Genetic Alterations in Aldosterone Producing Adenomas

To my family for all their love and support

List of Papers

This licentiate thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Åkerström T**, Crona J, Delgado Verdugo A, Starker LF, Cupisti K, Willenberg HS, Knoefel WT, Saeger W, Feller A, Ip J, Soon P, Anlauf M, Alesina PF, Schmid KW, Decaussin M, Levillain P, Wängberg B, Peix JL, Robinson B, Zedenius J, Bäckdahl M, Caramuta S, Iwen KA, Botling J, Stålberg P, Kraimps JL, Dralle H, Hellman P, Sidhu S, Westin G, Lehnert H, Walz MK, Åkerström G, Carling T, Choi M, Lifton RP, Björklund P. (2012) Comprehensive re-sequencing of adrenal aldosterone producing lesions reveal three somatic mutations near the KCNJ5 potassium channel selectivity filter. *PLoS One*, 7: e41926

II **Tobias Åkerström***, Elena A B Azizan*, Rajani Maharjan, Holger Sven Willenberg, Kenko Cupisti, Julian Ip, Ana Moser, Bruce Robinson, Alexander K. Iwen, Henning Dralle, Martin K. Walz, Hendrik Lehnert, Stan Sidhu, Per Hellman, Morris J Brown¤ and Peyman Björklund¤. Activating Mutations in *CTNNB1* in Aldosterone Producing Adenomas. *In manuscript.*

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 *and¤ contributed equally to this work.

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Abbreviations

AAG=Adjacent adrenal gland

ACTH=Adrenocorticotropic hormone

Ang II=Angiotensin II

APA=Aldosterone producing adenoma

ARR=Aldosterone to renin ratio

AT1R=Angiotensin II receptor 1

BAH=Bilateral adrenal hyperplasia

Ca^{2+}=Calcium

$[Ca^{2+}]_{IC}$=Intracellular calcium concentration

CaMKI=Calmodulin-dependant protein kinase

CaV1.x=High-voltage activated L-type Ca^{2+} channel

CaV3.x=Low-voltage activated T-type Ca^{2+} channel

CCT=Cortical collecting tubules

CPA=Cortisol producing adenomas

DAG=Diacylglycerol

ENaC=Amiloride-sensitive epithelial Na^{+} channel

FH=Familial hyperaldosteronism

GIRK=G-protein-activated inward rectifier potassium channels

IP3=Inositol triphosphate

NHPA=Non-hormone producing adenomas

miRNA=Micro RNA

MR=Mineralocorticoid receptor

PA=Primary Aldosteronism

PLC=Phosphoinositide-specific phospholipase C

PLD=Phospholipase D

PIP2=Phosphatidylinositol bisphosphate

PMCA=Plasma membrane Ca^{2+}- calmodulin dependent ATPase

SERCA=Sarcoplasmic reticulum Ca^{2+}-ATPase pump

SFRP2=Secreted Frizzled-related protein II

StAR=Steroid acute regulatory protein

TASK-1=TWIK-Related Acid-Sensitive K^+ Channel-1

VDCC=Voltage-dependent calcium channels

ZF=Zona Fasciculata

ZG=Zona Glomerulosa

$[K^+]_{EC}$=Extracellular K^+ concentration

1. Introduction

The adrenal glands are small hormone producing organs located retroperitoneal and cranial to the kidneys. These organs are essential for the normal stress response, glucose homeostasis, fluid- and electrolyte balance, and disruption of its normal function is lethal to the organism[1]. The first description of the adrenal organs comes from Bartolomeo Eustachio (15?-1574)[2,3]. Eustachio, an Italian anatomist first published drawings of the adrenals in "Opuscola Anatomica" in 1563[3]. The drawings ended up in the Vatican library and were forgotten[4]. It was not until 150 years later, long after his death, that the drawings surfaced and were published[4]. Georges Cuvier (1769-1832) continued the anatomical research and was the first one to describe that the adrenals consisted of an outer cortex and an inner medulla[5,6]. Further characterization of these layers was later made by Kölliker in the mid-19th century[7]. In 1866, Arnold was the first to describe the three distinct layers of the adrenal cortex[8]. We now denote these layers as the Zona Glomerulosa, Zona Fasciculata and Zona Reticularis, and we know now that these layers have distinct functional properties along with their different microscopic appearances (Figure 1).

In 1912 Harvey Cushing described a new clinical syndrome characterized by skin manifestations, short stature, round face, and fast weight gain[9]. He hypothesized that this was due to "hyperadrenalism"[9]. The disease, which was later named Cushing's disease, where shown to be caused by increased glucocorticoid action through raised concentrations of the adrenal hormone cortisone. The term glucocorticoid stems

from the ability of these compounds to promote glycogen deposition in the liver[10]. For many decades the majority of the research on the adrenal gland involved its production of cortisone and other glucocorticoids[11]. However it was noted that besides from the glucocorticoid action, adrenal extracts had a mineralocorticoid effect i.e. caused sodium retention by the kidney[12]. Because most of the adrenal compounds found in adrenal blood were glucocorticoids it was postulated that these likely were responsible for the mineralocorticoid effects[11]. However, as time went by, more started to question this theory and believe that there were other hormones responsible for the mineralocorticoid action[13,14]. It was noted that patients with Addisons disease (i.e. adrenal hypofunction) were not controlled with only cortisone supplementation[15] and that liquidized adrenal was a more effective way of treating these patients[16]. In 1952 Grundy, Simpson and Tait analyzed beef adrenal extracts and observed, through chromatographic separation, a new compound, different from cortisone, with potent mineralocorticoid activity, which was named *Electrocortin*[12]. Simpson and Tait and a professor of chemistry Tadeus Reichstein continued the research on this newly found compound and could later isolate 21 mg of electrocortin from 500kg of beef adrenal[11,17]. This made it possible to determine its crystalline structure in 1953[17], and in 1954 the final structure of Aldosterone was announced[18]. Personal communications between Simpson, Tait and Reichstein are saved at the Contemporary Medical Archives Center in London and depicts the struggle of obtaining the final structure of the hormone and also shows when the name Aldosterone is mentioned for the first time:

TR to S-T

"Dr. Wettstein told TR that some American chemists already know the structure of electrocortin therefore the publication of a preliminary note is advisable. TR raises

the question of a definitive name for electrocortin. He suggests aldosterone. Asks whether contribution of Sir Charles Dodds should be acknowledged."[19]

TR= T. Reichstein

S-T= Simpson and Tait

As research on aldosterone progressed, interest in its role in different clinical disorders grew. First it was shown that increased amounts of aldosterone in urine could be found in patients with edema due to cardiac failure, kidney disease and liver failure[20,21]. However, it was not until 1954 that a major breakthrough on the clinical effects of aldosterone could be appreciated[22]. The breakthrough came when a 34 year old woman was admitted to the metabolic research laboratory in Ann Arbor, Michigan, USA. The patient had suffered from muscle spasms, weakness and paralysis for several years. Her blood pressure was known to be in excess of 180 mmHg and she had metabolic alkalosis, hypokalemia and hypernatremia[22]. The investigation and treatment was led by a man named Jerome Conn. Conn had previously studied how humans cope and adapt to warm and humid conditions[23]. He had found that the body acclimatized by increasing a "salt-retaining hormone" via increased adrenal activity[23]. To prove that this hormone was involved in this patients disease, Conn meticulously collected laboratory data during 227 days and could later show that the disease was due to abnormal mineralocorticoid activity through hyperfunctioning adrenals[22]. On December 10, 1954 the woman was operated with an right adrenalectomy removing a 4 cm tumor[22]. Postoperatively she was cured, with normalization of pH, normalized potassium and sodium, and normalized blood pressure[24]. Conn had been the first to describe and treat an aldosterone producing adenoma (APA) and the disease was later named Conn's syndrome after its discoverer[22,25]. After its initial discovery it was further recognized that APAs represented a

subgroup of the disorder primary aldosteronism (PA)[26] which also included multiple unilateral adenomas, unilateral hyperplasia or bilateral disease[26,27]. PA was characterized by the symptoms caused by overproduction of aldosterone, with the most common manifestations being hypertension, muscle weakness, hypokalemia, alkalosis and in some edema and cardiomegaly[26]. The reason for these symptoms could be explained by the effects of aldosterone on the cardiovascular system, and renal salt and water handling that was elucidated in detail years later. The problem that faced Conn and many doctors after the discovery of PA was that a high aldosterone level could either be due to an aldosterone producing lesion in the adrenal (PA) or by a functional consequence of an altered Na^+ concentration in the kidney (secondary hyperaldosteronism)[26]. Today we discriminate between these two disorders by measuring renin[27,28].

1.1 Aldosterone production and regulation

The cells that are responsible for aldosterone secretion are located in the outermost adrenal cortex where they form the Zona Glomerulosa (ZG)(Figure 1). These are separate from cells in the Zona Fasciculata (ZF) which produce cortisol. This functional zonation is derived from the cell specific expression of the enzyme aldosterone synthase (CYP11B2) in ZG and cholesterol side-chain cleavage (CYP11B1) in ZF[29,30]. Aldosterone synthase catalyzes the final step in the production of aldosterone whereas CYP11B1 is the final enzyme in the production of cortisol[30]. The production of aldosterone starts with the mobilization of cholesterol to the outer mitochondrial membrane of the ZG cell[31]. The rate limiting step occurs when cholesterol is transferred from the outer mitochondrial membrane to the inner membrane and converted to pregnelone by the steroid acute regulatory protein (StAR)[31,32]. Further

conversions are made by HSD3B2 and CYP21 in the endoplasmatic reticulum and the final step is completed in the mitochondria through hydroxylation and oxidation by CYP11B2[29]. Regulating the activity or expression of some of these enzymes represents important control steps in the production of aldosterone[29,33].

Aldosterone is the most important regulator of sodium and potassium homeostasis[33]. By this regulation it directly controls the electrolyte balance and indirectly the extracellular volume, blood pressure and acid-base balance[33,34]. In sodium depleted states, hyperkalemia and hypotension the signal for aldosterone production increases[33]. The main stimulating signals for this increase are ACTH, Angiotensin II (Ang II) and potassium[33,35,36] while atrial natriuretic peptide is the main negative regulator[37]. ACTH only increases aldosterone during severe fluid loss while the main regulators during physiologic conditions are potassium and Ang II[33]. Ang II regulates aldosterone production by binding to the Angiotensin II receptor 1 (AT1R) (Figure 3a)[33,38,39]. The production of Ang II starts with the release of renin from the kidney[36]. Renin is stored in granular cells in the kidneys juxtaglomerular apparatus[40]. The juxtaglomerular apparatus is a complicated sensory and protein-producing organ that contains three cell types; the granular cells, mesangial cells and macula densa cells[41,42]. The macula densa cell cluster are in close contact with the tubular fluid of the thick ascending limb of Henle, and senses its Na^+ content[40]. In states of decreased flow of tubular fluid, like in hypotensive conditions, or in situations with low Na^+ levels, the Na^+ concentration in the tubular fluid is low, triggering increased activity of the Macula densa cell which signals the granular cells to release renin[40]. Upon release, renin starts the conversion of Angiotensinogen to Angiotensin I, which is later transported to the lungs where it is converted by Angiotensin converting enzyme (ACE) to Ang II[43]. Binding of Ang II to the AT1R can stimulate aldos-

terone production by different pathways. It can activate phosphoinositide-specific phospholipase C (PLC) which catalyzes the conversion of Phosphatidylinositol bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG)[44,45]. It can also activate phospholipase D (PLD) which similarly increases PLC and DAG[46]. The up-regulation of IP3 and DAG elicit emptying of intracellular Ca^{2+} (Ca^{2+}_{IC}) stores which increase $[Ca^{2+}]_{IC}$ and activates calmodulin and calmodulin-dependant protein kinases (CaMKI)[47-49]. These signals cause increased activity of StAR, which is responsible for the acute rise in aldosterone production[47,50]. Besides its effects on the PLC- and PLD-pathway, Ang II also increases influx of Ca^{2+} by inhibition of ion channels and subsequent depolarization of the ZG membrane (Figure 3a)[33,51,52]. Depolarization leads to increased cytosolic Ca^{2+} in two ways; either increased activity of voltage-dependent Ca^{2+} channels at the cell membrane, or through emptying of intracellular Ca^{2+} stores[53]. The increased Ca^{2+} activates CaMKI which promotes CYP11B2 expression[54]. Continuous activation of Ang II also stimulate ZG cell proliferation, which by increased volume lead to increased aldosterone secretion[29,55,56]. In vivo models also show that sustained activity by Ang II up-regulates the AT1R itself, leading to augmented signaling[57].

1.2 Regulation of aldosterone through ion channels

Potassium is the other main regulator of aldosterone at physiologic concentrations (Figure 3b)[58]. Due to the deleterious effects of both high and low K^+ levels the body needs to regulate $[K^+]$ within a tight span[59]. Potassium even has the ability to override Ang II mediated aldosterone signaling in hypokalemic states, which protects against further K^+ loss[60]. During K^+ loading the increased extracellular levels of K^+ changes the membrane potential towards more positive voltages[61], directly regulat-

ing aldosterone production through depolarization of the ZG cell membrane[62]. Similar to the depolarizing effects of Ang II, K^+-induced membrane depolarization activates voltage-dependent calcium channels and raise intracellular calcium, activating CaMKI and subsequently increase aldosterone production through increased CYP11B2 expression[62]. To protect the glomerulosa cell against overproduction of aldosterone, the membrane potential is kept at a hyperpolarized state[61]. This negativity is determined by the high conductance of K^+[61,63]. Due to this K^+-permeability the membrane potential of the ZG cell approaches the equilibrium potential for K^+ (E_K) reaching >-80mV[64]. This makes the glomerulosa cells the most K^+ sensitive cells in the body[33] and an important way of regulating aldosterone production[65].

Establishing the hyperpolarized glomerulosa cell membrane

The highly negative membrane potential is first established through the activity of a Na^+/K^+-ATPase (Figure 3a)[66]. This protein is expressed ubiquitously, is vital for the organism, and consumes around 20-30% of all ATP at rest[67]. It is a member of the P-type ATPases which are characterized by the transfer of a phosphate from ATP to an aspartate residue on the ion channel[68]. The protein contains two subunits, one α- and one β-subunit[68]. Either of four isoforms makes up the α-subunit which is approximately 1000 amino acids in length with ten transmembrane segments[68]. Among the different subunits the α1-subunit encoded by the *ATP1A1* gene is expressed in the ZG cells but also in many other tissues throughout the body[68,69]. The β-subunit has four isoforms about 370 amino acids in length[68]. Through conformational changes driven by ATP, the affinity for Na^+ and K^+ changes, leading to the pumping action of the channel[70]. In the first conformational stage E_1 there is high affinity for cytoplasmic Na^+ and ATP. Phosphorylation by ATP after binding of three Na^+ leads

to occlusion of the Na^+ and release of ADP. The channel is now in an E_1P conformation, which leads to the release of the bound Na^+ to the extracellular side. Trough this release, the protein changes to an E_2P structure, with high affinity for K^+. Two K^+ now bind on the extracellular side causing dephosphorylation and K^+ occlusion (E_2K_2). In this state the protein can bind ATP, which will trigger the release of the bound K^+ to the inside of the cell (E_1)[68].

Because the ATPase increases the negativity of the membrane potential it lowers aldosterone production. Therefore one of the actions of Ang II is to inhibit the channel (Figure 3a)[66]. Importantly, the affinity for Na^+ and K^+ and the occlusion of these ions are vital for its normal function[69,71,72]. Mutagenesis studies and recent discoveries made in aldosterone producing adenomas, have shown that specific amino acids located in highly conserved parts of the α1-subunit are critical for its normal function[69,71-74].

TWIK-Related Acid-Sensitive K^+ Channel (TASK)

The TASK channels or K^+ leak channels are important for maintaining the membrane potential in glomerulosa cells (Figure 3a,b)[75,76]. These have a dimeric structure of two subunits[77], with each subunit containing two pore-forming P-domains[78]. These channels are termed leak K^+ channels due to their dominant conductance of K^+[78]. During physiologic concentrations of K^+ the channel is active and carry a large outward current that hyperpolarize the cell[52]. This can be reversed by increasing the extracellular K^+ or blocked by increasing the proton concentration[78]. By inhibiting or reversing the conductance of K^+ the membrane potential increase, leading to depolarization and aldosterone production[52,79]. In human glomerulosa, TASK-1 seems to be the most abundantly expressed[76,79]. Ang II is known to inhibit TASK-1 making

it an important regulatory step for aldosterone production (Figure 3a)[52]. TASK-1 has not been implicated in human hyperaldosteronism. However, disruptive mutations have been found in patients with familial pulmonary hypertension[80]. Interestingly, female TASK-1 -/- mice develop hyperaldosteronism with low renin levels, resembling primary aldosteronism[81]. This phenotype is sex dependent due to protective mechanisms from testosterone[81].

G-protein-activated inward rectifier potassium channels (GIRK)

The G-protein-activated inward rectifier potassium channels (GIRK) are important for the membrane potential in many different cell types[82,83]. Of the different GIRK channels GIRK4 and GIRK1 is expressed in the human glomerulosa cells and GIRK4 is essential for its normal function (Figure 3a)[84]. The name rectifier stems from the field of electronics and refers to a current led in only one direction[85]. The GIRK channels help to maintain the membrane potential close to the E_K, partly through a dominant inflow of K^+ at more negative potentials, and also through a slight outflow at more positive potentials[83]. As the name of the channels indicates they are activated by the Gγβ-subunit of G-proteins[86]. Apart from this regulation, intracellular Na^+ is an activator of GIRK channels[87]. This potentially represents an important feedback mechanism during Ang II induced Na^+/K^+-ATPase inhibition where Na^+ levels increase[66]. This increase in Na^+ would therefore activate the GIRK channel and return the membrane to its negative resting potential[87]. Another important regulator of GIRK activity is PIP2, which stimulates the Gγβ-subunit and GIRK interaction leading to increased GIRK activity[88]. Importantly, PLC hydrolyzes PIP2 and therefore decreases GIRK conductance[88] suggesting that Ang II by induc-

ing PLC mediated PIP2 hydrolysis, can inhibit GIRK channels[89]. This implies that GIRK channels in the glomerulosa protect against aldosterone overproduction[89].

The structure of the GIRK channels are similar among the different subunits[85]. They consist of two transmembrane domains, a pore helix and a selectivity filter[83-85]. Each ion channel has a tetrameric structure of four GIRK subunits[90,91]. These can either be homotetramers or consist of different subunits forming heterotetramers[92,93]. The assembly of GIRK subunits in the glomerulosa cells is not fully elucidated, but the expression of both GIRK1 and GIRK4, and *in vitro* patch clamp studies suggests that it functions as a heterotetramer[84,94]. The cytoplasmic pore of the GIRK proteins are highly conserved, and contain negative amino acids that are both responsible for concentrating K^+ to the pore entrance, and for the blocking action by cations from the inside of the cell, leading to its rectification[85]. The selectivity filter of the GIRK proteins contains a conserved TXGYGFR motif, which is essential for the K^+ specificity of the channel[84,85,95]. Interestingly, mutations affecting the selectivity filter of GIRK4 (*KCNJ5*), lead to loss of selectivity for K^+ and a dominant Na^+ influx, promoting depolarization and hyperaldosteronism[84](Figure 4).

After the discovery of *KCNJ5* mutations, interest in the normal function of GIRK4 has grown. Experiments using the human adrenocortical carcinoma cell line HAC15 have to some extent elucidated this function[89]. Ang II stimulation of these cells lowered the expression of GIRK4 at both the mRNA and protein level[89], while overexpression of GIRK4 decreased the membrane voltage and intracellular Ca^{2+} with subsequent lowering of StAR, CYP11B2 and aldosterone production[89]. This suggests that Ang II can regulate the membrane potential both by regulating the expression of GIRK4, and its activity through PIP2 hydrolysis, implying that GIRK4 promotes hyperpolarization of the glomerulosa membrane through K^+ efflux[89].

Regulation of intracellular calcium

As discussed above, increasing the intracellular levels of Ca^{2+} ($[Ca^{2+}]_{IC}$) lead to increased aldosterone production[47]. Therefore, controlling the $[Ca^{2+}]_{IC}$ represent an important way of regulating aldosterone[96].

Plasma membrane Ca^{2+}- calmodulin dependent ATPase (PMCA)

PMCA channels use ATP to excrete Ca^{2+} from the cytosol of the cell (Figure 3a)[97]. Like the Na^+/K^+-ATPase, PMCA belong to the P-type ion channels, characterized by a phosphate group transfer from ATP to an aspartate residue of the channel[68,98]. The crystalline structure of PMCA have not been established, instead deduction of its structure has been drawn from the sarcoplasmic reticulum Ca^{2+}-ATPase pump (SERCA)[99,100]. This has shown that PMCA share common structural elements with the Na^+/K^+-ATPase[68]; both have 10 transmembrane segments and both operate through ATP driven E_1 to E_2 conformational changes[98,99]. One of the main positive regulators of the PMCA pump is calmodulin[101]. During Ang II-signaling the calmodulin levels increase, which suggest another protective mechanism against increased $[Ca^{2+}]_{IC}$. There are different isoforms of PMCA, some of them are expressed ubiquitously while others (PMCA2 and PMCA3) are only expressed in certain tissues[102]. PMCA3 is expressed in the adrenal cortex[69]. Mutations affecting conserved amino acids in *ATP2B3* (encoding PMCA3) located at similar positions as those mutated in the Na^+/K^+-ATPase gene *ATP1A1*, have been found in APAs[69].

Voltage-dependent calcium channels (VDCC)

One of the most important ion channels expressed on the glomerulosa membrane are voltage gated calcium channels[96]. Two channel types are mainly expressed; the low-voltage activated T-type Ca^{2+} channel (CaV3.x) and the high-voltage activated L-type Ca^{2+} channel (CaV1.x)[103,104,105]. T-type channels are activated at more negative potentials while the L-type is activated at more positive potentials[33]. The channels are comprised of; α_1-, α_2-, δ-, β-, and γ-subunits[106]. The α_1-subunit has four repeated domains, each containing six transmembrane segments (S1-S6)[107](Figure 5). The interaction of the S5 and S6 segments is important for the normal function of the channel pore[107]. Different isoforms of the α_1-subunit are expressed in different tissues[108]. The T-type channel have three different isoforms; CaV3.1, CaV3.2 and CaV3.3,with CaV3.2 being the most abundant in glomerulosa cells[109]. The L-type channel have four different isoforms[110], in glomerulosa cells two isoforms are expressed; the CaV1.2 and the more abundantly expressed CaV1.3[111]. The T-type channel is activated by small increases in $[K^+]_{EC}$, stimulating Ca^{2+} influx and aldosterone production (Figure 3b)[53,112]. It is also positively regulated by Ang II (Figure 3a)[33]. The L-type channel is also activated by increased $[K^+]_{EC}$, but requires higher $[K^+]_{EC}$ than the T-type channel[33]. Interestingly, contrary to the T-type channel, it seems like its activity is inhibited by Ang II, suggesting a mechanism for protection against too high Ca^{2+} concentrations (Figure 3a)[33] . Also, all voltage gated channels contain an inhibitory Ca^{2+} sensing domain in their C-terminal, which is activated by calmodulin, representing another protective mechanism against increased Ca^{2+} signaling[113]. The function of the voltage gated Ca^{2+} channels and the importance of certain amino acids have been largely elucidated through the discovery of different mutations leading to mendelian syndromes[113,114] . These have shown that arginine residues in the S4 segment are important for voltage sensing[110]. That amino acids in

segment S5 and S6 are vital for the channel pore[110,113] and that mutations in the calmodulin inhibitory domain lead to increased channel activity[113]. The importance of L-type channels in the glomerulosa have also been elucidated through the discovery of mutations[72,111]. Alterations in the *CACNA1D* gene (encoding the CaV1.3 α_1-subunit) were recently found in APAs at similar positions as in previously described mendelian syndromes[113,114]. Most mutations found in APAs lead to an increased activation of the pump, increased [Ca^{2+}_{IC}] and subsequent hyperaldosteronism[72,111](Figure 4).

1.3 Effects of aldosterone

The Mineralocorticoid Receptor (MR)

After aldosterone has been produced by the glomerulosa cells it is released into the bloodstream and transported to its effector organs. The aldosterone response is mediated through its binding to the mineralocorticoid receptor (MR)[10]. This steroid receptor is abundantly expressed in the cortical collecting tubuli (CCT) of the kidney and in the hippocampus, brain, sweat gland, salivary gland, vessel wall and heart[10,115], interestingly it is also expressed in the glomerulosa, suggesting a feedback mechanism on aldosterone production[116]. The MR share many features with the Glucocorticoid receptor[10], and both aldosterone and cortisone activates it *in vitro*[10,117]. However, in tissues where aldosterone exerts most of its mineralocorticoid effects, there is increased specificity for aldosterone[117]. This specificity is partly accomplished by the enzyme 11β-hydroxysteroid dehydrogenase, which converts cortisol to cortisone[117]. Ablation of this enzyme give symptoms of mineralocorticoid excess[118]. The effects of this enzyme also explain why licorice causes sodium retention, hypertension and hypokalemia. Licorice contains glycyrrhetinic acid, which is

a potent inhibitor of 11β-hydroxysteroid dehydrogenase[117]. Inhibition of the enzyme leads to an increase in cortisol in the CCT cells and increased activation of the MR[34,117]. This is clinically important because intake of licorice will create more false negatives in the screening for PA[27]. Although a higher degree of specificity is elicited by this enzyme, there is still a large (up to 90%) binding of glucocorticoids to the MR, suggesting that other glucocorticoid-protective mechanisms occur[115,119]. Because aldosterone exerts its effects by binding to the MR one could expect that increased activity of this receptor lead to symptoms mimicking aldosteronism[34]. In some families with early onset hypertension, gain of function mutations in the MR has been observed[120,121]. Normally the MR is rather specific to aldosterone in the CCT, partly through the above-mentioned protection from cortisol, but also by subtle differences in its steroid-binding domain[121]. Mutations in the MR have been shown to alter its specificity for aldosterone, and cause activation by other steroid hormones, including progesterone[121]. In preeclampsia there are reports on lowered aldosterone and renin levels[122-124]. This makes it tempting to speculate that increased activity of the MR, through the action of pregnancy hormones, raise blood pressure and feedback inhibits renin and aldosterone, leading to this disease[121]. After aldosterone binding to the MR, the receptor elicits its response through a highly conserved DNA binding domain that binds to hormone response element (HRE) on target genes[125]. This will activate genes that are responsible for the effects of aldosterone[120].

The organs affected by aldosterone

Each day the kidney is flushed with almost 200 liters of plasma and 23 moles of salt, yet we only excrete a very small percentage of these due to the mineralocorticoid

effect of aldosterone[34]. Losing only a few percent of our electrolytes or fluid volume cause severe symptoms and may be lethal. Therefore the body needs to tightly control the mineralocorticoid effects on the kidney[34]. Most reabsorption of the primary urine occurs in the proximal tubule and in the thick ascending limb of Henle, where ~90% of the filtrate is reabsorbed[34,126]. Only 2% is reclaimed in the CCT, but it is here that the main regulation of fluid and electrolyte balance occurs through aldosterone[34,127]. In 1963, Liddle et al described a syndrome mimicking primary aldosteronism; the patients had severe hypertension, hypokalemia but with only trace amounts of detectable aldosterone[128]. Genetic investigation of these families showed that the syndrome was caused by mutations in the amiloride-sensitive epithelial Na^+ channel (ENaC), triggering increased activity of the channel, mimicking primary aldosteronism[129]. This suggested that upon binding of aldosterone to the MR, there is an increase in ENaC channel activity[127].

ENaC is expressed at the apical membrane of the renal tubule cells[127,130], and its principal effect is the establishment of an osmotic driving force through passive Na^+ reabsorption[130]. The electrochemical gradient that drives Na^+ through the ENaC channel is created by a Na^+/K^+-ATPase at the basolateral side of the cell which actively excretes Na^+ [131]. Entry of Na^+ through the ENaC cause depolarization and excretion of potassium into the urine[131]. Hyponatremia or fluid loss will increase renin levels and subsequently aldosterone, which by MR-mediated signaling, leads to increased activity of the ENaC channels, and subsequent Na^+ saving and osmotic fluid reabsorption from the urine, leading to raised blood pressure[128].

Aldosterone also exerts effect on the cardiovascular tree[132,133]. This effect is mediated either through genomic events, or through rapid non-genomic events[134,135]. The non-genomic response by aldosterone acts to rapidly increase blood pressure[135,136].

Moving from a recumbent to a supine position increases aldosterone, which protects against postural hypotension[137]. Chronic signaling by aldosterone through the MR in endothelium leads to hypertension[138]. Also, by blunting MR signaling in smooth muscle cells (SMC), no age-dependent hypertension develops[139]. This suggests that the mechanism for aldosterone induced hypertension is both due to renal actions and from activation of the MR in vascular SMC and endothelium. Also, aldosterone stimulates collagen deposition throughout the cardiovascular system leading to atrial fibrillation, mycocardial infarction and stroke, independent of blood pressure[132,133,140,141].

1.4 Primary aldosteronism

Since the first description of primary aldosteronism by Conn[22] large strides have been made in the understanding of this disease. Primary aldosteronism either develops sporadically or is a part of a familial syndrome[84,142,143]. The sporadic PA includes seven different pathological states, the most frequent being an aldosterone producing adenoma (APA, Figure 2) (30-50% of PA cases) and bilateral adrenal hyperplasia (BHA) (40-60%)[144-146]. The familial forms of PA are divided into three different subgroups depending on their distinct genetic etiology[84,142,143]. All subgroups of PA are characterized by an autonomous overproduction of aldosterone despite low renin levels[26]. Through this overproduction there is increased activity in tissues with MR expression, the most clinically important being the heart, blood vessels and the kidney[26,133]. Increased MR activity in the kidney leads to increased ENaC activity, K^+ wasting and Na^+ retention which by osmosis also increases water retention, subsequently leading to hypertension[34]. Due to MR activation in the cardiovascular system there is increased deposition of collagen, explaining why the mor-

bidity and mortality in PA patients are higher than in essential hypertensive[132,133]. Interestingly, there seems to be a need for a high Na^+ intake along with the increased aldosterone for the cardiovascular problems to develop[140]. In parts of New Guinea, the salt intake is much lower than in western societies, and despite having high levels of aldosterone they seldom present with hypertension or other cardiovascular problems[147].

Conn first proposed that PA accounted for 10% of hypertensive patients[26], while others claimed that it was much rarer[148,149]. Today, through new diagnostic techniques (i.e. using the aldosterone to renin ratio, "ARR"[150]) PA is diagnosed in 5-15% of hypertensive[144,146,151-153] and up to 20% in therapy resistant hypertensive[144]. In Sweden alone 1.8 million have hypertension[154], if 10% of these have PA, and 30% of these have an APA, this would mean that 54.000 have a surgically correctable form of hypertension[155].

Diagnosing PA is difficult[27]. The clinical picture is essentially the same as in those with primary hypertension[26]. Hypokalemia was previously thought to be a prerequisite for the PA diagnosis[149], but with the implementation of the ARR, a large proportion of PA patients have been shown to be normokalemic (50-70% of all cases)[144,152]. Therefore hypokalemia is no longer part of the diagnosis but is still highly indicative of PA[150,156]. The first screening test for PA is the ARR, and a high ratio implies that there is renin independent aldosterone production, suggesting an autonomous state[150]. However, there are a number of factors that can affect this ratio that needs to be taken into consideration[27]. All hypertensive medications known to affect the ratio are suspended for at least 4 weeks, hypokalemia needs to be corrected, and the patient should not eat licorice[27,157]. Still this test is too uncertain for establishing the diagnosis[27]. To validate that there is a truly autonomous overproduc-

tion, the patient is loaded with a substance that lowers renin[27,158] either; oral sodium[159], iv. sodium[160], fludrocortisone[145], or captopril[161]. Among these tests, no gold standard exits, the choice is rather determined by availability, the clinical status of the patient and cost[27,161]. If the confirmatory test is positive the patient is diagnosed with PA[145]. When the diagnosis is established the clinician needs to classify the subtype to correctly choose the treatment[27,145]. The management of the most common subtypes APA and BHA differs[27,145]. Because BHA is bilateral it is treated with aldosterone-inhibiting drugs[27,145], commonly spironolactone or eplerenone[162]. APAs are unilateral and therefore are suitable for adrenalectomy[27,145]. In the rare cases that the PA is due to an aldosterone producing carcinoma, the need for radical excision is much greater and other medical treatments are warranted[163]. Because of these factors the clinician first needs to perform a computerized tomography (CT)[27,145], which will give some hints as to what histopathology underlines the PA[27,145]. However, this should be interpreted with caution; it may sometimes be hard to differentiate benign from malignant tumors[164]. But more commonly, it may be hard to separate BHA from APA[165]. In the elderly, hormonally silent adrenal masses increases with age and may be found in 6% of the population[166]. This could potentially lead to a false diagnosis of an APA, and an unnecessary operation with removal of a silent adrenal mass, while the other adrenal is still hyperfunctional[27,145]. Also, the size of some APAs is smaller than what is detectable by CT, which would lead to lifelong medical treatment instead of a potentially curable operation[27]. Because of these difficulties the gold standard for lateralization is adrenal vein sampling[167]. With this procedure the adrenal veins are cannulated and the aldosterone/cortisol ratio between the different veins are measured[167]. A significant difference between the veins suggests a unilateral lesion, most often an APA[27,145,167].

The outcome of treatment also differs between APA and BHA[27]. Medical treatment will never cure the patient[27] but will both reduce blood pressure and potassium levels[168]. Medical treatment is also associated with side effects; including gynecomastia, muscle cramps and decreased libido[27] with the sexual side effects being caused by the antiandrogen effect of spironolactone[169]. Compared to medical treatment, surgical treatment of the APA may cure up to 60% of patients[155,170-172]. The results of surgery is dependent on patient characteristics; where negative predictive markers for outcome is; a family history of hypertension, >2 antihypertensive medications, older age at diagnosis, and the time from diagnosis to surgery[155,173]. Interestingly, the genetic alterations in the tumor also predicts outcome of surgery[174].

1.5 Genetic alterations in Primary aldosteronism and Aldosterone producing adenomas

In recent years the field of cancer and tumor biology has made great strides forward. Using techniques based on "next generation sequencing" (NGS) the genetic landscape and its functional consequences in tumors and mendelian disorders have been elucidated in increasing detail[175-178]. By discovering mutations we may also draw conclusions on how the affected proteins function in our body[69,72,84,111]. The field of endocrinology is well suited for these studies. Endocrine tissues are characterized by their production of hormones. Tumors originating from these cells usually give early clues about their existence through increased production and functional consequences of their individual hormones[84,176]. Subsequently, the overall somatic mutation rate in these tumors is low compared to other tumors[84,179]. This makes it easier and more cost effective to find driver mutations responsible for the development of these tumors.

One of the first aberrancies described in PA was in a family with bilateral adrenal hyperplasia with the peculiar finding that aldosterone was under the control of ACTH and suppressible by glucocorticoid administration[142]. This was given the name familial hyperaldosteronism type 1 (FH-1) or glucocorticoid remediable aldosteronism[142]. The cause of this syndrome was shown to be a fusion between the ACTH regulatory region of the *CYP11B1* gene and *CYP11B2*, causing ectopic production of aldosterone in fasciculata cells through ACTH stimulation[142]. The prevalence of FH-1 among PA patients is around 1% and should be excluded in young hypertensive with evidence of familial disease[27,180]. There are two other familial forms of PA; FH-2 and FH-3[143]. Both these are different from FH-1 by being non-suppressible by glucocorticoids[143]. The genetic cause of FH-2 is still unknown, but a linkage to chromosome 7p22 is usually found in these pedigrees[143]. Interestingly, a high ARR among hypertensive, like FH-2 families, seems to show linkage to 7p21-22[181]. Compared to FH-2, FH-3 families share mutations in the *KCNJ5* gene encoding GIRK4[84,182-184]. Mutations affecting this gene have been shown to increase Na^+ influx, leading to depolarization, increased intracellular Ca^{2+}, and aldosterone production[84]. The FH-3 patients usually develop bilateral adrenal hyperplasia and aldosteronism but their clinical phenotype differs depending on the amino acid residue affected[184]. Increasing the Na^+ influx, increases cell death, therefore the mutations with high Na^+ influx have a milder phenotype and do not show increased adrenal mass[184].

PA may also be a part of syndromes not classified as FH. *MEN1* encodes the protein menin, a tumor suppressor gene that causes Multiple endocrine neoplasia type 1[185,186]. This syndrome in rare occasions includes aldosterone producing tumors[187]. Also, patients with Familial Adenomatousis Polyposis, which is caused by mutations

in the APC gene leading to increased WNT signaling, have developed APAs[188,189]. Finally, two children with a syndrome, including primary aldosteronism and neuromuscular disturbances, were shown to have mutations in the *CACNA1D* gene, encoding the L-type voltage dependent Ca^{2+} channel isoform CaV1.3[111], important for regulating Ca^{2+} influx and aldosterone production in the glomerulosa cell[111,112].

Mutations in *KCNJ5*

In 2011, Choi et al. using next generation sequencing discovered mutations in *KCNJ5*, encoding GIRK4[84]. This study and multiple worldwide follow up studies have shown a mutational prevalence of 2-65%[71,174,190-193,194]. *KCNJ5* mutations are restricted to APAs and absent from surrounding peritumoral tissues and other adrenocortical tumors[191], however *KCNJ5* mutations have been detected in cortisol- and aldosterone co-secreting tumors[195], indicating that *KCNJ5* mutated APAs contain a mixed population of ZF and ZG. Accordingly, *KCNJ5* mutated tumors often show a predominance of "fasciculata-like" cells with a high cytoplasm to nuclear ratio[190,191]. This has led to speculations regarding the origin of cells with *KCNJ5* mutation, which is still not elucidated[196].

Interestingly, compared to APAs without known mutation, *KCNJ5* mutation is predominantly found in larger tumors, younger female patients and in those with higher aldosterone levels[191]. The reason for this female bias is unknown, but may include protective mechanisms from testosterone[81].

The normal function of GIRK4 in glomerulosa cells was until recently unknown[76]. The discovery of these mutations and ensuing *in vitro* studies have given new insights into its role in these cells[84,89,197]. The mutations in the GIRK4 gene usually are located in and around the selectivity filter and the highly conserved TXGYGFR

motif[71,84,174,190-193]. Mutant channels with a disrupted selectivity filter show Na^+ influx and increased membrane voltage[84,89,197](Figure 4). Increasing cell voltage in glomerulosa cells activates Ca^{+2} channels on the cell membrane leading to Ca^{2+} influx and aldosterone production[53,112]. Expressing *KCNJ5* WT channels lead to lower membrane voltage, reduced $[Ca^{2+}]_{IC}$ and lowered CYP11B2 mRNA[197]. Expressing mutant channels instead increased Ca^{2+}/calmodulin mediated CYP11B2 expression[197]. These results imply that GIRK4 is important for hyperpolarizing the cell membrane and protecting it from aldosterone production.

There still exits some controversy if mutated *KCNJ5* increases cell mass[84,197]. One important indication that *KCNJ5* mutations are enough for proliferation comes from FH-3 kindreds in which germline *KCNJ5* mutations lead to bilateral hyperplasia[84,182]. Despite this, *in vitro* studies have failed to show that *KCNJ5* mutation increases proliferation, but rather increase cell death[184,197]. This suggests that there may exist *in vivo* mechanisms that protect the adenoma cells from Ca^{2+} and Na^+ induced cell death[198] or that the proliferation is caused by another genetic event[199]. Interestingly, increased WNT signaling is found in 2/3 of all APAs[200,201], and WNT signaling is a known activator of proliferation[202].

The WNT signal pathway

A majority of APAs have active WNT signaling[200,201]. In the normal adrenal cortex active WNT signaling is restricted to glomerulosa cells[200]. Disrupting this pathway results in adrenal developmental disturbances and lowered aldosterone levels[203,204]. This suggests that the WNT pathway is involved in normal glomerulosa function. The WNT pathway signals trough the canonical and the non-canonical pathways[205,206]. The canonical pathway regulates β-catenin levels[207]. In this pathway

absence of WNT ligand binding to the frizzled and LRP co-receptor starts a degradation process of β-catenin[207]. This is carried out by a complex of proteins consisting of Dishevelled, AXIN, APC, CKI, and GSK3β[207]. This complex marks β-catenin for degradation by phosphorylating certain serine/threonine amino acids, leading to its ubiquitination and subsequent degradation in the proteasomes[207]. In active WNT signaling; WNT ligands bind to frizzled and LRP co-receptors, leading to LRP phosphorylation and sequestering of the destruction complex at the cytoplasm membrane, leaving it unable to stimulate ubiquitination causing accumulation of β-catenin[207]. β-catenin is then able to translocate to the cell nucleus, where it increases transcription of different genes, including LEF/TCF transcription factors[207]. In the glomerulosa cell, this β-catenin accumulation increases the expression of CYP21, ATIR and CYP11B2, promoting aldosterone production[201].

Aberrant WNT signaling is involved in tumors[208]. There is also evidence that suggests that abnormal signaling is involved in APAs[72,111,201,209]. *In vitro* cell models shows that β-catenin stimulates the transcription of key regulatory enzymes in the production of aldosterone and the AT1R receptor[201]. Animal models show that aberrant activation leads to primary aldosteronism, tumor formation and malignancy[209]. In human APAs, active WNT signaling is seen in 2/3 of tumors[200,201]. However, if this depicts an regulated active state, or a deregulated aberrant state is not completely elucidated. Also, the cause of this activation is not fully known. In some APAs decreased expression of the negative WNT regulator, Secreted Frizzled-related protein II (SFRP2) have been found[201,210]. This could explain the increased WNT activation in some tumors, but not all. Mutations in exon 3 in the β-catenin gene (*CTNNB1*) cause active and aberrant WNT signaling[208]. These mutations disrupt specific serine and threonine residues important for the phosphorylation of β-

catenin, leading to its accumulation and an abnormal signaling cascade[208]. Mutations are therefore indirect evidence of aberrant signaling, which in other tissues and mouse models induce tumor formation[208]. Mutations in *CTNNB1* seems to occur in APAs, however the frequency is still unknown[72,111,200]. If *CTNNB1* mutations are established in APAs it would indicate that abnormal WNT signaling is important in the formation of these tumors.

Somatic mutations in *ATP1A1*

ATP1A1 encodes the α1-subunit of the ubiquitously expressed Na^+/K^+-ATPase[68]. Mutations in this gene have been found in 5.8% of APAs, while being absent from BHA patients and FH[69,71,72,192]. The mutations observed are commonly found in smaller adenomas, with a male gender bias and with higher aldosterone production compared to *KCNJ5* mutated tumors[69,71,72]. In vitro studies of mutant *ATP1A1* shows that it stimulates depolarization, increase NURR1[71] and CYP11B2 expression (Figure 4)[71,72]. The mutations found affect highly conserved residues in transmembrane segments M1, M4 and M9[69,71,72,192]. Mutations in certain amino acids in the M1 and M4 affect the ATPase affinity for K^+, and subsequently its normal function[73]. The mutated residues in the M9 domain include Gly961, which has previously been described as important for the release of Na^+ from the third Na^+ binding site of ATP1A1[74].

In vitro studies of the mutant ATP1A1 have consistently shown an increased cell depolarization; however there is some disagreement as to how this is accomplished[69,71,72]. As mentioned previously, Ang II can inhibit the ATPase, which increases aldosterone production[66]. This suggests that disruption of the normal function of the ATPase would lead to aldosterone production. Interestingly, the muta-

tions found in *ATP1A1* are located at highly specific amino acid residues which would indicate a gain of function mechanism, like *KCNJ5* mutations[84]. Some argue that the mutations are disruptive[69,71], while others argue that the mutations cause a gain of function[72]. Future studies will further elucidate the consequences of *ATP1A1* mutations.

Somatic mutations in *ATP2B3*

ATP2B3 encodes PMCA3, which uses ATP to excrete Ca^{2+} from the cytoplasm[97]. Like the Na^{+}/K^{+}-ATPase it has 10 transmembrane segments, operates in similar ways[98] and is also highly expressed in the Zona Glomerulosa[69]. Exome sequencing has revealed mutations in this gene[69] and subsequent follow up studies have shown a mutation frequency of 1.5% in APAs[69,71,192]. Interestingly, all mutations observed are located in the M4 domain corresponding to the location of mutated amino acids in the M4 domain of ATP1A1[69]. Projecting the mutated residues on the highly similar SERCA protein shows that they probably are important for Ca^{+2} binding[69]. Also, *in vitro* studies show that cells expressing the mutant channel have a more positive membrane potential (Figure 4)[69]. In other cell models this would increase aldosterone production[69,71,84]. Like mutations in *ATP1A1*, no mutations in *ATP2B3* where found in familial PA or in BHA[69]. Importantly, a germline *ATP2B3* mutation has been found in a family with X-linked congential cerebellar atrophy[211]. *In vitro* studies showed that the mutant ATP2B3 had decreased ability to extrude Ca^{2+} through impaired binding of calmodulin to the pump[211], leading to increased $[Ca^{2+}]_{IC}$ (Figure 4).

Somatic mutations in *CACNA1D*

Like *KCNJ5*, *ATP1A1* and *ATP2B3*, exome sequencing of APAs have detected genetic alterations in *CACNA1D* with a total mutational frequency of 7%[72,111]. *CACNA1D* encodes the α1-subunit of the L-type voltage dependent Ca^{2+} channel (CaV1.3)[212]. This α1-subunit forms the channel pore and is vital for its normal function[113]. Activation of the channel increases the influx of Ca^{2+}, which stimulates aldosterone synthesis in the glomerulosa cells (Figure 4)[33,72,111,213]. Interestingly, a gain of function mutation in a Gly403 residue in region I transmembrane segment 6 (IS6), was seen in the isoform CaV1.2, causing Timothy syndrome and in CaV1.4 leading to X-linked recessive congenital stationary night blindness (CSNB)(Figure 5)[214,215]. The corresponding mutation was also found in APAs, suggesting that the mutations increased the channel activity, which was confirmed by *in vitro* experiments[72,111,216,217]. Seven additional mutations have been observed in APAs (Figure 5)[72,111]. Interestingly, many of these mutations like the Gly403Arg have been observed in other isoforms of the α1-subunit, other Ca^{2+} channels, and shown to be associated with other mendelian syndromes[113,114]. The Phe747and Ile750 residues were found to be mutated in APAs[72,111]. These are located in the corresponding S6 segment, but in region II of the protein, and both cause increased channel activity[72,111]. Similar residues at this position are mutated in CaV1.4 and CaV2.1 leading to CSNB and familial hemiplegic migraine[113,114,217]. Also, a gain of function mutation in the linker between IS4 and IS5 (Val259Asp) was observed in one APA[72] and at a similar position in CSNB[214]. Another mutation Arg990His in IIIS4 was also observed in one tumor[72]. Although it has not been functionally studied in APAs, *in vitro* experiments in the structurally similar NaV1.4 channels showed that mutations in arginine residues in the S4 segment lead to an inward leak of positive ions and

depolarization[218]. Lastly, three mutations were detected in IVS5 in similar locations previously observed in CaV2.1[72,111,217].

Importantly, *CACNA1D* germline mutation was also detected in a family with a new syndrome characterized by neuromuscular disease and hyperaldosteronism[111]. Interestingly, treatment with a Ca^{2+} channel blocker rescued the patient from the effects of the autonomous aldosterone production, suggesting that a genetically based treatment for a subgroup of APAs may be advocated[111,219]. A specific CaV1.3 channel antagonist has recently been produced, which may display additional therapeutic benefits in APAs and with less side effects[220].

1.6 Figures

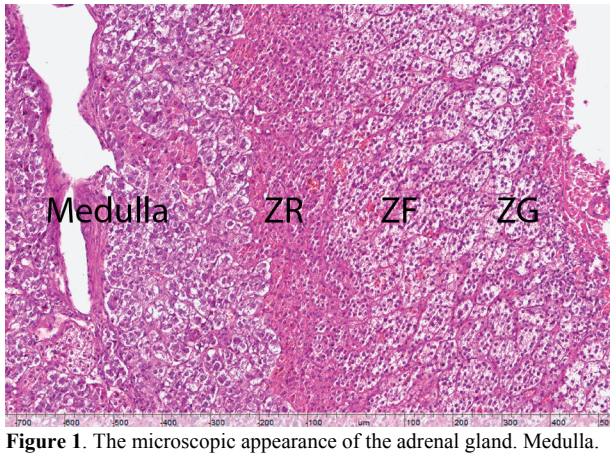

Figure 1. The microscopic appearance of the adrenal gland. Medulla. ZR=Zona Reticularis. ZF=Zona Fasciculata. ZG=Zona glomerulosa.

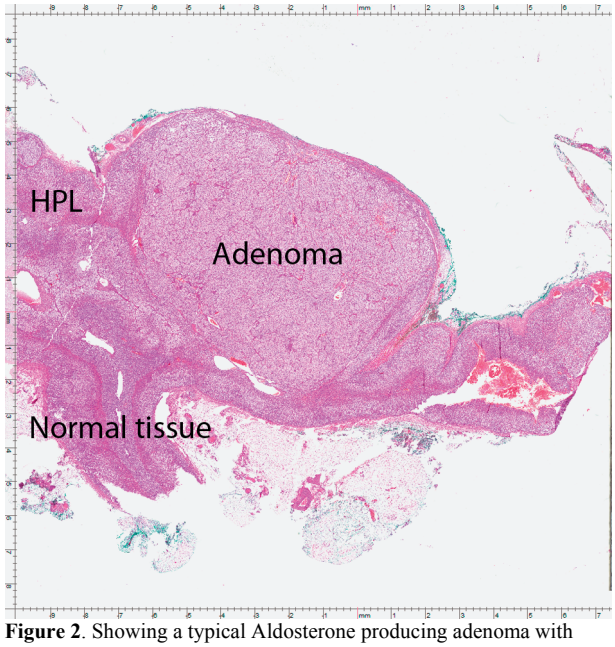

Figure 2. Showing a typical Aldosterone producing adenoma with surrounding normal tissue and hyperplasia (HPL).

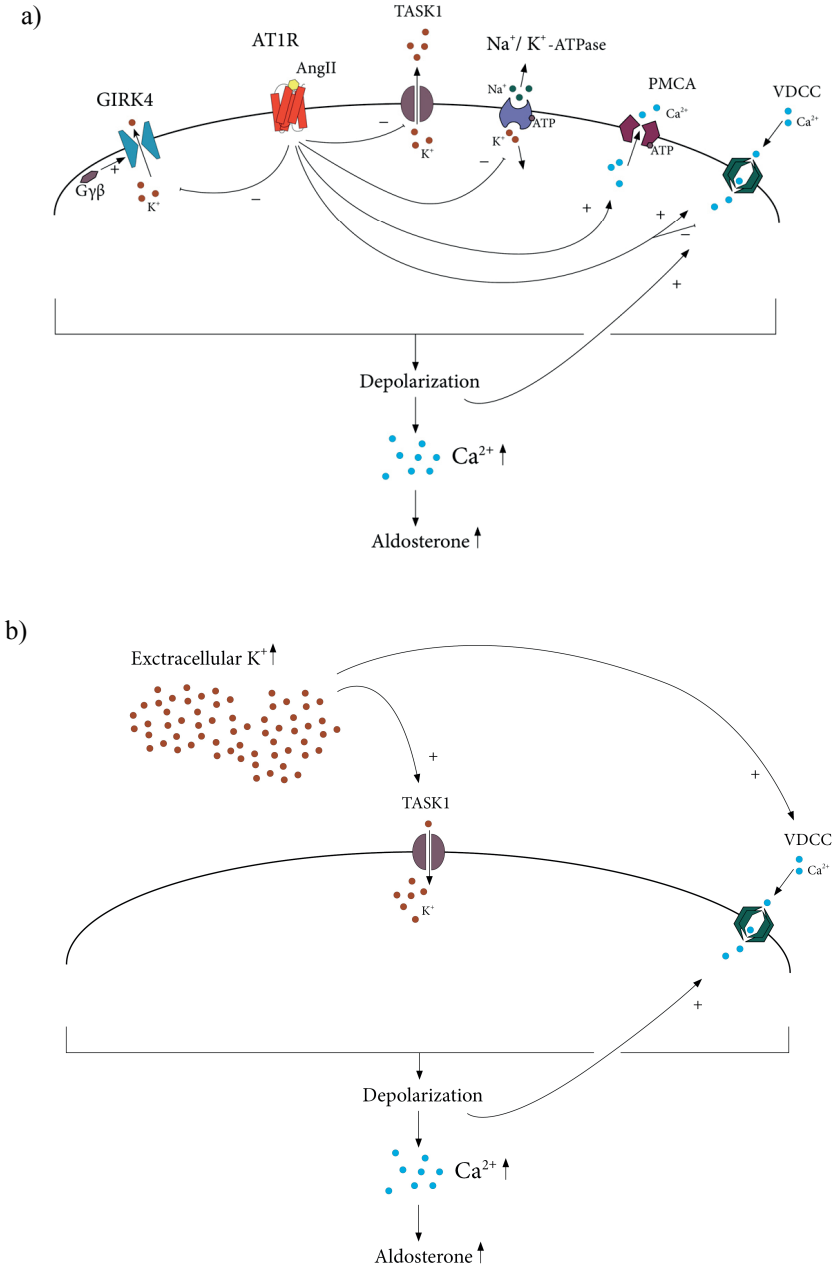

Figure 3. Schematic illustration showing a typical glomerulosa cell responding to a) Angiotensin II and b) increased extracellular K^+.

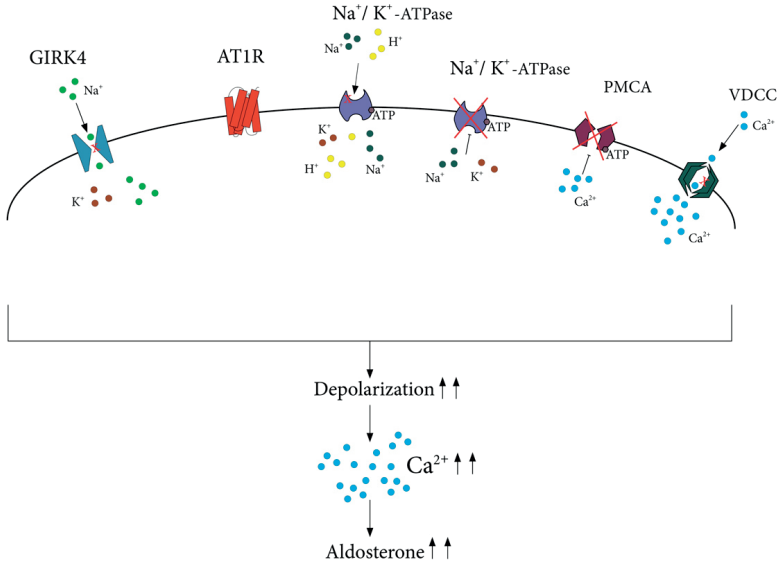

Figure 4. Schematic illustration showing proposed mechanisms for the increased aldosterone production in APAs due to different somatic mutations in ion channels.

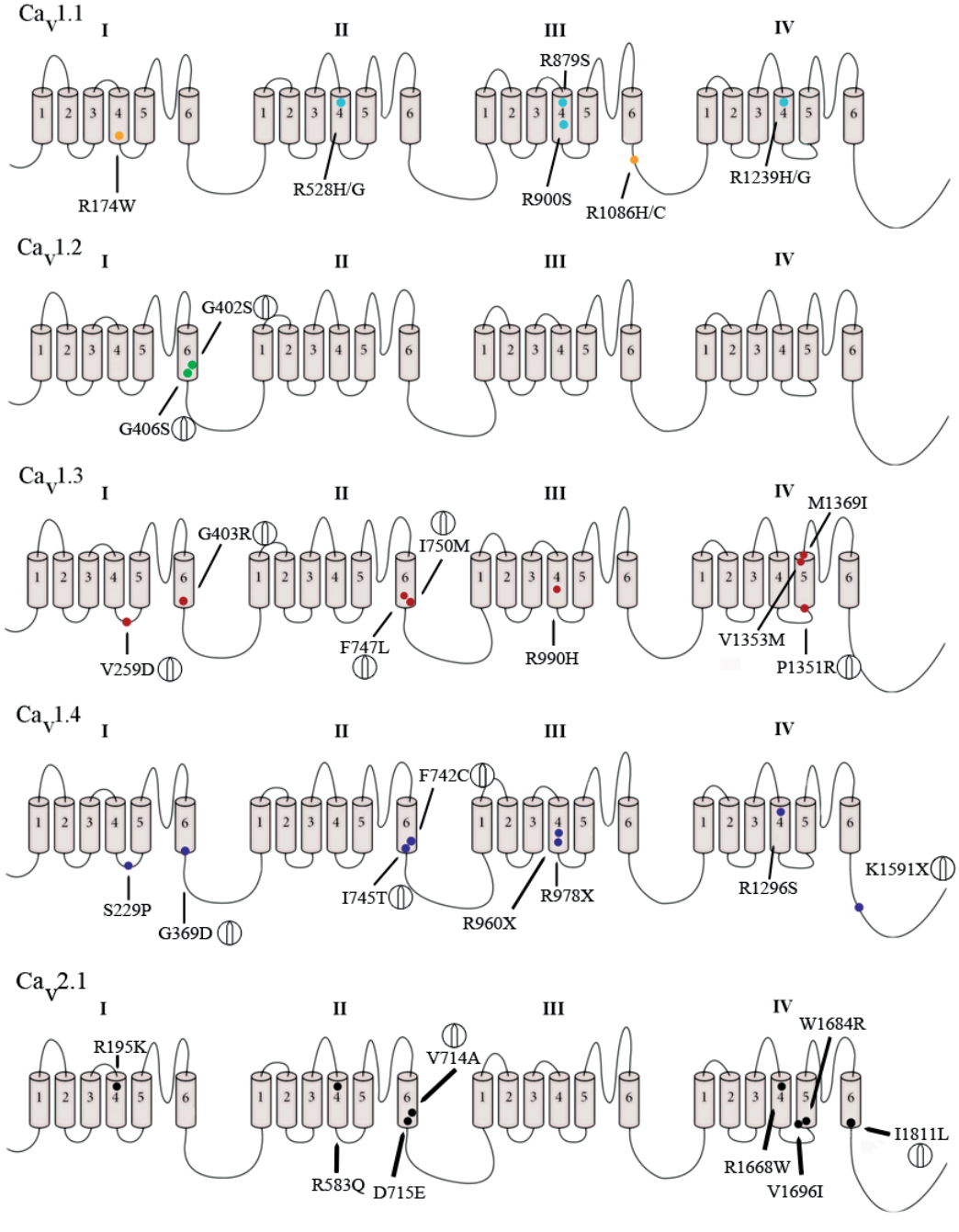

Figure 5. Schematic illustration depicting mutations in different voltage-dependent calcium channels α1-subunits. Color indicates different disorders associated with each isoform and mutation. Light blue=Hypokalemic periodic paralysis type 1. Orange= Malignant hyperthermia susceptibility. Green= Timothy syndrome. Red=Primary Aldosteronism. Dark blue=Congenital stationary blindness. Black dots= Familial hemiplegic migraine. A circle with an arrow indicates a gain of function mutation shown by *in vitro* studies[72,110,111,113,114].

2. Materials and Methods

Patients and tumor tissue

In paper I, a total of 348 Aldosterone producing adenomas and three adrenocortical carcinomas were collected from 10 different centers (Sweden; Uppsala and Stockholm, Germany; Hamburg, Lübeck, Düsseldorf, Essen, and Halle, Australia; Sydney, France; Lyon and Poitiers. Also, 30 cortisol producing adenomas (CPA), 20 cortisol producing carcinomas, 50 non-functional adenomas (NHPA) and 30 non-functioning carcinomas, that had been removed at Uppsala University hospital, were used. All patients gave written informed consent and approval from the local ethics committees were obtained.

The diagnosis of APA was based on a positive ARR screening test, followed by a confirmatory test and positive lateralization on either CT or AVS, and postoperative cure or considerable improvement. After removal the sample was diagnosed as an adrenocortical adenoma/carcinoma by the local pathologist. In paper I we also subdivided the specimens into; adenoma, adenoma with surrounding hyperplasia and unilateral hyperplasia.

In paper II we analyzed 135 of the APAs, previously screened for *KCNJ5* mutations, for mutations in *CTNNB1* (these were selected based on what centers wanted to participate in the study). We also included 57 APAs from our collaborating group from Cambridge. Four NHPA, Four CPA and 15 AAG from different tumors were used for CYP11B2, CYP11B1, CYP17A1, SLC24A3, HNT expression analysis.

In paper III we used 165 APAs from the initial cohort. We also conducted CYP11B2 expression analysis on five ATPase mutated tumors, 14 KCNJ5 mutated, four NHPA and four CPA.

DNA and RNA extraction (Paper I, II, III).

DNA and RNA from 20 µm sections were obtained using Allprep DNA/RNA kit, (Cat no: 80204, QiAgen) or FFPE tissue sections using AllPrep DNA/RNA FFPE Kit (Cat no: 80234, Qiagen) and from blood using DNeasy Blood & Tissue Kit (Cat no: 69506, Qiagen). cDNA was prepared using the First-Strand cDNA Synthesis kit according to the manufacturer's instructions (Cat no: K1631, Fermentas).

DNA Sequencing (Paper I, II, III)

PCR was performed on exons, and intron-exon boundaries from *KCNJ5* (all), *ATP1A1* (4, 8 and 21), *ATP2B3* (8) and *CTNNB1* (3). Amplicons were analyzed by gel-electrophoresis to verify that the reactions were specific and without primer dimers. Direct Sanger sequencing was performed at Beckman Coulter Genomics, Tackeley, UK. Obtained chromatograms were analyzed in house using CodonCode Aligner software (CodonCode Corporation, Dedham, MA).

Immunohistochemistry (IHC) (Paper I, II)

IHC sections (6 µm) from formalin fixed paraffin embedded tissue were deparaf-finized and incubated with polyclonal anti-KCNJ5 (#HPA017353, Sigma; 1:100 dilution) or β-catenin goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; catalog no. sc-1496, 1:10 dilution). IHC for CYP11B2, CYP11B1, KCNJ5 and ATP1A1was performed by our collaborators in Cambridge.

Radioimmunoassay (Paper II)

Fifteen 20 µm sections of frozen tumor tissue; from two *CTNNB1* mutants, one *KCNJ5* mutant and one CPA, were weighed with a highy sensitive scale and ho-

mogenized in 500µl of milliQ water. The lysate was then weighed and a concentration of mg of tumor/mg of water was calculated. The samples were diluted 1:10 and subjected to an aldosterone radioimmunoassay analysis at the clinical chemistry department at Uppsala University hospital. A final concentration of pmol/l was obtained and normalized to each tumors corresponding weight/mg of water.

RTqPCR (Paper II and Paper III)

The expression of CYP11B2 was measured using a custom assay (Applied Biosystems) with mRNA specific primers as previously described[221]. An mRNA specific GAPDH assay was used as the reference gene (Applied biosystems, Part no: 4352934E). The relative CYP11B2 expression was compared using a calculated ΔΔCT value. Expression analysis on SLC24A3, HNT, CYP11B1, CYP17A1 and another measurement of CYP11B2 was performed in Cambridge using 18S rRNA as the reference gene. AXIN2 mRNA expression was quantified using SYBR green with mRNA specific primers for AXIN2 (FW: 5'-GGTCCACGGAAACTGTTGACA and RW: 5'-GGCTGGTGCAAAGACATAG CC) and the chosen reference gene, GAPDH.

Statistics (Paper I, II, III)

The overall group effect was analyzed using a parametric ANOVA or non-parametric Kruskal Wallis test. If significant group effects were present the overall analysis was followed by Bonferroni corrected post hoc comparisons between groups using either; Students T-test for normally distributed or Mann-Whitney U test for non-normally distributed. Categorical data was analyzed by Chi-square test. Non-normally distributed data were log transformed. For statistical analysis SPSS

18 and 22 (IBM, NY, USA) were used.

3. Summary of Paper I

Background

In 2011 Choi et al. discovered mutations in *KCNJ5*[84]. This gene encodes the GIRK4 ion channel that is involved in the regulation of the glomerulosa cell membrane potential[89]. The mutations were located in and near the GIRK4 selectivity filter and *in vitro* studies showed that they caused a loss of selectivity and depolarization, the normal signal for aldosterone production and possible proliferation in glomerulosa cells[29,54]. A total of 22 sporadic APAs were screened, with a total frequency of 8/22 being mutated.

Aim

In this report we screened 348 APAs from an unbiased cohort collected from multiple research centers around the world. The main aim of the study was to verify the previous findings in a larger cohort. Also, with such a large sample size we hoped that we would be able to find a distinct clinical phenotype in patients with *KCNJ5* mutated APAs. To explore if these mutations were restricted to APAs we also screened for *KCNJ5* mutations in other adrenocortical tumors, which included; 80 benign adenomas and 50 adrenocortical carcinomas. We also biopsied different lesions from the same patient to investigate where *KCNJ5* mutations occurred. Lastly, we wanted to compare GIRK4 expression between mutated and non-mutated tumors.

Results and discussion

We observed a frequency of 45% *KCNJ5* mutations in our cohort of APAs. These occurred in all sizes of tumors (6-47mm). No mutations were found in other adreno-

cortical tumors, surrounding HPL or in non-dominant nodules. Interestingly, we were the first and only group to observe a mutation in an aldosterone producing carcinoma. We also described a novel E145Q mutation. This mutation was previously investigated by *in vitro* patch clamp measurements and shown to cause a loss of selectivity for K^+ and depolarization[222]. Comparing phenotypes, we observed a large female bias. We also found that male tumors with G151R were larger and got operated at an earlier age, suggesting that tumor mass depended on mutation. Lastly, we found that KCNJ5 expression was independent of mutational status.

Today almost 1000 APAs have been screened for *KCNJ5* mutation[223]. These have shown a mutational frequency between 2-65%, with a mean frequency of 40%[190,191,193,194,196]. Similar to our results, others have also shown a predominant occurrence in younger patients, in females and in those with larger adenomas[182,191,193,196]. Also, more FH-3 families have been discovered, and their clinical presentations have been shown to depend on which missense mutations they carry[184].

The discovery of *KCNJ5* mutations represent a major breakthrough in our understanding of human pathology and suggests that not only APAs but also other endocrine pathologies and even hypertension may have abnormalities in the tight regulation of the glomerulosa membrane potential and/or intracellular Ca^{2+}.

4. Summary of Paper II

Background

In the canonical WNT pathway, WNT ligand activation stabilizes β-catenin which translocates to the cell nucleus and activates transcription[207]. Both normal ZG cells[200] and APAs[200,201] show active WNT signaling. Disruption of normal signaling lead to disruption of the adrenal gland and lowered aldosterone production[203,204]. Abnormal active WNT signaling is involved in ACTs[224] and other tumors[208]. Mutations in the β-catenin gene (*CTNNB1*) stabilize β-catenin and lead to aberrant WNT activation[208].

Aim

We wanted to elucidate if mutations in the phosphorylation sites in exon 3 of the *CTNNB1* gene occurred in APAs. Through this we would gain more evidence that aberrant WNT activation is involved in APA tumor formation.

Results and discussion

Mutations in *CTNNB1* were observed in 14/192 APAs (7.3%). These were found in hot spot residues p.Ser33Cysp, p.Thr41Ala, p.Ser45Phe, p.Ser45Pro, p.Gly38Asp and p.Gly48Asp, affecting highly conserved residues, known to be mutated in other tumors and stabilize β-catenin[208,225-227]. All mutations occurred in non-mutated APAs (WT for *KCNJ5, ATP1A1, ATP2B3*), which suggested that stabilized β-catenin might be a driver in tumor formation, as previous mouse models have indicted[209]. Importantly, non-hormone producing ACTs occur in 6% of the elderly population[166], and many of these carry *CTNNB1* mutation[224]. To confirm that our mutated tumors were aldosterone producing we measured the relative expression of

CYP11B2 on available *CTNNB1* mutated tumors (n=5) and compared this to both *KCNJ5* mutated (n=16), NHPA (n=4), CPA(n=4) and AAG (n=19). *CTNNB1* mutated APAs had higher expression than the other adrenocortical tumors, AAG and *KCNJ5* mutated tumors. We also directly analyzed aldosterone levels in tumor lysate from two of the *CTNNB1* mutants, one *KCNJ5* mutant and one NHPA. This showed that *CTNNB1* mutated APAs (S45P and S45F) contained considerably higher aldosterone levels than the NHPA control, which had almost undetectable levels. To verify that the mutations in *CTNNB1* were functional we performed β-catenin IHC and AXIN2 mRNA expression analysis. Both IHC and expression analysis suggested that the mutation caused aberrant WNT activation. We also performed expression analysis and IHC for known ZG/ZF specific mRNAs/proteins. Results from this, suggested that *CTNNB1* mutants had a ZG like phenotype compared to *KCNJ5* mutants, which had a more ZF like phenotype.

Previous studies, including two separate exome sequencing projects have observed *CTNNB1* mutations in APAs[72,111,228,229]. Interestingly, two other studies have failed to detect any alterations[200,201]. This suggests that *CTNNB1* mutations occur in APAs, but are a rare cause of the activated WNT signaling seen in these tumors.

5. Summary of Paper III

Background

By utilizing exome sequencing, mutations in *ATP1A1* and *ATP2B3* were observed in APAs[69,72]. *ATP1A1* encodes the α1-subunit of a Na^+/K^+-ATPase that is important for establishing the membrane potential in glomerulosa cells[66]. The mutations were shown to increase depolarization, an important signal for aldosterone production[69,71,72]. *ATP2B3* encodes PMCA3 a plasma membrane Ca^{2+}- calmodulin dependent ATPase, important for the regulation of intracellular Ca^{2+}[97]. Like the mutations in *ATP1A1*, *ATP2B3* mutations lead to increased cell depolarization[69]. Both the ATPases share structural similarities and the mutations found are located in similar domains and residues in both proteins[69]. Interestingly, these tumors more often occurred in older male patients[69,71,72].

Aim

We wanted to confirm the presence of *ATP1A1* and *ATP2B3* mutation in a large cohort of APAs previously screened for *KCNJ5* mutation.

Results/Discussion

The main findings in this study were the confirmation of somatic mutations in both *ATP1A1* and *ATP2B3*. We analyzed a cohort of 165 APAs and detected a total of 16 (9.7%) mutations, which is similar to previous results[69,71,72,192]. In total we observed nine novel deletions, all located in highly conserved areas previously shown to be important for the normal protein function. We could confirm the phenotype difference discovered in the previous studies, except for the higher aldosterone values and the gender difference in *ATP2B3*. Despite this our CYP11B2 expression results

indicated that these tumors may in fact have higher aldosterone production than *KCNJ5* mutated tumors. The discovery of mutations in these genes improves our knowledge on APA tumorigenesis and also improves our understanding of the normal function of these two ion channels. This could potentially lead to development of new therapies in a wide variety of diseases.

6. Future perspectives

The main goal of my research has been to elucidate the genetic events that are involved in Aldosterone producing adenomas (APA). We have found that almost 2/3 of our APAs have a possible pathogenic mutation. This raises the question what other genetic aberrations are involved in the non-mutated tumors. From preceding work we know that these mutations often occur in ion channels that are involved in the tight regulation of the glomerulosa cell membrane potential or intracellular Ca^{2+}. Therefore it is tempting to speculate that other proteins affecting this could be mutated in APAs. We are currently sequencing *KCNK3* and *KCNK9* (encoding TASK-1 and TASK-3) and *KCNJ3* for mutations, but have currently not found any abnormalities. We are also sequencing *CACNA1D* in which we have found three mutations that have previously been described as pathogenic.

In our collaboration with Yale University, *KCNJ5* mutations were detected[84]. In the exome sequencing results we observed a mutation in another ion channel. We are currently sequencing this gene and have detected one additional mutation. We are planning to conduct functional studies on this mutant to see whether it has any functional consequences.

A majority of APAs also show up-regulated WNT signaling. This activation could either be due to down regulation of SFRP2 or *CTNNB1* mutations. However, only a proportion of tumors show these aberrancies, suggesting that other regulators of WNT are involved. The adrenocortical carcinoma cell line H295R, that produces aldosterone, carries both a β-catenin mutation (S45P) and also an *AXIN2* mutation[230]. AXIN2 is a negative regulator of β-catenin[231]. It associates with the β-catenin degradation complex that in the absence of WNT signaling targets β-catenin for degradation[231]. Mutations in *AXIN2* have previously been described in one APA[230].

The functional consequence of this mutation is unknown but it may cause disruption of the β-catenin degradation complex and activate WNT. We screened APAs for mutations in exon 6, 8 and 10 of the *AXIN2* gene, where most of the known mutations occur, without observing any potential pathological changes. Also, one of the main questions that remain to be answered is why *CTNNB1* mutation is not as common in APAs as in other ACTs. Active WNT signaling will lead to a ZG differentiation and higher aldosterone levels in mouse and cell models[201,209]. Interestingly, in the mouse model with constitutive activated β-catenin, most cells showed increased CYP11B2 expression, but cells with the highest levels of activation were more undifferentiated[201,209]. This suggests that the level of WNT activation could determine cellular fate. Importantly, *CTNNB1* mutations are common in adrenocortical carcinomas. Whether patients carrying APAs with *CTNNB1* mutations have an increased risk of developing malignancy is not known.

We have also performed a single nucleotide polymorphism array to investigate if there are common LOH events and/or imbalances of the genome in these tumors. The results of this are expected in the beginning of 2014. This could potentially identify parts of the genome that are important for tumor progression through either loss of tumor suppressor genes or gain of oncogenes. Through this we may gain further knowledge of the pathogenic events involved in these tumors.

Today 27 APAs have had their exome sequenced and common pathogenic variants have been detected[69,72,84,111]. This means that other potential genetic variants may not be as common. Importantly, by only sequencing the exome, disease causing alterations in introns may be overlooked. Also, the importance of non-DNA altering events like methylation and micro RNAs has been established in a variety of tumors. One study has previously looked into the methylation profile of APAs[232]. However,

the authors did not use glomerulosa cells as normal control but rather the whole adrenal cortex making the interpretation of their data difficult. Recently a micro RNA (miRNA) analysis of adrenocortical tumors (including APAs) was conducted[233]. This showed that APAs have a distinct miRNA profile compared to CPA and NHPA. It also showed that individual miRNAs were differentially expressed in APAs compared to the other tumors. These results indicate that epigenetic mechanisms may also be important for APA tumorogenesis.

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