

REVIEW

EJE PRIZE 2013

Regulation of aldosterone secretion: from physiology to disease

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This article is based on the presentation for the *European Journal of Endocrinology* Prize Lecture 2013 at the European Society of Endocrinology Meeting at Copenhagen, Denmark

Abstract

Arterial hypertension is a major cardiovascular risk factor that affects between 10 and 40% of the population in industrialized countries. Primary aldosteronism (PA) is the most common form of secondary hypertension with an estimated prevalence of around 10% in referral centers and 4% in a primary care setting. Despite its high prevalence until recently, the underlying genetic and molecular basis of this common disease had remained largely obscure. Over the past decade, a number of insights have been achieved that have relied on *in vitro* cellular systems, wild-type and genetically modified *in vivo* models, as well as clinical studies in well-characterized patient populations. This progress has been made possible by a number of independent technical developments including that of specific hormone assays that allow measurement in small sample volumes as well as genetic techniques that enable high-throughput sequencing of a large number of samples. Furthermore, animal models have provided important insights into the physiology of aldosterone regulation that have served as a starting point for investigation of mechanisms involved in autonomous aldosterone secretion. Finally, national and international networks that have built up registries and biobanks have been instrumental in fostering translational research endeavors in PA. Therefore, it is to be expected that in the near future, further pathophysiological mechanisms that result in autonomous aldosterone secretion will be unraveled.

European Journal of Endocrinology 168 R85–R93

Clinical background

Hypertension is a major cardiovascular risk factor that affects between 10 and 40% of the general population in an age- and population-dependent manner (1, 2). The renin–angiotensin–aldosterone system (RAAS) is regulating blood pressure, fluid volume, and the vascular response to injury and inflammation (3). Chronic RAAS activation leads to persistent hypertension, setting off a cascade of inflammatory, thrombotic, and atherogenic effects eventually leading to end-organ damage (4, 5). Accordingly, numerous studies have demonstrated that elevated aldosterone levels are predictors of adverse outcome in hypertension (6), heart failure (7, 8), myocardial infarction (9), and renal insufficiency (10). Primary aldosteronism (PA) is the most common secondary form of hypertension with an estimated prevalence of around 4% in hypertensive patients in primary care and around 10% in those referred to specialized centers (11). Accordingly, a high proportion (between 11 and 20%) of PA is present in patients that are resistant to combined antihypertensive medical

treatment (12, 13). PA is currently the most common curable form of hypertension (14). Given the detrimental cardiovascular adverse effects of aldosterone excess that are independent of high blood pressure levels (15, 16, 17, 18), early detection of PA has an important impact on clinical outcome and survival.

The two predominant causes of autonomous aldosterone secretion are aldosterone-producing adenomas (APA), treated by adrenalectomy, and idiopathic hyperaldosteronism, currently managed by chronic mineralocorticoid antagonist therapy. Despite progress in the management of PA patients, critical issues related to diagnosis, subtype differentiation, and treatment of not surgically correctable forms still persist. To date, the definitive diagnosis of PA is a multistep procedure requiring expert knowledge. For example, while adrenal venous sampling is recommended to assess whether aldosterone hypersecretion is lateralized in PA patients (19), this procedure is invasive, poorly standardized, and not widely available (20, 21). Overall, compared with its importance as the major secondary cause of hypertension, the currently available tools for diagnosis

and treatment of PA are cumbersome and quite inefficient. These shortcomings relate in part to the heterogeneity of Conn's syndrome. In epidemiological terms, there appears to be a continuous spectrum from low renin hypertension, normokalemic Conn's syndrome to hypokalemic PA that makes cutoffs used for screening arbitrary. Likewise, based on histopathology of adrenal tissues resected because of unilateral PA, heterogeneity exists at multiple levels: aldosterone excess may be caused by micro- or macronodular hyperplasia or by typical adrenal adenoma (22); the adjacent adrenal cortex may be atrophic, diffuse hyperplastic, or nodular hyperplastic (23).

Physiology of aldosterone regulation

Until recently, functional data on the regulation of aldosterone secretion in rodent models had been scarce. This shortcoming had been at least in part due to the high sample volumes necessary for available commercial assays and by the lack of sensitivity to detect the very low concentrations of aldosterone. To improve this situation, we had developed a highly sensitive and non-isotopic, time-resolved fluorescence, competitive immunoassay that requires only a small sample volume for a duplicate measurement (24). Using a multiplex immunoassay technology, we could also demonstrate the feasibility of simultaneous measurement of steroid hormones in rodents (25). It is to be expected that steroid profiling by tandem mass spectrometry will further improve the determination of adrenal phenotypes in animal models (26, 27).

Following these technical requirements, we studied in detail normal aldosterone values of not only male and female animals of two common mouse strains, the C3HeB/FeJ and the C57BL/6, but also all offspring after crossbreeding of these strains (F1 and F2 generations) (28). This investigation identified C57BL/6 animals with significantly higher values than those of C3HeB/FeJ mice. Interestingly, aldosterone levels in offspring animals were also different in dependence of maternally or paternally conferred phenotype, providing indirect evidence for epigenetic mechanisms that modulate baseline aldosterone release. Likewise, it has been demonstrated that RAAS components can be a target of epigenetic modifications such as AT(1b) angiotensin receptor gene expression, which is highly dependent on promoter methylation (29). Therefore, these mechanisms could contribute to hyperaldosteronism and development of hypertension also in the adult organism.

The storage capacity for aldosterone within the adrenal zona glomerulosa cell is very limited. Therefore, tight regulation of steroidogenesis from cholesterol is required to maintain the organism's fluid and electrolyte homeostasis (30). Within this process, transcriptional activation as well as posttranscriptional

modification of steroidogenic enzymes are the major regulatory mechanisms for acute and chronic adrenocortical steroid production and release (31). The major rate-limiting steps for the synthesis of aldosterone are twofold: first, cholesterol needs to be transported to the inner mitochondrial membrane by the StAR protein (STAR) (32, 33) and secondly, conversion of 11-deoxycorticosterone to aldosterone is required by aldosterone synthase (34). Expression of *CYP11B2* that encodes aldosterone synthase is mainly regulated by extracellular potassium concentration as well as angiotensin II. Already, very subtle changes in extracellular potassium (35, 36) set off a signaling cascade that is initiated by calcium influx through T- and L-type channels. Likewise, angiotensin II increases intracellular calcium content through calcium release from intracellular stores (37, 38). Through the calcium-binding protein, calmodulin increases in intracellular calcium activates calcium/calmodulin-dependent protein kinase I and IV (CaMK I/IV) (39), which translates in the activation of a number of transcription factors such as *NURR1* (*NR4A2*), *NGFIB* (*NR4A1*), *ATF1*, and *CREB* (*CREB1*) (30, 40). These transcription factors in turn activate promoter sites of the *CYP11B2* gene and increase transcription of aldosterone synthase (30, 41). While these transcriptional-based mechanisms had initially been reported in the context of chronic stimulation experiments in a time frame of hours to days (41, 42), we could demonstrate that *in vivo* adrenal *CYP11B2* expression is upregulated even within minutes upon specific stimulation experiments (43). As activation of CaMK-dependent pathways is detectable in Conn's adenomas (44), it is prudent to expect that these pathways also play a pathophysiological role in autonomous aldosterone secretion.

In addition to these well-defined molecular pathways, we could recently demonstrate that potassium challenge of experimental animals induces a whole array of transcriptional changes in the adrenal cortex (45). Upon *in vitro* verification of transcriptional regulation of some candidate genes, we further investigated the expression pattern in Conn's adenomas. Up to this point, the available information on the candidate genes in relation to adrenal physiology is limited: *LPHN3* had been reported to be highly expressed in fetal as in adult adrenal glands (46). Another of the candidate genes, *SPP1*, coding for secreted phosphoprotein 1 had been identified in a meta-analysis of genome-wide gene expression analysis as a gene with lower expression during development of hypertension (47). As *SPP1* was also found to be expressed less in Conn's adenomas, these findings might point toward a functional relevance of *SPP1* in the pathophysiology of hypertension. While shortcomings of experimental models clearly have to be taken into account, the definition of candidate genes can serve as the starting point for further functional and translational studies.

Mouse models of PA

Potassium channels

As alluded earlier, the particular relevance of calcium-dependent signaling within the zona glomerulosa cell for the regulation of aldosterone secretion has long been appreciated (48). However, the functional significance of these molecular findings for PA only became clear when mouse models with targeted gene deletions affecting electrophysiological properties and intracellular Ca^{++} content of glomerulosa cells were evidently affected by autonomous aldosterone secretion (49, 50). A number of K^+ channels of the 2-pore domain (K2P) family are expressed in zona glomerulosa cells including TREK1, TASK1, and TASK3. In rodents, only TASK1 and TASK3 appear to be major determinants of the membrane potential of adrenal glomerulosa cells. In keeping with this notion, knockout of *Task1* alone or in combination with *Task3* (*Kcnk9*) resulted in severe sex-dependent hyperaldosteronism that was restricted to female animals. Elevation of aldosterone secretion and concomitant low renin activity could not be normalized by changes in salt intake. As a consequence of PA, female *Task1* knockout mice developed arterial hypertension and hypokalemia that could be normalized by blockade of the mineralocorticoid receptor with canrenoate (49, 50). Interestingly, on a morphological level, adrenals from *Task1* knockout animals were characterized by an ectopic expression of *Cyp11b2* within the zona fasciculata. Accordingly, some extent of ACTH dependency of aldosterone secretion could be demonstrated. While deletion of *Task3* in knockout mice also resulted in some level of aldosterone autonomy, the effects were found to be milder rather resembling those found in patients with low renin hypertension (51, 52). Taken together, these findings clearly point out that potassium channels have an impact on aldosterone secretion and can further affect adrenocortical zonation. Furthermore, the reports highlight that gender-dependent differences in phenotypic penetrance have to be taken into account in this pathophysiological scenario.

Circadian clock genes

Phenotypic characterization of mice lacking the core clock components cryptochrome-1 (*Cry1*) and *Cry2* (*Cry*-null mice) revealed the presence of salt-sensitive hypertension (53). CRY proteins act as potent transcriptional repressors that downregulate transcription of E-box enhancer-containing clock genes as well as a wide variety of clock-controlled targets. In *Cry*-null mice, autonomous aldosterone secretion was associated with chronic overexpression of *Hsd3b6* within the adrenal cortex. This particular isoform of *Hsd3* was found to be exclusively expressed in the zona glomerulosa and its overexpression to result in high

aldosterone secretion and elevated blood pressure that was inducible by high salt intake and suppressible by mineralocorticoid receptor blockage. The human equivalent of the murine *Hsd3b6*, *HSD3B1*, also displayed a glomerulosa specificity of expression providing indirect evidence for a functional significance for aldosterone secretion. So far, no evidence for transcriptional dysregulation of CRY genes or *HSD3B1* in APA has been published. However, recently it was demonstrated that with a constant daily salt intake Na^+ excretion exhibits aldosterone-dependent weekly rhythms, resulting in periodic changes in Na^+ storage (54). Therefore, it is possible that circadian clock gene-related effects within the adrenal gland could also affect aldosterone secretion in humans and disruption of this system could be involved in the molecular mechanisms underlying, for example, the bilateral form of PA.

Mutagenesis screen

An approach to develop new mouse models for a specific phenotype is to induce genetic variation by random mutagenesis of the mouse genome using *N*-ethyl-*N*-nitrosourea (ENU) as the mutagen (55). ENU is an alkylating agent that causes ethylation of nucleic acids that ultimately result in point mutations. Specifically, ENU exerts mutagenic action on DNA of pre-meiotic spermatogonial stem cells (56), i.e. A–T base pair substitutions and/or small intragenic lesions. Many of the mutant offspring produced by ENU-treated mice will, therefore, be hypomorph (partial loss of function), although gain-of-function as well as complete loss-of-function mutants can also be expected (57, 58). Several studies have demonstrated that the number of induced mutations depends on the dosage of ENU administered (59, 60) and on the number of exons and length of coding sequences (61). In the case of phenotype-driven genetics, the genomic association is accomplished using common single nucleotide polymorphisms (SNPs).

Following definition of normal values of aldosterone in wild-type mice (28), we established a mutagenesis screen for the parameter aldosterone. C3HeB/Fej male animals were treated with three injections of ENU causing random point mutations in the genome. These animals were then mated to untreated female animals and all the F1 offspring were screened for their aldosterone levels. Animals that showed increased plasma aldosterone upon repeated measurement underwent further breeding. Therefore, these founders gave rise to F2 pedigrees from which eight lines with different patterns of inheritance of hyperaldosteronism could be established (62). Phenotypic characterization of unselected offspring of ENU-treated animals demonstrated elevation of aldosterone:renin ratio and lower potassium levels in affected animals in a gender-dependent manner. These biochemical changes were associated with higher adrenal expression of *Cyp11b2* and

increased zona glomerulosa area, indicating a phenotype of glomerulosa hyperplasia. Ongoing research aims at the identification of the underlying genetic event through SNP analysis of F2 pedigrees and exome sequencing of affected inbred animals.

Genetic studies on PA in patient populations

PA patient registries

An example for a national registry dedicated to the study of PA is the German multicenter Conn's registry (www.conn-register.de). Since its inauguration in 2006 within the prospective part of the registry, detailed clinical data and high-quality biosamples for genetic, metabolic, and steroid analysis have been generated. By now, 550 patients have been enrolled, and annual recruitment is currently around 100 new patients. Based on the annotation of this cohort, a number of clinically relevant questions could be addressed including prevalence of cardiovascular (63) or renal (64) comorbidities, improvement of diagnostic algorithms (65, 66), and description of long-term outcome of PA patients (67). As the European equivalent, the European Network for the Study of Adrenal Tumors (ENS@T, www.ensat.org) has set up a registry and associated biobank and aims to improve the understanding of the genetics, tumorigenesis, and hypersecretion in patients with adrenal tumors and associated familial syndromes (68, 69). The funding by the European Science Foundation (www.ensat.org/esfensat.html) and by the European Community (www.ensat-cancer.eu) has allowed the implementation of a European database and also biobanking for PA (70), with the German Conn's registry being one of the major contributors.

Genome-wide association studies

Similar to investigations in inbred mouse strains (71) in population-based studies, the aldosterone-to-renin ratio has been recognized as an inherited trait and predictor of increased blood pressure (72, 73). Technological advances in human genomics now allow the study of a large entity of the allelic spectrum from the frequent to the very rare inherited or *de novo* genetic variants. Genome-wide association studies (GWAS) are powerful to identify common genetic susceptibility loci in human heredity and disease. GWAS uses genotyping arrays of a dense set of common SNPs to generate genotypes in large populations of cases and controls (74). This method has greatly improved our knowledge about human genetics, especially for common diseases, where both genetic and environmental factors contribute to disease susceptibility (75). In fact, in a recent GWAS,

systolic and diastolic blood pressure could be correlated with 16 novel genetic loci (76). It is to be expected that this method – when applied to large enough and well-characterized patient cohorts – will also contribute to the elucidation of genetic events resulting in idiopathic bilateral hyperplasia.

Identification of candidate genes by exome sequencing

Despite its high prevalence, so far, the genetic causes of PA have been elucidated only for the rare familial forms of the disease. Worldwide, only a few families have been identified with familial hyperaldosteronism (FH) type 1, also known as glucocorticoid-remediable hyperaldosteronism (77). The condition reflects the presence of a hybrid gene resulting from unequal crossing over between the highly homologous genes *CYP11B2* and *CYP11B1*, coding for aldosterone synthase and steroid 11 β -hydroxylase (77). This fusion produces a chimeric gene, with activity of aldosterone synthase, but tissue specificity and regulation of 11 β -hydroxylase, so that the synthesis of aldosterone is under control of ACTH rather than plasma potassium concentrations and the renin–angiotensin system.

FH2 summarizes several forms of genetically determined PA. Likewise, the morphological and functional phenotype is variable from APA to bilateral hyperplasia. A locus has been mapped on chromosome 7p22 in some but not all families (78), but the linkage area has not been resolved to any causative mutation.

KCNJ5 Next-generation sequencing applies massively parallel sequencing of clonally amplified or single-DNA molecules that are spatially separated in a flow cell. This design has allowed scaling-up of sequencing by orders of magnitude and has been used to identify mutations in the context of inherited disorders, complex human diseases, and cancer research among others (79). As benign tumors such as APAs are considered genomically stable, only a low number of somatic mutations have to be expected, further increasing the likelihood of identifying functionally relevant genetic alterations. As exemplified by the identification of *KCNJ5* mutations in APAs, exome sequencing is a powerful tool to reveal point mutations in genes that would not have necessarily been detected within a candidate gene-driven approach (80).

KCNJ5 encodes the G protein-activated inward rectifier potassium channel GIRK4. In parallel with the elucidation of FH3, the recent detection of *KCNJ5* point mutations in sporadic APAs has been a major scientific breakthrough (81). The different hot spot mutations identified in APA (p.G151R and p.L168R) and FH-3 (p.T158A) are clustered near or within the selectivity filter of GIRK4 and affect the ion selectivity of the channel, with increased sodium conductance leading to chronic membrane depolarization (Fig. 1).

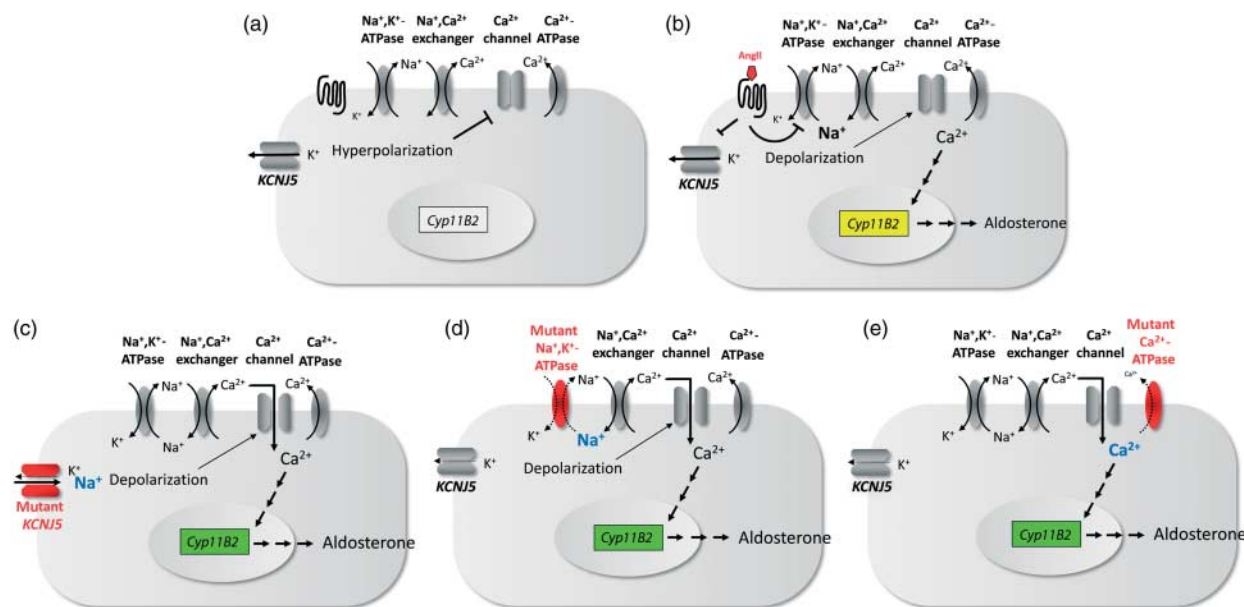


Figure 1 Schematic model of normal aldosterone regulation and genetic abnormalities leading to autonomous aldosterone secretion. (a) In the normal adrenal gland, potassium channels, calcium channels, and ATPases are involved in maintaining the hyperpolarized state of the glomerulosa cell membrane. (b) Among other stimuli, angiotensin 2 acts in part by inhibiting function or expression of potassium channels and Na^+, K^+ -ATPase, which results in cell membrane depolarization, opening of voltage-gated Ca^{++} channels, and activation of calcium signaling, the main trigger for increase in *Cyp11B2* expression and aldosterone production. Mutations in *KCNJ5* (c) and *ATP1A1* (d) result in inappropriate depolarization of glomerulosa cells, while mutations in *ATP2B3* (encoding the Ca^{++} -ATPase) (e) are predicted to lead to elevation of intracellular Ca^{++} through abnormal calcium recycling.

These changes are responsible for increased calcium influx into the cell leading to constitutive secretion of aldosterone and possibly cell proliferation (80, 82). Since its first publication, large collections of sporadic APAs have been screened worldwide demonstrating – with one exception (83) – that *KCNJ5* mutations are present in 34–65% of APAs (for references, see Table 1).

Notably, *KCNJ5* mutations appear to be isolated events in the progression toward APA, as they were not identified within the adjacent adrenal cortex of an APA carrying a mutation. These findings are in line with the concept that *KCNJ5* mutations may occur within a proliferating and hyperplastic cortex, leading to growth advantage, clonal expansion, and APA formation in a considerable number of cases. Transcriptome analysis performed on a large number of APA samples showed that *KCNJ5* mutations were not associated with a unique molecular signature, as could have been expected from mutations acting on key signaling pathways or master transcriptional regulators (81). Rather, they may represent one of several possible mechanisms triggering, via increased Ca^{2+} signaling and/or other pathways, increased cell proliferation, and aldosterone production.

ATP1A1 and ATP2B3 We recently set out to identify additional genetic causes of autonomous aldosterone secretion. To achieve this goal, we implemented exome sequencing in APA and matched control tissue from

nine male patients that were characterized by hypokalemic PA and did not possess somatic *KCNJ5* mutations (84). Within this small patient group, we identified somatic mutations in two genes that had not yet been related to PA: *ATP1A1*, coding for the α subunit of the Na^+, K^+ -ATPase; and *ATP2B3*, coding for the plasma membrane calcium-transporting ATPase 3 (PMCA3). The identified mutations affected highly conserved regions within the proteins required for direct interaction with transported potassium for Na^+, K^+ -ATPase and calcium for PMCA3 (Fig. 1). *In vitro* studies showed that the identified *ATP1A1* mutations

Table 1 Prevalence of somatic *KCNJ5* mutations in aldosterone-producing adenomas.

References	n	<i>KCNJ5</i> -mutated tumors (%)	Special clinical features
(81)	380	34	Preferentially females; lower age
(85)	351	47	Preferentially females; larger tumors
(86)	73	41	Larger tumors, lack of postural aldosterone response
(83)	53	2	–
(87)	47	38	–
(88)	46	43	Tumors larger, lower age
(89)	23	65	–
Total	973	38.8	

significantly reduced the physiological Na^+,K^+ -pump activity, as well as the apparent affinity of Na^+,K^+ -ATPase for K^+ . In *ex vivo* experiments, electrophysiological measurements on primary cultured adenoma cells with different *ATP1A1* mutations revealed inappropriate depolarization of cells with ATPase mutations. Given the highly specific position of mutations identified in both genes, a gain-of-function mechanism would be predicted from a genetic point of view. Based on the *in vitro* and *ex vivo* results, deleterious impact of the identified mutation on the pump activity of the Na^+,K^+ -ATPase enzyme is evident. However, it is conceivable that similar to the mechanisms observed for *KCNJ5*, the mutations could, in addition, result in cation leakage of the affected ATPases, followed by cellular depolarization. As overexpression of mutated ATPases is toxic in most cellular systems, this hypothesis needs to be further tested in appropriate models.

In a large collection of 309 APA samples, we demonstrated that somatic *ATP1A1* mutations were present in 5.2% and *ATP2B3* mutations in 1.6% of patients. We were not able to identify germline mutations in *ATP1A1* or *ATP2B3* in patients with FH or with the sporadic bilateral form of the disease. Both Na^+,K^+ -ATPase and Ca^{2+} -ATPase are required for a multitude of vital cellular processes that rely on the generation of electrochemical gradients. Therefore, it is to be expected that mutations in these genes would not be compatible with life, which explains the lack of systemic mutations.

No Conn adenoma was identified that carried both *KCNJ5* and ATPase mutations, providing indirect evidence that either mutation is sufficient to bring about the clinical phenotype. Although mutation carriers showed increased plasma aldosterone and lower potassium compared with non-carriers, similar to what has been observed in *KCNJ5* mutation carriers, ATPase mutations displayed male dominance. Consistent with a more severe phenotype, carriers of ATPase-mutated tumors had higher preoperative aldosterone levels and significantly lower serum potassium concentrations. In contrast, other clinical features such as blood pressure, tumor size, and urine albumin secretion among others were not different between the groups.

Taken together, these findings highlight the power of high-throughput techniques that are able to identify new genetic mechanisms involved in PA. In addition to the mechanistic insights, these findings might hold the promise for potential therapeutic targets as well as novel biomarkers that could aid in personalized treatment decisions.

Conclusion

The last years have witnessed the elucidation of molecular and genetic mechanisms that ultimately result in autonomous aldosterone secretion and PA.

A number of rodent models with a PA phenotype could be added to the experimental armamentarium for future functional and interventional studies. In parallel, a new form of FH could be elucidated and an increasing proportion of Conn's adenomas could be attributed to specific somatic genetic alterations. This development opens up entirely different areas of research, which might ultimately lead to new biomarkers and molecularly targeted treatment for PA patients. It is to be expected that further candidate genes will be identified in patients with type II FH as well as in sporadic APA.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

Funding

This work was supported in part by the Deutsche Forschungsgemeinschaft (Re 752/17-1).

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Received 18 March 2013

Accepted 8 April 2013