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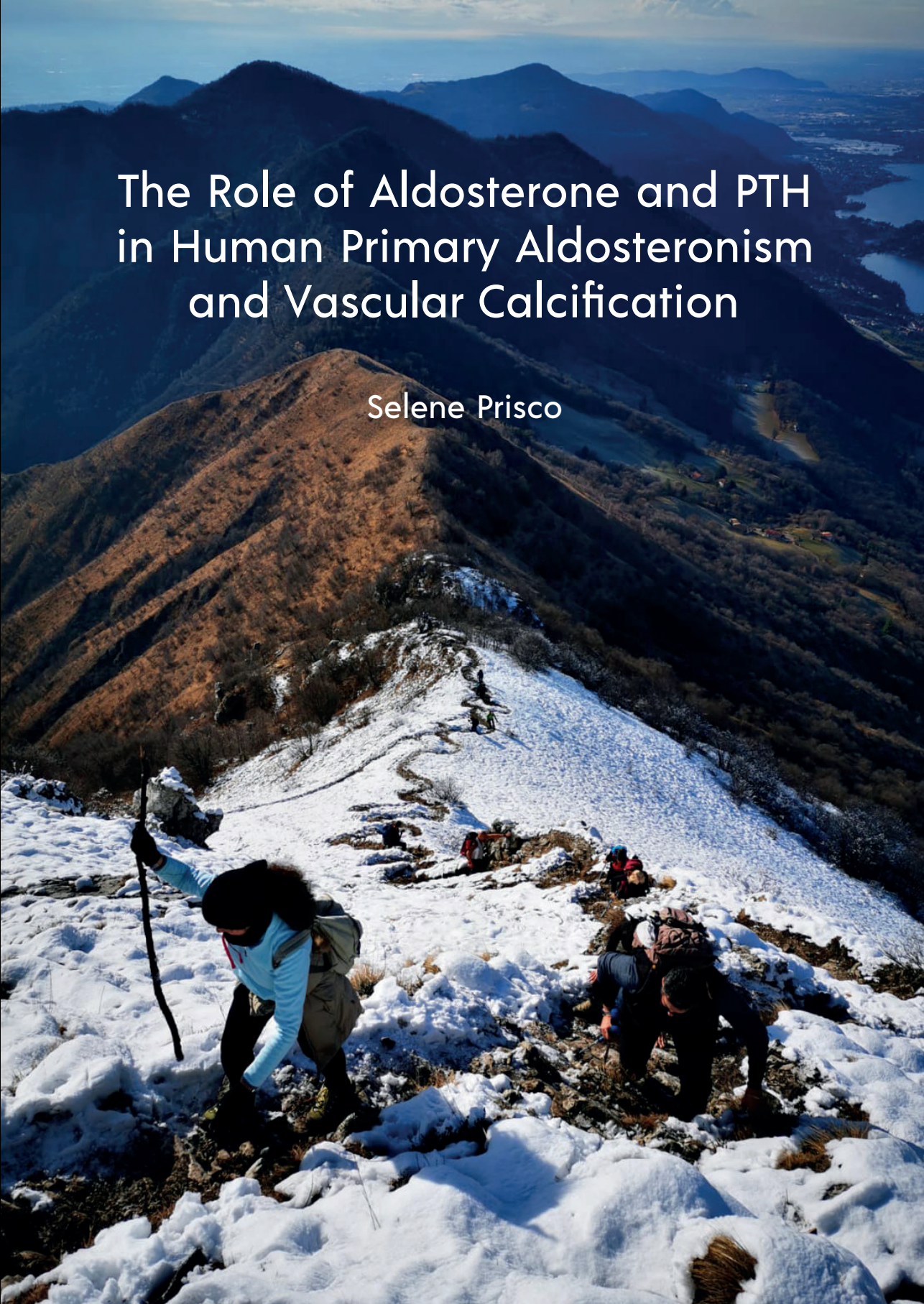

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Selene Prisco

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## Promotor

Prof. Dr. L.J. Schurgers

Prof. Dr. T. Hackeng

## Copromotoren

Prof. Dr. Gian Paolo Rossi

## Beoordelingscommissie:

Prof. Dr. Coen Stehouwer

Prof. Dr. Edwin Mariman

Dr. Sebastien Foulquier

Prof. Dr. Ralf Westenfeld

Prof.Dr. Michele Antonello

# Contents

# General Introduction and thesis outline

# 1. ADRENAL GLANDS

## 1.1 Anatomy and zonation

The adult adrenal glands are a pair of triangular or round bodies located at the superior pole of each kidney which display a combined weight of about 10–15 g in humans. In mammalian the adrenal gland is characterized by its anatomic zonation (Figure 1), where each zone can be distinguished from the other based on structural and anatomic characteristics[1]. The adrenal gland is composed of two distinct tissues; the cortex and the medulla. A thick capsule consisting of connective tissue surrounds the entire adrenal gland[2]. From the capsule, moving centripetally towards the medulla, the cortex comprises the zona glomerulosa (ZG) composed of densely packed cells with comparatively scant cytoplasm forming irregular cords or glomeruli. The zona fasciculata (ZF) is made of cells with abundant cytoplasm containing numerous lipid droplets organized in columns or fascicles. The innermost part of the cortex is the zona reticularis (ZR) where cells are arranged like cords that project in different directions to form a net. The adrenal medulla is composed of neural crest cells are arranged in small nests and cords separated by prominent vasculature[1,3].

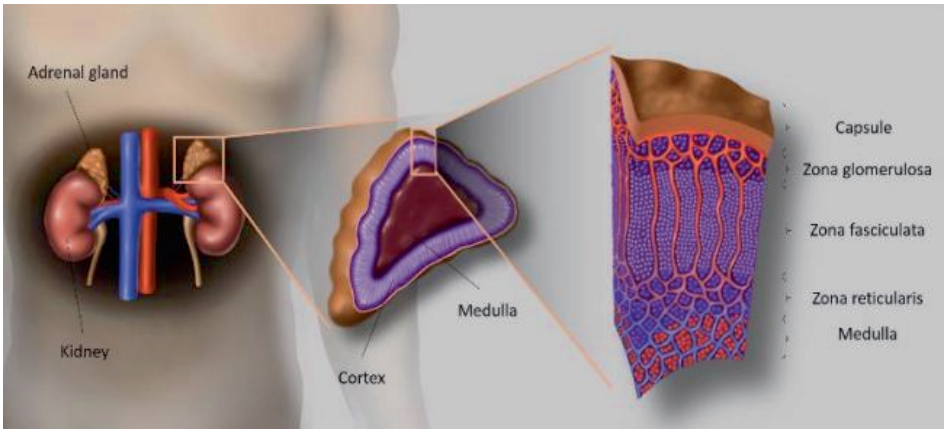


**Figure 1. Anatomy and cross section of human adrenal gland.** Figure reproduced with permission of the copyright author P.E.Vanderriele.

## 1.2 Steroidogenesis

Adrenal steroidogenesis is a dynamic process that recognizes cholesterol as the common precursor for all steroids produced in each cortex zone. To initiate steroidogenesis, cholesterol is mobilized from a pool in the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) through the steroidogenic acute regulatory (StAR) protein. In IMM the side-chain cleavage enzyme (CYP11A1, P450scc) catalyzes the first and rate-limiting step of steroidogenesis: the conversion of cholesterol to pregnenolone followed by a cascade of enzymes regulating hormonal production peculiar for each zone (Figure 2)[4]. The ZR is mainly regulated by adrenocorticotropic hormone and produces adrenal androgens, including dehydroepiandrosterone and dehydroepiandrosterone sulfate. The ZF is responsible for the glucocorticoids synthesis under the regulation of adrenocorticotropic hormone. In ZG, synthesis of aldosterone occurs through the action of a number of enzymes. Of these, CYP11B2 is expressed specifically in the zona glomerulosa, whereas expression of 11β-hydroxylase (CYP11B1), which is required for the final step in the biosynthesis of cortisol, is limited to the zona fasciculata.

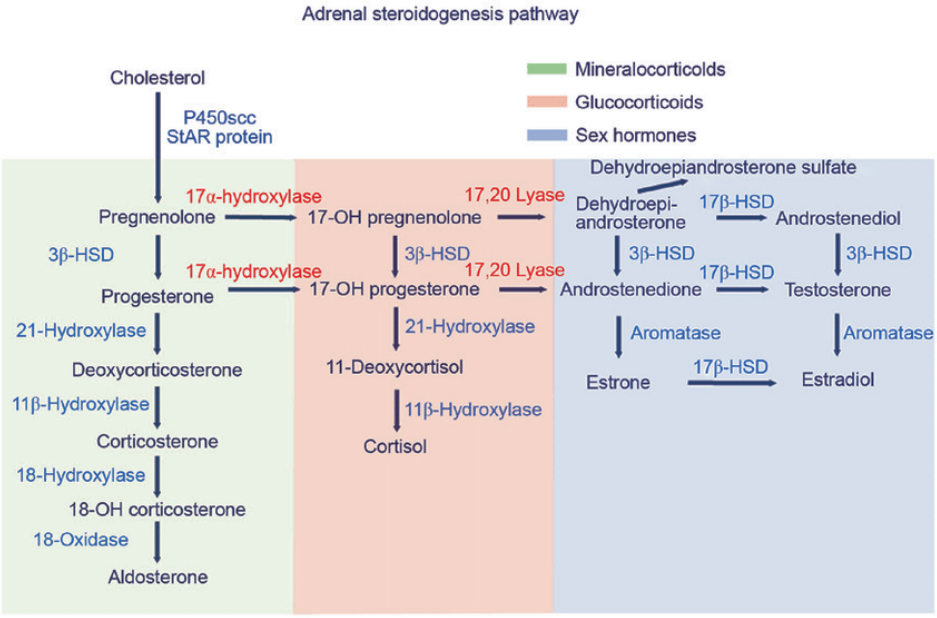


**Figure 2. The adrenal steroidogenesis pathway.** Figure adapted from Xu S.17α-hydroxylase/17,20-lyase deficiency in congenital adrenal hyperplasia: A case report. Molecular Medicine Reports, 2016

## 1.3 Aldosterone biosynthesis regulation

Aldosterone, the main mineralocorticoid hormone, is fundamental for maintaining body fluid balance, vascular resistance, and thereby blood pressure under conditions of salt/water depletion. Aldosterone secretion is mainly regulated by the activity of Angiotensin II (Ang II), serum potassium (K+) and adrenocorticotropin (ACTH). Aldosterone production can be upregulated within minutes through increased expression and phosporylation of StAR protein and chronically (over hours to days) by augmented expression of the steroidogenic enzymes, in particular CYP11B2[5,6].

## 1.4 Primary aldosteronism and its molecular mechanisms

Primary aldosteronism (PA) is the most common endocrine cause of secondary arterial hypertension with an estimated prevalence that occurs in 5% to 20% [7,8]. In hypertensive patients, PA is often not diagnosed because patients are not screened for it. Aldosterone producing adenoma (APA) are responsible for more than half of PA cases. Although the molecular mechanisms of PA are not completely understood, investigating the adrenal cortex is instrumental for understanding physiological and pathological aldosterone synthesis (Figure 3).
The regulation of cell membrane potential in the ZG is crucial to maintain cells in a hyperpolarized state in the absence of a secretagogue stimulus. Cell membranes in the ZG are selectively accessible for $K^+$ due to the expression of a large number of potassium channels[9]. During the last decade, several somatic mutations in or surrounding the selectivity filter of these channels have been identified to decrease both $K^+$ selectivity and permeability to $Mg^{2+}$ with ensuing greater influx of $Na^+$ into the cell, resulting in chronic cell depolarization. As a consequence, this is followed by opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production. More recently an excess “autonomous” aldosterone secretion was attributed to other somatic mutations in genes encoding for ATPases and Cav 1.3 calcium channel (CACNA1D)[10]. Furthermore, germline mutations in both cation and anion channels, downregulation of TASK2 potassium channel, via GPER activation as well as elevated serum levels of PTH were identified as cause of PA[11–13].

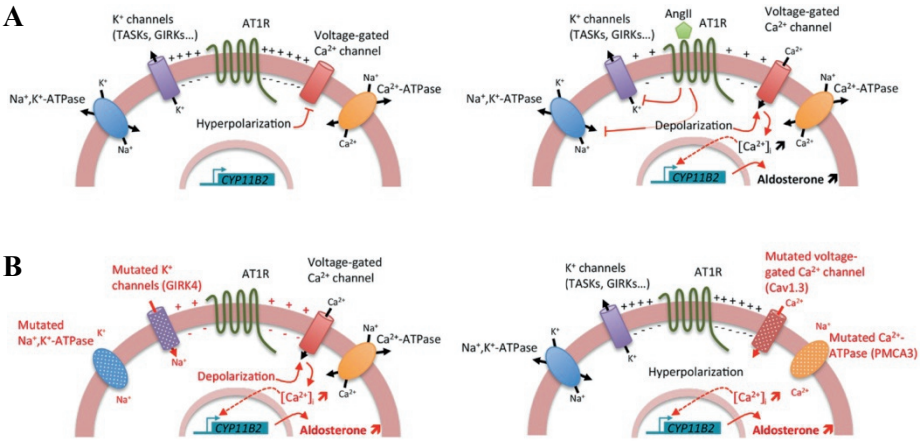


**Figure 3. Regulation of aldosterone biosynthesis under physiological (A) and pathological conditions (B).** Figure adapted from Zennaro C, Molecular and cellular mechanisms of aldosterone producing adenoma development

# 2. PARATHYROID GLANDS

## 2.1 Anatomy

The parathyroid glands are four nodular structures typically located on the dorsum of the thyroid at each of its four poles. Each parathyroid gland is about 6 millimeters long, 3 millimeters wide, and 2 millimeters thick. Its glandular tissue is separated from that of the thyroid by a fibrous capsule. Its parenchyma is primarily composed of chief cells and of a small number of oxyphil cells. The chief cells are believed to secrete most, if not all, PTH. Although the function of oxyphil cells remains unclear, there is some evidence supporting that they secrete PTH with aging or upon exposure functional stress[14,15].

## 2.2 Function

The principal physiological function of the parathyroid glands is sensitivity to small changes in serum concentration of calcium ion ($Ca^{2+}$), through calcium sensing receptor (CaSR) located on the cell surface, which leads to large changes in PTH secretion.

## 2.3 Parathyroid hormone

Parathyroid hormone is a polypeptide that is synthesized on the ribosomes and cleaved into an active form within the parathyroid gland. The initial structure is a pre-pro-PTH, a 115 amino acid polypeptide that is cleaved to form pro-PTH comprised of 90 amino acids. It is then cleaved a second time, again at the amino-terminal portion to form active parathyroid hormone comprising of 84 amino acids at the endoplasmic reticulum and Golgi apparatus. In addition to 1-84PTH, which actually induces hormonal action within the target cells, various PTH fragments of other lengths are also found in the systemic circulation. Among these fragments, 7-84PTH is directly secreted from parathyroid cells, and binds to PTH receptors with a binding affinity comparable to that of 1-84PTH[16]. The extracellular $Ca^{2+}$ level critically affects the switching of 1-84PTH/7-84PTH secretion in parathyroid cells. That is, 7-84PTH is predominantly secreted from parathyroid cells under a hypercalcemic condition, whereas 1-84PTH secretion is promoted in these cells under a hypocalcemic condition. Smaller compounds of 34 amino acids adjacent to the N terminus of the molecule were also isolated from the parathyroid glands. Because the

kidneys rapidly remove the whole 84-amino acid hormone within minutes, but fail to remove many of the fragments for hours, a large amount of the hormonal activity can be caused by the fragments[17].
The release of this hormone, essential for the regulation of calcium and phosphate metabolism, occurs in three different contexts (Figure 4):

## a. Effects of PTH in the Bones

PTH has two main effects on bone in causing absorption of $Ca^{2+}$ and phosphate. The first, rapid, phase (mins to hours) results from activation of osteocytes to promote $Ca^{2+}$ and phosphate reabsorption. The second, much slower, phase (days to weeks) results from proliferation of the osteoclasts, followed by increased osteoclastic reabsorption of the bone itself, not merely, absorption of the calcium phosphate salts from bone. This leads to destroy bones and minimize developing of new ones.

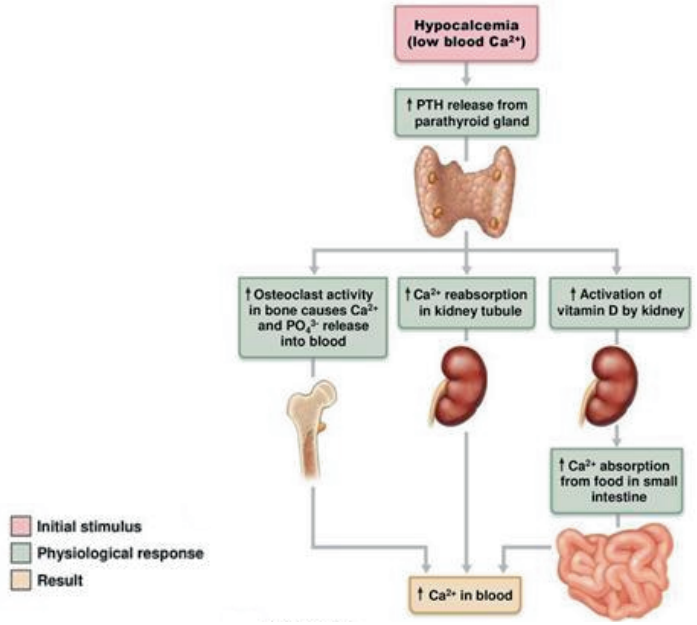


**Figure 4. Effects of parathyroid hormone on bone, kidneys and intestine.** Figure from 2013 Pearson Education

## b. Effects of PTH on the Kidneys

PTH increases renal tubular reabsorption of $Ca^{2+}$ mainly in the late distal tubules, the collecting tubules and to a lesser extent the ascending loop of Henle minimizing the calcium loss in the urine. Moreover, it increases the rate of reabsorption of $Mg^{2+}$ and $H^{-}$ ions while decreases phosphate reabsorption at the proximal convoluted tubule.

## c. PTH Indirect Effects on the Intestine

Starting in the proximal convoluted tubule, PTH stimulates the production of 1alpha-hydroxylase enzyme. This enzyme is responsible for catalyzing the synthesis of active vitamin D-1,25-dihydroxycholecalciferol from the inactive form, 25-hydroxycholecalciferol. PTH greatly enhances both $Ca^{2+}$ and $PO^{4-}$ reabsorption in the intestine by food through the increased formation of the active form of vitamin D.

# 3. VASCULATURE

## 3.1 Vascular calcification

Physiological calcification is a normal developmental process that is essential for the formation and functioning of various tissues such as skeletal bone and teeth. Ectopic calcification is usually prevented by local and systemic inhibitors of calcification. However, when dysregulated or inappropriate environmental stimuli occur in tissues that do not normally calcification (in the vasculature or soft tissue), it may lead to adverse consequences.

Vascular calcification (VC) is a highly regulated pathological process characterized by deposition of hydroxyapatite crystals within the blood vessel wall. This includes large arteries such as aorta, coronary arteries, carotid arteries, peripheral arteries, capillaries and heart valves. The presence of VC reduces arterial wall elasticity and alters the hemodynamic profile, thereby contributing to an increased risk of cardiovascular events.

Although the exact molecular basis still remains elusive; at least 4 key mechanisms are put forward to contribute to VC. These include: 1. circulating mineral complex, 2. loss of calcification inhibitors, 3. apoptosis and 4. phenotypic change of vascular smooth muscle cells (VSMCs)[18,19].

### Types of vascular calcification:

Vascular calcification can occur, based by its anatomical location, in the tunica media or in tunica intima of the vessel wall (Figure 5)[20].

#### a. Intimal calcification

In the setting of atherosclerosis, calcification of the intima has long been thought to occur as a result of an inflammatory response to lipid accumulation and plaque formation. More in detail, the long exposure to cardiovascular risk factors provoke oxidative stress and endothelial dysfunction, leading to expression of adhesion molecules, diapedesis of monocytes and subsequent transformation into macrophages. Macrophages internalize oxidized-LDL, become foam cells and release proinflammatory cytokines that lead to VSMC differentiation and migration into the atherosclerotic plaque[21].

Depending on the stage of the atherosclerosis, the migration of VSMC can exercise two opposite effects. On one hand it can make the plaque more prone to rupture by contributing to inflammation and lipid retention. On the other hand VSMC migration can stabilize the atherosclerotic plaque by depositing extracellular matrix protein and contribute to formation of a fibrous cap[22]. The stability of the plaque is influenced by the level of calcification. Spotty microcalcification within the fibrous caps of the atherosclerotic plaques is thought to promote local stress and increases the chance of plaque rupture[23]. Conversely, macrocalcification is thought to have a stabilizing effects on the atherosclerotic lesion[24].
Recently, clinical trials demonstrated a direct effect of aldosterone on the vessel wall inducing a proatherogenic and plaque-destabilizing effect. This is supported by the recent identification of pathways linking aldosterone to increased oxidative stress and metalloproteinase-9 activation. These pathways which might occur in monocytes/macrophages are well-known inducers of plaque instability and subsequent CV events.

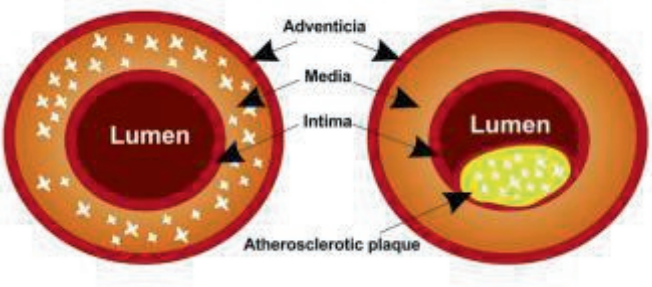


**Figure 5. Representation of the different types of vascular calcification occurring in the vessel well, i.e., medial (on the left), intimal (on the right).** Figure adapted from Viegas C, Inflammation and Calcification in the Vascular Tree; Insights Into Atherosclerosis. Immunity and Inflammation in Health and Disease, 2018

### b. Medial calcification

Medial calcification is driven by age-related changes in the vascular wall and is strongly associated with chronic kidney disease and diabetes mellitus. In this process of calcification, amorphous mineral deposits are formed along the elastin fibers of the tunica media then causing the vessel wall to stiffen. Medial calcification occurs in the absence of lipid infiltration or inflammation and is thought to depend on the local stress on VSMCs. Phenotypic change of VSMCs results in the increased release of extracellular vesicles which provide the nidus for calcification[25]. These small crystalline structures are phagocytosed by

surrounding VSMCs causing VSMC apopotosis[26]. They also promote secretion of chemokines and cytokines, attracting macrophages into the vessel wall[27].

Finally, both atherosclerosis (intimal calcification) and arterial stiffening or arteriosclerosis (medial calcification) likely contribute to each other, accelerating the process of vascular damage[28].

## 3.2 VSMC and their phenotypical transdifferentation

VSMCs are stromal cells of the vessel wall and control blood pressure by regulating contraction and dilation and thus vascular tone. Mature differentiated VSMC still have a strong plasticity, with significant and reversible phenotypic changes upon local environment stress. The elongated and spindle-shaped VSMCs exhibit features of the contractile phenotype by showing high expression of alpha-smooth muscle actin(α-SMA), calponin, and SM22alpha that enable them to perform contraction of the vessel wall. In the atherosclerotic context described above, as well as in hypertension, aneurysm, restenosis and postangioplasty, VSMCs can quickly transform from a contractile phenotype to a synthetic phenotype changing their morphology, protein expression, migration and proliferation capacity. The latter phenotype express lower levels of the proteins involved in contraction but is more prone to secrete extracellular vesicles. Continued extracellular stress signals such as inflammatory cytokines, uremic toxins and mainly exposure to elevated phosphate concentrations trigger the differentiation of VSMCs into an osteo-chondrogenic phenotype[29–31]. Both extracellular vesicle release and osteochondrogenic differentiation of VSMCs contribute to vascular remodeling, as a consequence, calcification of extracellular matrix. Finally, when vessel wall homeostasis is restored, VSMCs can regain the contractile phenotype.

## 3.3 A link between endocrine and vascular systems

In the last years, important strides have been made in understanding the pathogenesis of vascular disease. Endocrine factors have been recognized to play an important role in the development and progression of these vascular diseases[32]. Increasing evidence suggests a bidirectional interplay between aldosterone and parathyroid hormone in increased risk of cardiovascular pathology. The link between both hormonal systems was supported by the

identification of PTH receptors on ZG cells and Mineralocorticoid Receptors (MR) on parathyroid chief cells. Moreover, the identification of PTH and MR receptors within the cardiovascular system (cardiomyocytes, VSMCs, and endothelial cells) indicate that their inappropriate secretion may alter the cardiovascular (CV) system directly.

The potential impact of aldosterone and PTH on CV system, has been investigated in 2 different scenarios (Figure 6):

### a. In Primary Aldosteronism

Recent evidence strongly suggests that PTH may sustain the excess production of aldosterone in PA despite suppression of the renin-angiotensin system, high blood pressure and hypokalaemia, all factors that are expected to blunt aldosterone secretion[33,34]. That PTH might play a role in PA was supporting by several studies. First, significantly increased serum concentrations of PTH were found in PA patients which decreased after 1 month of MR blockade, along with an increase in serum-ionized calcium[35]. More recently, it was shown that patients with PA had significantly higher plasma PTH than patients with primary (essential) hypertension. Of interest, adrenalectomy normalized PTH levels and increased ionized $Ca^{2+}$ in APA patients[36]. The mechanism that might explain a clinically relevant interaction between aldosterone and PTH, presumably potentiating the CV risk in patients with primary aldosteronism can be summarized as follows. Elevated aldosterone levels in PA correlate with increased urinary and faecal loss of $Mg^{2+}$ and $Ca^{2+}$. The resulting lowering of serum calcium concentration further stimulates production of PTH which in turn amplifies adrenal aldosterone synthesis. PTH excess in turn induces calcium overload and oxidative stress in cardiomyocytes and vascular smooth muscle cells and aggravates reduction in intra mitochondrial ATP levels resulting in subsequent necrotic cell death and myocardial fibrosis.
Thereby, a vicious circle between aldosterone and PTH might potentiate CV damage[32].

### b. In Hyperparathyroidism

Primary hyperparathyroidism (PHPT) is the third most common endocrine disorder and is characterized by excess PTH secretion. In PHPT, chronic excess secretion of PTH is associated with the development of osteoporosis, diabetes, and hypertension. Remarkably, patients with chronic PHPT have a higher risk of dying from CV disease compared with to general population[37,38]. In the majority of humans studies that evaluated RAAS in PTH excess, a significant

decline of plasma renin activity, angiotensin II, and of aldosterone levels was documented after parathyroidectomy[39–41]. Other studies documented that the stimulatory effect of PTH on RAAS may potentiate the risks of development and progression of arterial hypertension as well as the risk of CVD in PHPT patients by stimulating the adrenal aldosterone synthesis. This effect can be direct by facilitating calcium entry into adrenal ZG cells via binding to PTH/PTH-related protein, adrenocorticotropic hormone-receptors and voltage gated L-type calcium channels. The indirect effect is facilitated by stimulation of renal renin release and increasing angiotensin II concentration thus sensitizing adrenal ZG cells.

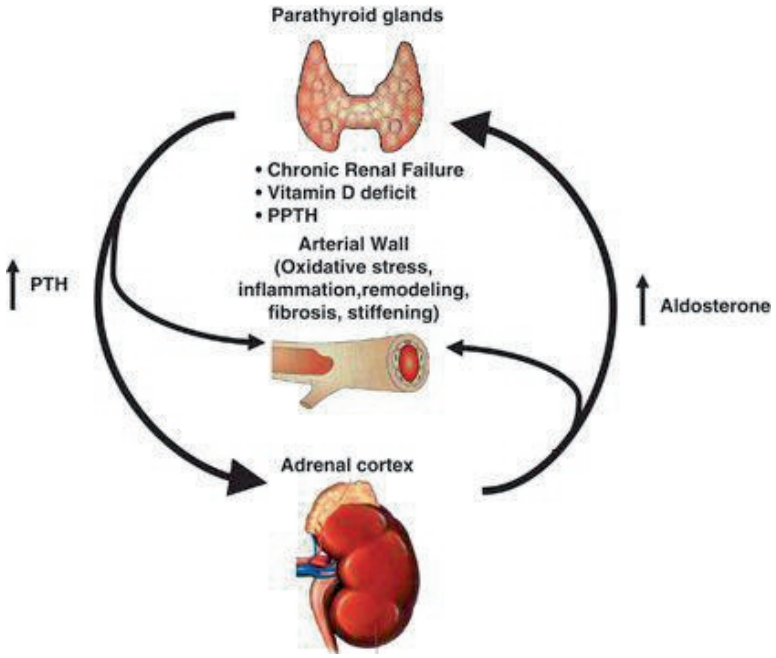


**Figure 6. Overview of the cardiovascular impact resulting from the interaction of aldosterone and parathyroid hormone.** Figure adapted from Rossi GP, Hyperparathyroidism, arterial hypertension and aortic stiffness: a possible bidirectional link between the adrenal cortex and the parathyroid glands that causes vascular damage? Hypertension Research, 2011

Given the emerging evidence implicating hyperparathyroidism as well as PA as a cardiovascular risk factors, it could be arguable that the high hormone levels in these patients contribute to the excess cardiovascular damage.

# Outline of the thesis

In **Chapter I** we provide an update of current literature on the genetic basis of familial forms of Primary Aldosteronism (PA), including recent discoveries, their molecular basis and their mechanistic implications. In **Chapter II** we pave the way towards personalized diagnosis and treatment of PA patients. More specific, patients with the most florid form of the disease, such as those with Aldosterone-Producing-Adenoma (APA) carrying KCNJ5 mutations. In **Chapter III** we show that aldosterone can modulate its own synthesis through GPER activation. The mineralocorticoid hormone acts in an autocrine-paracrine manner in GPER overexpressing APA, thereby generating high local concentration of mineralocorticoid hormone and thus self-perpetuating the hyperaldosteronism, whereas the renin angiotensin system is blunted. In **Chapter IV** we explore mechanisms underlying the decreased expression of TASK2 channels in APA. In particular, by sequencing the TASK2 gene promoter region we identified mutations that explain some 25% of APA cases. **Chapter V** is based on a brief letter that highlights important limitations of a double blinded randomized study that evaluates the interaction between the renin angiotensin-aldosterone system (RAAS) and parathyroid hormone (PTH) system in a claiming the possible influence of AngII in PTH secretion via AT1-R. We investigated the role of the RAAS system, particularly of aldosterone and AngII in the regulation of PTH in **Chapter VI**. In vivo, we examined the effect of Ang II blockade on PTH secretion in both essential hypertensive and APA patients and ex vivo we investigated direct effects of aldosterone and Ang II on PTH production. In **Chapter VII** we demonstrate that use of phosphate binders in combination with high intake of vitamin K significantly decreases vascular calcification in CKD rats as compared to vitamin K or phosphate binders alone. This might have great clinical implications since phosphate binder therapy is common standard therapy for dialysis patients and has recently been shown to further lower vitamin K status. Combining phosphate binder therapy with high vitamin K intake might bypass this and benefit patients by lowering vascular calcification. **Chapter VIII** concludes this thesis with a general discussion of all presented results.

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# Chapter 1

# The Saga of Familial Hyperaldosteronism: yet a new channel

Livia Lenzini, Selene Prisco, Brasilina Caroccia, Gian Paolo Rossi

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# 1. Introduction

In 1953, Litynski[1] reported in a Polish journal the first case of an adrenocortical adenoma associated with hypertension and hypokalemia that was cured by adrenalectomy. Three years later, Conn[2] reported a similar case and described the full characterization of the syndrome of primary aldosteronism (PA). Compelling evidence is now established that PA, albeit deceiving diagnosis in the majority of the cases because of the lack of hypokalemia, is the most common endocrine cause of hypertension. Moreover, if adrenal vein sampling is systematically used, PA is surgically curable in about two thirds of the cases[3,4] . Exceptions to this rule entail the familial cases of PA because of germline mutations that, by definition, involve both adrenals and, therefore, cannot be cured by unilateral adrenalectomy. The familial occurrence of PA has been known for decades since the discovery of PA in 1966 by Sutherland et al.[5] Of note, they reported that in some pedigrees of patients with PA, the syndrome could be corrected by glucocorticoids, which led them to define this condition glucocorticoid-remediable aldosteronism[5]—a condition today redefined as familial hyperaldosteronism (FH) type 1 (FH-1). The molecular mechanisms of FH-1 remained unknown until 1992 when a chimeric gene deriving from unequal crossing over of the CYP11B1 and CYP11B2 genes was identified as the culprit of FH-1[6]. Genetic testing for this form based on long polymerase chain reaction[7] rapidly followed, which allowed the identification of familial cases of PA without the chimeric gene that by default were provisionally classified as FH type 2. A long quest for responsible genes was unsuccessful[8] until a few months ago. Moreover, an impressive amount of research work in the past 6 years has allowed the discovery of additional germline mutations in multiple genes and to a new classification of FH (Table). Familial forms of PA with germline mutations account now from 1% to 5% of PA cases, depending on the cohorts studied, and are transmitted as an autosomal dominant trait. Therefore, the purpose of this review is to provide an update on FH taking account of the recent discoveries, their genetic basis, and their mechanistic implications.

# 2. Familial hyperaldosteronism type 1 (FH-1)

As mentioned, the first familial forms of PA reported in the 60s were defined glucocorticoid-remediable aldosteronism (OMIM [Online Mendelian Inheritance in Man]: 103900) because they were found to be corrected by low-dose dexamethasone treatment[5,9]. More recently, they were renamed as FH-1,

after the discovery of other familial forms. FH-1, inherited as an autosomal dominant disorder, is present in ≤1% of familial cases among patients with PA and has phenotypic heterogeneity (Table). In fact, although hypertension is often of early onset and may be sufficient to result in early death, commonly because of intracerebral hemorrhage, it has been reported that the degree of hypertension varies widely even within the same family. Moreover, the severity of hypertension correlates with the sex: female subjects show a less-severe phenotype and a better prognosis[10]. Its molecular basis was clarified in 1992 when Lifton et al[6] identified a fusion of the promoter sequence of the CYP11B1 gene with the coding region of the CYP11B2 gene in an affected pedigree. This chimeric structure of the gene is held to originate from an unequal crossing over of the CYP11B1 and CYP11B2 genes, two 95% homologous genes located on chromosome 8q24. The regulatory region of the chimeric gene is altered in that the CYP11B1 promoter ends upstream of the CYP11B2 coding sequences, which explains why the latter gene becomes responsive to adrenocorticotropic hormone. The resulting phenotype features ectopic expression of aldosterone synthase encoded by CYP11B2 in the adrenal zona fasciculata under the control of adrenocorticotropic hormone instead of angiotensin II (Figure 1A). Moreover, the chimeric enzyme catalyses the C-18 hydroxylation and C-18 oxidation of cortisol made by the zona fasciculata, thus causing the production of the hybrid steroids 18OH-cortisol and 18-oxo cortisol[6]. This also explains why in affected individuals specific treatment with low-dose dexamethasone usually normalizes blood pressure and corrects completely the hyperaldosteronism—a clear-cut example of molecularly targeted precision medicine. A genetic test based on long polymerase chain reaction soon followed the discovery of the chimeric gene,[11] thus allowing a conclusive diagnosis of FH-1. It was thereafter ascertained that FH-1 is a rare disease with a prevalence <1% in PA[12].

## 3. Familial hyperaldosteronism type 2 (FH-2)

Familial cases of PA that do not respond to glucocorticoid treatment were known for years, but only the availability of the genetic test for FH-1 allowed the determination that they did not have the chimeric gene. These cases were, therefore, by exclusion defined as FH type 2 (OMIM: 605635). They have a prevalence of 5% in PA cases and comprise either adrenocortical hyperplasia or aldosterone-producing adenoma (APA) and are clinically indistinguishable from sporadic PA except than for the familial occurrence[13,14].

Of note, although by linkage analysis a quantitative trait locus was identified on chromosome 7p22,[8] the responsible gene(s) remain unknown until recently when Lifton et al starting from a multiplex kindred featuring autosomal

dominant FH type 2 originally reported by Stowasser et al[14] in 1992, identified a recurrent functional variant (R172Q) in the CLCN2 gene in 8 of the probands with early-onset PA (Table). This gene encodes the ClC-2 (chloride channel), which is expressed in many tissues, including brain, kidney, lung, intestine, and the adrenal gland. Using exome sequencing in 80 additional probands with unsolved early-onset PA, the authors identified 2 de novo mutations (M22K and R172Q) and 4 independent occurrences of the R172Q (Figure 2).[15] In some of the probands carrying the mutations, the phenotype ameliorated with age, suggesting an incomplete penetrance. When tested in vitro in zona glomerulosa cells (H295R), these germline mutations showed gain of function, for example, enhanced chloride efflux at physiological membrane potentials, as compared with wild-type channels and, therefore, caused membrane depolarization, increased CYP11B2 expression, and aldosterone overproduction (Figure 1B).

| Type | Subtype | Prevalence, %* | Cytogenetic Location | Gene Mutation | CT Findings | Treatment | Drug-Resistant Hypertension | Clinical Features |
|---|---|---|---|---|---|---|---|---|
| FH-1 | | 0.5–1 | 8q24 | *CYP11B2*/*CYP11B1* Chimeric | BAH or APA | Low-dose dexamethasone | Yes (with drugs other than dexamethasone) | Early-onset PA, hybrid steroids, cerebrovascular events |
| FH-2 | | 5 | 3q27 | *CLCN2* (R172Q, M22K, G24D, S865R, Y26N) | BAH or APA or no adrenal abnormalities | MRA | No | Early-onset PA |
| FH-3 | Type A | 0.3 | 11q23 | *KCNJ5* (T158A, I157S, E145Q) | BAH | Bilateral adrenalectomy | Yes | Severe early-onset PA |
| | Type B | 0.3 | 11q23 | *KCNJ5* (G151E, Y152C) | No | MRA | No | Mild PA |
| FH-4 | | NA | 16p13 | *CACNA1H* (M1549V, S196L, P2083L, V1951E) | Little or no adrenal abnormalities | MRA | No | Early-onset PA, mental retardation, social and development disorders |
| FH-5 (PASNA) | | NA | 3p14.3 | *CACNA1D* (I770M, G403D) | No adrenal abnormalities | Calcium channel blockers | No | Early-onset PA, seizures, neurological abnormalities |

**Table. Clinical and Molecular Classification of FH**. APA indicates aldosterone-producing adenoma; BAH, bilateral adrenal hyperplasia; CT, computed tomography; FH, familial hyperaldosteronism; MRA, mineralocorticoid receptor antagonist; NA, not available; PA, primary aldosteronism; and PASNA, primary aldosteronism with seizures and neurologic abnormalities. *In patients with PA.

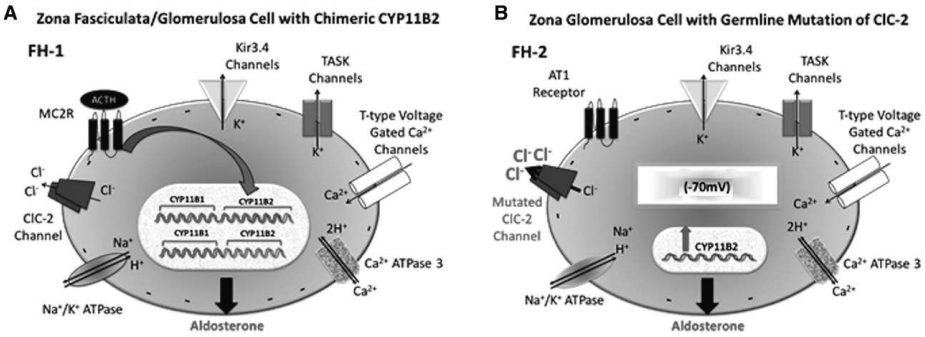
In the same journal issue, by analyzing 12 patients with young-onset hypertension and hyperaldosteronism diagnosed by 25 years of age, Zennaro et al[16] reported an additional de novo germline CLCN2 variant (G24D) in a highly conserved inactivation site of the N-terminal cytoplasmic domain (Figure 2). They explored the impact of the G24D variant on the membrane potential of H295R cells transfected with this mutation under basal and Ang II- and K+-stimulated conditions. According to their findings, the mutation conferred higher aldosterone production under all conditions. Moreover, they could

demonstrate that whereas in wild-type H295R, aldosterone synthesis depended only on T-type calcium channel activity, in mutated cells, it involved both L-type and T-type calcium channel activity.[16] Thus, CLCN2 variants result in a strong gain of function, in line with the dominant clinical phenotype caused by the mutations even if present only in the heterozygous state.

Noteworthy, the identification of these chloride channel mutations pointed for the first time to a role of anion channels in the regulation of cell membrane potential and aldosterone biosynthesis in adrenal zona glomerulosa. Thus, these seminal discoveries will likely generate new possibilities for the diagnosis and, possibly, treatment of early-onset PA cases.

# 4. Familial hyperaldosteronism type 3 (FH-3)

In 2011, alongside the identification of somatic mutations in APA in the KCNJ5 gene encoding the Kir3.4 $K^+$ channel (OMIM: 600734), new forms of FH were discovered[17] and defined as FH type 3 (FH-3). A novel clinical molecular classification was then proposed,[18] which defines FH-3 as a genetic disease made of 2 distinct subtypes: 1 severe (type A) requiring bilateral laparoscopic adrenalectomy and 1 milder (type B) usually responding well to antihypertensive therapy (Table). The prevalence of these forms is low, and it was reported to be ≈0.3% of all patients with PA.[19]



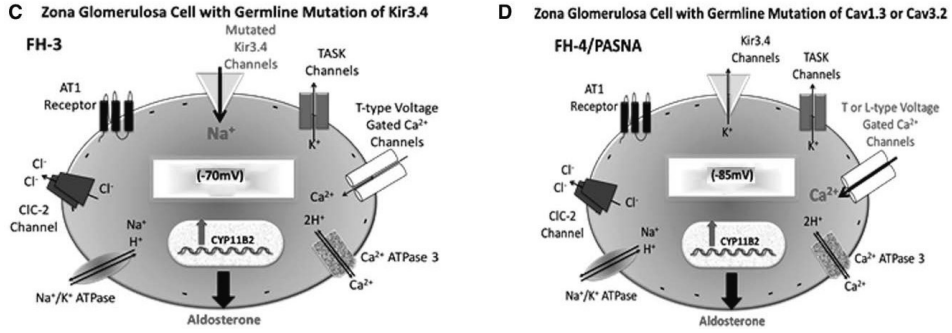



**Figure 1. Genetic causes of familial aldosteronism (FH)**. A, FH-1 is because of the fusion of the promoter sequence of the CYP11B1 gene with the coding region of the CYP11B2. The regulatory region of the resulting chimeric gene is altered, and the CYP11B2 gene becomes responsive to the adrenocorticotropic hormone (ACTH). This translates in an ectopic expression of aldosterone synthase in the adrenal zona fasciculata under the control of ACTH. No data exist about alterations of the activity of ion channels in this disease. **B**, Germline gain-of-function mutations were identified in cases with FH-2 in the gene CLCN2 coding for the CIC-2 chloride channel. These cause enhanced chloride efflux, membrane depolarization, increased CYP11B2 expression, and aldosterone overproduction. **C**, In FH-3 cases, the disease occurs as a result of Kir3.4 (KCNJ5) mutations, which cause a loss of selectivity for $K^+$ , $Na^+$ influx, depolarization of the cell, with ensuing entry of $Ca^{2+}$ via voltage-gated T-type $Ca^{2+}$ channels and autonomous production of aldosterone. **D**, Two additional genetic causes of FH are mutations in CACNA1H (FH-4) and CACNA1D (primary aldosteronism with seizures and neurologic abnormalities [PASNA]) genes coding for the T-type Cav3.2 and L-type Cav1.3 voltage-gated calcium channels, respectively. These mutations activate channels at less-depolarized potentials with ensuing increased $Ca^{2+}$ influx.

*FH-3 type A*

Choi et al[17] examined a pedigree characterized by severe aldosteronism and massive bilateral adrenal hyperplasia, which required bilateral adrenalectomy because of drug-resistant hypertension. The index case and his 2 daughters had a mutation (T158A) in the KCNJ5 gene, mapping near to the Kir3.4 selectivity filter. The mutation causes a threonine-to-alanine substitution at codon 158 resulting in reduced $K^+$ selectivity, increased $Na^+$ conductance, and cell membrane depolarization (Figure 1C).

Another heterozygous mutation at position 470, resulting in isoleucine-to-serine substitution at amino acid 157 (I157S), was thereafter found in a mother and daughter, who presented with severe PA, bilateral massive adrenal hyperplasia, and early-onset hypertension refractory to drug treatment.[20]

A further E145Q germline mutation previously known to occur in APA was reported in a white girl presenting at the age of 2 years with polydipsia, polyuria, and failure to thrive.[21,22] She was found to have profound hypokalemia, severe hyperaldosteronism with renin suppression, and arterial hypertension resistant to treatment, which led to bilateral laparoscopic adrenalectomy in spite of negative computed tomography and magnetic

resonance imaging. Like for other KCNJ5 mutations, functional characterization of this mutation showed $Na^+$-dependent cell membrane depolarization and increased intracellular $Ca^{2+}$ concentration causing high CYP11B2 expression.[23]

*FH-3 type B*

In 2012, 2 studies independently reported the G151E germline mutation in patients with a milder form of hyperaldosteronism.[19,24] These patients had no evidence of adrenal hyperplasia, and their hypertension could be easily controlled with drugs.[24] In vitro, the mutation showed prominent effects featuring a large $Na^+$ conductance with rapid $Na^+$-dependent cell lethality. To explain these paradoxical findings, it was contended that the $Na^+$ influx-dependent cell death could limit the expansion of zona glomerulosa cell mass, thus preventing the development of hyperplasia with ensuing less-prominent PA. Hence, it was suggested that the overproduction of aldosterone in the surviving zona glomerulosa cells might be sufficient to raise blood pressure but not high enough to render hypertension resistant to drug treatment.
Another germline mutation (Y152C) was detected in a patient with mild form of hyperaldosteronism because of an adrenal adenoma, but it remains unclear whether this is another familial form or a sporadic de novo mutation because no information on other family members was given.[23]

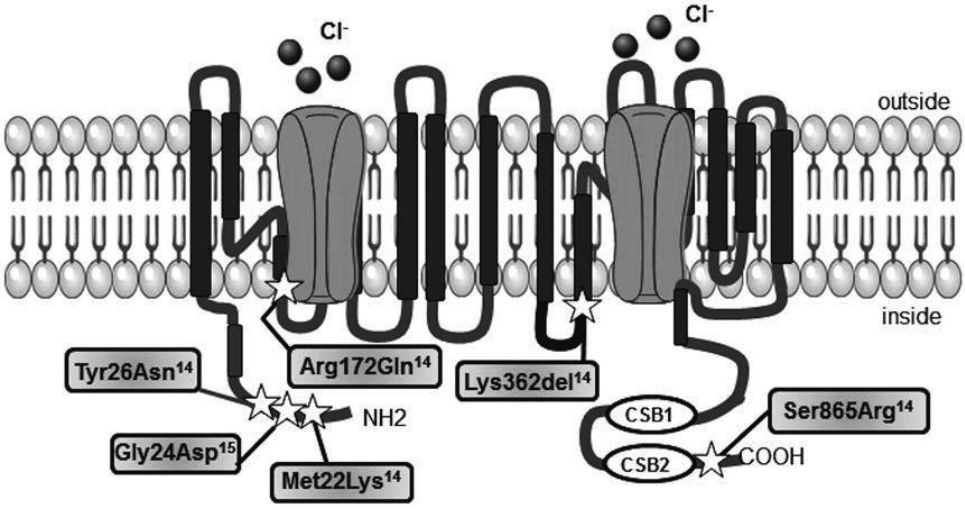



**Figure 2. Gain-of-function variants inthe CIC -2 chloride channel identified in patients with early-onset primary aldosteronism.** Stars indicate variant positions in the N terminus, D helix, K helix, and C terminus. Intracellular loop of CIC-2 is shown in black. CSB1 and CSB2 are cystathionine-β-synthase domains that can affect gating of CIC channels, here shown with white ellipses.

## 5. Familial hyperaldosteronism type 4 (FH-4)

A recurrent germline gain-of-function mutation in CACNA1H gene (M1549V) was identified in 5 children with PA before 10 years of age as the cause of FH type 4 (Table). The mutation was inherited in 3 of the cases and occurred de novo in 2.[25] All patients showed hyperaldosteronism with low plasma renin activity but no evidence of mass or hyperplasia on adrenal imaging at the time of presentation. There were no recurrent or distinctive features in the index cases, for example, history of seizures or neurological or neuromuscular disorders.

CACNA1H gene is located on chromosome 16p13 and encodes the pore-forming α1 subunit of the T-type voltage-dependent calcium channel Cav3.2. CACNA1H is highly expressed in the adrenal zona glomerulosa and is activated at slightly depolarized potentials. Of note, the Cav3.2 channel is involved in the zona glomerulosa membrane potential oscillations and aldosterone production, according to Barrett et al.[26]

Whole-cell patch-clamp experiments in human embryonic kidney-293 cells showed that the M1549V CACNA1H channel exhibited activation to less-depolarized potentials and slow inactivation—2 features held to cause enhanced $Ca^{2+}$ influx in adrenal glomerulosa cells. The overexpression of M1549V in HAC15 adrenocortical cells mutant channel increased CYP11B2 gene expression and aldosterone production in basal conditions (Figure 1D), whereas cotreatment with the T-type calcium channel blocker mibefradil abolished aldosterone production, indicating that M1549V CACNA1H mutation induces autonomous aldosterone production via T-type calcium channels.

Four additional germline CACNA1H mutations in patients with PA and different clinical features were identified by Daniil et al.[27] A M1549I de novo mutation occurred in the same position of M1549V and caused hypertension and hyperaldosteronism alongside mild mental retardation, social skill alterations, learning disabilities, and development disorders. The S196L and P2083L were identified in 2 families affected by hypertension and PA. The V1951E germline variant was also identified in a patient with APA, cured by unilateral adrenalectomy.

In vitro electrophysiological experiments demonstrated that these new CACNA1H mutations changed the electrophysiological properties of the channel similar to M1549V.[25] Furthermore, transfections of mutant in H295R-S2 cells induced high aldosterone levels and overexpression of genes coding for steroidogenic enzymes after $K^+$ stimulation.

## 6.Primary Aldosteronism with Seizures and Neurologic Abnormalities syndrome (PASNA)

The primary aldosteronism with seizures and neurologic abnormalities syndrome (OMIM: 615474) phenotype (Table) was the first calcium channel mutation found to be associated with extra adrenal symptoms.[28] Two de novo germline mutations (I770M and G403D) in the CACNA1D gene were detected in 2 children with severe hypertension diagnosed at birth, hypokalemia, and neurological manifestations, including seizures and cerebral palsy.[28] This gene, located on chromosome 3p14.3, encodes for Cav1.3—the α subunit of the L-type voltage-gated calcium channel. Both are gain-of-function mutations and had already been found in sporadic forms of APA.[28,29] They cause channel activation at membrane potentials close to the resting of the zona glomerulosa cells (−80 mV) and increase $Ca^{2+}$ influx and stimulation of aldosterone production (Figure 1D).

## 7. Conclusions and Perspectives

The last decade has witnessed enormous progresses in understanding the molecular basis of human PA, both its sporadic (reviewed elsewhere[18]) and its familial forms. Genetic testing is already available for FH-1—the first familial form to be clarified at the molecular level—as well as for the other forms. In some forms of FH, this progress already opened the way to personalized treatment. For example, as mentioned above, the reason why low-dose dexamethasone is highly effective in correcting PA and lowering blood pressure in FH-1 not only received an explanation at the molecular level but also has allowed pinpointing which patients with familial PA should receive this therapy. This field has received further impulse from the more recent discovery that the altered electrophysiology caused by the KCNJ5 mutations L168R and G151R can be specifically corrected in vitro[30] and ex vivo[31] with macrolides antibiotics. In fact, these findings can open the way to personalized diagnosis and treatment of PA caused by these mutations—a hypothesis now being tested in the MAPA study (Macrolides for KCNJ5-Mutated Aldosterone-Producing Adenoma)[32] and to be further explored in the same patients with FH-3. As molecular, electrophysiological, and pharmacological research will be further expanded, there is no doubt that the development of specific drugs interfering with the function of mutated channels could be foreseen in the near future as a personalized treatment for the other familial forms.

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# Chapter 2

# Macrolides blunt Aldosterone Biosynthesis: A Proof Of Concept Study in KCNJ5 Mutated Adenoma Cells Ex Vivo

Brasilina Caroccia, Selene Prisco, Teresa Maria Seccia, Maria Piazza, Giuseppe Maiolino and Gian Paolo Rossi

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Aldosterone-producing adenoma, a major subtype of primary hyperaldosteronism, the main curable cause of human endocrine hypertension, involves somatic mutations in the potassium channel Kir3.4 (KCNJ5) in 30-70% of cases, typically the more florid phenotypes. Since KCNJ5 mutated channels were reported to be specifically sensitive to inhibition by macrolide antibiotics, which concentration-dependently blunt aldosterone production in HAC15 transfected with the G151R and L168R mutated channel, we herein tested the effect of clarithromycin on aldosterone synthesis and secretion in a pure population of aldosterone-secreting cells obtained by immunoseparation ($CD56^+$ cells) from aldosterone-producing adenoma tissues with/without the two most common KCNJ5 mutations. From a large cohort of patients with an unambiguous aldosterone-producing adenoma diagnosis we recruited those who were wild-type (n=3), or had G151R (n=2) and L168R (n=2) mutations. We found that clarithromycin concentration-dependently lowered CYP11B2 gene expression (by 60%) and aldosterone secretion (by 70%) ($p<0.001$ for both) in $CD56^+$ cells isolated ex vivo from KCNJ5-mutated aldosterone-producing adenomas, while it was ineffective in $CD56^+$ cells from wild-type aldosterone-producing adenomas. By proving the principle that the over secretion of aldosterone can be specifically blunted in aldosterone-producing adenoma cells ex vivo with G151R and L168R mutations, these results provide compelling evidence of the possibility of specifically correcting aldosterone excess in patients with aldosterone-producing adenoma carrying the two most common KCNJ5 somatic mutations.

**Key words**: Aldosterone, synthesis, ion channel, macrolides, aldosteronism, hypertension

# Introduction

In 2011 Choi et al.[1] identified mutations in the Kir 3.4 (KCNJ5) $K^+$ channel in about one third of 24 aldosterone-producing adenoma (APA), which were shown to lead to a loss of selectivity for $Na^+$ and thus to $Ca^{2+}$ influx, via opening of T-type calcium channel and activation of the $Na^+/Ca^{2+}$ exchange,[2,3] and thus increased aldosterone production. Of note, these mutations were found to involve from 30% to 70% of all APA in a very large meta-analysis, albeit with marked geographic variations.[4]

Some macrolide antibiotics and their derivatives were recently shown to correct the altered electrophysiology of HEK293T cells transfected with the L168R and G151R KCNJ5 mutated Kir 3.4 channel, and to concentration-dependently lower aldosterone synthesis in HAC15, an adrenocortical carcinoma cell line transfected to overexpress these mutated channels.[5] Notably, these effects were suggested to be specific in that they were not seen in the same cells carrying the wild type Kir 3.4 channel.[5] These seminal discoveries might open the way to personalized diagnosis and treatment of primary aldosteronism, the most common, albeit often overlooked, form of secondary arterial hypertension,[6] which carries an excess cardiovascular damage, as well as a worse outcome.[7,8]

In fact, if these in vitro findings could be confirmed in vivo, the fall of plasma aldosterone concentration (PAC) after administration of a macrolide antibiotic might represent a proxy for the presence of an APA carrying a KCNJ5 mutation. Likewise, if associated with a blood pressure lowering, this PAC fall would suggest the feasibility of controlling the aldosteronism and arterial hypertension with a macrolide or derivatives devoid of antibiotic activity.

As APA are known to be highly heterogeneous at the molecular level,[9] and therefore could respond differently to stimuli and drugs, a first inevitable step toward these clinical applications is to prove that the effects seen in these two models of genetically engineered cell lines, which are far from APA cells, do occur also in the APA cells taken ex vivo from APA patients. Therefore, by taking advantage of the availability of primary culture of aldosterone-producing cells isolated ex vivo from wild-type APA and from G151R and L168R mutated APA, we conducted a pilot study to test the effect on aldosterone synthase (CYP11B2) gene expression and aldosterone production of clarithromycin, one of the macrolides that effectively corrected the altered electrophysiology of G151R and L168R overexpressing HEK293T cells.[5]

# Methods

## Patients

From a large databank of cells from APA patients we selected for this study a total of 7 patients. These patients received an unambiguous diagnosis of APA by the "four corners criteria".[6] Approval for this study was obtained from the Ethics Committee of the University of Padova and all participants gave informed written consent to the study and to the use of their adrenocortical specimen; all procedures followed the Helsinki Declaration Principles.

## Detection of KCNJ5 mutations

DNA was extracted from APAs removed from the consecutive APA patients referred to our specialized hypertension center with a standard procedure as reported.10 PCR was performed on 250 ng DNA in a final volume of 50 µl containing 300 nM MgCl2, 400 nM of each primer, 200 µM deoxynucleotide triphosphate, and 2.6 U expand high-fidelity enzyme mix (Roche Applied Science, Milan, Italy). Purified PCR products underwent direct Sanger sequencing using the ABI Prism Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

## Isolation of aldosterone producing cells

Dispersed APA cells were obtained by sequential enzymatic digestion, mechanical disaggregation and incubation in 5 mL of Krebs Ringer solution supplemented with 2 mg/mL collagenase-I, 0.1 mg/mL deoxyribonuclease-I, 4% bovine serum albumin at 37°C for 40 min with gentle shaking. Single-cell suspension obtained was washed with PBS containing 0.1% BSA and then incubated with CD56 pre-coated magnetic beads by gentle shaking for 1 hour at 4°C. Beads were used at a ratio of 5 beads per cell.[5] After separating the bead-bound $CD56^-$positive ($CD56^+$) cells with a magnet, the $CD56^+$ cells were seeded at a density of $10^4$ cells/$cm^2$ into 24-well plates and treated with 5 umol/L, 20 umol/L or 50 umol/L of clarithromycin.

## Electron microscopy

Freshly immunoseparated $CD56^+$ APA cells were fixed in 3% phosphate-

buffered glutaraldehyde and processed following a standard protocol, as described.[12]

| Variable | Wild-Type APA | | G151R Mutated APA | | L168R Mutated APA | |
|---|---|---|---|---|---|---|
| | Baseline | Post-Adrenalectomy | Baseline | Post-Adrenalectomy | Baseline | Post-Adrenalectomy |
| Age, y | 52±4 | N.A. | 49±3 | N.A. | 49±7 | N.A. |
| Systolic BP, mm Hg | 139±4 | 124±5 | 168±32 | 128±4 | 154±20 | 130±0 |
| Diastolic BP, mm Hg | 91±15 | 73±6 | 96±8 | 80±7 | 90±14 | 77±4 |
| Serum $K^+$, mmol/L | 3.3±0.1 | 4.2±0.3 | 3.0±0.5 | 4.0±0.4 | 3.4±0.3 | 4.0±0.1 |
| U-$Na^+$ excretion, mEq/24 h | 150±164 | 113±60 | 100±14 | 127±84 | 174±26 | 100±24 |
| DRC, mUI/L | 4.0 (2.0–8.0) | 15.4 (2.0–34.0) | 2.5 (2.1–2.8) | 5.0 (3.0–7.0) | 2.4 (2.0–2.9) | 6.7 (2.8–11.0) |
| PAC, ng/dL | 69.7 (27.1–152.4) | 9.0 (2.6–12.3) | 48.9 (44.7–52.1) | 4.0 (3.0–5.1) | 35.8 (15.0–56.6) | 3.0 (1.4–4.5) |
| ARR, (ng/dL)/(mUI/L) | 60.3 (10.8–93.9) | 1.0 (0.4–1.3) | 143.6 (21.3–265.9) | 0.9 (1.0-0.8) | 84.7 (28.3–141.1) | 0.6 (0.4–0.7) |
| APA diameter, mm* | 12±11 | | 12±2 | | 19±16 | |
| Histopathology (%), ZF like/ ZG like/mixed | 33/0/66 | | 50/0/50 | | 50/0/50 | |

**Table. Demographic and Clinical Features of the APA Patients and Diagnostic Indexes**
Data presented as mean±SD or median (IQR), as appropriate. Normal values: $SK^+$:3.5–4.5 mmoL/L; on a $Na^+$ intake 200–300 mmoL/d: DRC: 2.5–32 mUL/L; PAC: <15.0 ng/dL; ARR <2.06 (ng/dL)/(mUI/L).[12] The predominant pattern at histopathology (% ZF like/ZG like/mixed) is also indicated. Statistical significance was not calculated because of the small number of cases. APA indicates aldosterone-producing adenoma; ARR, aldosterone–renin ratio; BP, blood pressure; DRC, direct renin concentration; N.A., not applicable; PAC, plasma aldosterone concentration; serum $K^+$ , serum potassium levels; U-$Na^+$ , urinary $Na^+$ ; ZF, zona fasciculata; and ZG, zona glomerulosa. *At pathology.

## CYP11B2 gene expression quantification

CYP11B2 mRNAs were measured in duplicate in $CD56^+$ APA cells after incubation for 18 hours in DMEM-F-12 medium with 5% FBS added with increasing concentrations of clarithromycin.
After treatment, the cells were lysed and mRNA was isolated using the high-pure RNA isolation kit (Roche Applied Science, Milan, Italy). Gene expression was quantified by real-time RT-PCR with universal probe library probes in the LightCycler 480 instrument (Roche Applied Science, Milan, Italy) using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method and the porphobilinogendeaminase (PBGD) as reference gene.

## Aldosterone measurement

Aldosterone levels were quantified by an aldosterone ELISA kit (Alpha Diagnostic International, San Antonio, Texas). A total of 50 µL of cell medium was added to aldosterone-coated wells following the manufacturer's instructions; the signal was detected in an ELISA Reader (Berthold

Technologies, Milan, Italy). Aldosterone levels were normalized to the amount of cell RNA content.

**Statistical analysis**

Data are expressed as mean and SD. One-way ANOVA followed by Bonferroni's post hoc test and repeated measure ANOVA were used to test the effect of clarithromycin and its increasing concentration amongst APA groups. The non-parametric Mann Whitney test was used to compare CD56+ and CD56- cells for quantitative variables. Significance was set at $p< 0.05$. The GraphPad for Mac (vers. 6.0 GraphPad Software, Inc., San Diego, CA) and SPSS (version 24 for Mac, IBM, USA) softwares were used for statistical analysis.

# Results

**Characteristics of the APA patients.**

We selected seven PA patients, who were unambiguously diagnosed with APA by the "four corners criteria";6 of them 3 were found to be wild-type, 2 had G151R and 2 had L168R mutations at sequencing. All showed the florid PA phenotype expected in APA patients, with no clear-cut differences among genotypes from the clinical standpoint (Table 1). By definition all were biochemically cured of PA and became normotensive without treatment after unilateral adrenalectomy.

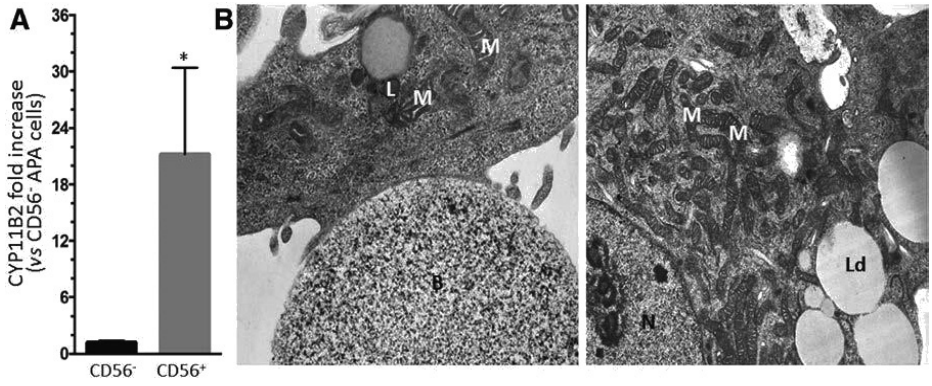

**Figure 1. Characterization of aldosterone-producing adenoma (APA) CD56$^+$ cells. A,** The bar graph shows that the relative expression of CYP11B2 gene expression was significantly higher in CD56$^+$ than in CD56$^-$ cells isolated from APA. Data represent mean±SD, n=7, *P=0.01 by Mann–Whitney test. **B**, Representative electron microscopy pictures of CD56$^+$ APA wild-type cells showing binding to CD56 precoated beads. CD56$^+$APA cells are characterized by the presence of mitochondria with tubule-vesicular cristae, abundant lipid droplets, some liposome, all of which are the typical features of steroidogenic aldosterone-producing cells (B, n=3). Magnification: ×20 000. B indicates bead; L, lysosomes; Ld, lipid droplets; M, mitochondria; and N, nucleus.

## Characterization of APA CD56$^+$ cells

Real-time PCR showed that expression of CYP11B2 was 21.23 ± 9.15-fold (p=0.01) higher in CD56$^+$ cells than in CD56$^-$ cells isolated from APA cells (Figure 1, panel A, n=7). Electron microscopy demonstrated that dispersed CD56$^+$ APA cells were selectively bound to CD56-precoated beads. These cells showed the classical features of steroidogenic aldosterone-producing APA cells,[13] e.g. abundant mitochondria with tubule-vesicular cristae, abundant lipid droplets, some liposomes, and smooth endoplasmic reticulum (SER) profiles (Figure 1, panel B).

## Effect of clarithromycin on CYP11B2 gene expression and aldosterone release

Compared to the CD56$^+$ cells isolated from the wild-type APA, the G151R and L168R mutated APA showed a significantly higher in vitro production of aldosterone (Figure 2, panels B and D). When CD56$^+$ cells isolated from G151R mutated (Figure 2 panel A, circle symbols) and L168R APAs (Figure 2 panel A, square symbols) were exposed to increasing concentrations [5 to 50 umol/L] of clarithromycin, we found a consistent concentration-dependent blunting effect of clarithromycin on both CYP11B2 gene expression (Figure 2, panel A) and aldosterone release (Figure 2, panel B), which did not occur in wild-type APA cells (Figure 2 panel A and B, triangle symbol). No differences in CYP11B2 gene expression changes and the aldosterone production lowering between the G151R and the L168R mutated APA cells were seen at post hoc Bonferroni's test. Therefore, the results obtained in the mutated APA cells were examined jointly and compared to the wild-type APA cells (Figure 2, panels C and D). This showed a highly significant (p<0.001) effect of clarithromycin concentration and group at repeated measures ANOVA.

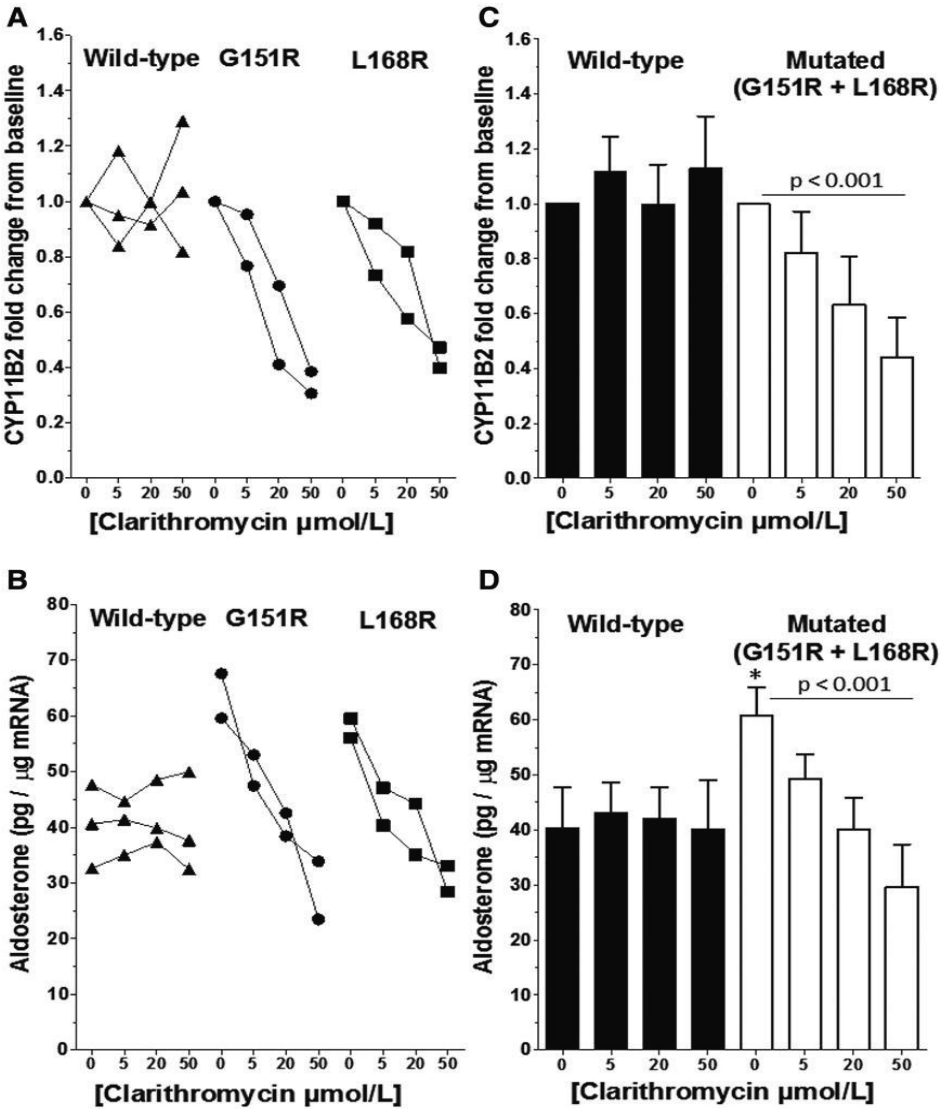


**Figure 2. Effects of clarithromycin on aldosterine in wild-type and mutated KCNJ5 aldosterone-producing adenoma (APA). A** and **B**, Average values of CYP11B2 gene expression (**A**) and aldosterone production (**B**) obtained from the $CD56^+$ APA cells. Clarithromycin concentration dependently blunts CYP11B2 gene expression and aldosterone release in $CD56^+$ cells isolated from G151R mutated (circle symbols, n=2) and L168R APAs (square symbols, n=2) but not in those wild-type APAs (triangle symbols, n=3). Each experiment was performed in duplicate: please note the consistency of the findings. Aldosterone production was significantly higher at baseline in the KCNJ5 mutated than in wild-type APA (*P<0.01). Given the similar response to clarithromycin of G151R and L168R APA cells, the downstream analysiswas performed by cumulating these 4 APA together (**C** and **D**). At repeated measures ANOVA, there was a highly significant (P<0.001) lowering of both CYP11B2 mRNA and aldosterone production in the mutated group and a highly significant (P<0.001) difference of the change when compared with wild- type APA cells. 0 denotes exposure to vehicle. Data represent mean±SD.

## Discussion

In this proof-of-principle study we selected seven patients, who were unambiguously diagnosed with APA by the "four corners criteria", as confirmed by biochemical cure of PA and normotension without treatment after unilateral adrenalectomy [6]. They showed the florid PA phenotype expected in APA patients, but no clear-cut differences among genotypes from the clinical standpoint. Of them, 3 were found to be wild-type, 2 had G151R and 2 had L168R mutations at sequencing. The APA tissue obtained at adrenalectomy was used to obtain dispersed APA cells from which a pure population of aldosterone-secreting cells could be immunoseparated using CD56 pre-coated magnetic beads, as described in detail.[11]

When exposed to increasing concentrations of clarithromycin the $CD56^+$ cells obtained from wild-type APAs showed no change of CYP11B2 gene expression and aldosterone secretion in response to the macrolide (Figure 2). By contrast, both G151R or L168R APA cells exposed to clarithromycin showed a concentration-dependent consistent blunted expression of the aldosterone synthase gene (CYP11B2), the step-limiting enzyme, which in a 3-step process converts deoxycorticosterone to aldosterone in the mitochondria. Moreover, both G151R and L168R mutated $CD56^+$ APA cells, which exhibited a higher *in vitro* production of aldosterone than wild-type APA cells at baseline in keeping with the literature,[4,14] showed a concentration-dependent consistent brisk fall also of aldosterone release (Figure 2, panels B and D). Therefore, these experiments extend the finding obtained *in vitro* in the HAC15 cell line engineered to over express the mutated G151R and L168R Kir3.4 channel,[5] to APA cells obtained *ex vivo*, which had these two common somatic mutations.

## Perspectives

These results, albeit obtained in a small dataset, provide compelling evidence of the feasibility of blunting aldosterone synthesis specifically in aldosterone-producing cells from tumors carrying two most common KCNJ5 mutations.

Whether these effects occur in all KCNJ5 mutated APA cells and whether they can happen in vivo in patients with KCNJ5 mutated APA, and whether the lowering of PAC and blood pressure after acute administration of a macrolide can be a proxy for pinpointing the PA patients harboring a mutated APA is the currently under investigation at our center. If proven, this principle will open the way to personalized diagnosis and treatment of the PA patients, who show the most florid forms of the disease, e.g. those with APA carrying KCNJ5 mutations.

**What Is New?**

This study extends to aldosterone-producing cells obtained ex vivo from tumors carrying two most common KCNJ5 mutations and to clarithromycin the observation that roxithromycin, another macrolide, blunts aldosterone synthesis specifically in genetically engineered HAC15 cells.

**What Is Relevant?**

Macrolides and their derivatives devoid of antibiotic activity may be a novel diagnostic and therapeutic tool for blunting aldosterone synthesis specifically in aldosterone-producing cells from tumors carrying two most common KCNJ5 mutations.

## Summary

In a pure population of aldosterone-producing cells isolated ex vivo from KCNJ5-mutated APAs, clarithromycin specifically blunted aldosterone secretion, while it was ineffective in CD56+ cells from wild-type APAs. This discovery opens new horizons for the diagnosis and treatment of APA with KCNJ5 mutations, which entail the most common and severe cases of PA.

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# Chapter 3

# Aldosterone stimulates its biosynthesis via a novel GPER mediated mechanism

Brasilina Caroccia, Teresa Maria Seccia, Maria Piazza, Selene Prisco, Sofia Zanin, Maurizio Iacobone, Livia Lenzini, Giorgia Pallafacchina, Oliver Domening, Marko Poglitsch, Rosario Rizzuto and Gian Paolo Rossi

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# Abstract

The G protein-coupled estrogen receptor (GPER) mediates an aldosterone secretagogue effect of 17β-estradiol in human HAC15 adrenocortical cells after estrogen receptor β blockade. As GPER mediates mineralocorticoid receptor-independent aldosterone effects in other cell types, we hypothesized that aldosterone could modulate its own synthesis via GPER activation.

HAC15 cells were exposed to aldosterone in presence or absence of canrenone, a mineralocorticoid receptor antagonist, and/or of the selective GPER antagonist G36. Aldosterone synthase (CYP11B2) mRNA and protein levels changes were the study endpoints. Similar experiments were repeated in strips obtained ex vivo from aldosterone-producing adenoma and in GPER-silenced HAC15 cells.

Aldosterone markedly increased CYP11B2 mRNA and protein expression (vs untreated samples, $p<0.001$) in both models by acting via GPER, as these effects were abolished by G36 ($p<0.01$) and not by canrenone. GPER-silencing ($p<0.01$) abolished the aldosterone-induced increase of CYP11B2, thus proving that aldosterone acts via GPER to augment the step-limiting mitochondrial enzyme (CYP11B2) of its synthesis. Angiotensin II potentiated the GPER-mediated effect of aldosterone on CYP11B2. Coimmunoprecipitation studies provided evidence for GPER-AT-1R heterodimerization. We propose that this autocrine-paracrine mechanism could enhance aldosterone biosynthesis under conditions of immediate physiological need where the renin-angiotensin-aldosterone system is stimulated as, for example, hypovolemia. Moreover, as aldosterone-producing adenoma overexpresses GPER this mechanism could contribute to the aldosterone excess that occurs in primary aldosteronism in a seemingly autonomous fashion from angiotensin II.

# Introduction

The mechanisms regulating aldosterone biosynthesis remain incompletely known notwithstanding several decades of investigation (for review[1]). For example, what causes the inappropriate over-secretion of aldosterone in primary aldosteronism (PA), the most common cause of endocrine hypertension where the renin-angiotensin system is shut off,[2] remained unknown until agonistic autoantibodies against the angiotensin type 1 receptor[3,4,] elevated serum levels of PTH[5] that stimulates aldosterone secretion[6], down-regulation of TASK-2 $K^+$ channel[7], and somatic and germline loss- and gain-of-function mutations in both cations and anions channels[8-13] were discovered (for review[1,14,15]).
Multiple observations have also suggested a role for estrogens in the regulation of aldosterone under physiological and pathophysiological conditions[16-19]. Accordingly, GPER, a G-protein coupled receptor initially described as an estrogen-specific receptor[20-22], but subsequently discovered to promiscuously bind other steroids[23], and to mediate mineralocorticoid receptor (MR)-independent aldosterone effects in different cell types[24-28], was found to be overexpressed in aldosterone-producing adenoma (APA), a main subtype of human PA[29]. In human adrenocortical cells we previously observed that GPER mediates a potent secretagogue effect of 17β-estradiol on aldosterone when the estrogen β receptor is pharmacologically blocked or molecularly silenced [29]. Based on evidences suggesting the possibility of promiscuous activation of GPER by steroids other than 17 β-estradiol, we hypothesized that aldosterone could activate GPER and act as its own secretagogue. If proven, this mechanism could be relevant for rapidly and potently enhancing the biosynthesis of aldosterone when the renin-angiotensin-aldosterone system is stimulated. It could be also important for understanding the autonomous aldosterone production in human PA[2,30]. Hence, aldosterone, by acting in an autocrine-paracrine fashion in GPER-overexpressing APA[29,31], which generate high local concentrations of the hormone, could self-perpetuate the hyperaldosteronism in spite of a blunted renin-angiotensin system. This study was, therefore, set up to test in vitro this hypothesis using two different models that are characterized by sizable differences of aldosterone production, i.e. a human adrenocortical carcinoma cell line that produces relatively small amounts of aldosterone, and strips obtained ex vivo from APA that produce substantial amounts of aldosterone.

# Materials and Methods

## Adrenocortical cell line HAC15

Adrenocortical cell line HAC15, a gift of William E. Rainey (University of Michigan at Ann Arbor, USA), was grown in Dulbecco's Modified Eagle Medium (DMEM F12) supplemented with 10% Cosmic Calf serum (CCS), 1% glutamine, and 1% antibiotic/antimycotic mixture. For the experiments, the cells were seeded in 12-well plates at $2*10^5$ cells per well and grown to sub-confluence (80%). Prior to experiment, they were synchronized by incubation for 24 hours with DMEM/F12 medium supplemented with 0.5% dextran-coated charcoal-stripped CCS, 1% glutamine, and 1% antibiotic/antimycotic. Cells were treated with increasing concentrations of aldosterone (from $10^{-11}$ to $10^{-7}$ M; Sigma-Aldrich, Milan, Italy) for 12 hours. The treatment with $10^{-7}$ M aldosterone was then performed at different time points (2, 8, 12, 24, 48 hours). To establish if aldosterone effect was mediated by mineralocorticoid receptor, glucocorticoid receptor or GPER receptor, the selective MR antagonist, canrenone ($10^{-5}$M; Sigma-Aldrich, Milan, Italy), or the selective GR antagonist RU486 ($10^{-6}$ M or $10^{-5}$ M; generous gift of Prof. Massimiliano Caprio), or the selective GPER antagonist G36[32] ($10^{-5}$ M; Tocris Bioscience, Bristol, UK), were added to fresh media 1 hour before aldosterone treatment.
To establish if AT1R and GPER activation interacted in altering CYP11B2 expression and if these two receptors formed heterodimers, HAC15 cells were treated for 12 hours with aldosterone $10^{-7}$ M on top of angiotensin II (Ang II) $10^{-7}$ M, in the absence or presence of the selective AT1R antagonist irbesartan, G36, and of both antagonists.

## Tissues

APA tissues were obtained from consenting patients with a conclusive diagnosis of APA, as established by the five corner criteria, which include i) biochemical evidence of PA; ii) lateralized aldosterone secretion by adrenal vein sampling; iii) detection of an adenoma by imaging (TC or MR) and at pathology; iv) biochemical correction of PA after adrenalectomy; v) detection of a CYP11B2-positive adenoma in the resected adrenal cortex at immunohistochemistry with a monoclonal antibody for human CYP11B2 [1,2].
Briefly, tissues were obtained under sterile conditions in the operating room immediately after excision. For the functional studies, APA tissues were cut into 2 to 3-mm strips, which were transferred into 48-well plates containing 500 uL of serum-free DMEM/F12 culture medium. After 6 hours of starvation, the strips were treated with $10^{-7}$ and $10^{-8}$ M aldosterone alone or in presence of $10^{-5}$ M G36. All procedures were approved by the local Ethics Committee and informed written consent was obtained from each individual patient.

## RNA extraction and quantitative real-time PCR

RNA from cells was extracted after treatments with the Roche RNeasy kit (Roche, Monza, Italy) and RNA from tissues with Qiagen RNA extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The quantity and quality were also determined by spectrophotometric readings at 260/280/230 nm. One µg total RNA from cells and from tissues was reverse transcribed in a final volume of 20 µL using the iScriptTM cDNA Synthesis kit (Bio-Rad, Milan, Italy) following the manufacturer's recommendations. The RT-PCR reactions were performed in Delphi 1000TM Thermal Cycler (Oracle BiosystemsTM Watertown, MA, USA). We measured the levels of aldosterone synthase (CYP11B2) mRNA with a real time RT-PCR by the comparative Ct ($2^{-\Delta\Delta Ct}$) method: each sample was quantified vs its housekeeping gene transcript, porphobilinogen deaminase (PBGD), and normalized to the control group. Each experiment was repeated at least 5 times in duplicate, and the results are presented as fold increase ± SD. Primers used in real time RT-PCR are reported in Supplemental Table 1[33].

## Immunoblotting

Immunoblotting for CYP11B2, GPER and AT1R was performed following a standard protocol. In brief, after treatment, cells were homogenized in lysis buffer (Thermo Scientific, Milan, Italy) and protein concentration was determined in the soluble supernatant with BCA (Thermo Scientific, Milan, Italy). Lysate fraction (50 µg) was separated in a polyacrylamide gel and then electro-blotted onto nitrocellulose or polyvinyldene fluoride (PVDF) membrane (Amersham-Hybond EC, GE Healthcare Life Sciences, Milan, Italy). The membranes were blocked for 30 minutes at room temperature in 5% non-fat dry blocking milk and thereafter incubated overnight at 4°C with a primary mouse monoclonal antibody CYP11B234 (diluted 1/500) or GPER or AT1R. After washing, membranes were incubated 1 hour with an anti-mouse secondary antibody. After that, the band intensity was measured in a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Images were analyzed by Image Processing and Analysis in Java (Image J-NIH). Bands for CYP11B2 were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

## Small interfering RNA (siRNA)

The expression of GPER was silenced in HAC15 cells by RNA interference using the Nucleofector technology (Amaxa Biosystems, Thermo Scientific, Milan, Italy) following the manufacturer's protocol. To silence GPER, HAC15 were transfected with 50 nM ON-TARGET plus GPER siRNA (GPER siRNA; Dharmacon, Carlo Erba Reagents, Milan, Italy). An ON-TARGET plus Non-targeting Pool (Dharmacon, Carlo Erba Reagents, Milan, Italy) transfection in

mock-transfected cells was used as control. Cells were seeded in 6-well culture plates at the density of $1 \times 10^6$ cells per well. After 24 hours of transfection, mock-transfected and silenced cells were treated with aldosterone. Protein extraction was performed after 24 hours of treatment and followed by immunoblotting analysis for GPER and CYP11B2.

### Cytosolic $Ca^{2+}$ measurements in $CD56^+$ cells

A pure population of aldosterone-secreting cells from APA was obtained by sequential enzymatic digestion and immune-separation of $CD56^+$ cells on magnetic beads pre-coated with an antibody specific for neural cell adhesion molecule tissues from 3 patients, as previously reported[35]. At electron microscopy we showed that these $CD56^+$ cells had a high content in mitochondria and lipid droplets, thus entailing the ultrastructural features of aldosterone-producing cell. Moreover, they also showed aldosterone production in vitro[35,36]. $CD56^+$ cells were plated on L-polylysine pre-coated coverslips two day before the experiments. Cells were loaded with 2 μM Fura-2-AM (Life Technologies, Milan, Italy) diluted in Krebs-Ringer modified buffer containing 0.02% pluronic acid and 250 μM sulfinpyrazone for 20 min at 37°C and then washed two times for 10 min with Krebs-Ringer modified buffer. Images were acquired every 2 s with a Nikon Ti-E microscope equipped with a Nikon 20x air objective (Nikon, Tokyo, Japan) and the software used for the acquisition was NIS Element AR (Nikon). Exposure time was set to 100 ms. Excitation was performed with a Cairn OptoScan equipped with a 150W Xenon arc lamp. Changes in Fura-2 fluorescence (340/380 nm ratio) were expressed as R/R0, where R is the ratio at time t and R0 is the ratio at the beginning of the experiment. $CD56^+$ cells from APA tissues were treated with aldosterone, Ang II and 17 β-estradiol (E2), the two latter used as control.

### Aldosterone measurement in human adrenocortical tissues

We measured aldosterone levels in APA-adjacent tissue and in APA to determine how the concentration of aldosterone that increased CYP11B2 expression in our in vitro experiments compared with those occurring in vivo in these tissues. To this end, adrenal tissue samples (10-20 mg wet weight per sample) were grinded under liquid nitrogen. The obtained frozen tissue powder was then rapidly dissolved in ice-cold guanidine hydrochloride (6 mol/L) supplemented with 1 % (v/v) TFA at a 100 mg tissue/mL concentration. Resulting homogenates were spiked with 500 pg of deuterated aldosterone (D4) for internal standardization. Following C18-based solid phase extraction, samples were analyzed by UPLC-MS/MS using a reversed phase analytical column operating in line with a Xevo TQ-S mass spectrometer (Waters, Milford, MA). The tissue concentration of aldosterone was calculated considering the corresponding response factors determined in calibration curves

in tissue extract matrix, under condition in which the integrated signals exceeded a signal to-noise ratio of 10.

### Co-immunoprecipitation (Co-IP) studies

Interaction between AT1R and GPER was investigated in HAC15 cells using Pierce Co-Immunoprecipitation Kit (Thermofisher, Milan, Italy) and following the manufacturer's protocol, as described in detail in the Supplemental Methods[33]. HAC15 cells harvested from four 15 cm culture dishes were centrifuged and pelleted. Cell pellet was dissolved into the solution for protein isolation. For co-immunoprecipitation studies, protein complexes were immunoprecipitated with anti-AT1R (Cusabio, Paris, France) or anti-GPER antibody (Novus biologicals, Milan, Italy). The presence of GPER in AT1R immunoprecipitated protein was investigated with immunoblot;the presence of AT1R in GPER immunoprecipitated protein was also verified with immunoblot.

### Statistical analysis

Statistical analysis was performed with GraphPad Software™ (vers. 8.02 for Mac OS X, La Jolla, CA). Non-parametric Mann Whitney test or one-way ANOVA test and repeated-measures ANOVA were used, as appropriate.

## Results

### Aldosterone treatment enhances CYP11B2

In concentration-response and time-course experiments for up to 48 hours in HAC15 cells, we found that aldosterone enhanced CYP11B2 gene expression at $10^{-8}$ and $10^{-7}$ M and that this effect was clearly evident after 12 hours (Figure 1 panel A and B). The latter concentration and time point were, therefore, used to analyze the changes of CYP11B2 mRNA and protein in the further experiments in HAC15 cells.

To investigate the role of the MR in mediating CYP11B2 gene expression changes we preincubated HAC15 cells with either the MR antagonist canrenone, or the GPER antagonist G36. At concentration between $10^{-6}$ - $10^{-4}$ M canrenone alone slightly, but significantly, increased CYP11B2 mRNA[33], but blunted CYP11B2 expression at $10^{-3}$ M [33].

Cell viability studies carried out in parallel in HAC15 cells showed a cytotoxic effect of canrenone at the latter concentration[33]. Importantly, at $10^{-5}$ M, i.e. a concentration 100-fold higher than that of aldosterone, but not cytotoxic, canrenone did not blunt aldosterone-induced CYP11B2 mRNA increase. By contrast, 1-hour pretreatment with $10^{-5}$ M G-36 abolished the CYP11B2 gene expression enhanced by aldosterone (Figure 1, panel D).

To rule out the possibility that at $10^{-8}$ M - $10^{-7}$ M aldosterone could activate the glucocorticoid receptor (GR)[37], we treated HAC15 cells with the GR antagonist RU486 at two different concentrations ($10^{-6}$ M - $10^{-5}$ M), alone or in the presence of aldosterone. RU486 neither affected by itself CYP11B2 gene expression, nor did it alter aldosterone-induced CYP11B2 gene expression[33].
The stimulatory effect of aldosterone on CYP11B2 was confirmed at protein levels: HAC15 cell exposure to aldosterone for 24 hours increased CYP11B2 protein expression, while G-36 abolished this increase, and canrenone left it unaffected (Figure 1, panel E).
Practically identical results were found in strips obtained ex vivo from two female and two male APA patients upon exposure to $10^{-7}$ and $10^{-8}$ M aldosterone concentrations: the hormone augmented CYP11B2 gene expression; this effect was prevented by G-36 but not by canrenone, that per se elicited no effects (Figure 1, panel C).
Importantly, in parallel experiments the changes in CYP11B2 gene expression were found to be consistently associated with aldosterone release in the medium[33].

## GPER silencing blunted aldosterone-induced CYP11B2 protein expression

We used siRNA technology to confirm the finding that GPER modulates aldosterone synthase expression after aldosterone treatment. After 48 hours transfection GPER-silenced HAC15 cells showed a 50% significant reduction of GPER protein expression, as compared to control HAC15 cells mock-transfected with non-targeting siRNA (Figure 2, panel A). In the GPER silenced cells $10^{-7}$ M aldosterone induced no increase of CYP11B2 protein expression after 24-hour, thus confirming that the aldosterone-activated CYP11B2 expression was GPER-mediated (Figure 2, panel B).

## Cytosolic $Ca^{2+}$ measurements

We measured cytosolic $Ca^{2+}$ levels after exposure to $10^{-7}$ M aldosterone to gain insight into the pathways downstream GPER activation, using the ratiometric Fura-2 $Ca^{2+}$ indicator and 17 β-estradiol and Ang II, as controls, in $CD56^{+}$ cells freshly isolated from APA. We found that, while exposure to $10^{-8}$ M Ang II caused a significant increase of cytosolic $Ca^{2+}$ that was followed by prominent $Ca^{2+}$ oscillations (Figure 2, panel C), cytosolic $Ca^{2+}$ was affected neither by E2 nor by aldosterone, indicating that the GPER-mediated stimulatory effect of these steroids on CYP11B2 gene expression does not involve detectable increases of cytosolic $Ca^{2+}$ (Figure 2, panel C).

## Aldosterone concentration in human adrenocortical tissues ex vivo

To investigate how the concentrations of aldosterone that elicited a clear-cut effect on CYP11B2 mRNA levels and aldosterone synthase protein expression compared with those present in the APA tissue, we measured tissue concentrations of aldosterone by using a state-of-the-art UPLC-MS/MS spectrometry after C18-based solid phase extraction. We found $3.9*10^4$ fmol/g ($1.8*10^4$- $9.1*10^4$ fmol/g, median, 95% CI) and $1.8*10^6$ fmol/g ($7.0*10^5$-$3.8*10^6$ fmol/g, median, 95% CI) of aldosterone in the APA-adjacent tissue and in APA obtained ex vivo, respectively (Figure 2, panel D). By assuming 1 g of adrenal tissue to correspond approximatively 1 ml of tissue, we estimated that $1.8*10^6$ fmol/g is equivalent to $1.8*10^6$ M, i.e. about 18-fold higher than the highest concentration ($10^{-7}$ M) of aldosterone used in our in vitro and ex vivo experiments in HAC15 cells and APA strips.

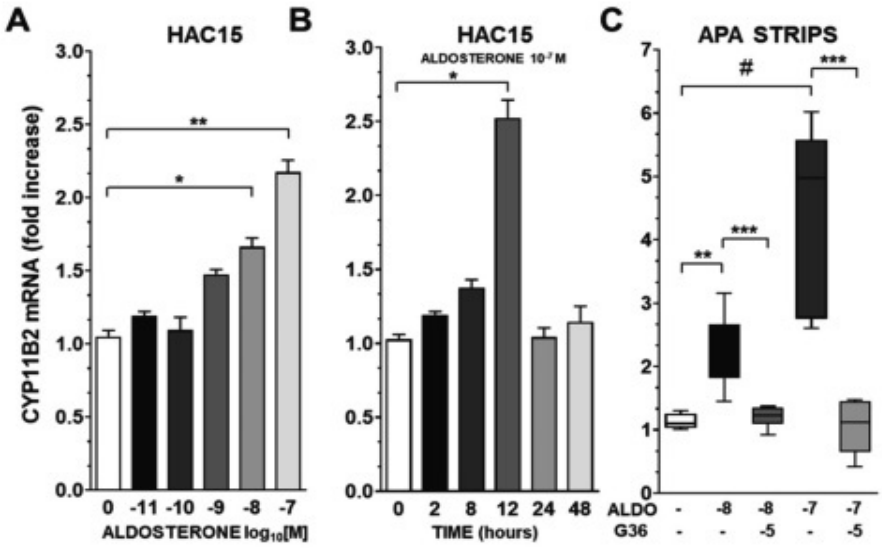


**Figure 1: Aldosterone treatment enhances CYP11B2 via GPER activation. Panel A:** CYP11B2 gene expression after exposure of HAC15 cells to increasing concentration of aldosterone. Data represent mean ± SD (n= 5). **Panel B**: CYP11B2 expression levels in HAC15 cell exposed for 2, 8, 12, 24 and 48 hours to aldosterone 10-7 M. Data represent mean ± SD (n= 5). **Panel C:** Aldosterone-induced CYP11B2 mRNA expression in APA strips obtained from 4 different patients. APA strips were treated with aldosterone $10^{-8}$ M and $10^{-7}$ M alone or on top of G36 $10^{-6}$ M or $10^{-5}$ M, respectively. Data represent median and 95% CI of 4 different experiments performed in triplicate.

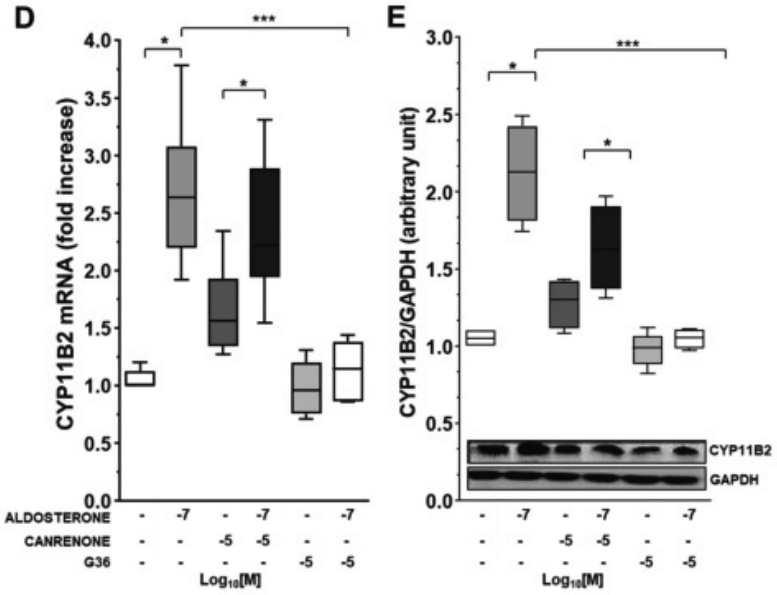


**Figure 1: Aldosterone treatment enhances CYP11B2 via GPER activation. Panel D**: CYP11B2 gene expression after treatment of HAC15 cells with aldosterone $10^{-7}$ M on top of canrenone $10^{-5}$ M or G36 $10^{-5}$ M. Data represent median and 95% CI of 7 different experiments performed in duplicate. **Panel E:** CYP11B2 protein expression after treatment with aldosterone 10-7 M alone or on top of canrenone $10^{-5}$ M or G36 $10^{-5}$ M for 24 hours. Data represent median and 95% CI of 5 different experiments performed in duplicate. ALDO: Aldosterone; * P < 0.01; ** P < 0.001,*** P < 0.0001; # P = 0.002

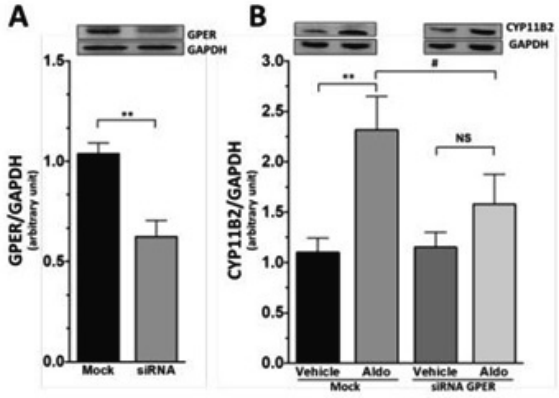


**Figure 2: GPER silencing blunted aldosterone-induced CYP11B2 protein expression. Panel A:** GPER-1 protein levels in HAC15 cells after transfection with GPER siRNA compared with mock-transfected cells. Data represent mean ± SD of 4 different experiments performed in duplicate. **Panel B:** CYP11B2 protein expression after exposure of mock transfected HAC15 cells and of silenced GPER-1 cells to aldosterone $10^{-7}$ M. Data represent mean ± SD (n= 4).

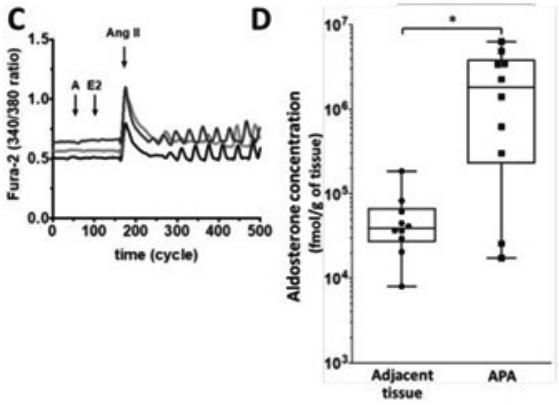


**Figure 2: GPER silencing blunted aldosterone-induced CYP11B2 protein expression. Panel C:** Representative traces of cytosolic Ca2+ measurement in $CD56^+$ cells obtained from APA performed with the $Ca^{2+}$ -sensitive fluorescent dye Fura-2 after treatment with $10^{-7}$ M aldosterone (A), $10^{-8}$ M 17 β-estradiol (E2) and $10^{-8}$ M angiotensin II (Ang II). Experiments were performed in $CD56^+$ cells obtained from 3 different APAs. **Panel D:** Tissue concentration of aldosterone in APA and in APA-adjacent tissue. Data represent median and 95% CI, n = 10. * P = 0.01; ** P < 0.01; # P < 0.001

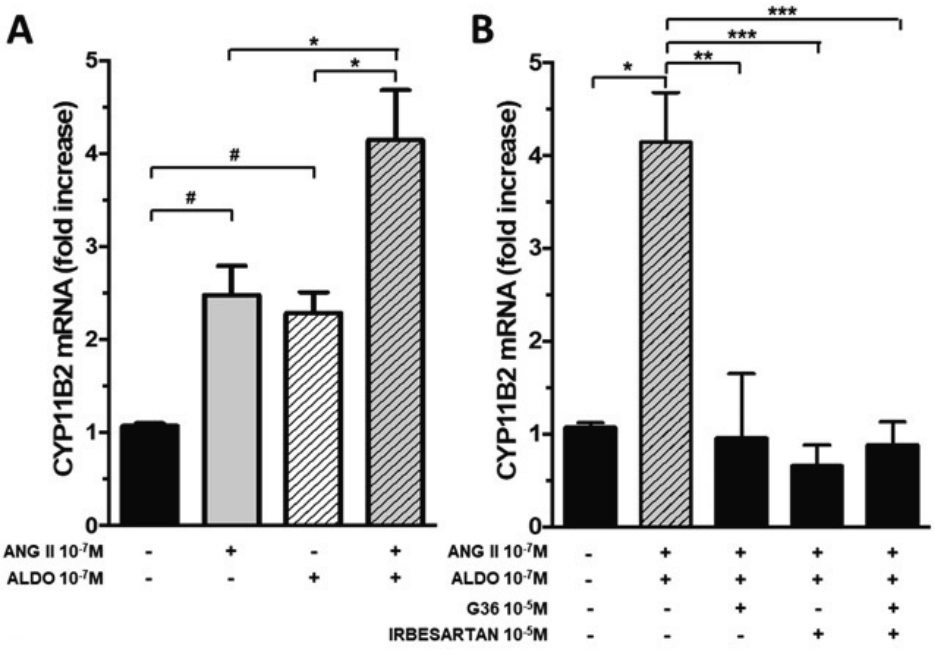


**Figure 3: Cross talk between G protein-coupled receptors AT1 and GPER-1. Panel A:** Aldosterone on top of Ang II increased the expression of CYP11B2 gene in APA strips. **Panel B:** CYP11B2 gene expression in APA strips after pretreatment with aldosterone and Ang II on top of irbesartan or G36, or both.

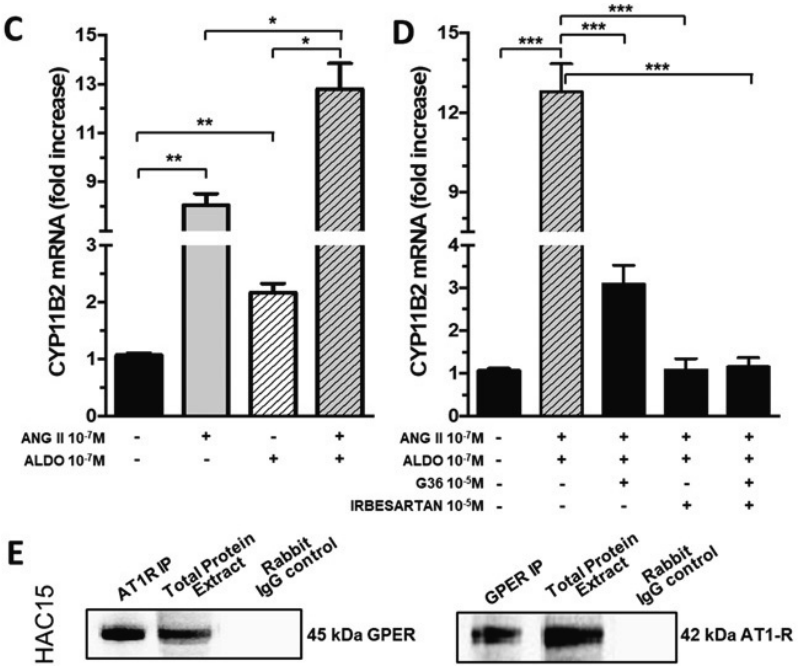


**Figure 3: Cross talk between G protein-coupled receptors AT1 and GPER-1. Panel C:** Aldosterone on top of Ang II increased the expression of CYP11B2 gene in HAC15 cells. **Panel D**: CYP11B2 gene expression in HAC15 cells after pretreatment with aldosterone and Ang II on top of irbesartan or G36, or both. For **panels A-D** data represent mean ± SD of 4 different experiments performed in duplicate. **Panel E:** Representative results of co-immunoprecipitation experiments performed in HAC15 cells; after AT1R immunoprecipitation, GPER protein expression was detected by immunoblot; the presence of heterodimers in GPER-immunoprecipitated protein was also confirmed (n=3). Rabbit IgG control: negative control. ALDO: Aldosterone; # P = 0.04; * P = 0.03; **P < 0.01; ***P < 0.001

## Cross-talk between G protein-coupled receptors AT1 and GPER-1

As functional interactions between GPCRs have been reported, we investigated if a cross-talk between angiotensin type-1 receptor (AT1R) and GPER could help understanding of the mechanism(s) by which aldosterone-induced GPER activation promoted CYP11B2 gene expression. We quantified GPER and AT1R gene and protein expression in APA and APA adjacent tissues by digital droplet PCR, a technique held to furnish absolute copies number of gene transcripts. We found that GPER and AT1R showed comparable mRNA levels; however, immunoblotting studies showed that only GPER was more abundant in APA than in APA adjacent tissue at the protein level [33]. Both aldosterone and Ang II increased CYP11B2 gene expression in APA strips (Figure 3, panel A); when given on top of Ang II, aldosterone potentiated the secretagogue effect of

the peptide. Moreover, the additive effect of aldosterone and Ang II was inhibited by either irbesartan or G36 (Figure 3, panel B). Similarly, in HAC15 cells aldosterone potentiated the effect of Ang II, whilst pre-treatment with irbesartan and/or G36 blunted this augmentation (Figure 3, panels C, D).
To investigate if the interaction between GPER and AT1R activation could be sustained by heterodimer formation, we performed co-immunoprecipitation experiments in HAC15 cells. GPER protein expression was detected by immunoblot after AT1R immunoprecipitation and vice versa, thus confirming the presence of heterodimers (Figure 3, panel E).

# Discussion

In this study we found that aldosterone stimulated the expression of CYP11B2, the mitochondrial $Ca^{2+}$ and NADH-dependent step-limiting enzyme of its biosynthesis. This effect occurred both in a female-derived adrenocortical carcinoma cell line (HAC15), and in strips obtained ex vivo at adrenalectomy from a mixed-gender cohort of primary aldosteronism patients with aldosterone-producing adenoma (Figure 1). These are novel important pieces of information on the regulation of aldosterone secretion under physiological an pathophysiological conditions, as several parallel studies[33] showed that increased levels of CYP11B2 mRNA and protein are proxies of enhanced aldosterone release[29]. They could also improve understanding of what happens in primary aldosteronism, the most common, albeit often overlooked, cause of endocrine hypertension[2,38].
By using pharmacological tools to specifically block the MR or the GPER, and also by means of molecular silencing, we could identify GPER as the main mediator of CYP11B2 activation in response to aldosterone (Figures 1 and 2). The selective MR antagonist canrenone chosen because it is an active metabolite of spironolactone, which, at variance with spironolactone, does not require cytochrome P450 activation[39]. In accordance with the notion that MR antagonists are not very potent drugs and require a high molar antagonist/agonist ratio to display their antagonistic effects[40], we used canrenone at concentrations 100-fold higher than those of aldosterone and we found that canrenone did not blunt the effect of aldosterone.
The effect of canrenone on CYP11B2 expression was further investigated in a concentration-dependent experiment that showed a dual action: at $10^{-6}$ and up to $10^{-4}$ M canrenone stimulated CYP11B2 gene expression, while at $10^{-3}$ M it blunted it[33]. We would like to contend that the stimulatory effect seen at lower concentrations might involve an off-target antagonist effect on the ER β[33],as previous studies showed that ER β-blockade unmasks the GPER-mediated

stimulatory effect on CYP11B2 expression of steroids as 17 β-estradiol[29]. Moreover, the fall of CYP11B2 seen at the highest concentrations of the MR antagonist likely occurred because of a cytotoxic effect as shown by our MTT experiments.

Of further note, the effect of aldosterone on CYP11B2 did not involve the glucocorticoid receptor (GR), as it was be unaffected by the GR antagonist RU486[33,37]. As the adrenocortical carcinoma cells HAC15 might not reflect what occurs in vivo in human PA, which is usually caused by an aldosterone-producing adenoma, we wished to corroborate the findings by using strips of these tumours obtained ex vivo at adrenalectomy from a mixed gender cohort of patients presenting with florid clinical PA phenotypes. These results provided additional proof that aldosterone increased CYP11B2 gene expression (Figure 1, panel C) via GPER, and not the MR or GR, as its effect was abolished by G36 and unaffected by canrenone and RU486[33].

As regards signaling pathways, we herein found no detectable changes of cytosolic $Ca^{2+}$ levels in response to aldosterone (Figure 2, panel C), consistently with the previous finding that 17 β-estradiol-induced GPER-mediated expression of CYP11B2 and aldosterone secretion does not involve cytosolic $Ca^{2+}$ mobilization, but the PKA-cAMP signaling pathway[29].

Some limitations are, however, to be acknowledged: first, experiments with mitochondria-specific $Ca^{2+}$ sensitive probes are necessary to investigate the possibility that GPER activation of CYP11B2 can involve $Ca^{2+}$ influx into the mitochondria. Second, although strips from APA represent the experimental model that is closest to the in vivo conditions, caution is advised before extrapolating our findings to what occurs clinically in PA patients. Finally, it might be argued that we have merely identified a pharmacologic effect of aldosterone, because we used high concentrations of the hormone, possibly higher than those in plasma or in adrenocortical tissue of patients with PA. However, as aldosterone is locally produced in the zona glomerulosa and in APA cells, and then released in the interstitial fluid, it might attain local concentrations greater than in the bloodstream. Given the lack of information on aldosterone concentrations in APA tissue, we measured aldosterone concentrations in APA by a state-of-the art tandem mass spectrometry technique. We found that the local tissue concentrations of aldosterone in aldosterone-producing adenoma tissue were about 18-fold higher than those enhancing CYP11B2 expression in vitro (Figure 2, panel D). As we used exogenous aldosterone as a secretagogue, we could not measure aldosterone release in the medium of our cell and strip preparations, because of the

impossibility of discriminating between endogenously made and exogenously added aldosterone, without using radiolabeled precursors, which are no longer allowed in our laboratory. However, in multiple parallel experiments with angiotensin II and 17 β-estradiol, as secretagogue, we found that changes in CYP11B2 mRNA were consistently followed by aldosterone secretion[33], finding consistent with the view that aldosterone is not stored but constitutively secreted.

One additional point deserves a comment: aldosterone-mediated GPER activation did not require prior ERβ receptor blockade at variance with results with 17 β-estradiol[29]. As data on relative affinity of aldosterone and 17β-estradiol for human adrenocortical cells lack, at present we could only speculate that this might be due to a higher affinity of aldosterone for GPER and/or to lower binding affinity of 17 β-estradiol for GPER than for ER β41.

A further novel and intriguing observation in this study was the finding that aldosterone potentiated in an additive fashion the secretagogue effect of angiotensin II in adrenocortical cells, which suggested a functional interaction between the angiotensin II type 1 receptor (AT1R) and GPER. This could be due to functional heterodimer formation between GPER and AT1R as shown in HAC15 cells (Figure 3), which were found to be stable at coimmunoprecipitation studies with proper controls in spite of no prior cross-linking (Figure 3, panel E).

Based on these in vitro and ex vivo studies, we would like to propose an autocrine-paracrine mechanism whereby aldosterone, acting via GPER, can swiftly increase its own biosynthesis and release, under conditions when the renin-angiotensin-aldosterone system needs to be rapidly activated to preserve vital organ perfusion as, for example, during acute hypovolemia and dehydration. This mechanism can play a pathophysiological role also in maintaining an inappropriately high secretion of aldosterone in PA patients, where the high blood pressure and volume expansion would also be expected to blunt renin and angiotensin II synthesis and to diminish aldosterone biosynthesis. Finally, we suggest that this autocrine-paracrine mechanism could explain the occurrence of aldosterone-producing cell clusters (APCCs), which can coexist with APA, and possibly represent an early stage of the latter tumor[42].

Whether this mechanism could be implicated in idiopathic hyperaldosteronism that might be sustained by APCCs[43], and whether this mechanism can be further amplified by angiotensin II remains to be investigated.

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# Chapter 4

# Mutations of The Twik- Related Acid-Sensitive $K^+$ Channel 2 (TASK-2) Promoter in Human Primary Aldosteronism

Livia Lenzini, Selene Prisco, Michele Gallina, Maniselvan Kuppusamy and Gian Paolo Rossi

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# Abstract

As a blunted expression of the twik-related acid-sensitive $K^+$ channel 2 (TASK-2) is a common feature of aldosterone producing adenoma (APA) causing primary aldosteronism (PA), we sequenced the promoter region of the TASK-2 gene (KCNK5) in APAs (n=76), primary hypertensive patients (n=98) and 20 years-old healthy volunteers (n=71), searching for variants that could affect expression of this channel.

We found TASK-2 promoter mutations in 24% of the APA: C999T in 6.6%, G595A in 5.3%, G36A in 5.3%, and C562T, Gins468, G265C, C1247T, G1140T and C1399T in 1.3% (each). The C999T mutation was found in only one of the 98 primary hypertensive patients, but mutations were detected also in 12% of volunteers: four carried the C999T, three G1288C, one the G1140T mutation and one the 468ins. After 16 years' follow-up none of these subjects developed hypertension or PA.

The effect of C999T mutation was investigated in H295R cells using reporter vectors with the mutated or the wild-type (WT) TASK-2 promoters.

TASK-2 gene expression was decreased by 31% (±18, p=0.01) in mutated, as compared to WT APA. Likewise, in transfected H295R cells, the C999T mutation decreased TASK-2 transcriptional activity by 35% (normalized luciferase signal fold change: 0.65±0.25 $p<0.001$).

Thus, mutations in the promoter region of the TASK-2 gene can account for the low expression in about 24% of APA. As these mutations did not result into hypertension or PA during long-term follow-up in healthy subjects, they do not seem to be a factor causing PA by themselves.

# Introduction

The detection of germ-line mutations in the KCNJ5 gene in rare familial forms of primary aldosteronism (PA) provided compelling evidence for a genetic predisposition to PA, the most common cause of endocrine high blood pressure1. Moreover, multiple seminal discoveries have recently pointed to altered function of ion channels due to gene mutations (KCNJ5, ATP1A1, ATP2B3, CACNA1H, and CACNA1D)[2-4] as a molecular mechanism of PA. By increasing cytosolic $Ca^{2+}$ levels, dysfunction of these channels, particularly those involved in $K^+$ handling, can account for enhanced constitutive aldosterone secretion in PA[5]. However, the rate of the most common and best characterized of such mutations in the KCNJ5 gene is highly heterogeneous in aldosterone-producing adenoma (APA), ranging from 12% in Western countries to 80% in Asia (median 45%), whereas that of the other genes (ATP1A1, ATP2B3, CACNA1H, and CACNA1D) is even lower (from 5% to 15% of the cases)[6]. Additional molecular mechanisms in cases that do not have such functional mutations remain, therefore, to be found. The TASK family generates background, or "leak," $K^+$ currents that are essential for maintaining a negative (-70 mV) resting membrane potential and low cytosolic and mitochondrial $Ca^{2+}$ levels in zona glomerulosa cells, thus precluding constitutive excess aldosterone production[7]. Accordingly, the knockout of TASK channels in mice created phenotypes similar to human sporadic PA and the familial type 1 form[8-12]. Moreover, a consistent blunted expression of the twopore-domain potassium (K2p) channel 2 [twik-related acid-sensitive $K^+$ channel 2 (TASK-2)] at the gene and protein levels was recently reported in APA[13]. In about one-third of APAs (30%), the underexpression of TASK-2 could be explained by enhanced expression of microRNA 23 and 34, which were shown to blunt TASK-2 gene expression by binding to the 3' UTR of the TASK-2 gene (KCNK5)[13]. However, in most of the cases, other yet unknown mechanisms can blunt the expression of TASK-2. Hence, we set up this study to investigate if variations in the promoter sequence of the KCNK5 gene can lead to a low expression of TASK-2 in APA.

# Materials and Methods

## Participants

Adrenocortical tumors from 76 consecutive patients with a diagnosis of APA unequivocally established by the "4 corners criteria" were studied [1]. Whenever necessary, treatment with mineralocorticoid receptor antagonists and oral $K^+$ supplementation made the patients normokalemic at the time of adrenalectomy.

Ninety-eight essential hypertensive patients and 71 healthy individuals comprising 20-year-old mariners on service on two ships of the Italian National Naval Force recruited in 2001 were also studied. In all patients and healthy volunteers, germ-line DNA was obtained from peripheral blood. All participants provided an informed consent, and the institutional ethics committees approved all procedures.

**KCNK5 promoter sequencing**

DNA was extracted from the APA tissues and the buffy coat with a kit (QIAquick DNA purification kit; Qiagen, Milan, Italy) following the manufacturer's instructions. The promoter region of the KCNK5 gene (coding for TASK-2 channel) was amplified using three sets of primers and then sequenced from position –1504 upstream of the ATG site to the +145 site. Polymerase chain reaction was performed on 100 ng DNA using the Expand HiFi PCR System dNTPack kit (Roche, Milan, Italy) in a final volume of 50 mL containing 2.5 mM MgCl2, 2.6 U Taq, and 300 nM primer. After purification with the GenElute PCR Clean Up Kit (Sigma, Milan, Italy), amplicons were submitted to Sanger sequencing at a dedicated facility (BMR, University of Padova). The mutations were also checked in germline DNA in mutated APA, extracting DNA from the peripheral blood buffy coat. All APAs were also checked for mutations in the selectivity filter regions of the KCNJ5 gene as reported [14].

**TASK-2 gene expression in mutated and wild-type APA**

The relative expression of the TASK-2 gene in APA vs a control pool of 10 normal adrenocortical tissues was measured using oligomicroarray 15 and compared between the KCNK5- promoter mutated and wild-type (WT) APA.

**Luciferase reporter assay**

To test the effect of the C999T, the most frequent mutation found in APA, we designed three different reporter vectors containing the TASK-2 promoter: one with the WT sequence, one carrying the C999T mutation and, as a negative control, one with a mutation in a different position (G/A mapped 200 bp downstream the C999T). These sequences were fused to the coding region of Gaussia luciferase (1.7 kb inserted in SgfI-MluI restriction sites) (GeneCopoeia, Rockville, MD). As a positive control, we used a vector containing the glyceraldehyde 3-phosphate dehydrogenase promoter sequence fused to the Gaussia luciferase gene (GeneCopoeia) and, as normalizer, the secreted alkaline phosphatase. H295R cells were transfected with 2 mg of vectors with the

Nucleofector device (Lonza, Milan, Italy) and the Cell Line Nucleofector® Kit R. After nucleofection, cells were kept for 48 hours in culture; the cell medium was then collected to measure the activities of Gaussia luciferase and of secreted alkaline phosphatase. The high-sensitivity protocol of the Dual Luminescence Assay kit (GeneCopoeia) was followed for the luminescent assay; luminescence was read in the Mithras LB940 luminometer (Berthold, Milan, Italy). Nucleofection efficiency was checked by transfecting cells with 2 mg of the pmaxGFP Vector (Cell Line Nucleofector® Kit R; Lonza). Each experiment was done three times in triplicate. The ratio of luminescence intensity of the Gaussia luciferase signal over the secreted alkaline phosphatase signal was calculated to minimize the impact of transfection efficiency variability.
Gaussia luciferase signal over the secreted alkaline phosphatase signal was calculated to minimize the impact of transfection efficiency variability.

### Transcription factors binding site prediction

The PROMO[16] and the TFBIND[17] software were used for the in silico prediction of transcription factors binding sites in or near to the C999T mutation. Gene expression of transcription factors identified to bind the region of the C999T mutation was checked in the Gene Expression Omnibus profiles database.

### Statistical analysis

For statistical analysis, we used SPSS (version 24 for Mac; SPSS, Inc., Milan, Italy) and the GraphPad Prism (version 6.00 for Mac; GraphPad Software, La Jolla, CA) software.

## Results

### KCNK5 promoter sequencing

We identified nine different heterozygous mutations in the TASK-2 (KCNK5) promoter region in 25% of APAs (19/76) (Table 1); of them, three recurred in more than one APA: C999T was present in five samples (6.5%) (Fig. 1A), G595A in four (5.2%), and G36A in three samples (3.9%). No APA showed multiple mutations. Germline DNA was sequenced in the 11 available specimens from patients with mutated APA. All variants (Table 1) were germline, except the G1140T, which was only somatic; for the G263C, the

germline mutational status could not be verified because the DNA was not available. No other PA cases could be identified in the kinships of mutated patients.

| Mutation Position | Nucleotide Change | Mutation Rate % | Reference SNP | Reported Mutation Rate, %[a] | Germline Mutation[b] | High Levels of miR23 or miR34 |
|---|---|---|---|---|---|---|
| −36 | G→A | 3.9 | | | Yes | No |
| −263 | G→C | 1.3 | | | NA | No |
| −468 | G insertion | 1.3 | | | Yes | No |
| −562 | C→T | 1.3 | | | Yes | No |
| −595 | G→A | 5.2 | | | Yes | In one case |
| −999 | C→T | 6.5 | rs115955810 | 0.66 | Yes | No |
| −1140 | G→T | 1.3 | rs538720853 | 0.24 | No | No |
| −1247 | C→T | 1.3 | | | Yes | No |
| −1399 | C→T | 1.3 | | | Yes | No |

**Table 1. Summary of the Mutations in the TASK-2 (KCNK5) Promoter Region Detected in APA**
Abbreviation: SNP, single-nucleotide polymorphism. [a] SNP database source. [b] Mutation detected in at least one of the available germline DNAs

A germline C999T mutation was detected in one of the 98 primary hypertensive patients: the patient had had a hemorrhagic stroke 27 years before at age 30 and was on multiple antihypertensive drugs because of stage III hypertension[18]. On computed tomography, he had a subcentrimetric lump in the left adrenal gland, but a lateralized aldosterone secretion could not be demonstrated on adrenal vein sampling. Among the 71 healthy volunteers, 12% had a germline mutation in the KCNK5 promoter region: 4 carried the C999T, 3 a new mutation in position G1288C, 1 the G1140T mutation, and 1 the 468ins mutation. None of these subjects, who had been recruited in 2001, developed arterial hypertension or PA after 16 years of follow-up. The C999T (rs115955810) and G1140T (rs538720853) mutations were already reported in the dbSNP (National Center for Biotechnology Information website), but their allele frequency was much higher in our APA cohort (6.5% for C999T and 1.3% for G1140T) than in the public-reported Minor Allele Frequency [0.66% and 0.24%, respectively; 1000 Genomes source[19]].

## Molecular and clinical characteristics of mutated and wild-type APA

The mutated APA showed a mean TASK-2 gene expression of 31% ± 18% lower than that of the WT APA (P = 0.01) (Fig. 1B). The clinical and biochemical features of patients with or without the KCNK5 promoter mutations showed no significant differences (Table 2). Of the 76 APA, 11 (14.5%) were also mutated in the KCNJ5 gene; of them, 4 carried both TASK-2

and KCNJ5 mutations (3 the G151R and 1 the L168R variants). No obvious differences in the clinical phenotype were detected between patients carrying both TASK-2 and KCNJ5 mutations compared with the WT or single mutation carriers.

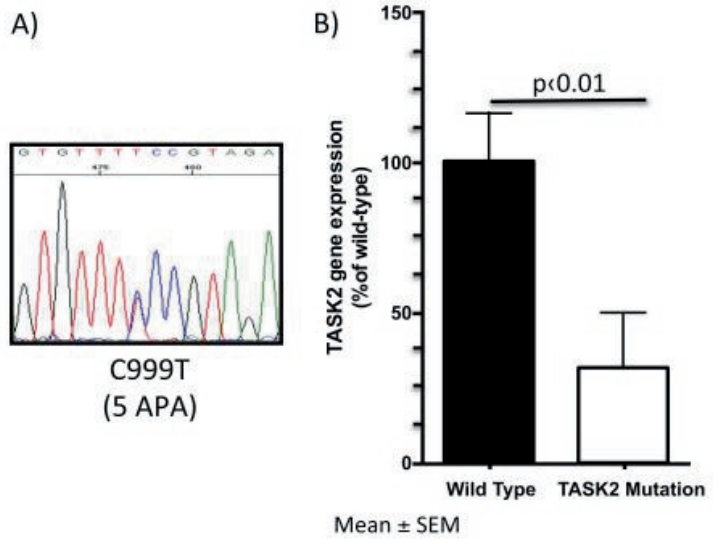


**Figure 1. (A) Example of sequencing result of an APA with the C999T mutation. (B)** TASK-2 gene expression in WT and mutated APA. Gene expression is calculated as percentage of fold change of TASK-2 in APA (n = 28) relative to a pool of 10 normal adrenal cortexes. SEM, standard error of the mean.

## In vitro effect of the C999T mutation

The luciferase signal in H295 cells transfected for 48 hours with a reporter vector containing the Gaussia luciferase coding gene fused to the TASK-2 mutated (C999T) promoter was 35% lower than in cells transfected with the WT promoter ($P < 0.001$) (fold change of normalized luciferase signal: 0.65± 0.25, of WT promoter sequence $P < 0.001$) (Fig. 2). The luciferase signal of the negative control carrying a mutation in a different position of the TASK-2 promoter showed no changes compared with the WT promoter sequence (Fig. 2). These findings indicate that only the C999T mutation is functional in that it lowers the transcription of the TASK-2 gene.

| Characteristic | Mutated APA (n = 19) | | Not Mutated APA (n = 57) | |
|---|---|---|---|---|
| | Preadrenalectomy | Postadrenalectomy | Preadrenalectomy | Postadrenalectomy |
| Age, mean ± SD, y | 48 ± 11 | | 51 ± 13 | |
| Sex (% f/m) | 62/38 | | 63/37 | |
| Systolic blood pressure, mean ± SD, mm Hg | 166 ± 12 | 126 ± 13 | 156 ± 16 | 134 ± 16 |
| Diastolic blood pressure, mean ± SD, mm Hg | 102 ± 15 | 81 ± 7 | 90 ± 14 | 80 ± 8 |
| Plasma aldosterone concentration, mean ± SD, ng/dL | 23.93 ± 7.76 | 9.94 ± 4.98 | 20.35 ± 18.23 | 8.15 ± 4.44 |
| Plasma renin activity, mean ± SD, μg/L/h | 0.363 ± 0.200 | 1.87 ± 2.29 | 0.752 ± 0.956 | 1.58 ± 1.94 |

**Table 2. Comparison Between Patients With APA With and Without TASK-2 Promoter Mutations**

## Prediction of transcription factor binding region on the C999T site

We performed an in silico prediction of the transcription factors binding site in the WT and mutated sequences to investigate if the functional effect of the C999T mutation was associated with loss of a transcription factor binding site. Both the PROMO [16] and the TFBIND [17] software predicted a consensus sequence for the Stat1 factor from position 2997 to 21006 (TTCCCGTAG); moreover, this site was disrupted by the presence of the C999T variant. Of note, the expression of Stat1 was reported in H295 and APA cells in the Gene Expression Omnibus profiles database (GDS3556/209969_s_at/STAT1 and GDS2860/209969_s_at/STAT1, respectively). In position 21291, we identified a response element for nuclear retinoic acid receptors, retinoid X receptors, and liver X receptor [20] (TGAGGTC) that in three healthy volunteers was mutated (G1288C).

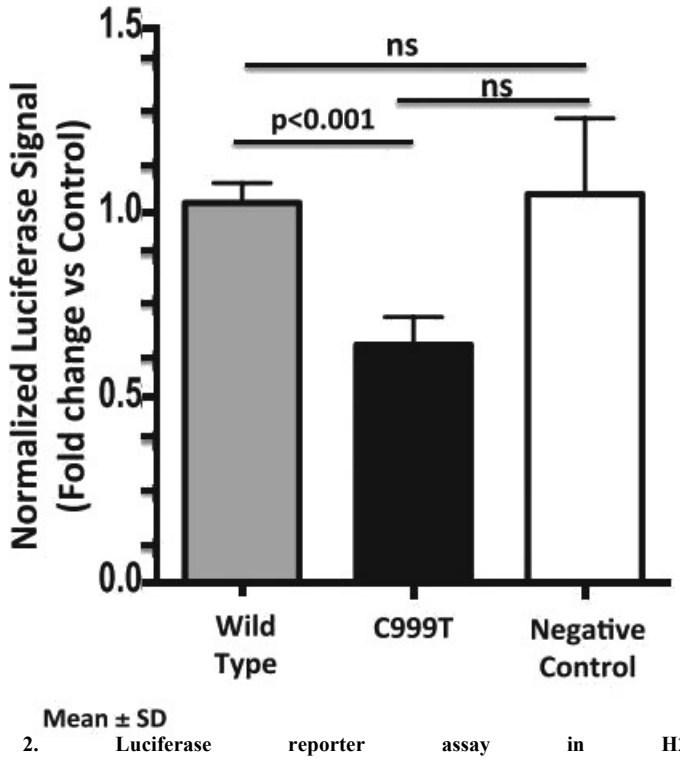


**Figure 2. Luciferase reporter assay in H295 cells.** Three different reporter vectors were transfected: one containing the WT sequence of the TASK-2 promoter (1.7 kb inserted in SgfI-MluI restriction sites), one carrying the C999T mutation, and one with a mutation in a different position of the TASK-2 promoter. Each sequence was fused to the Gaussia luciferase. To normalize transfection data, the secreted alkaline phosphatase was used as a second reporter gene. Experiments were done three times in triplicate. The ratio of luminescence intensities of the Gaussia luciferase signal over the secreted alkaline phosphatase signal was calculated with the normalized luciferase signal. ns, not significant; SD, standard deviation.

# Discussion

The blunted expression of the TASK-2 channel identified as a consistent molecular feature of APA can derive from several potential mechanisms [13,21]. We previously found that the expression of TASK-2 in APA was inversely related with the expression of 13 microRNAs[13], only three of which were predicted to bind the TASK-2 3' UTR. Moreover, of these microRNAs, two—miR23 and miR34—were shown to significantly blunt the expression of TASK-2 in vitro in a reporter gene assay. An increased expression of miR was, however, found in only 30% of the APAs, which indicated that other

mechanisms were implicated. The present results identify one such additional mechanism: by sequencing the TASK-2 gene (KCNK5) promoter region, we identified mutations in 25% of the APAs, of which two (C999T, rs115955810 and G1140T, rs538720853) were already present in the dbSNP database, and seven were novel. The reported allele frequency of the known mutations in the general population in the 1000 Genomes project [19] was consistent with the percentage (1%) of the C999T mutation found in our cohort of primary hypertensive patients but about 10-fold lower than in our APA cohort. To investigate the functional relevance of the most prevalent mutation detected in APA, C999T, we compared the transcriptional activity of the TASK-2 promoter mutant with that of the WT TASK-2 promoter in a reporter in vitro assay. By this approach, we could show that introduction of the C999T mutation significantly decreases the transcription activity of the gene by 35% (Fig. 2), an extent remarkedly similar to the percentage decrease of TASK-2 expression of APA carrying the mutations compared with WT APA. The prediction of the binding site loss for Stat1 transcription factor in the presence of the C999T variant and the reported expression of this factor in both H295 cells and in APA (GDS3556/209969_s_at/ STAT1 and GDS2860/209969_s_at/STAT1, respectively) offer a possible mechanism for the decreased in vitro transcriptional activity. It is worth pointing out that most of the KCNK5 promoter variants involved both the APA and the surrounding adrenal cortex because they were germline, thus suggesting that they are a genetic cause of PA and/or hypertension. In contrast with this hypothesis, we did discover these mutations in 12% of healthy normotensive mariners, who remained normotensive and did not develop overt PA after 16 years of follow-up. Thus, these mutations could not be causative of PA per se, and other factors likely need to occur for PA to develop. The latter could comprise somatic mutations in other genes involved in the hyperaldosteronism in APA as, for example, KCNJ5. Interestingly, four of our patients with mutated APA have also a somatic functional variant of the KCNJ5 gene (three the G151R and one the L168R), which, however, seemingly did not markedly change their clinical phenotype. In this cohort of APA, the frequency of KCNJ5 mutations was lower than that found in a recent large metaanalysis (median 43%; range, 12% to 80%)[6], possibly reflecting the occurrence of other, yet unknown, gene variants or a selection bias. This could be because with the systematic screening of hypertensive patients for PA exploited in our center, we pinpoint the less florid cases, whereas KCNJ5 mutations are held to imply a more florid clinical

phenotype[6]. Thus, the low expression of TASK-2 can be associated with different genetic and epigenetic predisposition factors, as sketched in Fig. 3. Of note, an additional novel mutation (G1288C) in the binding site for the retinoid X and retinoic acid receptors, which in a cell model were recently shown to regulate aldosterone production[22], was not found in any of our APAs but was detected in three healthy mariners, who remained normotensive for 16 years. Thus, whether this mutation could play a functional role, either promoting PA or protecting from it, remains to be explored. As for mutations in other genes associated with PA, the TASK-2 gene (KCNK5) variants were detected in only 25% of the APAs, in agreement with the current view that they are highly heterogeneous tumors[15,23]. Although the mechanisms causing TASK-2 low expression in nonmutated samples remain to be clarified, the finding that 30% of APAs have high levels of two microRNAs (hsa-miR-23 and hsamiR-34) and that these microRNAs are able to cause the degradation of the TASK-2 transcript[13], together with the present results, collectively suggest that different molecular mechanisms can eventually result in the same molecular feature (e.g., low expression of TASK-2 with ensuing aldosteronism) (Fig. 3). Of note in our present series, only one subject had high levels of hsa-miR-23 and hsa-miR-34 and a mutation in the TASK-2 promoter (Table 1).

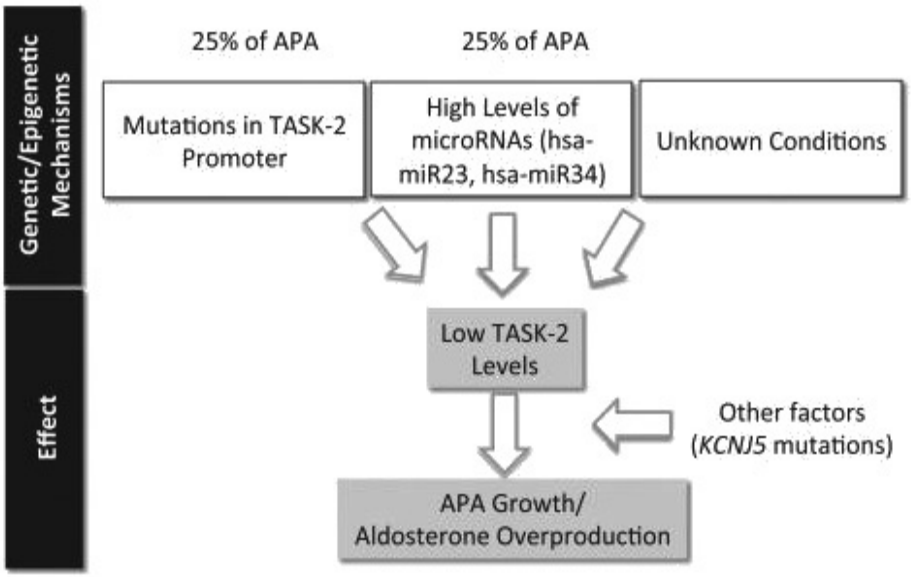

**Figure 3. Schema of proposed mechanisms causing TASK -2 low levels in APA.** Different mechanisms may result in the TASK-2 low expression that features all APAs. This may be due to genetic causes (mutations in TASK-2 promoter), epigenetic factors (high levels of microRNA hsa-miR23 and hsa-miR34), or as-yet-unknown conditions.

# Conclusions

In summary, this study shows that in about one-fourth of APAs, the low expression of TASK-2 is associated with functional mutations in the promoter region of the TASK2 gene (KCNK5). These variants could thus act as predisposing factors that, when associated with other genetic variations acquired by the adrenal gland (e.g., somatic mutations in other genes involved in the autonomous production of aldosterone) and/or with environmental factors, can eventually coincide with the development of PA in a relevant proportion of the cases.

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# Chapter 5

# Does Angiotensin II regulate Parathyroid Hormone secretion or not?

Gian Paolo Rossi and Selene Prisco

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In this issue of the Journal, a study by Bislev et al evaluated the interaction between the renin-angiotensin-aldosterone system (RAAS) and parathyroid hormone (PTH) system in a double-blinded randomized placebo-controlled trial. The authors found no effects of RAAS blockade with valsartan on PTH levels. However, in spite of the strength of this approach, their study has important limitations that could have affected their conclusions as discussed below.

Aldosterone, the main mineralocorticoid hormone, plays a key role in regulating salt and water balance, and thus blood pressure (BP). Its synthesis in the adrenocortical zona glomerulosa (ZG) is regulated by multiple factors, including angiotensin II acting via the AT- 1 receptor (AT-1R), endothelin – 1, urotensin II, K+, and ACTH. The parathyroid hormone (PTH), secreted by the parathyroid gland when the $Ca^{2+}$ sensing receptor (CaSR) is activated by a decrease in ionized $Ca^{2+}$ in serum, acts by promoting $Ca^{2+}$ reabsorption in the kidney and by enhancing mobilisation of $Ca^{2+}$ from the bone. This restores the $Ca^{2+}$ serum concentration, which is tightly regulated within a narrow physiological range (1.19-1.29 mmol/L at pH 7.40) in humans.

The adrenocortical ZG and the parathyroid gland were thought to serve indipendent physiological functions, but recent discoveries have challenged this view and propose a bidirectional link between these glands [1]. In 2011 a patient with drug-resistant hypertension caused by primary aldosteronism (PA) due to bilateral nodular adrenocortical hyperplasia, was cured with unilateral adrenalectomy, but thereafter manifested primary hyperparathyroidism that was surgically cured by removal of a single parathyroid adenoma. This unique case provided an opportunity to examine mineralocorticoid receptor (MR) expression in the excised parathyroid, and the type 1 PTH receptor in adrenocortical tissue, which we could unambiguously demonstrate for the first time at both the mRNA and protein level[2]. Subsequent independent studies confirmed the presence of the MR in normal and pathologic parathyroid tissue[3].

A systematic measurement of serum PTH in consecutive PA patients thereafter showed that mild hyperparathyroidism is a consistent feature of PA[4], particularly of the more florid subtype sustained by aldosterone-producing adenoma (APA)[5]. Experimental studies in rats and observational studies in humans collectively support the concept that in the setting of hyperaldosteronism an increased urinary calcium loss lowers serum ionised calcium levels, thus inducing PTH secretion and leading to secondary hyperparathyroidism[6-8]. Accordingly, correction of PA with unilateral adrenalectomy raises the low serum ionised $Ca^{2+}$ levels and normalizes serum

PTH in APA patients, thus indicating an in vivo link between aldosterone and PTH.

These observations extended previous in vitro studies by Mazzocchi et al. which showed that PTH raises aldosterone secretion in a concentration-dependent fashion by acting on a specific PTH receptor via activation of PKA and PLC [9,10]. Further human intervention studies showed that blunting of Ang II or aldosterone (with captopril or spironolactone) lowered PTH and also that the human parathyroid gland expresses the AT-1R, which led to the suggestion that Ang II and aldosterone regulate PTH secretion in acute and chronic conditions, respectively . By examining the Multi-Ethnic Study of Atherosclerosis database Brown et al. reported a direct association of higher serum levels of PTH with higher serum levels of aldosterone and, conversely, lower PTH concentrations associated with use of RAAS inhibitors [11].

Bislev et al used a pharmacologic approach to further investigate this hypothesis. They are to be commended for undertaking a randomized clinical trial where they assigned normotensive postmenopausal women to receive either the AT-1R blocker (ARB) valsartan, or a placebo, with/without vitamin D supplementation. This robust design of study provided an opportunity of testing for interactions between valsartan and vitamin D, which is relevant since that the action of ARBs is known to be subjected to interactions with other drugs. Interestingly, they could find no evidence for such interactions by examining serum PTH, aldosterone, serum 25(OH)D, 1.25(OH)2D, and BP as endpoints.

Notwithstanding their strong experimental design and a sizable cohort of women, Bislev et al found no effects of valsartan on PTH, which led them to contend that Ang II has no place in regulating PTH release, a conclusion that contradicts previous results[3], but which deserves some scrutiny before it can be taken for granted. The first relates to the selection of the study population: the authors recruited normotensive women with no hyperparathyroidism, an unfortunate choice for at least two reasons. Firstly, normal PTH levels are unlikely to exhibit a detectable decrease with any interventions. Moreover, even if a PTH fall could be demonstrated, its clinical relevance would remain unclear as discussed below. Secondly, given their choice of normotensive women, the authors could not use a full BP-lowering dose of valsartan: the dose that they used (80mg) is that usually prescribed for treatment of heart failure, not of arterial hypertension[12- 14]. In the largest randomised clinical trial of valsartan in hypertension, the Value trial, the average dose of valsartan was almost two-fold higher (151.7 mg/day; IQR 83.2-158.5), and treatment lasted for a mean of 3-6 years. Furthermore, the poor (19%) oral bioavailability of valsartan capsules and the short (two-weeks) treatment suggest that the dose of valsartan was too

low and administered for too short period of time to detect changes [12]. Hence, the women in the valsartan group exhibited a fall neither of systolic blood pressure nor of aldosterone, two expected effects of ARB administration. In fact, the only proof of adherence to the prescribed treatment was the increase of plasma renin.

The lack of standardisation of food, calcium, and sodium intake during the study could also have influenced their assessment of PTH changes, in that a high calcium intake, not uncommon in post-menopausal women who are prone to osteoporosis, could have blunted the PTH levels at baseline, thus further precluding the possibility of demonstrating a fall in response to therapy.

Finally, as the RAAS is known to be progressively blunted by aging, it is conceivable that the effect of valsartan was low in the 60-80 years-old women selected by Bislev et al [15].

Therefore, the possibility that Ang II affects PTH secretion via AT1-R cannot be conclusively ruled out based on this study. Moreover, this phenomenon might be clinically relevant when PTH secretion and/or the RAAS are overtly activated, neither of which conditions occurred in the population of normotensive postmenopausal women studied by Bislev et al[15].

In summary, the role of the RAAS, and particularly of Ang II and aldosterone, in the regulation of PTH, needs further investigations in populations where the activation of both hormonal systems is clear-cut and their blunting clinically relevant as, for example, patients with primary and secondary aldosteronism.

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# Chapter 6

# PTH modulation by Aldosterone and Angiotensin II is blunted in Hyperaldosteronism and rescued by adrenalectomy

Livia Lenzini, * Selene Prisco,* Paul E. Vanderriele,Silvia Lerco,Francesca Torresan,Giuseppe Maiolino, Teresa Maria Seccia,Maurizio Iacobone and Gian Paolo Rossi

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# Abstract

Accumulating evidence suggests a link between adrenocortical zona glomerulosa and parathyroid gland through mechanisms that remain unexplored. Objectives: To test the hypothesis that in vivo angiotensin II blockade affects PTH secretion in patients with hypertension and that aldosterone and angiotensin II directly stimulate PTH secretion ex vivo.

We investigated the changes of serum PTH levels induced by oral captopril (50 mg) administration in patients with primary essential hypertension (EH) and with primary aldosteronism (PA) caused by bilateral adrenal hyperplasia (BAH) or aldosterone-producing adenoma (APA), the latter before and after adrenalectomy. We also exposed primary cultures of human parathyroid cells from patients with primary hyperparathyroidism to angiotensin II ($10^{-7}$ M) and/or aldosterone ($10^{-7}$ M).

Captopril lowered PTH levels (in nanograms per liter) both in patients with EH (n= 63; 25.9 ± 8.3 baseline vs 24.4 ± 8.0 postcaptopril, P= 0.0001) and in patients with APA after adrenalectomy (n= 27; 26.3 ± 11.6 vs 24.0 ± 9.7 P= 0.021). However, it was ineffective in patients with full-blown PA caused by APA and BAH. In primary culture of human parathyroid cells, both aldosterone (P< 0.001) and angiotensin II (P= 0.002) markedly increased PTH secretion from baseline, by acting through mineralocorticoid receptor and angiotensin type 1 receptor, as these effects were abolished by canrenone and irbesartan, respectively.

These results collectively suggest an implication of the renin-angiotensin-aldosterone system in PTH regulation in humans, at least in PTH-secreting cells obtained from parathyroid tumors. Moreover, they further support the concept that mild hyperparathyroidism is a feature of human PA that is correctable with adrenalectomy.

# Introduction

Primary aldosteronism (PA) is the most common cause of endocrine arterial hypertension[1]. In addition to low active renin, apparently autonomous aldosterone secretion, and often hypokalemia, patients with PA show increased levels of serum PTHcompared with matched primary (essential) hypertension (EH)[2-5], particularly when they have the most florid clinical phenotype, usually caused by an aldosterone-producing adenoma (APA)[6]. This hyperparathyroidism, which is usually mild, can be important mechanistically because both PTH and the PTH-related peptide were shown to concentration dependently stimulate aldosterone secretion in vitro[7-10]. Because most determinants of PTH secretion, including ionized serum calcium, magnesium, phosphate, and 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxycholecalciferal (1,25OH) vitamin D, did not differ between patients with PA and patients with EH[4], the mechanisms underlying the increase of serum PTH remain unknown. Of note, a four-week administration of aldosterone to normotensive rats on 1% sodium intake increased PTH, likely by causing hypercalciuria and thereby lowering serum ionized calcium ($Ca^{2+}$) levels [11]. The contention was made that this can also occur in human PA because of the hyperfiltration-induced calciuretic effect of hyperaldosteronism; therefore, patients with PA could initially experience a slight decrease of serum ionized calcium, which thereafter becomes barely detectable being offset by the elevated PTH levels mobilizing $Ca^{2+}$ from the bones [2,11,12]. In agreement with this, we found that in patients with APA, adrenalectomy and the ensuing correction of hyperaldosteronism increased ionized $Ca^{2+}$ and decreased serum PTH [5,6]. Based on the finding that human parathyroid cells express functional mineralocorticoid receptor (MR) [13], and on the aforementioned secretagogue effect of PTH on aldosterone secretion in vitro , the existence of a bidirectional functional loop between the $Ca^{2+}$ regulating parathyroid gland and the adrenal cortex was suggested and it was proposed that hyperparathyroidism can be one of the drivers of persisting hyperaldosteronism in spite of a blunted renin-angiotensin system in patients with PA[14]. According to this hypothesis, in this loop the key players would be aldosterone, acting via MR in the parathyroid cells, and PTH, acting via type 1 PTH receptors in the adrenocortical zona glomerulosa and in APA cells. However, information on the effect of in vivo manipulation of the renin-angiotensin-aldosterone system, either in healthy

subjects or in patients with primary hyperparathyroidism, is conflicting, thus leaving unknown the role of angiotensin II, the other major mediator of this system. In healthy subjects, infusion of angiotensin II stimulated PTH secretion in a dose-dependent manner within one to two hours, whereas captopril lowered PTH by 12% from baseline[15]. Moreover, in primary hyperparathyroidism patients and in normal subjects, one week angiotensin converting enzyme (ACE) inhibition with lisinopril caused a modest marginally significant decrease of serum PTH levels [16.] Therefore this study set out to investigate the in vivo effect on PTH secretion of angiotensin II blockade in hypertensive patients with PA who have overtly elevated PTH levels, and in patients with EH who have normal PTH levels. In the former group we examined not only the PTH response to captopril according to the PA subtype, e.g. unilateral APA and bilateral adrenal hyperplasia (BAH), but also the impact of cure of hyperaldosteronism provided by adrenalectomy on this response. Finally, as we succeeded in obtaining primary culture of human parathyroid cells ex vivo from primary hyperparathyroidism surgically treated patients, we investigated the direct effect of aldosterone and angiotensin II on PTH production ex vivo in the presence and absence of specific antagonists.

# Materials and Methods

### Patient study

We prospectively recruited hypertensive patients referred to our specialized center for hypertension for the work-up of arterial hypertension. In those with PA and with primary (essential) hypertension (EH) we assessed the acute effect of captopril on plasma PTH. PA was diagnosed according to the 2016 Endocrine Society Practice guidelines; APA was confirmed by biochemical cure and cure/improvement of hypertension after adrenalectomy following the experts' recommendations [17] and the recently published five- corners criteria, which also entails immunochemical demonstration of CYP11B2 in the node(s) of the resected adrenal [18]. Blood was collected under basal conditions after one-hour supine rest and again in the same position one hour after oral captopril administration (50 mg). The test was repeated in an identical manner one month after surgery in patients with APA, and after one month of medical therapy, entailing a MR antagonist, in cases of BAH. PTH, plasma aldosterone concentration (PAC), and plasma renin activity (PRA) concentrations were measured as previously described [19]. All procedures followed the principles of

the Declaration of Helsinki and the institutional guidelines; the protocol was approved by the local Institutional Review Board and written consent was obtained from all participants.

**Cell isolation and primary culture of human parathyroid cells**

Tissue samples were obtained in the operating room immediately after excision under sterile conditions from consenting patients with primary hyperparathyroidism undergoing surgery for parathyroid adenoma. After removal of all visible fat and capsule tissues, specimens were minced and digested in 1 mg/mL collagenase type II, 0.05 mg/mL DNAse I (Roche, Milan, Italy) in RPMI-1640 media (Sigma-Aldrich, Milan, Italy) for one cycle of 20 minutes at 37°C, followed by mechanical disaggregation using the GentleMACS™ Dissociator (Miltenyi Biotec, Milan, Italy). Cells were filtered through a 40-mm cell strainer, and then centrifuged for 5 minutes at 800g. Their viability was assessed by the trypan blue exclusion test. After tissue dispersion the cell suspension was washed with RPMI-1640 medium (Sigma-Aldrich, Milan, Italy) and incubated with CD90-coated magnetic beads for 30 minutes at 4°C. Beads were used at five beads per cell. After separating the $CD90^+$ bound-bead cells with a magnet, $CD90^+$ cells were cultured at a density of $1x\ 10^5$ cells into six-well plates and maintained in a humidified 5% carbon dioxide/air atmosphere at 37°C for six days. Fresh medium was added every 48 hours.

**Immunocytochemistry of parathyroid primary cells**

Primary cells were stained directly on the glass slide. Samples were fixed for 10 minutes with 2% paraformaldehyde in PBS at pH 7.4, and then washed in PBS. After endogenous peroxidases inhibition with 0.5% hydrogen peroxide, slides were incubated overnight at 4°C with the following antibodies: anti-PTH (1:500, GTX39458; GeneTex, Inc., Irvine, CA), anti-calcium sensing receptor (CaSR) (1:70, ab19347; Abcam, Cambridge, UK), anti-chromogranin A (CGA) (1:300, ab15160; Abcam, Cambridge, UK), anti-glial cell missing-2 homolog (GCM2) (1:80, ab96063; Abcam). After repeated PBS washes, antigens were detected by incubation with a secondary antibody (1:100) and Advance HRP Detection System Kit–Envision System (Dako Corp., Carpinteria, CA) for mouse and rabbit primary antibodies for 60 minutes at room temperature. The immunecomplexes were visualized with 3,30 -diaminobenzidine (Dako Corp.) and the reaction was stopped with distilled water. Negative controls were

carried out by omitting and also immunoprecipitating the primary antibody. The images were acquired with a Leica DMR microscope.

**Phenotypic characterization of primary cells**

The functional responsiveness of parathyroid primary cells to extracellular $Ca^{2+}$ was verified by exposure to a calcium-free low serum concentration medium (fetal bovine serum 0.5%) with/without physiological (1.25 mM) $Ca^{2+}$ for one hour. PTH was measured with an ELISA for human bioactive PTH 1-84 (Immutopics, San Diego, CA). Results were normalized per microgram RNA to adjust for among-well cell number differences.

**Cells stimulation with aldosterone and angiotensin II**

After five days in culture, primary parathyroid cells were starved overnight and then stimulated for six hours with aldosterone ($10^{-7}$ M) or angiotensin II ($10^{-7}$ M) at a (1.25 mM) calcium in a low serum concentration (fetal bovine serum 0.5%). Parallel experiments were performed by adding to the fresh medium either the MR antagonist [canrenone ($10^{-5}$)] or the angiotensin type 1 receptor (AT-1R) antagonist [irbesartan ($10^{-5}$)] 30 minutes before stimulation. These concentrations of canrenone and irbesartan, which did not affect PTH secretion per se, were 100-fold higher than those of their relative agonists. Moreover, they were found to abolish the effects of their agonist in other cell models [20]. All experiments were performed in duplicate and repeated eight times.

**AAT-1R and 11-b hydroxysteroid dehydrogenase type 2 (11bHSD2) protein expression**

We measured the total protein concentration with BCA (Thermo Scientific, Milan, Italy) and the AT-1R and the expression of 11-b hydroxysteroid dehydrogenase type 2 (11bHSD2) by immunoblotting in the soluble supernatant of tissue samples obtained from those patients who underwent surgery for parathyroid adenoma and APA. To this aim, normal adrenal cortex surrounding the APA (n=3), parathyroid gland (n=3), human kidney from our tissue bank (n=4), and H295 cells were homogenized in 600 mL lysis buffer (Thermo Scientific, Milan, Italy) using a MagNALyser Instrument (Roche, Milan, Italy). Lysate fraction (50 mg) was separated from acrylamide gel and then electro-blotted onto nitrocellulose membrane (Hybond ECL-Amersham Biosciences

Europe, Berlin, Germany). The membranes were blocked for one hour at room temperature in 0.5% Tween 20 in Super Blocker (ThermoScientific, Rockford, USA) and then incubated overnight at 4°C with a primary sheep polyclonal antibody against AT-1R (1:100 dilution) (NovusBio, Milan, Italy) and 11bHSD2 (1:100 dilution) (a gift from Prof. Paul Stewart, University of Leeds, Leeds, UK). Blots were analyzed by the QuantityOne Program of VersaDOC 1000 (BioRad, Milan, Italy).

**Gene expression of MR and AT-1R in parathyroid tissues**

From the parathyroid tissue samples (n=8), total RNA was extracted using the RNeasy Mini kit (Qiagen, Milan, Italy). Total RNA (1 mg) was reverse transcribed with Iscript (Bio-Rad) in a final volume of 20 mL. The gene expression was measured with a real-time quantitative RT-PCR with universal ProbeLibrary probes in the LightCycler 480 Instrument (Roche) using specific primer for AT-1R, MR, and actin. Standard curves were preliminarily performed to assess the reaction efficiency of each primers set. The expression was calculated relative to the housekeeping gene actin used as an internal control.

**Statistical analysis**

Results were expressed as mean ± SD of eight separate experiments in which samples were assayed in duplicate. Differences between groups were analyzed by unpaired t test. Stepwise regression (Wald backward) and Pearson correlation were used for correlation analyses. Significance level was set at $P< 0.05$. Statistical analyses were performed using Graphpad/ prism 6 (Graphpad Software 6™, La Jolla, CA) and SPSS (version 24, IBM, Armonk, NY).

# Results

**In vivo study: baseline findings**

The PA patients with APA (n=27) and BAH (n=15) had overtly elevated values of PAC, suppressed PRA, and thus elevated levels of the aldosterone-to-renin ratio (ARR; Table 1). The patients with APA showed higher serum PTH levels (34.6 ± 14.2 ng/L) than the patients with EH (n =63; 25.9 ± 8.3 ng/L, $P< 0.0001$) (Fig.1). Moreover, in 25% of the former patients the levels exceeded

the upper normal range. Adrenalectomy provided biochemical cure of PA, e.g., a normalization of PAC, PRA, and hypokalemia when present at baseline in all except in two patients who were removed from the analysis. Moreover, it lowered serum PTH to levels that were similar to those seen in patients with EH (26.3 ± 11.6 ng/L; Fig. 1 and Table 1). The patients with PA with BAH had serum PTH levels ;10% lower than patients with APA at baseline, but showed higher serum PTH levels (31.6 ± 12.1 ng/L; Table 1) than patients with EH whose PTH, PAC, PRA, and ARR were within the normal range (Table 1).

| | APA | | BAH | | EH |
|---|---|---|---|---|---|
| Variable | Baseline (n = 27) | Post Adrenalectomy (n = 25) | Baseline (n = 15) | Follow-Up (n = 7) | Baseline (n = 63) |
| Age, y | 52 ± 11 | — | 52 ± 12 | — | 52 ± 10 |
| Sex, % (M/F) | 56/44 | — | 61/39 | — | 58/42 |
| Systolic BP, mm Hg | 149 ± 18 | 140 ± 24 | 150 ± 22 | 149 ± 16 | 143 ± 14 |
| Diastolic BP, mm Hg | 93 ± 13 | 94 ± 13 | 92 ± 12 | 89 ± 13 | 91 ± 6 |
| Heart rate, bpm | 68 ± 11 | 76 ± 12 | 71 ± 14 | 63 ± 8 | 76 ± 15 |
| BMI, kg/m$^2$ | 25.4 ± 4.1 | 23.7 ± 2.5 | 26.2 ± 4.6 | 26.7 ± 3.9 | 25 ± 3 |
| ARR, ng/dL/μg/L/h[a] | 93 ± 96 | 20 ± 22 | 44 ± 29 | 40 ± 22 | 19 ± 14 |
| PRA, μg/L/h | 0.24 (0.10– 0.57)[b] | 0.49 (0.12–2.89)[b] | 0.24 (0.10–7.16) | 0.24 (0.24–0.26) | 0.5 (0.1–4.4) |
| PAC, ng/dL | 14.2 (4.7–93.7)[b] | 5.4 (2.9–22.0)[b] | 13.7 (2.2–24.9) | 13.5 (7.2–18.9) | 8.4 (1.5–15) |
| PTH, ng/L[c] | 34.6 ± 14.2[b] | 26.3 ± 11.6[b] | 31.6 ± 12.1 | 29.7 ± 7.3 | 25.9 ± 8.3 |

**Table 1. Clinical and biochemical data of the surgically (APA) and medically (BAH) treated patients with PA at baseline and follow-up visits and of patients with EH**

Data presented as mean ± SD or median (IQR), as appropriate. Abbreviations: ARR, aldosterone to renin ratio; BP, blood pressure; BMI, body mass index; IQR, interquartile range.

a Normal range: <26 ng/dL/mg/L/h for a Na+ intake between 100–300 mmoL/d.

b P value baseline vs post adrenalectomy <0.05.

c Normal range: 6.5–36.8 ng/L

## In vivo effects of captopril

In the PA patients with APA, captopril lowered PAC both before (from 23.9 ± 21.5 to 20.7 ± 20.9 ng/dL; P= 0.0009, not shown) and after adrenalectomy (from 7.7 ± 5.2 to 6.2 ± 3.8 ng/dL; P< 0.0001 Fig. 1A). The changes that occurred in patients with BAH did not achieve statistical significance either at baseline (from 12.7 ± 5.5 to 10.3 ± 4.5 ng/dL;, P > 0.05) or at follow-up (from 12.4±4.4 to 11.6±7.3 ng/dL; P>0.05). PRA was unaffected by captopril in APA before adrenalectomy (0.38 ± 0.47 vs 0.41±0.39 ng/mL per hour; P > 0.05) and also in BAH, at baseline (0.95 ± 1.80 vs 1.38 ± 2.66 ng/mL per hour; P >0.05). In the patients with APA, removal of the tumor restored captopril responsiveness of PRA, which increased (from 0.63 ± 0.61 to 1.79 ± 3.73 ng/mL per hour; P= 0.0006), similar to that seen in patients with EH (from 0.72 ± 0.69 to 1.13 ± 1.41 ng/mL per hour; P < 0.0001). At variance, at follow-up PRA remained unresponsive to captopril in patients with BAH (0.60± 0.91 vs

0.73 ± 1.23 ng/mL per hour; P > 0.05). Captopril administration did not affect serum PTH levels at baseline in the whole group of patients with PA, as well as in patients with APA (from 34.6 ± 14.2 to 34.7 ± 16.3 ng/L; P > 0.05) and in patients with BAH (from 31.6 ± 12.1 to 30.4 ± 12.2 ng/L; P > 0.05) patients. However, captopril decreased PTH levels (from 25.9±8.3 to 24.4 ± 8.0 ng/L; P< 0.0001) (Fig.1D) in patients with EH. After adrenalectomy captopril lowered PTH (from 26.3 ± 11.6 to 24.0 ± 9.7 ng/L; P = 0.021) in the patients with APA (Fig.C); no such change could be detected in the smaller group of patients with BAH (29.7 ± 7.3 vs 31.4 ± 11.5 ng/L; P > 0.05) available at follow-up[21].

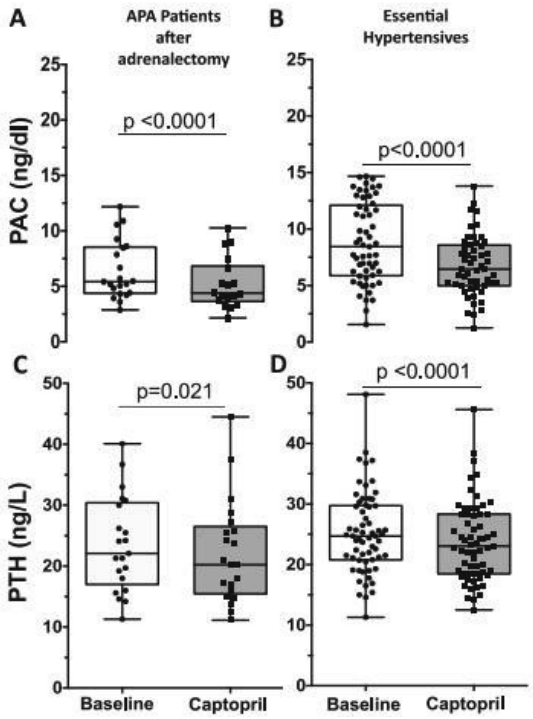


**Figure 1. Effect of captopril administration on PAC and PTH levels**. Plasma levels of PAC and PTH before and after captopril administration (50 mg orally) in 25 patients after the removal of an APA (**A** and **C**) [and in 63 patients with EH (**B** and **D**)].

## Predictors of the PTH response to captopril

We performed a stepwise backward regression analysis. We used the backward (Wald) technique to not miss any relevant covariates and to identify the variables that predicted the post-captopril within-patient Δ change of PTH from baseline. We entered in the regression model age, diagnosis, body mass index, serum ionized calcium, systolic and diastolic blood pressure, and captopril induced changes of PRA and PAC. The only variable that remained in the model was the decrease of PAC levels from baseline (F= 12.15; P< 0.0001), whereas all other variables were not useful to the model. A bivariate correlation analysis confirmed a direct correlation between with the decreased aldosterone secretion and that of PTH post captopril stimulation (r= 0.296; P= 0.0007; Fig. 2).

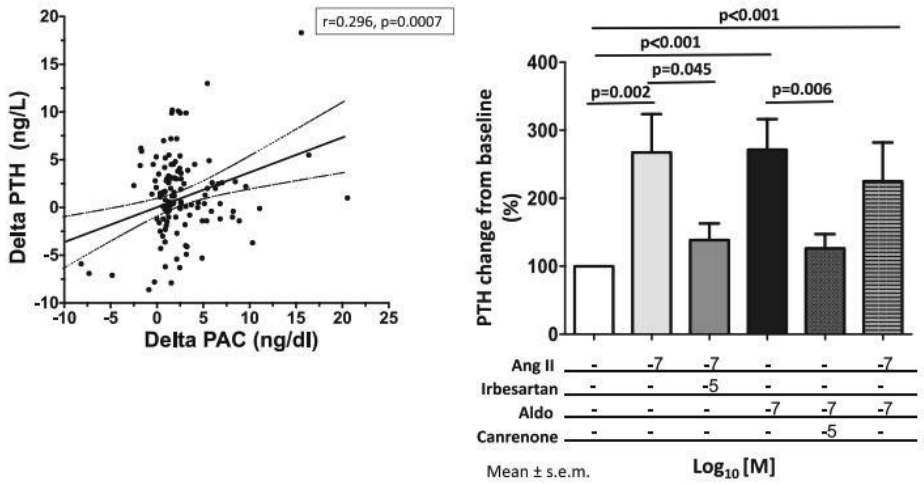


**Figure 2(on the left). Regression model for identification of variables correlated to the response of PTH secretion after captopril**. Considering all patients, the decrease in PTH was correlated with the response of aldosterone secretion after captopril stimulation. **Figure 3(on the right). Effects of aldosterone and angiotensin II stimulation on PTH secretion**. PTH levels after 6 h stimulation of primary parathyroid cells with angiotensin II ($10^{-7}$ M), AT-1R antagonist irbesartan ($10^{-5}$ M), aldosterone ($10^{-7}$ M), and MR($10^{-5}$ M). Experiments were repeated eight times in duplicate.

## Primary cultures of human parathyroid cells ex vivo

Eight parathyroid glands from consecutive consenting primary hyperparathyroidism patients were collected[21]. At histology, seven were

adenoma and one carcinoma. All cells were cultured to obtain a primary culture adherent after 24 hours. More than 95% cells were viable and grew in columnar aggregates, small clusters or pseudo-follicles. No difference in terms of cell growth and structure was seen in cells isolated from the carcinoma as compared with the adenomas. Immunocytochemistry confirmed the protein expression of PTH, CaSR, CGA, and GCM2 in primary cells up to seven days in culture[21]. The ability of the parathyroid cells to produce PTH was investigated after seven days of culture by incubating them at (1.25 mM) $Ca^{2+}$ concentrations [21]. These studies show that the capability to secrete PTH was maintained until seven days in presence of physiological (1.25 mM) $Ca^{2+}$ concentrations.

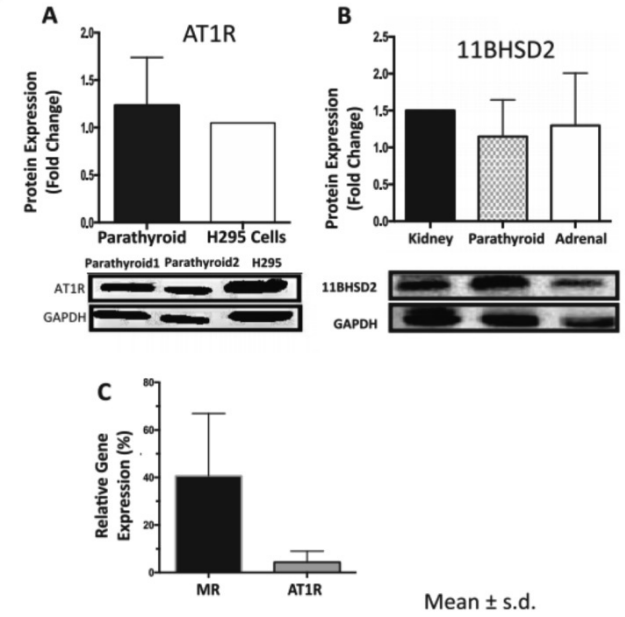


**Figure 4. Expression of AT-1R and 11bHSD2 in parathyroid tissue. (A)** Protein levels of AT-1R were assessed by immunoblotting in parathyroid and in H295 cells (positive control). **(B)** Expression of 11bHSD2 was measured in parathyroid, kidney, and adrenal gland (positive controls). Protein fold change was normalized using glyceraldehyde phosphate dehydrogenase (GAPDH). Representative examples of blotted membrane are reported for both proteins. **(C)** Relative expression of AT-1R and MR in parathyroid tissues measured by real time RT-PCR.

## Effects of aldosterone and angiotensin II stimulation on PTH secretion in human primary parathyroid cells

Cells exposure to ($10^{-7}$ M) aldosterone at a physiological $Ca^{2+}$ concentration (1.25 mM) increased PTH secretion after six hours (+271% ± 179% vs control,

P < 0.001). MR blockade with canrenone ($10^{-5}$ M), which had no effects per se, abolished the increase induced by aldosterone (P = 0.006; Fig. 3). Exposure of the cells to ($10^{-7}$ M) angiotensin II under identical conditions also stimulated PTH production (+267 ± 226% vs control, P= 0.002); this effect was abolished by the AT-1R antagonist irbesartan ($10^{-5}$ M) (138± 24% vs angiotensin II, P= 0.045; Fig. 3), which had no effect on PTH when given alone. Costimulation of the parathyroid cells with both angiotensin II and aldosterone had no additive effect on PTH secretion: PTH production (+225% ± 58%) did not differ from that seen with aldosterone (P=0.54) and angiotensin (P=0.64) alone.

**Expression of renin-angiotensinaldosterone system in parathyroid tissue**

We found that the human parathyroid tissues expressed both AT-1R and 11bHSD2 immunoreactive proteins (Fig.4A and 4B), as well as the AT-1R gene mRNA, which, however, was expressed at levels about 100-folds lower than the MR (Fig.4C).

# Discussion

Several studies have suggested a link between hyperaldosteronism and PTH secretion, but the relative importance of aldosterone and angiotensin II in modulating PTH release in humans remains uncertain. Immunohistochemistry and gene expression studies provided unambiguous evidence for the presence of the MR in the human parathyroid gland and the type 1 PTH receptor in the adrenocortical zona glomerulosa[13]. However, a randomized clinical trial failed to show any effect of the angiotensin II type 1 receptor (AT-1R) antagonist valsartan on serum PTH in postmenopausal women [22], possibly because these women showed normal serum PTH levels and a blunted renin-angiotensin-aldosterone system [23]. The present in vivo and ex vivo studies provided further evidence that can be useful for deciphering the interactions between adrenal and parathyroid glands, in that they showed that both aldosterone and angiotensin II regulate PTH biosynthesis, albeit with prominent differences between patients in whom aldosterone production is upregulated, as in PA, and those in whom it is not, as in patients with EH and adrenalectomized patients with APA cured of the hyperaldosteronism. In the patients with PA, and particularly in those with the florid clinical phenotype, the acute inhibition of angiotensin II generation with captopril had no effect on the overtly elevated serum PTH levels, thus further confirming previous findings in an expanded series [4,6]. Collectively, these results suggest that the parathyroid gland loses its ability to respond to acute angiotensin II inhibition when hyperaldosteronism coexists with increased PTH secretion. This responsiveness was instead evident in the hypertensive

patients without hyperaldosteronism: captopril lowered serum PTH, albeit slightly in patients with EH (Fig. 1), and also in patients with APA biochemically cured by adrenalectomy. Thus, the decrease of angiotensin II and/or of aldosterone induced by the ACE inhibition modulates PTH secretion in these patients. To get insight into the relative weight of angiotensin II and aldosterone, we examined the relation between the (delta) changes from baseline induced by captopril in the levels of PTH and aldosterone and renin, the latter taken as a proxy for angiotensin II inhibition. This analysis highlighted a correlation between changes of PTH and aldosterone, but not renin (Fig. 2), thus pointing to aldosterone as the prevailing modulator of PTH secretion in vivo in hypertensive patients. Further observations support a predominant role of aldosterone. First, in patients with PA who show oversecretion of aldosterone (despite blunted renin and angiotensin II), acute ACE inhibition did not lower PTH levels. Whether this was because of blunted angiotensin II levels, or because of failure to acutely correct the hyperaldosteronism, is an issue that deserves further ad hoc research. Second, cure of the hyperaldosteronism with adrenalectomy in APA resulted in a clear-cut captopril induced decrease of PTH, indicating restoration of the responsiveness of PTH when aldosteronism was corrected and/or with the upregulation of renin and angiotensin II (Fig. 1). Third, captopril did not lower PTH in the patients with PA with a non-lateralized aldosterone excess, i.e., with BAH in whom hyperaldosteronism was not abrogated (Table 1); moreover, the same was seen in two patients, who were adrenalectomized because of drug-resistant hypertension, in whom blood pressure resistance was resolved but PA was not biochemically cured. We cannot rule out at present a type error occurring in the smaller BAH group. However, considering the results seen in the PA group at baseline and in the BAH group at follow-up, we assert that the presence of hyperaldosteronism attenuates or abolishes the responsiveness of PTH to captopril-induced lowering of angiotensin II and aldosterone. Thus, these findings, in addition to confirming the association of hyperaldosteronism with hyperparathyroidism in humans, showed that the acute angiotensin II and aldosterone lowering decreases serum PTH levels only slightly in patients with PA unless hyperaldosteronism is surgically cured. It was claimed that the elevated PTH secretion would represent a compensatory mechanism to the PA-associated hypercalciuria, maintaining normal serum levels of ionized $Ca^{2+}$ at the expenses of bone reabsorption[11,24,25]. At variance with this contention, the present results obtained ex vivo, in addition to supporting the in vivo evidence involving the renin-angiotensin-aldosterone system in the regulation of PTH release, provided unambiguous evidence for a direct secretagogue effect of aldosterone on PTH secretion. It is worth noting that these experiments were made possible because for the first time to our knowledge, we succeeded in obtaining primary cultures of human parathyroid cells that maintained their capability to produce PTH [21]

and express specific parathyroid markers, including the CaSR, PTH, GCM2, and CGA in culture for the time frame of our experiments [21] . Notably, these cells also express MR [13] and AT-1R, which suggested their ability to respond to aldosterone and angiotensin II. Moreover, they showed detectable levels of 11bHSD2 (Fig. 4B), the enzyme inactivating cortisol to cortisone in aldosterone target tissues thus preventing MR activation by cortisol, which supports the conclusion that the human parathyroid gland entails a target tissue of aldosterone action. The effects on PTH production of the two main effectors of the renin-angiotensin-aldosterone system in these primary culture of human PTH-secreting cells ex vivo are to be emphasized (Fig. 3): when exposed to aldosterone these cells showed an increase of PTH secretion that was mediated by the MR, as it was cancelled by canrenone (Fig. 3). As mentioned above, this is consistent with the observation that the human parathyroid cells express MR predominantly in the nucleus [13], which suggests agonist-induced translocation of the receptor. However, in vitro aldosterone was not the only component of the renin-angiotensin-aldosterone system that affects PTH secretion. Angiotensin II also markedly stimulated PTH secretion from human parathyroid cells in primary culture through a mechanism that involved the AT-1R receptor inasmuch as this effect was abrogated by irbesartan, a specific AT-1R antagonist (Fig. 3). There are several possible reasons underlying this divergence between the in vivo and the ex vivo findings, which at present remain speculative and need further research. The limitations to be acknowledged in this study include: (i) the small number, which exposed the possibility of a type II error, of medically treated patients with PA challenged with captopril; (ii) the acute nature of our experiments that might raise questions concerning their relevance for chronic human diseases; and (iii) the fact that parathyroid cells from PTH-secreting neoplasms might not reflect the behavior of normal human PTHsecreting cells. However, this type of viable cells is not available for ethical reasons and no human cell line of PTH cells exists. Hence, our success in obtaining human PTH-secreting cells ex vivo suitable for an in vitro functional proof-of-concept study is a major accomplishment and strength of this investigation.

In summary, in addition to confirming that patients with PA have a mild form of hyperparathyroidism that in the long term can lead to osteoporosis, our ex vivo results also demonstrated that both angiotensin II and aldosterone stimulated PTH secretion by acting via the AT1R and the MR, respectively, albeit with no additive effect, which suggests that they might act through the same postreceptor signaling pathway as described in other models [27]. The acute inhibition of ACE with captopril lowered PTH in patients with EH and in patients with PA, but only after they were surgically treated. The correlation between the captopril-induced changes of aldosterone and PTH, along with the lack of such correlation between the changes of PTH and renin, collectively

implicate aldosterone as a predominant in vivo regulator of PTH secretion. On the whole, the correction of hyperparathyroidism in adrenalectomized patients with APA further supports the need to pursue definitive surgical cure of PA in a timely manner whenever possible.

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# Chapter 7

# Combining phosphate binder therapy with high vitamin K2 inhibits vascular calcification in an experimental animal model of kidney failure

Aegida Neradova[#] , Grzegorz Wasilewski[#] , Selene Prisco , P. Leenders Marjolein Caron , Tim Welting , Bert van Rietbergen, Rafael Kramann, Juergen Floege, Marc Vervloet*, Leon Schurgers*

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# Abstract

Hyperphosphatemia is a common feature in end stage renal disease and is strongly associated with cardiovascular disease and mortality. Therefore, phosphate binders (PBs) are widely used to lower phosphate levels however proof of risk reduction is lacking. Recently, PBs have been shown to bind vitamin K, thereby aggravating vitamin K deficiency which may offset beneficial effects of phosphate level reduction on vascular calcification (VC). Here we assessed whether combining PBs with vitamin K2 supplementation inhibits VC by controlling hyperphosphatemia and improving vitamin K-dependent inhibition of vascular calcification.

We performed 3/4 nephrectomy (3/4Nx) in Sprague-Dawley rats (male, n=90). Vitamin K-antagonist (warfarin) was given for three weeks to induce vitamin K deficiency. Next, animals were fed a high phosphate diet in the presence of low (5 μg/gram; n=45) or high (100 μg/gram; n=45) vitamin K2 and were randomized to either control or one of four different PBs (calcium carbonate, lanthanum carbonate, sevelamer carbonate and sucroferric oxyhydroxide; 1000 mg/kg/day; n=9/group) for eight weeks, after which the rats were sacrificed. Blood chemistry was evaluated to verify the validity of the chronic kidney disease (CKD) model. Thoracic and abdominal aorta were analyzed for VC using high resolution micro Computed Tomography (μ-CT), and by histochemical analysis with Alizarin Red and Von Kossa staining. Vitamin K status was analyzed using ucMGP specific antibodies.

3/4Nx resulted in a significant increase in both creatinine (from 37.1 to 75.7 μmol/L; $p < 0.01$) and urea levels (from 8.7 to 16.1 mmol/L; $p < 0.01$). PB treatment alone as well as high vitamin K2 alone did not reduce VC. However, the combination of high vitamin K2 diet and PB treatment significantly reduced VC compared to either treatment as monotherapy for the thoracic ($p=0.026$) as well as abdominal aorta ($p=0.023$). VC was measured by μ-CT and verified by Alizarin Red and Von Kossa staining. UcMGP staining was merely present at sites of VC especially in the low vitamin K2 treated groups in the thoracic ($p<0.01$) and abdominal aorta ($p<0.01$), indicating severe vascular vitamin K deficiency.

Here we provide evidence that in an animal model of kidney failure with vitamin K-deficiency, PB therapy nor vitamin K2 supplementation alone is

sufficient to prevent VC. However, our data indicate that vitamin K2 treatment in combination with PB treatment might provide a novel combination therapy to combat VC in chronic kidney disease. Whether this treatment also improves clinical outcome has to investigated.

# Introduction

Cardiovascular mortality increases progressively with advancing chronic kidney disease (CKD). Traditional risk factors for cardiovascular disease only partially explain these observations, pointing to a role of non-traditional risk factors such as uremia, hyperphosphatemia, oxidative stress and vitamin K deficiency [1]. Many of these risk-factors contribute to vascular calcification (VC), which is an important contributor to morbidity and mortality especially in late stage chronic kidney disease (CKD) [2]. One major established contributing factor for VC and mortality is a high serum phosphate concentration [3]. In late stage CKD both dietary interventions and phosphate binders (PBs) are used to target hyperphosphatemia. Generally, studies consistently show a slower progression of VC if calcium-containing PBs are avoided [4,5,6,7]. This is in line with in vitro data, showing that a slight increase of calcium on top of increased phosphate levels induces vascular smooth muscle cell calcification [8]. Lowering phosphate levels with non-calcium-containing PBs such as lanthanum carbonate and sevelamer carbonate has been shown to attenuate VC progression [4,5,6,7]. However, even with the use of non-calcium-containing PBs VC frequently progresses [9]. One explanation for this observation may be the overlooked effect of PBs on vitamin K status. Vitamin K is a key player in protection against VC, as it is a mandatory cofactor for the activation of matrix Gla protein (MGP). MGP is a vitamin K dependent protein produced and secreted by vascular smooth muscle cells and an important local inhibitor for calcification of the vessel wall [10]. In late stage CKD the prevalence of vitamin K deficiency is high [11]. This is, in part, due to dietary restrictions in potassium-rich dietary products (leafy green vegetables rich in K1) and in phosphate-containing food (dairy products such as cheeses rich in vitamin K2) [12]. Vitamin K deficiency is aggravated in patients using vitamin K antagonists [13]. Additionally, it has been established that vitamin K is bound by several PBs in vitro [14,15] as well as in vivo [16]. Recently it was demonstrated that clinical use of PB therapy was associated with increased serum levels of dephosphorylated undercarboxylated MGP (dp-ucMGP), a biomarker indicative of vascular vitamin K deficiency [17]. Increased serum levels of dp-ucMGP have been associated with increased VC and mortality [18,19,20]. We hypothesised that combining PB therapy with vitamin K2 supplementation will abolish the impact of PBs on vitamin K status, thereby preventing vitamin K deficiency and providing optimal therapy to reduce or hold progression of VC.

# Materials and Methods

## Ethical statement

The study was approved by Animal Experiments Committee of the University of Amsterdam and Maastricht animal care committee. All relevant licenses were obtained, and the study was performed according to national guidelines for the care and use of animals.

## Animals

Ninety male Sprague Dawley (Charles River, Ecully, France) weighing 220-250 gram at start of the experiment were used. Animals were maintained under conventional laboratory conditions, allowed to acclimatize one week before the experiments started and had full access to water and food (Teklad Diets, Madison WI, USA).

## 3/4 nephrectomy

Renal insufficiency was induced by a 3/4 nephrectomy. At least 24 hours prior to surgery, blood was drawn from the tail vein for serum reference levels. During a single surgical procedure, rats were anaesthetized with Isoflurane® (2,5%, with 40% oxygen) and received a s.c. injection of Temgesic® (0,03 mg/kg buprenorfinehydrochloride) 30 min before surgery to ensure analgesia during the procedure. The left kidney was exposed through an abdominal incision after which half of the arterial branches were ligated, directly followed by full ligation and removal of the right kidney. 6-8 hours after the first injection, administration of Temgesic® was repeated to prolong analgesia during recovery. Animals were kept individually for the first 24 hours post-surgery and daily checked to monitor recovery.

## Experimental design

One week after 3/4 nephrectomy, rats received a purified diet for three-weeks (Altromin, Lage, Germany), containing calcium 0,76%, phosphate 0,45%, 3 mg/g warfarin (Sigma) and 1,5 mg/g vitamin K1 (Sigma). This treatment model consists of concomitant administration of vitamin K1 and warfarin to overcome vitamin K antagonism in liver but not in extrahepatic tissues [21]. This enables us to study VC yet prevents major bleeding in the animals. Next, the diet was switched to a purified diet, calcium 1,34%, phosphate 1,2%, either with or without PB. The following PBs were used: calcium carbonate, lanthanum

carbonate, sevelamer carbonate and sucroferric oxyhydroxide. The diets contained 1000 mg PB per kilogram body weight per day, in both the high vitamin K2 (100 μg/gram; Nattopharma ASA, Oslo, Norway) groups and low vitamin K2 (5 μg/gram) groups for another 8 weeks (Figure 1). After the treatment period, rats were sacrificed and blood was collected from the portal vein into 105 mM trisodium citrate. Plasma was prepared and aliquots were frozen at -80°C until analysis. After bleeding, the rat vasculature was washed by injecting a sterile isotonic buffer (40 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 2.5 mM CaCl2, pH 7.3) via the left ventricle. Aortas were harvested, dissected and fixed overnight into 1% (v/v) Hepes-buffered paraformaldehyde, containing 150 mM saline at 4˚C, before embedding into paraffin.

**Biochemical measurements**

Before surgery and before sacrifice the following data were collected: serum creatinine, urea, calcium and phosphate levels. Measurements were conducted by standard laboratory techniques.

**Quantification of calcification by high resolution micro Computed Tomography**

All groups of rats (5-9 per group) underwent ex vivo high resolution scanning of the thoracic and abdominal aorta including renal artery and cartilage of tibias using micro-computed tomography (micro-CT) at 55 kV and 200 μA (microCT100 Scanco Medical, Bruettisellen, Switzerland) of paraffin embedded aortas. Thoracic and abdominal aorta were scanned and the resolution was set to 24.5 μm isotropic. Tissue with mineralization exceeding 50 milligrams of hydroxyapatite per cubic centimeter (mgHA/$cm^3$) was segmented from the image using a single threshold and the total volume of this mineralized tissue was calculated (Figure 2). This method is sensitive to measure early signs of calcification, takes the whole tissue into account, and leaves the tissue intact for further histochemical analysis.

**Immunohistochemical analysis**

Aortic arches including carotid arteries and abdominal arteries, including renal arteries were collected upon sacrifice, fixed in formalin embedded paraffin. Paraffinized tissues were cut in tissue sections of 4 μm. The tissues were stained

for Alizarin Red, Von Kossa and uncarboxylated MGP (ucMGP). For antibody based immunohistochemical imaging, sections were stained with primary antibodies for ucMGP (1:25; IDS, Boldon, UK). Secondary antibodies used were goat anti-mouse HRP conjugated IgG (1:1000, Dako). Antibodies were visualized by red alkaline substrate kit I (Vector SK-5100; Vector Laboratories, Burlingame; CA); nuclei were counterstained with hematoxylin. Semi-quantitative analysis of ucMGP was performed by a blinded single reader. Scores for 2 tissue sections with 4 independent regions of the thoracic and abdominal aorta per animal group were determined. The extent of ucMGP signal was quantified on a 'absent' (0), 'light' (+), 'moderate' (++), or 'heavy' (+++).

### Statistical analysis

Statistical analysis was performed using Prism version 6 (GraphPad Software INC; San Diego, CA, USA). Data are presented as mean ±SD unless stated otherwise. All data were analyzed using Mann-Whitney for the difference between two groups and Kruskal-Wallis for the difference between three or more groups since the data were not normally distributed.

## Results

### Blood chemistry

At the end of the study, 3/4Nx surgery resulted in increased serum creatinine and urea concentrations from 37.1 μmol/L to 75.7 μmol/L and from 8.7 mmol/L to 16.1 mmol/L, respectively ($p < 0.01$ for both). Both calcium and phosphate levels did not change significantly (2.52 mmol/L to 2.39 mmol/L; $p= 0.131$ and 2.14 mmol/L to 2.09 mmol/L; $p= 0.586$, respectively). There were no significant differences between the groups in phosphate levels, calcium levels or kidney function at the begin or the end of the study (Table 1 and 2).

| | Vitamin K2 high group | | Vitamin K2 low group | |
|---|---|---|---|---|
| | Control (mean ±SD) | PB group (mean ±SD) | Control (mean ±SD) | PB group (mean ±SD) |
| Creatinine (µmol/L) (SD) | 28.0 (10.5) | 49.9 (68.5) | 53.6 (48.1) | 34.8 (16.0) |
| Urea (SD) (mmol/L) | 6.9 (1.6) | 11.4 (14.0) | 12.1 (9.2) | 8.5 (2.9) |
| Calcium (SD) (mmol/L) | 2.43 (0.65) | 2.49 (0.45) | 2.84 (0.24) | 2.5 (0.43) |
| Phosphate (SD) (mmol/L) | 2.20 (0.44) | 2.24 (0.94) | 2.56 (0.85) | 2.19 (0.49) |

**Table 1** Baseline data for creatinine, urea, calcium and phosphate in animals on high or low vitamin K diet with or without phosphate binder use. There were no significant differences between the groups.

| | Vitamin K high | | Vitamin K low | |
|---|---|---|---|---|
| | Control (mean ±SD) | PB group (mean ±SD) | Control (mean ±SD) | PB group (mean ±SD) |
| Creatinine (µmol/L) (SD) | 63.67 (42.7) | 75.7 (38.4) | 72.8 (61.1) | 79.7 (45.6) |
| Urea (SD) (mmol/L) | 12.9 (4.8) | 16.1 (6.9) | 16.0 (11.2) | 17.0 (45.6) |
| Calcium (SD) (mmol/L) | 2.39 (0.22) | 2.39 (0.23) | 2.27 (0.37) | 2.40 (0.20) |
| Phosphate (SD) (mmol/L) | 2.11 (0.42) | 2.09 (0.58) | 2.12 (0.78) | 2.11 (0.80) |

**Table 2** End of study data for creatinine, urea, calcium and phosphate in animals on high or low vitamin K diet with or without phosphate binder use. There were no significant differences between the groups.

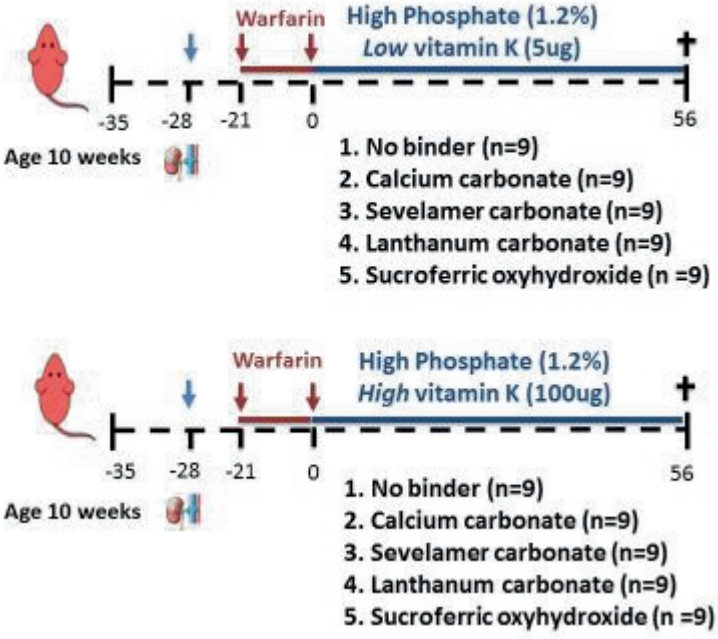


**Figure 1** Study set up, one week after 3/4 nephrectomy animals were started on 3 weeks course warfarin and subsequently on a high or low vitamin K diet combined with a phosphate binder or no binder

## Vascular calcification detected by aortic micro-CT thoracic and tissue staining

In the low vitamin K2 group, all tested PBs failed to inhibit VC as measured by micro-CT in the thoracic ($p=0.057$) and abdominal aorta ($p=0.82$) compared to animals not taking PB (Figure 3). In animals not treated with PBs there was no statistically significant difference in VC between animals treated with high or low vitamin K2 diet ($p=0.95$). On the contrary, rats in de high vitamin K2 group on PBs developed significantly less VC as measured by micro-CT for both the thoracic ($p=0.026$) and abdominal aorta ($p=0.023$; Figure 3) compared to control. There was no statistically significant difference among the different binders tested, nor compared to the control for individual binders (supplemental Figures 1 and 2). The presence of VC, as quantified by micro-CT, was confirmed by von Kossa and Alizarin Red staining after sectioning the same tissues. Both von Kossa and Alizarin Red staining revealed calcification in line with the micro-CT results (Figure 4).

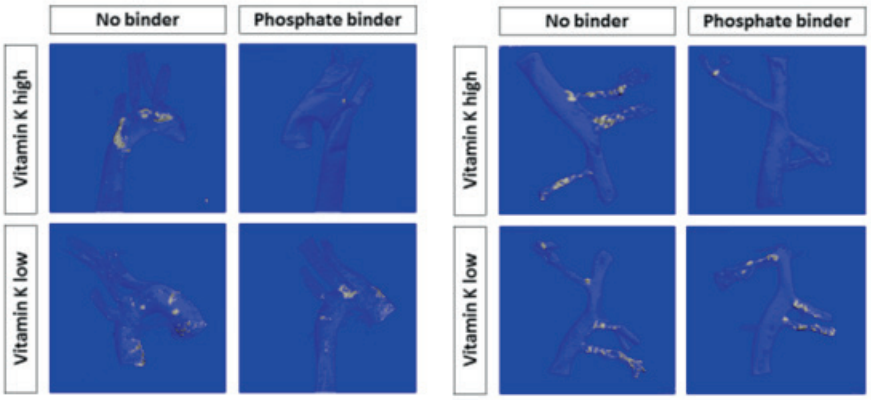


**Figure 2** Pictures of vascular calcification as captured by micro-CT scan, aortic arch and thoracic aorta and abdominal aorta with renal arteries

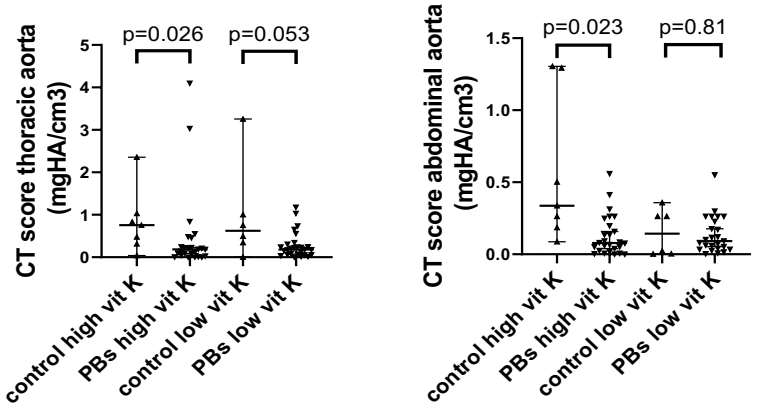


**Figure 3** Vascular calcification score measured by micro-CT scan for thoracic and abdominal aorta, the score is depicted in hydroxyapatite per cubic centimeter (mgHA/cm3) with a median and 95% confidence interval for control and pooled phosphate binder groups

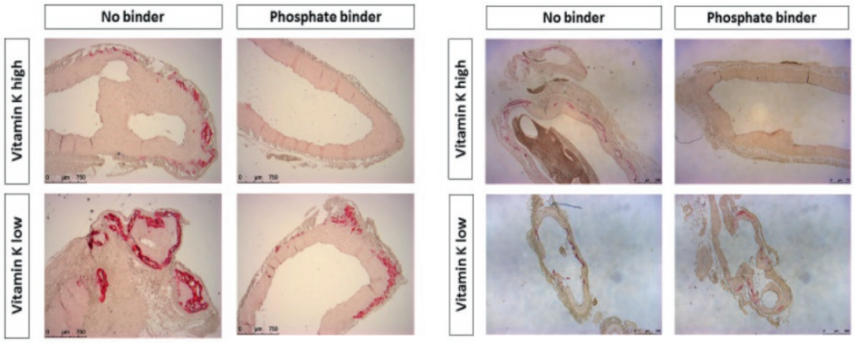


**Figure 4** A Alizarin red staining of the thoracic and abdominal aorta in vitamin K2 high and low groups in control or in phosphate binder group .

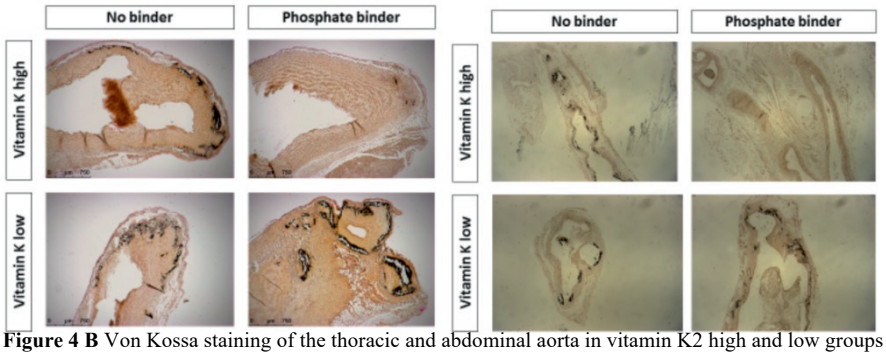


**Figure 4 B** Von Kossa staining of the thoracic and abdominal aorta in vitamin K2 high and low groups in control or in phosphate binder group

## ucMGP accumulation in the vascular wall

Immunohistochemical staining for ucMGP in the vessel wall revealed ucMGP present at sites of VC. Presence of ucMGP was mainly detectable in the low vitamin K2 treated groups. The low vitamin K2 group had significantly higher expression of ucMGP in both the thoracic ($p<0.01$) as well as abdominal aorta ($p<0.01$) compared to the high vitamin K2 group (Figure 5). There was no difference between the control and PBs in either the high ($p=0.95$) or low vitamin K2 groups ($p=0.58$; Figure 6). However there was less VC in the group on PBs on the high vitamin K diet compared to the group on PBs on the low vitamin K diet respectively for thoracic ($p$ 0.013) and abdominal ($p< 0.01$) aorta.

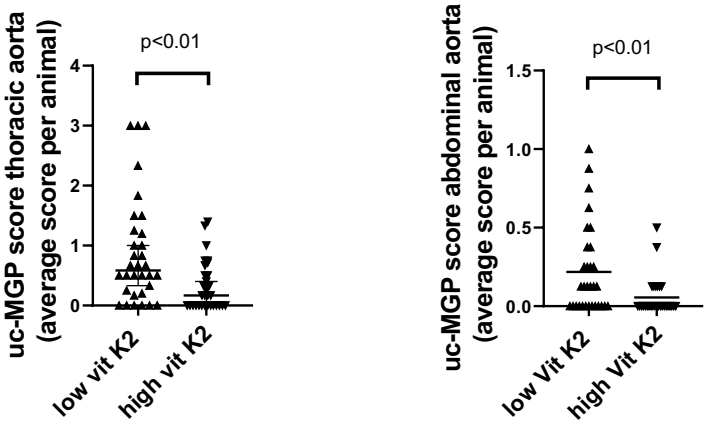


**Figure 5** ucMGP expression in vitamin K high and low groups, the score is depicted in median and 95% confidence interval for thoracic and abdominal aorta

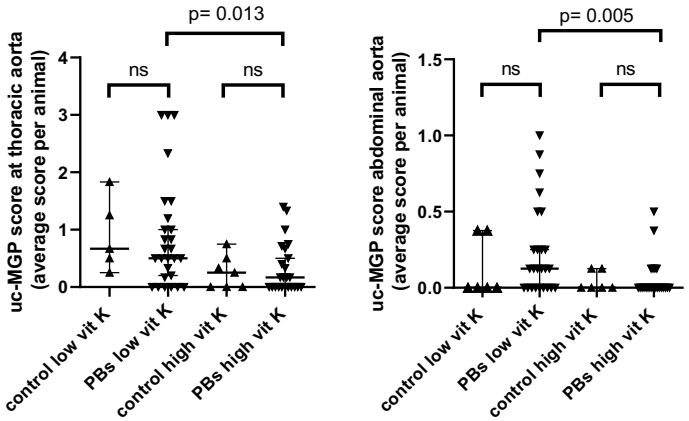


**Figure 6** ucMGP expression in vitamin K high and low groups and control versus phosphate binders the scor is depicted in median and 95% confidence interval for thoracic and abdominal aorta

### Micro-CT cartilage

Besides being produced by vascular smooth muscle cells, MGP is being produced by chondrocytes. Therefore, to further validate the protective effect on calcification of PB treatment in combination with high vitamin K2, we analyzed the amount of cartilage calcification of the tibia. In the PB groups on low vitamin K diet, cartilage calcification was evenly present and not different between groups (p=0.90). However, PB treatment in combination with high vitamin K2 resulted in significantly less cartilage calcification compared to control (p<0.01; supplemental data Figures 3).

## Discussion

In this study we demonstrate that the use of PB treatment in a kidney failure (CKD) animal model including vitamin K deficiency is not sufficient to prevent ectopic calcification. However, combining PBs with high vitamin K2 supplementation strongly attenuated VC. This protective effect on calcification is likely accomplished by the synergistic effect of combined PB treatment and vitamin K2 supplementation. To the best of our knowledge, this is the first in vivo preclinical study combining PB therapy with vitamin K2 supplementation. In animal models without CKD 21 and even in animals with CKD [22,23] vitamin

K supplementation has been shown to inhibit VC. Additionally, PB therapy in CKD animals demonstrated reduction in VC to some extend [24,25,26]. However, none of these animal models combined CKD with vitamin K deficiency, as is the clinical situation for the majority of late stage CKD patients 11. Both vitamin K deficiency [27] and increased phosphate levels [28] are associated with increased morbidity and mortality in CKD. In our model, CKD animals were primed vitamin K deficient by pretreatment with warfarin and provided a high phosphate diet. Additionally, animals were treated with different PBs in combination with low intake of vitamin K2, thereby mimicking the clinical situation in late stage CKD [27,28]. Patients with late stage CKD are recommended to lower both phosphate and potassium intake. These recommendations, however, also limit intake of vitamin K as leafy green vegetables (as source of potassium) are rich in vitamin K1 and cheeses (rich in phosphate) are a major source of vitamin K2 [12]. On top of these untoward effects of dietary intervention on vitamin K intake, different PBs have shown, both in vitro and in vivo, to bind also vitamin K, thereby limiting its bioavailability [14,15,16]. Moreover, in patients on renal replacement therapy and after transplantation, an association of sevelamer carbonate use and increased dp-ucMGP levels was observed, suggesting that PB therapy aggravates vitamin K deficiency [24]. However, in our study there was no difference in VC or ucMGP levels between the distinct PB's. This may be due to a type II error, given the small number of animals per phosphate binder. Nevertheless, the use of calcium -containing PBs in our study did not aggravate VC, as seen in clinical studies with calcium-containing PBs compared to calcium free PBs [4,5,6,7] either. The relative short duration of our experiment, compared to clinical CKD, may explain the absence of difference in calcification with use of calcium-containing and non-calcium-containing PBs. To test whether vascular vitamin K deficiency was present, we quantified ucMGP in thoracic and abdominal aorta tissue. In the vessel wall of CKD animals treated with PBs in combination with low vitamin K, ucMGP colocalized extensively with VC. However, when PBs were combined with high vitamin K2 intake, significantly less ucMGP and VC was present in the vessel wall. Our findings are in line with previous data in experimental animals and suggest that vitamin K deficiency is a risk factor for developing VC [21]. However, both PB treatment and vitamin K supplementation are required to reduce VC in our CKD model. VC in CKD patients is predominantly linked to

medial calcification [29], a condition that is more present in the abdominal aorta and peripheral arteries [30]. VC in CKD is also located at the aortic arch and the thoracic aorta [31]. Therefore, we analyzed calcification in both the thoracic and abdominal aorta and found VC to be increased at both vascular sites in CKD animals treated with PBs and low vitamin K2 intake. UcMGP expression could be detected at both vascular anatomical sites, suggesting involvement of vitamin K at both locations. Vitamin K antagonist treatment, inducing a vitamin K deficiency, is known to induce vascular as well as cartilage calcification 32. MGP is produced by both vascular smooth muscle cells and chondrocytes. Additionally, cartilage calcification is a feature which is present in dialysis patients [38,39] suggesting a process similar to VC. Therefore, we analyzed calcification of cartilage to confirm the impact of vitamin K2 treatment in combination with PB on MGP. Indeed, also in cartilage we found that the combination therapy resulted in significantly less calcification as compared to PB treatment in combination with low vitamin K2 intake.

There are a few limitations of this study. In our rat model the severity of CKD was less pronounced as compared to other CKD models [24.] This could be due to 3/4 nephrectomy procedure compared to animal models using adenine [24] or 5/6 nephrectomy [35] to induce CKD. In our model, there was no change in phosphate levels after the 3/4 nephrectomy as compared to baseline. This is, however, in line with what has been described previously [36,37], and also in clinical CKD hyperphosphatemia is a late stage feature [38]. Also, we did not collect urine so we cannot exclude a difference in phosphate balance. Finally, this is an experimental animal study and thus the results can be translated to clinical practice with great caution only and need to be confirmed in the clinical setting.

Our study also has several strengths. We have included a broad variety of PB's in this model and measured a great number of parameters, which are known to have an effect on VC. We have used CT as highly sensitive and specific technique for measurement of VC ex vivo. In this way we analyzed total VC throughout the vasculature and kept the tissue available for (immuno)histochemical analysis.

In this study the use of PB monotherapy, effective in lowering serum phosphate levels, hardly inhibits progression of VC [8,39]. Also, vitamin K2 supplementation alone seems not to be efficient in reducing the progression of VC in CKD [40].

Because vitamin K absorption is likely to be reduced by PBs, monitoring of vitamin K status should be considered to assess optimal treatment dosage. We conclude that high dose vitamin K2 may be needed in combination with PB use to significantly counteract VC. Future clinical studies should analyze the combination of vitamin K2 supplementation with PB therapy to hold progression of VC, changes in arterial stiffness and cartilage calcification.

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**Supplemental data**

**Figure 1**

Vascular calcification score in the low and high vitamin K group measured by micro-CT scan depicted in hydroxyapatite per cubic centimeter (mgHA/cm3) with a median and 95% confidence interval for thoracic aorta. Abbreviations caco calcium carbonate, lanco lanthanum carbonate, sevel sevelamer carbonate and sfoh sucroferric oxyhydroxide, low: low vitamin K diet, high: high vitamin K diet

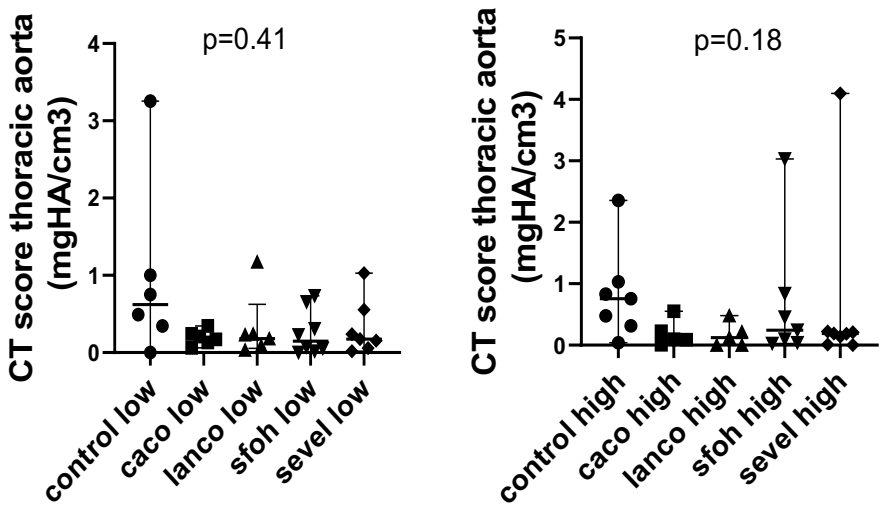



**Figure 2**

Vascular calcification score in the high vitamin K group measured by micro-CT scan depicted in hydroxyapatite per cubic centimeter (mgHA/cm3) with a median and 95% confidence interval abdominal aorta. the score is depicted in hydroxyapatite per cubic centimeter (mgHA/cm3) with a mean and 95% confidence interval. Abbreviations caco calcium carbonate, lanco lanthanum carbonate, sevel sevelamer carbonate and sfoh sucroferric oxyhydroxide, low: low vitamin K diet, high: high vitamin K diet.

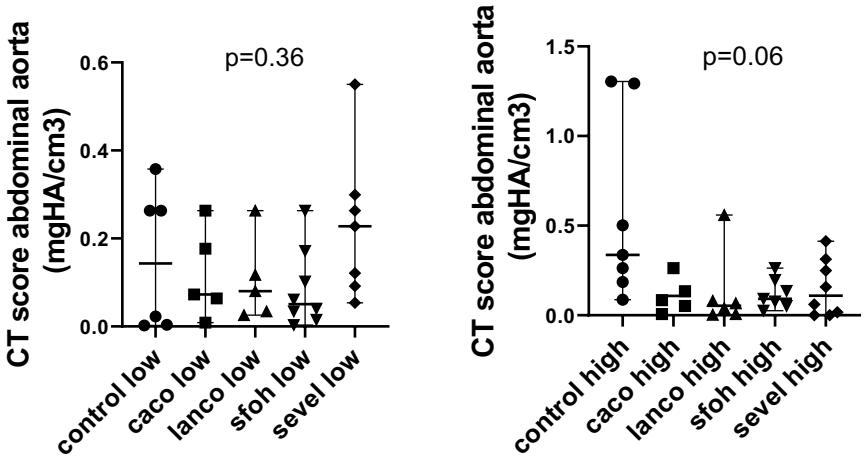
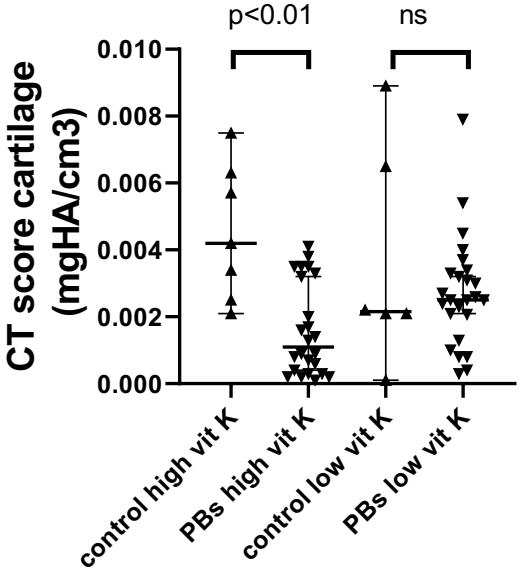



**Figure 3**

Ectopic calcification in the cartilage of the tibia (knee joint) measured by micro-CT scan depicted in hydroxyapatite per cubic centimeter (mgHA/cm3) with a median and 95% confidence interval.



# Chapter 8

# General Discussion

# General Conclusion

Arterial hypertension is considered a major cardiovascular risk factor with a prevalence of 24.1% in adult males and 20.1% in adult females[1]. Detection of primary aldosteronism, the most common form of secondary hypertension, is pivotal for targeted management of the underlying disease and prevention of cardiovascular damage. For this purpose, animal and human studies are elemental for both supporting clinical relevance between aldosterone and parathyroid hormone (PTH) levels and the impact of this interaction on cardiovascular (CV) health. The identification of PTH-receptors on zona glomerulosa (ZG) cells and mineralcorticoid receptor (MR) on parathyroid chief cells further supported the close interplay between these hormonal systems. This finding is further reinforced by clinical data demonstrating that treatment of both hyperparathyroidism and hyperaldosteronism is effective in normalizing levels of both PTH and aldosterone. The interaction of aldosterone and PTH was also shown to have therapeutic implications in different groups of CVD patients with adverse renal, cardiovascular, metabolic and bone outcomes. Manipulating the aldosterone and PTH association by interfering with either synthesis or target receptors may potentially improve CV health. Accordingly, the aim of my thesis was to elucidate mechanisms by which aldosterone synthesis is regulated, the pathological context of primary aldosteronism with a focus on PTH modulation and the overall impact on the cardiovascular system.

**Aldosterone stimulates its biosynthesis via several mechanisms**

Numerous novel data generated in the last decades has elucidated the molecular bases of human Primary Aldosteronism (PA) and prompted us to investigate the regulating mechanisms of aldosterone synthesis.

The work presented in my thesis starts from the identification of the chimeric gene responsible for FH-1 to the most recent discovery of mutations in the gene CLCN2 coding for ClC-2 chloride channels defined as FH-2. Next, my interest shifted from these inherited mutations to the identification of somatic mutations involving modifications of the intracellular ionic equilibrium and regulation of cell membrane potential. Alterations that result in autonomous aldosterone over-production[2].

Interestingly, a recent European study performed on 474 Aldosterone producing adenoma (APA) identified somatic mutations in 54.3% of these APA and 38% of these mutations were found in the KCNJ5 gene[2]. Thus, it is of great importance to provide novel therapeutic tools blunting aldosterone synthesis specifically in tumors carrying the two most common KCNJ5 mutations, L168R and G151R. In our study, we showed that clarithromycin-antibiotic was instrumental for this purpose. In fact, it was recently shown in vitro that the macrolide antibiotic corrected the altered electrophysiology of cells transfected with L168R and G151R mutations in Kir 3.4 channel (KCNJ5)[3]. Over the last years, an additional family of potassium channels (TASK2) was found that play a role in the underlying molecular mechanisms responsible for primary aldosteronism. This class of channels encoded by KCNK5 genes, generates constitutive or leaky $K^+$ currents that are essential for maintaining a negative resting membrane potential. Previous data from our group showed that in 30% of APA cases the decreased expression of TASK2 could be explained by enhanced expression of miRNA23 and miRNA34. The enhanced expression of miRNA23 and miRNA34 were shown to blunt TASK2 gene expression by binding to the 3'UTR of the KCNK5[4]. In Chapter 4, we investigated genetic variations in the promoter region of TASK2 gene and concluded that these could explain the development of PA in some 25% of APA cases.

This thesis cannot be read as a single monograph, and the fil rouge that runs through the chapters presents discoveries of factors responsible for the most common form of secondary hypertension, namely primary aldosteronism.

Intriguingly, our observations reported in Chapter 3 show that estrogens have a novel role in the regulation of aldosterone synthesis via GPER activation. Thus, our findings might explain the rapid and potent enhanced concentration of aldosterone via self-perpetuating hyperaldosteronism despite the blunted RAAS. Moreover, the progress in understanding the mechanisms of the pathogenesis of PA highlight the importance of a diagnostic workup from screening to subtyping. Nonetheless, our work encourages better education of primary care providers and to implement the knowledge of PA screening in clinical practice.

**Aldosterone and PTH exercise a role in the PTH secretion**

The detection of the MR in parathyroid cells, alongside recent implications of PTH in aldosterone production in primary aldosteronism, suggests a relationship between parathyroid and the adrenocortical zona glomerulosa. Several experimental studies have been performed trying to clarify mechanisms underlying the effect of PTH on adrenal aldosterone secretion from the adrenal. It is known that PTH stimulates the entry of cytosolic $Ca^{2+}$ into the mitochondrial matrix which is essential for the initiation of steroidogenesis within the mitochondria[5,6]. However, it is still unclear whether PTH stimulates adrenal aldosterone synthesis directly.

The effect of PTH on $Ca^{2+}$ mediated aldosterone secretion in isolated rat adrenocortical zona glomerulosa (ZG) cells was investigated and aldosterone release significantly increased above baseline values in cells exposed to PTH (1–34), suggesting that PTH exerts $Ca^{2+}$ ionophore-like effects in the ZG causing increased $Ca^{2+}$ stimulated aldosterone secretion[7]. More recently, Mazzocchi et al. demonstrated in human adrenocortical cells that PTH and PTH-related protein increased aldosterone production by binding to the PTH/PTH-rP receptor, subsequently activating adenylate cyclase/PKA and PLC/PKC-dependent signaling cascades[8].
In chapter 6 we show the importance of the hormonal systems in development of PA. Clarifying the role of aldosterone and angiotensin II on RAAS in the regulation of PTH provides further deciphers the interactions within the endocrine system. Our group is the first that succeed in culturing primary human parathyroid cells for an in vitro proof of concept study. Accomplishing the in vitro use human parathyroid cells can be considered a pioneering study that might inspires future and more in-depth studies.

**The precarious equilibrium between endocrine and vascular system plays a role in CVD progression**

The endocrine system plays a pivotal role in regulating and controlling the vascular system. An endocrine dysfunction may have a significant impact on the vascular system. Moreover, normalizing endocrine activity correlates with reversal of adverse cardiovascular changes. In this context, it has been recently

reported that administration of PTH after myocardial infarction attenuates ischemic cardiomyopathy by increasing migration of bone marrow-derived stem cells to the ischemic myocardium[9].

Other studies reported that deregulation of aldosterone or PTH plays an important role in the development and progression of CV disease. In fact, it has been shown that long-term PTH excess in primary hyperparathyroidism (PHPT) is linked to adverse effects such as bone loss and increased fracture risk, coronary microvascular dysfunction, aortic valve calcification, increased aortic stiffness, endothelial dysfunction and arterial hypertension [10–13]. Accordingly, patients with PHPT have a remarkably higher CV mortality risk as compared to the general population[14,15].

Elevated PTH levels in patients with declining kidney function has been related to vascular calcification and subsequent adverse CV disease [16]. Therefore, in the last part of my thesis, we have addressed how hormonal imbalances contribute to CV changes. Among the most prevalent complications of CV diseases is vascular calcification. In CKD, increased PTH levels are a compensatory mechanism for dietary calcium deficiency, disorders of vitamin D and increased levels of phosphate[17].

In chapter 7, we investigated vitamin K as key player in vascular calcification. Vitamin K is a cofactor for the carboxylation of vitamin K dependent proteins which are crucial to prevent VC. ESRD is characterized by increased phosphate levels and vitamin K deficiency, both contributing to progression of VC [18]. Adequate levels of vitamin K are mandatory to activate MGP a vascular calcification inhibitor produced by vascular smooth muscle cells[19]. Phosphate binders (PBs) are used to lower phosphate absorption thereby lowering circulating levels of phosphate[20,21]. However, PBs have limited effect on inhibition of vascular calcification and vascular mortality. One explanation is that PBs also bind vitamin K, thereby aggravating the vitamin K deficiency. Therefore, we tested the combination of PBs and vitamin K in a CKD rat model. In our experiment, we demonstrate that PB treatment combined with high vitamin K2 inhibits vascular calcification whereas both high vitamin K2 or PB treatment alone did not. This effect of combined PB and high vitamin K2 probably lies in the reduced levels of inactive ucMGP. Our results in Spradue-Dawley rats support a proof of principle study with PB and vitamin K2 in ESRD patients to inhibit progression of VC.

Although we are just at the beginning of unraveling the complex relationship between aldosterone and PTH, there is no doubt that our findings propel the search for novel treatment strategies to prevent CV disease. Considering that CV disease is still the number one killer in the Western world, it would be valuable to measure circulating levels of PTH and aldosterone in subjects with increased CV risk.

## Conclusion remarks and future perspectives

The aim of this thesis was to gain further insight in the role of aldosterone and parathyroid hormone (PTH) in the pathological context of primary aldosteronism and vascular calcification. We provided new insights in the association of endocrine factors in the development and progression of vascular disease. Our work also highlights to carefully reconsider the use of phosphate binders in CKD patients because of vitamin K-deficiency. This has strong clinical implications since phosphate binder therapy is the recommended treatment in dialysis patients. Finally, our results suggest that phosphate binder therapy combined with high vitamin K2 prevent the progression of vascular calcification in patients with ESRD. Interestingly, a recent study demonstrated a strong association between inactive dp-ucMGP and high levels of aldosterone[22]. Furthermore the relationship between aldosterone and increased arterial stiffness has already been reported[23,24]. Thus, our findings could result in studies investigating the potential role of MGP on arterial stiffening in patients with high aldosterone levels.

Taken together, the research presented in this thesis provides further insight into the mechanisms regulating aldosterone synthesis with a focus on PTH modulation and the overall impact on the cardiovascular system.

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# Addendum

Summary

The present thesis focuses on mechanisms describing the bidirectional interaction between aldosterone and PTH and the impact on the cardiovascular system.
Although only a small proportion of cases of primary aldosteronism (PA) are familial, investigation of these cases has been elemental for understanding the molecular basis of the disease. One such example is the mechanism of apparent autonomy in hyperaldosteronism by the renin-angiotensin system. **Chapter 1** summarizes current knowledge on familial forms of PA and the association with main gene mutations. These familial genetic abnormalities are present as germline mutations in PA and account for up to 5% of cases and are inherited in an autosomal dominant manner. To date, four different forms of familial hyperaldosteronism (FH) have been described. FH-1 is caused by an unequal crossing-over of the promoter sequence of the CYP11B1 gene with the coding region of CYP11B2. As a consequence, the regulatory region of the resulting chimeric gene is altered such that the CYP11B2 gene becomes responsive to the adrenocorticotropic hormone (ACTH). FH-2 is caused by gain of function mutations in the gene CLCN2 coding for ClC-2 chloride channels. These mutations result in an enhanced chloride efflux and membrane depolarization causing increased CYP11B2 expression and aldosterone overproduction. In FH-3, Kir3.4 (KCNJ5) mutations cause a loss of selectivity for $K^+$, $Na^+$ influx, depolarization of the cell, with subsequent increase in intracellular $Ca^{2+}$ via voltage-gated T-type $Ca^{2+}$ channels and autonomous production of aldosterone. Finally, in FH-4, the gene encoding for T-type Cav3.2 (CACNA1H) activates channels at less depolarized potentials with ensuing increased $Ca^{2+}$ influx.

**Chapter 2** describes new horizons for diagnosis and treatment of aldosterone-producing adenoma caused by a somatic mutation in the potassium channel Kir 3.4 (KCNJ5), which explains for some 30% to 70% of PA cases. We tested effects of clarithromycin-macrolide on aldosterone synthesis and secretion in a population of aldosterone-secreting cells, obtained by immuno-separation of $CD56^+$ cells from APA tissue, with or without the 2 most common KCNJ5 mutations (G151R and L168R). When exposed to increasing concentrations of clarithromycin, $CD56^+$ cells obtained from wild-type APAs showed no change of CYP11B2 gene expression and aldosterone secretion in response to the antibiotic macrolide. Conversely, both G151R and L168R APA cells exposed to clarithromycin showed a concentration-dependent consistent blunted expression of the aldosterone synthase gene. Our results provide compelling evidence of the feasibility of blunting aldosterone synthesis specifically in aldosterone-

producing cells from tumors carrying the 2 most common KCNJ5 mutations, paving the way towards personalized treatment.

**Chapter 3** highlights the role of estrogens in the context of autonomous over-secretion of aldosterone in PA. GPER, a G protein coupled estrogen receptor, is highly expressed in the normal human adrenocortical zona glomerulosa. Our previous results suggest GPER to have a potent secretagogue effect of 17b-estradiol on aldosterone when the estrogen β receptor is blocked. Multiple observations subsequently showed GPER to promiscuously bind other steroids, and mediate MR-independent aldosterone effects in various cell types. Based on these findings, we set up a study to investigate whether aldosterone actives GPER and acts as its own secretagogue. This was studied both in vitro using a human adrenocortical cell line (HAC15) and ex vivo using APA tissues (cut into 2-3mm) obtained at adrenalectomy from a cohort of primary aldosteronism patients with aldosterone-producing adenoma (APA). We found that aldosterone stimulated expression of CYP11B2 mRNA, mitochondrial $Ca^{2+}$ and NADH-dependent step responsible for its biosynthesis. This effect occurred both in vitro and ex vivo in APA. Our findings provide important information on the regulation of aldosterone secretion under pathological and physiological conditions. Furthermore, using a pharmacological approach to block the MR and the GPER, we found that only GPER is the main mediator of CYP11B2 activation in response to aldosterone. Based on our data we propose an autocrine-paracrine mechanism whereby aldosterone, acting via GPER, increases its own biosynthesis and release.

In **Chapter 4** we present mechanisms underlying APA associated with a blunted expression of the twik-related acid-sensitive $K^+$ channel 2(TASK-2). For that, we investigated functional mutations in the promoter sequence of the KCNK5 gene known to lead to low expression of TASK-2. C999T, the most prevalent mutation detected in APA, significantly decreases transcriptional activity of the TASK-2 gene by some 35%, being a significant decrease compared to the percentage observed in WT. It is worth mentioning that the blunted expression of TASK-2 channel was also inversely related to the expression of 13 microRNAs. From these, only miR-23 and miR-34 significantly blunted expression of TASK-2, explaining only 25% of APAs. Thus, the low expression of TASK2 is associated with other genetic variations

acquired by the adrenal gland and/or with environmental factors, coinciding with development of PA.

In **Chapter 5** we report important limitations of a published double-blind placebo-controlled trial investigating whether PTH levels are lowered by short-term treatment with an Ang-II receptor blocker (valsartan) in postmenopausal women with low 25(OH)D levels. The hypothesis comes from evidence supporting the relevant interaction between parathyroid hormone (PTH) and the renin-angiotensin-aldosterone-system (RAAS). Interestingly, they found no effects of RAAS blockade with ARB treatment on PTH levels. Our objection to this statement was that Ang-II has a role in regulating PTH release, yet cannot be conclusively ruled out based on this trial. Firstly, the authors decided to recruit normotensive woman with no hyperparathyroidism, and thus cannot use a full BP lowering dose of valsartan. Secondly, the assessment of PTH changes can be influenced by lack of standardization of food, calcium and sodium intake during the study. Finally, considering that RAAS is progressively blunted by aging, it is conceivable that the effect of valsartan was low in 60-80 year old women that were selected for this study.

**Chapter 6** describes the interaction between the adrenocortical zona glomerulosa and the parathyroid gland in both in vivo and ex vivo experimental data. In vivo, we examined PTH secretion when blocking angiotensin II in hypertensive patients with PA and in patients with EH. Interestingly, captopril, an inhibitor of angiotensin-converting enzyme, lowered PTH levels both in patients with EH and in patients with APA after adrenalectomy. Next, we used primary human parathyroid cells isolated from patients receiving surgery for primary hyperparathyroidism. We showed that both angiotensin II and aldosterone stimulated PTH secretion by acting via the AT-1R and the MR, respectively.

In **Chapter 7** we present the first in vivo study that investigates the combination of phosphate binders (PBs) with high vitamin K2 intake in a rat model of chronic kidney disease (CKD). It is known that patients with ESRD receive phosphate binders to lower circulating levels of phosphate, a known inducer of vascular calcification and vascular disease. However, although phosphate binders lower circulating phosphate, they do not decrease vascular calcification. One explanation is that phosphate binders also bind vitamin K, thereby further

aggravating the vitamin K deficiency of ESRD patients. Animals were given high phosphate combined with high or low levels vitamin K2 in their diet. Intriguingly, we found less VC in animals on a phosphate binder plus high vitamin K2 diet compared to that of phosphate binder with low vitamin K2. Our findings suggest a novel treatment of phosphate binder therapy combined with high vitamin K2 for patients with CKD that can prevent the progression of vascular calcification.

**Chapter 8** discusses the findings of this thesis and gives an overview of the conclusions. The results were analyzed in light of published literature.

# Addendum

Valorisation

Academic research is the most important repository of cutting-edge knowledge. A joint community of scientists share discoveries, being frontier of and expand knowledge that benefits society. The social impact of research is obvious when it provides a product. The findings presented within this thesis do not offer an immediate commercialized solution to cardiovascular problems but provide detailed pieces of a larger puzzle that might result in an explanation for the cause and consequences of cardiovascular disease.

In the era where CVD represent 31% of global deaths[1] I would like to cite Paul Dudley White, the 'Father of US Cardiology' who loved to say "Heart disease's death, before 80, is our fault, not God's or Nature's will". This somewhat bold statement does connect however to hypertension, a condition that is a major risk factor for CVD and which can effectively be prevented by proper management.

Because of its high prevalence and poor rate of blood pressure control, arterial hypertension (HT) is the major cause of mortality and early disability worldwide [2]. Most forms of arterial hypertension include primary aldosteronism (PA), secondary aldosteronism (due to renin-producing tumors, renovascular stenoses, and high-renin essential hypertension), hypertension associated with overweight-obesity[3,4], and drug-resistant forms[5]. All forms of arterial hypertension recognize aldosterone as main determinant of blood pressure. PA, albeit often overlooked, is the most common form of arterial hypertension, implicated in 5-13% of hypertensives[6–8]. Lack of mechanistic knowledge has impeded the development of effective preventive strategies and timely diagnostic strategies[9]. This results in late, or even missed diagnoses with ensuing development of drug-resistant hypertension and cardiovascular complications. The key objective of my thesis was to gain further insight into the mechanisms by which aldosterone synthesis is regulated with an overall impact on the cardiovascular system.

Molecular studies performed in the last decade have highlighted that most of the familial and sporadic cases of PA involve mutations in the selectivity filter of the KCNJ5 gene[10], encoding for the Kir3.4 potassium channel. It has been shown that H295 cells overexpressing mutant Kir3.4 have altered channel function causing: i) permeability to $Na^+$; ii) cell depolarization; and iii) $Ca^{2+}$ influx[10]. Increased intracellular $Ca^{2+}$ levels were also discovered in other gene mutations controlling ZG cell cation homeostasis, such as ATP1A1, ATP2B3, CACNA1H and CACNA1D[11–13]. Our work embarked on the recent discovery of two Kir3.4 mutations (G151R and L168R) that render the KCNJ5 channel in HAC-15 cells specifically sensitive to inhibition by macrolides, and that these agents concentration-dependently blunt Aldosterone production. This is indicative of an altered physiology of this channelopathy underlying PA that can be corrected[14]. However, the mechanisms whereby macrolides and their derivatives work are unknown, and our work laid down the fundament for further personalized treatment of PA. Developing better investigative tools will

be a prerequisite for novel diagnostic tests. It is conceivable that these will have a positive economic and technological impact on society at large in partnership with industry.

Our team was the first to analyze the molecular signature of aldosterone-producing adenoma using a whole transcriptome analysis. This led to the discovery that increased levels of microRNA-23 and microRNA-34 via direct binding to the 3' UTR of the type 2 TWIK-related acid-sensitive $K^+$ channel (TASK-2) gene cause a consistent under-expression of this channel in APA15. These genetic variations in the promoter region of TASK2 gene could explain development of PA in 25% of APA cases. Our work explains some 25% of APA cases, however in most of the cases the mechanisms have still to be unraveled.

To further understand the mechanisms leading to the pathogenesis of PA we investigated the intertwined hormonal implications. Intriguingly, our observations show that estrogens have a novel role in the regulation of aldosterone synthesis via GPER activation. Aldosterone acts in an autocrine-paracrine manner in GPER overexpressing APA, thereby generating high local concentration of mineralcorticoid hormone. Thus, self-perpetuating hyperaldosteronism, whereas the renin angiotensin system is blunted. These findings might have implications for the vast population women with breast or ovarian cancer, who are being treated with ER modulators (SERM). Although there is no knowledge of the off-target effects of SERM it is conceivable that these GPER agonists increase aldosterone production, and thus subsequently increase BP. Our data reveal a yet unknown side effect of ER modulatory cancer treatment on the cardiovascular system.

Single patient cases can shed light on molecular pathways leading to cardiovascular disease. Analyzing a patient with concurrent aldosterone-producing adenoma and primary hyperparathyroidism (PHPT) due to a parathyroid- hormone-secreting adenoma revealed that hyperparathyroidism is a feature of primary aldosteronism and particularly of aldosterone-producing adenoma16. In addition to primary aldosteronism, PHPT is known to be related to hypertension, which is not surprising, given that parathyroid hormone stimulates aldosterone secretion[17], promotes vascular calcification and affects arterial stiffness[18]. Thus, a possible genetic link between PHPT, hypertension and the feasibility of genetic testing is mandatory and currently under further investigation.

Considering the vast impact of cardiovascular disease on health care systems, it is fundamental to investigate the most prevalent complication of CV disease, such as vascular calcification. Vascular calcification is predominantly present in chronic kidney disease due to the imbalance in calcium and phosphate homeostasis. Moreover, chronic kidney disease patients suffer from severe vitamin K deficiency, in part induced by phosphate lowering strategies. We

developed a novel animal model of kidney failure with additional vitamin K-deficiency, thereby mimicking the clinical situation. We showed that PB therapy in combination with vitamin K2 supplementation strongly attenuated VC, whereas the single treatments were not effective. Our in vivo preclinical study combining PB therapy with vitamin K2 supplementation shows that combination therapies might be superior compared to single therapies. This finding might have a great clinical impact since phosphate binder therapy is common standard therapy for end stage kidney patients.

Translational medicine projects like described in my thesis are known to be most challenging but also most rewarding. The reward comes from the drive to generate knowledge that will potentially provide solutions for better care. This project, dissecting the molecular effectors of hyperaldosteronism and use of animal models in combating vascular calcification is, therefore, relevant not only for hypertensive patients, but also for those affected by other common cardiovascular diseases. Hence, a better understanding of the mechanisms underlying the complex regulation of aldosterone synthesis represents a major scientific achievement, with far reaching implications for general cardiovascular health.

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# Addendum

About the author

Selene Prisco was born in Bergamo, Italy on the 21st of March in 1991.
She attended the secondary education at the Scientific Lyceum in Bergamo and received her degree in 2011.
Upon completion of the Bachelor's program in Milan University, Selene started a 2 year Master in Biology in a joint programme (double degree) with Paris Diderot 7 University. She completed her master's degree in October 2016 with great honour under the supervision of prof. Silvia Kirsten and Vanessa Ribes.
In November 2016 she started her PhD programme named "arterial hypertension and vascular biology" at the Department of Medicine of Padua University, under the supervision of prof. Gian Paolo Rossi. In April 2019 Selene started her collaboration in Biochemistry Department at the Maastricht University under the supervision of prof. Leon Schurgers.
During her PhD program, Selene has published her data in international scientific journals and presented it at international conference.
She currently works in the Technology Transfer unit at Thermofisher Scientific in Milan.

# Addendum

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# Addendum

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