Investigations of somatic and germline mutations in primary aldosteronism

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1 Abstract

Primary hyperaldosteronism is one subform of secondary hypertension that can be caused amongst others by bilateral adrenal hyperplasia or aldosterone-producing adenomas (APAs). Recurrent somatic mutations in the genes *KCNJ5*, *CACNA1D*, *ATP1A1, ATP1A2* and *CTNNB1* have been identified in APAs. While some APA mutations are well characterized, relatively small amounts of data are available on others. This work contains a study in which the mutational status of APAs is matched to the corresponding patient and tumor characteristics. It identifies *KCNJ5* as the most frequently mutated gene in our cohort (37.1%). *KCNJ5* mutant tumors were larger, appeared less dense in a CT scan and had a zona fasciculata-like appearance. Patients with *KCNJ5*-mutated APAs were more often female, while male predominance was observed for the other mutations.

Many studies concur with our finding that *KCNJ5* is the most frequently mutated gene in APAs. Thereby, the G151R and L168R alterations account for most cases, while other mutations are very rare. The second study included in this work identifies macrolides as blockers of KCNJ5 G151R and L168R channels, albeit the KCNJ5 wild type remains unaffected by these compounds. Roxithromycin, the most promising substance out of this class, is able to reduce both aldosterone production and aldosterone synthase expression in adrenocortical cells expressing KCNJ5 mutant channels. Aldosterone synthase expression was similarly reduced by the non-antibiotic macrolide PLUX 38. In the future, blockers of mutant KCNJ5 channel might present an option in the diagnosis and treatment of patients with *KCNJ5* mutant APAs.

Familial forms of hyperaldosteronism have been traced back to alterations in the aldosterone synthase gene *CYP11B2* as well as to mutations in the genes *KCNJ5*, *CACNA1D* and *CACNA1H*. However, sometimes the underlying genetic causes remain unknown. The third study included in this work substantiates the role of the germline *CACNA1H M1549V* mutation as a cause of familial hyperaldosteronism type IV. The variant was first discovered in patients with early-onset primary hyperaldosteronism and electrophysiological studies suggested a gain of function of the mutant channels. Our investigations show that aldosterone production and aldosterone synthase expression is increased in adrenocortical cells transfected with mutant *CACNA1H* in comparison to the wild type. This effect can be reduced or even abrogated by the T-type calcium channel blocker mibefradil. Another mutation studied in this work is *CACNA1H S1073C*, which

was identified in monozygotic twin sisters with hyperaldosteronism and their family members. Transfection of adrenocortical cells with *CACNA1H S1073C* does not result in differences in aldosterone synthase expression or aldosterone production in comparison to the wild type, which suggests that the mutation is not disease-causing.

The studies included in this work enhance our understanding of the underlying mechanisms of primary hyperaldosteronism, by confirming the role of the *CACNA1H M1549V* mutation in familial hyperaldosteronism and by linking APA mutations to a specific phenotype. They provide new impulses for diagnosis and treatment of primary hyperaldosteronism, especially through the identification of macrolides as blockers of mutant KCNJ5 channels. Nevertheless, in some occasions, the underlying genetics of primary hyperaldosteronism remain unclear, as in the case of the patients with the *CACNA1H S1073C* mutation.

2 Zusammenfassung

Primärer Hyperaldosteronismus ist eine Form der sekundären Hypertonie, die beispielsweise durch bilaterale Nebennierenhyperplasie oder Aldosteron-produzierende Adenome (APA) verursacht wird. Wiederkehrende somatische Mutationen in den Genen *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3* und *CTNNB1* wurden in APA identifiziert. Für einige dieser Mutationen ist ein typischer klinischer Phänotyp klar etabliert, wohingegen für andere die Datenlage dünn ist. Diese Arbeit beinhaltet eine Studie, die eine Verbindung zwischen dem APA Genotyp und den entsprechenden Tumor- und Patientenmerkmalen herstellt. Die Studie identifiziert *KCNJ5* als das am häufigsten mutierte Gen in unserer Kohorte (37.1%). Die betreffenden Tumoren waren größer, hatten ein weniger dichtes Erscheinungsbild im CT Scan und ähnelten in ihrer Histologie häufig der Zona fasciculata. APA-Patienten mit *KCNJ5* Mutation waren häufiger weiblich, wohingegen die Patienten mit anderen Mutationen überwiegend männlich waren.

Zahlreiche Studien stimmen darin überein, dass *KCNJ5* das am häufigsten mutierte Gen in APA ist. Dabei decken die *G151R* und die *L168R* Mutation die meisten Fälle ab, während andere Mutationen selten sind. Diese Arbeit beinhaltet eine zweite Studie, die Makrolide als Blocker mutierter KCNJ5 Kanäle identifiziert, wobei Wildtypkanäle nicht beeinträchtigt werden. Roxithromycin, die vielversprechendste Substanz aus dieser Stoffklasse, senkt sowohl die Aldosteronproduktion als auch die Aldosteron-Synthase Expression in Nebennierenkarzinomzellen mit mutiertem KCNJ5. Die Aldosteron-Synthase Expression wurde durch das nicht-antibiotische Makrolid PLUX38 in ähnlicher

Weise gehemmt. In der Zukunft könnten Blocker mutierter KCNJ5 Kanäle in der Diagnose und Behandlung von APA Patienten mit *KCNJ5* Mutationen eingesetzt werden.

Familiäre Formen des primären Hyperaldosteronismus konnten zum Teil auf Veränderungen im Aldosteron-Synthase Gen *CYP11B2*, sowie in *KCNJ5*, *CACNA1D* und *CACNA1H* zurückgeführt werden. Nichtsdestotrotz sind die zugrundeliegenden genetischen Faktoren in manchen Fällen unbekannt. Die dritte Studie in dieser Arbeit untermauert die Rolle von *CACNA1H M1549V* als Ursache von familiärem Hyperaldosteronismus Typ IV. Die Mutation wurde in Patienten entdeckt, die bereits in ihrer Kindheit an primärem Hyperaldosteronismus erkrankt waren. Elektrophysiologische Studien deuteten auf einen Gain of Function der mutierten Kanäle hin. Unsere Untersuchungen zeigen, dass Aldosteron-Synthase Expression und Aldosteronproduktion in Nebennierenkarzinomzellen mit CACNA1H M1549V im Vergleich zum Wildtyp erhöht sind. Dieser Effekt kann durch den T-Typ Calciumkanalblocker Mibefradil abgeschwächt oder gar eliminiert werden. Eine weitere Mutation, die in dieser Arbeit untersucht wird, ist *CACNA1H S1073C*. Diese Variante wurde in eineiigen Zwillingen mit primärem Hyperaldosteronismus und deren Familienmitgliedern entdeckt. Die Transfektion von Nebennierenkarzinomzellen mit *CACNA1H S1073C* resultiert weder in einer erhöhten Aldosteronproduktion noch in einer erhöhten Aldosteron-Synthase Expression im Vergleich zum Wildtyp. Daher ist anzunehmen, dass die *CACNA1H S1073C* Mutation nicht krankheitsverursachend ist.

Die in dieser Arbeit enthaltenen Studien verbessern unser Verständnis der dem primären Hyperaldosteronismus zugrundeliegenden Mechanismen, indem sie die Rolle der *CACNA1H M1549V* Mutation in familiärem Hyperaldosteronismus bestätigen und eine Verknüpfung zwischen Mutationen in APA und einem spezifischen Phänotyp herstellen. Sie vermitteln neue Impulse für die Diagnose und Behandlung von primärem Hyperaldosteronismus, insbesondere durch die Identifizierung von Makroliden als Blocker mutierter KCNJ5 Kanäle. Nichtsdestotrotz bleiben die zugrundeliegenden genetischen Faktoren in primärem Hyperaldosteronismus zuweilen ungeklärt, wie im Fall der Patienten mit einer *CACNA1H S1073C* Mutation.

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5 Symbols and Abbreviations

ACE	angiotensin-converting enzyme
ACTH	adrenocorticotropic hormone
Ad-4,5	adrenal 4,5
ADH	anti-diuretic hormone
AngI/II	angiotensin I/II
ANP	atrial natriuretic peptide
APA	aldosterone-producing adenoma
APCC	aldosterone-producing cell clusters
ARMC5	armadillo repeat-containing 5
ARR	aldosterone-to-renin ratio
$AT_{1/2}$	angiotensin receptor 1
ATP1A1	sodium/potassium-transporting ATPase subunit alpha-1
ATP2B3	plasma membrane calcium-transporting ATPase 3
AVS	adrenal vein sampling
CACNA1x	voltage-gated calcium channel subunit alpha1x
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
Ca_v	voltage-gated calcium channel
CCS	Cosmic Calf Serum
cDNA	complementary deoxyribonucleic acid
C_T	threshold cycle
CT	X-ray computed tomography
CTNNB1	beta-catenin
CYP	cytochrome P450
DACH1	dachshund homolog 1
DAG	diacylglycerol
dbSNP	Database of Single Nucleotide Polymorphisms
DHEA	dehydroepiandrosterone
DNA	deoxyribonucleic acid
EA	East Asia
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENaC	apical epithelial Na^+ channel
ER	endoplasmic reticulum
ESC	European Society of Cardiology
ESH	European Society of Hypertension
EU	European Union
EU	Europe
ExAC	Exome Aggregation Consortium
FH	familial hyperaldosteronism
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GIRK	G protein-coupled inwardly-rectifying potassium channel
GNRHR	gonadotropin-releasing hormone receptor
GRA	glucocorticoid-remediable hyperaldosteronism
HSD3B2	3-beta- hydroxysteroid-dehydrogenase type 2
IMM	inner mitochondrial membrane
IP_3	inositol-1,4,5-trisphosphate
IP_3R	inositol trisphosophate receptor
KCNJ	*gene name encoding members of the Kir family*
Kir	inwardly-rectifying potassium channel
LH-CGR	luteinizing hormone–chorionic gonadotropin receptor
MC2R	melanocortin 2 receptor
N/A	not available
N/D	not determined
NA	North America
NBRE	nerve growth factor IB response element
NCC	sodium chloride symporter
NGFIB	nerve growth factor IB
NURR	nuclear receptor-related protein
OMM	outer mitochondrial membrane
ORAI1	calcium release-activated calcium channel protein 1
p.	page
PAC	plasma aldosterone concentration
PASNA	primary hyperaldosteronism with seizures and neurologic abnormalities
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	primary hyperaldosteronism
PIP_2	phosphatidylinositol 4,5-bisphosphate
PK	protein kinase
PL	phospholipase
PRA	plasma renin activity
RAAS	renin-angiotensin-aldosterone system
RGS	regulator of G protein signaling
RNA	ribonucleic acid
ROMK	renal outer medullary potassium channel
rRNA	ribosomal ribonucleic acid
rt-qPCR	real time quantitative polymerase chain reaction
SEM	standard error of the mean
SNP	single nucleotide polymorphism
StAR	steroidogenic acute regulatory protein
STIM1	stromal interaction molecule 1
STR	short tandem repeat
TM	transmembrane
TRPC	canonical transient receptor potential channel
UKD	Universitätsklinikum Düsseldorf
ZF	zona fasciculata
ZG	zona glomerulosa
ZR	zona reticularis
α_{1x}	voltage-gated calcium channel subunit alpha1x

6 Introduction

This work addresses genetic and pharmacological aspects of primary hyperaldosteronism (PHA), one of the known underlying causes of hypertension. This requires an understanding of the normal regulation of blood pressure, especially aldosterone homeostasis, and its dysregulation. Furthermore, background information on different known causes of PHA and their underlying genetics, considering the physiological and pathophysiological roles of the most important genes, is crucial. Putting the topic into perspective by gaining an overview about the current state of the art in medical care, information on epidemiology, diagnosis and treatment options is also important. In addition, a review of suitable models for the investigation of PHA is vital for the purpose of this work. All these aspects will be covered in this introductory section.

6.1 Blood pressure and its regulation

The force circulating blood exerts on the surface of arterial walls is referred to as blood pressure. It is usually measured in mmHg and expressed as systolic and diastolic blood pressure, the former referring to the maximum pressure during a heartbeat and the latter representing the minimum pressure between two heartbeats (see e.g. [1,2]).

Blood pressure is determined by stroke volume and heart rate, as well as resistance. The latter is strongly dependent on blood vessel diameter and viscosity of the blood[1]. The stroke volume is the volume of blood ejected from the heart during one beat[3].

6.1.1 Systemic regulation in the normotensive body

Blood pressure homeostasis can occur via fast acting mechanisms and more slowly acting ones. One example of a rapid response mechanism is the baroreflex, which is mediated by stretch sensors located in the carotid bodies as well as the aortic arch. These receptors affect blood pressure through regulation of sympathetic and parasympathetic activity, affecting vasoconstriction, heart rate and contractile force[4].

For this work mechanisms exerting more sustained effects are of special interest, in particular the renin-angiotensin-aldosterone system (RAAS).

6.1.1.1 Renin-angiotensin-aldosterone system (RAAS)

The RAAS is one of the slower acting mechanisms of blood pressure homeostasis. Renin is synthesized from its precursor prorenin, and it is stored in granules of juxtaglomerular cells[5]. Juxtaglomerular cells in the kidney release renin in response to: a) low blood pressure detected by a renal baroreceptor mechanism, most likely through reversal of

high pressure-induced suppression, b) sympathetic signaling through ß1 adrenergic receptors, and c) as a reaction to prostaglandins released by macula densa cells of the distal convoluted tubule upon detection of a decreased luminal Na^+ load[5] (Figure 1).

Renin is an aspartyl protease, which converts angiotensinogen, a 452 amino acid protein secreted into the bloodstream mostly by liver cells, into angiotensin I (AngI)[6]. AngI, a decapeptide, is cleaved into the 8 amino acid peptide angiotensin II (AngII) by the angiotensin-converting enzyme (ACE) expressed on the surface of a variety of cells, e.g. vascular endothelial cells in the lung[6,7] (Figure 1). AngII acts as a direct vasoconstrictor (Figure 1), affecting smooth muscle cells in blood vessels by binding to the angiotensin receptor 1 (AT_1), causing G_q-mediated activation of phospholipase C (PLC), which generates inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol-4,5-bisphosphate (PIP_2). This then leads to an inositol-1,4,5-trisphosphate (IP_3)-dependent rise in cytoplasmic Ca^{2+} levels, causing contraction. Ca^{2+} influx through channels in the membrane contributes to that effect[8]. In addition, AngII increases Na^+ and therefore water retention in the kidney by activating the Na^+/H^+-exchanger in the proximal tubules[9] (Figure 1). It also mediates a negative feedback mechanism via AT_1, which directly inhibits renin secretion from juxtaglomerular cells[10] (Figure 1). Besides this autoregulatory effect, RAAS inhibition can be achieved through atrial natriuretic peptide (ANP), a hormone secreted from the heart upon detection of low blood volume, which i.a. modulates secretion of renin[11] (also see 6.1.2.1.4).

Moreover, AngII stimulates the adrenal gland via AT_1 binding to produce the mineralocorticoid hormone aldosterone, which then promotes retention of Na^+ and thus water into the blood as well as K^+ excretion into the urine in the collecting duct and late distal convoluted tubule of the kidney[12] (Figure 1). Via interaction with the mineralocorticoid receptor, aldosterone induces expression and activation of basolateral Na^+/K^+-ATPase as well as upregulation of the apical epithelial Na^+ channel (ENaC), thus promoting the reabsorption of Na^+ into the bloodstream with water reabsorption as a compulsory result due to osmosis[12–14]. In addition, aldosterone increases the expression of serum and glucocorticoid-induced kinase SGK1, which, besides activating the Na^+/K^+-pump and ENaC, also increases membrane abundance of the renal outer medullary potassium channel (ROMK) and thus facilitates K^+ clearance into the urine[12,14,15]. Furthermore, SGK1 plays a role in upregulation of sodium chloride symporter (NCC) activity, a transporter whose activity and expression have been linked to aldosterone stimulation in

the distal convoluted tubule, thus increasing the intracellular Na^+ concentration followed by reabsorption into the bloodstream by Na^+/K^+-ATPase[16–18]. A more detailed description of aldosterone secretion mediated by a range of stimuli as well a more extensive insight into the underlying molecular mechanisms will be provided in section 6.1.2.

Furthermore, AngII stimulates the pituitary gland to release the anti-diuretic hormone (ADH), also called vasopressin. ADH is a vasoconstrictor hormone, which promotes water retention in the kidney through insertion of aquaporin 2 water channels into the apical membranes of epithelial cells in the collecting duct. Additionally, ADH mediates increased aquaporin 2 transcription[19,20] (Figure 1).

To summarize, the RAAS affects blood pressure by regulation of overall vascular resistance through vasoconstriction and by controlling stroke volume though retention of water, either directly or indirectly as a side effect of Na^+ retention. In addition, it provides a powerful autoregulatory mechanism through inhibition of renin release by AngII.

6.1.2 Molecular mechanisms of aldosterone production on cellular level

The adrenal gland is structured into an inner medulla and an outer cortex, which is divided into three zones (from the center outwards): the zona reticularis (ZR), zona fasciculata (ZF) and zona glomerulosa (ZG)[21]. The zones of the adrenal cortex are involved in steroidogenesis: The glucocorticoid cortisol is synthesized in the ZF, while the human ZR produces precursor androgens, like dehydroepiandrosterone (DHEA) and androstenedione. Nevertheless, ZR cells are also able to produce cortisol. The mineralocorticoid aldosterone is produced in ZG cells[21,22].

6.1.2.1 Modulators of aldosterone production

The most important modulators of aldosterone secretion under physiological conditions are AngII, adrenocorticotropic hormone (ACTH), as well as increased extracellular K^+ concentration acting as positive stimuli, while ANP acts as an inhibitor (compare e.g. [23]).

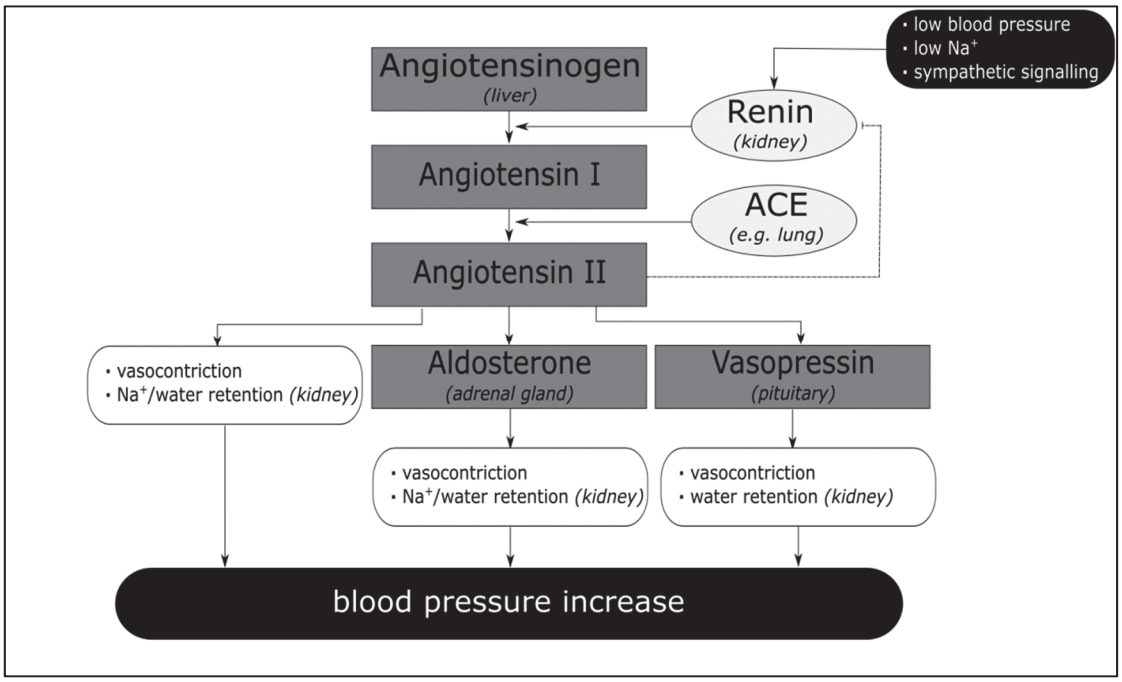


Figure 1. The renin-angiotensin-aldosterone system (RAAS). Angiotensinogen is released by cells of the liver. It is converted to angiotensin I by renin. Renin release from the juxtaglomerular cells of the kidney is promoted by low blood pressure, a low luminal Na^+ concentration and sympathetic signaling. Angiotensin I is converted to angiotensin II by angiotensin-converting enzyme (ACE) found e.g. in the lung. Angiotensin II promotes vasoconstriction and Na^+/water retention in the kidney as well as secretion of aldosterone from the adrenal gland and release of vasopressin from the pituitary. These hormones promote vasoconstriction, as well as Na^+ and/or water retention. These effects mediated by angiotensin II ultimately lead to an increase in blood pressure. Furthermore, angiotensin II-mediated inhibition of renin release constitutes a negative feedback mechanism (compare section 6.1.1.1). Adapted from Dutta, Söderkvist and Grimm, 2016[24].

6.1.2.1.1 Angiotensin II

AngII mediates its effects via binding to G protein-coupled angiotensin receptors, most importantly AT_1 and AT_2. Both AT_1 and AT_2 are expressed in the human adrenal gland. While AT_1 is mainly expressed in the ZG, AT_2 is chiefly expressed in the medulla, but it can also be detected in the ZG[25]. On the one hand, AT_1 activation promotes aldosterone production and cell growth as well as proliferation[26]. AT_1-mediated growth-promoting effects have been detected in adrenal cells, including the ZG[27–29]. On the other hand, binding to AT_2 inhibits cell growth and stimulates apoptosis and has been suggested to play a role in fetal development and tissue regeneration, since its expression is elevated under these circumstances[26,30]. In the human adrenal, AT_2 distribution differs between fetal and adult glands: While in adults, it is predominantly expressed in the medulla, it is expressed throughout the human fetal adrenal gland[31].

The effects of AT_1 binding in the ZG cell are mediated by the heterotrimeric G proteins G_i and G_q. They cause inhibition of adenylyl cyclase and affect voltage-gated calcium channels[23,26,32–34]. They also activate phospholipase C (PLC), resulting in formation of

IP_3 and diacylglycerol (DAG)[23,26,35,36]. Formation of IP_3 results in Ca^{2+} release from intracellular stores through binding to inositol-3-phosphate receptors (IP_3R) found on endoplasmic reticulum (ER) vesicle membranes with light attachment to the plasma membrane in adrenocortical cells[23,37–39]. Depletion of intracellular Ca^{2+} stores leads to Ca^{2+} influx from the extracellular space and expression of proteins thought to be involved in this process, i. e. stromal interaction molecule 1 (STIM1), calcium release-activated calcium channel protein 1(ORAI1) and canonical transient receptor potential channels (TRPC), has been reported in the H295R adrenal carcinoma cell line[23,40].

AngII also induces Ca^{2+} influx from extracellular space via voltage-gated Ca^{2+} channels, which requires depolarization of the cell membrane. Depolarization is achieved by AngII-induced inhibition of the Na^+/K^+-ATPase, increasing open probability of non-selective cation channels and inhibition of K^+ conductance through channels active at resting potential. Inhibition of TASK channels by AngII has been suggested to be an important contributor to the latter effect[23,41–43].

Modulation of receptor-mediated aldosterone production can occur via sensitization or desensitization, which can be achieved via regulation of receptor abundance on the cell surface or controlling the sensitivity of the receptor itself[23]. Both effects have been observed with regard to the AT_1 receptor[44–47]. However, *in vivo*, AngII preexposure, as well as sodium depletion appear to increase the aldosterone response to AngII[48,49]. This has been suggested to be an indirect effect that requires the presence of other circulating hormones, like ACTH[23].

6.1.2.1.2 Adrenocorticotropic hormone

ACTH stimulates the ZG cell by binding to the melanocortin 2 receptor (MC2R), which is G protein-coupled[22]. G proteins then mediate activation of adenylyl cyclase, thereby upregulating production of cAMP[22,50]. ACTH-induced cAMP production can be inhibited by AngII in ZG cells[51]. In addition to increasing cAMP production, ACTH binding to MC2R leads to depolarization, partly attributed to inhibition of K^+ conductance, and thus causes Ca^{2+} influx through activation of L-type voltage-gated calcium channels, which can be mediated by cAMP and protein kinase A (PKA)[22,52–54]. ACTH-dependent cAMP formation and aldosterone production in the ZG appear to be greatly relying on Ca^{2+} [ref23,55]. Interestingly, ACTH stimulation has been shown to induce a short-term increase in aldosterone levels, but *in vivo* experiments in rats and humans suggest that it can reduce aldosterone production in the long term[56–58].

6.1.2.1.3 Potassium

Increasing extracellular K^+ concentration leads to membrane depolarization. This causes Ca^{2+} influx from extracellular space through voltage-gated calcium channels, generating an intracellular Ca^{2+} signal[23,59,60]. The highly sensitive response of ZG cells to changes in the extracellular K^+ concentration appears to be based mainly on their large K^+ conductivity, as well as on the involvement of low voltage-gated calcium channels, which generate oscillations in the membrane potential[61–63].

6.1.2.1.4 Atrial natriuretic peptide

ANP, a negative regulator of aldosterone production, increases K^+ conductance of the cell through activation of potassium channels. Accordingly, ANP secures membrane hyperpolarization. It further opposes Ca^{2+} signaling by inhibition of T-type voltage-gated calcium channels[23,64–66].

6.1.2.2 Enzymes involved in aldosterone production and their regulation

Ca^{2+} signaling is a common prerequisite of aldosterone production induced by different stimuli. ZG cells do not have hormone stores but produce steroids upon stimulation[23]. The steps of aldosterone production in the adrenal ZG are depicted in Figure 2. The general rate-limiting step of steroidogenesis is the formation of pregnenolone from cholesterol. This requires transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, which is mediated by the steroidogenic acute regulatory protein (StAR)[23]. Expression, as well as activating phosphorylation of StAR are promoted by ACTH, AngII and K^+, while ANP reduces *StAR* transcription[23,67–70].

6.1.2.2.1 Aldosterone synthase (*CYP11B2*)

Aldosterone synthase (CYP11B2) catalyzes the second rate-limiting step in aldosterone production (compare [71]). *CYP11B2* is expressed in the ZG alone, thus limiting aldosterone production to this region of the adrenal cortex[56]. In the adrenocortical carcinoma cell line H295R, stimulation with AngII and K^+ leads to increased *CYP11B2* expression[72,73] (compare Figure 5 A and B for a schematic depiction of AngII-mediated effects). Induction by both stimuli appear to follow different kinetics: Upon stimulation with 100 nm AngII, *CYP11B2* expression in H295R cells peaks after around 12 h and then declines again, while upon stimulation with 10 mM KCl, upregulation of expression was still observed after 42 h[74].

Ca^{2+}-mobilizing agents have been shown to increase expression of the encoding gene *CYP11B2*, an effect that is mediated by Ca^{2+}, calmodulin and finally Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI), and to a lesser extent CaMKIV[75–77].

CaMKI might activate transcription factors that bind to CRE-elements in the *CYP11B2* promoter region through phosphorylation. However, other regulatory elements also play a role (Ad-5, Ad-4 and NBRE-1)[56,73,75,76,78,79]. While overexpression of NGFIB and NURR1 (binding both Ad-5 and NBRE-1) increase *CYP11B2* reporter expression, SF1 (binding to Ad-4) does not appear to be a major regulator, but reports on its effects vary[78,80–82*]. Other steroidogenic enzymes that are upregulated by NGFIB transcription factors are HSD3B2 and CYP21 (encoding 3-beta-hydroxysteroid-dehydrogenase type 2 and 21-hydroxylase, respectively)[83].

Protein kinase C (PKC) appears to inhibit *CYP11B2* expression and aldosterone production[23,82,84]. However, protein kinase D (PKD), a positive regulator of *CYP11B2* expression and aldosterone production, is activated through phosphorylation by PKC upon stimulation with AngII in H295R cells[85].

Another gene that has recently been suggested to play a role in the regulation of *CYP11B2* expression and aldosterone secretion is the putative tumor suppressor gene *dachshund homolog 1* (*DACH1*). Silencing of *DACH1* in H295R cells increased *CYP11B2* expression and aldosterone production while overexpression of *DACH1* decreased aldosterone production[86]. A mutation in this gene has been identified in two patients with primary aldosteronism and their family members, whose cases will be discussed in this work.

6.2 Classes of ion channels of major interest for this work

Ion channels play an important role in ion transport across cell membranes and their function heavily influences cellular ion homeostasis, i.e. the maintenance of an ion's concentration gradient within the cell and its surroundings[87,88]. This balance is the basis for the membrane resting potential of each cell[87]. Ion channel activity is closely linked to the activation of signaling cascades within the cell, either through adapter molecules or directly by increasing intracellular concentrations of the messenger ion Ca^{2+} [ref87,88]. Consequently, ion channel activity is crucial in cellular function.

* NGFIB: nerve growth factor IB; NURR1: nuclear receptor-related protein 1; NBRE-1: nerve growth factor IB response element 1; Ad-4,5: adrenal 4,5

This work comprises functional and pharmacological studies of ion channels involved in blood pressure regulation and dysregulation. These channels, i.e. Kir3.4/GIRK4/KCNJ5 and $Ca_v3.2$/CACNA1H, belong to the classes of G protein-coupled inwardly-rectifying potassium channels and voltage-gated calcium channels. A brief introduction into these classes of ion channels as well as their assumed functions in the normal adrenal gland will be presented in the following sections.

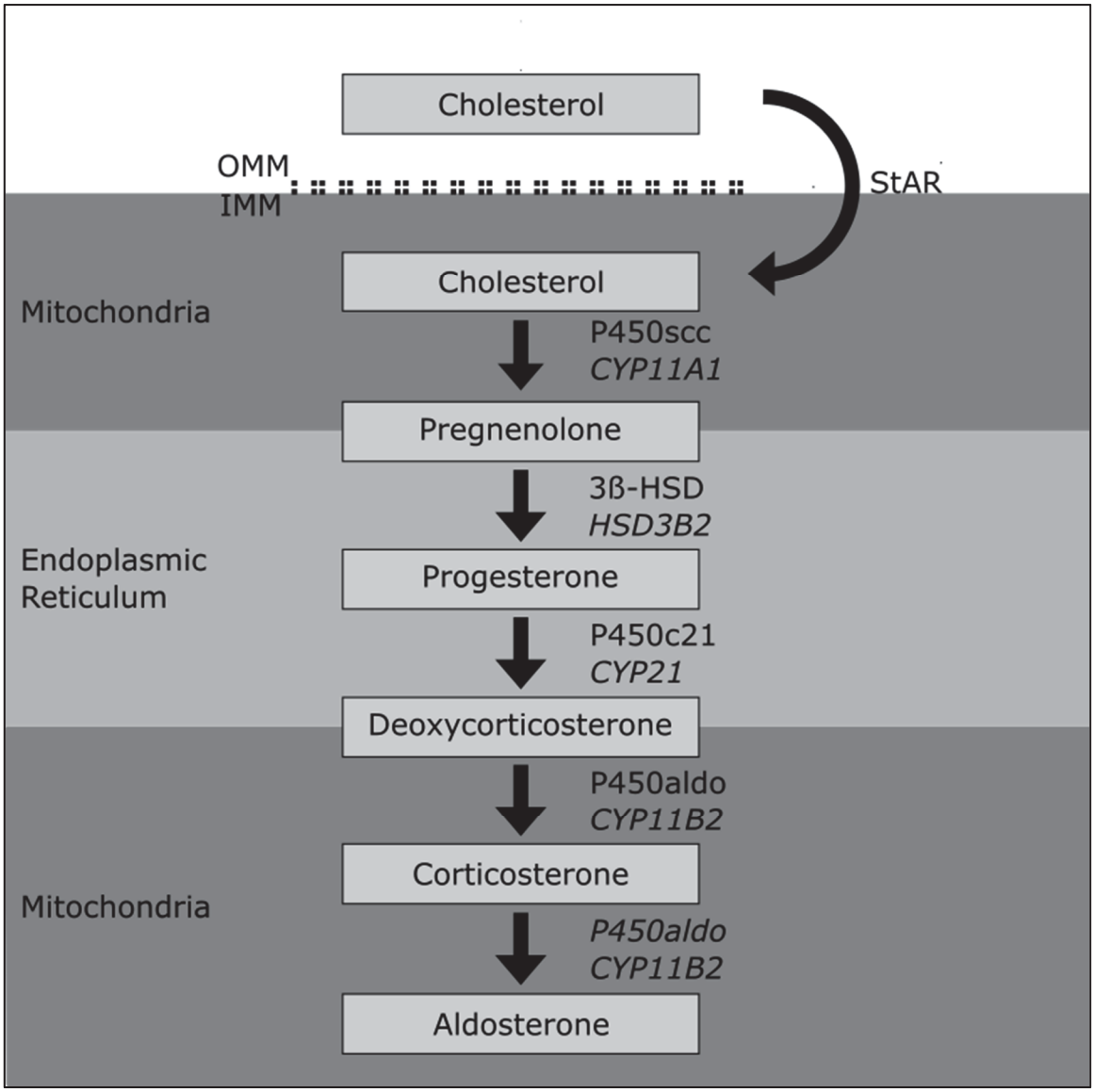


Figure 2. Steps of aldosterone synthesis in the ZG cell, enzymes involved and subcellular localization. StAR transports cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM). Cholesterol side chain cleavage by P450scc/CYP11A1 results in formation of pregnenolone. 3ß-HSD and P450c21/CYP21 are localized in the ER. Aldosterone synthase (P450aldo/CYP11B2) catalyzes the last two steps of aldosterone synthesis in the mitochondria. Adapted from Gallo-Payet, 2016 under consideration of Payne and Hales, 2004[22,89].

6.2.1 Voltage-gated calcium channels

Intracellular Ca^{2+} serves as an important ubiquitous second messenger. It is plays a crucial role in a variety of mechanisms such as neurotransmitter release, muscle contraction as well as cellular differentiation and proliferation[90]. Calcium-dependent signaling cascades are triggered upon an increase of intracellular Ca^{2+} levels. This can either occur through Ca^{2+} release from intracellular stores in the ER or through influx from the extracellular space[90].

Voltage-gated calcium channels can increase intracellular Ca^{2+} levels through the latter mechanism[90]. Their ability to conduct ions is dependent on membrane potential, opening at depolarized potentials[91]. They exist in 3 stages: closed, activated and inactivated. Activation refers to the opening of the channel as a result of membrane depolarization, while inactivation describes a state from which the open state cannot be immediately reached but requires a reactivation period. Like activation, it is also brought on by depolarization[23]. Deactivation is the process of channel closing in response to repolarization of the membrane[92].

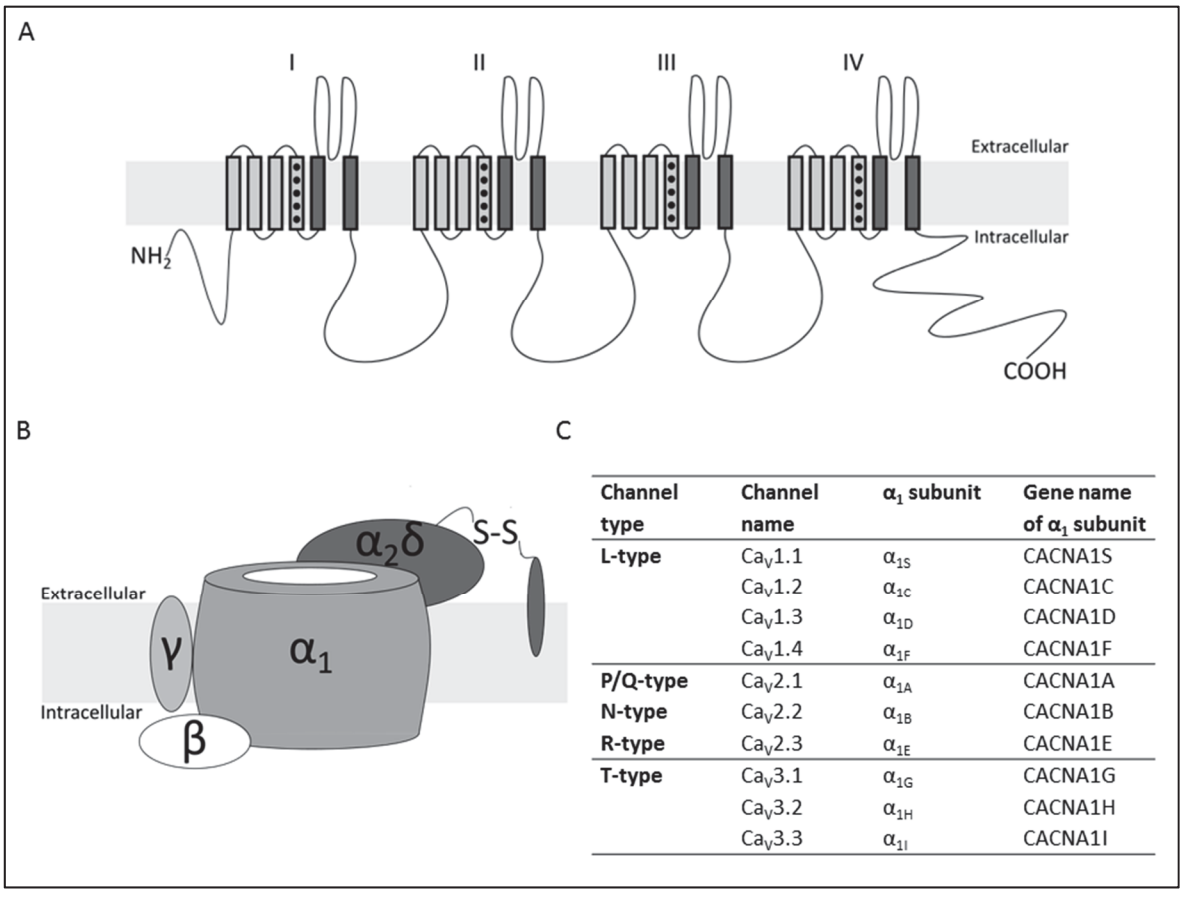
Voltage-gated calcium channels generally form heteromultimers, consisting of different subunits: α_1, $\alpha_2\delta$, β, and γ[91] (Figure 3 B). Channel composition can vary among different types of voltage-gated calcium channels[93,94]. While the α_1 subunits form the channel's pore, other subunits may affect gating and channel expression levels[93]. Five different classes of α_1 subunits can be distinguished depending on the type of current they generate: L-type, N-type, P/Q-type, R-type and T-type (Figure 3 C). While L, N, P/Q and R channels are high voltage-activated, T-type channels are low voltage-activated[91,95]. It should be noted that in contrast to the heteromultimeric high voltage-activated calcium channels, the α_1 subunits of low voltage-activated calcium channels appear to be able to form fully functional calcium channels on their own, which closely mirror the gating behavior of native T-type channels without requiring association with auxiliary subunits[96,97].

Structurally, α_1 subunits consist of 4 homologous repeats (I-IV), each consisting of six transmembrane segments (S1-S6)[91]. While S5 and S6 line the pore, the other transmembrane segments are involved in gating and voltage sensing. S4 is positively charged due to a pattern of basic arginine or lysine residues altering with uncharged residues (Figure 3 A). Changes in the electric field cause the segment to move, a mechanism enabling its function as a voltage sensor[23,91,98]. The pore loop between S5 and S6

controls channel conductance and selectivity. Calcium selectivity is mediated by glutamate residues in this region[23,99].

L-type channels sensitive to the compound class of dihydropyridines were first purified from skeletal muscle[95,100]. In addition, they can be found in a large variety of cells, including cardiac myocytes, neurons and endocrine cells, where they facilitate hormone release [93,101–103]. Neurons are also an important site of action for N-type channels[92,104]. P-type, Q-type and R-type channels are present in neuronal cells of the cerebellum[105,106]. T-type channels were first identified in guinea pig neurons[93,107]. They are also expressed in cardiac and smooth muscle myocytes[91,108,109].

Both L-type and T-type currents have been detected in the adrenal ZG of different species. Furthermore, N-type currents have been recorded in rat ZG cells and expression of the N-type channel $Ca_V2.2$ was detected in the normal human ZG[23,110–114].



Channel type	Channel name	α_1 subunit	Gene name of α_1 subunit
L-type	$Ca_V1.1$	α_{1S}	CACNA1S
	$Ca_V1.2$	α_{1C}	CACNA1C
	$Ca_V1.3$	α_{1D}	CACNA1D
	$Ca_V1.4$	α_{1F}	CACNA1F
P/Q-type	$Ca_V2.1$	α_{1A}	CACNA1A
N-type	$Ca_V2.2$	α_{1B}	CACNA1B
R-type	$Ca_V2.3$	α_{1E}	CACNA1E
T-type	$Ca_V3.1$	α_{1G}	CACNA1G
	$Ca_V3.2$	α_{1H}	CACNA1H
	$Ca_V3.3$	α_{1I}	CACNA1I

Figure 3. Voltage-gated calcium channels. A) The α1 subunit consists of 4 homologous repeats (I-IV), each comprising 6 transmembrane segments (S1-S6). S5 and S6 line the pore. S4 forms the voltage sensor. It is positively charged due to an altering pattern of basic and uncharged residues[23,91]. Depiction adapted from Catterall, 2011 and Daniil *et al.*, 2016[96,115]. B) Subunit composition of a high voltage-activated Ca^{2+} channel. Adapted from Catterall, 2000 and Buraei and Yang, 2010[93,116] C) Overview of the different voltage-gated Ca^{2+} channels, their corresponding α_1 subunits and genes[93].

6.2.1.1 L-type and T-type channels and their role in aldosterone production

T-type channels activate at more hyperpolarized membrane potentials than L-type channels (positive to -70 mV versus positive to -10 mV, respectively). Furthermore, T-type channels inactivate faster, but deactivate and reactivate more slowly than L-type channels[23,92].

T-type channels are formed by the α subunits α_{1G}, α_{1H} and α_{1I}, also referred to as $Ca_v3.1$, 3.2, 3.3, respectively[93] (Figure 3 C). In the rat and bovine ZG, α_{1H} is highly expressed, while only low expression levels of α_{1G} can be detected and α_{1I} appears to lack expression completely[23,117]. α_{1H}, encoded by the gene *CACNA1H*, is also expressed in the normal human adrenal ZG, as well as in the adrenal carcinoma cell line H295R[110,118,119]. In this cell line, exposure to aldosterone induces α_{1H} expression as well as T-type current[119].

L-type channels can consist of the α subunits α_{1S}, α_{1C}, α_{1D} and α_{1F} ($Ca_v1.1$, 1.2, 1.3, 1.4 respectively, compare Figure 3 C)[91]. In the rat ZG and H295R cells, both α_{1D} and α_{1C} are expressed, with α_{1D} being the most abundant[23,119,120]. α_{1D} and α_{1C} expression has also been confirmed in the normal human ZG[110,121]. The α_{1D} subunit is encoded by the gene *CACNA1D*[91]. From here on, voltage-gated calcium channels and their α_1 subunits will be referred to by the gene name of the corresponding α_1 subunit - e.g. $Ca_v1.3$ and α_{1D} will be called CACNA1D.

Both L-type and T-type channels manifest differential pharmacological properties: L-type channels are more sensitive than T-type channels to blocking by Cd^{2+}, dihydropyridines like nifidepine, as well as phenylalkylamines like verapamil[104,122,123]. Their currents can be enhanced by Bay K8644[124]. Relatively selective inhibition of T-type channels over L-type channels can be achieved with Ni^{2+}, amiloride and mibefradil[104,125–127]. The latter is a pharmaceutical that was introduced for the treatment of hypertension and angina pectoris. Shortly after its approval by the FDA, it was withdrawn from the market due to possible severe drug interactions[128–130].

In the ZG cell, both L-type and T-type channels appear to be involved in production of aldosterone (compare e.g. [23]). Increased extracellular K^+ concentrations activate voltage-gated calcium channels through membrane depolarization[23,131]. At lower K^+ concentrations, the effect appears to be mostly dependent on T-type channels. At increasing physiological concentration, L-type channels, requiring stronger membrane depolarization for activation, are involved in the process as well. At concentrations above

the physiological range, the signal is mainly dependent on L-type channels[23,126,131,132]. Increased sensitivity of T-type channels to K^+-mediated depolarization caused by hypoosmosis, and thus increased cell volume, as well as a Ca^{2+}-dependent shift of T-type channels to be activated at more hyperpolarized potentials induced by CaMKII has been suggested in some cell models[23,133–136].

AngII-stimulated aldosterone production has been suggested to mostly involve T-type channels, while L-type channel activity appears to be inhibited, an effect possibly involving simultaneous G protein-mediated activation of T-type channels and inactivation of L-type channels[23,34,131,137,138], In concert with these observations, T-type channel inhibition by mibefradil and tetrandrine reduce AngII-stimulated Ca^{2+} influx and aldosterone production[126,139]. However, some studies support an involvement of L-type channels. For instance, AngII and K^+ stimulated production of aldosterone can be blocked by L-type channel blocker nifedipine in H295R cells[140]. Nevertheless, the role of L-type channels might be minor. In their studies of the dual L-type/T-type channel inhibitor efonidipine, Imagawa *et al.* report that mibefradil was able to reduce AngII-induced aldosterone production in H295R cells more potently than nifedipine. Nifedipine, however, was a more potent inhibitor of K^+-mediated aldosterone production. Mibefradil reduced both AngII and K^+-stimulated *CYP11B2* expression, while nifedipine only diminished K^+-mediated effects. Efonidipine was the most potent inhibitor with both stimuli[141]. These results suggest a more pronounced influence of T-type channels on AngII-mediated aldosterone production, without fully dismissing a putative function of L-type channels. Of note, it has been suggested that L-type voltage-gated calcium channels modulate IP_3-dependent Ca^{2+} release via interaction with IP_3R[23].

In this work, the effects of mutations in the T-type channel CACNA1H on aldosterone production will be investigated. Effects of K^+ and AngII stimulation as well as inhibition by mibefradil will play a role in some experiments.

L- and T-type voltage-gated calcium channels have been implicated in a variety of diseases: For instance, loss of function in CACNA1D has been associated with deafness and brachycardia[142–144]. Both mutations in *CACNA1D* and *CACNA1H* might play a role in autism spectrum disorders and epilepsy[145–149]. Furthermore, studies in *CACNA1H* knockout and knock-down mice suggest that it plays a role in nitric oxide (NO)-mediated smooth muscle relaxation in the heart as well as in the perception of pain[150–152]. The latter effect is corroborated by studies using the inhibitor mibefradil[153,154].

A detailed description of *CACNA1H* and *CACNA1D* mutations in primary aldosteronism will be given in sections 6.3.2.1.2, 6.3.2.2.4 and 6.3.2.2.5.

6.2.2 G protein-coupled inwardly-rectifying potassium channels

K^+ is the most abundant cation in the intracellular space, while Na^+ is the highest concentrated one in the extracellular space[155]. This gradient is maintained mainly by function of the Na^+/K^+ ATPase and acts as the driver for K^+ efflux out of the cell, which is largely dependent on potassium channels. This system is one of the main contributors to the membrane resting potential[155]. Notably, while the main direction of K^+ flux through potassium channels is outward, under some conditions K^+ influx might play a role[155–157]. Potassium channels are linked to a variety of functions throughout the body, e.g. control of the heart rate, smooth muscle contraction and neurotransmitter release[158].

G protein-coupled inwardly-rectifying potassium channels (GIRKs) belong to the family of inwardly-rectifying potassium channels (Kir)[159] (compare Figure 4 D). These channels are characterized by a greater inward current than outward current due to physically blocked K^+ efflux, mediated by Mg^+ and polyamines upon depolarization. This blockage is reversed upon hyperpolarization, leading to "activation"[156]. GIRKs are classified as strong inward rectifiers[156,160]. Kir channels are complexes formed by four subunits, which all have the same basic structure: Each subunit consists of two transmembrane (TM) helices: The outer TM1 helix and the inner TM2 helix with the pore loop containing the selectivity filter in between (compare Figure 4 A, B and C). A salt bridge behind the selectivity filter mediates stability in the majority of Kir channels, including GIRKs[156,160]. Different sites of channel opening and closing have been suggested: First, the bundle crossing, referring to a section where the TM2 domains of the subunits intersect near the intracellular side of the channel, which blocks the cytoplasmic pore for K^+ ions; second, the selectivity filter, containing the T-X-G-Y-G or T-X-G-F-G motif which mediates K^+ selectivity[160,161]. Either one of the elements might form the physical gate or both elements might be involved[160]. Kir channels generally show two types of gating: A slow gate of opening bursts altering with long closed periods and fast gate consisting in rapid opening and closing, which has been described as "flickering"[156,160].

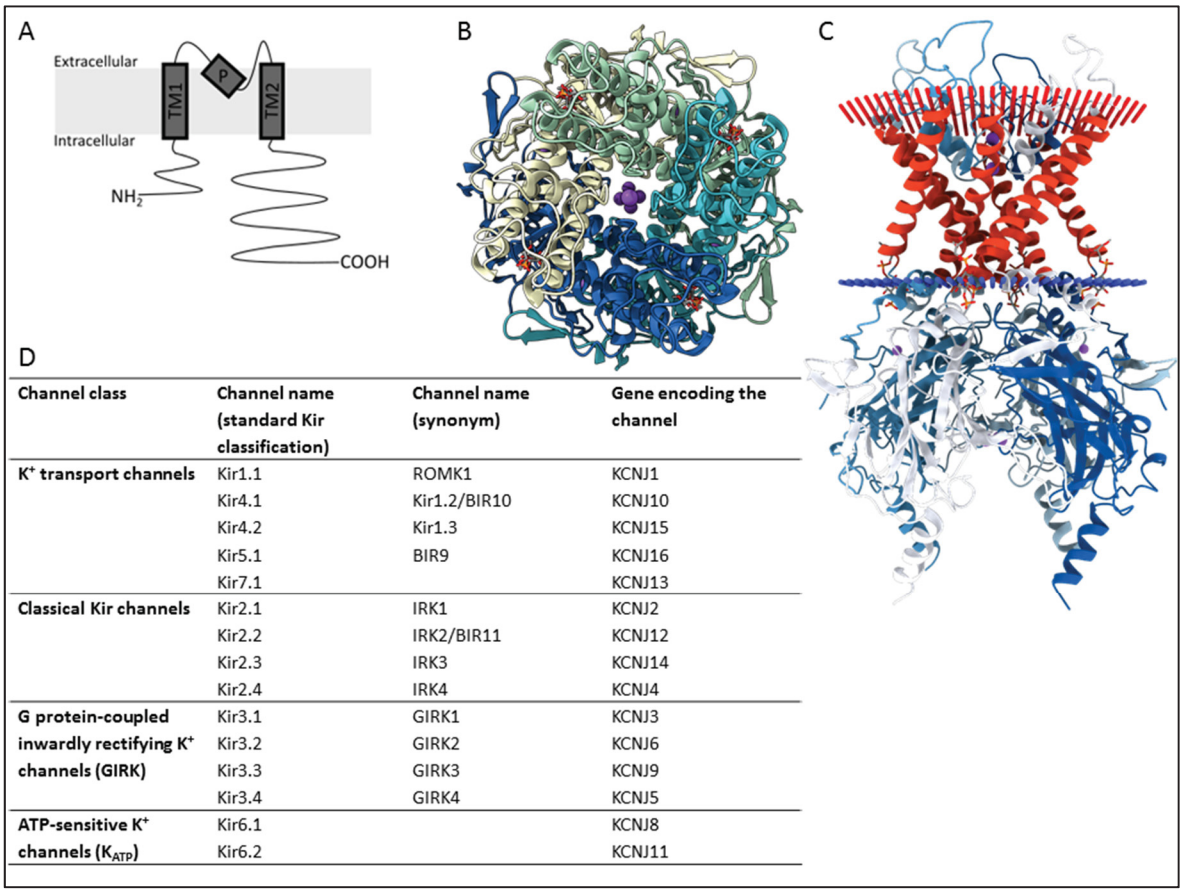
GIRKs are a class of Kirs that can interact with $G_{ßγ}$ heterodimeric G proteins, released upon activation of G protein-coupled receptors by ligand binding. This induces conformational changes that lead to channel opening. More exactly, $G_{ßγ}$ binding might induce rotation of the inner pore helix. It might also induce bending at a glycine hinge in TM2. These events lead to opening of the cytosolic side of the pore[156]. This is the result of direct interaction with -most likely- several cytosolic amino acid residues of the channel[160]. Availability of free $G_{ßγ}$ and thus GIRK activity might be modulated by $G_α$ and regulator of G protein signaling (RGS) proteins[156]. Presence of PIP_2 is also vital for GIRK activity[156,162]. Furthermore, channels containing GIRK2 and GIRK4 can be activated by intracellular Na^+, since these subunits contain a Na^+ sensor[156,163–165]. Intracellular acidification is able to inhibit GIRK channels independent of G protein signaling[166].

GIRKs can be found in a variety of tissues, where they can be activated by a variety of G protein-coupled receptors, such as the M2-muscarinic receptor in the heart, the α2-adrenergic, D2-dopamine, μ-, δ-, and κ-opioid, glutamate and $GABA_B$ receptors in neurons, smooth muscle and cells of the endocrine system, leading to membrane hyperpolarization[156,167–173]. They can form either homotetramers or heterotetramers with other GIRK family members (compare Figure 4 B and C). Complex composition depends on the tissue or cell type[156]. GIRK membrane localization is dependent on an ER forward trafficking signal as well as a post ER signal[174].

6.2.2.1 G protein-coupled inwardly-rectifying potassium channel isoforms

There are four subforms of GIRKs. GIRK1, also referred to as Kir3.1, is encoded by the *KCNJ3* gene[159]. Its cDNA was first isolated from the rat heart[175]. GIRK1 usually forms heterotetramers with other GIRK family members in native cells[156]. When GIRK1 is heterologously expressed in absence of other subunits, it remains in the ER. Membrane trafficking can be reconstituted by coexpression with GIRK2 and GIRK4[174].

GIRK2 (Kir3.2) is encoded by the *KCNJ6* gene[159]. It comprises several isoforms of different lengths, which are the product of alternative splicing and/or differential transcription[156,176,177]. GIRK2 can be found in a variety of tissues such as the brain, pituitary gland, pancreas and testis[156,178–181]. They are often co-expressed with other GIRK subunits and have been described to form heterotetramers with other GIRK family members[156,178,182–185]. However, they also build homotetramers in some cells[156,180,182].



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Channel class	Channel name (standard Kir classification)	Channel name (synonym)	Gene encoding the channel
K^+ transport channels	Kir1.1	ROMK1	KCNJ1
	Kir4.1	Kir1.2/BIR10	KCNJ10
	Kir4.2	Kir1.3	KCNJ15
	Kir5.1	BIR9	KCNJ16
	Kir7.1		KCNJ13
Classical Kir channels	Kir2.1	IRK1	KCNJ2
	Kir2.2	IRK2/BIR11	KCNJ12
	Kir2.3	IRK3	KCNJ14
	Kir2.4	IRK4	KCNJ4
G protein-coupled inwardly rectifying K^+ channels (GIRK)	Kir3.1	GIRK1	KCNJ3
	Kir3.2	GIRK2	KCNJ6
	Kir3.3	GIRK3	KCNJ9
	Kir3.4	GIRK4	KCNJ5
ATP-sensitive K^+ channels (K_{ATP})	Kir6.1		KCNJ8
	Kir6.2		KCNJ11

Figure 4. Inwardly-rectifying potassium channels. A) Kir subunits consist of two transmembrane subunits and a pore domain. Schematic depiction adapted from Bichet, Haass and Jan, 2003[160]. B) Crystal structure of the of the tetrameric assembly of GIRK2 in complex with PIP_2 and Na^+ obtained from protein database (PDB ID: 3SYA, provided by Whorton *et al.*, 201[186]). C) Transmembrane view of the crystal structure of tetrameric GIRK2 in complex with PIP_2 and Na^+ obtained from protein database (PDB ID: 3SYA, provided by Whorton *et al.*, 2011[186]). D) Different classes of Kir channels and their members[156].

Besides an ER export signal and a post ER signal promoting surface expression, GIRK2 might contain a motif promoting channel internalization, which modulates its membrane abundance[174].

GIRK3/Kir3.3/*KCNJ9* was first identified in cDNA from the murine brain[159,181]. It has been suggested to form heterotetramers with other GIRK subunits[156,184,187]. However, conflicting results have been published with regard to functionality of these channels[156]. GIRK3 lacks an ER forward trafficking signal but contains a motif for lysosome targeting[174]. Coexpression of other GIRK1-containing heterodimers with GIRK3 might promote their ER retention and thus reduce membrane expression and/or activity[156,174,185].

6.2.2.1.1 Kir3.4/GIRK4/KCNJ5

GIRK4 (Kir3.4) is encoded by *KCNJ5*[159]. It was first identified due to its co-immunoprecipitation with GIRK1 in preparations of the bovine atrial membrane[188]. It forms heterotetramers with GIRK1 in a 1:1 ratio[189]. However, it also seems to exist in a homotetrameric form in the bovine atrium[190]. GIRK4 protein was also identified in the axon terminals in the rat brain[191]. Heterodimers of GIRK4 and GIRK2 have been implicated to play a role in the modulation of hormone release from pancreatic islet cells[156,178]. Muscarinic K^+ (K_{ACh}) channel currents, likely generated by GIRK1/4 channels, might be involved in atrial fibrillation[156,192]. GIRK4 knockout mice show less variability in their heart rate at rest and after stimulation than wild type mice, suggesting that GIRK4 is involved in murine heart rate regulation[193]. These mice are also prone to develop late-onset obesity, suggesting a role of GIRK4 in maintaining energy balance and regulation of body weight[194]. Behavioral studies of GIRK4 knockout mice might imply slightly decreased abilities in spatial learning and memory, hinting at an involvement of GIRK4 in these functions[195]. A *KCNJ5* loss-of-function mutation (G387R) has been identified in a family with long QT syndrome[196]. Furthermore GIRK4 is expressed in the human adrenal ZG as well as the adrenal carcinoma cell line H295R[197,198]. However, functionality of native, but not of overexpressed, GIRK4 channels in this cell line has been disputed[199]. Mutations in or close to the G-Y-G selectivity filter have been found in patients with familial forms of hyperaldosteronism and aldosterone-producing adenoma (see sections 6.3.2.1.1 and 6.3.2.2.3).

Studies in the adrenal carcinoma cell line HAC15 show that *KCNJ5* overexpression increases membrane hyperpolarization and reduces basal as well as AngII-stimulated Ca^{2+} influx, *CYP11B2* expression and aldosterone production. Similar effects on AngII-mediated depolarization, *CYP11B2* expression and aldosterone production could be observed upon treatment with GIRK channel activator naringin[200,201]. However, *KCNJ5* knockdown did not have any effects, which the authors attributed to compensation by other potassium channels. Furthermore, *KCNJ5* expression was reduced by AngII stimulation or treatment with a Ca^{2+} ionophore. These results implicate a possible role of KCNJ5 as a negative regulator of AngII-mediated aldosterone production in the ZG, acting through interference with membrane depolarization and Ca^{2+} influx. *KCNJ5* downregulation might constitute a positive feedback, enhancing AngII-mediated aldosterone production[201].

Several inhibitors of GIRK4-containing channels have been described: The bee venom-derived potassium channel inhibitor tertiapin Q is able to inhibit GIRK1/4 channels[202]. Studies in H295R cell overexpressing *KCNJ5* wild type also suggest homotetramer inhibition[203]. Furthermore, the calcium channel blocker verapamil blocks K_{ACh} currents in the heart, which are likely generated by GIRK1/4 channel activity[156,204]. Like for most other Kir channels, inorganic blockage of GIRK4 can be achieved with Ba^{2+} and Cs^{+}(compare e.g. [156,197,205]). Pharmacologic properties of GIRK4 mutant channels will be described in section 6.3.2.1.1. Inhibition of mutant GIRK4 channels while avoiding wild type blockage to prevent symptoms associated with loss of function will be an important topic of this work.

From here on, GIRK channels and their subunits will be referred to by the gene name of the respective subunit or subunits (e.g. Kir3.4 and GIRK4 are called KCNJ5).

6.3 Hypertension

The term arterial hypertension refers to a medical condition in which the pressure in the arterial vascular system is chronically increased. In their 2013 guidelines for the management of arterial hypertension, the European Society of Hypertension (ESH) and European Society of Cardiology (ESC) classify a systolic blood pressure ≥140 mmHg or a diastolic blood pressure ≥90 mmHg as hypertensive when measured in a doctor's office[206]. It was suggested that in the year 2000 26.4% of the world population suffered from hypertension with a predicted tendency towards increase (29.2% in 2025). While the disease also affects a great number of people in developing countries, it has an especially high prevalence in the western industrialized countries[207]. A study from 2003 suggests that 27.6% of North Americans over the age of 35 are hypertensive. For Germany, the percentage of affected individuals was higher than the European average (55.3% versus 44.2%, respectively)[208]. Considering different studies, the ESH proposed in 2013 that 30-45% of the general European population might be hypertensive[206]. Worldwide, the percentage of affected individuals increases with age. The overall percentage of affected men is higher at a younger age, whereas the proportion of hypertensive women is larger at an older age. However, the overall difference between male and female appears to be small and sex prevalences seems to differ between regions[207].

Hypertension is associated with an increased risk of cardiovascular and cerebrovascular fatality, atherosclerosis being an important factor that raises the risk for coronary

heart disease, stroke and myocardial infarction[209–213]. Ischemic heart disease and cerebrovascular disease are among the leading causes of death in high-income countries (27.2% of deaths in 2001)[214]. Hypertension can also damage the heart muscle, leading to hypertensive heart disease, which can include heart failure[215]. Another risk associated with hypertension is renal failure caused by damage to the blood vessels in the kidneys[216]. Moreover, hypertension can lead to visual impairment due to damage of blood vessels in the retina (hypertensive retinopathy)[217].

If the cause of hypertension cannot be identified, it is referred to as essential hypertension, idiopathic hypertension, or primary hypertension. Affecting over 90% of hypertensive patients, this is the most prevalent form of the disease[218]. It is supposed that both genetic and environmental factors, such as obesity, high sodium intake, lack of exercise and excessive alcohol consumption play a role in the development of essential hypertension[219,220]. Hypertension caused by known underlying factors is referred to as secondary hypertension. Causes can consist of a variety of renal or endocrine disorders[206].

White coat hypertension describes a condition in which blood pressure is elevated in a clinical setting, while the patient is normotensive in out-of-the-office measurements. The contrasting effect - normal blood pressure in in-office measurements and hypertension in a domestic setting - is referred to as masked hypertension[206].

6.3.1 Antihypertensives

One important aspect of this work is to provide new impulses for targeted treatment strategies in patients with known underlying causes of hypertension. Therefore, a generalized overview about current approaches is of interest. There are different common classes of compounds suitable to treat hypertension, which can be used alone or in combination. They target different elements of blood pressure homeostasis. Well established families of antihypertensives are ACE inhibitors, angiotensin receptor blockers, beta blockers, diuretics, as well as calcium antagonists[206].

ACE inhibitors and angiotensin receptor blockers directly interfere with the RAAS, the former by inhibiting the ACE, thus leading to reduced production of AngII and the latter by impeding activation of the AT_1 receptor[221]. While ACE inhibitors appear to be more effective than angiotensin receptor blockers in preventing myocardial infarction, they seem to be less effective than other classes of hypertensives in counteracting stroke[206].

Beta blockers are competitive inhibitors of adrenergic beta receptors. Their classic main antihypertensive mode of action is reduction of cardiac output, although some drugs of this class mainly act through reduction of vascular resistance[222]. Despite their reported inferiority to some other families of antihypertensives in ameliorating certain secondary complications like stroke in some studies, the ESH/ESC still consider beta blockers an option for the therapy of hypertension[206].

Diuretics are generally a first choice of medication when it comes to hypertension treatment[206]. Diuretics promote Na^+ and water excretion by the kidney, thus decreasing plasma volume and consequently blood pressure[223,224]. Thiazide diuretics prevent Na^+ reabsorption by inhibiting the NCC in the distal convoluted tubule of the kidney[225]. Another class of diuretic compounds are potassium sparing diuretics such as the mineralocorticoid receptor antagonist spironolactone. These are an effective treatment in subjects with hypokalemia[206,226].

Calcium antagonists are pharmaceuticals that are able to block different classes of voltage-gated calcium channels, whereas many chiefly or exclusively act on L-type channels, with verapamil being a prominent example[123,227,228]. They cause vasodilation and reduce contractility of the heart[227,228]. Furthermore, compounds of this class have been reported to reduce aldosterone levels in patients, likely through direct interference with aldosterone production in the adrenal gland[110,229,230]. On the one hand, calcium antagonists may be slightly more effective in preventing stroke than other classes of antihypertensives, on the other hand, their inferiority in counteracting heart failure has been suggested in some studies[206].

Besides medication, lifestyle changes such as cessation of smoking, reduction of salt intake and alcohol consumption, weight loss as well as increase in physical activity are crucial in the treatment or prevention of hypertension and may reduce the risk for secondary complications[206].

6.3.2 Primary hyperaldosteronism

Primary hyperaldosteronism (PHA), also referred to as primary aldosteronism, is an important form of secondary hypertension and affects around 6-11% of patients in hypertension referral centers - its prevalence increasing in patient groups with higher blood pressure[231–233]. Prevalence of PHA among hypertensive patients in primary care practices is suggested to be ~4-6%[231,234]. PHA is characterized by an aldosterone production that becomes relatively independent of the normal physiological stimuli, which

leads to high serum aldosterone and suppressed plasma renin, increased water and Na^+ retention as well as increased potassium excretion. The latter effect, if severe and untreated for a prolonged amount of time, can lead to hypokalemia in some patients[231].

One important value for the diagnosis of PHA is the aldosterone-to-renin ratio (ARR). When measured in the morning in seated patients, common ARR cutoff values are in between 20-40 ng/dl per ng/mL/h. For patients with an ARR above that range confirmatory testing is recommended for conclusive diagnosis of PHA. However, it can be omitted in subjects presenting with severe signs indicating PHA[231].

Widely accepted testing procedures are fludrocortisone suppression, oral sodium loading, saline infusion, or captopril challenge[231]. In general, these tests are designed to disclose autonomous aldosterone production. They expose the patient to conditions that would normally cause aldosterone suppression by the RAAS. If aldosterone levels remain elevated despite these conditions, PHA is a likely diagnosis[235]. For example, the saline infusion test asses aldosterone suppression by increased blood volume and Na^+ retention[235]: The patient is infused with usually 2 liters of 0.9% saline over 4 hours followed by a measurement of plasma aldosterone[231].

The most common cause of PHA is bilateral idiopathic hyperplasia of the adrenal gland, affecting around 60% of patients followed by adrenal aldosterone-producing adenoma, making up approximately one third of PHA cases. In bilateral adrenal hyperplasia both adrenals contribute to the increase in aldosterone production, which might involve micro- or macronodular enlargement of both adrenal glands[236]. Aldosterone-producing adenomas are benign tumors of the adrenal gland marked by sustained aldosterone production independent of regulation by the RAAS[237]. Their subforms and underlying mechanisms will be elucidated in section 6.3.2.1. Unilateral hyperplasia (~2%), involving enlargement of one adrenal gland, adrenal aldosterone-producing adrenocortical carcinoma (<1%) as well as ectopic aldosterone producing adenoma and carcinoma (<0.1%), hormone-producing tumors located outside the adrenal gland, are very rare[236,238]. Reports on the occurrence of familial hyperaldosteronism among PHA patients vary: Recent studies from Germany and Italy suggest a prevalence from 1% to over 6% (compare[239–241]).

To test which subtype of PHA a patient presents with, a CT scan is recommended as a first step. Imaging techniques are helpful to detect large masses, such as adrenal carci-

noma. However, they seem insufficient in the detection of aldosterone-producing adenoma, which might be hard to distinguish from hyperplasia and cannot be differentiated from non-functioning adenoma or can be small[231].

Since a reliable diagnosis of lateralization cannot be achieved via imaging techniques, bilateral adrenal vein sampling (AVS) is recommended for this purpose. In this procedure, the right and left adrenal vein are catheterized[231]. Then plasma aldosterone concentration (PAC) is determined in the adrenal vein blood samples and the cortisol-corrected lateralization index is determined. For procedures performed without cosyntropin (synthetic ACTH 1-24) stimulation, an index of 2:1 is generally considered positive for lateralization[231,242]. Despite its benefits, adrenal vein sampling is a costly as well as invasive procedure and requires a skilled practitioner. Therefore, it is not consistently used in referral centers[231,243].

6.3.2.1 Aldosterone-producing adenoma

In adrenal aldosterone-producing adenomas (APAs) constitutive aldosterone production, which can cause PHA, has been traced back to a variety of genetic alterations in the tumorigenic tissue. Genetic analysis of APAs showed somatic mutations in the genes *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3* and *CTNNB1*[121,197,244,245].

This work includes a study which investigates the mutational status of APAs considering clinical phenotype of the patients, as well as histological characteristics of the tumor. An extensive overview of previous reports on APA mutations and their distinctive features can be found in Table 6, Table 7, Table 8 and Table 9 in the appendix (section 11.5).

6.3.2.1.1 *KCNJ5* mutations in aldosterone-producing adenomas

KCNJ5 mutations are very common; they are found in around 40% of APAs although it has been suggested that the percentage of APAs with *KCNJ5* mutations is lower when less stringent diagnosis criteria for lateralization are applied[237,246,247] (Table 1 and Table 2). APA patients with *KCNJ5* mutations tend to be younger than those without these mutations (compare e.g. [198,246,248,249] and Table 2). Studies done in European or Australian centers often report *KCNJ5* mutations to be more frequent in women than in men (compare e.g. [157,246,250,251] and Table 2). It has been argued that this bias appears to be less pronounced in East Asia[157,248,252]. However, several studies from the region still report female predominance (compare e.g. [198,253]). Also, prevalence of *KCNJ5* mutations in APAs appears to be higher in East Asian centers (~60-70%, Table 1), which

has been suggested to be a "selection bias" resulting from diagnostic criteria shifted towards patients with a more severe phenotype and procedural differences[157,247,248,252–255]. As a notable exception, a Taiwanese study analyzing 69 APAs reports a prevalence of *KCNJ5* mutations similar to European studies (37%)[198]. Some studies report higher plasma aldosterone levels[246,248] as well as lower serum potassium levels[248,250] in patients with *KCNJ5*-mutated APAs (Table 2). Tumors with *KCNJ5* mutations are often described to have fasciculata-like appearance[256–258] as well as larger size (compare e.g. [198,248,251,253,257,259] and Table 2). A meta-analysis comprising 1636 APAs patients in 13 studies confirmed key characteristics of tumors with *KCNJ5* mutation in comparison to those without: younger age, larger tumors, higher plasma aldosterone levels as well as female bias[247]. Moreover, a study by Kitamoto *et al.* found that patients with *KCNJ5*-mutated APAs display a higher improvement in determinators of cardiovascular risk after adrenalectomy than patients with tumors without known mutations[248].

Somatic *KCNJ5* mutations in APAs are usually accompanied by only a few additional mutations. Two *KCNJ5* mutations, namely *G151R* and *L168R*, account for most of the cases; other mutations in *KCNJ5* are rare[197,237] (Table 2). Both the G151R and L168R variant are located in or in vicinity to the selectivity filter, leading to a loss of selectivity and allowing for Na^+ influx into the cell. The pathomechanism suggested is that this Na^+ influx leads to depolarization of the cell membrane, causing voltage-gated calcium channels to open, thereby increasing intracellular Ca^{2+} levels. This stimulates cell proliferation as well as aldosterone synthesis through increased expression of *CYP11B2*[197] (Figure 5 C). Cell depolarization and Na^+ conductance of mutant channels were initially shown in electrophysiological studies in HEK cells. *In vitro* studies in adrenocortical carcinoma cell lines further confirm the different aspects of this mechanism: Tauber *et al.* described more depolarized membrane potentials in H295R cells with the *L168R* mutation in comparison to the wild type. Moreover, the *G151R* and *L168R* mutations lead to elevated intracellular Na^+ and Ca^{2+} concentrations in these cells[203]. The effect of KCNJ5 G151R and L168R increasing *CYP11B2* expression and aldosterone production has been confirmed in cell culture studies using the cell line HAC15[250].

Although there is a general consensus that *CYP11B2* and *KCNJ5* are expressed in APAs with mutations in the latter gene, there are conflicting reports about levels of expression in comparison to other APAs: Whereas Taguchi *et al.* describe increased levels of both *KCNJ5* mRNA and protein in APAs with *KCNJ5* mutation in comparison to those

without[254], Boulkroun *et al.* found no significant correlation of *KCNJ5* mRNA levels and mutational status of the tumors, but describe the ratio of KCNJ5 protein in tumorigenic tissue compared to normal adjacent tissue to be lower in tumors carrying *KCNJ5* mutations[260]. The latter results are confirmed by another study[198]. However, Akerström *et al.* found no correlation between mutational status and KCNJ5 protein expression in their 2012 study[251]. Expression of *CYP11B2* was reported to be higher in tumors with *KCNJ5* mutation than other APAs in some studies, while others report no differences (compare [246,250,261,262]). Another gene that was found to be upregulated in APAs with *KCNJ5* mutations in comparison to those without is *VSNL1*, which codes for the neuronal calcium sensor protein visinin-like 1. *In vitro* experiments have shown that its overexpression increases *CYP11B2* expression in the adrenal carcinoma cell line H295R and appears to also reduce Ca^{2+}-mediated apoptosis in these cells[261].

Mutant KCNJ5 channels generally show altered pharmacological qualities in comparison to the wild type: The Kir channel blocker tertiapin Q (L168R) as well as the potassium channel blocker Ba^{2+} (G151R, L168R) hardly affect mutant channels[197,203]. Tauber *et al.* identified the calcium channel blocker verapamil as an inhibitor of KCNJ5 L168R at concentrations that are in the range of attainable blood plasma levels. Moreover, other blockers of Ca^{2+}- and Na^{+}-conducting proteins, among them the diuretic amiloride, seem to act as inhibitors of KCNJ5 L168R channels, while some might also affect the wild type[203]. Since *KCNJ5* mutations account for a high percentage of APAs, finding pharmacological inhibitors of these channels might be beneficial in the treatment of affected patients. They might also counteract tumor progression by inhibiting proliferation mediated by mutant KCNJ5 channels. While normally considered to have effects on aldosterone production that are negligible during indicative testing, verapamil administered in high doses (3x120 mg/d) might hamper the diagnosis of PHA in some patients by affecting the ARR in blood tests due to its effect on KCNJ5 L168R[203,231]. Inhibition of *KCNJ5* wild type might also elicit undesired effects, since a loss-of-function mutation in the gene has been associated with long QT syndrome, stressing the importance of drug specificity[196]. An extensive pharmacological study of mutant KCNJ5 channels is included in this work.

Attempts at *in vivo* investigations of *KCNJ5* mutations found in APAs have been of limited success: Characteristics of a mouse model harboring the human *KCNJ5 G151R* and *L168R* mutations did not replicate the human phenotype[263]. Notably, *KCNJ5* is neither

expressed in the murine nor in the rat adrenal gland[264,265]. This constitutes an important limitation for studies investigating the role of mutant *KCNJ5* in PHA and stresses the importance of *in vitro* studies or, wherever applicable, studies on human test subjects for this purpose.

6.3.2.1.2 *CACNA1D* mutations in aldosterone-producing adenomas

Somatic mutations in *CACNA1D* have also been repeatedly found in APAs, with a prevalence of ~9.3% reported in the so far largest European cohort (compare [262] and Table 1). A lower percentage of *CACNA1D* mutations has been described in Asian cohorts (~2%, compare e.g. [248] and Table 1). Of note, some studies have not detected mutations in *CACNA1D* at all (compare [266,267] and Table 1). It should be noted, that most studies investigate only mutation hotspots, although not all specify their approach (compare e.g. [266,267]). Since *CACNA1D* mutations in APAs have been identified throughout the gene, it is possible that mutations were overlooked in the process (also compare [268] and section 8.1.1). Especially when grouped with ATPase mutations, *CACNA1D*-mutated APAs have been described to be smaller and patients tend to be older in comparison to APAs with *KCNJ5* variants. In addition, there appears to be no female bias (compare [121,249,257,262] and Table 2). Statements on the histological appearance of this APA group have been contradictory so far (e.g. [255,262,269]).

Similar to APAs with *KCNJ5* mutations, few additional protein-altering somatic mutations in other genes were found in the discovery cohort[121]. A variety of mutations have been found in different areas of the protein. They frequently affect segment 6 as well as the linker between segments 4 and 5 in different repeats. Mutations have also been found in segments 4 and 5. The afflicted areas are involved in the forming of the channel pore (e.g. G403R, I750M, F747L, compare Figure 6 C), the voltage sensor (R990H) or the S4-S5 linker coupling the voltage-sensor and the pore (e.g. P1336R, V259D)[121,262,269]. The model of the pathomechanism, derived from electrophysiological data for some of the most common mutations, relies on influx of Ca^{2+} through activation of CACNA1D channels at more hyperpolarized potentials and/or reduced channel inactivation[121,269] (Figure 5 D). H295R cells transfected with *CACNA1D P1336R* and *V259D* show an increase in aldosterone production in comparison to the wild type, which can be reduced with high concentrations of L-type channel blockers[268].

Table 1. Mutation frequencies in APAs according to different studies. Adapted from Seidel and Scholl, 2016[270].

Study	Region	Study size	Mutation frequencies (%)				
			KCNJ5	*CACNA1D*	*ATP1A1*	*ATP2B3*	*CTNNB1*
Akerström, 2012[251]	EU, AU	339	46.3	N/A	N/A	N/A	N/A
Akerström, 2015[a,249]	EU, AU	165	54.5	3	6.1	3	N/A
Akerström, 2016[b,271]	EU, AU	198	46.5	1.5	3	1.5	5.1
Arnesen, 2013[272]	EU	28	35.7	N/A	N/A	N/A	N/A
Azizan, 2012[259]	EU, AU	73	41.1	N/A	N/A	N/A	N/A
Azizan, 2013[c,269]	EU	152	N/A	7.9	7.9	N/A	N/A
Beuschlein, 2013[244]	EU	308	38.3	N/A	5.2	1.6	N/A
Boulkroun, 2012[246]	EU	380	33.9	N/A	N/A	N/A	N/A
Cheng, 2015[198]	EA	69	37.7	N/A	N/A	N/A	N/A
Choi, 2011[197]	EU	22	36.4	N/A	N/A	N/A	N/A
Dekkers, 2014[256]	EU	53	41.5	1.9	1.9	9.4	N/A
Dutta, 2014[273]	EU	35	31.4	N/A	5.7	8.6	N/A
Fernandes-Rosa, 2014[262]	EU	474	38	9.3	5.3	1.7	N/A
Hong, 2016[274]	EA	66	71.2	0	0	0	N/A
Kitamoto, 2015[248]	EA	108	69.4	1.9		2.8[d]	N/A
Kitamoto, 2016[e,255]	EA	159	73.6	2.5	0.6	2.5	N/A
Kuppusamy, 2014[266]	EU	195	24.6	0	1.5	0.5	N/A
Monticone, 2012[250]	EU, NA, EA	47	38.3	N/A	N/A	N/A	N/A
Scholl, 2013[f,121]	EU, NA	64	32.8	10.9	1.6	3.1	3.1
Tadjine, 2009[245]	NA	11	N/A	N/A	N/A	N/A	9.1
Taguchi, 2012[254]	EA	23	65.2	N/A	N/A	N/A	N/A
Williams, 2013[275]	EU	112	39.3	N/A	6.3	0.9	N/A
Wu, 2015[267]	EA	148	59.5	0	1.4	0.7	N/A
Wu, 2017[g,276]	EA	219	53	0	1.4	0.5	3.7
Xekouki, 2012[277]	NA	16	12.5	N/A	N/A	N/A	N/A
Zheng, 2015[253]	EA	168	76.8	0.6	2.4	0.6	N/A

AU: Australia, EA: East Asia, EU: Europe, NA: North America, N/A: not available [a]overlap with Akerström *et al.,* 2012 and 2017, [b]overlap with Akerström *et al.*, 2012 and 2015, [c]overlap with Azizan *et al.*, 2012, [d]ATPase mutation frequency, since individual *ATP1A1* and *ATP2B3* mutation frequencies are not available, [e]possible overlap with Kitamoto *et al.*, 2015, but not specified by authors, [f]overlap with Choi *et al.*, 2011, [g]likely overlap with Wu *et al.*, 2015, but not specified by authors, number of *CACNA1D* mutations concluded from publication by process of elimination

6.3.2.1.3 ATPase mutations in aldosterone-producing adenoma

Recurrent somatic mutations in *ATP1A1*, coding for the Na^+/K^+-ATPase α1 subunit, and *ATP2B3,* encoding the plasma membrane Ca^{2+}-ATPase 3, have been found in APAs[244]. In total, ATPase mutations have been reported in around ~6% of APAs with *ATP1A1* mutations being generally more prevalent than *ATP2B3* mutations (~1-5% vs. ~0.5-1.5%, respectively (compare e.g. [244,262,267], Table 1 and Table 2). Some, but not all, studies report an older age of the patients and male predominance in tumors with ATPase mutations, especially when grouped with *CACNA1D*-mutated APAs[244,249,262,269,273]. However, upon discrimination between *ATP1A1* and *ATP2B3* mutations, *ATP2B3* mutations might actually occur more frequently in female patients[24]. Moreover, a higher *CYP11B2* expression than in *KCNJ5*-mutated APAs and a glomerulosa-like appearance of the tumor have been described by some authors but not by others (compare [249,262,269,275]).

Generally, ATP1A1 mutations are often found in regions associated with ion binding. L104, located in the M1 helix of the protein, is the most frequently mutated ATP1A1 residue in APAs, leading either to a missense mutation (generally L104R) or a deletion (100_104del, compare Figure 6 B and Table 2). This residue is supposed to interact with another residue in the M4 helix, namely E334, thus enabling K^+-binding and occlusion[244,249]. Another recurrent mutation, V332G, lies near the latter residue[244,249]. The L104R mutation has been described to cause not only severe disturbance of K^+-binding and ATPase activity, but also H^+ influx into the cell[244,269]. The 100_104del mutation however appears to create inward Na^+ currents[269]. Transfection of mutated *ATP1A1* leads to depolarization, as well as increased *CYP11B2* expression and aldosterone production in comparison to the wild type in adrenocortical carcinoma cells at basal level, as well as after treatment with AngII[269,275,278] (Figure 5 E). Acidification of the cell has been confirmed in H295R cells transfected with *ATP1A1 L104R* and *V332G*[278].

ATP2B3 mutations are usually in-frame deletions affecting the positions 423-429 of the protein, i. e. the M4 helix[244,249,255,262] (Figure 6 B). It is assumed that these mutations cause alterations in regions involved in Ca^{2+} binding[24,244]. A study in which the L425_V426del variant was expressed in H295R cells suggests Na^+ conductance of mutant ATP2B3, leading to membrane depolarization. The cells expressing the

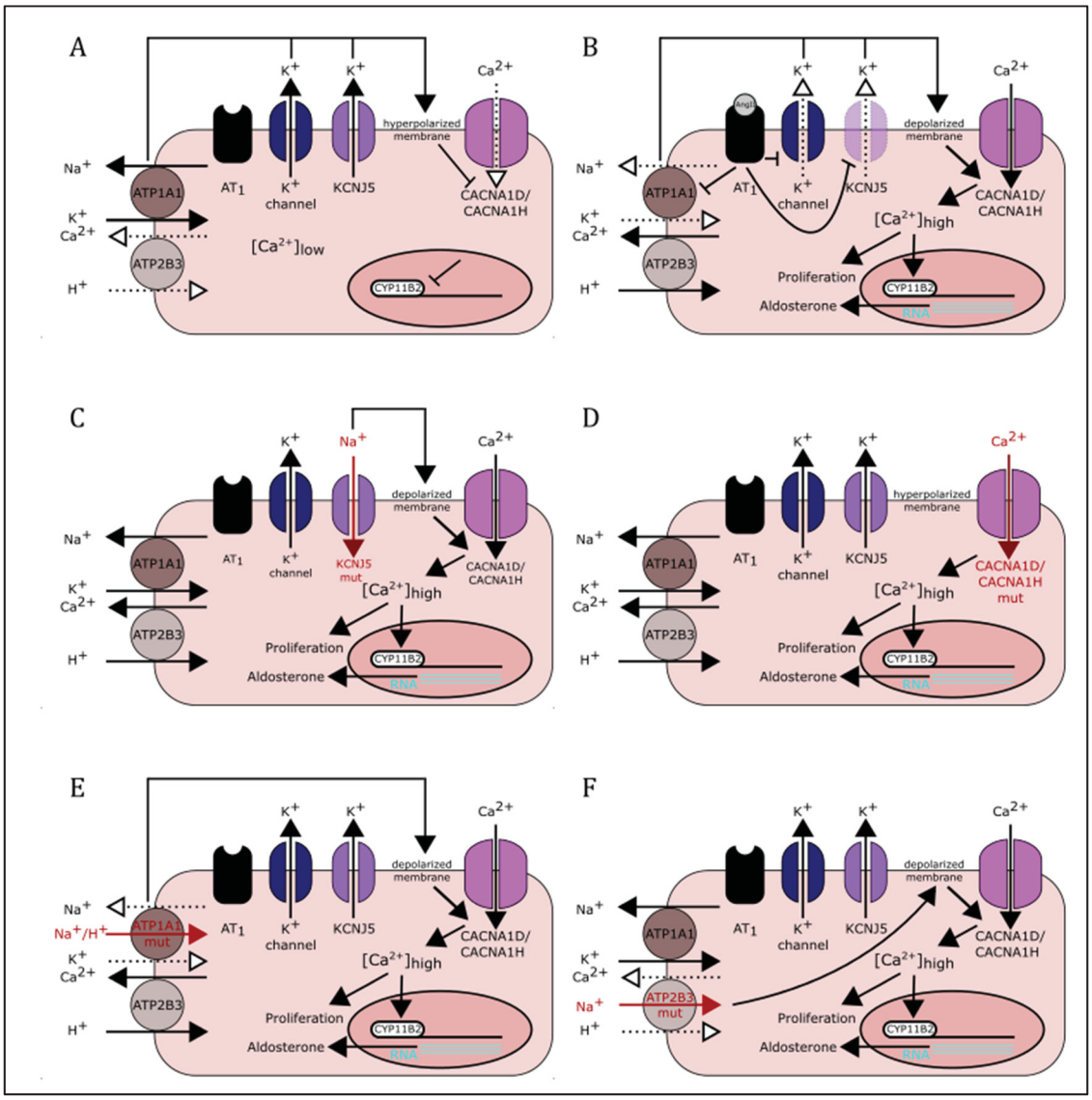

Figure 5. Suggested pathomechanisms of ion channel and pump mutations implicated in PHA. A) In the unstimulated ZG cell, activity of Na^+/K^+-ATPases, here referred to as ATP1A1 after the α-subunit isoform implicated in APAs, as well as outward potassium currents contribute to maintaining membrane hyperpolarization. Voltage-gated calcium channels like CACNA1D and CACNA1H remain closed and Ca^{2+}-ATPase ATP2B3 is nearly inactive at low intracellular Ca^{2+}. *CYP11B2* expression is suppressed. B) Upon binding of AngII to the AT_1 receptor in the normal ZG cell, Na^+/K^+-ATPase and outward K^+ currents are blocked. Downregulation of KCNJ5 has also been described[201]. These alterations lead to membrane depolarization, causing the opening of voltage-gated calcium channels and increased intracellular Ca^{2+} levels, stimulating *CYP11B2* expression, and thus aldosterone production, as well as proliferation. (Contribution of IP_3-dependent Ca^{2+} release from intracellular space and the suggested blockage of L-type Ca^{2+} channels have been omitted here for simplicity, for review see [23]). ATP2B3 becomes active at elevated intracellular Ca^{2+}-concentrations, promoting Ca^{2+} clearance. C) Sodium influx through mutant KCNJ5 channels leads to membrane depolarization, Ca^{2+} influx through voltage-gated calcium channels and thus *CYP11B2* expression, subsequently aldosterone production and proliferation. D) Alterations in CACNA1D and CACNA1H allow for Ca^{2+} influx at more hyperpolarized membrane potentials, promoting *CYP11B2* expression, aldosterone production and proliferation. E) Na^+ or H^+ influx through mutant ATP1A1 leads to membrane depolarization and therefore the opening of voltage-gated calcium channels. The resulting Ca^{2+} influx leads to *CYP11B2* expression, aldosterone production and proliferation. F) Na^+ influx through mutant ATP2B3 induces aldosterone production and proliferation in a similar fashion as mutant ATP1A1. Decreased Ca^{2+} clearance by the mutant protein might contribute to these effects. Adapted from Choi *et al.*, 2011, Oki *et al.*, 2012, Beuschlein *et al.*, 2013, Azizan *et al.*, 2013, Korah and Scholl 2015, Tauber *et al.*, 2016, Seidel and Scholl, 2016, and under consideration of Di Leva *et al.*, 2008[197,201,244,269,270,279–281].

mutant display high intracellular Ca^{2+} levels, likely caused by Ca^{2+} influx through voltage-gated calcium channels due to depolarization as well as reduced Ca^{2+} clearance by the mutant ATPase. The mutant cells also showed increased *CYP11B2* expression and aldosterone production[281] (Figure 5 F).

6.3.2.1.4 *CTNNB1* mutations in aldosterone-producing adenomas

Somatic mutations in the gene encoding beta-catenin (*CTNNB1*) have been found in different types of adrenocortical adenomas (i.e.: APAs, cortisol-secreting and non-functional) as well as adrenocortical carcinoma (for frequencies in APAs compare Table 1). Different types of mutations (substitutions, deletions and insertions) have been identified, all of them affecting exon 3 of the gene. The residue S45 is the most frequently altered one in missense mutations[245,271,282] (Table 2). The adrenocortical carcinoma cell line H295R harbors a *S45P* mutation in the *CTNNB1* gene, which was suggested to lead to constitutive activation of the wnt pathway[245,282]. A recent study of 198 APAs has described a mutation frequency of 5.1%, which remains comparable among different national cohorts. In this report, tumors with *CTNNB1* mutation were also found to be significantly larger than those without known mutations. Increased expression of *AXIN2*, hinting at activation of the wnt pathway, was found in tumors with *CTNNB1* mutation in comparison to those with *KCNJ5* mutation[271]. Another study published in 2017 found a mutation frequency of 3.7% in a cohort of 219 APAs[276]. It reports older age of patients with *CTNNB1* in comparison to *KCNJ5* mutant APAs. Furthermore, patients with APAs carrying *CTNNB1* mutations had an increased risk of residual hypertension after adrenalectomy in comparison to patients with wild type or *KCNJ5* mutant tumors[276]. Of note, *CTNNB1* mutations were not only both found in areas expression *CYP11B2* positive and negative areas but also in cells expressing the 11β-hydroxylase gene *CYP11B1*, crucial for cortisol production in the adrenal ZF[22,276]. This indicates that the role of *CTNNB1* mutations in APAs might primarily be based on its pro-proliferative effects, leading to tumor formation, instead of directly affecting aldosterone production[276].

In mice, constitutive beta-catenin activation was shown to lead to increased adrenal proliferation, hyperplasia and dysplasia, as well as primary hyperaldosteronism over time. Over an extended timespan, transgenic murine adrenal glands developed malignant characteristics[283].

In their 2015 study, Teo *et al.* reported increased levels luteinizing hormone–chorionic gonadotropin receptor (LH-CGR) and gonadotropin-releasing hormone receptor (GNRHR) in three female patients with *CTNNB1* mutated APAs. PHA in these patients manifested either during pregnancy or menopause, situations generally marked by high concentrations of circulating chorionic gonadotropin, luteinizing hormone, or gonadotropin- releasing hormone[284]. Increased expression of receptors for luteinizing hormone and gonadotropin-releasing hormone in APAs of unknown mutational status haven been described previously[285–287]. *In vitro* studies using H295R cells implicate luteinizing hormone and gonadotropin- releasing hormone as positive regulators of *CYP11B2* expression[285,287]. However, it should be noted that *CTNNB1* mutations have been detected in APAs from both female and male patients[271,276]. Interestingly, while Wu *et al.* report increased expression of LH-CGR and GnRHR in APAs carrying *CTNNB1* mutations in comparison to those with mutant *KCNJ5*, they make this observation in samples from both sexes. Furthermore, they confirm that receptor expression is not unique to *CTNNB1* mutant tumors[276]. Of note, in the relatively large cohort presented by Wu *et al.*, female PHA patients were not diagnosed during pregnancy, but at older age, i.e. during menopause or in post-menopause[276]. Consequently, cases of *CTNNB1* mutant APAs manifesting during pregnancy, as described in the study by Teo *et al.,* which comprised only three subjects, seem to be rather singular events[276,288]. This is in line with a comment in which researchers state that pregnancy did not play a role in the cases of *CTNNB1* mutant APAs they have encountered[289].

6.3.2.1.5 APA origins and the role of aldosterone-producing cell clusters

So far, our understanding of APA formation is limited. Notably, aldosterone-producing cell clusters (APCCs) have been suggested to play a role in APA development. They are clusters of cells located right below the adrenal capsule, which are marked by high expression levels of *CYP11B2*[290,291]. Aldosterone production in these cells has been suggested to be autonomous, i.e. independent of regulation by the RAAS[290]. APA-associated somatic mutations in *ATP1A1* and *CACNA1D* mutations have been identified in these cell aggregates, suggesting that they might be progenitors of at least a subset of APAs[291]. A recent report describes APCC containing a microAPA portion, which were interpreted as a possible transitional stage between APCCs and APAs. Mutations in genes implicated in APAs were found in the APA portion, but not in the APCC portion of two large transitional lesions (out of three), suggesting their

Table 2. Gene mutations in APAs and their characteristics. Approximate mean frequencies of somatic mutations in larger studies, the most frequently recurrent mutations as well as distinctive patient and tumor features repeatedly reported as statistically significant by different studies. Based on the studies listed in Table 6, Table 7, Table 8 and Table 9 (section 11.5) and Azizan *et al.* 2012[257]; Monticone *et al.*, 2015[258] for ZF-like appearance of *KCNJ5* mutant APAs . Layout adapted from Stowasser, 2013[351] and Dutta, Söderkvist and Grimm, 2016[24].

Gene	Frequency in larger cohorts	most common mutations	Repeatedly reported patient and tumor characteristics					comment
			Sex	Age	APA size	PHA severity	Tumor histology	
KCNJ5	~40%	G151R[a] L168R	female	younger	larger	higher	ZF-like	compared to wt EA cohorts: higher mutation frequency (~70%)
CACNA1D	~5%	G403R[a] F474L[a] I750M[a]	no female bias	N/A[b]	N/A[b]	N/A[b]	N/A[b]	EA cohorts: lower frequency (~2%) Western cohorts: higher frequency (~8%)
ATPase	~6%	ATP1A1 L104R ATP2B3 deletions	often male	N/A[b]	N/A[b]	N/A[b]	ZG-like	*ATP2B3* in frame deletions most frequently affect L425 and V426
CTNNB1	~4%	S45P	no bias	N/A	N/A	no difference	N/A	compared to wt and *KCNJ5* Only two large studies available comparing patient and tumor characteristics

N/A not available, EA: East Asian, [a] the three most frequently reported mutations in *CACNA1D* with G403R being the most common one (mutations have been identified throughout the gene), [b]for ATPase and *CACNA1D* mutations combined male prevalence, older age and smaller tumors reported[249,269].

relevance in APCC to APA progression[292]. Notably, one of these mutations was *KCNJ5 G151R*, the most frequent mutation in APAs[292] (compare Table 2).

6.3.2.2 Familial hyperaldosteronism

Inherited forms of hyperaldosteronism have been traced back to germline mutations in a variety of genes. Ion channel mutations identified in familial hyperaldosteronism and central patient characteristics are listed in Table 3.

6.3.2.2.1 Glucocorticoid-remediable hyperaldosteronism – crossing-over between *CYP11B1* and *CYP11B2*

The form that has been known the longest is glucocorticoid-remediable hyperaldosteronism (GRA) also referred to as familial hyperaldosteronism type I (FH I). It was first described in 1966 in a father and son with hypertension, elevated aldosterone and suppressed renin levels as well as hypokalemia, which were relieved by administration of dexamethasone[279,293]. Glucocorticoid remediable hyperaldosteronism is very rare, affecting <1% of PHA cases[238]. It is inherited in an autosomal dominant fashion and affects men and women equally. Onset of hypertension is early, usually by the age of 20. Patients also have an increased risk of cerebral hemorrhage, often leading to death[279,294]. Genetically, the disorder is based on a crossing-over event between the genes *CYP11B2* and *CYP11B1*, causing aldosterone-synthase to be expressed under control of the *CYP11B1* promoter, and therefore under regulation of ACTH in the ZF of the adrenal gland[295,296]. Administration of dexamethasone leads to suppression of ACTH and accordingly, in these patients, to reduction of aldosterone levels[294]. The diagnosis can be confirmed via PCR amplification of the mutated gene[297]. GRA reportedly has an increased prevalence among people of Irish ancestry[296].

6.3.2.2.2 Familial hyperaldosteronism type II – familial hyperaldosteronism with unknown causes

After the discovery of GRA, evidence of other forms of familial hyperaldosteronism emerged, which were not remediable by administration of dexamethasone[298–301]. In order to distinguish between the different familial forms of PHA, the term familial hyperaldosteronism type II (FH II) was established[299]. It refers to familial cases of hyperaldosteronism in which the underlying cause has not been identified (compare e.g. [231,240,279]). It includes both patients with APAs as well as bilateral hyperplasia[240,298]. They are virtually indistinguishable from non-familial cases[240,298,302]. FH II was re-

ported to be more prevalent than FH I[231,240]. This is at least in part a result of the relatively relaxed diagnosis criteria applied for FH II, including cases of APA and bilateral hyperplasia and simply focusing on multiple occurrences of PHA within one family without requiring identification of common underlying genetics[279]. A primarily autosomal dominant mode of inheritance has been suggested[298,303,304]. Linkage analysis has tied FH II to the chromosomal locus 7p22, but without identifying causal variants[303–305]. However, genetic heterogeneity has been suggested[304].

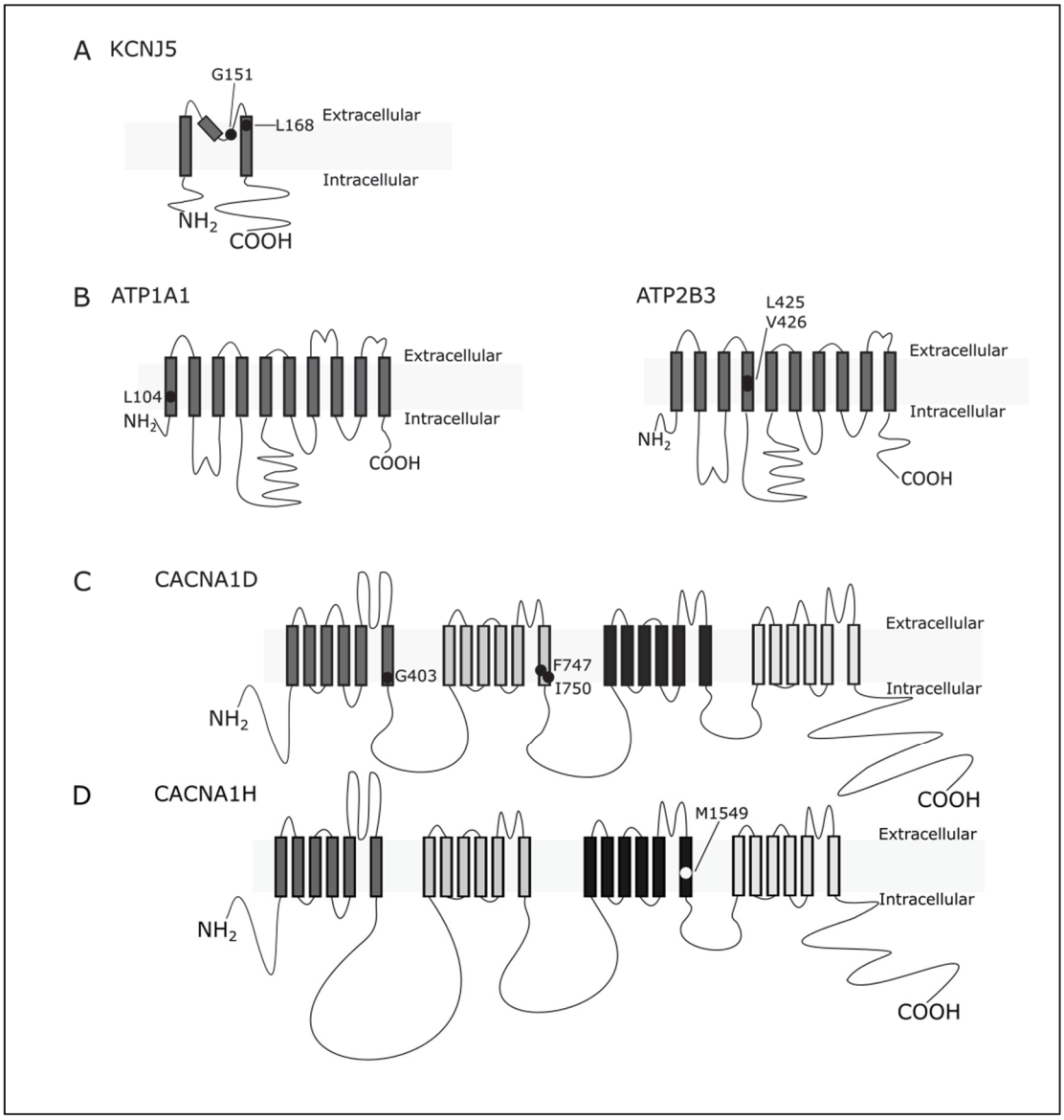

Figure 6. Frequently mutated residues in ion channels implicated in PHA and their position within each protein. A) KCNJ5 G151R is located in between the pore helix and TM2 and L168R is located in the upper part of TM2, which localizes both mutations near the selectivity filter of the channel pore. B) ATP1A1 L104 is located in the M1 helix of the protein. ATP2B3 L425-V426 are located in the M4 helix of the channel. C) CACNA1D G403 is located in segment S6 of repeat I and CACNA1D F747 and I750 are located in segment S6 of repeat II. The mutations depicted here represent the three most common mutations in CACNA1D. Nevertheless, mutations have been discovered throughout the gene (compare Table 7). D) CACNA1H M1549 is located in segment S6 of repeat III. Adapted from Dutta, Söderkvist and Grimm, 2016, Choi *et al.* 2011, Beuschlein *et al.* 2013, Scholl *et al*., 2013, Scholl *et al.,* 2015 and Daniil *et al*., 2016[24,115,118,121,197,244].

6.3.2.2.3 Familial hyperaldosteronism type III – *KCNJ5* germline mutations

With the rise of next generation sequencing, it was possible to shed further light on the underlying genetics of familial hyperaldosteronism, especially through identification of candidate genes established in investigations of APAs[279]. Familial hyperaldosteronism type III (FH III) is an inheritable form of PA caused by mutations in *KCNJ5* (compare e.g. [279]). One of the earlier cases of non-glucocorticoid remediable familial hyperaldosteronism was later related to the *KCNJ5 T158A* mutation[197,306,307]. This mutation is also occasionally found in APAs (compare [252,275,308]). Like the most common mutations described in APAs, G151R and L168R, it is located near the selectivity filter and causes Na^+ conductance in the mutant channel, which leads to membrane depolarization and Ca^{2+} influx[197,203,309]. Studies in adrenal carcinoma cell lines have also confirmed that transfection with the mutant channel leads to increased *CYP11B2* expression and aldosterone production, which can be reduced by inhibitors of calcium signaling[201,310]. Other *KCNJ5* mutations found in familial forms of hyperaldosteronism are *G151R, I157S,* and *E145Q*. Patients with these mutations usually present with a severe phenotype and generally require adrenalectomy, since they often suffer from massive bilateral hyperplasia and their PA cannot be managed by medication[279,311–313] (Table 3). Again, pathophysiology is based on a loss of K^+ selectivity in the mutant channel, leading to Na^+ influx into the cell[197,312,313] (Figure 5 C). For the *E145Q* mutation, *in vitro* studies have shown increased intracellular Ca^{2+} levels under control conditions in mutant cells, presumably due to Na^+ influx-mediated depolarization, as well as increased *CYP11B2* and *NR4A2* (*NURR1*) expression. E145Q mutant channels were neither sensitive to tertiapin Q, an inhibitor of Kir channels, nor to verapamil, which was previously shown to inhibit the T158A, G151R and L168R mutations[203,313].

Patients with *KCNJ5 G151E* and *Y152C* mutations usually present with a milder phenotype and do not necessarily require surgery[279,308,311,314] (Table 3). Notably, the G151E mutation was shown to cause more severe cellular depolarization and stronger Na^+ currents upon overexpression *in vitro*, which was accompanied by increased cellular lethality in comparison to cells carrying the G151R mutation. Therefore, it has been suggested that the milder phenotype manifesting in patients with these mutations might be due to high lethality of cells expressing the mutant channel[311]. The Y152C mutation, however, seems to have only a small effect on the channel's Na^+ conductance, which would explain the mild phenotype the patients present with. *In vitro* studies also show increased intracellular Ca^{2+} levels in cells harboring the Y152C mutation, but they

are substantially lower than for G151E mutant channels. These investigations also confirm increased *CYP11B2* expression that can be reduced by the calcium channel blocker nifedipine[314].

Not all FH III cases follow the patterns described above: One exception from the severe PHA phenotype usually caused by the *KCNJ5 G151R* germline mutation is a young female Japanese patient. By age 11, her hypertension had been successfully managed by spironolactone and adrenal hyperplasia was not detected[315]. Recently, the first FH III case with a combination of PA and Cushing's syndrome was reported in a young Chinese man with a germline *KCNJ5 E145Q* mutation. He suffered from early-onset hypertension, adrenal hyperplasia and started to develop Cushing's syndrome in his twenties. Cushing's syndrome is characterized by high cortisol levels, causing symptoms such as hypertension, abdominal obesity, a round face and striae. After bilateral adrenalectomy, his health improved and investigation of his adrenal tissue suggested that the excess cortisol was caused by adrenal hyperplasia[316]. Co-secretion of aldosterone and cortisol is an established phenomenon in patients with tumors carrying *KCNJ5* mutations[317,318].

In addition, Tamura *et al.* have recently described a case of early-onset PHA and bilateral adrenal hyperplasia, in which hyperplastic lesions displayed a *KCNJ5 G151R* mutation. This phenotype was most likely the product of genetic mosaicism, with the mutation occurring in an adrenal precursor mesodermal cell and being passed on to its daughter cells[319]. Notably, these findings support the previously suggested proliferative effects of the *KCNJ5 G151R* mutation (compare [197,288], Figure 5 C and section 8.2.1).

6.3.2.2.4 Primary hyperaldosteronism with seizures and neurologic abnormalities – germline *CACNA1D* mutations

De novo mutations in *CACNA1D* have been discovered in two cases of early-onset hypertension[121] (Table 3). Besides hyperaldosteronism and hypokalemia, these patients also showed a severe neurological phenotype, both suffering from seizures and cerebral palsy. Moreover, one of the patients displayed congenital heart anomaly, including a ventricular septal defect at birth. Due to the wide spectrum of symptoms with severe neurological impacts, this form of familial hyperaldosteronism is referred to as primary hyperaldosteronism with seizures and neurologic abnormalities (PASNA)[121,279]. However, it is sometimes included in the category of familial hyperaldosteronism type IV (FH IV) together with mutations in *CACNA1H* (further elucidated in section

6.3.2.2.5)[241]. The locations of the mutations found in these two patients match somatic ones found in APAs (G403D, I750M) and lie in regions involved in formation of the channel pore (compare Figure 6 C). Both show activation at more hyperpolarized potentials, and, like G403R, G403D also leads to decreased channel inactivation[121]. Thus, the suggested pathomechanism is similar to the one suggested for somatic *CACNA1D* mutations: Increased Ca^{2+} influx due to activation at less depolarized potentials leads to increased aldosterone secretion[121,279] (Figure 5 D).

A *de novo* occurring germline G403D mutation was recently described in a child with hyperinsulinaemic hypoglycaemia, heart defects, axial hypotonia, seizures and developmental delay. The patient's aldosterone levels within the normal range, while renin levels were not reported[320]. Interestingly, transient hypoglycaemia was also described in the PASNA patient carrying the same mutation. The differences in the phenotypes displayed by the two patients are currently not understood but might be explained by additional genetic or environmental factors[121,320].

In addition, Pinggera *et al.* recently described a *de novo CACNA1D* gain-of-function mutation (V401I) in an infant patient with autism spectrum disorder, epilepsy and generalized seizures. The authors recommend close monitoring of the patient for emerging signs of primary hyperaldosteronism[321].

Besides the adrenal gland, *CACNA1D* is also expressed in the brain, heart and pancreas accounting for the multisystemic nature of the symptoms[121,279,322,323].

6.3.2.2.5 Familial hyperaldosteronism type IV - *CACNA1H* germline mutations

A recurrent germline mutation in the gene *CACNA1H* was reported to cause early-onset hyperaldosteronism and hypertension by Scholl *et al.* in 2015[118] (Table 3). This subform of familial hyperaldosteronism is referred to as FH IV (compare e.g. [279]). The heterozygous *CACNA1H M1549V* mutation was discovered via exome sequencing in a cohort of 40 individuals that were diagnosed with hypertension by age 10 and showed high aldosterone levels with suppressed plasma renin activity. Some of the patients also presented with hypokalemia[118]. Furthermore, one was diagnosed with developmental delay and one with attention deficit disorder. Sequencing of family members showed that the mutation occurred *de novo* in some cases and was inherited from a parent in others. Gene analysis of the family members showed incomplete penetrance for the mutation, with two individuals being normotensive despite being carriers, hinting at a role of life-style factors like salt-intake or age-dependent activity of the

RAAS[118]. Furthermore, one of the index cases' mothers - a mutation carrier - underwent adrenalectomy for suspected unilateral PHA. Her adrenal gland showed microscopic hyperplasia of the ZG with nodules that expressed CACNA1H[118].

An additional case of FH IV with a different mutation in the same locus, *CACNA1H M1549I*, was recently described by Daniil *et al.*: The heterozygous *de novo* mutation was found in a patient who presented with primary aldosteronism and hypokalemia when two months old. Improvement of hypertension and hypokalemia could be achieved by spironolactone treatment. Treatment could be discontinued for a time in the patient's early teens, but needed to be reintroduced later due to reoccurrence of hypertension. No hyperplasia was identified in a CT scan. The patient was also diagnosed with multiplex developmental disorder[115].

CACNA1H M1549 mutations affect segment 6 of domain III of the protein, and the methionine residue at this position is highly conserved across species (compare Figure 6 D)[118]. It is also conserved across low voltage-activated paralogues, while it is substituted with isoleucine in high voltage-activated calcium channels. In the low voltage-gated calcium channel CACNA1G, the corresponding residue has been shown to be part of a M-F-V motive that is involved in voltage-gated calcium channel inactivation[118,324,325]. Electrophysiological studies show slower inactivation as well as activation at more hyperpolarized potentials for CACNA1H M1549V in comparison to the wild type[118]. Similar findings have been reported for the M1549I mutation. Daniil *et al.* also show an increased aldosterone production as well as *CYP11B2* expression in H295R cells with the mutation in comparison to the wild type after stimulation with K^+ [ref115]. Accordingly, the suggested pathomechanism is similar to the one proposed for CACNA1D[279] (compare Figure 5 D). In this work, we further elucidate the effects of *CACNA1H M1549V* on aldosterone production in an *in vitro* study.

Daniil *et al.* further implicate other germline mutations in *CACNA1H*, namely *S196L* and *P2083L,* in patients diagnosed with PA during adulthood with a family history of hypertension. The electrophysiological properties of these two mutations differ markedly from those found in modifications of *M1549* with activation at similar potentials like the wild type and small differences in inactivation pace. The P2083L mutation showed markedly lower window currents than the wild type. However, increased facilitation was found for both mutations[115]. Facilitation describes an increased current in response to two consecutive pulses in comparison to a single one[326]. Increased aldosterone production, as well as *CYP11B2* expression was found in H295R cells with S196L

mutation in comparison to the wild type after K^+ stimulation[115]. Furthermore, in a conference contribution, Roomp *et al.* describe a *CACNA1H G1064R* mutation in a family with late-onset PHA. In H295R cells, this mutation caused increased aldosterone production and *CYP11B2* expression in comparison to the wild type after K^+ stimulation. Although the authors conclude that their Ca^{2+} signaling studies in HEK cells are indicative of increased Ca^{2+} influx through the mutant channels, no differences between wild type and mutant were found in electrophysiological studies of the mutation[327].

Another *CACNA1H* germline mutation (V1951E) was found in a patient presenting with APA with no somatic mutations in the implicated genes and who was completely cured after adrenalectomy, which suggests that the tumor was the actual underlying cause of PHA. Electrophysiological studies showed increased channel facilitation. In H295R cells with this mutation, *CYP11B2* expression was increased after stimulation with K^+ in comparison to the wild type. The authors suggest that mutations in *CACNA1H* might also act as a "driver" in the formation of APA[115]. To summarize, mutations in other CACNA1H residues besides M1549 have been implicated in familial hyperaldosteronism, but the evidence for them to be disease-causing is rather inconclusive. A presumed role in APA formation remains to be established by further studies.

In this work, we performed an *in vitro* analysis of another germline *CACNA1H* mutation located in the vicinity of the one described by Roomp *et al.*[327]. It was found in monozygotic twins with a family history of hypertension. The same patients also carried mutations in *armadillo repeat-containing 5* (*ARMC5*) and *DACH1* (see section 6.1.2.2.1 and 6.3.2.2.6).

6.3.2.2.6 Putative roles of *ARMC5* in familial hyperaldosteronism

In the past, mutations in the putative tumor suppressor gene *armadillo repeat-containing 5* (*ARMC5*) have been found in patients with macronodular adrenal hyperplasia with Cushing's syndrome. In these patients, germline mutations are accompanied by a somatic "second hit" mutation in the nodule. The suggested pathomechanism is that inactivating ARMC5 mutations lead to decreased apoptosis and thus increased steroid secretion due to increased cell mass[328].

Recently, heterozygous germline mutations in *ARMC5* have also been implicated in primary aldosteronism. In one study, mutations were found in ~39% of patients, while alterations in ~10.7% of the patients were predicted to be damaging *in silico*. No evi-

dence of a somatic "second hit" was present in the analyzed samples[329]. All of the patients with putatively damaging mutations were African Americans, a group with an increased burden of rare variants[279,330]. Another study, which investigated *ARMC5* mutations in mostly Caucasian patients with PA, identified sequence variation in 18 out of 39 patients, none of which were predicted to alter protein function *in silico*[331]. Hence, despite some indications, the exact role of *ARMC5* mutations in familial hyperaldosteronism remains unclear[279,331].

6.3.2.3 Cell lines as models for primary hyperaldosteronism

Although over time many different approaches have been used to investigate adrenocortical behavior *in vitro*, established steroidogenic cell lines have the important advantage that they can provide the researcher with large numbers of readily available functional steroidogenic cells[332].

Despite the existence of a variety of human adrenocortical cell lines, several fail to produce steroids and are therefore of limited use in the investigation of PA (compare [332–336]). One prominent exception is the human cell line H295 and its derivatives, which originated from a tumor in a 48 year old female patient presenting amongst others with high urinary levels of aldosterone and cortisol as well as symptoms of virilization[337]. The original cells, growing in only slightly surface-attached clusters, were selected for adherence, which yielded the H295R cell line. Substrains of these cells, optimized for growth in different media, were selected[332,338]. HAC15 cells are monoclonal H295R-derived cells. Originally generated with the aim to create a novel steroid-producing adrenal carcinoma cell line derived from a different patient, they were later identified to be derived from contaminating H295R cells[333,339].

Similar to the tumor they originated from, H295-derived cells produce steroids and express genes coding for steroidogenic enzymes that originate from different zones of the healthy adrenal gland (compare e.g. [332,337,339–343]). Expression of the aldosterone synthase *CYP11B2* and aldosterone production in these cells can be stimulated with AngII and K^+ ref[72,339,342,343]. However, the various H295-derived cell lines and substrains differ markedly in their capacity to produce aldosterone and to express *CYP11B2*[344]. Aldosterone production might further be influenced by modifications of culture conditions[345].

HAC15 and H295R cells constitute a well-established cell culture model for PHA. They have been transfected using a variety of methods, stable cell lines have been generated,

cell culture supernatants have been assessed for steroid content and expression of steroidogenic enzymes has been monitored in numerous studies to investigate the underlying effects in hyperaldosteronism (compare e.g. [115,203,250,309,310]). Most importantly for this work, they have been used by multiple studies to investigate the role of *KCNJ5* and voltage-gated calcium channel mutations in PHA (e.g. [115,250,268,309]). The *in vitro* experiments featured in this work will rely on HAC15 and H295R cells as models for PHA involving ion channel mutations.

Table 3. Characteristics of FH patients with germline mutations in genes encoding ion channels. Adapted from Korah and Scholl, 2015[279].

FH type	Mutation	Study	Total number of subjects	Median age at diagnosis (years)	Patients with macroscopic hyperplasia (x/total)	Patients <u>not</u> responding to medication (x/total)	Patients with adrenalectomy (x/total)
FH III	KCNJ5 T158A	Choi, 2011[197]	3	5	3/3	3/3	3/3
	KCNJ5 G151R[a]	Scholl, 2012[311] Adachi, 2014[315]	5	1	2/4 (1 N/A)	3/4 (1 N/A)	3/4 (1 N/A)
	KCNJ5 I157S	Charmandari, 2012[312]	2	4.5	2/2	2/2	2/2
	KCNJ5 E145Q	Monticone, 2015[313] Tong, 2016[316]	2	2	2/2	2/2	2/2
	KCNJ5 G151E	Scholl,2012[311] Mulatero, 2012[308]	8	3	0/7 (1 N/A)	1[g]/7(1 N/A)	2[g]/8
	KCNJ5 Y152C	Monticone, 2013[314]	1	48	1/1	1/1	1[i]/1
PASNA	CACNA1D G403D[b]	Scholl, 2013[121]	1	0	N/A	0/1	0/1
	CACNA1D I750M	Scholl, 2013[121]	1	5	0/1	0/1	0/1
FH IV	CACNA1H M1549V	Scholl, 2015[118]	8[d]	7.5	0/6 (2 N/A)	0/8	1[i]/8
	CACNA1H M1549I	Daniil, 2016[115]	1	0.2	0/1	0/1	0/1
	CACNA1H S196L[c]	Daniil, 2016[115]	2	44	1/2	0/1[h], 1 N/A[h]	1/2
	CACNA1H P2083L[c]	Daniil, 2016[115]	2	41.5	1[f]/2	0/1[h], 1 NA	0/1, 1 N/A
	CACNA1H G1064R[c]	Roomp, 2016[327]	4 affected[e]	4 N/A (late-onset)	4 N/A	4 N/A	4 N/A

N/A: not available, Median for even values expressed as the mean of the two middle values, when all numbers are listed smallest to greatest, [a]also reported in a PHA patient with genetic mosaicism[319], [b]also reported in a patient with hypoglycaemia, seizures and developmental delay[320], [c]electrophysiological data (activation/inactivation or general) poorly support PHA-causing properties of the mutation, [d]excluding two adult normotensive carriers, [e]exact number of sequenced family members (including deceased) not specified, [f]left adrenal, [g]before antimineralocorticoids became accessible, [h]resistant hypertension, [i]unilateral adrenalectomy, non-curing

7 Projects

7.1 Novel somatic mutations in primary hyperaldosteronism are related to the clinical, radiological and pathological phenotype

7.1.1 Objectives of the work

Recurrent somatic mutations in *KCNJ5*, *CACNA1D*, *ATP1A1*, *ATP2B3* and *CTNNB1* have been described in APAs[121,197,244,245]. An increasing number of studies have tried to link the mutational status of the tumor to patient and tumor characteristics, in part producing contradictory results (compare Table 6, Table 7, Table 8, Table 9). The goal of the study at hand is to link mutations found in APAs from three different centers to a corresponding clinical, radiological and pathological phenotype. Thereby, this study aims at broadening our understanding of the distinctions between different APA subtypes. This could contribute to facilitation in the diagnosis of APA and therefore improve medical care for the affected patient group.

7.1.2 Manuscript

U. I. Scholl, J. M. Healy, A. Thiel, A. L. Fonseca, T. C. Brown, J. W. Kunstman, M. J. Horne, D. Dietrich, J. Riemer, S. Kücükköylü, E. N. Reimer, A. C. Reis, G. Goh, G. Kristiansen, A. Mahajan, R. Korah, R. P. Lifton, M. L. Prasad, and T. Carling, "Novel somatic mutations in primary hyperaldosteronism are related to the clinical, radiological and pathological phenotype," Clinical Endocrinology, vol. 83, pp. 779–789, 2015.

For a description of the contributions made to this manuscript by E. N. Reimer please refer to section 11.1.1 in the appendix.

In accordance with regulations, publication reprints were removed from this dissertation for the final print version.

7.2 Macrolides selectively inhibit mutant KCNJ5 potassium channels that cause aldosterone-producing adenoma

7.2.1 Objectives of the project

Somatic mutations in the potassium channel KCNJ5 are found in around 40% of APAs[247]. G151R and L168R are the most common variants by far, while other mutations are rare[24,237]. These alterations have been shown to increase aldosterone production in adrenocortical carcinoma cells[250]. The goal of this project was to identify compounds that block KCNJ5 channels with G151R and L168R mutations without inhibiting the wild type. Substances meeting these criteria could potentially be used in the diagnosis and treatment of patients with *KCNJ5* mutant APAs and thus improve medical care for this group. After screening over 73,000 substances, the effects of the most promising compound on aldosterone production and *CYP11B2* expression were assessed in studies of adrenocortical carcinoma cells expressing mutant KCNJ5. A reduction of aldosterone production upon inhibitor treatment would be a first indicator that a similar effect might be achieved *in vivo*, substantiating the potential benefits of mutant KCNJ5 channel blockers for APA patients.

7.2.2 Manuscript

U. I. Scholl, L. Abriola, C. Zhang, E. N. Reimer, M. Plummer, B. I. Kazmierczak, J. Zhang, D. Hoyer, J. S. Merkel, W. Wang, and R. P. Lifton, "Macrolides selectively inhibit mutant KCNJ5 potassium channels that cause aldosterone-producing adenoma," Journal of Clinical Investigation, vol. 127, no. 7, pp. 1–12, 2017.

For a description of the contributions made to this manuscript by E. N. Reimer please refer to section 11.1.2 in the appendix.

In accordance with regulations, publication reprints were removed from this dissertation for the final print version.

7.3 CACNA1H M1549V mutant calcium channel causes autonomous aldosterone production in HAC15 cells and is inhibited by mibefradil

7.3.1 Objectives of the project

The germline *CACNA1H M1549V* variant was first described in a group of patients with early-onset hyperaldosteronism. Electrophysiological studies suggested a gain of function of the mutant channels, showing slower inactivation and activation at more hyperpolarized potentials in comparison to the wild type[118]. The goal of this project was to further elucidate the role of the mutation as a cause of FH IV. Therefore, we investigated the effects of the mutation on *CYP11B2* expression and aldosterone production, which might be indicative of its *in vivo* effects. In accordance with previous reports, a gain-of-function mutation causing PHA was expected to increase both *CYP11B2* expression and aldosterone production in adrenocortical cells (compare e.g. [250,309]). To obtain an overview over potential effects, the impact of the mutation was analyzed under a wide range of conditions (basal, stimulatory and inhibitory). Thereby, T-type channel inhibition served to two purposes: First, to analyze whether any observed effect was indeed the result of low voltage-activated calcium channel activity and second, to explore the pharmacology of CACNA1H M1549V channels, which could lead to new impulses for the treatment of FH IV patients.

7.3.2 Manuscript

E. N. Reimer, G. Walenda, E. Seidel, and U. I. Scholl, "CACNA1H M1549V mutant calcium channel causes autonomous aldosterone production in HAC15 cells and is inhibited by mibefradil," Endocrinology, vol. 157, no. 8, pp. 3016–3022, 2016.

For a description of the contributions made to this manuscript by E. N. Reimer please refer to section 11.1.3 in the appendix.

In accordance with regulations, publication reprints were removed from this dissertation for the final print version.

7.4 Investigations of the *CACNA1H S1073C* variant: Effects on *CYP11B2* expression and aldosterone production in the adrenocortical cell line HAC15 in comparison to *CACNA1H M1549V*

7.4.1 Objectives of the project

Different mutations in the voltage-gated calcium channel CACNA1H have been implicated in familial forms of PHA[115,118,327,346]. Therefore, when the *CACNA1H S1073C* variant was discovered in a family with a history of hypertension, including two PHA patients, the mutation was considered a putative cause of the disease. *In vitro* studies in an adrenocortical carcinoma cell line were conducted in order to assess the possibility of increased aldosterone production by mutant cells, which might lead to the development of PHA *in vivo*. To this end, the effects of the mutation on aldosterone secretion and aldosterone synthase expression were measured in HAC15 cells transfected with wild type and mutant channels. A similar approach has already supported the role of *CACNA1H M1549V* as a cause of FH IV[346].

7.4.2 Materials and Methods

7.4.2.1 Patients and mutation discovery

Clinical, biochemical and genetic testing, including testing for FH I, FH III, FH IV (*CACNA1H M1549* mutation) and exome sequencing, was performed by the groups of Dr. M. Naruse, Dr. F. Beuschlein and Dr. T.-M. Strom with the patients' informed written consent. The patients were diagnosed with PHA at Ogona Hospital (Japan) in accordance with both national and institutional guidelines. The local ethics committee approved of the study. A case report on the index cases has been published by Yoshida *et al.* in 2017[347].

7.4.2.2 Chemicals, buffers and primers

If not indicated differently, standard chemicals were purchased at Sigma-Aldrich (St. Louis, MO, USA), Merck-Millipore (Billerica, MA, USA) or Carl Roth (Karlsruhe, Germany).

Buffer recipes will be indicated in the respective sections of Materials and Methods. Sterile phosphate-buffered saline (PBS) used for cell culture purposes was purchased from Merck-Biochrom.

Primers were ordered at Eurofins Scientific (Luxembourg, Luxembourg).

7.4.2.3 Cell culture

The H295R subclone HAC15 was a kind gift of Dr. W. Rainey[333,339]. The cells were cultured at 37°C and 5% CO_2 in DMEM:F12 (1:1, Gibco) containing 5% Cosmic Calf Serum (CCS, Hyclone, South Logan, UT, USA), 1% Penicillin/Streptomycin, 1% non-essential amino acids, 1% Insulin-Transferrin-Selenium as well as 0.1% lipid mixture (all Gibco).

Cell line identity was confirmed via short tandem repeat (STR) profiling using the ATCC cell line authentication service (ATCC, Manassas, VA, USA).

7.4.2.4 Plasmids and cloning

To generate vectors carrying the *S1073C* mutation, the vector pCMV6-Entry-*CACNA1H* wild type (*wt*) was digested with EcoRI (New England Biolabs, Ipswich, MA, USA) and the larger fragment was religated. Primers for Quikchange were designed using the Primer X tool (fw: GAGGCAGCCTGTGCCCTCCCCTCATC; rv: GATGAGGGGAGGG-CACAGGCTGCCTC). The site-directed mutagenesis was performed using the Pfu ultra high fidelity enzyme (Agilent Technologies, Santa Clara, CA, USA) following the instructions given by the manufacturer. Betaine was added to the reaction mix to increase reaction efficiency (final concentration: 1M). Briefly, Quikchange PCR was performed on the religated plasmid (conditions: 5 minutes at 95°C, 18 cycles of 95°C 50 s, 55°C 50 s, 68°C 13 min, and a final elongation step of 68°C for 16 min) followed by DpnI digest for several hours at 37°C and transformation into *E.coli* StbL3 (Invitrogen by Thermo Fisher Scientific). The transformants were screened for the desired mutation by Sanger sequencing (Beckman Coulter/Genewiz, Essex, UK) with primer 1H_seq4 (Table 5 in the appendix). Plasmids carrying the S1073C mutation were digested with the restriction enzymes XhoI and BspEI (New England Biolabs) and ligated into pCMV6-Entry-*CACNA1H wt* (used for electrophysiology by Dr. Gabriel Stölting, data not shown) and pCMV6-AC-*CACNA1H* IRES-GFP-Puro previously cut at the same restriction sites. Generation of pCMV6-AC-*CACNA1H wt/M1549V* -IRES-GFP-Puro was described previously[346].

Plasmids used for transfection via the Amaxa Nucleofector system were prepared with the Qiagen Endofree Maxi Kit (Qiagen, Hilden, Germany). For all other purposes the Qiagen Maxi Plus Kit was used. Plasmid identity was confirmed in a control restriction digest and absence of aberrant sequence was confirmed via Sanger sequencing using the primers listed in Table 5 in the appendix (section 11.3).

7.4.2.5 Transfection

To assess the effects of *CACNA1H* mutations *in vitro*, two million HAC15 cells, counted with a Juli Br counting module (NanoEnTek, Seoul, South Korea), were transfected with 1 µg vector DNA using the program X-05 on an Amaxa Nucleofector 2B and the Nucleofector kit R (all Lonza, Cologne, Germany) following the protocol provided by the manufacturer. After transfection, cells recovered for 15 minutes at 37°C in RPMI medium (Gibco by Thermo Fisher Scientific, Waltham, MA, USA) prior to seeding. Vectors used for transfection were AC *CACNA1H* IRES GFP Puro *wt, S1073C, M1549V* as well as an empty vector control. For ELISA, two million cells were plated per well of a 6-well-plate. For qPCR, 1.5 million cells were plated per well of a 12-well-plate. Successful transformation was confirmed the next day via GFP fluorescence microscopy.

Serum concentration was reduced to 0.1% CCS and Penicillin/Streptomycin was removed from the medium 24 hours after transfection. Another 24 hours later, the medium was changed again to the same serum deprivation medium. 72 hours after transfection, either supernatants were harvested for ELISA and cells were lysed for protein assay or cells were washed with PBS and harvested for qPCR.

7.4.2.6 Aldosterone ELISA

Supernatants were sent to the laboratory of Dr. F. Beuschlein for analysis with an automated chemiluminescent immunoassay (Liaison® DiaSorin, Saluggia, Italy). Aldosterone concentration was normalized to total soluble protein. For determination of protein concentrations, cell lysates were diluted 1:10 in water and analyzed using the Micro BCA Protein Assay Kit (Thermo Scientific by Thermo Fisher Scientific) following the instructions given by the manufacturer.

7.4.2.6.1 Isolation of total soluble protein from cells

For isolation of total soluble protein, 400 µL of protein lysis buffer (20 mM Tris pH 7.5, 150 mM KCl, 1% Triton-X-100, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM sodium-orthovanadate, 0.25 mM Phenylmethanesulfonyl fluoride (PMSF) and 1 complete mini inhibitor cocktail tablet (Roche, Basel, Switzerland) per 50 mL) was added to cells previously washed in PBS. Lysis was performed for 10 minutes on ice. Then, cells were scraped off and the whole lysate was transferred into a microcentrifuge tube. Lysates were centrifuged at full speed and 4°C for 20 minutes. Supernatants were transferred to a new microcentrifuge tube and stored at -20°C.

7.4.2.7 qPCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) following the instructions given by the manufacturer and including a DNaseI digestion step. Reverse transcription was performed using 300 ng RNA and the Quantitect Reverse Transcription Kit (Qiagen) in accordance with the instructions. Expression levels were determined on a 7300 RT-PCR system using the TaqMan Gene Expression Mastermix as well as a commercial assay for *GAPDH* (Hs02758991_g1) and *CYP11B2* (Hs01597732_m1, machines and materials by Applied Biosystems, Foster City, CA, USA). Samples were measured in triplicates and results were analyzed using the $2^{-\Delta\Delta C_T}$ (C_T: Threshold Cycle) method[348]. GAPDH was used as the reference gene and $2^{-\Delta\Delta C_T}$ values were calculated relative to empty vector-transfected cells.

7.4.2.8 Statistical analysis and software

Data was analyzed using the Prism software (GraphPad, La Jolla, CA, USA) and Excel (Microsoft, Redmond, WA, USA). All error bars represent standard error of the mean (SEM). Data analysis was performed with an unpaired, two-tailed Student's t-test.

Drawings were made using Inkscape (Free Software Foundation, Boston, MA, USA) and PowerPoint (Microsoft). *In silico* analysis of the *CACNA1H S1073C* and *ARMC5 A656S* mutation to predict their potential for damage of protein function was performed by our partner groups using the Polymorphism Phenotyping v2 tool (available from http://genetics.bwh.harvard.edu/pph2/). Sequencing results were analyzed with Sequencher 4.7 (demo version, Gene Codes Corporation Ann Arbor, MI, USA). If not stated differently, allele frequencies were obtained from the Exome Aggregation Consortium (ExAC) database, available from http://exac.broadinstitute.org/[349]. If not stated otherwise, single nucleotide polymorphism (SNP) IDs were obtained from the Database of Single Nucleotide Polymorphisms (dbSNP). Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. Available from: http://www.ncbi.nlm.nih.gov/SNP/. Genomic positions of mutations were obtained from the UCSC genome browser accessing human genome build 19 (available from http://genome.ucsc.edu/).

7.4.3 Results

In order to identify putative disease-causing variants, exome sequencing was performed on monozygotic twins diagnosed with PHA and three of their children after exclusion of three established forms of FH, namely FH I, FH III and FH IV (mutation in

CACNA1H M1549). A history of hypertension was established in the family (Figure 7 A). Clinical and biochemical parameters of the index patients have already been published[347]. The children of the index cases are all normotensive, with the exception of one son (IV-3, Figure 7 A) presenting with white coat hypertension. Notably, all the index cases were first diagnosed with hypertension when approximately 40 years old and all of their children were in their thirties when this study was conducted[347].

Exome sequencing revealed a *CACNA1H S1073C* mutation (chromosome 16:1258076 C>G, based on human genome build 19) in four family members: i.e. the two index cases and two of their children (Figure 7 A, B, C). The mutation is located in the linker region between tansmembrane segments S6 of homologous repeat II and S1 of repeat III of the CACNA1H protein (Figure 7 D). The *CACNA1H S1073C* variant (rs369474500) is listed in the ExAC database, where it was reported in 1 member of an East Asian population (allele frequency: $1.645 \cdot 10^{-5}$). No clinical relevance has been reported on this SNP. *In silico* analysis classified the mutation as possibly damaging to protein function.

To investigate a suspected role in PHA, we performed an *in vitro* analysis to investigate the effects of the mutation on *CYP11B2* expression and aldosterone production in the human adrenocortical cell line HAC15 (Figure 8). *CYP11B2* expression was slightly increased (~1.8-fold) in cells transfected with *CACNA1H wt* in comparison with empty vector-transfected cells, which is in line with our previous report[346]. No difference could be detected in cells transfected with *CACNA1H wt* in comparison to *CACNA1H S1073C*. Cells transfected with *CACNA1H M1549V* as a positive control show a significant increase in *CYP11B2* expression (~3.7-fold, p=0.0008) in comparison with *wt*-transfected cells, which is in agreement with our previous study on CACNA1H M1549V[346] (Figure 8 A). Studies on aldosterone production in these cells provide similar results, showing an increase in *CACNA1H wt*-transfected cells in comparison to those transfected with empty vector (~2.4-fold). No difference was detected between *CACNA1H wt* and *S1073C*, while transfection with *CACNA1H M1549V* resulted in a significant increase in aldosterone production in comparison to the *wt* (~4.6-fold, p<0.0001, Figure 8 B).

Furthermore, no difference between CACNA1H wt and S1073C was found in electrophysiological studies investigating channel activation and deactivation/inactivation in HEK293T cells (performed by Dr. Gabriel Stölting, data not shown).

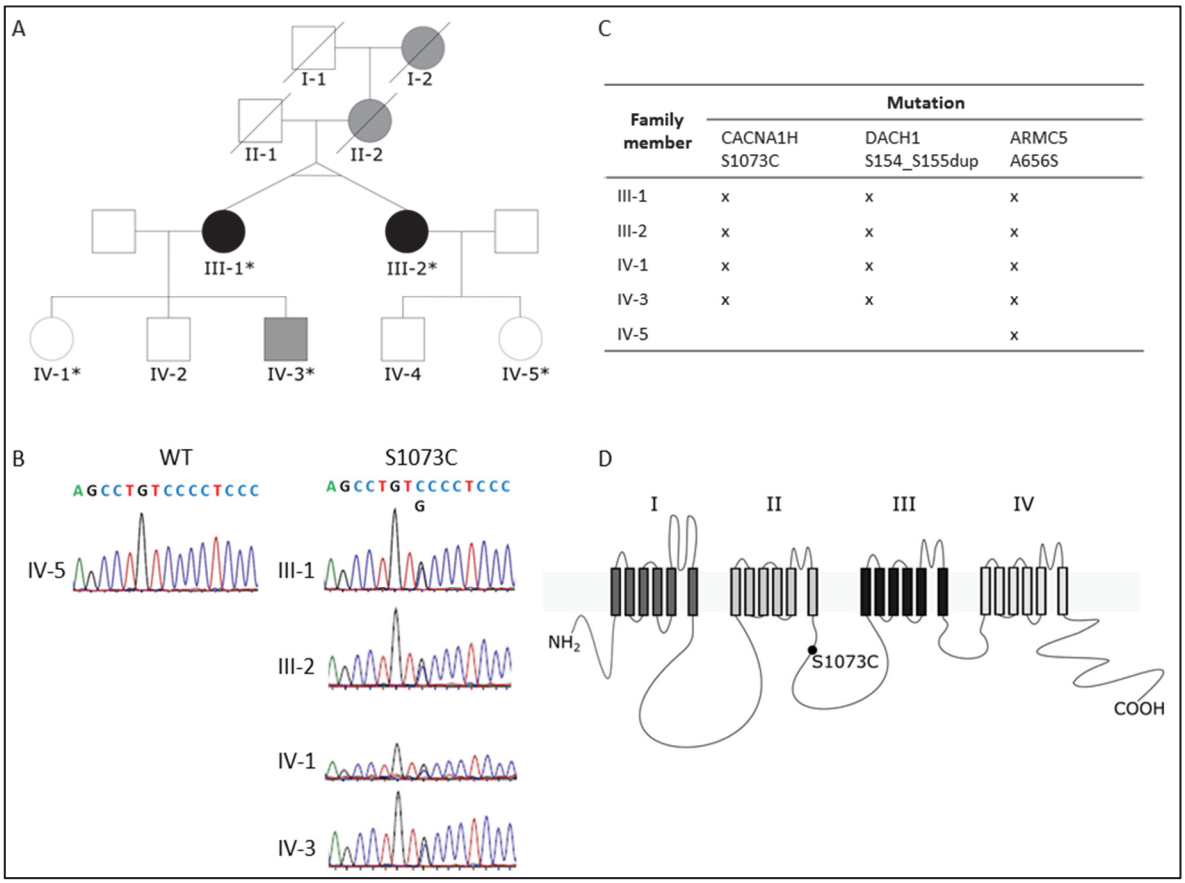

Figure 7. CACNA1H S1073C. A) Pedigree of the kindred investigated in this work. Round symbols represent females, square symbols represent males. Vertical lines indicate descent. Tilted lines with a vertical connector stand for monozygotic twins. III-1 and III-2 are the index cases. Individuals marked in black have been diagnosed with PHA, other hypertensive individuals are marked in grey. Genetic analysis, including exome sequencing, has been performed on individuals marked with asterisks. B) DNA sequencing results of all 5 family members obtained by Sanger Sequencing. Four family members carry the *CACNA1H S1073C* mutation, while one displays the wild type sequence at this position (Sequencing was performed by our cooperation partners and this depiction was included here with permission of Dr. F. Beuschlein). C) Mutations in *CACNA1H*, *DACH1* and *ARMC5* found in the investigated family members by exome sequencing. D) CACNA1H S1073C is located in the linker region between transmembrane segments S6 of homologous repeat II and S1 of repeat III.

Exome sequencing revealed the further mutations, i.e. *ARMC5 A656S,* (chromosome 16:31477272 G>T, found in all five family members, Figure 7 C) and *DACH1* S154_S155dup (chromosome 13:72440443 A>ACTGCTG based on human genome build 19, found in 4 family members, Figure 7 C), genes previously implicated in PHA or regulation of aldosterone production[86,329]. *In silico* analysis classified the *ARMC5* mutation as probably damaging to protein function. The mutation is referenced as rs753088639 in dbSNP. The ExAC Database reports a frequency of $8.306 \cdot 10^{-6}$ for this variant. It should be noted that the ExAC Database references only the genomic position of the SNP but not its SNP ID. The *DACH1 S154_S155dup* variant (rs551935140) can be found in the ExAC Database, where it has a reported allele frequency of $0.1261 \cdot 10^{-3}$. Of note, this mutation is referenced as rs773023074 in dbSNP, with the

UCSC genome browser stating an allele frequency of 0.96273. We were unable to identify the origins of this discrepancy. Clinical relevance has not been reported for this variant.

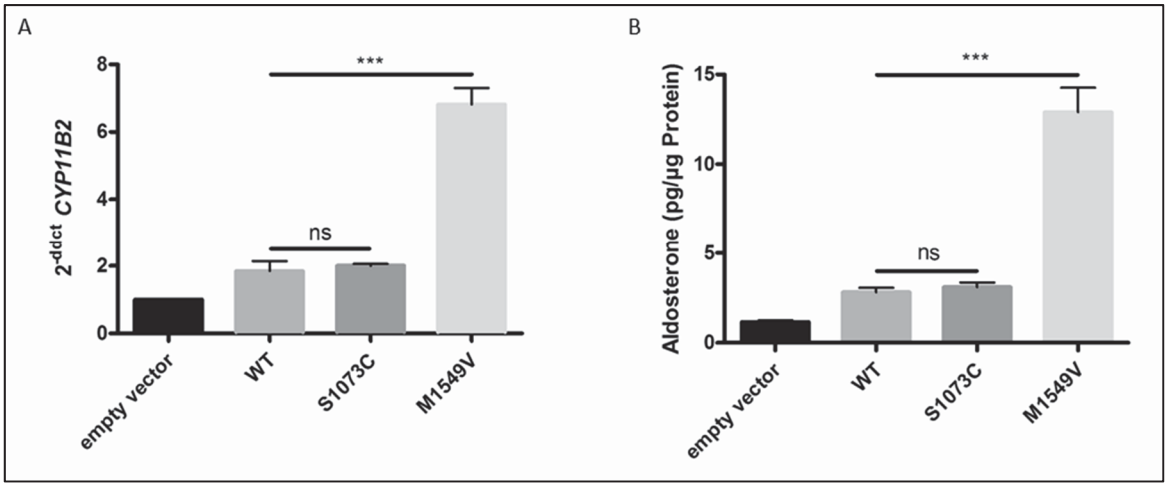

Figure 8. *CYP11B2* expression A) and aldosterone production B) in HAC15 cells transfected with empty vector, *CACNA1H wt*, *S1073C* or *M1549V*. Error bars represent standard error of the mean. Significances of results are indicated by ns (not significant) for $p > 0.05$, *** $P \leq 0.001$. Number of biological replicates analyzed are for A) n=4 for empty vector, wt, S1073C and n=2 for M1549V, for B) n=10 for empty vector, wt, S1073C and n=6 for M1549V.

7.4.4 Conclusions

In this study, the variant *CACNA1H S1073C* was identified in members of a family with a history of hypertension, including two family members diagnosed with PHA. Since mutations in *CACNA1H* have previously been implicated in FH and *in silico* analysis classified CACNA1H S1073C as possibly damaging, the mutation was considered a putative cause of the disease[115,118,346]. However, transfection with *CACNA1H S1073C* did not change either aldosterone production or *CYP11B2* expression in adrenocortical carcinoma cells in comparison to *CACNA1H wt*, while the previously implicated variant *CACNA1H M1549V* clearly did. Therefore, it is likely that *CACNA1H S1073C* is not the cause of PHA in these patients. This conclusion is supported by the lack of effect of the S1073C mutation on CACNA1H channel activation and inactivation, properties that have been shown to be altered in CACNA1H M1549 mutant channels in previous studies[115,118]. Two other mutations in genes previously implicated to play a role in aldosterone production or PHA, namely *DACH1 S154_S155dup* and *ARMC5 A656S*, have not been the subject of *in vitro* analyses in this study and might be the cause of FH in the

patients described above. However, a possibly high allele frequency of the *DACH1 S154_S155dup* variant challenges its position as a likely candidate.

8 Discussion

The studies included in this work all share the common goal of broadening our understanding of the underlying mechanisms of PHA. In this process, the investigation of PHA causes and their distinctive characteristics as well as the exploration of options for diagnosis and treatment are of particular interest. The following sections will discuss how our main findings fit in with other reports, how they contribute to our understanding of PHA and in what ways they might influence diagnosis and treatment options for PHA patients.

8.1 APA mutations and their correlation with patient and tumor characteristics

In recent years, recurrent somatic mutations in a variety of genes have been described in APAs (compare section 6.3.2.1 of the introduction). An increasing amount of studies of varying size has been performed on different populations (for an overview see Table 1 in the introduction). However, findings have not always been in agreement with each other, which might for example be due to differing diagnosis criteria, inherent population differences or insufficient statistical power (compare e.g. [157,237,247]).

While *KCNJ5* mutations in APAs have been extensively covered in the literature, with a meta-analysis of over 1600 patients available, other mutations that occur more rarely have been studied to a lesser extend (compare [247] and Table 1 in the introduction). The 2015 study by Scholl *et al.* featured in this work covers mutations in the five main genes currently implicated in APAs: *KCNJ5*, *ATP1A1*, *ATP2B3, CACNA1D* and *CTNNB1* (compare section 6.3.2.1 of the introduction).

8.1.1 Mutation frequencies are largely in accordance with previous reports

Mutation frequencies found in our study are mostly in line with other reports: *KCNJ5* mutations were found in 37.1% of APAs, which is in accordance with other findings in Western cohorts (compare Table 1). Also concurring with other reports, *G151R* was the most frequently found mutation in *KCNJ5* (23.7%), followed by the *L168R* mutation (9.3%). An unusually high number of additional novel *KCNJ5* mutations was found, i.e. *F154C*, *I150_G151insM*, *I157K*, *S143_I144insIA* (see [350]/section 7.1.2 and for comparison with other studies Table 6 in the appendix).

Mutations in *CACNA1D* were discovered in 10.3% of APAs, which is on the higher end of what other studies report (compare Table 1 in the introduction). It should be noted that in this study, two *CACNA1D* mutations became only apparent in exome sequencing. Since the general procedure for the detection of *CACNA1D* mutations in our cohort was

Sanger sequencing of mutation hotspots, it is possible that the detected mutation frequency is an underestimate[350]. The same is true for other studies that mostly or exclusively rely on a similar strategy, which applies to all studies listed in Table 7 in the appendix. This might be an indicator that *CACNA1D* mutations actually occur more frequently in APAs than the current literature suggests. Similar to previous reports, *G403R* was the most frequently found mutation in *CACNA1D* (6.2%). All other mutations we identified in *CACNA1D* have previously been reported[121,262] (also compare Table 7 in the appendix).

The frequency of *ATP1A1* mutations (8.2%) in our report is on the higher end of frequencies reported by other studies, while the occurrence of alterations in *ATP2B3* (3.1%) fits in the middle of the previously observed range (compare Table 1 in the introduction). Similar to previous reports *L104R* is the most commonly found mutation in *ATP1A1* (7.2%). Mutations in *ATP2B3* found in APAs are mostly deletions affecting the T123-V129 region (compare [244,249,255,262] and Table 8 in the appendix). Therefore, the novel V426G_V427Q_A428_L433del variation, consisting of a combined missense mutation and deletion, is somewhat distinctive (compare [350]/section 7.1.2).

Not many reports on mutation frequencies in *CTNNB1* in larger APA cohorts are available. The 2.1% mutation frequency found in our study is slightly below those reported by others, but taking into account limited sample size and the relatively rare occurrence of *CTNNB1* mutations, this is still within the margin of error (compare [271,276] and Table 1 in the introduction). In accordance with previous reports, all mutations are located in exon 3[245,271,276]. The most frequently reported S45P alteration was also detected in our cohort (compare e.g. [271,276] and Table 9 in the appendix).

8.1.2 Mutations relate to patient and tumor characteristics

Establishing correlations between known APA mutations and the clinical and radiological phenotype of the patients might facilitate diagnosis and treatment of the patients. One critical factor in APA diagnosis is adrenal vein sampling (AVS), performed to establish unilaterally increased aldosterone production. This invasive technique is complicated to perform and therefore requires an experienced radiologist[231]. Therefore, it would be desirable to avoid it in cases where reliable diagnosis of APA can be achieved by other means.

With regard to patient and tumor characteristics, we found a significant female predominance among patients with *KCNJ5*-mutated tumors. Tumors carrying *KCNJ5* mutations were also larger than both wild type tumors and tumors carrying mutations in other genes[350]. This is in line with a variety of previous reports and was confirmed by meta-analysis (compare e.g. [157,237,247,351] and Table 6 in the appendix). Furthermore, *KCNJ5* mutant tumors were found to be more likely of ZF-like appearance, consisting of mostly large clear cells with lipid droplets (compare [350]/section 7.1.2). This coincided with lower pre-contrast Hounsfield Units in CT analysis, which is indicative of lower tumor density (compare [350]/section 7.1.2). ZF-like appearance in *KCNJ5* mutant tumors has previously been described in some studies[256–258].

Based on these findings together with the frequent observation of younger age in patients with *KCNJ5* mutant APAs, which was confirmed by meta-analysis, our study suggests that AVS could be omitted in the diagnosis of young female patients with large adenoma presenting with low Hounsfield units in a CT scan[350]. For cases with a severe APA phenotype, this would be in line with current guidelines of the endocrine society[231]. Of note, a severe PHA phenotype is a feature that has also been described to be more common in patients with *KCNJ5* mutant tumors than in those without in the past (compare e.g. [247,351] and Table 6 in the appendix).

In addition, in our study, patients with non-*KCNJ5* mutations were more frequently male, which is in accordance with previous reports analyzing ATPase and *CACNA1D* mutations[249,269]. No correlation with a ZG- or a ZF-like phenotype was found for non-*KCNJ5*-mutated tumors in comparison to wild type tumors. Reports on the histological appearance of ATPase and *CACNA1D* mutant tumors vary, either reporting ZG-like appearance for one or both types of gene mutations or no correlation (compare [255,262,269] and Table 7, Table 8 in the appendix).

8.1.3 Conclusion and outlook

This study has broadened the information on correlation between APA mutational status with tumor and patient characteristics, further confirming a variety of observations made by previous studies. However, likely due to their high prevalence, *KCNJ5* mutant APAs remain the best-characterized ones. More detailed information on the distinctive features of tumors carrying ATPase and *CACNA1D* mutations in comparison to each other and to wild type tumors would be desirable to improve our knowledge of APAs

with the benefit of patients with these rarer mutations in mind. Especially *CTNNB1* mutations in APAs are not well-characterized so far, but more extensive studies have been conducted recently[271,276].

8.2 Macrolides are blockers of KCNJ5 mutant channels

In the 2017 study by Scholl *et al.* included in this work, a high-throughput screen of over 73,000 compounds was performed to identify inhibitors of KCNJ5 G151R and L168R channels which do not inhibit the wild type. Multiple compounds belonging to the family of macrolides were found to meet these criteria, roxithromycin being the most effective one[352].

Macrolides are a family of antibiotic compounds. Their main structural element is a 12-16 atom lactone ring[353]. Their antibiotic activity is based on inhibition of bacterial protein synthesis through binding of 23s rRNA in the 50s ribosome[353,354]. Macrolides mostly affect gram-positive bacteria, but are also effective treatments for infections with *Legionella, Chlamydia* and *Mycoplasma*. They are a frequent choice in the treatment of respiratory tract infections as well as soft tissue and skin infections[353,354]. Resistance to macrolides can be acquired by susceptible bacteria and has been observed in multiple strains[353,354].

Natural macrolides tend to be instable in gastric acid and to have undesirable pharmacokinetics. To improve drug performance, chemical processing such as salt formation or the introduction of structural modifications have been performed, resulting in the development of semisynthetic macrolides such as clarithromycin and roxithromycin[353]. In contrast to other macrolides, roxithromycin has a relatively high bioavailability (72-80% versus 30-55%). Macrolides are able to inhibit the enzyme CYP3A4, which is a member of the cytochrome P450 family and is involved in the metabolization of a variety of drugs. This can lead to problematic drug interactions[355]. The most common adverse effects of macrolides are gastrointestinal reactions, caused by motilin induction[356]. This motilide function has been successfully separated from antibiotic function by chemical modification[357]. Besides antibacterial and motilide function, anti-inflammatory effects of macrolides have been described[358].

The mechanisms through which macrolides inhibit KCNJ5 channels are unknown so far, although an interaction with the channel pore would be conceivable. Of note, it appears that the lactone ring and cladinose sugar of roxithromycin are critical elements

in KCNJ5 blockage[352]. GIRK channel structures have been investigated by protein crystallography in the past, which might also prove a feasible option here (compare e.g. [160,186]).

8.2.1 KCNJ5 channel blockers reduce aldosterone production and give new impulses for the diagnosis and treatment of PHA patients

In our study, roxithromycin as well as the non-antibiotic compound PLUX38 reduced *CYP11B2* expression in H295R cells expressing *KCNJ5 G151R* and *L158R* without affecting the wild type ([352]/section 7.2.2). Roxithromycin also reduced aldosterone production in stably transfected HAC15 cells, again not producing changes in the wild type[352] (section 7.2.2). This indicates that reduced channel activity is directly linked to reduced aldosterone production, suggesting that a similar effect could be achieved *in vivo*. Plasma concentrations of roxithromycin within the concentration range that induced significant effects *in vitro* have been reported in patients (up to 19.5 µM measured shortly after single dose administration, compared to significant *in vitro* effects being observed between 5 and 20 µM roxithromycin[352,359], section 7.2.2).

Two possible uses for macrolides in the course of APA diagnosis and treatment are imaginable: First, they could be used in the diagnosis of PHA patients with suspected APA. In theory, a drop in aldosterone levels in response to a short term administration of roxithromycin would be indicative of *KCNJ5* mutant APA. Consequently, it might be possible to forgo AVS in these patients[352]. Second, macrolides could serve as a non-surgical treatment option in patients with *KCNJ5* mutant APAs. However, to avoid resistances, non-antibiotic macrolides should be used to this end. The *in vitro* experiments using PLUX38 show that an effective blockage of KCNJ5 mutant channels can be separated from antibiotic properties in macrolides[352] (section 7.2.2). It has been suggested that APA formation in *KCNJ5* mutant tumors is due to proliferation in response to increased intracellular Ca^{2+} caused by influx through voltage-gated calcium channels upon membrane depolarization (compare [197] and Figure 5 in the introduction). Reports of massive adrenal hyperplasia in patients carrying germline *KCNJ5 G151R* mutations or a patient displaying hyperplastic lesions due to genetic mosaicism further support that notion (compare e.g. [311,319,352], Table 3 and section 6.3.2.2.3 in the introduction). Therefore, prevention of depolarization through blockage of mutant KCNJ5 channels might stop this effect. APA size might even been reduced or the tumor might disappear completely[352]. Such an effect has been observed in pituitary prolactinoma, where re-

duced prolactin output as well as tumor shrinkage are often achieved by dopamine agonists. In some patients, tumors recede completely, which allows for discontinuation of treatment[360].

The benefits of macrolide treatment for FH III patients are difficult to predict. While patients with *G151R* mutations might likely profit from macrolide treatment, effects on other mutations remain to be investigated. Of note, preliminary experiments in our lab have suggested that *CYP11B2* expression in T158A mutant cells might not be affected by roxithromycin (data not shown). Another important factor to consider is that, because FH III patients carry germline mutations, KCNJ5 mutant channels will not only be expressed in the adrenal gland, but also in other tissues like the heart in these patients (compare section 6.2.2.1.1 in the introduction).

When suggesting the prescription of macrolides to hypertensive patients, it should be ensured that a possible preceding calcium channel blocker treatment is discontinued. Combined administration of these drugs would not only interfere with diagnostic findings, but could also lead to severe hypotension due to increased plasma concentrations of calcium channel blockers, which tend to be subject to metabolization by CYP3A4[361]. Therefore, strict instructions and ensuring patient compliance is important.

8.2.2 Comparison with previous studies

Previous attempts at the identification of mutant KCNJ5 channel blockers have been made. In their 2014 study Tauber *et al.* identified e.g. verapamil and amiloride as blockers of mutant KCNJ5 channels[203]. Nevertheless, the scope of the study was much smaller, reporting tests on less than 20 compounds. Furthermore, the focus was on L168R inhibition, while G151R inhibition was not investigated for most substances. Verapamil did inhibit KCNJ5 L168R channels at pharmacological concentrations, while the G151R and T158A mutants required higher concentrations[203]. In our study, this substance was eliminated as a candidate in the initial screen due to its limited ability to block KCNJ5 G151R. Similarly, none of the other compounds tested by Tauber *et al.* were among the substances inhibiting KCNJ5 G151R with >33.3%[352]. A later study disclosed that the KCNJ5 E145Q channel is not blocked by verapamil, which suggests that pharmacological properties can largely vary depending on the mutation[313]. Inhibition of the wild type was also not investigated by Tauber *et al.*[203]. However, verapamil is a known blocker of K_{ACh} currents in the heart, which are generated by KCNJ5/KCNJ3 heterotetramers[156,204]. Therefore, it can be assumed that KCNJ5 wild type channels will be

affected by verapamil. This was an aspect deliberately excluded in our study to avoid undesired cardiac side effects, since loss of function in KCNJ5 wild type has been linked to long QT syndrome[196,352]. Notably, verapamil has been described to shorten, rather than prolong QT intervals and thus to protect from cardiac arrhythmias[362,363]. This protective effect has been suggested to be the result of its blockage of L-type voltage-dependent calcium channels[364,365]. In contrast to our study, Tauber *et al.* did not investigate the inhibitory effects on aldosterone production or *CYP11B2* expression[203,352].

8.2.3 Outlook: Future studies of channel inhibitors

Further studies are warranted to investigate the effects of KCNJ5 mutant channel blockers on aldosterone production and cell proliferation more closely. However, the lack of a suitable rodent model for mutant *KCNJ5* complicates *in vivo* studies. *Kcnj5* is expressed in neither mouse nor rat adrenal gland and a generated mouse model did not replicate the human phenotype[263–265].

To investigate the short-term effects of roxithromycin on aldosterone levels, a human study would be feasible, since the antibiotic is an approved drug in the EU. Since aldosterone has a short half-life of under 20 minutes, a response is expected to be measurable quickly[352,366]. Roxithromycin would be administered to APA patients for a few days and plasma aldosterone levels would be monitored. In patients eligible for unilateral adrenalectomy, the measured response could be matched with the genotype of the tumor after surgery[352].

Investigating the effects of macrolides on cell proliferation is a bigger challenge. Long-term administration of roxithromycin to patients would not be recommendable because it could lead to bacterial resistance[352]. Cell culture studies are complicated, since overexpression of mutant *KCNJ5* reduces cell viability and can cause death as a result of massive ion influx[311,352]. This notion is further illustrated by Oki *et al.*, who report that cell proliferation in HAC15 cells stably transfected with *KCNJ5 T158A* is reduced despite the fact that adrenal hyperplasia has been observed in mutation carriers *in vivo*[197,309]. Reduced viability due to strong ion influx would explain this effect. It should be noted that the authors do not consider this interpretation in the discussion of their findings[309]. The limitations of carcinoma cell lines as models for normal cell proliferation will be further discussed in section 8.3.1.3.

Primary cell culture of tumor cells would be another suitable approach to investigate the effects of macrolides on *KCNJ5* mutant APAs. However, while such *ex vivo* cultures

have been used in investigations of aldosterone production and cell proliferation in the past, limited availability, challenges in the isolation process and variation between samples from different patients might cause difficulties[268,333,367].

Additionally, establishing non-rodent animal models which resemble the human phenotype more closely in terms of adrenal *KCNJ5* expression might prove useful to test the long-term effects of roxithromycin. Alternatively, novel non-antibiotic macrolides would have to pass all stages for approval by pharmaceutical regulatory agencies.

To conclude, our findings will potentially improve the diagnosis and the treatment of APAs. Nevertheless further *in* and *ex vivo* studies are warranted on the way and might bring additional hurdles. Investigations of the structural interactions between macrolides and KCNJ5 would also be of interest.

8.3 *In vitro* investigations of the effects of *CACNA1H* mutations on *CYP11B2* expression and aldosterone production

As of yet, several germline mutations in *CACNA1H* have been implicated in FH IV[115,118,327] (compare section 6.3.2.2.5 in the introduction). The studies featured in this work improve our understanding of the underlying genetics of FH IV and give hints towards suitable treatment options. They also alert to the pitfalls researchers might encounter in genetic investigations of PHA.

8.3.1 CACNA1H M1549V increases aldosterone production and *CYP11B2* expression in HAC15 cells

The germline *CACNA1H M1549V* mutation was first identified in patients with early-onset PHA and is characterized by incomplete penetrance, i.e. not every mutation carrier displays the expected phenotype. Electrophysiological studies of mutant channels show slowed inactivation and activation at more hyperpolarized potentials[118]. The 2016 report by Reimer *et al.* featured in this work comprises *in vitro* studies of the *CACNA1H M1549V* mutation conducted in adrenocortical carcinoma cell lines[346]. Our investigations support the suspected role of the mutation as the cause of FH IV, since transfection with *CACNA1H M1549V* leads to an increase in *CYP11B2* expression and aldosterone production in comparison to wild type transfected cells[346]. This is true under unstimulated conditions, mimicking the *in vivo* situation in PA patients with a suppressed RAAS most accurately, as well as after stimulation with AngII. A similar tendency can be observed when extracellular K^+ concentrations are increased. Interestingly, while both wild type and *M1549V* transfected cells increase their *CYP11B2* and

aldosterone production upon stimulation, the relative increase is smaller in cells carrying the mutation[346] (section 7.3.2). We originally attributed this to the fact that the *M1549V* mutation and the other stimuli enhance aldosterone production via similar pathways[346].

8.3.1.1 Comparison of our findings on *CYP11B2* expression and aldosterone production with other studies

The role of mutations in CACNA1H M1549 in FH IV was further confirmed in a study by Daniil *et al.*. They discovered a germline *CACNA1H M1549I* mutation in a patient with early-onset PHA and learning disability[115]. Electrophysiological properties are similar to *CACNA1H M1549V* and cell culture experiments confirm increased aldosterone production and *CYP11B2* expression, but only after stimulation with K^+ ref.[115,118]. The latter observation has also been made for other CACNA1H mutations with different electrophysiological characteristics[115,327].

Interestingly, the requirement of K^+ to see the effect of the mutation is directly opposite to the findings of our study. Since CACNA1H M1549V and M1549I affect the same amino acid residue and display comparable electrophysiological properties, similar effects on aldosterone production and *CYP11B2* expression would be expected to be found in *in vitro* investigations of these mutations. When exploring possible reasons for the observed divergence, it should be taken into account that a tendency towards an increase in *CYP11B2* expression and aldosterone production in cells transfected with *CACNA1H M1549I* in comparison to the wild type can be perceived without prior stimulation in the study by Daniil *et al.*[115]. While the difference in the presented data lies within the margin of error, increased sample size might provide significant results under basal conditions, making the requirement for K^+ stimulation obsolete.

However, differences in experimental setups might also contribute to the observed divergence: First, different H295R-derived cell lines were chosen for the investigation of aldosterone production and *CYP11B2* expression, which might differ in levels of *CYP11B2* expression, aldosterone production as well as response to AngII[344]. Second, different transfection methods were used: While we transfected the cells via electroporation, Daniil *et al.* used a chemical transfection method[115,346]. This might result in differences in transfection efficiency and cell viability. It is possible that the experimental setup chosen by Daniil *et al.* relies on a synergistic effect of the transfected T-

type channels and endogenous L-type channels activated by K^+ stimulation. Stimulation with AngII, often described to mainly activate T-type channels, does not appear to provide this effect[23,115]. Contrastingly, maximum levels of aldosterone production and *CYP11B2* expression might have been reached already in mutant cells at the given overexpression rates in our study. Alternatively, strong ion influx through mutant channels might affect cell viability in mutant cells in comparison to the wild type at comparable transfection efficiencies. This might be especially relevant in cells transfected by a relatively harsh method, such as electroporation. Lower viability in mutant cells, negatively affecting cell function as a whole, could then reduce response to further stimulation in comparison to the wild type.

Several studies analyzing mutant KCNJ5 channels show that strong ion influx is able to reduce viability and proliferation in transfected cells (compare [309,311,352] and section 8.2.3). A milder transfection method or a markedly lower transfection rate would avoid or at least attenuate these mechanisms and possibly allow for synergistic effects of K^+ and the mutation on aldosterone production. To conclude, the seemingly contradictory findings on the effects of stimulators of aldosterone production on *CACNA1H* mutant-transfected cells can likely be reconciled by considering statistical power and procedural differences of the two studies.

8.3.1.2 CACNA1H M1549V can be inhibited by T-type channel blocker mibefradil

In our study, we also investigated the effects of the T-type channel inhibitor mibefradil, which prevents Ca^{2+} influx through CACNA1H channels[125]: The compound significantly reduced both *CYP11B2* expression and aldosterone production in cells overexpressing *CACNA1H* wild type and *M1549V*. However, upon inhibitor treatment aldosterone production was still elevated in *CACNA1H M1549V*-transfected cells in comparison to the wild type, while there was no difference observed in *CYP11B2* expression[346] (section 7.3.2). We attributed this divergence to the short half-life of *CYP11B2* mRNA in comparison to the relatively high stability of aldosterone[346,368,369]. Hence, residual elevated hormone levels from before the inhibitor exerted its full effect on aldosterone production would still be measurable via ELISA, while *CYP11B2* expression would represent the more recent situation in the cell after the full effect of the inhibitor has already been reached. First, the observed effects of mibefradil substantiate that the increases in aldosterone production and *CYP11B2* expression in our study were indeed the product of T-type channel function. Second, they demonstrate that *CACNA1H M1549V* mutant

channels are still susceptible to pharmacological T-type channel inhibition. This indicates that T-type channel blockers might constitute a suitable treatment option in patients with FH IV. However, mibefradil is not an eligible choice, since it was retracted from the market due to severe drug interactions[129].

Compounds achieving a relatively selective blockage of low voltage-activated calcium channels over high voltage-activated ones are rare (for review compare [370]). However, while many calcium antagonists frequently prescribed as antihypertensives primarily act on L-type channels, some can also block T-type channels at micromolar concentrations (for review compare [97,370]). Furthermore, the drug efonidipine, currently approved in Japan, is a dual T-type and L-type channel blocker that has been shown to effectively reduce aldosterone production *in vitro* as well as in human test subjects[141,229,371,372]. In addition, the efonidipine R(-) isomer seems to be a selective inhibitor of T-type channels and its ability to block CACNA1H channels has been confirmed[373,374].

Since the informative value of cell culture studies in the assessment of treatment options is limited, an animal model might help to explore calcium channel blockers as treatment options for patients with FH IV. *CACNA1H* is expressed in the murine ZG, which might make the mouse a suitable model organism[63].

8.3.1.3 Outlook: Investigating the effects of *CACNA1H M1549V* on cell proliferation

In this study, we only investigated short term effects of the *CACNA1H M1549V* mutation on aldosterone production and *CYP11B2* expression *in vitro*[346]. Micronodular adrenal hyperplasia has been observed in one FH IV patient who underwent adrenalectomy. Nodules were positive for the expression of CACNA1H[118]. This poses the question whether CACNA1H M1549V might also promote cell proliferation or prevent cell death. In the past, the antiproliferative effects of mibefradil led to the conclusion that Ca^{2+} influx through T-type voltage-gated calcium channels might be linked to cell proliferation[375,376]. However, these assumptions were challenged upon the discovery of several off-target effects of the compound[97,377]. Not much is currently known about the role of T-type channels in adrenal cell proliferation and growth (compare [110]).

It should be noted that cell culture studies investigating the impact of CACNA1H M1549V on adrenal proliferation might have several limitations. Generally, continuous cancer cell lines are often generated from fast growing tumors[378]. A comparison of cell

lines with tumor cells and cells from normal tissue originating from six different tissue types conveyed frequent alterations in genes involved in the cell cycle[379]. These findings challenge the notion that an accurate representation of normal proliferation can be achieved by the use of cancer cell lines, which would be desired to investigate the proliferative effects of a mutation in either normal or benign tumor tissue. Nevertheless, H295R cells have been used to study cell growth and proliferation in the past. However, these studies were often targeted at the characterization or treatment of adrenocortical cancer (compare [332,333]). In addition to adrenocortical carcinoma cell lines, primary cells from animals have been used to study adrenal proliferation (compare e.g. [380]). However, these cells have a limited life span and their properties might change over time. Therefore, their use requires constant supply with fresh tissue and isolation of the desired cells (compare [332,333]). In addition, they might prove difficult to transfect (compare e.g. [381]).

The expression system also poses problems: massive ion influx created by overexpression of mutant ion channels might already affect cell viability, masking other effects (compare sections 8.3.1.1 and 8.2.3). Therefore, an animal model might prove a more suitable tool to investigate the effects of *CACNA1H M1549V* on cell proliferation. These animals could be kept for an extended period of time and adrenals could be screened for hyperplasia after death. Environmental factors which might affect the development or severity of PHA and hyperplasia, such as salt intake, could also be monitored in this experiment. This might help to understand environmental factors contributing to incomplete penetrance of the mutation.

Despite the fact that our studies of the *CACNA1H M1549V* variant have substantiated its role in FH IV and have provided insight into pharmacological options, further in-depth studies are needed. These should include the long term study of animal models carrying the mutation.

8.3.2 *CACNA1H S1073C* does not affect aldosterone production and *CYP11B2* expression in HAC15 cells

While *in vitro* investigations of *CACNA1H M1549V* support its role in FH IV, similar studies of the *S1073C* mutation do not reveal any effect of the mutation on channel function or aldosterone production (section 7.4.3, Figure 8). This indicates that it is most likely not the primary cause of PHA in the family it was discovered in.

8.3.2.1 Sequence conservation and structural considerations

High conservation across orthologues and paralogues, as observed for CACNA1H M1549 and the surrounding residues, can serve as an indicator of the region's importance in protein function[118]. The CACNA1H S1073 residue is not strongly conserved across species. Only other primates carry a serine in the corresponding position, while other animals, including a variety of mammals, carry a proline residue. However, the surrounding region appears to be at least in part conserved (compare Figure 9 A). This might hint at an existing, but not very stringent sequence-function relationship of this region. While the residue corresponding to CACNA1H M1549 is conserved across different low voltage-activated calcium channel α_1 subunits, CACNA1H S1073 is not. Residues surrounding CACNA1H S1073 do not show any homology to other classes of voltage-gated calcium channel α_1 subunits either (compare Figure 9 B).

The CACNA1H S1073C mutation is located in the linker between segments IIS6 and IIIS1 (Figure 7 D). This region is involved in channel modulation by a variety of second messengers (for review see [382]). Mutations in this region have been discovered in patients with epilepsy. While the A1059S mutation appears to mediate a loss of CACNA1H channel function, other mutations found in this region have been found not to change channel characteristics[383]. Another mutation in this region, CACNA1H G1064R, has been implicated in PHA[327]. However, the evidence supporting the role of this mutation as the cause of PHA is less compelling than for mutations in CACNA1H M1549. Similar to the results found in our study on CACNA1H S1073C, electrophysiological properties did not reveal any difference between wild type and mutant channels. Furthermore, aldosterone production and *CYP11B2* expression did only differ between wild type and mutant cells upon stimulation with K^+, but not at basal conditions[327]. The latter observation is in line with the previously discussed study by Daniil *et al.*[115] (compare section 8.3.1.1).

8.3.2.2 Limitations of our study

Unlike Daniil *et al.* and Roomp *at al.,* we did not investigate aldosterone production or *CYP11B2* expression under elevated extracellular K^+ in our study on *CACNA1H S1073C*[115,327]. Considering our investigations of *CACNA1H M1549V*, conducted similarly to the ones of *CACNA1H S1073C*, it is unlikely that our experimental setup would conceal an effect of the mutation under basal conditions that would become evident after stimulation. In contrast, differences between wild type and M1549V became less pronounced after stimulation under our experimental conditions[346] (compare section

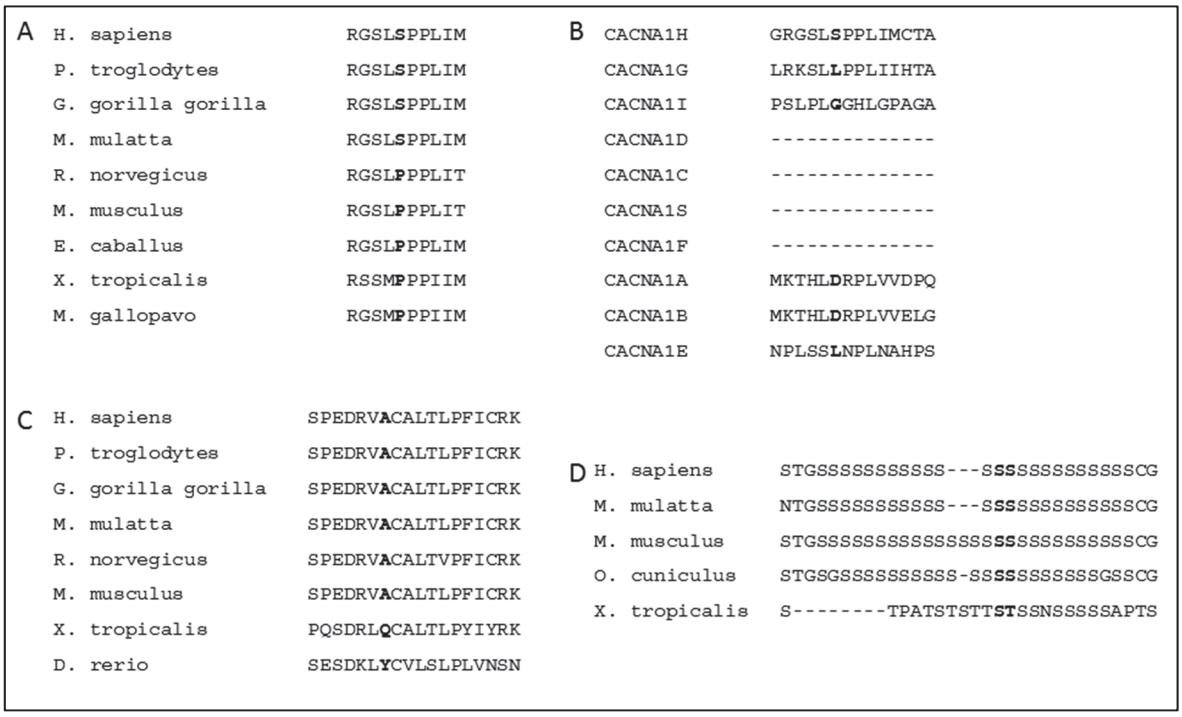

Figure 9. Homologues of the protein regions containing human CACNA1H S1073, ARMC5 A656 and DACH1 S154_S155. A) Orthologues of the CACNA1H S1073C-containing protein region across different species. S1073 is conserved in primates, but not in other mammalian species. B) Paralogues of the CACNA1H S1073C-containing protein region across the different human voltage-gated calcium channel α_1 subunits. The S1073 residue is not conserved. C) Orthologues of the ARMC5 A656-containing region. The residue is conserved across different mammalian species. D) Orthologues of the DACH1 S154_S155-containing region. In mammals, this region contains multiple consecutive serine residues, but their number varies. Alignments were done via the uniprot website (http://www.uniprot.org/). Uniprot references used: O95180, H2QZZ9, G3RZS6, F7DCZ9, Q9EQ60, O88427, F6VZM1, F6U0H3, G1N160, O43497, Q9P0X4, Q01668, Q13936, Q13698, O60840, O00555, Q00975, Q15878, Q96C12, K7APD1, G3QWL1, F6T7B4, Q5PQP9, Q5EBP3, F6XYH0, F1QIU6, Q9UI36, F6XES1, Q9QYB2, G1SGW7, Q0PNF7

8.3.1). Furthermore, unstimulated conditions represent the situation in PHA patients most accurately, where the RAAS is suppressed and extracellular K^+ is in or below the normal physiological range. Nevertheless, it is possible that small effects on aldosterone production or effects based on a different underlying change in channel properties might not be identified under our experimental conditions. *In vivo*, they might still contribute to a PHA phenotype, especially in conjunction with other genetic or environmental factors.

For some CACNA1H mutations (S196L, P2083L and V1951E) found in PHA patients, Daniil *et al.* described no marked effects on channel activation or inactivation, but reported significant differences in channel facilitation[115]. Increased channel facilitation would allow for increased Ca^{2+} influx into the cell upon multiple consecutive depolarizing pulses. Neither channel facilitation nor measurements of intracellular Ca^{2+} con-

centrations were subject of our investigation. Therefore, effects of the S1073C mutation on other channel properties but activation and inactivation cannot be excluded. They might influence intracellular Ca^{2+} levels without marked effects on aldosterone production at basal level.

8.3.2.3 Outlook: Investigations of mutations in *ARMC5* and *DACH1*

Since our investigations did not provide any evidence that the *CACNA1H S1073C* mutation might cause PHA, other germline mutations found in genes implicated in aldosterone homeostasis or PHA should be investigated. DACH1 has been suggested to be a negative regulator of *CYP11B2* expression and aldosterone production[86]. Therefore, a loss-of-function mutation would be expected to increase aldosterone production. A duplication of serine residues (*Dach1 S154_S155dup*) was found in four of five family members. S154_S155 are located in a sequence of multiple consecutive serine residues. A comparison of orthologues reveals that their number appears not to be conserved across species (compare Figure 9 D). This indicates that the exact number of serine residues in this region might not have a vital effect on protein function and that the effect of a serine duplication at this position might be minor. Another fact supporting this idea is that the region surrounding *DACH1 S154_S154* is rich in SNPs, which affect the number of consecutive serines in this region and therefore render a clinical relevance rather unlikely (Table 4). Of note, database reports on the frequency of DACH1 S154_S155dup vary ($0.1261 \cdot 10^{-3}$ in the ExAC Database and 0.96273 according to the UCSC genome browser, compare section 7.4.3). In case of an actual allele frequency of ~0.96, a disease causing effect of the variant would be improbable.

Another mutation found in five out of five exome-sequenced family members was *ARMC5 A656S.* This residue, as well as the surrounding residues, are conserved in mammals, but can differ markedly in other vertebrates (compare Figure 9 C). This complicates evaluation of the region's role in protein function. In the vicinity of A656, a T643M mutation has previously been described in patients with PHA. The mutation was predicted to be detrimental to protein function[329]. No mutations in the direct vicinity of A656 were discovered in macronodular adrenal hyperplasia, but some are located roughly in the same region of the protein (150 amino acids upstream or downstream of A656, namely L548P, R593P, R619X, A702_S706del)[328,384,385].

To evaluate the role of *ARMC5 A656S* and *DACH1 S154_S155dup* as a putative cause of FH, *in vitro* investigations similar to those performed for *CACNA1H S1073C* or *M1549V*

should be conducted. Given the aforementioned information, *ARMC5 A656S* could be considered a stronger candidate than *DACH1 S154_S155dup,* so the former mutation might represent a suitable starting point for further experiments. Of note, carriers of other voltage-gated calcium channel mutations implicated in FH do not always display a PHA phenotype[118,121,320]. While environmental factors affecting penetrance are difficult to simulate in cell culture experiments, co-transfection of two or all three of the discovered mutations might mimic the patients' genetic predispositions more closely, and reveal cumulative effects of the variants on aldosterone production that might not be detected in an analysis of the individual mutations.

Despite our investigations of the *CACNA1H S1073C* mutation, the origin of PHA in the patients it was discovered in remains unclear. Further studies of additionally identified mutations in *DACH1* and *ARMC5* might provide further insight into the underlying genetic causes.

Table 4. Known variants in *DACH1* altering the number of consecutive serine residues surrounding S154_S155. Based on information from the ExAC Database. Genomic position in human genome build 19. Effects on the DACH1 protein based on the genomic position using the UCSC genome browser. *Marks the *DACH1 S154_S155dup* mutation found in the patients discussed in this work.

Chromosome	Position	Base change	Effect on protein	Annotation	Allele frequency
13	72440443	ACTG>A	S155del	inframe deletion	0.006518
13	72440443	ACTGCTGCTG>A	S153_S155del	inframe deletion	0.0001261
13	72440443	A>ACTG	S155dup	inframe insertion	0.0005046
13	72440443	A>ACTGCTG	S154_S155dup*	inframe insertion	0.0001261
13	72440467	ACTG>A	S147del	inframe deletion	0.01603
13	72440467	A>ACTG	S147dup	inframe insertion	0.0002324
13	72440467	A>ACTGCTG	S146_S147dup	inframe insertion	0.00007746

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11 Appendix

11.1 Projects featured in this work and contributions

11.1.1 Project 1 (Section 7.1)

U. I. Scholl, J. M. Healy, A. Thiel, A. L. Fonseca, T. C. Brown, J. W. Kunstman, M. J. Horne, D. Dietrich, J. Riemer, S. Kücükköylü, E. N. Reimer, A. C. Reis, G. Goh, G. Kristiansen, A. Mahajan, R. Korah, R. P. Lifton, M. L. Prasad, and T. Carling, "Novel somatic mutations in primary hyperaldosteronism are related to the clinical, radiological and pathological phenotype," Clinical Endocrinology, vol. 83, pp. 779–789, 2015.

Esther Reimer's contribution to the publication: ~5% (FFPE DNA from tissue, PCR analysis and confirmation of *KCNJ5* mutations for 8 of 62 newly analyzed patients, initial PCRs to screen for *CACNA1D*, *ATP1A1*, *ATP2B3* and *CTNNB1* mutations for 8 of 62 newly analyzed patients with minor help by Anne Thiel, *CACNA1D*, *ATP1A1*, *ATP2B3* and *CTNNB1* confirmatory PCRs and mutation analysis for these 8 patients performed by Anne Thiel)

11.1.2 Project 2 (Section 7.2)

U. I. Scholl, L. Abriola, C. Zhang, E. N. Reimer, M. Plummer, B. I. Kazmierczak, J. Zhang, D. Hoyer, J. S. Merkel, W. Wang, and R. P. Lifton, "Macrolides selectively inhibit mutant KCNJ5 potassium channels that cause aldosterone-producing adenoma," Journal of Clinical Investigation, vol. 127, no. 7, pp. 1–12, 2017.

Esther Reimer's contribution to the publication: ~15% (performance and data analysis of experiments to test the effects of roxithromycin on *CYP11B2* expression and aldosterone production and the effects of PLUX38 on *CYP11B2* expression)

11.1.3 Project 3 (Section 7.3)

E. N. Reimer, G. Walenda, E. Seidel, and U. I. Scholl, "CACNA1H M1549V mutant calcium channel causes autonomous aldosterone production in HAC15 cells and is inhibited by mibefradil," Endocrinology, vol. 157, no. 8, pp. 3016–3022, 2016.

Esther Reimer's contribution to the publication: ~70% (cloning, performance and data analysis of experiments to test the effects of *CACNA1H M1549V* on *CYP11B2* expression and aldosterone production with minor help by Eric Seidel and Gudrun Walenda)

11.1.4 Project 4 (Section 7.4)

Unpublished

Esther Reimer's contributions: Generation of pCMV6-Entry-*CACNA1H S1073C* and pCMV6-AC-*CACNA1H S1073*-IRES-GFP-Puro, performance and data analysis for experiments testing the effects of CACNA1H S1073C on *CYP11B2* expression, performance and data analysis of experiments to test the effects of CACNA1H S1073C on aldosterone production together with members of Felix Beuschlein's group (performance and analysis of aldosterone ELISA by the group of Felix Beuschlein, Klinikum der Universität München, performance of transfection, treatment, harvest as well as performance and analysis of protein assay by Esther Reimer)

Additional contributions by others mentioned in this work: patients, genetic analysis, *in silico* analysis of mutations by Yara Rhayem, Tetsuro Yoshida, Thomas Schwarzmayr, Martin Bidlingmaier, Martin Reincke, Tim-Matthias Strom, Mitsuhide Naruse, Felix Beuschlein, electrophysiology by Gabriel Stölting, Forschungszentrum Jülich

11.2 Plasmid maps

Maps of the most important plasmids used in this work, generated with SerialCloner 2.6.1

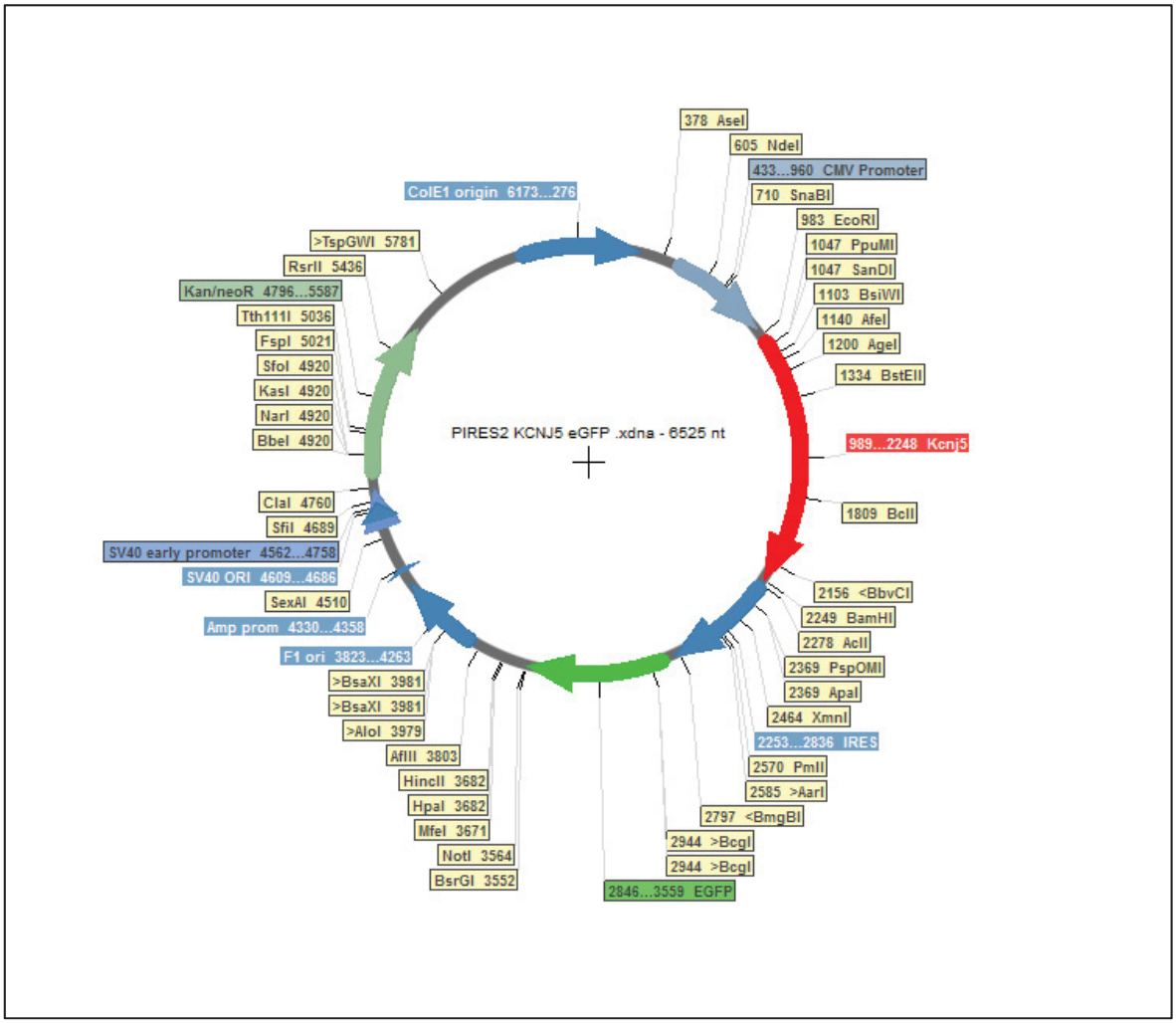

Figure 10. Plasmid map of pIRES2-*KCNJ5*-eGFP.

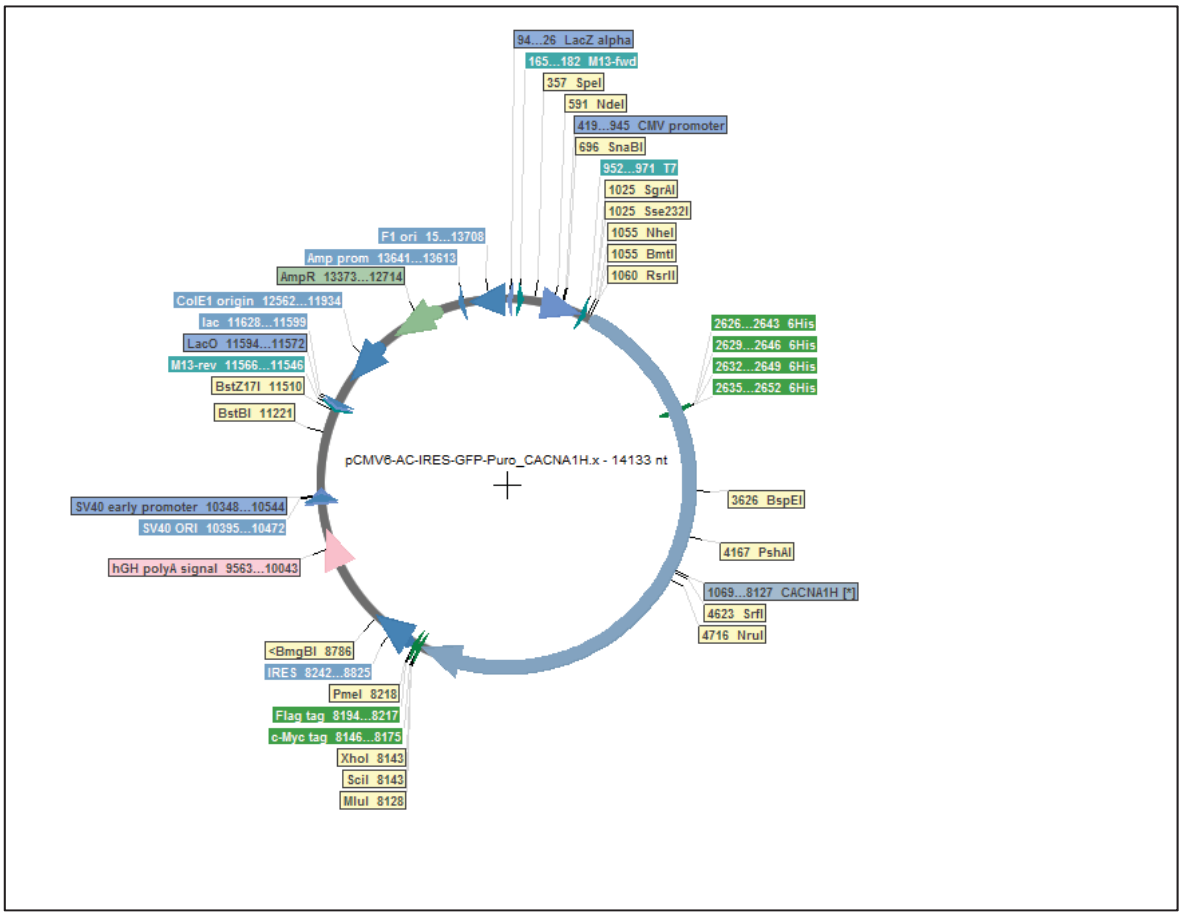

Figure 11. Plasmid map pCMV6-AC-*CACNA1H*-IRES-GFP-Puro.

11.3 Sequencing Primers

The following primers were used for Sanger sequencing of *CACNA1H* cDNA in this work.

Table 5. Primers used for plasmid sequencing. All primers target the human cDNA.

Primer name	Sequencing target		Sequencing direction	Primer sequence
1H-seq 0	Human cDNA	CACNA1H	rv	TTCTCCTCGCCCTCCTCCGTCT
1H-seq1	Human cDNA	CACNA1H	fw	TCTCGGCTATCAGGACCGTG
1H-seq2	Human cDNA	CACNA1H	fw	AAGAGCTGCTGAAGTACGTGG
1H-seq3	Human cDNA	CACNA1H	fw	AGCGGCTCGGAAAGTGGA
1H-seq4	Human cDNA	CACNA1H	fw	TCCTGTACAACGGCATGGC
1H-seq5	Human cDNA	CACNA1H	fw	ACTCGGAGGACAGCTGCT
1H-seq6	Human cDNA	CACNA1H	fw	TGAACATCATGTACGACGGGC
1H-seq7	Human cDNA	CACNA1H	fw	TTTTTTATCTATGCTGCGCTGGG
1H-seq8	Human cDNA	CACNA1H	fw	TGCACACCGATTCCTTGGAAG
1H-seq9	Human cDNA	CACNA1H	fw	ACAGTGTGACCCCAGAATCCAGAG

11.4 Software used for the generation of this thesis

This thesis was written in Microsoft Word (Microsoft, Redmond, WA, USA). If not indicated otherwise, figures were created using Inkscape (Free Software Foundation, Boston, MA, USA) and Microsoft PowerPoint. SerialCloner 2.6.1 (Serial Basics) was used for the management of plasmids and cloning. Plasmid maps included in this work were generated with this program.

11.5 Summary of APA studies

The following tables summarize numerous studies investigating mutations in APAs and findings with regard to patient and tumor characteristics.

Table 6. Studies of *KCNJ5* mutant APAs. Adapted from Stowasser *et al.* 2013[351].

Study	Region	Mutation frequencies	Characteristics of patients and tumors with *KCNJ5* mutation					
			Sex	Age	APA size	PHA severity	Histology	Comments
Akerström, 2012[a,251]	EU, AU	46.3% (157/339)	G151R: 24.8% (84/339) L168R: 20.9% (71/339) E145Q: 0.6% (2/339)	female[b]	N/D[c]	N/D[c]	N/A	N/A	[a]overlap with Akerström, 2015 [b]compared to wt [c]younger/larger in males without hyperplasia
Akerström, 2015[a,249]	EU, AU	54.5% (90/165)	N/A	female[b]	younger[b]	N/D[b]	N/A	N/A	[a]overlap with Akerström 2012 [b]compared to non-KCNJ5 mutant
Arnesen, 2013[272]	EU	35.7% (10/28)	G151R: 21.4% (6/28) L168R: 14.3% (4/28)	N/D[a]	N/D[a]	N/A	lower[a,b]	N/A	[a]compared to wt [b]lower systolic and diastolic blood pressure
Azizan, 2012[259]	EU, AU	41.1% (30/73)	G151R 26.0% (19/73) L168R 13.7% (10/73) I157del 1.4% (1/73)	N/A	N/A	larger[a]	N/A	N/A	[a]compared to wt
Boulkroun, 2012[246]	EU	33.9% (129/380)	G151R: 20% (76/380) L168R: 13.9% (53/380)	female[a]	younger[a]	N/D[a]	higher[a,b]	N/A	[a]compared to wt [b]higher PAC
Cheng, 2015[198]	EA	37.7% (26/69)	G151R: 15.9% (11/69) L168R: 13% (9/69) E145Q: 1.4% (1/69) R115W: 4.3% (3/69) E246G: 2.9% (2/69)	female[a]	younger[a]	larger[a]	N/D[a,b]	N/A	[a]compared to wt [b]PAC/Blood pressure
Choi, 2011[a,197]	EU	36.4% (8/22)	G151R: 9.1% (2/22) L168R: 27.3% (6/22)	female[b]	younger[b]	N/A	N/A	N/A	[a]overlap with Scholl, 2013 [b]according to Scholl and Lifton, 2013[237]

Study	Region	Mutation frequencies	Characteristics of patients and tumors with *KCNJ5* mutation					
			Sex	Age	APA size	PHA severity	Histology	Comments
Dekkers, 2014[256]	EU	41.5% (22/53)	G151R: 18.9% (10/22)[a] L168R: 22.6% (12/22)[a] E1545Q: 1.9% (1/22)	female[b]	N/D[c]	N/A	N/D[c]	ZF-like[c]	[a]1 multinodular hyperplasia with 2 nodules containing different *KCNJ5* mutations [b]compared to male [c]compared to wt
Dutta, 2014[273]	EU	31.4% (11/35)	G151R: 22.9% (8/35) L168R: 8.6% (3/35)	7/11 female	N/D[a]	N/D[a]	N/D[a,b]	N/A	[a]compared to wt and different mutant groups [b]PAC
Fernandes-Rosa, 2014[262]	EU	38% (180/474)[a]	G151R: 23.8% (113/474) L168R: 13.7% (65/474) T158A:0.2% (1/474) W126R:0.2% (1/474)	female[b]	N/D[b]	N/D[b,c]	N/A	N/D[d]	[a]for patient characteristics only 365 APAs [b]compared to wt [c]compared to *CACNA1D* mutant [d]78 APAs, compared across groups
Hong, 2016[274]	EA	71.2% (47/66)	G151R: 47% (31/66) L158R: 24.2% ((16/66)	female[a]	younger[a,b]	N/D[a]	N/D[a,c]	N/A	[a]compared to wt [b]higher portion under 35, no difference in age [c]PAC, blood pressure
Kitamoto, 2015[248]	EA	69.4% (75/108)	G151R 42.6% (46/108) L168R 22.2% (24/108) E145Q 1.9% (2/108) T158A 0.9% (1/108) I157del 1.9% (2/108)	N/D[a]	younger[a]	larger[a]	higher[a]	N/A	[a]compared to wt [b]PAC, urinary aldosterone
Kuppusamy, 2014[266]	EU	24.6% (48/195)	G151R 13.8% (27/195) L168R 8.7% (17/195) T158A 1.5% (3/195) insT149 0.5% (1/195)	N/A	N/A	N/A	N/A	N/A	
Monticone, 2012[250]	EU, EA, NA	38.3% (18/47)	G151R 17% (8/47) L168R 21.3% (10/47)	female[a]	N/D[a]	N/A	N/D[a,b]	N/A	[a]compared to wt [b]PAC, blood pressure

Study	Region	Mutation frequencies	Characteristics of patients and tumors with *KCNJ5* mutation					
			Sex	Age	APA size	PHA severity	Histology	Comments
Scholl, 2013[a,121]	EU, NA	32.8% (21/64)	G151R: 18.8% (12/64) L168R: 14.1% (9/64)	15/21 female	N/A	N/A	N/A	N/A	[a]overlap with Choi, 2011
Taguchi, 2012[254]	EA	65.2% (15/23)	G151R: 52.2% (12/23) L168R: 13.0% (3/23)	8/15 female	N/A	N/A	N/A	N/A	
Williams, 2013[275]	EU	39.3% (44/112)	G151R: 31.3% (35/112) L168R: 8.9% (10/112) T158A: 0.9% (1/112) W126R: 0.9% (1/112)	female[a]	N/D[b]	N/D[b]	N/D[b]	N/A	[a]male vs. female [b]compared between groups
Wu, 2015[267]	EA	59.5% (88/148)	G151R: 30.4% (45/148) L168R: 27.7% (41/148) I157del: 0.7% (1/148) T158A: 0.7% (1/148)	N/A	N/A	N/A	N/A	N/A	
Wu, 2017[276]	EA	53% (116/219)	G151R 29% (64/219) L168R 21.9% (48/219) T158A 1.4% (3/219) I157del 0.5% (1/219)	N/A	N/A	N/A	N/A	N/A	
Xekouki, 2012[277]	NA	12.5% (2/16)	G151R: 12.5% (2/16)	all female	N/A	N/A	N/A	N/A	
Zheng, 2015[253]	EA	76.8% (129/168)	G151R 39.9% (67/168) L168R: 35.7% (60/168) T158A: 0.6% (1/168) T148-T149insR 0.6% (1/168)	female[a]	N/D[a]	larger[a]	higher[a]	N/A	[a]compared to wt

N/A: not available, N/D: no difference, AU: Australia, EA: East Asia, EU: Europe, NA: North America, PAC: plasma aldosterone concentration

Table 7. Studies of *CACNA1D* mutant APAs. Layout adapted from Stowasser *et al.* 2013[351].

Study	Region	Mutation frequencies		Characteristics of patients and tumors with *CACNA1D* mutation					
				Sex	Age	APA size	PHA severity	Histology	Comments
Akerström, 2015[249]	EU, AU	3% (5/165)	G403R: 1.2% (2/165) F474L: 0.6% (1/165) V1353M: 0.6% (1/165) V401L: 0.6% (1/165)	3/5 male[a]	N/A[a]	N/A[a]	N/A	N/A	[a]male, older and smaller for *CACNA1D* + ATPase compared to *KCNJ5*
Azizan, 2013[269]	EU	7.9% (12/152)	G403R 1.3% (2 152) V259D 1.3% (2/152) F747L 1.3% (2/152) I750M 1.3% (2/152) P1336R 1.3% (2/152) M1354I 0.7% (1/152) R990H 0.7% (1/152)	10/12 male[a]	N/A[a]	N/A[a]	N/A	N/A[a]	[a]male, older, smaller and ZG-like for *CACNA1D* + ATPase compared to *KCNJ5*
Dekkers, 2014[256]	EU	1.9% (1/53)	I1750M: 1.9% (1/53)	1/1 male	N/A	N/A	N/A	ZG-like	

Study	Region	Mutation frequencies	Characteristics of patients and tumors with *CACNA1D* mutation					
			Sex	Age	APA size	PHA severity	Histology	Comments
Fernandes-Rosa, 2013[262]	EU	9.3% (44/474)	G403R (8A + 8B): 2.3% (11/474) F747L: 1.3% (6/474) V1338M: 1.3% (6/474) I750M: 0.8% (4/474) A998I: 0.6% (3/474) V1151F: 0.2% (2/474) V259R: 0.2% (1/474) S652L: 0.2% (1/474) L655P: 0.2% (1/474) Y741C: 0.2% (1/474) F747V: 0.2% (1/474) I750F: 0.2% (1/474) V979D: 0.2% (1/474) L981N: 0.2% (1/474) A998V: 0.2% (1/474) I1152N: 0.2% (1/474) P1336R: 0.2% (1/474) M1354I: 0.2% (1/474)	male[b,c]	older[b,c]	smaller[a,b,c], N/D[d]	higher[b,c,e]	N/D[a,b,c]	[a]compared to wt [b]compared to *KCNJ5* [c]78 APAs, Paris cohort [d]compared to wt and *KCNJ5* in 365 APAs from 3 cohorts [e]systolic blood pressure
Kitamoto, 2016[255]	EA	2.5% (4/159)	G403R: 1.9% (3/159) I750M: 0.6% (1/159)	N/D[a]	N/D[a]	N/D[a]	N/D[a]	ZF-like[c]	[a]compared to wt [c]similar to wt
Scholl, 2013[121]	EU, NA	10.9% (7/64)	G403R (8A+8B): 6.3% (4/64) I750M 1.6% (1/64) F747V 1.6% (1/64) V1373M 1.6% (1/64)	N/D[a]	N/D[b]	smaller[b]	N/A	N/A	[a]no tendency detected [b]compared to *KCNJ5*
Zheng, 2015[253]	EA	0.6% (1/168)	G403R: 0.6% (1/168)	N/A	N/A	N/A	N/A	N/A	

N/A: not available, N/D: no difference, AU: Australia, EA: East Asia, EU: Europe, NA: North America

Table 8. Studies of ATPase mutant APAs. Layout adapted from Stowasser *et al.* 2013[351].

Study	Region	Mutation frequencies	Characteristics of patients and tumors with ATPase mutation					
			Sex	Age	APA size	PHA severity	Histology	Comments
Akerström, 2015[249]	EU, AU	9.1% (15/165)	<u>ATP1A1: 6.1% (10/165)</u> L104R 2.4% (4/165) M102_I106del 0.6% (1/165) F959_G961del 0.6% (1/165) G960_L964del 0.6% (1/165) F956_G961del 0.6% (1/165) M102_L103del 0.6% (1/165) L103_L104del 0.6% (1/165) <u>ATP2B3 3% (5/165)</u> V424-L425del 1.2% (2/165) T423-L425del 0.6% (1/165) V424-V426del 0.6% (1/165) V426-V429del 0.6% (1/165)	10/15 male[a]	N/A[a]	N/A[a]	N/A[b]	N/A	[a]male, older and smaller for *CACNA1D* + ATPase compared to *KCNJ5* [b]no difference in PAC levels for *CACNA1D* + ATPase compared to *KCNJ5*
Azizan, 2013[269]	EU	7.9% (12/152)	<u>ATP1A1 7.9% (12/152)</u> L104R 6.6% (10/152) F100_L104del 0.7% (1/152) EETA963S 0.7% (1/152)	11/12 male[a]	N/A[a]	N/A[a]	N/A	N/A[a]	[a]male, older, smaller and ZG-like for *CACNA1D* + ATPase compared to *KCNJ5*

Study	Region	Mutation frequencies		Characteristics of patients and tumors with ATPase mutation					
				Sex	Age	APA size	PHA severity	Histology	Comments
Beuschlein, 2013[244]	EU	6.8% (21/308)	ATP1A1 5.2% (16/308) L104R: 3.9% (12/308) F100_L104_del 1% (3/308) V332G: 0.3% (1/308) ATP2B3 1.6% (5/308) L425_V426_del: 1.3% (4/308) V426_V427_del: 0.3% (1/308)	male[a]	older[a,b]	N/D[a,b,c]	higher[c,d]	N/A	[a]compared to *KCNJ5* [b]patients from 2 cohorts [c]compared to wt [d]PAC
Dekkers, 2014[256]	EU	11.3% (6/53)	ATP1A1 L104R: 1.9% (1/53) ATP2B3 L425_V426del: 9.4% (5/53)	5/6 male	N/A	ATP2B3 smaller[a]	N/A	ZG-like	[a]compared to *KCNJ5*
Dutta, 2014[273]	EU	14.3% (5/35)	ATP1A1 L104R: 5.7% (2/35) ATP2B3: 8.6% (3/35) L425_V426del: 5.7% (2/35) R428_V429del: 2.9% (1/35)	4/5 male	N/D[a]	N/D[a]	N/D[a,b]	N/A	[a]between wt and different mutant groups [b]PAC

Study	Region	Mutation frequencies	Characteristics of patients and tumors with ATPase mutation					
			Sex	Age	APA size	PHA severity	Histology	Comments
Fernandes-Rosa, 2014[262]	EU	7% (33/474)	ATP1A1 5.3% (25/474) L104R 3.6% (17/474) F100_L104del 1.1% (5/474) V332G 0.4% (2/474) G99R 0.2% (1/474) ATP2B3 1.7% (8/474) L425_V426del 1.1% (5/474) L424_V425del 0.4% (2/474) V426_V427del 0.2% (1/474)	N/A	N/A	N/A	N/A	N/A	
Kitamoto, 2016[255]	EA	3.1% (5/159)	ATP1A1 L104R 0.6% (1/159) ATP2B3 2.5% (4/159) L425_V426del 1.3% (2/159) V427_A428del 0.6% (1/159) V424_L425del 0.6% (1/159)	all male	younger[a]	smaller[a]	higher[a,b]	ZG-like	[a]compared to wt, tendencies [b]high urinary aldosterone
Kuppusamy, 2014[266]	EU	2.1% (4/195)	ATP1A1 L104R 1.5% (3/195) ATP2B3 V126_V127del 0.5% (1/195)	N/A	N/A	N/A	N/A	N/A	

Study	Region	Mutation frequencies	Characteristics of patients and tumors with ATPase mutation					Comments
			Sex	Age	APA size	PHA severity	Histology	
Scholl, 2013[121]	EU, NA	4.7% (3/64)	ATP1A1 L104R 1.6% (1/64) ATP2B3 3.1% (2/64) V426_V427del 1.6% (1/64) L425_V426del 1.6% (1/64)	N/A	N/A	N/A	N/A	N/A	
Williams, 2013[275]	EU	7.1% (8/112)	ATP1A1: 6.3% (7/112) L104R: 5.4% (6/112) G99R: 0.9% (1/112) ATP2B3 L425_V426del: 0.9% (1/112)	N/D[a]	N/D[b]	N/D[b]	N/D[b,c]	N/A	[a]compared male to female [b]between wt and different mutant groups [c]PAC, PRA, preoperative blood pressure
Wu, 2015[a,267]	EA	2% (3/148)	ATP1A1 L104R: 1.4% (2/148) ATP2B3 Y410D: 0.7% (1/148)	N/A	N/A	N/A	N/A	N/A	[a]likely overlap with Wu, 2017, but not specified
Wu, 2017[a,276]	EA	1.8% (4/219)[b]	ATP1A1 L104R: 1.4% (3/219[b]) ATP2B3 Y410D. 0.5% (1/219[b])	N/A	N/A	N/A	N/A	N/A	[a]likely overlap with Wu, 2015, but not specified [b]number calculated from percentage
Zheng, 2015[253]	EA	3% (6/168)	ATP1A1: 2.4% (4/168) L104R 1.8% (3/168) M102_L103del: 0.6% (1/168) ATP2B3 delV422_V426insSTL 0.6% (1/168)	all male	N/A	N/A	N/A	N/A	

N/A: not available, N/D: no difference, AU: Australia, EA: East Asia, EU: Europe, NA: North America, PRA: plasma renin activity, PAC: plasma aldosterone concentration

Table 9. Studies of *CTNNB1* mutant APAs. Layout adapted from Stowasser *et al.* 2013[351].

Study	region	Mutation frequencies	Characteristics of patients and tumors with *CTNNB1* mutation					
			Sex	Age	APA size	PHA severity	Histology	Comment
Akerström, 2016[271]	EU, AU	5.1% (10/198)	S45P 4.0% (8/198) S45F 0.5% (1/198) T41A 0.5% (1/198)	N/D[a,b]	N/D[a,b]	larger[a]	N/D[a,b]	N/D	[a]compared to wt [b]compared to *KCNJ5*
Scholl, 2013[121]	EU, NA	3.1% (2/64)	S45P 3.1% (2/64)	all female	N/A	N/A	N/A	N/A	
Tadjine, 2008[245]	NA	9.1% (1/11)	26 995 del 271 bp 9.1% (1/11)	1/1 female	N/A	N/A	N/A	N/A	
Wu, 2017[276]	EA	3.7% (8/219)	S45P 2.3% (5/219 S45F 1.4% (3/219)	N/D[a,b]	older[b]	N/D[a,b]	N/D[a,b,c]	N/A	[a]compared to wt [b]compared to *KCNJ5* [c]blood pressure, PRA, log PAC, log ARR

N/A: not available, N/D: no difference, AU: Australia, EA: East Asia, EU: Europe, NA: North America, PRA: plasma renin activity, PAC: plasma aldosterone concentration, ARR: aldosterone-to-renin ratio

12 Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Außerdem versichere ich, dass ich diese Dissertation an keiner anderen Fakultät eingereicht habe. Ich habe bisher keinen anderen erfolgreichen oder erfolglosen Promotionsversuch unternommen.

Düsseldorf, 02.02.2018

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