThrAla963Ser	None
ATP2B3	Xq28	plasma membrane calcium-transporting ATPase 3, PMCA3	p.Val424_Leu425del [#] p.Leu425_Val426del (c.1272_1277delGCTGGT and c.1273_1278delCTGGTC) p.Val426_Val427del [#]	None
CACNA1D	3p14.3	Voltage-dependent L-type calcium channel subunit alpha-1D Voltage-gated calcium channel alpha subunit Cav1.3	p.Val259Asp p.Gly403Arg p.Ser652Leu [#] p.Leu655Pro [#] p.Tyr741Cys [#] p.Phe747Leu [#] p.Ile750Phe [#] p.Ile750Met p.Phe747Val ^{*#} p.Val979Asp [#] p.Arg990His [#] p.Lys981Asn [#] p.Ala998Ile [#] p.Ala998Val [#] p.Val1151Phe [#] p.Ile1152Asn [#] p.Pro1336Arg p.Val1338Met ^{*#} p.Met1354Ile [#]	p.Gly403Asp p.Ile770Met [*]

* Mutations p.Ile750Met, p.Phe747Val and p.Val1338Met reported in Azizan et al. and Fernandes-Rosa et al. correspond to p.Ile770Met, p.Phe767Val and p.Val1373Met respectively in Scholl et al. because of the use of different reference sequences (NM_001128839.2; NM_000720.3).

[#] Mutations that have not been functionally characterized