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CICLO: XXIX

Role of the protective Renin-Angiotensin System in aldosterone and cortisol production in human adrenocortical cell lines (HAC15 and NCI-H295R), in human adrenal cortex, and in aldosterone-producing adenoma (APA)

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" Dans la vie, rien n'est à craindre, tout est à comprendre."

" In life, nothing is to be afraid, everything is to be understood"

Marie Curie Sklodowska

Contents

Abbreviations

A- Angiotensin and angiotensin receptors

ACE1: angiotensin converting enzyme 1

ACE2: angiotensin converting enzyme 2

Ang: angiotensin

AT1R: angiotensin II type 1 receptor

AT2R: angiotensin II type 2 receptor

MasR: angiotensin-(1-7) receptor

MrgprD or MrgD: alamandine receptor

B- Adrenal tissue

AGP: adrenogonadal primordium

APA: aldosterone-producing adenoma

NAG: normal adrenal tissue/cortex

ZG : zona glomerulosa

ZF : zona fasciculata

ZR : zona reticularis

C- Housekeeping genes

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

PBGD: porphobilinogendeaminase

D- Receptors

GPCR: G-protein-coupled receptor

GR: glucocorticoid receptor

MR: mineralocorticoid receptor

E- Diverse

11βHSD: 11β-hydroxysteroid dehydrogenase

C21: compound 21

CAMK: calcium/calmodulin-dependent protein kinase

CRH: corticotropin release hormone

DIZE: diminazene aceturate

FH: familial hypertension

IL: interleukine

IP_3: inositol 1,4,5-triphosphate

Irbe: irbesartan

KCNJ5: G-protein-activated inward rectifier potassium channel

KDa: kilo Dalton

K_i: inhibitory constant

KI: kinase inhibitor

KO: knock-out

LV: left ventricular

MC2R: ACTH receptor

NO: nitric oxide

PA: primary aldosteronism

PAC: plasma aldosterone concentration

PKC: protein kinase C

RAS: renin-angiotensin system

SER: smooth-endoplasmic reticulum

SF1: steroidogenic factor 1

SHR: spontaneous hypertensive rat

StAR: steroidogenic acute regulatory protein

TGF: transforming growth factor

VSMC: vascular smooth muscle cells

WT: wild type

Acknowledgments

Traditionally, in France a student begins the PhD manuscript with acknowledgments. Of course, science in general and PhD in particular is not a ride alone but a shared-worked.

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Abstract

Background: Angiotensin II (Ang-II) regulates aldosterone and cortisol secretion via type 1 angiotensin II receptor (AT1R) in the adrenal cortex. Although the type 2 angiotensin II receptor (AT2R) and Mas receptor (MasR), form an important part of the so-called protective renin-angiotensin system (RAS), and counter regulate AT1R-mediated effects in various tissues, their function in the adrenal cortex remains largely unknown. Furthermore, the angiotensin-converting enzyme 2 (ACE2), another component of the protective RAS, cleaves Ang II and is also known to regulate effects of AT1R in several tissues.

Aim: Therefore, our aim was to investigate:

- the presence of AT2R, MasR, and ACE2 in human adrenocortical tissue and in aldosterone-producing adenoma (APA);

- the role of Ang-(1-7) and compound 21 (C21), an AT2R agonist, and diminazene aceturate (DIZE) in modulation of aldosterone and cortisol production in humans.

Methods: We detected AT2R, MasR, and ACE2 by immunoblotting and RT-PCR. Furthermore, using immunohistochemistry we localized AT2R and MasR in aldosterone-producing adenoma (APA) and in normal adrenal glang (NAG). We used Ang-(1-7) to stimulate MasR, C21 to stimulate AT2R, Ang II to stimulate AT1R and AT2R, and DIZE to activate ACE2. Irbesartan and A779 were used as blockers for AT1R and MasR respectively. RT-PCR was used to quantify *CYP11B1/CYP11B2* genes expression at baseline and after stimulation.

Results: AT2R and MasR were found to be heterogeneously express in human adrenal cortex and in APA. At low concentrations [nM], Ang-(1-7) had no effect on *CYP11B1* and *CYP11B2* gene expression, but at high concentrations [μM] it increased them. A779 did not significantly decrease the Ang-(1-7) effects, whereas irbesartan blunted them.

In human adrenocortical cell lines (HAC15 and NCI-H295R), the AT2R-agonist C21 had no effect at low concentrations [nM], but at high concentrations [μM] it increased *CYP11B1* and *CYP11B2* expression. Irbesartan blunted these effects

Finally, DIZE effect was no potent in *CYP11B1* and *CYP11B2* gene expression, but it may be involved in *ACE2* gene expression increase.

Conclusions: The expression of AT2R and MasR in the human adrenal cortex is, respectively 10-fold and 150-fold lower than AT1R. Moreover, whereas *AT1R* and *MasR* genes are expressed at the same level in the healthy adrenal cortex and in APA, *AT2R* gene expression is significantly lower in APA. The secretagogue effect of Ang-(1-7) and C21 on aldosterone and cortisol production seen with high concentrations likely occurs via AT1R. Thus, Ang-(1-7) and C21 may not be used as therapeutically tool to decrease aldosterone and cortisol production in human.

Riassunto

Background: L'angiotensina II (Ang-II) regola aldosterone e secrezione di cortisolo via di tipo 1 del recettore dell'angiotensina II (AT1R) nella corteccia surrenale. Anche se il tipo 2 del recettore dell'angiotensina II (AT2R) e del recettore Mas (Masr), costituiscono parte importante del cosiddetto sistema renina-angiotensina protettivo (RAS), e il contatore di regolare effetti AT1R mediata in vari tessuti, la loro funzione nel corteccia surrenale rimane in gran parte sconosciuta. Inoltre, l'enzima di conversione dell'angiotensina 2 (ACE2), un altro componente della RAS di protezione, si unirà Ang II ed è noto anche per regolare gli effetti di AT1R in diversi tessuti.

Obiettivo: Pertanto, il nostro obiettivo era quello di indagare:

- La presenza di AT2R, Masr e ACE2 nel tessuto adrenocorticale umana e aldosterone adenoma (APA);

- Il ruolo di Ang (1-7) e composto di 21 (C21), un agonista AT2R, e Diminazene aceturato (Dize) nella modulazione di aldosterone e cortisolo produzione negli esseri umani.

Metodi: Abbiamo rilevato AT2R, Masr, e ACE2 da immunoblotting e RT-PCR. Inoltre, utilizzando l'immunoistochimica abbiamo localizzato AT2R e Masr in aldosterone producono adenoma (APA) e in normale gLang surrene (NAG). Abbiamo usato Ang (1-7) per stimolare Masr, C21 per stimolare AT2R, Ang II per stimolare AT1R e AT2R, e Dize di attivare ACE2. Irbesartan e A779 sono state usate come bloccanti rispettivamente per AT1R e Masr. RT-PCR è stato utilizzato per quantificare l'espressione dei geni *CYP11B1 / CYP11B2* al basale e dopo stimolazione.

Risultati: AT2R e Masr sono risultati essere eterogeneo espressa in corteccia surrenale umana e in APA. A basse concentrazioni [Nm], Ang (1-7) non ha avuto effetti sulla espressione genica e *CYP11B1*/*CYP11B2*, ma ad alte concentrazioni [mM] li aumenta. A779 non è diminuito in modo significativo il Ang (1-7) gli effetti, mentre irbesartan loro smussati.
In linee cellulari umane surrenalici (HAC15 e NCI-H295R), il AT2R-agonisti C21 non ha avuto effetto a basse concentrazioni [Nm], ma ad alte concentrazioni [micron] è aumentata *CYP11B1* e *CYP11B2* espressione. Irbesartan smussati questi effetti
Infine, effetto Dize non era potente nel *CYP11B1* e l'espressione genica *CYP11B2*, ma può essere coinvolto in aumento dell'espressione genica ACE2.

Conclusioni: L'espressione di AT2R e Masr nella corteccia surrenale umano è, rispettivamente di 10 volte e 150 volte inferiori rispetto AT1R. Inoltre, mentre AT1R e Masr geni sono espressi allo stesso livello nel sano corteccia surrenale e in APA, AT2R espressione genica è significativamente più bassa in APA. L'effetto secretagogo di Ang (1-7) e C21 sulla aldosterone e cortisolo produzione visto con alte concentrazioni probabilmente avviene attraverso AT1R. Così, Ang (1-7) e C21 non possono essere utilizzate come strumento terapeutico per diminuire aldosterone e cortisolo.

Introduction

I- Anatomy and zonation of human Adrenal gland

I-1 Structure of adult adrenal cortex

The adult adrenal glands are a pair of triangular or round bodies located at the superior pole of each kidney (Figure 1), which display a combined weight of about 10–15 g in humans. They have anatomically been described by Barthlomeo Eustachi in 1563 (Bestetti RB *et al.*, 2014), but the first report of it physiological role was presented almost three centuries later by Thomas Addison in 1855, and the first histologically description dated of 1866.

In mammalian the adrenal gland is characterized by its anatomic zonation (Figure 1), which can be appreciated at the microscopic level, where each zone can be recognized and distinguished from one another based on structural and anatomic characteristics (Belloni AS *et al.*, 1987). The most outer layer is the ZG composed of ovoid shaped cells and are arranged in irregular cords. In particular, ZG contains outer undifferentiated small cells and inner well-differentiated larger elements, which display mitochondria with tubule-laminar cristae, abundant smooth endoplasmic reticulum (SER) and sparse lipid droplets (Belloni AS *et al.*, 1987). The ZF constitutes the majority of the adrenal cortex, sitting directly under the ZG. ZF cells are bigger than ZG cells and are organized into bundles, which led to its name "fascicles". The innermost of the cortex, between the ZF and the adrenal medulla, is the ZR. ZR cells are arranged like cords that project in different directions, resulting in the net-like appearance that gives the zone its name (Xing Y *et al.*, 2015; Belloni AS *et al.*, 1987) (Figure 1).

In rodent, the adrenal cortex is also divided into three morphologically and functionally zones. However, in 1994 it has been noticed the presence of a fourth zone between ZG and ZF (Mitani F *et al*., 1994). This zone has no immunoreactivity with anti-CYP11B1 and anti-CYP11B2 antibodies and appears like a white ring surrounding ZF suggesting the absence of both CYP11B1 and CYP11B2 (Mitani F *et al*., 1994). The newly found zone can be characterized as lacking adrenal androgen synthesis. Thus, the cells in this zone appeared to have no significant endocrine function specific to zones of the adrenal cortex and, therefore, could be regarded as undifferentiated cells with respect to corticosteroidogenesis (Mitani F *et al*,. 2014). This zone is designated as the "undifferentiated cell zone" and is considered to be a stem/progenitor cell zone in the adrenal cortex.

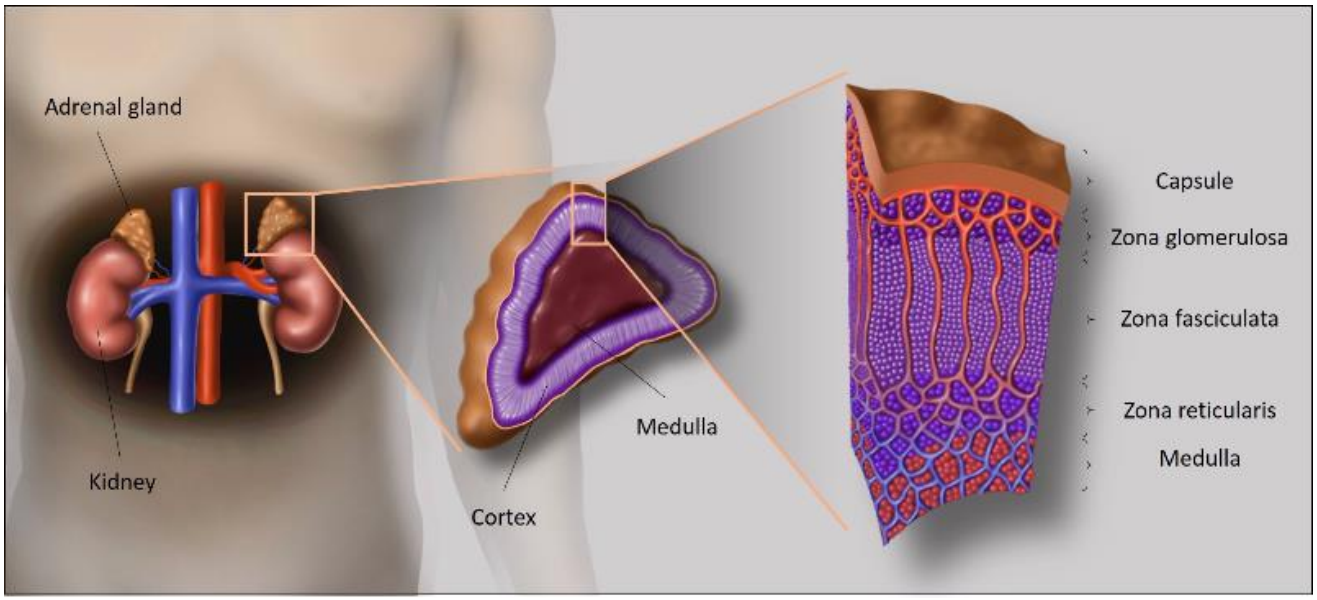

Figure 1: Anatomy of human adrenal gland

The adrenal medulla is composed of neural crest cells known as chromaffin cells, medullary cells or pheochromocytes cells. They are arranged in small nests and cords separated by prominent vasculature. They comprise large polygonal cells with poorly outlined borders, abundant granular and usually basophilic cytoplasm. Surrounded by a meshwork of blood vessels called venous sinusoids, the chromaffin cells secrete into the sinusoids noradrenaline and adrenaline, which are delivered to the rest of the body via the bloodstream. However, specific immunostaining for the neuroendocrine protein chromogranin-A unambiguously showed that adrenomedullary chromaffin cells are in all three zones of the human adrenal cortex (Bornstein SR *et al.*, 1990; Bornstein SR *et al.*, 1992). Medullary cells, especially in the ZG, frequently spread into the subcapsular region, forming large nests of chromaffin cells (Bornstein SR *et al.*, 1990; Bornstein SR *et al.*, 1992). Chromaffin cells produce a wide variety of autocrine/paracrine regulatory factors, including neuropeptides, classic neurotransmitters, and cytokines that stimulate adrenocortical steroidogenesis (Ehrhart-Bornstein M *et al.*, 1998; Nussdorfer GG *et al.*, 1996; Nussdorfer *et al.*, 1986). Furthermore, a local corticotropin-releasing hormone (CRH)-corticotropin system in the adrenal medulla participates in the intra-adrenal regulation of steroidogenesis (Nussdorfer GG *et al.*, 1996).

To note, after the functional adult adrenocortical zones are established, they are maintained by stem or progenitor cells (Xing Y *et al.*, 2015). Several studies showed that most of proliferating cells are located in the outer layer of the matural adrenal gland, the so called “cambium layer”,

while most cell death occurs near the boundary of cortex and medulla (Stachowiak A *et al.*, 1990; Sasano H *et al.*, 1995; Morley SD *et al.*, 1996).

I-2 Fetal and early adult development of the adrenal cortex

The adrenal glands develop from two separate embryological tissues: the medulla is derived from neural crest cells, while the cortex originates from the intermediate mesoderm (Gruenwald P *et al.*, 1946; Mitani F *et al.*, 2014; Xing Y *et al.*, 2015). At 28-30 days post coitum, the embryonic adrenal gland appears as "adrenogonadal primordium" (AGP), and is marked by the expression of steroidogenic factor 1 (SF1), which is a nuclear receptor essential for adrenal development and steroidogenesis (Figure 1) (Hatano O *et al.*, 1996; Luo X *et al.*, 1994). AGP appears first between the urogenital ridge and the dorsal mesentery, and contains a mixed population of adrenocortical and somatic gonadal progenitor cells. SF1-positive AGP cells delaminate from the epithelium and invade the underlying mesenchyme of the intermediate mesoderm (Morohashi K, 1997). After delamination, the majority of AGP cells migrate dorsolaterally to form the gonadal *anlagen*, whereas AGP cells that express higher levels of SF1 migrate dorsomedially to form the adrenal *anlagen* (adrenal primordial) (Hatano O *et al.*, 1996). At about 48 days post coitum neural crest cells migrate to the area where the adrenal primordial is developing (Le Douarin NM *et al.*, 1974). Neural crest cells persist as discrete islands spread all around the embryonic adrenal until birth and ultimately differentiate into the catecholamine-producing chromaffin cells of the adrenal medulla (Doupe AJ *et al.*, 1985; Hillarp NB *et al.*, 1953). At the same time the adrenal gland starts to separate from surrounding mesenchym and becomes encapsulated. The encapsulation phase is largely complete by 52 days post coitum (Figure 2) (Xing T *et al.*, 2015; Keegan CE *et al.*, 2002), and when it is over, the embryonic adrenal cortex expands rapidly. At term, the fetal adrenal is one of the largest organs with 80% of the gland composed of fetal zone (FZ) cells (Johannisson E *et al.*, 1968). Steroidogenic cells of the fetal adrenal robustly express cytochrome P450 17 alpha (CYP17), and so produce high quantity of dehydroepiandrosterone (DHEA) and DHEA-S, that are then converted by the placenta into oestrogens for the maintenance of normal pregnancy (Xing Y *et al.*, 2015). During encapsulation, by the 8th week of gestation, new adrenocortical cells emerge between the capsule and FZ, forming the definitive zone (DZ) which will develop into the adult cortex. Moreover, as gestation advances, inner cells of the DZ form arched cords with finger-

like columns of cells reaching the outer rim of the FZ. The cells of this cord will continue to expand and will begin to produce cortisol under the regulation of adrenocorticotropin hormone (ACTH). This zone is the future Zona Fasciculata (ZF) of the adult adrenal cortex (Xing Y *et al.*, 2015).

As for several others tissues, the adrenal gland continues to undergo significant remodeling during neonatal and pubertal periods. Immediately after birth, under the influence of angiotensin II (Ang II) and ACTH, zona glomerulosa (ZG) and fasciculata (ZF) mature in parallel with an involution of the remaining FZ (Ishimoto H *et al.*, 2011). Later, between 6 to 9 years old, the zona reticularis (ZR) begins to form between the ZF and the medulla (Havelock J *et al.*, 2004). This phenomenon, which is characterized by increased proliferation and production of adrenal androgens is known under the name of adrenarche.

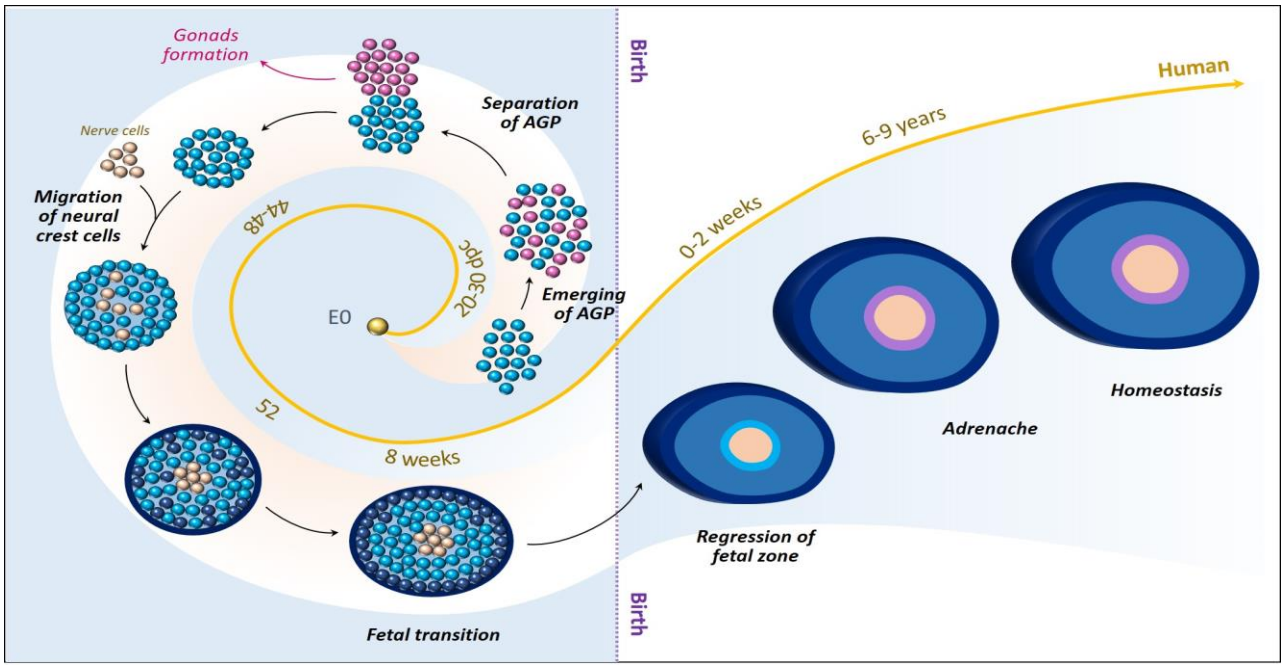

Figure 2: Development and zonation of human adrenal gland
(adapted from Xing Y *et al.*, 2015)

II- Steroidognesis

Like other steroidogenic tissues, each adrenal cortex zone synthesizes its steroid products from the same substrate: cholesterol; the high cholesterol content give them a yellowish colour. Steroidogenic cholesterol can arise from endogenous cholesterol stores, from serum derived lipoprotein or by *de novo* synthesis.

The rate-limiting step in steroid hormone biosynthesis is the translocation of substrate cholesterol from the outer mitochondrial membrane to cytochrome P450 side-chain cleavage (P450ssc) enzyme (Privalle CT *et al.*, 1983). P450ssc is the first enzyme in the steroidogenic pathway, it is located on the matrix side of the inner mitochondrial membrane (Farkash Y *et al.*, 1986). The steroidogenic acute regulatory protein (StAR) gene was cloned in Stocco laboratory in 1994. This enzyme mediates the transfer of cholesterol from the outer mitochondria membrane to the inner membrane through the aqueous intermembrane space (Stocco DM *et al.*, 2001). However, the mechanisms promoting the specific *StAR* gene expression, in both temporal and spatial manner, and the transfer of cholesterol to the inner mitochondrial membrane, are yet poorly understood.

Within the human adrenal cortex, steroid synthesis involves the coordinated actions of five forms of cytochrome P450 and the enzyme 3-β-hydroxysteroid dehydrogenase (3βHSD) (Figure 3), which are respectively in the inner membrane of mitochondria and in endoplasmic reticulum. It is the differential expression of these enzymes within the three adrenocortical zones that allows for the wide array of steroid hormones secreted by this gland.

Once at the inner mitochondrial membrane cholesterol is converted to pregnelonone by P45011A1 (P450scc). P45011A1 catalyzes three separate reactions, 20 α-hydroxylation, and C20-22 bond scissions, converting the C27 cholesterol to the C21 pregnenolone. Once pregnenolone is formed, it can be 17 β-hydroxylated by P450c17 to form 17 α-hydroxyprenenolone or converted to progesterone by 3-β-hydroxysteroid dehydrogenase (3 β-HSD), the only non-P450 enzyme in the pathways. P450c17 can also form 17 β-hydroxyprogesterone from progesterone. P450 17α also catalyzes a 17-20 lyase reaction on 17-hydroxypregenolone and 17-hydroxyprogesterone to form the adrenal androgens dehydroepiandrosterone and androstenedione, respectively. Both progesterone and 17-hydroxyprogesterone can be 21-hydroxylated by P450 C21 to yield deoxycorticosterone and

11-deoxycortisol, respectively. The final reactions in adrenocortical steroid hormone biosynthesis involve the hydroxylation of 11-deoxycortisol and deoxycorticosterone by P45011B1 to form cortisol and coorticosterone, respectively. Corticosterone, in zonaglomerulosa, can then be converted to aldosterone by P45011B2 (CYP11B2 or aldosterone synthase) in a series of hydroxylation steps (Miller WL and Auchus RJ, 2011).

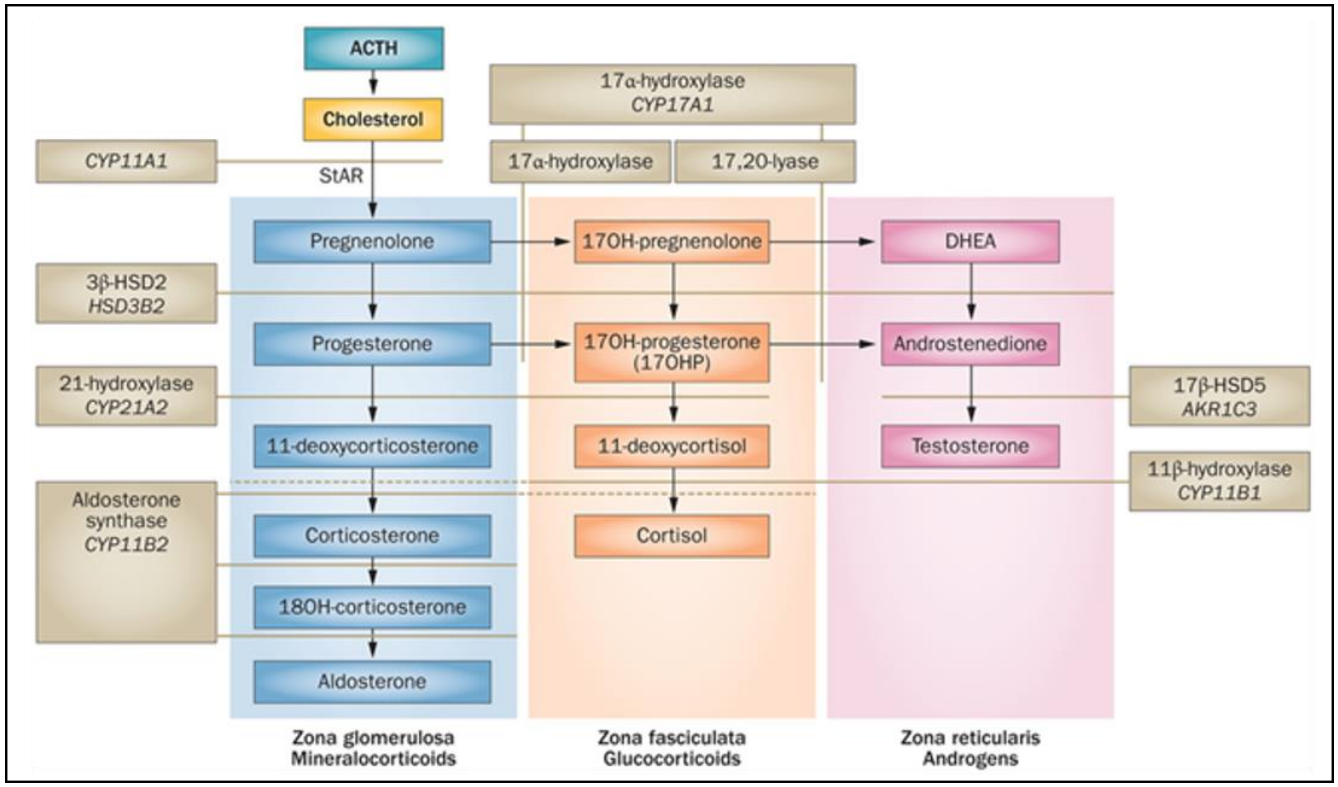

Figure 3: Steroidogenesis pathways (from Han TS *et al*,. 2013)

III- Aldosterone biosynthesis regulation and role in cardiovascular disease

III-1 Biosynthesis regulation of aldosterone

Mllions years ago, when life forms moved from salty to fresh water, and finally to predominantly terrestrial existence, some mechanisms have emerged to conserve salt and water under this evolutionary pressure. The mineralocorticoid pathway and its prototype hormone, aldosterone, came out as vital axes for survival of several land forms, including Homo sapiens (Xanthakis V *et al.*, 2013). However, our prehistoric ancestors were hunter-gatherers who ate a sodium-restricted, potassium-enriched diet consisting of fruits and vegetables. Moreover, they also lived in arid environment, and they were losing volume and sodium through swearing, exsanguinating hemorrhage, or diarrheal disorders. Thus, mechanisms for conserving sodium and water and excreting potassium was vital, and the renin-angiotensin-aldosterone system (RAS), with aldosterone as final product, emerged as the much needed mechanism for survival.

Aldosterone is the least hormone of the adrenal cortex, and is made exclusively in the ZG where aldosterone synthase is located (Hattangady NG, 2012; Natsukawa N *et al.*, 1990; Shibata I *et al.*, 1991; Foster RH *et al.*, 1997; Conley AJ *et al.*, 1997). The biosynthesis of aldosterone is regulated by more than a dozen factors. Among them dopamine (Missale C *et al*,. 1988; Missale C *et al*,. 1989; Missale C *et al*,. 1998; Wu KD *et al*,. 2001; Pivonello R *et al*,. 2004; Chang HW *et al*,. 2008), vasopressin (Perraudin V *et al*,. 1993; Guillon G *et al*,. 1995; Gallo-Payet N and Guilon G, 1998 ; Perraudin V *et al*,. 2006), endothelin-1 (Nussdorfer GG *et al*,. 1999 ; Rossi GP *et al*,. 1997 ; Rossi GP *et al*,. 2003 ; Hu CW *et al*,. 2004), urotensin II (Feuilloley M *et al*,. 1994 ; Takahashi K *et al*,. 2001 ; Albertin G *et al*,. 2006 ; Giuliani L *et al*,. 2009), AT1R auto-antibody (Wallukat G *et al*,. 1999 ; Rossito G *et a*l,. 2012 ; Yang J *et al*,. 2015). However, the three main secretagogues that can modify aldosterone secretion are angiotensin II (Ang II), potassium (K^+), and circulating adrenocorticotrophic hormone (ACTH). Furthermore, the regulation of aldosterone biosynthesis is divided into two key events in the steroidogenic pathway (Clark AJ *et al.*, 1992; Müller J *et al.*, 1998) (Figure 4). The acute aldosterone production is controlled by rapid signaling pathways that increase the movement of cholesterol into the mitochondria where it is converted to pregnenolone. This “early regulatory step” is mediated by phosphorylation of the StAR protein (Arkane F *et al.*, 1997; Cherradi N *et al.*,

1998; Fleury A *et al.*, 2004). The "late regulatory step" modulates the chronic aldosterone production (from hours to days) at the enzymatic expression level (Bassett MH *et al.*, 2004). To note, the chronic mechanisms involve changes in the glomerulosa cell ability to produce aldosterone as well as the size of the zona glomerulosa.

III-1 A) Acute effects of Ang II

After binding to angiotensin II type 1 receptor (AT1R), Ang II activates three different pathways. The first one, is a phosphoinositide-specific phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two second messengers: the inositol 1,4,5-triphosphate (IP_3) and the diacylglycerol (DAG) (Barrett PQ *et al.*, 1989; Bird IM *et al.*, 1993; Bollag WB *et al.*, 1991; Farese RV *et al.*, 1981; Ganguly A *et al.*, 1994; Hunyady L *et al.*, 1990; Kijima I *et al.*, 1984). Whereas IP_3 initiates aldosterone secretion by eliciting a transient increase in the cytosolic calcium-concentration and activating calcium/calmodulin-dependent protein kinases (CaMK) (Ganguly A *et al.*, 1992; Pezzi V *et al.*, 1996; Spät A and Hunyady L, 2004), DAG stimulates protein kinase C (PKC) (Bollag WB *et al.*, 1991; Bollag WB *et al.*, 1992; Kapas S *et al.*, 1995).

A second pathway involves an increase in calcium influx, in part through CaM kinase II and GTP-binding proteins. This calcium enhancement acts synergistically with PKC-activating agents to stimulate secretion (Barrett PQ *et al.*, 2000; Lu HK *et al.*, 1994; Yao J *et al.*, 2006). Calcium influx occurs through two main mechanisms: first, through the voltage-dependent calcium transient (T)-and long-lasting (L)-type channels, whose activities are maintained through appropriate membrane polarization (Spät A and Hunyady L, 2004; Aptel HB *et al.*, 1999; Burnay MM *et al.*, 1998); and second, from store-operated calcium channels (Hattangady NG, 2012; Spät A and Hunyady L, 2004). Using pharmacological agents, it has been shown that the two parts of this pathway are needed (Kojima I *et al*,.1984; Bollag WB *et al*,. 1990; Kijima I *et al*,. 1985; Barrett PQ *et al*,. 1986) (Figure 4).

The last signaling pathway activated by Ang II is phospholipase D (PLD), which can also indirectly increase DAG (Bollag WB *et al*,. 1990; Zheng X *et al*,. 2003). PLD (2 mammals isoforms: PLD1 and PLD2) hydrolyzes phospholipids to yield phosphatidic acid (phosphorylated DAG), which can be then converted into DAG by the action of lipid phosphate

phosphatases (Bollag WB *et al*,. 2007; Qin H *et al*,. 2010). Phosphatidic acid is an important element in several pathways. It can directly be a second messenger acting as a slow-release reservoir of DAG for sustained cellular responses (Bollag WB *et al*,. 1990; Bollag WB *et al*,. 2007; Bollag WB *et al*,. 2008). Moreover, it can also be deacylated by phospholipase A2 to produce lysophosphatidic acid (LPA) which activates G protein-coupled LPA receptors (Hattangady NG, 2012). The most important for our purpose, Phosphatidic acid can also be metabolized to additional lipid signals known to stimulate aldosterone secretion (Natarajan R *et al*,. 1988; Natarajan R *et al*,. 1990). Besides, it has been demonstrated in bovine glomerulosa cells that PLD activity may be sufficient to stimulate aldosterone secretion (Bollag WB *et al*,. 1990). Furthermore, the overexpression of wild-type PLD1 or PLD2 in human adrenocortical cells (NCI-H295R), allowed to prove that PLD2 is the isoform mediating aldosterone secretion in response to Ang II (Qin H *et al*,. 2010).

III-1 B) Acute effects of potassium

Similar to Ang II, small increases in extracellular potassium levels also stimulate calcium influx, via depolarization of the glomerulosa cell plasma membrane and activation of the voltage-dependent calcium channels (L or T-type) (Kojima I *et al*,. 1984), with ensuing an influx, which is required for the response to potassium. Others signaling pathways have been proposed but are still controversial. For instance, some investigators have observed an ability of potassium to increase cAMP levels (Fujita K *et al*,. 1979; Ganguly A *et al*,. 1990). Others reported that potassium can stimulate PLD activity through voltage-dependent calcium channels in bovine glomerulosa cells (Betancourt-Cale S *et al*,. 2001).

III-1 C) Acute effect of ACTH

ACTH stimulates acutely aldosterone production by binding to the ACTH receptor (MC2R) which activates adenylate cyclase via the heterotrimeric G protein, Gs. Adenylate cyclase produces the second messenger cAMP, thereby stimulating the activity of cAMP-dependent protein kinase or protein kinase A (PKA). The latter can then phosphorylate and activate StAR (Betancourt-Cale S *et al*,. 2001) leading to increase cholesterol delivery to the inner mitochondria membrane. Furthermore, PKA can regulate the transcriptional activity of CREB transcription factor family and so rapidly increases expression of StAR and further enhances acute steroid production (Jo Y *et al*,. 2005; Johannessen M *et al*,. 2007). In addition, ACTH is capable to promoting calcium influx, likely through PKA-mediated phosphorylation of L-type calcium channels (Sculptoreanu A *et al*,. 1953). This influx increases cytosolic calcium concentration and further enhances adenylate cyclase production of cAMP and aldosterone secretion (Gallo-Payet N *et al*,. 1996). Finally, the involvement of the guanine nucleotide exchange factor is probably involved in ACTH pathway (Gambaryan S *et al*,. 2006).

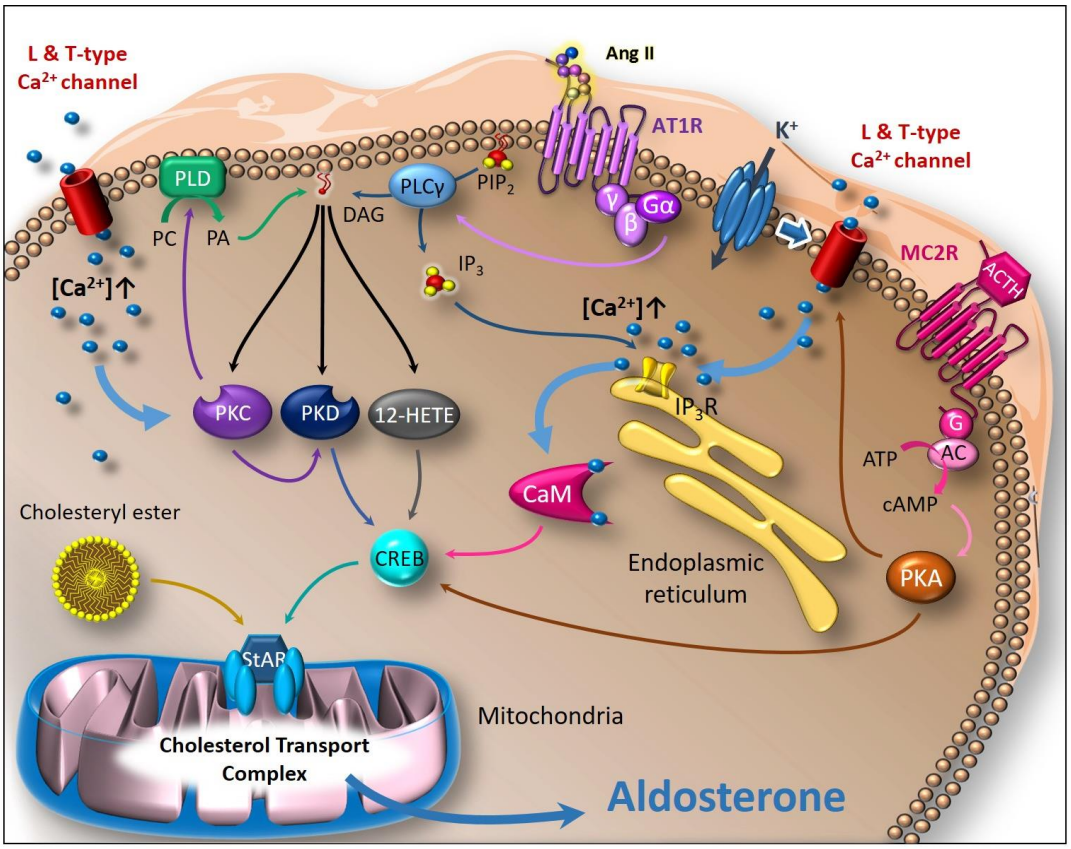

Figure 4: Acute actions of Ang II, K^+ and ACTH on adrenal glomerulosa cell aldosterone production (adapted from (Hattangady NG, 2012)

III-1 D) Chronic effects of Ang II

Ang II increases the expression of enzymes required for aldosterone synthesis, particularly CYP11B2.

Several *in vivo* experiments, notably using sodium restriction indicated that activation of the renin-angiotensin system (RAS) induces the expression of CYP11B2 in glomerulosa cells without affecting CYP11B1 (Adler GK *et al*,. 1993; Holland OB *et al*,. 1993; Tremblay A *et al*,. 1992). This result confirms the ability of Ang II to specifically increase the production of aldosterone but not glucocorticoid secretion (Holland OB *et al*,. 1993; Tremblay A *et al*,. 1992). This zone-specific effect is due to the greater expression of AT1R in ZG versus ZF (Breault L *et al*,. 1996). However, these data are challenged by recent studies (Oki K *et al*,.2013). Besides, it is widely admitted that Ang II can also increase CYP11B2 expression by increasing glomerulosa cell sensitivity to Ang II through up-regulation of its own receptor (AT1R) (Du Y *et al*,. 1996; Wang DH *et al*,. 1995).

In addition to these *in vivo* models, some *in vitro* models have also been developed in order to understand intracellular mechanisms involved in chronic Ang II effects. And as for acute stimulation, it appeared that one of the main pathway leading to aldosterone production is the PLC-mediated generation of DAG and IP_3, which increases intracellular calcium and acts via CaMK (Pezzi V *et al*,. 1997). Furthermore, Ang II increases CYP11B2 expression through the activation of its transcription. Several studies have revealed that *cis*-elements in the *CYP11B2* promoter are essential for both the basal and the AngII-mediated *CYP11B2* promoter activity (for review see Hattangady NG, 2012). Among these three key regulatory *cis*-elements: one cAMP response element (CRE)/Ad1 and two distal *cis*-element (Ad5 and NBRE) (Bassett MH *et al*,. 2004a; Bassett MH *et al*,. 2004b; Romero DG *et al*,. 2010; Szekeres M *et al*,. 2010). Of note, the overexpression of one such transcription factors, NURR1, is implicated in the development of aldosterone-producing tumors (Lu L *et al*,. 2004).

Finally, Ang II also increases aldosterone production by expansion of the ZG via hypertrophy and hyperplasia. This effect has been shown *in vivo* and *in vitro* (McEwan PE *et al*,. 1999; Tian Y *et al*,. 1998), and may be attributed to the ability of Ang II to induce the expression of cyclin D1 (Watanabe G *et al*,. 1996). This phenomenon allows the hypothesis that the side effects of Ang II on cardiovascular system is not only due to the secretion of aldosterone, but also to glomerulosa cells hyperplasia.

III-1 E) Chronic effects of potassium

Potassium induces "early events" and regulates chronic events. Nowadays, it is widely admitted that high potassium diets in rats increase the expression of aldosterone synthase (CYP11B2) and aldosterone production.

As mentioned before, potassium increases calcium influx via depolarization of the cells leading to extracellular calcium influx through the T-and L-type calcium channels. This increase in calcium influx through the binding of calcium to calmodulin upregulates CYP11B2 expression. The calcium-calmodulin complex activates several enzymes and kinases, many of which are expressed in a tissue-specific manner. Among the different CaMK, two types (I and IV), are the most likely involved in chronic stimulation of Ang II and potassium (Condon JC *et al*,. 2002), and were found to be differentially expressed in APA (Lenzini L *et al*,. 2007).

Furthermore, as Ang II, potassium is able to activate several transcription factors, such as NURR1, ATF1, ATF2 or CREB, which bind the proximal promoter of CYP11B2 at key *cis*-elements to enhance transcription (Bassett MH *et al*,. 2004a; Bassett MH *et al*,. 2004b; Nogueira EF *et al*,. 2010).

In addition, it has been observed in rats that a high potassium diet leads to an upregulation of several genes as Mtus 1, Smoc 1, and Grp 48. The latest, is associated with a down-regulation of the cyclin-dependent kinase inhibitor p27 (Kip1) (Gao Y et al,. 2006). This upregulation is correlated to an increase in the thickness of the ZG (Dierks A et al,. 2010).

III-1 F) Chronic effects of ACTH

During decades, ACTH was thought to be a cortisol regulator and only a secondary stimulus for aldosterone. However, it has been shown, and is now well accepted, that ACTH can acutely stimulate aldosterone secretion. Nevertheless, over time ACTH causes cultured glomerulosa cells to switch their phenotype from an aldosterone producing to that of a cortisol-producing fasciculata cell phenotype (Crivello JF *et al*,. 1983). Thus, chronically ACTH decreases aldosterone secretion. In agreement with this first observation, it has been demonstrated that in isolated rat glomerulosa, ACTH causes an initial increase in mRNA levels of CYP11B2 in the first 3 hours and a decrease of CYP11B2 when infused chronically (Holland OB *et al*,.

1993). Moreover, *in vivo* ablation of the pituitary pre-pro-opiomelanocortin-secreting cells that produce ACTH, and the resultant low ACTH level, was accompanied by an acute decrease in the transcript levels of CYP11B1, but not of CYP11B2 (Allen RG *et al*,. 1995).

However, the mechanism by which ACTH downregulates CYP11B2 when infused chronically remains unknown. One hypothesis is a possible hormone direct induction of CYP11B1 and CYP17, whose activities direct the precursors of the steroidogenic pathway away from the production of aldosterone and toward that of cortisol (Hattangady NG, 2012; Xanthakis V *et al*,. 2013).

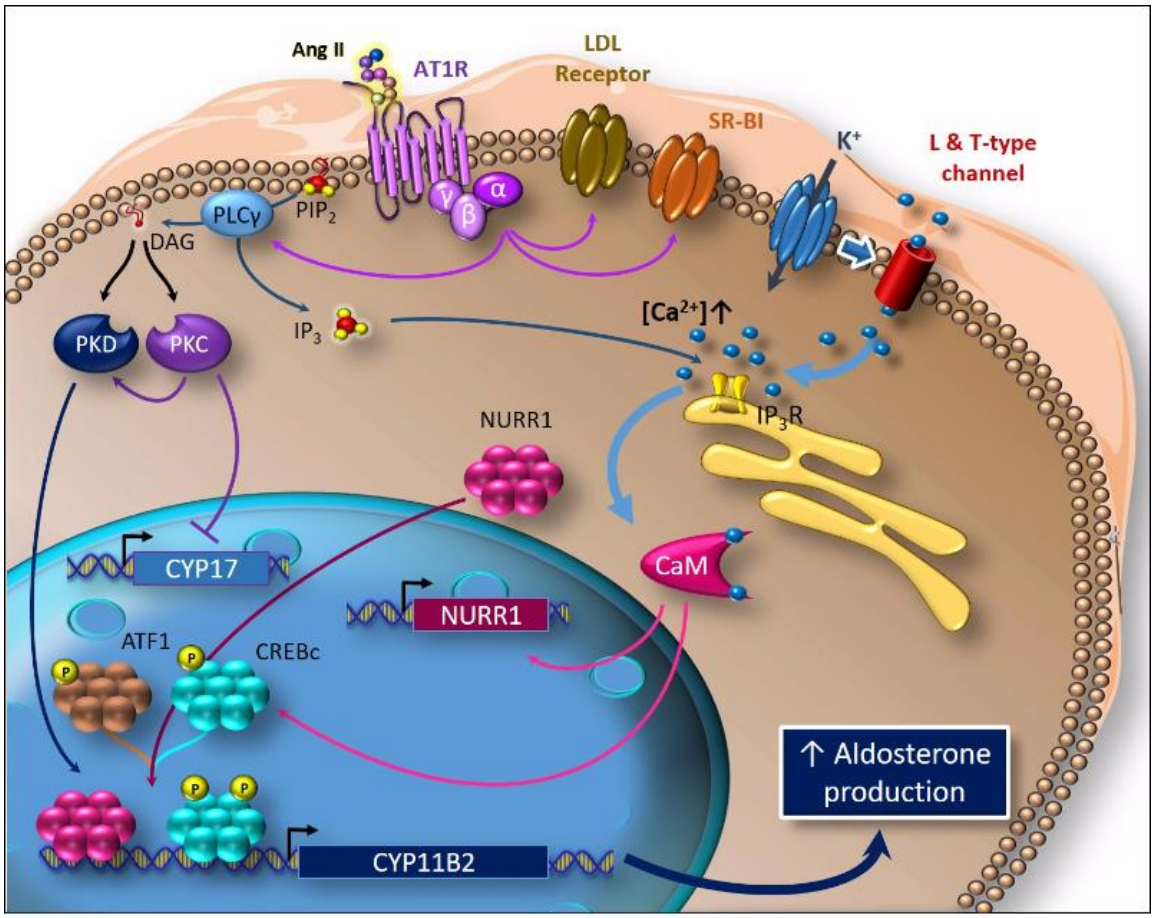

Figure 5: Chronic aldosterone regulation with angiotensin II, K^+ and ACTH (adapted from (Hattangady NG, 2012)

III-2 Aldosterone role in cardiovascular disease

Aldosterone binds to the mineralocorticoid receptors (MR) in the epithelial cells in the distal convoluted tubule and the collecting duct of the nephrons (Xanthakis V *et al*,. 2013; Briet M and Schiffrin EL, 2012; Shibata H and Itoh H, 2012). By up-regulating the basolateral sodium-potassium exchange pump, the epithelial sodium channel (ENaC), and the outer medullary renal potassium channels, aldosterone promotes sodium and water retention, which contributes directly to increase blood pressure, via genomic and nongenomic mechanisms (Xanthakis V *et al*,. 2013; Shibata H and Itoh H, 2012; Hattangady NG, 2012). The genomic mechanisms increase the transcription of genes regulating these channels and the exchange pump. The nongenomic effects are mediated either by intracellular receptors (G protein coupled receptor 30) or via non-receptor second messenger pathways that can alter intracellular ionic concentrations (including the sodium-hydrogen exchanger) (Xanthakis V *et al*,. 2013; Briet M and Schiffrin EL, 2012).

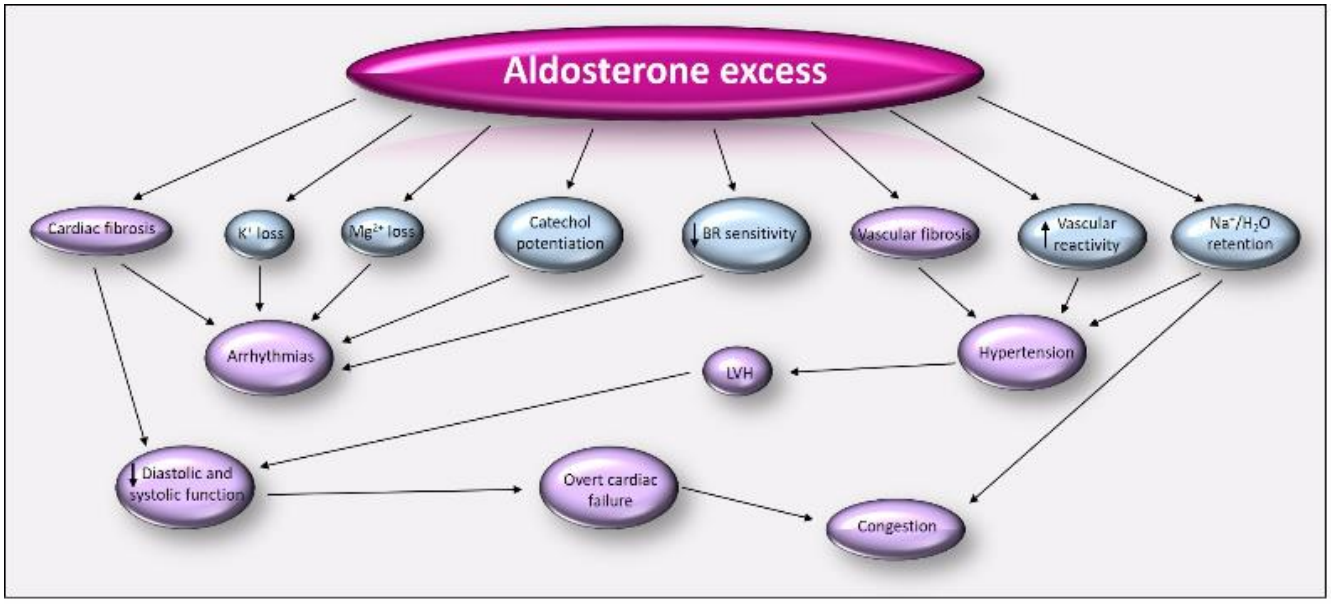

Figure 6: Adverse cardiovascular effects of aldosterone (adapted from Stowasswer M, 2011; Gaddam KK *et al*,. 2013)

BR= baroreceptor, LVH: left ventricular hypertrophy.

Nonetheless, some years ago it has been demonstrated that the physiological effects of aldosterone are not limited to the MR on the renal epithelial cells (Shibata H and Itoh H, 2012; Hattangady NG, 2012). MR is also present on several nonepithelial cells, including cardiomyocytes, cardiac fibroblasts and vascular smooth muscle cells. In the myocardium

aldosterone elicits a fibrotic response. In the vasculature this hormone leads to a profibrotic, an hypertrophic and inflammatory response. Moreover, it also reduces nitric oxide bioactivity thereby promoting endothelial dysfunction (Xanthakis V *et al*,. 2013; Hattangady NG, 2012). Aldosterone influences endothelial cell function adversely by causing the cells to swell and become stiff due to its effect on the antioxidant enzyme glucose-6-phosphate dehydrogenase and epidermal growth factor, mostly by activating phospholipase C.

Other possible effects of aldosterone that may contribute to the pathogenesis of cardiovascular disease include increase in sympathetic activation, impairment of baroreflex function, and increase in thrombogenesis (Gaddam KK *et al*,. 2013) (Figure 6).

IV- Cortisol dysregulation

Cortisol is key for maintaining physiologic homeostasis; it is involved in many metabolic and immune processes, but also in the diurnal sleep-wake cycle, in the human stress response, and in blood pressure regulation (Baudrand R and Vaidya A, 2015).

With evolutionary pressure, different cortisol regulatory mechanisms and modes of action were established, including: input from the brain and hypothalamic-pituitary apparatus, a highly specific circadian secretagogue pattern, some intricate adrenal steroidogenic pathways, but also tissue specific conversion enzymes such as 11β-hydroxysteroid dehydrogenases, or the capacity to activate more than one steroid (mineralocorticoid and glucocorticoid) receptor. Several environmental factors, mainly in western countries, can disturb these established cortisol regulatory and effector pathways, resulting in, or aggravating some human diseases. Among them, obesity may be one of the best example of a disease state as a consequence of our modern life style. Indeed, increased availability and consumption of high caloric foods in combination with decreased physical activity and sleep lead to cardiometabolic disorders as type 2 diabetes milletius or hypertension (Cronise RJ *et al*,. 2014).

A growing body of evidences suggests that metabolic and cardiovascular morbidities in obesity may be partially explained by a dysregulation in cortisol physiology (Shulman GI *et al*,. 2014; Baudrand R *et al*,. 2011; Varughese AG *et al*,. 2014; Stomby A et *al*,. 2014). Glucocorticoid secretion not only depends on the hypothalamic-pituitary-adrenal (HPA) circadian rhythm, but, as mentioned before, also on the pre-receptor intracellular regulation of cortisol by 11β-hydroxysteroid dehydrogenases (11 β-HSD) (Cooper MS *et al*,. 2009). This local regulation due to 11 β-HSD is important because it represents a key tissue-level source of cortisol, and a potential ligand of both the GR and MR, that may not be reflect circulating cortisol levels alone. Furthermore, one isoform of this enzyme, 11 β-HSD1, is expressed in adipose tissue and, thus, could be an important component of excessive GR and/or MR activation (Cooper MS *et al*,. 2009; Baudrand R *et al*,. 2010; Valsamakis G *et al*,. 2004). In contrast, another isoform, 11 β-HSD2, inactivates cortisol into the inactive metabolite cortisone, thereby reducing the activation of the GR and improving the selectivity of the MR for aldosterone, which generally circulates in a 100-to-1000 concentration fold lower than cortisol. Thus, a local balance exists,

which regulates cortisol action. The latter can play a role in metabolic disorders if it is tilted towards increased local-tissue cortisol production (Baudrand R *et al*,. 2015; Stamby A *et al*,. 2014). Therefore, the possible influence of cortisol in obesity relies on interplay between adrenocortical cortisol secretion in response to the HPA axis, and local cortisol activation or inactivation in adipose and other tissues.

Based on available information it has been proposed that cortisol is a double-face hormone; acting mainly as a glucocorticoid but also a mineralocorticoid. In epithelial tissues, the enzyme 11βHSD2 converts cortisol to corticosterone leading to a cortisol binding inhibition to the MR. At this stage, aldosterone is so the only steroid binding the MR. However, when intracellular redox state changed by inhibition of the 11βHSD2, cortisol is not metabolized and can bind to MR (Funder JW, 2005). Thus, the MR can be activated by both aldosterone and cortisol. In addition, excess adiposity and obesity are known to be associated with the development of elevated blood and urinary aldosterone levels and increased renin-angiotensin system activity (Bentley-Lewis R *et al*,. 2007; Byrd JB and Brook RD, 2014), as well as a lack of normal suppressibility and stimulation of adrenal aldosterone secretion (Vaidya A *et al*,. 2013). The explanation for this excess of aldosterone level is probably a combination of inappropriate adrenal aldosterone secretion, local adipose-tissue aldosterone production (Briones AM *et al*,. 2012), and the result of adipose-derived adrenal aldosterone secretagogues (Byrd JB and Brook RD, 2014; Pojoga LH *et al*,. 2013).

V- Endocrine hypertension and primary aldosteronism

V-1 Definition and diagnosis of primary aldosteronism

Hypertension, defined as a systolic and diastolic blood pressure respectively superior to 140 mmHg and 90 mmHg, is a physical consequence reflecting a variety of genetic and environmental factors, including renal sodium retention and increased vascular resistance. In the majority of cases, hypertension is primary (idiopathic or essential), but an important subgroup has secondary hypertension.

Secondary hypertension, a term used for the hypertension for which there is an identifiable cause, account for 10% of all patients with hypertension in general practice (Vega J, Bisognano JD, 2014; Velasco A and Vongpatanasin W, 2014; Thomas RM *et al*,. 2015), but up to 50% at referred centers (Azizi M *et al*,. 2015). The endocrine conditions causing hypertension are primary aldosteronism, pheochromocytoma, Cushing's syndrome, acromegaly, hyperparathyroidism, congenital adrenal hyperplasia, hypothyroidism, hyperthyroidism, and renin-secreting tumors (Thomas RM *et al*,. 2015).

Primary aldosteronism (PA) is one of the most common causes of secondary hypertension with an estimated prevalence from 6 to 12% (11,2% in PAPY study) in referred patients, 4,8% in primary care, and as high as 20% in patients with resistant hypertension (Hannemann A *et al*,. 2012; Bakris G *et al*,. 2002; Boulkroun S *et al*,. 2015; Rossi GP *et al*,. 2006; Calhoun D, 2016). PA exists in two main forms: idiopathic hyperaldosteronism (IHA) and aldosterone-producing adenoma (APA), which is a benign adrenocortical tumor. IHA involves both adrenals and accounts for an estimated 60 (57,2% in PAPY study) of diagnosis. The second one, discovered by Conn over 60 years ago (Conn JW *et al*,. 1964), is a unilateral adrenal adenoma and is responsible for the majority of remaining cases of PA (42,8% in PAPY study) (Young WF *et al*,. 2004; Young WF *et al*,. 2007). Nevertheless, the subtypes vary depending on the access to confirmatory testing, notably adrenal vein sampling (AVS): more patients are diagnosed with bilateral than unilateral disease if there was no access to AVS and vice versa (see below). For instance, in PAPY study, if centers into those with and without AVS are splitted, the results obtained are the opposite: around 60% APA and 40% IHA (Rossi GP *et al*,. 2006).

The typical patient with PA presents hypertension with elevated plasma aldosterone and low plasma renin levels, often associated with hypokalemia (Boulkroun S *et al*,. 2015). If severe, hypokalemia may be accompanied by muscle weakness, cramping, headaches, palpitations, and polyuria (Thomas RM *et al*,. 2015). Further, hypokalemia must be unmasked with the addition of diuretics. In addition, patients with PA have been reported to exhibit more severe left ventricular hypertrophy and diastolic dysfunction than patients with essential hypertension and a high prevalence of myocardial infarction, stroke, and atrial fibrillation (Boulkroun S *et al*,. 2015; Mulatero P *et al*,. 2013; Savard S *et al*,. 2013). Patients with PA tend to be younger and present severe symptoms in terms of degree and frequency of hypertension and hypokalemia respectively. Biochemical analysis reveals high plasma concentration aldosterone (PAC) usually 15 ng/dL) (Phillips JL *et al*,. 2000; Espiner EA *et al*,. 2003).

The diagnosis for PA traditionally includes the following three steps: screening (case detection), confirmation, and diagnosis subtype (Young WF *et al*,. 2007) (Figure 6).

According to the last Endocrine Society Guidelines (Funder JW *et al*,. 2016), screening for PA should be considered for hypertensive patients presenting spontaneous or diuretics-induced hypokalemia, or those with adrenal incidentaloma, or those with sleep apnea, or with a family history of early onset hypertension or cerebrovascular events at less than 40 years, and all hypertensive first degree relatives of patients with PA (Funder JW *et al*,. 2016; Thomas RM *et al*,. 2015). Initial screening of patients suspected to have hyperaldosteronism should be conducted with a morning plasma aldosterone and renin values. Following the Endocrine Society guidelines, for proper interpretation, aldosterone and renin testing should be performed in the morning on a seated ambulatory patient. To note the tests are, in general, affected by medications, including many antihypertensive agents, renal function, upright posture, age, sex and pregnancy (Stowasser M *et al*,. 2012; Tomaschit A *et al*,. 2010). Ideally, to avoid interference with screening test, hypertensive drugs interfering with renin and aldosterone measurements should be discontinued at least 2 weeks prior to laboratory testing. Nevertheless, because it could be unsafe in much cases, the Endocrine Society suggest the following medications: verapamil, hydralazine, prazosin hydrochloride, doxazosin and terazosin as alternatives during screening because of their minimal impact on screening assays (Funder JW *et al*,. 2016).

Even if an increased ratio of plasma aldosterone to plasma renin is highly suggestive of the diagnosis, some experts advocate for confirmatory testing. Currently there are no gold standard

confirmatory tests. Previous Endocrine Society guidelines suggested the following ones as potential confirmatory test: oral sodium loading test, saline infusion test, fludrocortisone suppression test, and the captopril challenge test. The selection of a confirmatory test should be based on cost, time, morbidity and conflicting data on sensitivity and specificity of the test (Aronova A *et al*,. 2014).

The last step is the subtyping classification. To reach this goal, three modalities can be used: CT imaging, scintigraphy, and, when feasible and desired by the patient, adrenal vein sampling (AVS). Among them, AVS is the most reliable technique used to distinguish a true unilateral adenoma (APA) from bilateral disease (Stowasser A *et al*,. 2004; Rossi GP *et al*,. 2014). Shortly, in AVS adrenal veins are accessed via the femoral vein. Blood sample are taken from both adrenal veins and compared to that found in the inferior vena cava (IVC) at the level below the renal veins.

Finally, the diagnosis of APA, the unique clinical form of PA identifiable with certainly, is established when the following "four corners criteria" are satisfied (Funder JW *et al*,. 2016; Rossi GP *et al*,. 2007; Rossi GP *et al*,. 2008):

1- Evidence of PA at the biochemical screening test (serum creatinine, serum and urine Na+ and K+ levels, plasma renin activity (PRA), aldosterone, cortisol, and glomerular filtration rate);

2- Lateralization of aldosterone secretion of AVS or at 131-I-norcholesterol dexamethasone-suppressed adrenocortical scintigraphy;

3- Evidence of adenoma at computed tomography, and/or magnetic resonance, and/or surgery, and/or pathology;

4- Demonstration of normokalemia and HT cure, or improvement, at follow-up after adrenalectomy

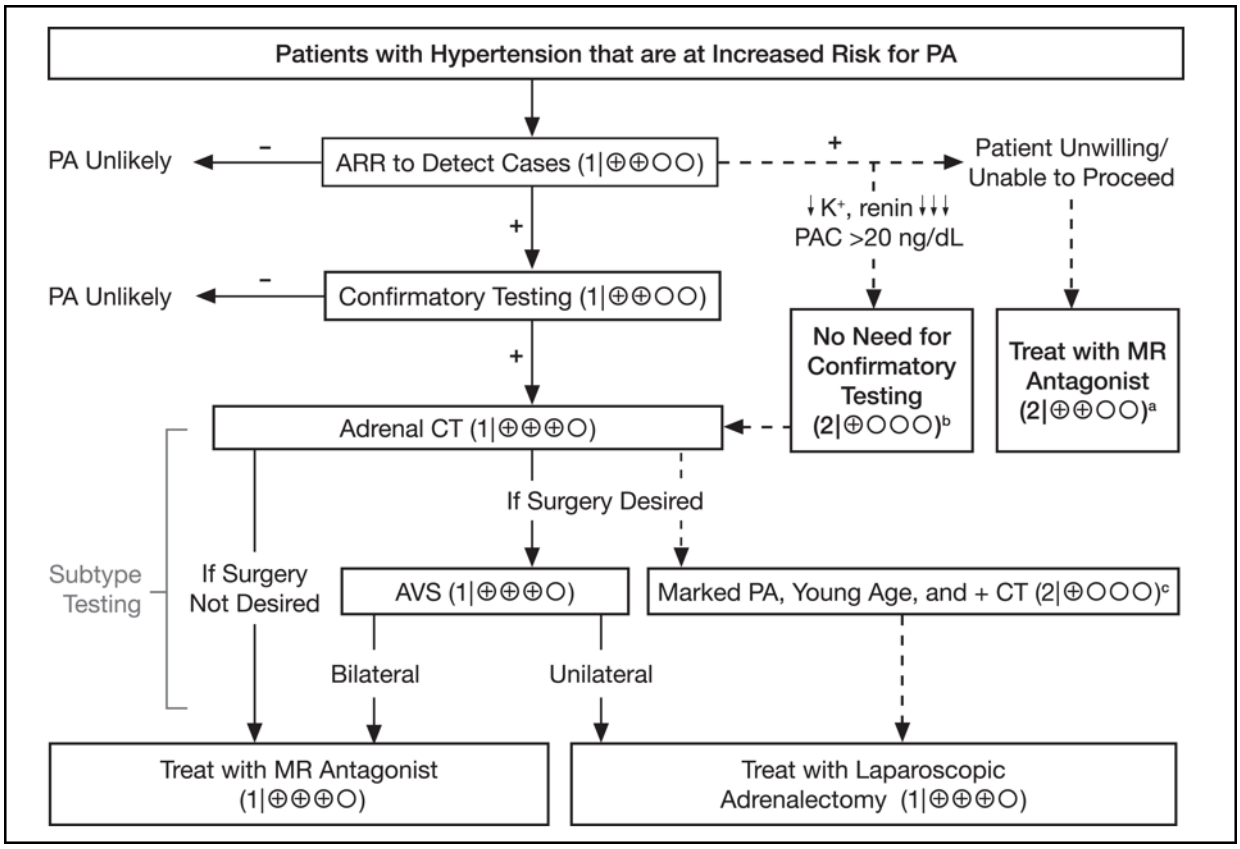

Figure 7: Algorithm for the detection, confirmation, subtype testin, and treatment of PA (from Funder JW *et al*,. 2016).

V-2 Morphological aspect of Aldosterone-Producing Adenoma and Molecular pathways

V-2 A) Morphological aspect

The great majority of APA is macroscopically a single, unilateral and well-circumscribed tumor with or without encapsulation. The size of APA varies from 0,5 cm to 6,0 cm, and usually appears to be with golden-yellow color on the surface (Nakamura Y *et al*,. 2014).

Microscopically, APA is composed mainly of clear cortical cells resembling to the ZF-like cells showing round to oval vesicular nuclei, often with small nucleus and lipid-laden cytoplasms, which gives the tumor its characteristic golden-yellow cut surface (Nakamura Y *et al*,. 2014; Neville AM *et al*,. 1966). Compact cells resembling the ZG-like may also be seen in APA in various ratio among APA cases. On the other hand, microadenoma is sometimes difficult to distinguish from non-neoplasic micronodular change in the adrenal cortex caused by localized ischemia and compensatory cortical cell hypertrophy (Nakamura Y *et al*,. 2014).

Interestingly, APA tissues express in general relatively high levels of CYP11B2, but StAR mRNA expression is usually not elevated in these tumor tissues (Bassett MH *et al*,. 2005). Moreover, it has been demonstrated that APA is generally composed of a heterogeneous mixture of cortical cells from different adrenal zones, which could explain the particular tumor tissue expression patterns: presence of CYP11B2, CYP11B1 and CYP17 (Azizan EA *et al*,. 2012), which indicated that the same APA tumor cells could secrete cortisol as well as aldosterone (Hiraishi K *et al*,. 2011; Fallo F *et al*,. 2011; Konosu-Fukaya S *et al*,. 2015).

V-2 B) Molecular pathways

The regulation of cell membrane potential of the ZG is crucial to maintain the cell in a hyperpolarized state in the absence of a secretagogue stimulus. The ZG cell membrane is selectively permeable to K^+ due to the expression of a large number of potassium channels (Nakamura Y *et al*,. 2014).

During the last decade, several somatic *KCNJ5* (G protein-activated inward rectifier potassium channel 4) mutations were identified (Choi M *et al*,. 2011). Some of these mutations are localized near or within the selective filter of the channel (as p.Gly151Arg or p.Leu168Arg). Furthermore, mutations in or surrounding the selectivity filter (i.e. p.Ile157Ser, p.Gly151Glu) have been identified (Charmandari E *et al*,. 2012; Murthy M *et al*,. 2012; Kuppusamy M *et al*,. 2014; Akerstrom T *et al*,. 2012). This kind of mutation results in a significant decrease in K^+ selectivity and permeability to Mg^{2+} with ensuing greater influx of Na^+ into the cell, resulting in chronic cell depolarization. Thus is followed by opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production (Nakamura Y *et al*,. 2014; Oki K *et al*,. 2012; Monticone S *et al*,. 2012). In addition, more recently, others somatic mutations have been found in genes coding for ATPases, namely ATP1A1 (Azizan EA *et al*,.2013 Beuschlein F *et al*,. 2013), ATP2B3 (Beuschlein F *et al*,. 2013), and the Cav1.3 calcium channel, CACNA1D (Azizan EA *et al*,.2013; Scholl UI *et al*,. 2013). Although mutations in KCNJ5 channel and ATP1A1 ATPase affect adrenal ZG cell membrane potential and intracellular ionic homeostasis, with chronic depolarization leading to opening of voltage-dependent calcium channels and activation of calcium signaling and aldosterone production (Oki K *et al*,. 2012; Azizan EA *et al*,. 2013; Beuschlein F *et al*,. 2013), mutations in ATP2B3

and CACNA1D directly modify intracellular calcium equilibrium, also leading to an activation of calcium signaling and aldosterone production (Nakamura Y *et al*,. 2014; Oki K *et al*,. 2012; Azizan EA *et al*,. 2013; Beuschlein F *et al*,. 2013) (Figure 8). Furthermore, germline KCNJ5 mutations were also identified as a causative event of familial hyperaldosteronism type III (FH-III) (Geller DS *et al*,. 2008; Gomez-Sanchez CE *et al*,. 2016).

Interestingly, *KCNJ5* mutations are the most frequent genetic abnormalities reported in APA with a prevalence of ~ 40% in Caucasian population, and as high as 70% in series from Japan (Boulkroun S *et al*,. 2012; Rossi GP *et al*,. 2014; Kitamoto T *et al*,. 2014; Taguchi R *et al*,. 2012). The less frequent are mutations affecting *ATP1A1* and *ATP2B3* genes, whereas mutations in the calcium voltage-gated channel subunit alpha1 D (CACNA1D), and in the calcium voltage-gated subunit alpha1 H (CACNA1H) genes are more frequent (Scholl UI *et al*,. 2015).

Even though the role of all these mutations in abnormal aldosterone secretion has been clearly established, their impact in adenoma formation and in abnormal cell proliferation is not well completely understood. Some elements of the response came at the beginning of the decade with the investigation of mouse models in which TASK1 and/or TASK3 potassium channels were invalidated to determine the contributions of TASK channels to background K^+ currents in adrenal ZG and their role in the control of aldosterone production (Davies LA *et al*,. 2008; Heitzmann D *et al*,. 2008). TASK1 and TASK3 are two-pore domain K^+ channels. In mouse adrenal cortex, whereas TASK1 expression is found throughout both the ZF and the ZG, TASK3 is solely present in the ZG (Davies LA *et al*,. 2008). Deletions, *in vivo*, of TASK1 and TASK3, respectively, lead to the development of hyperaldosteronism or low-renin hypertension (Davies LA *et al*,. 2008; Heitzmann D *et al*,. 2008; Penton D *et al*,. 2012; Guagliardo NA *et al*,. 2012). This hyperaldosteronism is due to abnormal depolarization of the ZG cell membrane resulting in increased intracellular Ca^{2+} concentration and stimulation of aldosterone biosynthesis. Nevertheless, formation of adrenal tumors has never been observed in these models indicating that other mechanisms are required to promote increased cell proliferation in APA (Nakamura Y *et al*,. 2014). Moreover, a reduced expression of TASK2, encoded by *KCNK5*, has been recently described in APA compared with normal adrenal gland, and the expression in NCI-H295R cells of a TASK2 dominant-negative mutant resulted in increased aldosterone production and CYP11B2 and StAR expression (Lenzini L *et al*,. 2014). Moreover, comparison of gene expression profiles of adrenal glands from $TASK1^{-/-}$ female and male mice

allowed the identification of a cluster of genes closely associated with hyperaldosteronism, among them dicckkopf3 (dkk3), a member of the dickkopf family of Wnt signaling modulators (Wakil A *et al*,. 2012). Inactivation of dkk3 in TASK1$^{-/-}$ mice resulted in the extension of the phenotype of hyperaldosteronism to male animals (Wakil A *et al*,. 2012); suggesting that Wnt/β-catenin pathway could play a role in the development of APA.

Briefly, the "canonical" Wnt signaling pathway acts through the regulation of the amount of the transcriptional regulator β-catenin, which controls the expression of specific genes involved in development. The "non-canonical" Wnt signaling pathway is independent of β-catenin. It implicates small GTPases/jun N-terminal kinase (JNK) and intracellular calcium signaling (Nakamura Y *et al*,. 2014; Rao TP *et al*,. 2010). It has been demonstrated that Wnt/β-catenin pathway is a key signaling pathway in adrenal gland development during embryonic phase. In human adult adrenal gland, while β-catenin expression was found in the entire cortex, its activated form was restricted to ZG cells (Boulkroun S *et al*,. 2011). One major component of this pathway, Wnt4, which plays a central role in differentiation of the definitive zone of the adrenal cortex (Jeays-Ward K *et al*,. 2003), is higher expressed in APA than in normal adult adrenocortical cells (Kuulasmaa T *et al*,. 2008). This overexpression leads to an increase of aldosterone biosynthesis. The activation of β-catenin pathway has also been recently reported in two-thirds of APA (Boulkroun S *et al*,. 2011; Kuulasmaa T *et al*,. 2008), but at variance with adrenocortical tumors and adrenocortical adenoma, only few mutations of β-catenin pathway were found in APA (Nakamura Y *et al*,. 2014; Tadjine M *et al*,. 2008), strongly suggesting that the activation of this pathway is not associated with the presence of mutations (Tissier F *et al*,. 2005; Gaujoux S *et al*,. 2005). Additionally, in adrenocortical carcinoma, the activation of β-catenin pathway is associated with a poor prognostic, whereas in APA it is not associated with specific tumor characteristics (Gaujoux S *et al*,. 2011). Hence, activation of this pathway differs between APA and adrenal cortex carcinoma and contributes to aldosterone hypersecretion rather than to cell proliferation (Berthon A *et al*,. 2014).

Cell proliferation is due to another pathway: the Sonic HedgeHog signaling (shh). As β-catenin pathway, shh pathway is essential for adrenal gland development and maintenance (Nakamura Y *et al*,. 2014). To make a long story short, shh encodes a secreted signal that belongs to the Hedgehog family. The activation of shh signaling occurs through its binding to a receptor complex formed by the protein patched-1 and the G-protein coupled receptor Smoothened. In human adult adrenals gland, the expression of SHH is restricted to few cells of the "cambium

layer" (Boulkroun S *et al*,. 2011; Kim AC *et al*,. 2009). Using mice shh$^{-/-}$, it has been shown that shh is essential for expansion of the adrenal cortex but not for zonation and differentiation (Huang CC *et al*,. 2010). Interestingly, the expression of shh has been found in APA as well as in the entire hyperplastic ZG (Boulkroum S *et al*,. 2011). The activation of this pathway in APA was confirmed by transcriptomic analysis (Boulkroum S *et al*,. 2011). All these results suggest that APA have acquired some characteristics of stem/precursor cells or, alternatively, that reexpression of fetal markers from the definitive zone in the adrenal cortex could underlie excessive proliferation and APA formation (Nakamura Y *et al*,. 2014; Boulkroum S *et al*,. 2011).

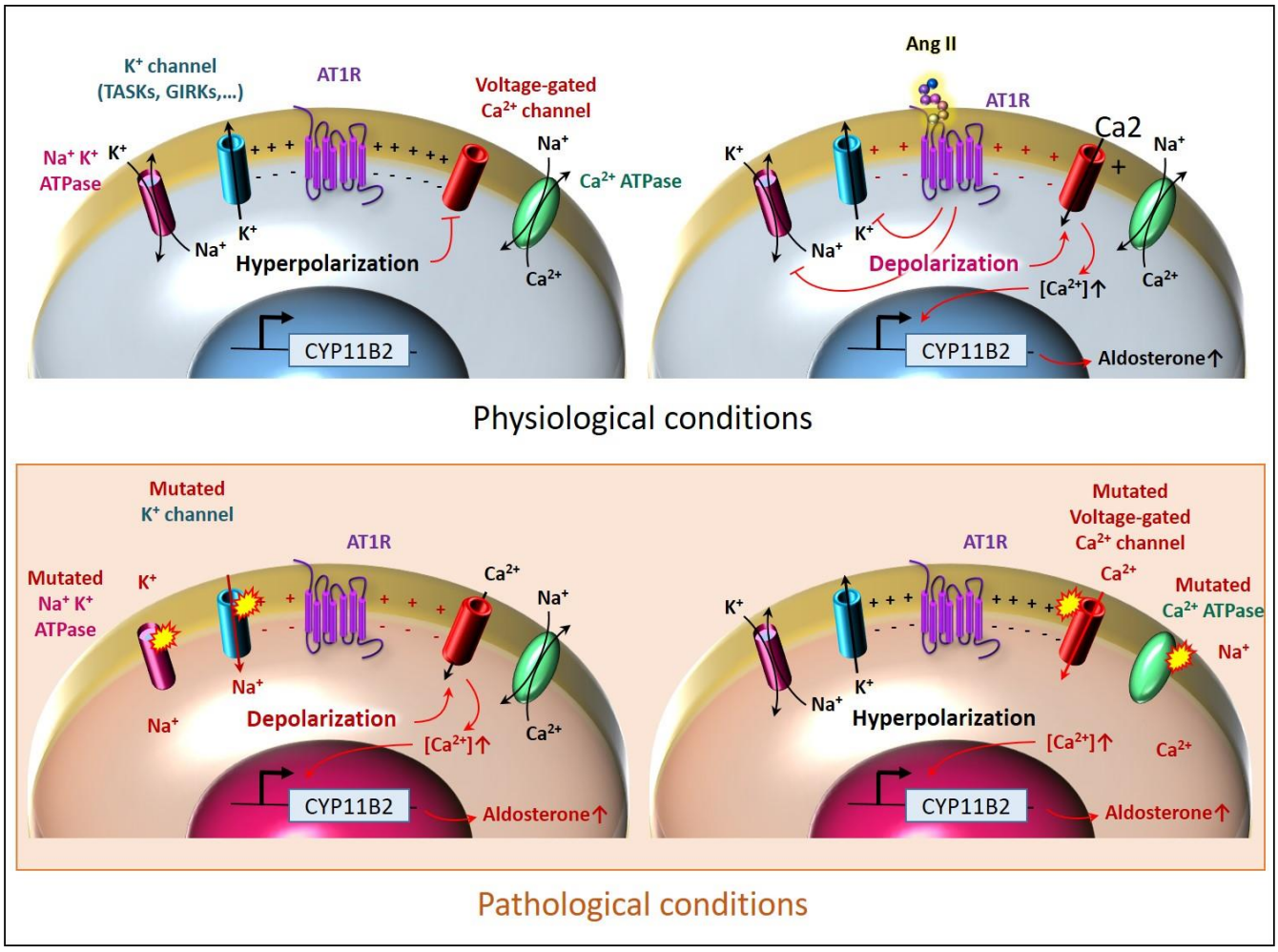

Figure 8: Regulation of aldosterone biosynthesis under normal (in blue) and pathological conditions (in pink) (adapted from Nakamura Y *et al*,. 2014).

VI- The Renin-angiotensin-aldosterone system

VI-1 From renin to angiotensin

Over a century ago after the identification of a "renin-like" activity in the canine kidney by Tigerstdtedt and Bergman, and more than 50 years after the purification of the vasoactive peptide angiotensin II (Ang II), carried out independently by Page and Braun-Menendez *et al*., the characterization of the renin-angiotensin system (RAS) continues relentless. Thus is broadening our understanding of the function of this key hormonal system, which has paramount clinical and therapeutic implications. The RAS is currently regarded as both an endocrine and a paracrine system (Rocha R and Stier CT,Jr, 2001). The circulating RAS entails a coordinated hormonal cascade, which begins with the biosynthesis of pro-hormone (pro-renin) that undergoes proteolytic cleavage of the prosegment N-terminus peptide to give the bioactive renin. The latter is a secretory glycoprotein mainly made by the juxtaglomerular cells (JG) that line the afferent arteriole of the renal glomerulus (Atlas SA, 2007). Renin is stored in granules of the JG cells and released first into the renal circulation, and then systemically, through a highly regulated exocytic process. Besides and in parallel to this, the kidney constitutively releases pro-renin, which can be activated at the level of the pro-(renin) receptor (Rocha R and Stier CT,Jr, 2001). Compelling evidence now indicates that the RAS also acts as a paracrine system, which is directly involved in regulating the function of many organs, as the adrenal gland, the reproductive system, the visceral adipose tissue, the vascular tissue, the eyes, the heart, and the brain.

Control of renin secretion is a key determinant of the RAS activity, because renin regulates the initial and rate-limiting step of the RAS by cleaving the N-terminal portion of the renin substrate (angiotensinogen) to form the biologically inert decapeptide angiotensin I (Ang I). Although mainly produced in the liver, angiotensinogen is also synthesized locally in tissues, as the vessel wall and adipocytes. These sites might attain importance in disease conditions, as shown by the observation that adipocyte angiotensinogen deficiency, while not affecting plasma angiotensinogen levels under normal conditions, greatly reduced circulating Ang II under high fat diets.

Ang I, the inactive decapeptide formed by renin, is hydrolyzed by the angiotensin-converting enzyme type 1 (ACE 1), which removes the C-terminal His-Leu dipeptide to form the octapeptideAng II (Ang 1-8), which, alongside aldosterone, is the major mediator of the RAS (Miura S and Karnik SS, 1999; Lautner RQ *et al*,. 2013; Hrenak J *et al*,. 2016) (Table 1).

Name	Amino-acids sequence
Angiotensin I (1-10)	H_2N-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
Angiotensin II (1-8)	H_2N-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
Angiotensin III (2-8)	H_2N-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
Angiotensin IV (3-8)	H_2N-Val-Tyr-Ile-His-Pro-Phe-COOH
Angiotensin-(1-9)	H_2N-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-COOH
Angiotensin-(1-7)	H_2N-Asp-Arg-Val-Tyr-Ile-His-Pro-COOH
Angiotensin A	H_2N-Ala-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
Alamandine	H_2N-Ala-Arg-Val-Tyr-Ile-His-Pro-COOH

Table 1: Amino-acid sequences of angiotensin peptides (adapted from Miura S and Karnik SS, 1999; Lautner RQ *et al*,. 2013; Hrenak J *et al*,. 2016)

VI-2 The angiotensin II type 2 receptor (AT2R)

VI-2 A) Knowledge before Compound 21 revolution

As mentioned before, the octapeptide hormone Ang II is an important mediator of RAS functions. Most of the physiological effects attributed to Ang II, such as regulation of mean arterial blood pressure, water-electrolyte balance, vasoconstriction, catecholamine release, aldosterone production, sodium and water reabsorption, cell growth and cardiac hypertrophy, are mediated by the AT1 receptor (AT1R) (Timmermans PB et al,. 1993; Kaschina E and Unger T, 2003). Nevertheless, another cell surface receptor, AT2R, can mediate physiological effect of Ang II.

As AT1R, AT2R is a 7-transmembrane G-protein-coupled receptor. It is composed of 363 amino acids for a molecular weight of 41,220 KDa, and shares 34% of sequence homology with AT1R (Padia AH and Carey RM, 2013; Miura S and Karnik SS, 1999). The sequence homology between the receptors occurs mainly in the transmembrane hydrophobic regions of the molecules which form their 7-transmembrane helical columns (De Gasparo M *et al*,. 2000). Interestingly, Ang II binds to AT1R and AT2R through different types of epitope recognition (Miura S and Karnik SS, 1999).

AT2R is expressed ubiquitously at very high levels in the fetus, where it plays a major role in organ development. For example, in human fetal adrenal gland, it leads to apoptosis (Chamoux E *et al*,. 1999), and antagonizes the stereoidogenic actions of AT1R in ovine fetal adrenal gland (Moritz KM *et al*,. 1999). In most tissues, AT2R expression dramatically decreases after birth, being restricted to few organs, including kidney, adrenal cortex, heart, vasculature, uterus, ovary, and discrete areas of the brain. However, recent data using immunobloting to measure total AT2R protein level in tissue, showed that adult rats exhibited a higher AT2R protein level compared with fetus or neonates' brainstem, liver and kidney tissue (Yu L *et al*,. 2010).

Notwithstanding this, its role in adult animals and humans remained uncertain for decades, owing to the fact that specific agonists for AT2R were not available. Therefore, concomitant stimulation of AT1R could not be avoided when using the AT2R natural ligand Ang II, which binds to AT1R and AT2R with almost equal affinity (Table 2). Hence, given the higher abundance of AT1R in many organs Ang II usually induces predominant AT1R effects. Moreover, the synthetic peptide CGP42112A, which was used to identify the AT2R, was thereafter found to have AT2R agonistic and antagonistic effects depending on its concentration (Kaschina E and Unger T, 2003; De Gasparo M *et al*,. 2000; Unger T *et al*,. 2010; Foulquier F *et al*,. 2012), pulling down difficulties to interpret results. In the late 1990's, generation of AT2R knockout animals leads to some progress in the understanding its role in adult. First, anatomical, target deletion of AT2R is followed by anomalies of the kidney and urinary tract, a malformation caused by delayed apoptosis of undifferentiated mesenchymal cells (Nishimura H *et al*,. 1999). Moreover, $AT2R^{-/-}$ mice have slightly elevated blood pressure as well as low basal levels of renal bradykinin, cGMP, and a low index of nitric oxide (NO) production (Siragy HM *et al*,. 1999). Notably, the loss of AT2R abolished left ventricular hypertrophy and cardiac fibrosis in mice with Ang II-induced hypertension (Ichihara S et al,. 2001). Interestingly, AT2R-deficient mice have increased sensitivity to pain and decreased levels of β-endorphin

(Sakagawa T *et al*,. 2000). This observation leads to hypothesize that AT2R may be involved in the regulation of the pain threshold. Unfortunately, in spite of these results, AT2R-knockout mice did not shed enough light on the various enigmata of this receptor, since the ensuing phenotype did not fully reveal its secrets (Unger T *et al*,. 2010).

An important step has been done in 2004 with the design and synthesis of compound 21 (C21) by the groups of Mathias Alterman and Anders Hallberg (Wan Y *et al*,. 2004). C21 is the first, and still unique, non-peptide orally active AT2R agonist. C21 K_i value is 25000 fold higher for the AT1R than for the AT2R (respectively 0.4 nM (2 nM in human receptor) and >10 µM) (Bosnyak *S et al*,. 2011) (Table 2). Unlike to CGP42112A, C21 can be used both *in vitro* and *in vivo*. For the first time Compound 21 gave opportunity to selectively stimulate the AT2R under several experimental conditions, and allowed important discoveries about role of AT2R.

	Angiotensin II type 1 receptor (AT1R)		**Angiotensin II type 2 receptor (AT2R)**		**AT2R selectivity**	
Peptide ligand	IC_{50} value (M)	Relative affinity to AngII (100%)	IC_{50} value (M)	Relative affinity to AngII (100%)	AT1R/AT2R	Foldselectivity
Ang II	$7{,}92 \times 10^{-9}$	100	$5{,}22 \times 10^{-10}$	100	1,18	15
Ang-(1-7)	$1{,}00 \times 10^{-5}$	> 1000	$2{,}46 \times 10^{-7}$	0,21	1,61	41
A-779	$1{,}00 \times 10^{-5}$	> 1000	$1{,}00 \times 10^{-5}$	> 1000	/	/
Candesartan	$1{,}56 \times 10^{-9}$	507	$1{,}00 \times 10^{-5}$	> 1000	3,81	6402
CGP42112	$1{,}00 \times 10^{-5}$	> 1000	$2{,}33 \times 10^{-10}$	224	4,63	42863
Compound 21 (C21)	$1{,}00 \times 10^{-5}$	> 1000	$2{,}29 \times 10^{-9}$	23	3,64	4367

Table 2: Affinity of several agonists and antagonists for angiotensin receptors (adapted from Bosnyak S *et al*,. 2011)

At this time, it is well established that expression of AT2R is upregulated in various pathological conditions associated with tissue remodelling or inflammation including hypertension, atherosclerosis, heart failure, myocardial infarction, tissue ischaemia, and diabetes mellitus (Miura S and Karnikk SS, 2000; Levy BI *et al*,. 1996; Matsubara H *et al*,.

1998; Savoia C *et al*,. 2007), suggesting that it serves a counter-regulatory role when over activation of the AT1R occurs.

V-2 B) Vascular tone and blood pressure

Concerning the control of vascular tone, there is now overwhelming evidence that AT2R opposes the AT1R-mediated vasoconstrictor action of Ang II. This regulation has been demonstrated in small arteries of the mesenteric, uterine, adrenal, coronary and peripheral circulations, both in animal models and in humans (Padia SH and Carey RM, 2013; Siragy HM *et al*,. 1999a; Siragy HM *et al*, 1999b; Carey RM et al,. 2001; Katada J *et al*,. 2002; Widdop RE *et al*,. 2002; Hannan RE *et al*,. 2004; Batenburg WW *et al*,. 2004; Bergaya S *et al*,. 2004; Savoia C *et al*,. 2006; Savoia C *et al*,. 2007). It also been determined in high capacitance vessels such as the aorta (Hiyoshi H *et al*,. 2004; Yayama K *et al*,. 2004), and in the fetal circulation (Gauthier KM *et al*,. 2005). The vasodilatator effect of AT2R is due to the release of nitric oxide (NO) via bradykinin production (Figure 9) (Katada J and Majima M, 2002; Lemarie CA and Schiffrin EL, 2010; Wiemer G *et al*,. 1993; Seyedi N *et al*,. 1995). Furthermore, the AT2R-mediated vasodilator action of Ang II is most easily demonstrated when AT1R have been inhibited using an AT1R blocker (Siragy HM and Carey RM, 1999; Carey RM *et al*,. 2001; Katada J and Majima M, 2002; Widdop RE *et al*,. 2002; Carey RM, 2005). Besides, it has been shown that the vasodilator and hypotensive effects of AT2R activation are both acute and long term, and are not associated with desensitization, making AT2R a potential therapeutic target in hypertension (Katada J and Majima M, 2002; Widdop RE et al,. 2002). Some studies have also demonstrated that the blood pressure-lowering effects of AT1R blockade might be mediated, at least in part by AT2R activation (Siragy HM *et al*,. 1999; Carey RM *et al*,. 2001). Along this line, others studies performed with C21 administration in rat demonstrated that an AT2R-mediated decrease in blood pressure occurred only when there was a concomitant low-dose blockade of AT1R (Foulquier S *et al*,. 2012; Carey RM *et al*,. 2001; Barber MN *et al*,. 1999; Unger T *et al*,. 2013). The observation that AT2R stimulation lowers BP only in the presence of low-level AT1R blockade indicates that *in vivo*, an AT1R-mediated tone predominates over any vasodilatory effect of AT2R. Consequently, it would seem unlikely that

AT2R agonists will become antihypertensive drugs suitable for monotherapy (Unger T *et al*,. 2013; McCarthy CA *et al*,. 2013).

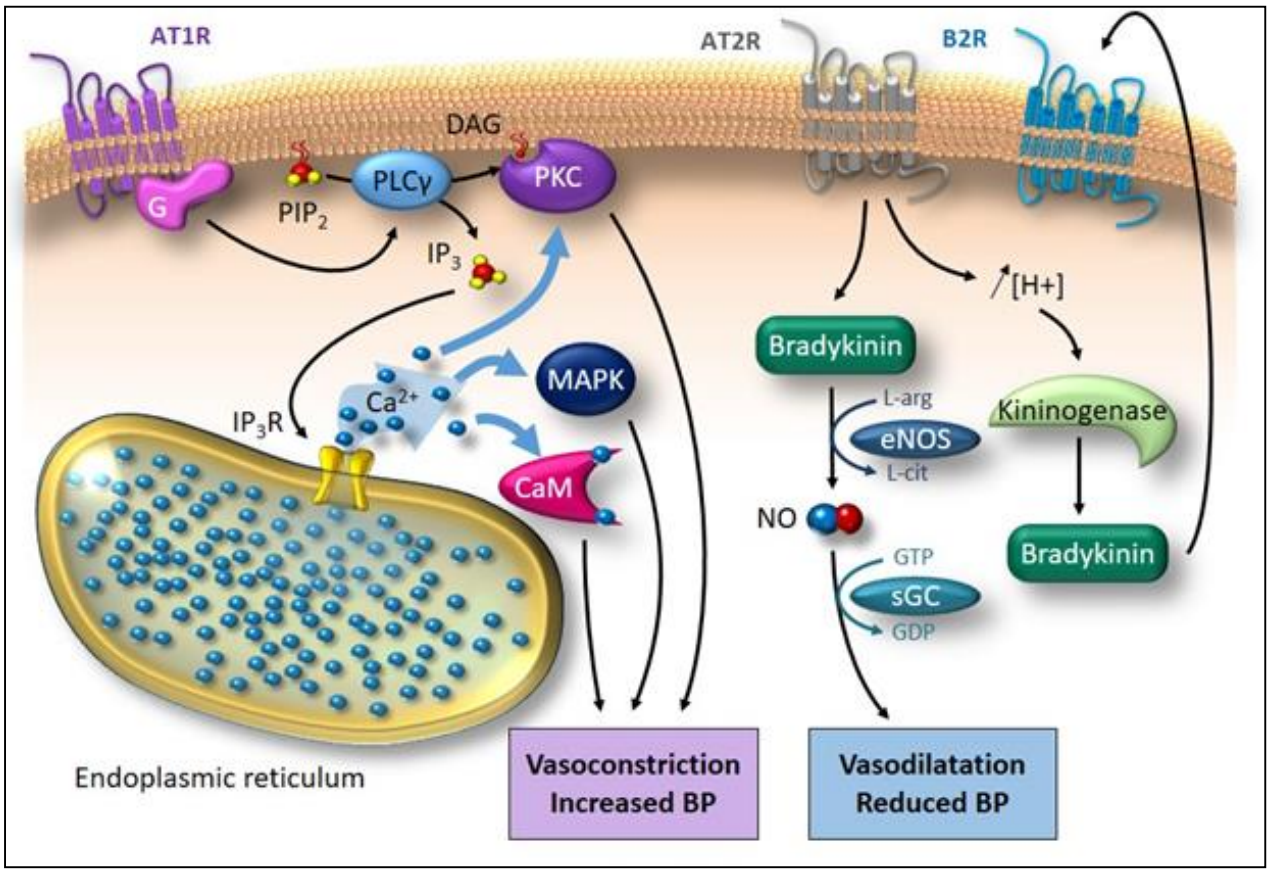

Figure 9: Interaction between the angiotensin II type 2 receptor and the kinin/bradykinin type 2 receptor (adapted from Lemarie CA *et* al,. 2010)

V-2 C) Renal actions

As mentioned before, abnormalities in anatomy and kidney functions have been observed in AT2R knockout mice (Nishimura H *et al*,. 1999; Danyel LA *et al*,. 2013). Compared to wild-type, these mice experience, inter alia, aggravated glomerular damage and impairment of renal function, a high albuminuria, and an increase of renal macrophage infiltration of glomerulus and interstitium. These states may result from a persistent impairment in renal function that evolved during fetal development, due to the absence of the AT2R, and may not represent missing counter-regulatory actions of the receptor itself in the adult organism.

In adult, AT2R is expressed throughout the kidney in both the vasculature and the tubular structures and are heavily expressed in renal proximal tubule cells (Ozono R *et al*,. 1997; Miyata

N *et al*,. 1999). With the use of the technique of direct renal interstitial microinfusion of pharmacological agents (Ang II and Ang III) (Padia AH *et al*,. 2007a; Padia SH *et al*,. 2007b; Salomone LJ *et al*,. 2007), and C21 in rats (Danyel LA *et al*,. 2013; Pulgar VM *et al*,. 2011) it has been strongly evidenced that AT2R mediates natriuresis. Furthermore, these studies showed that Ang III is the natural and preferred AT2R agonist in the regulation of renal Na^+ excretion. Mechanistically, renal AT2R mediates natriuresis mostly via a NO/cGMP signaling cascade operating in the renal proximal tubule (Kemp BA *et al*,. 2012). In addition, using the 5/6 nephrectomy mice, model of renal injury, it has been shown that AT2R protects the kidney from ischemic damage (Hashimoto N *et al*,. 2004; Vazquez E *et al*,. 2005). Additionally, with the use of C21, the kidneys protective effect from inflammation of AT2R has been strongly demonstrated (Matavelli LC *et al*,. 2011; Abadir PM *et al*,. 2011). Last but not least, it is well admitted now that AT2R suppress renin biosynthesis and secretion in response to high Ang II levels (Carey RM *et al*,. 2013; Siragy HM *et al*,. 2005).

V-2 D) Vascular remodeling and anti-inflammatory effects

Chronically elevated BP triggers an inflammatory response followed by an excess synthesis and accumulation of extracellular matrix mainly in the left ventricle (LV), the kidney, and the vascular wall. This fibrotic response to hypertension leads to deteriorated organ function and resulting in heart failure, renal disease, or vascular stiffening (Danyel LA *et al*,. 2013). On top of that, renal disease and vascular stiffening reinforce the development of hypertension. The first studies showed a positive effect of AT2R due to an indirect stimulation of this receptor (Jones ES *et al*,. 2004; Tea BS *et al*,. 2000). Here too, the beneficial effects of AT1R blocker were, at least in part, due to indirect AT2R stimulation.

The proof of a direct beneficial role of AT2R on hypertension-induced vascular remodeling was separately obtained by two teams (Paulis L *et al*,. 2012; Rehman A *et al*,. 2012). With a chronic infusion of C21 in Nω-nitro-L-arginine-methyl-ester hydrochloride-induced hypertension and in stroke-prone spontaneously hypertensive rats (SHR-SP) respectively, they demonstrated that AT2R agonist prevented the development of vascular hypertrophy and

fibrosis. Remarkably, in both studies, this reversal of hypertension-induced pathology was achieved without any significant effect on blood pressure.

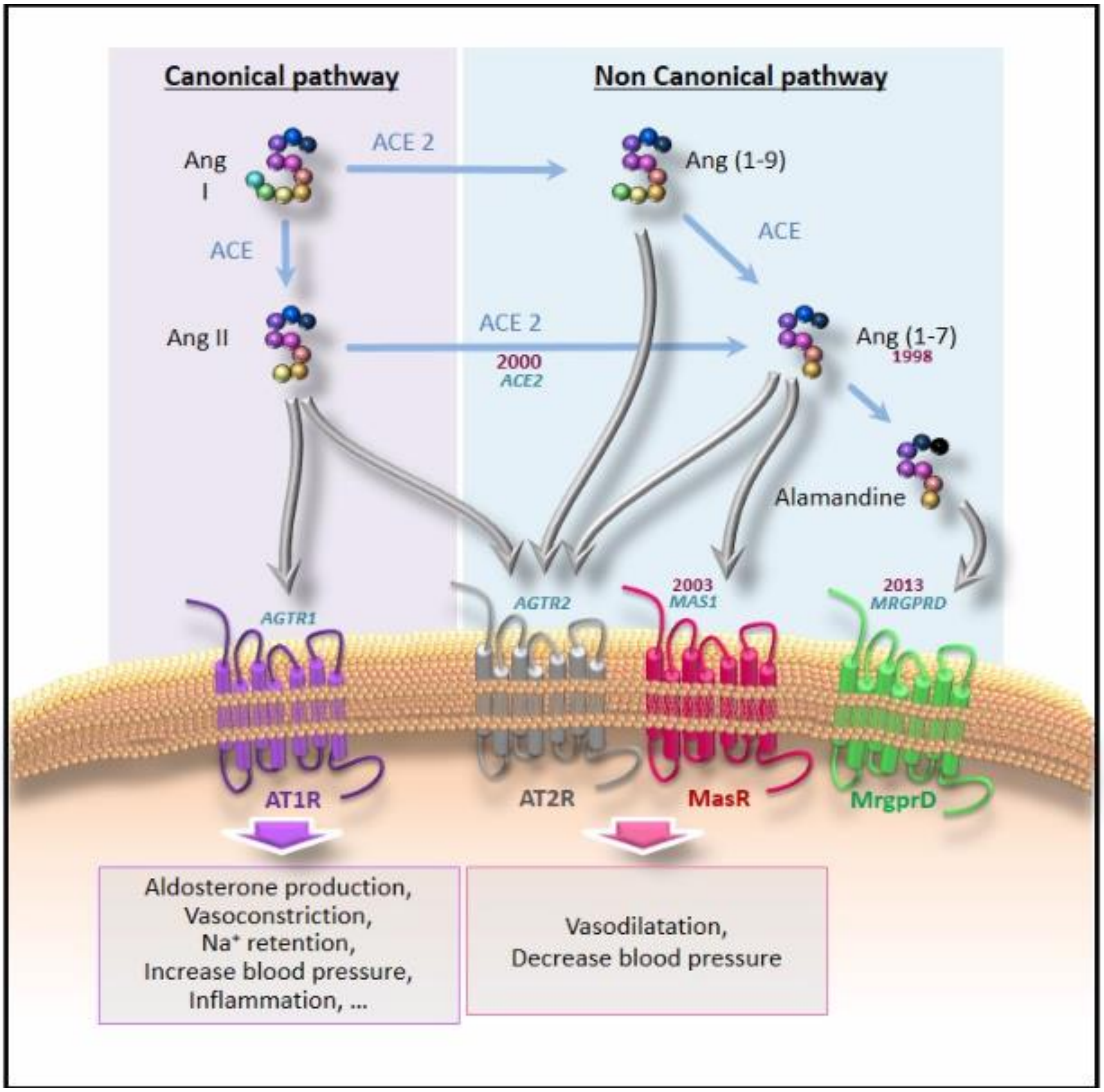

Figure 10: Canonical and non-canonical (also named protective pathway) pathway of the renin-angiotensin system - In blue: genes; in red: date of discovery

For decades it has been though that AT2R could play a role against inflammation, but this function remained uncertain. However, with C21 this effect has been evidenced in different models. For example, in a model of myocardial infarction (MI), treatment with C21 improved cardiac function and decreased scar size after 7 days of treatment (Kaschina E *et al*,. 2008). In a model of hypertension-induced renal dysfunction (salt-loaded SHR-SP), C21 delayed the occurrence of brain damage and reduced proteinuria, and attenuated the inflammatory and the fibrotic processes in the kidneys (Gelosa P *et al*,. 2009). In another rat model of hypertension

(two-kidney-one-clip model) presenting an upregulated inflammatory status, C21 significantly decreased several inflammatory markers as Interleukine-6, tumor necrosis factor-α or TGF-β1 (Matavelli LC *et al*,. 2011). The role of AT2R in decreasing inflammatory markers has been also shown *in vitro* (Rompe F *et al*,. 2010; Menk M *et al*,. 2015).

Finally, AT2R is one component of the protective RAS, a new pathway discovered less than 2 decades ago (Figure 10). Another important component of this RAS non-canonical pathway is angiotensin-(1-7) receptor (MasR), which often acts in association with AT2R.

V-3 Angiotensin-(1-7) and Mas receptor

V-3 A) Mas receptor

As the two other angiotensin receptors described before, Mas receptor (MasR) is a GPCR discovered in 1986 and initially misidentified as a proto-oncogene (Westermeier F *et al*,. 2015; Sharif M *et al*,. 1994; Young D *et al*,. 1986). The human MasR is a 325 amino acid polypeptide and was, in 2003 (Santos RA *et al*,. 2003), the first member identified of the Mas-related GPCR protein (Bader M *et al*,. 2014), which also include the alamandine receptor. MasR is highly expressed in brain and testis tissues and to a less expand in the heart, kidney, lung, liver, spleen, tongue and skeletal muscle (Alenina N *et al*,. 2008). Interestingly, as AT2R, MasR is differentially expressed in different cardiac physiological and pathological states (Dias-Peixoto MF *et al*,. 2012). Functionally, MasR was thought to be an Ang II receptor for decades (Jackson TR *et al*,. 1988). It has been completely identified as Ang-(1-7) receptor using radioligand assays and functional analyses (Santos RA *et al*,. 2003).

Ang-(1-7) binding to MasR exerts different actions, and therefore, activates diverse intracellular pathways, of which the most relevant one is probably activation of the phospholipase A2 (PLA2)/arachidonic acid (AA) pathway. The relaxing properties of Ang-(1-7) on blood vessels is possibly due to this pathway (Westermeier F *et al*,. 2015). The second important one is the activation of the PI3K/Akt cascade. Through this pathway, MasR signaling induces eNOS phosphorylation and activation, and the concomitant NO release in several models (Sampaio WO *et al*,. 2007; Zhao J *et al*,. 2014). Furthermore, through this pathway, Ang-(1-7) increases PPARϒ activity promoting adipogenesis (Than A *et al*,. 2013). Additionally, in several tissues and cells MasR is related to insulin signaling and modifies the phosphorylation state of

JAK/STAT, PI3K/Akt, and IRS proteins signaling networks, thus increasing insulin sensitivity and tissue glucose uptake (Giani JF *et al*,. 2007; Munoz MC *et al*,. 2010; Verano-Braga T *et al*,. 2012). Finally, MasR induces intracellular Ca^{2+} signals that are activated in response to the coupling of the receptor to Gq/G11 proteins, stimulating the activation of PKC, which in turn increases the expression of AT1R (Canals M *et al*,. 2006).

V-3 B) Cardiovascular effects of MasR/Ang-(1-7) axis

A cardiovascular effect of Ang-(1-7) on cardiovascular biology was observed for the first time in dog hearts thirteen years before the MasR discovery (Santos RA *et al*,. 1990). After the identification of MasR, the cardiovascular protective effect of Ang-(1-7) has been strongly demonstrated. MasR KO mice exhibited reduced heart function (Simoes AC *et al*,. 2016; Gava E *et al*,. 2012; Dias-Peixoto *et al*,. 2008) associated with smaller peak of Ca^{2+} and slower uptake of Ca^{2+}, probably due to a decreased SERCA2 expression in cardiomyocytes (Simoes AC *et al*,. 2016; Gomes ER *et al*,. 2012). Moreover, the NO production is increased in parallel with a high significant increase in the expression of caveolin 3 and a decrease in heat shock protein 90 (Dias-Peixoto MF *et al*,. 2008). Because caveolin 3 prevents calmodulin interaction with NO synthase and heart shock protein 90 acts as a scaffold protein for the recruitment of Akt to the endothelial NO synthase complex, these effects reduce the activity of endothelial NO synthase (Wu KK *et al*,. 2002; Takahashi S *et al*,. 2003).

Notably, chronic infusion of Ang-(1-7) prevented the development of heart failure after experimental myocardial infarction (Chamsi-Pasha MA *et al*,. 2014) and also cardiac dysfunction in spontaneously hypertensive rats. It also reduced NF-κB activity and the expression of Nalp12, caspase 1, and several interleukines IL-1β and IL-6, in response to acute ischemia-reperfusion injury (Al-Maghrebi M *et al*,. 2009). Incidentally, in mice with genetic deletion of *ACE2*, Ang-(1-7) administration in a pressure overloaded induced heart failure model suppressed NADPH oxidase, reduced cardiac hypertrophy and recovered systolic dysfunction (Patel VB *et al*,. 2012). Additionally, Ang-(1-7) reduced pathological cardiac remodeling by attenuating interstitial and perivascular fibrosis (McCollum LT *et al*,. 2012).

In addition to its cardiac effects, it has been shown that Ang-(1-7) and MasR have a positive role on renal diseases in different animal models (Simoes AC *et al*,. 2016). In experimental

glomerulonephritis, the infusion of Ang-(1-7) reduced glomerulosclerosis (Simoes AC *et al*,. 2016). In adriamycin-induced nephropathy, oral administration of AVE 0991, a potent MasR agonist, improved renal function parameters, reduced urinary protein loss, and attenuated histological changes (Silveira KD *et al*,. 2013). Furthermore, in a mice model of acute renal injury, administration of AVE 0991 decreased kidney dysfunction, histological damage and tissue inflammation (Barroso LC *et al*,. 2012). In 5/6 nephrectomized mice, chronic treatment with Ang-(1-7) improved heart and renal function, and reduced blood pressure and left ventricular (Li Y *et al*,. 2009). In type 2 diabetes models as the kk-Ay/Ta mouse, the treatment with Ang-(1-7) counteregulated the effects of Ang II by reducing glomerulosclerosis, oxidative stress and cell proliferation, and mesangial expansion, TGF-β, fibronectin mRNA and NOX activity (Moon JY *et al*,. 2011). Moreover, in Zucker diabetic fatty rats, 2 weeks infusion of Ang-(1-7) lead to a recovery of creatinine clearance and to a reduction of systolic blood pressure, plasma triglycerides and urinary protein loss (Giani JF *et al*,. 2012).

Nevertheless, in spite of these beneficial effects, according to few studies Ang-(1-7) would exacerbate renal injury in certain experimental conditions (Shao Y *et al*,. 2008; Esteban V *et al*,. 2009; Velkoska E *et al*,. 2011): in these three studies, the infusion of Ang-(1-7) worsened renal function in diabetic rats (Shao Y *et al*,. 2008), elicited an inflammatory response in wild-type mice (Esteban V *et al*,. 2009) and was associated with deleterious effects on blood pressure and heart function in 5/6 nephrectomized rats (Velkoska E *et al*,. 2011). These side effects could be due to the dose of Ang-(1-7), the route of administration, the state of local RAS activation, the cell-specific signaling or the non-Mas-mediated pathways in the kidney (Zimmerman D *et al*,. 2012). For instance, Ang-(1-7) displays growth inhibitory properties and antagonizes the effects of Ang II in the proximal tubule (Su Z *et al*,. 2006), whereas in human mesanglial cells, the heptapeptide was described to stimulate cell growth pathways (Zimpelmann J *et al*,. 2009).

V-3 C) Non-cardiovascular effects of MasR/Ang-(1-7) axis.

Beyond its cardio-renal effects, the Ang-(1-7)/MasR axis regulates several others pathways in different tissues (Figure 11) (Passos-Silva DG *et al*,. 2013). Mostly in cellular proliferation and in inflammation.

Treatment with Ang-(1-7) inhibits the growth of several lung cancer cells *in vitro* (Gallagher PE *et al*,. 2004). This effect can be explained, at least in part, as a consequence of the ERK (MAPK) signal transduction pathway inhibition, or by the reduction in cyclo-oxygenase 2 (COX-2) (Gallagher PE *et al*,. 2004; Menon J *et al*,. 2007). Indeed, the two pathways have a central role in lung cancer cell proliferation. Furthermore, Ang-(1-7) is also involved in the volume reduction of orthopic human breast tumours by decreasing the growth of cancer in associated fibroblasts and fibrosis (Cook KL *et al*,. 2010). Additionally, Ang-(1-7) desactivates the histone deacetylase 1 (HDAC1), overexpressed in solid tumours and known for its role in tumorigenesis, through the dephosphorylation of its regulatory sites (Verano-Braga T *et al*,. 2012). On the other hand, Ang-(1-7) is able to induce the activation of the transcriptional factor FOXO1, via the dephosphorylation of its regulatory site, which led to the activation of genes involved in apoptosis, cell-cycle arrest and oxidative stress resistance (Verano-Braga T *et al*,. 2012 ; Calnan DR and Brunet A, 2008). Moreover, in a mouse sponge model of angiogenesis daily injection of Ang-(1-7) was shown to significantly inhibit angiogenesis via NO release (Calnan DR and Brunet A, 2008).

More importantly, Ang-(1-7) can also counter-regulate the AT1R-mediated inflammatory effects. For instance, mouse peritoneal macrophages treated with Ang-(1-7) presented a reduction in pro-inflammatory cytokines expression as TNF-α or IL-6 after lipopolysaccharide stimulation (Souza LL et al,. 2012).

Nonetheless, Ang-(1-7)/MasR axis plays a beneficial role in atherosclerosis, another clinically important inflammatory condition, which is characterized by endothelial dysfunction, vascular inflammation, and the accumulation of lipids within the intima of the vessel wall (Corrado E *et al*,. 2010). In the atherosclerotic model alipoprotein E-/- (ApoE-/-) KO mice, chronic incubation with Ang-(1-7) improved endothelial function and attenuated the lesion progression as shown by a reduction in fatty deposits and intima/media ratio (Tesanovic S *et al*,. 2010). This could be due to the inhibition of NADPH oxidase expression, which is involved in production of reactive oxygen species (ROS) and in the co-stimulatory molecules on antigen-presenting cells' expression diminution (Jawien J *et al*,. 2012).

Finally, the effects of Ang-(1-7) have also been evaluated in inflammatory conditions of the respiratory tract. For example, a mouse model of allergic asthma challenged with ovalbumin and treated with Ang-(1-7) resulted in attenuation of ovalbumin-induced perivascular and peribronchial inflammation and fibrosis (Matavelli LC *et al*,. 2011), probably via the

diminution of ERK1 and IκBα (inhibitor of NF-κB) phosphorylation induced by ovalbumine (El-Hashim AZ *et al*,. 2012). Interestingly, it is abolished by the use of the MasR antagonist A779.

To note, in the kidney conflicting results have been observed concerning the effect of Ang-(1-7) on inflammation (Esteban V *et al*,. 2009; Zimmerman D *et al*,. 2012).

V-3 D) Interplays between AT1R and MasR

In addition to these positive effects of Ang-(1-7) via the MasR, Ang-(1-7) can also counter-regulate the side effects of Ang II through a direct antagonism of AT1R (Santos RA *et al*,. 2013).

Before MasR was discovered it was shown that Ang(1-7) caused a concentration-related rightward displacement of the Ang II curve and depressed the maximum response to Ang II in rabbit isolated endothelium intact aortic rings (Mahon JM *et al*,. 1994), indicating that at very high concentration (from 10 to 100 µg/kg/min) Ang-(1-7) can bind to AT1R. This observation has been done in rat brain, in which at a concentration superior to 106 mol/L, Ang-(1-7) binds to AT1R (Rowe BP *et al*,. 1995). Later, using human embryonic kidney (HEK-293) cells stably transfected with either AT1R or AT2R binding affinities of all the major angiotensin peptides were calculated (Bosnyak S *et al*,. 2011) (Table 2). This study demonstrated that up to 10 µM Ang-(1-7) could bind AT1R. Nevertheless, it has also been reported that Ang-(1-7) may compete for the binding of AT1R with high affinity in kidney slices (K_i = 8,0 ± 3,2 nM) (Gironacci MM *et al*,. 1999).

Therefore, Ang-(1-7) can modulate Ang II actions through an effect on AT1R mRNA synthesis and/or translation (Santos RA *et al*,. 2013). Indeed, the AT1R expression has been shown to be downregulated in Chinese hamster ovary (CHO) cells stably expressing ectopic AT1a receptor and pretreated with 1 or 10 µM Ang-(1-7) (Clark MA *et al*,. 2001), but also in rat aortic vascular smooth muscle cells (VSMC) (Clark MA *et al*,. 2001), and in the cortical tubule-interstitial area of the kidney (Clark MA *et al*,. 2003). However, two others studies presented opposite results: the first showed that Ang-(1-7) upregulates the mRNA expression of AT1R

in VSMC (Neves LA *et al*,. 2000), while the second indicated the same effects in overexpressing MasR (Canals M *et al*,. 2006).

Finally, it has been reported in few studies that MasR co-expressed with the AT1R (Kostenis E *et al*,. 2005; Lyngso C *et al*,. 2009). To assess whether the functional interaction could result from dimerization, a series of Bioluminescence Resonance Energy Transfer (BRET) experiments were performed leading to the conclusion of heterodimerization between AT1R and MasR (Kostenis E *et al*,. 2005). However, two years later it was shown that their control was not appropriate (Marullo S *et al*,. 2007). Studying the functional interaction between the two receptors, another team reported that MasR expression also results in a decreased AT1R coupling G-protein accompanied by a simultaneous increase in the receptor surface expression (Canals M *et al*,. 2006). Yet, these data do not indicate a dimerization but rather are consistent with a constitutive activity of the MasR (Lyngso C *et al*,. 2009). Later, with the use of wild types (WT) and mutants AT1R- and MasR-transfected CHO cells, Santos El *et al*,. demonstrated that MasR expression uncoupled the WT AT1R from the G-protein to a similar extent to previous reports (Santos EL *et al*,. 2007). Furthermore, they observed that MasR can result in a gain-of-function of the mutant AT1R. Indeed, this mutant receptor does not travel to the cell surface when it is expressed alone, but surface expression, ligand binding and G-protein coupling is restored to AT1R WT levels upon MasR expression (Santos EL *et al*,. 2007). What is more, they also reported that the two receptors co-localize and therefore might form dimers.

V-3 E) Interplays between AT2R and MasR

AT2R and MasR share only 31% identical sequence homology and are localized on different chromosomes (Villela D *et al*,. 2015). Notwithstanding this structural difference, they have very similar physiological and pathophysiological actions. As described above, AT2R and MasR activate phosphatases, in particular Src homology 2 domain-containing protein tyrosine phosphatase SHP-1 and SHP-2 leading to inhibition of MAPKs or NF-κB. Moreover, both receptors increase NO synthesis and subsequent accumulation of cGMP, which mediates the vasodilatory effects with activation of both receptors. Secondly, Ang-(1-7) can bind AT2R (Table 2). For example, it has been shown that Ang-(1-7)-induced vasorelaxation in

preparations of rat thoracic aortic rings (Roks AJM *et al*,. 2004) or in porcine coronary arteries (Gorelik G *et al*,. 1998) could be blocked by PD123319, an AT2R antagonist. The Ang-(1-7) effects inhibition with AT2R antagonist was observed *in vitro* and *in vivo*. Interestingly, the reverse is also true: in male Sprague-Dawley rats stroke was induced by injection of endothelin-1 (ET-1) into the vicinity of the right middle cerebral artery, causing transient occlusion of this vessel. Cerebral infarct size and neurological deficit were significantly ameliorated 3 days after occlusion by C21 (Gorelik G *et al*,. 1998). This effect of C21 was abolished by central administration of A779.

One of the hypothesis to explain the blockade of Ang-(1-7) actions by AT2R antagonists and vice versa is the dimerization of AT2R and MasR, which has been observed in HEK 293 cells, with fluorescence resonance energy transfer (FRET) method (Villela D *et al*,. 2015). Nevertheless, this observation need confirmation.

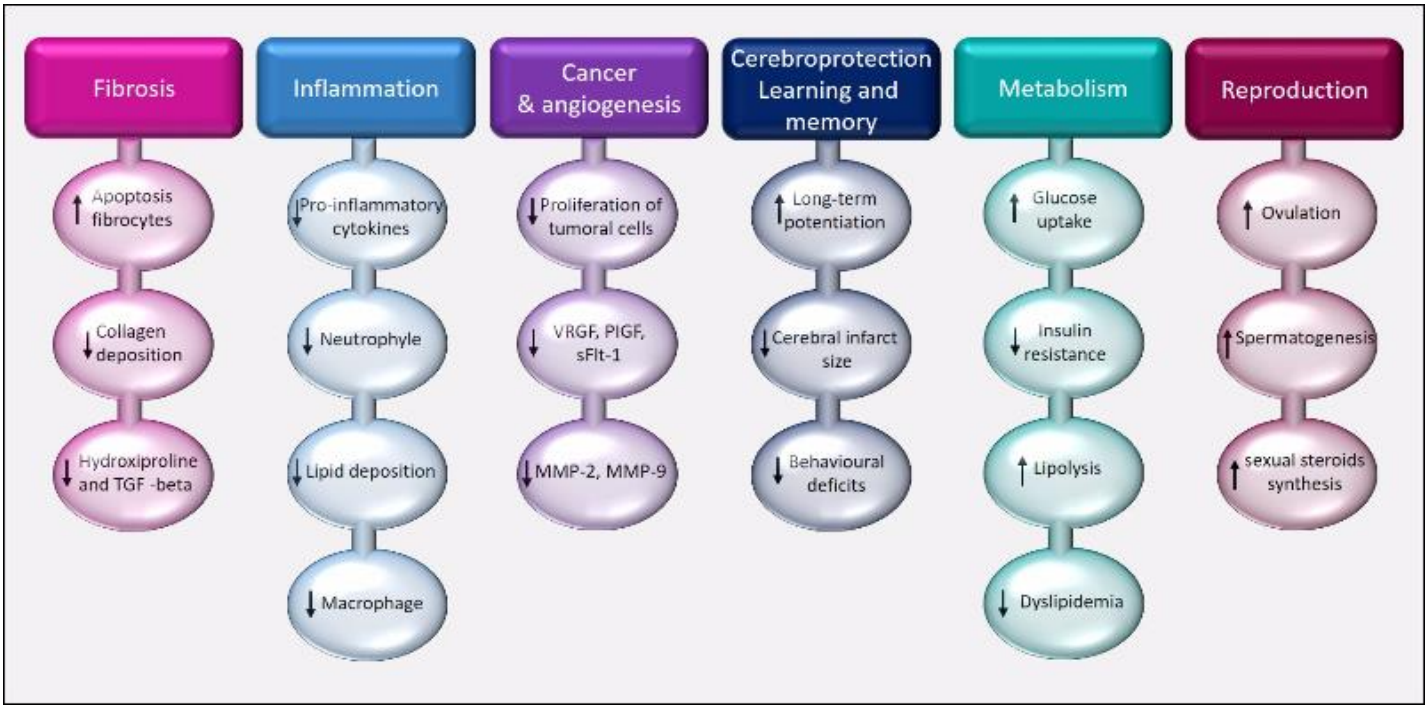

Figure 11: Main non cardiovascular effects of angiotensin-(1-7) via its receptor in different tissues and processes (adapted from Passos-Silva DG *et al*,. 2013).

V-4 Angiotensin converting enzyme 2 (ACE2) and diminazeneaceturate (DIZE)

The other key element of the so-called "protective RAS" is the angiotensin-converting enzyme isoform 2 (ACE2).

Angiotensin converting enzyme 2 (ACE2) is the first known homologue of ACE, and is an integral cell membrane protein with its catalytic domain on the extracellular surface exposed to vasoactive peptides (Velkoska E *et al*,. 2016; Donoghue M *et al*,. 2000). ACE2 is a monocarboxypeptidase and cleaved Ang I to form Ang-(1-9) and Ang II to form Ang-(1-7) (Figure 10). Strikingly, its catalytic efficiency is 400-higher with Ang II as a substrate than with Ang I (Patel VB *et al*,. 2016). ACE2 was initially thought to be expressed only in heart, kidney and testis (Tipnis SR *et al*,. 2000), but is currently known to be widely expressed in various organ systems including the cardiovascular system, kidneys, lungs, and brain, where it exerts important actions to maintain cardiovascular homeostasis (Hamming I *et al*,. 2004; Paizis G *et al*,. 2005; Doobay MF *et al*,. 2007; Wong DW *et al*,. 2007; Kuba K *et al*,. 2006; Gembardt F *et al*,. 2005). In the heart, ACE2 is localized in cardiomyocytes, cardiac fibroblasts, and the coronary vascular endothelium (Patel VB *et al*,. 2013; Patel VB *et al*,. 2014).

The cardiovascular regulatory effects of ACE2 mostly involve and result in the cleavage of Ang II, leading to the formation of Ang-(1-7). Whether the positive effects of ACE2 are due to Ang-(1-7) increase or to Ang II decrease remains to be determined (Ferrario CM, 2011). However, it is now widely accepted that an alteration of ACE2 levels can affect not only blood pressure, but also cardiac structure and function. In first, it has been demonstrated that targeted disruption of ACE2 resulted in increased Ang II levels and impaired cardiac function (Crackower MA *et al*,. 2002). Moreover, in spontaneous hypertensive rats (SHR) ACE2 overexpression attenuated left ventricular hypertrophy and myocardial fibrosis (Diez-Freire C *et al*,. 2006). Furthermore, reduced levels of cardiac ACE2 have been reported in hypertension, diabetic heart disease, Ang II-induced cardiac dysfunction, and with the development of kidney disease (Patel VB et al,. 2013; Diez-Freire C *et al*,. 2006; Ferreira AJ et al,. 2011; Tikellis C et al,. 2012; Burrell LM et al,. 2012). Further, serum ACE2 activity was higher in hypertensive patients compared to healthy individuals (Patel VB *et al*,. 2016). In addition, in hypertensive patients with type 1 diabetes mellitus, serum ACE2 activity was positively correlated with systolic blood pressure improvement (Soro-paavonen A *et al*,. 2012). Based on these observations, it has been hypothesized that elevated ACE2 may be a compensatory response to hypertension. Likeness, in heart cells ACE2 expression is highly affected by pathological disease conditions (Gallagher PE *et al*,. 2008), suggesting that ACE2 plays a counter-regulatory role in the development of cardiac diseases. Interestingly, in human population, genetic variations in *ACE2* gene correlate with susceptibility to cardiovascular disease. Indeed, single-nucleotide polymorphisms of

ACE2 are associated with variation in septal wall thickness (Lieb W *et al*,. 2006), ventricular hypertrophy, and coronary artery disease (Keidar S *et al*,. 2007).

Hence, as activation of ACE2 might represent an important therapeutic approach in cardiovascular disease. Three possible strategies are currently having investigated: recombinant ACE2, orally available ACE2 and specific compounds that activate endogenous ACE2. The last strategy seems to be the most promising and, therefore, will be detailed below.

An *in silico* screening strategy has been used to identify ACE2 activators (Hernandez Prada JA *et al*,. 2008). The first activator found is xantherone (XNT). Acute administration of XNT in spontaneous hypertensive rats (SHR) resulted in reduced blood pressure, whereas chronic infusion reversed cardiac and renal fibrosis (Hernandez Prada JA *et al*,. 2008). Furthermore, XNT infusion increased cardiac gene and protein ACE2 levels in SHR. With the same *in silico* screening, other existing compounds that would enhance the catalysis of ACE2, were discovered. Diminazeneaceturate (DIZE), already used as anti-trypanosomal drug since decades (Kulemina LV *et al*,. 2011), is the most effective and is structurally similar to XNT (Velkoska E *et al*,. 2016). In spite of its structural similarities, DIZE has a greater bioavailability than XNT, is pharmacologically more effective, and has better physiochemical characteristics (Da Silva Oliveira GL *et al*,. 2015).

While XNT is hypotensive compound, whether DIZE affects blood pressure is controversial. Indeed, its effects are dose, mode of injection and model-dependent (Velkoska E *et al*,. 2016). However, several studies reported that DIZE reduced cardiac hypertrophy, cardiac fibrosis, and reduced proinflammatory cytokines effects (Shenoy V *et al*,. 2013; Rigatto K *et al*,. 2013). Furthermore, it has been strongly evidenced in rat model of ischemic stroke a decrease of infarct volume and an improvement of the neurological deficit of ischemic stroke with infusion of DIZE (Mecca AP *et al*,. 2011; Bennion DM *et al*,. 2015). As abolished with A779 administration, these effects are a consequence of Ang-(1-7) formation. Furthermore, in atherosclerosis model, DIZE enhanced plague stability but did not reduce atherosclerotic lesion size (Fraga-Silva RA *et al*,. 2015). DIZE has also beneficial effects in hyperglycemic rat model induced by streptozotocin by improving cardiac electrical function in these rats, whereas, it does not reverse hyperglycemia in diabetic rats (Coutinho DC *et al*,. 2014).

Finally, ACE2 is a main component of the “protective RAS”. Although, it is difficult to stimulate its activity. The best therapeutically approach is to use chemical ACE2 activators.

Until now, DIZE is the most potent activator of ACE2 even though conflicting results regarding its effects.

V-5 Alamandine/MrgD axis

Alamandine has been discovered three years ago (2013) by Lautner RQ *et al,*. (Lautner RQ *et al,*. 2013). Using mass spectrometry analysis they observed that alamandine circulates in human blood and can be formed from angiotensin-(1–7), through decarboxylation of N-terminal aspartate amino acid residue, in the heart. Alamandine is also product from Ang A via ACE2 which hydrolyzes the C-terminal amino acid (Etelvino GM *et al,*. 2014). Little is known about alamandine degradation, but ACE may remove the N-terminus-Ala residue, leading to the formation of Ang-(2-7) considered as an inactive peptide (Paula RD *et al,*. 1999).

Alamandine is structurally closed to Ang-(1-7), but it binds to a distinct receptor: the Mas-related receptor type D (MrgD), a G-protein coupled receptor (Lautner RQ *et al,*. 2013; Etelvino GM *et al,*. 2014). MrgD is widely expressed in sensory neurons of the dorsal root spinal ganglia (Dong X *et al,*. 2001; Zylka MJ *et al,*. 2003; Shinohara T *et al,*. 2004). Moreover, it has been found, in lower levels, in testis, urinary bladder, arteries, uterus, brain, cerebellum, eyeball, spinal cord, trachea, thymus, heart, lung, diaphragm, peritoneum, gastrointestinal tract, skeletal muscle, prostate, seminal vesicle, and write and brown adipose tissue (Lautner RQ *et al,*. 2013; Zylka MJ *et al,*. 2003; Zhang L *et al,*. 2005).

It is quite understandable that the effects of alamandine are not well known. Nonetheless, some studies demonstrated that alamandine has protective effects in several pathologies (Hrenak J *et al,*. 2016). In FVB/N mice aortic rings, alamandine induced endothelial-dependent vasorelaxation similar to the effect of Ang-(1-7) (Lautner RQ *et al,*. 2013; Le Tran Y *et al,*. 1997). Though, contrary to Ang-(1-7), alamandine can counteract the vasoconstriction induced by Ang A without affecting Ang II-induced vasoconstriction (Habiyakare B *et al,*. 2014). Furthermore, alamandine increases the bioavailability of acethycholine and, thus promotes acethylcholine-mediated vasodilation in the thoracic aorta and iliac artery (Le Tran Y *et al,*. 1997). This effect was not observed in carotid artery. Furthermore, in MasR knockout-mice, alamandine induces vasorelaxation, indicating a signaling pathway distinct from the Ang-(1-7)/MasR axis (Lautner RQ *et al,*. 2013). Moreover, alamandine induced-release in MrgD-transfected cells but not in MasR-transfected cells (Villela DC *et al,*. 2014). Interestingly, in spontaneous hypertensive rats, a single dose of alamandine/β-hydroxypropylcyclodextrin

compound decreased blood pressure for a long-term period (Lautner RQ *et al*,. 2013). Finally, alamandine seems to exert antiremodeling effects. Indeed, a chronic administration of alamandine to isoproterenol-treated Wistar rats was associated with a decreased accumulation of collagen I, collagen III, and fibronectin in the heart (Lautner RQ *et al*,. 2013; Mendoza-Torres E *et al*,. 2015).

Nowadays, alamandine is seen as a potential therapeutic compound to treat hypertension.

VII- *In vitro* models to study steroidogenesis in human

As mentioned before, the adrenal gland is an endocrine gland compound composed of two developmentally unrelated tissues: the adrenal medulla and the adrenal cortex. The adrenal cortex is divided into three concentric zones which have distinct roles in steroid hormone production. The regulation of adrenocortical function is extremely complex and the mechanisms regulating production of steroids from each zone are different. This complexity has made the development of *in vitro* cell models an attractive alternative to studies using whole animals. Several *in vitro* systems, mostly with rat cells, have been investigated as cell suspensions from acutely dispersed tissue (Kloppenborg PW *et al*,. 1968; Swallow RL *et al*,. 1969), primary cultures from normal adrenal glands and adrenal tumors (Buanassisi V *et al*,. 1962; O'Hare MJ *et al*,. 1973; Gospodarowicz D *et al*,. 1977), cell lines from tumors (Yasumura Y *et al*,. 1966; Rodriquez H *et al*,. 1997), or from immortalized cells (Auersperg N *et al*,. 1990; Mellon SH *et al*,. 1994; Pan J *et al*,. 1995; Mukai K *et al*,. 2002).

One of the first, and still routinely used, adrenocortical human cell line is the NCI-H295. In 1980, an adrenocortical carcinoma was identified in a 48-year-old, black female patient (Gazdar AF *et al*,. 1990; Rainey WE *et al*,. 2003). The large excised tumor has been used to establish the original NCI-H295 cell line. Of the 30 steroids detected, approximately 20 were identified. Based on secreted steroids, these cells appeared to contain all of the adrenocortical enzyme systems which presumably were present in the original tumor, including CYP11A, HSD3B2, CYP11B1, CYP21, CYP17, CYP11B2, 3β-hydroxy-steroidsulfo-transferase, and low levels of aromatase (CYP19) (Rainey WE *et al*,. 2003).

Later, other substrains have been adapted from the original NCI-H295 cell line using alternative growth conditions. The goal, reached, was to encourage substrate attachment and shorter cell cycle times. To get into details, NCI-H295 demonstrated increased growth rate, but the cells continued to grow as floating aggregates or weakly attached cells (McAllister JM *et al*,. 1988). Over a three-month period, during which culture medium was changed three times a week and unattached cells were discarded, cells were selected for attachment to plastic culture dishes. After it characterization, this adapted strain was designated as H295R (NCI-H295R) to differentiate it from the original cells (Rainey WE *et al*,. 2003). Compared to the primordial H295 cell line, H295R cells grow as a tightly adherent monolayer with a population doubling

time reduced from five to two days. To note, this strain will be more or less sensitive to angiotensin II and potassium stimuli, depending on the serum used. Moreover, using the same method for isolation, a new stain has been selected. NCI-H295A cells grow as a monolayer while removing non-attached cells with medium changes (Rodriquez H *et al*,. 1997). In spite of the lack of steroidogenesis status characterization of this strain, NCI-H295A are used to study mechanisms affecting steroidogenesis pathway (Rainey WE *et al*,. 2003). Furthermore, the original strain is a model for adrenal cell proliferation.

Basically, the first study done in NCI-H295R was the regulation of angiotensin II type 1 receptor (AT1R), because it exhibits readily detectable levels of AT1R mRNA (Bird IM *et al*,. 1993a; Bird IM *et al*,. 1993b). In addition, K^+ increases intracellular calcium levels in NCI-H295R cells which appears to be the mechanism to increase aldosterone biosynthesis (Rainey WE *et al*,. 1994; Pezzi V *et al*,. 1996). Studies shown 15 years ago demonstrated that K^+ stimulation increases the production of both angiotensin I and II, suggesting that these cells may provide a model to study the hypothesis that local (i.e. intra adrenal) renin/angiotensin system exists (Hilbers U *et al*,. 1999). Nowadays, NCI-H295R is the most often cell line used to study steroidogenesis and hyperaldosteroidism (Lichtenauer UD *et al*,. 2012; Nakamura Y *et al*,. 2015), because it is considered to be an appropriate model to study several of the major and minor physiologic regulators of aldosterone biosynthesis (Rainey WE *et al*,. 2003). Indeed, there are strongly sensitive to angiotensin II, K^+, and, important point, to parathormone (PTH) (Lichtenauer UD *et al*,. 2012; Nakamura Y *et al*,. 2015). However, this cell line is only mildly responsive to adrenocorticotropic hormone (ACTH), which is an important regulator of steroidogenesis (Staels B *et al*,. 1993).

That is why, in 2008, a new cell line has been created (Rainey WE *et al*,. 2008). Human adrenocortical carcinoma (HAC) cells were isolated after surgical removal of an adrenocortical carcinoma from an 11-month-old female with hypertension and hirsutism (Rainey WE *et al*,. 2008). Conversely, HAC cells are sensitive to ACTH (Rainey WE *et al*,. 2008). The most sensitive clone to this ACTH stimuli was clone 15 (HAC15) (Rainey WE *et al*,. 2008; Wang T and Rainey WE, 2012). Thus, HAC15 cells are sensitive to K^+, angiotensin II, forskoline, and ACTH. Compared to NCI-H295 cells, HAC cell clones are monoclonal and may provide a more stable steroidogenic phenotype. In addition, it has been shown that these cells can be stimulated with angiotensin III (Oki K *et al*,. 2013). But, qRT-PCR analysis showed that the NCI-H295 cell line had higher expression levels of CYP11B2. In the NCI-295 cell line, mRNA

expression of CYP11B2 was almost two-fold compared to that was observed in the HAC 15 cell line (Wang T *et al*,. 2012). However, aldosterone production was lower than in other cell lines, including the HAC 15 cell line, because of the lower expression of HSD3B2 observed in the NCI-H295 cell line (Wang T *et al*,. 2012). When all is said and done, HAC15 are a promising model to study steroidogenesis pathways.

Propose of the study, materials and methods

Aims

My PhD work was set out to answer the following questions:

1- Are the main components of the protective renin-angiotensin system (RAS) present in the human adrenal cortex and in aldosterone-producing adenoma?

2- If these components are expressed what role do they play in the regulation of aldosterone and cortisol?

3- What are the effects of angiotensin-(1-7), compound 21 and diminazene aceturate on aldosterone and cortisol production in human adrenocortical cell lines (HAC15 and NCI-H295R), in normal adrenal gland (NAG), and in aldosterone-producing adenoma (APA) under baseline conditions and when aldosterone production is up-regulated?

Materials and methods

Cells

NCI-H295R were grown in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine, and 1% antibiotic/antimycotic mixture. HAC15 were 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, they were seeded in 12-well plates at 20. 10^4 cells per well and grown to subconfluence (80%). Before treatment, cells were starved for 24 hours with RPMI/DMEM F12 medium supplemented with 0,5 % FBS/CCS, 1% glutamine, and 1% antibiotic/antimycotic. This deprivated medium is also used for the stimulation.

After starvation, the cells were treated with stimuli alone and/or with selective antagonists for 12 hours. When receptor antagonists were used, they were added in fresh media 30 minutes before stimulation. Antagonists are irbesartan (from Sigma-Aldrich) and A779 (from Abcam).

Tissues

We obtained aldosterone-producing adenoma (APA) tissue, and APA-adjacent tissue from patients. The diagnosis of APA was confirmed by the "4 corner criteria" described before. All tissues were obtained under sterile conditions in the operating room immediately after excision. Small pieces were frozen and stored in liquid nitrogen. The creation of a biobank of human adrenal tissues was approved by the Ethics Committee; informed consent was obtained from each patient.

Pieces enough large were cut into slices of 20 mg in average. Slices are put in well of a 24-wells plate and starved with DMEM F12 stravation medium for 6 hours. Then each piece is stimulated with stimuli alone and/or with selective antagonists for 12 hours.

RNA extraction and quantitative real-time PCR

After stimulation, with angiotensin-(1-7) (from Sigma), Compound 21 (from Vicore), and Diminazene Aceturate (from Sigma), the supernatant was stored at -20°C. RNA from cell was extracted with the Roche RNeasy kit, and RNA from tissue with Qiagen RNA extraction kit following the manufacter's protocol. The quantity and quality were also determined by spectrophotometric readings at 260/280/230 nm.

One µg of total RNA from cells, and 300 ng total RNA from tissues, were reverse transcribed in a final volume of 20 µL, using the iScript^{TM}cDNA Synthesis kit (Bio-Rad, Milan, Italy), and following the manufacter's recommendations. The RT-PCR reactions were performed in Delphi 1000^{TM} Thermal Cycler (Oracle Biosystems).

	Name and gene code	Sequences
Angiotensinreceptors	AT1R NM_000685.4	For : 5'-atgattccagcgcctgac- 3' Rev : 5'- ggtccagacgtcctgtcact- 3'
	AT2R NM_000686.4	For: 5'- ggtttctagcatatacatcttcaacct- 3' Rev: 5'- ttgcccatagaggaagagtagc- 3'
	MasR NM_002377.2	For: 5'- ttcgctatgcccatgagact- 3' Rev: 5'- tggtgtaggttcccaaaggt- 3'
	MrgD NM_198923.2	For: 5'- cgtggaccttgtcagtggta- 3' Rev: 5'- ttcctcttcagcatggcttc- 3'
Angiotensin converting enzymes	ACE 1 NM_000789.3	For: 5'- aggagcagaaccagcagaac- 3' Rev: 5'- tcagcctcatcagtcaccag- 3'
	ACE 2 NM_021804.2	For: 5'- aaagtggtgggagatgaagc- 3' Rev: 5'- gagatgcggggtcacagtat- 3'
Steroid genes	CYP11B2 NM_000498.3	For: 5'-gtgaccgcaggttgcttt-3' Rev: 5'-cccttattcctttcccatgc-3'
	CYP11B1 NM_000497.3	For: 5'- ttcagccgccctcaaca- 3' Rev: 5'- ggatgttcactgatgctgg- 3'
Housekeeping genes	PBGD NM_000190.3	For: 5'-tgccctggagaagaatgaag-3' Rev: 5'- agatggctccgatggtga -3
	GAPDH NM_001256799.2	For: 5' gggaaggtgaaggtcggagtc- 3' Rev: 5'- sgcagaggggcagagatgat- 3'
	β-actin NM_001101.3	For: 5'- tggcaatgagcggttcc- 3' Rev: 5'- gatgccacaggactcca- 3'

Table 3: Primers sequences

The relative expression levels of aldosterone synthase (*CYP11B2* gene), cortisol synthase (*CYP11B1*gene) mRNAs were measured with a real time RT-PCR. Primers and probes (Universal ProbeLibrary, Roche, Monza, Italy) for the amplification of the genes of interest

were designed using ProbeFinder Software (Roche, Monza, Italy, www.lc480.it). Sequences are shown in table 3.

The reaction efficiency was optimized by preliminarily performed standard curves. After treatment, *CYP11B2* and *CYP11B1* gene expression were calculated by the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak KJ and Schmittgen TD, 2001): each sample was quantified against its housekeeping gene transcript content and normalized to the control group. Each experiment was repeated at least 3 times in duplicate, and the results are presented as mean percentage fold increase +/- SD. Moreover, for each experimental condition, three housekeeping genes (porphobilinogendeaminase (*PBGD*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and *β-actin*) were preliminary tested to determine the more efficient.

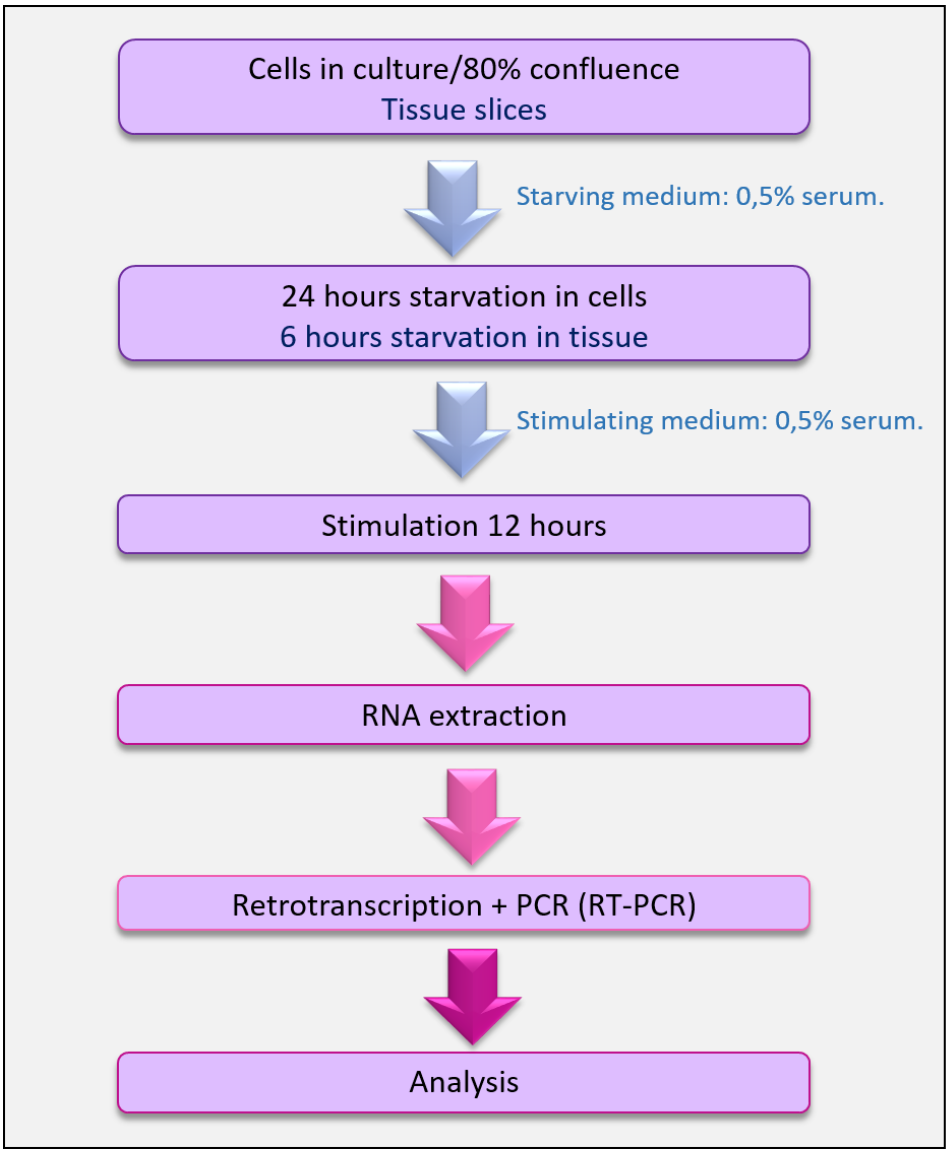

Figure 12: Flow-chart summarizing the experimental procedure

To quantify gene expression of angiotensin receptors (AT1R, AT2R, MasR), alamandine receptor, and angiotensin converting enzyme 1 and 2, the same RT-PCR protocol described above has been used.

Immunoblotting

Immunoblotting for AT1R, AT2R, and MasR, and ACE2 was performed following standard protocol. In brief, cells were homogenized in lysis buffer (Thermo Scientific, Italy) and protein concentration was determined in the soluble supernatant with BCA (Thermo Scientific, Italy). Lysate fraction (50 µg) was separated in a polyacrylamide gel and then electro-blotted onto nitrocellulose or polyvinyldene fluoride (PVDF) membrane (Hybond ECL-Amersham Biosciences Europe, Germany). The membranes were blocked for 30 minutes at room temperature in 5% non-fat dry Blocking Milk or with 5% bovine serum albumin (BSA) and thereafter incubated overnight at 4°C with a primary rabbit monoclonal antibody against human AT1R (diluted 1/700), AT2R (diluted 1/500), MasR (diluted 1/1000), and ACE2 (diluted 1/500). All antibodies are from NovusBio (1 mg/mL size). After washing, the membrane was incubated 1 hour with an anti-rabbit secondary antibody. Then, band intensity were measured with VersaDoc Imaging System (Biorad).

Protein samples were obtained from APA tissues (n = 3) and the adjacent normal adrenal gland (n = 3). Images were analyzed by Image Processing and Analysis in Java (Image J- NIH). Bands for AT1R, AT2R, MasR, and ACE2 were normalized to glyceraldehyde 3-phosphate dehydrogenase.

HAC15 adrenocortical cells or immortalized proximal tubule epithelial cell line from normal adult human kidney (HK2) proteins were used as positive controls.

Aldosterone measurement

Aldosterone levels were quantified with the Aldosterone Elisa kit (Alpha Diagnostic International) following the manufacter's protocol. Briefly, a total of 50 µL of cell medium was added to aldosterone-coated wells following producer's protocol, and the signal was detected in an ELISA Reader (PerkinElmer). Aldosterone levels were normalized to the amount of cell RNA content.

Immunohistochemistry

We performed immunohistochemistry using AT1, AT2, and Mas receptors antibodies.

As for immunoblot, human adrenal tissues are from patients. The three tissues choose were already used in a previous study and immunostained with CYP11B1 and CYP11B2 antibodies to determine aldosterone and cortisol secretion zones (Gioco F *et al*,.2015).

Paraffin-embedded adrenal was cut at 5 m and the sections dried and then melted at 56°C for at least 3 hours. After deparaffination through alcohols, slides were subjected to antigen retrieval using Trilogy (Cell Marque Corp) in autoclave, 15 minutes at 121°C, and then treated with phenylhydrazine 0.1% for 20 minutes to inhibit endogenous peroxidases. Slides were blocked with Tris0.1 M, goat serum 5%, or horse serum 5%, SDS 0.5% (pH 7.4) for 1 hour, and then incubated with angiotensin receptors antibodies (AT1R diluted 1/150, AT2R diluted 1/100, and MasR diluted 1/200) overnight at 4°C. After washing, slides were incubated with secondary antibodies for 1 hour at room temperature.Slides were developed using diaminobenzidine and HighDef green immunohistochemistry chromogen AP (Enzo Life Sciences). All the samples were counterstained with Meyer hematoxylin (Vector Laboratories) before mounting.

Negative control was immunostained only with secondary antibody. Kidney tissue was used as positive control for AT1R and MasR. Placenta tissue was used as positive control for MasR.

Statistical analysis

Statistical analysis were performed with PrisGraphPad Software, version 7.

Each group was compared with non-parametric Mann Whitney test. Moreover, results from ELISA assay were compared with one-way ANOVA test.

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Results

I- Presence of the main components of the protective renin-angiotensin system in human normal adrenal gland (NAG) and in aldosterone-producing adenoma (APA)

I-1 Gene expression level

To determine the presence of angiotensin receptors in human adrenal (NAG) and in aldosterone-producing adenoma (APA), we firstly checked gene expression with RT-PCR.

Angiotensin II type 1 (*AT1R*), type 2 (*AT2R*) and angiotensin-(1-7) receptor (*MasR*) genes are expressed in both NAG and in APA. Of note, *AT1R* and *MasR* gene expression did not differ significantly in NAG and in APA (Figure 1), while that of the *AT2R* gene was significantly lower in APA than in NAG (Figure 1).

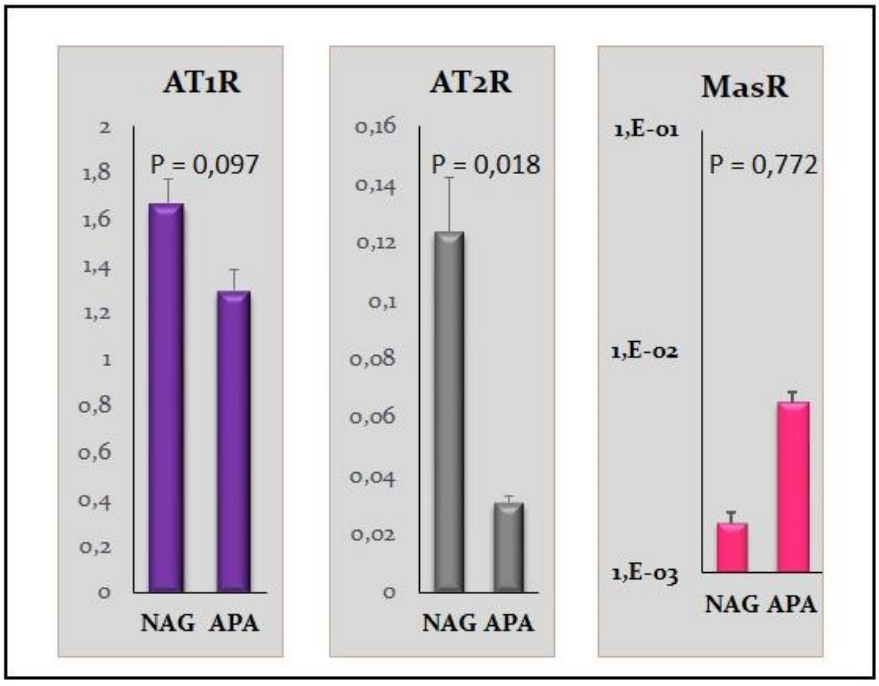


Figure 1: Gene expression of angiotensin II type 1 receptor, type 2 receptor, and angiotensin-(1-7) receptor in normal adrenal cortex (n=3) and in aldosterone-producing adenoma (n=6) (in arbitrary units)

AT1R= angiotensin II-type 1 receptor; AT2R= angiotensin II-type 2 receptor; MasR= angiotensin-(1-7) receptor; NAG= normal adrenal gland; APA = aldosterone-producing adenoma

As regards the amount of the different angiotensin receptor mRNA the *AT1R* gene was significantly more expressed in NAG and in APA than the *AT2R* gene and the *MasR* gene. Moreover, the *AT2R* gene is also significantly more expressed in both tissue than *MasR* gene

(Figure 2). On average, *AT2R* gene is 10-fold less expressed than *AT1R* and *MasR* 150-fold less.

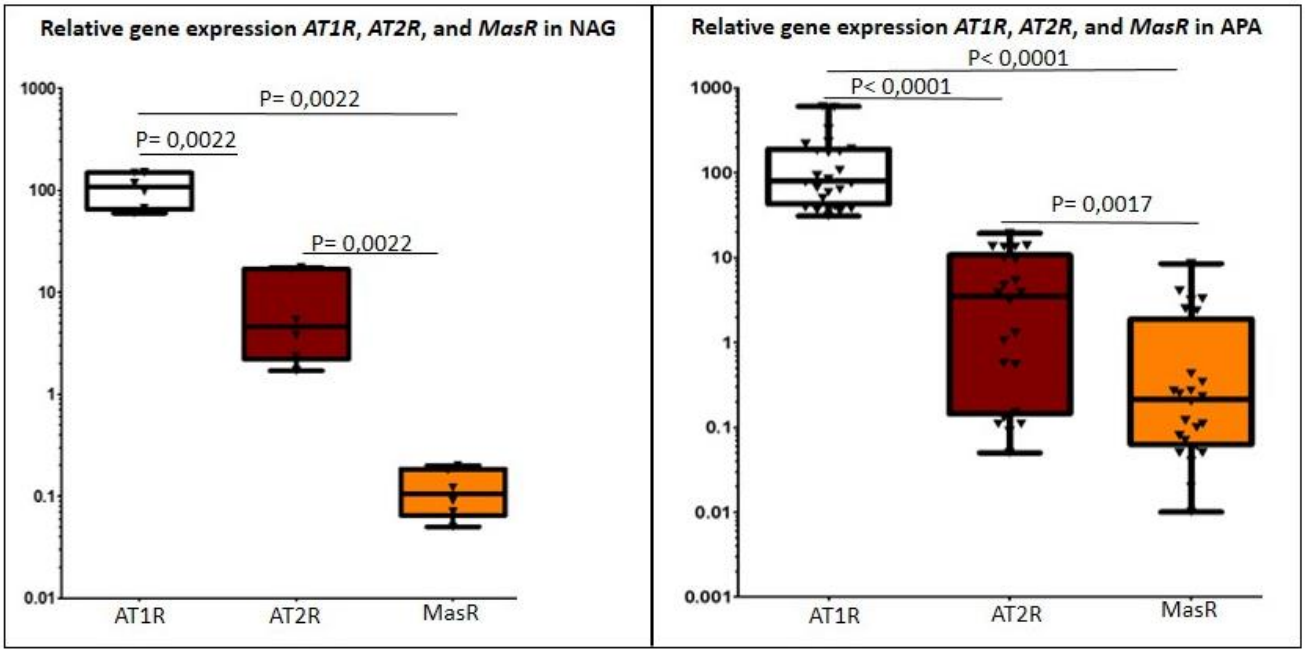


Figure 2: Relative gene expression of angiotensin II type 1 receptor, type 2 receptor, and angiotensin-(1-7) receptor in normal adrenal cortex (n=3) and in aldosterone-producing adenoma (n=6)

AT1R= angiotensin II-type 1 receptor; AT2R= angiotensin II-type 2 receptor; MasR= angiotensin-(1-7) receptor; NAG= normal adrenal gland; APA = aldosterone-producing adenoma

Furthermore, alamandine receptor gene (*MrgD*) is also expressed in NAG and in APA. Eventhough, it is not differentially expressed in NAG and APA. However, as for *AT2R* and *MasR* genes, *MrgD* gene is significantly lower expressed than *AT1R* in both tissue (Figure 3). Its expression is, on average, at the same level than *AT2R* gene expression.

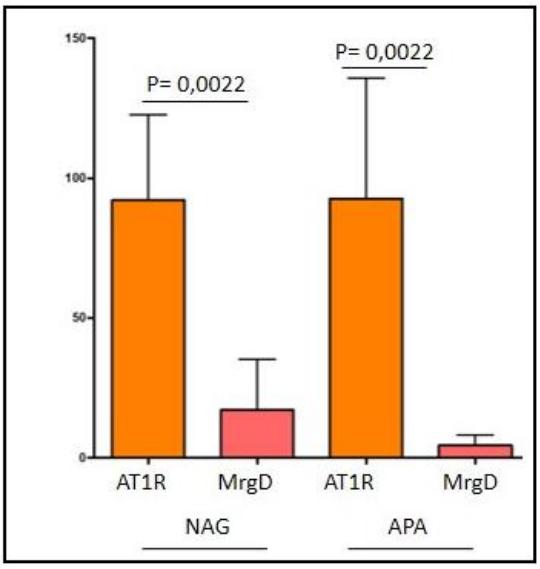


Figure 3: Relative gene expression of angiotensin II type 1 receptor and alamandine receptor in normal adrenal gland (n=4) and in aldosterone-producing adenoma (n =3)
AT1R= angiotensin II-type 1 receptor; MrgD= alamandine receptor
NAG= normal adrenal gland; APA = aldosterone-producing adenoma

Finally, the genes of the two converting enzymes (*ACE* and *ACE2*) were found to be expressed in NAG and in APA, without significant differences between tissues (Figure 4). Furthermore, no significant difference between *ACE* and *ACE2* gene expression in both tissues were found. Thus, ACE/ACE2 ratio was found to be close to 1. Nevertheless, this ratio was quite impossible to calculate because of the heterogeneity of gene expression in these tissues. This variation in expression level was found in all the genes studied.

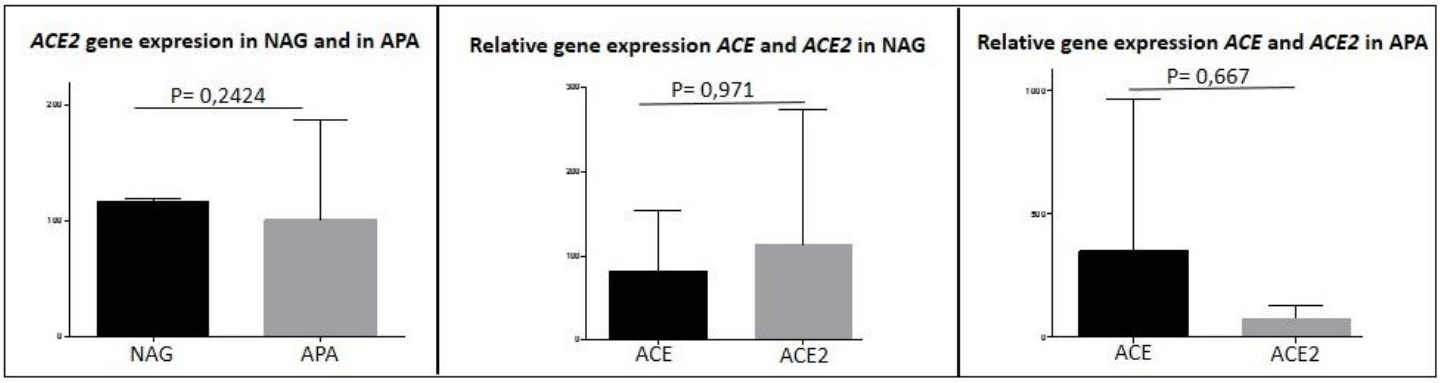


Figure 4: Relative gene expression of angiotensin converting enzyme 1 and 2, in normal adrenal gland (n=4) and in aldosterone-producing adenoma (n=3)

ACE = angiotensin converting enzyme 1; ACE2 = angiotensin converting enzyme 2; NAG= normal adrenal gland; APA = aldosterone-producing adenoma

I-2 Protein expression level

After determination of gene expression, we performed immunoblots to verify the expression of the three angiotensin receptors and the angiotensin converting enzyme at the protein level, and immunohistochemistries to evaluate their intracellular localization.

As expected, all three angiotensin receptors as well as angiotensin converting enzyme 2 (ACE2) were found to be expressed at the protein level in both NAG and in APA as shown in figure 5.

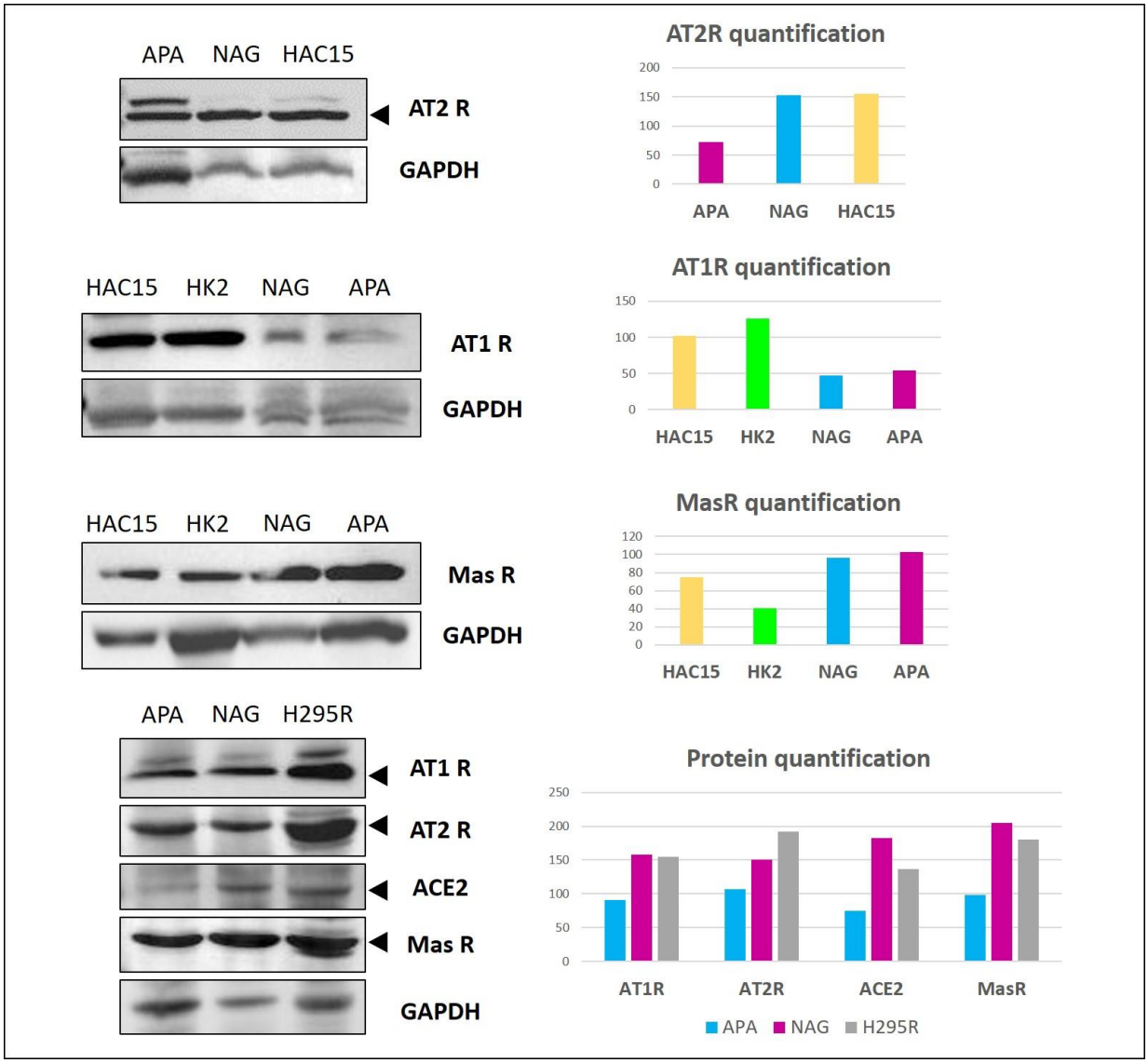


Figure 5: Detection of angiotensin receptors and angiotensin-converting enzyme protein with immunoblot

AT1R= angiotensin II type 1 receptor; AT2R= angiotensin II type 2 receptor; MasR = angiotensin-(1-7) receptor; HAC15/H295R= human adrenocortical cells; HK2= human kidney cells

For immunohistochemistry, tissues were obtained as described in methods. For all, APA and APA-adjacent tissue were visible.

Negative control is tissue immunostained only with the secondary antibody. As shown in figure 6, no positive staining was found.

Positive controls are tissues already published to contain AT1R, AT2R, or MasR. Kidney tissue was used as a positive control for AT1R (figure 6- B) and MasR (figure 6- D), and placenta tissue for AT2R (figure 6- C).

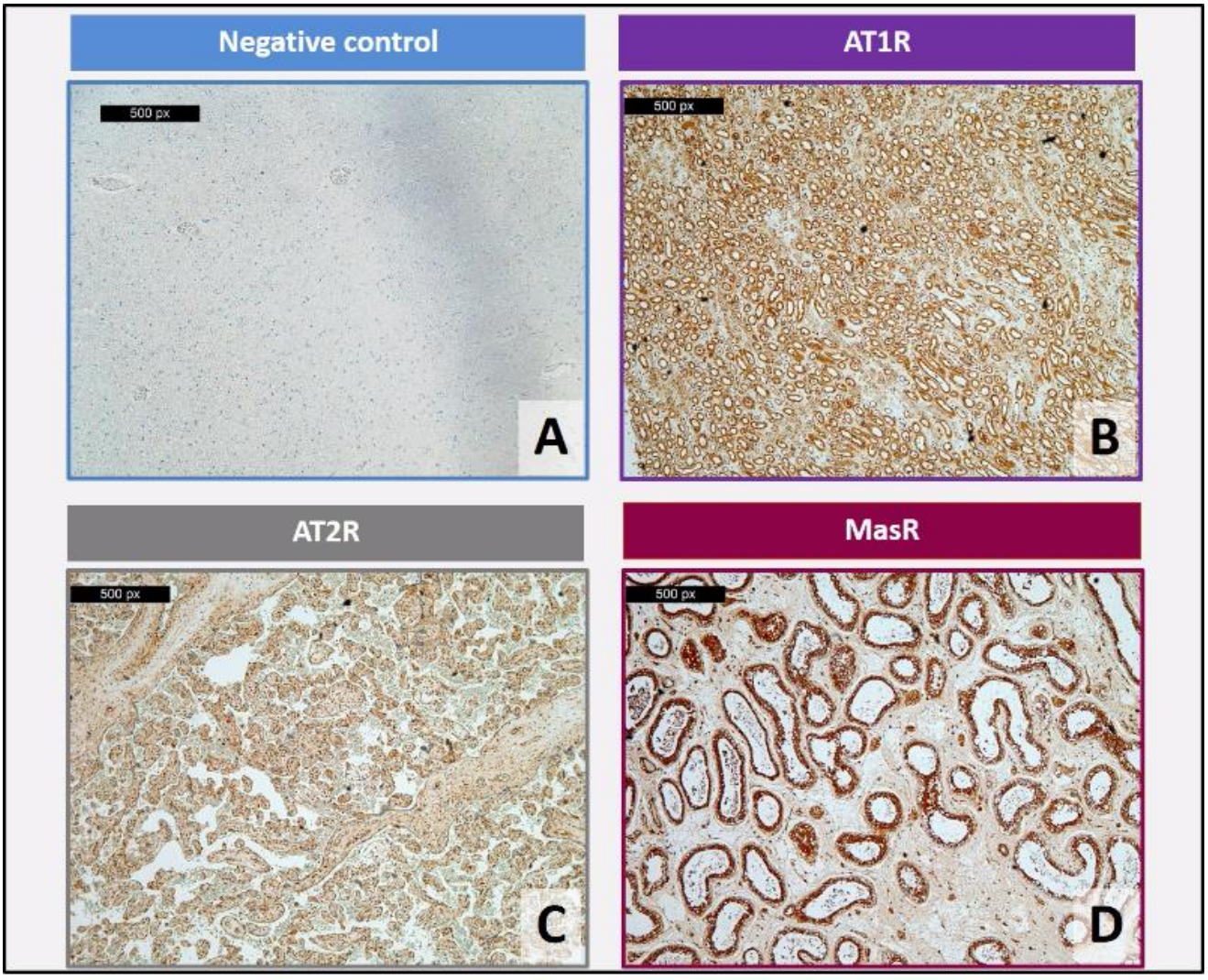


Figure 6: Representative immunohistochemical staining for angiotensin receptors positive and negative controls.

Panel A: negative control; Panel B: positive control for angiotensin II type 1 receptor (AT1R); Panel C: positive control for angiotensin II type 2 receptor (AT2R); Panel D: positive control for angiotensin-(1-7) receptor (MasR)

The immunohistochemistry experiments showed that the three receptors were expressed in NAG and in APA, but were heterogeneously distributed in both tissues (Figures 7 and 8). Nevertheless, AT1R staining was much stronger in glomerulosa layer than in fasciculata (Figure 7-F). Moreover, with a 20-fold magnification we observed that all angiotensin receptors were expressed in cellular membrane (Figure 8- L, M, N, O, P, Q).

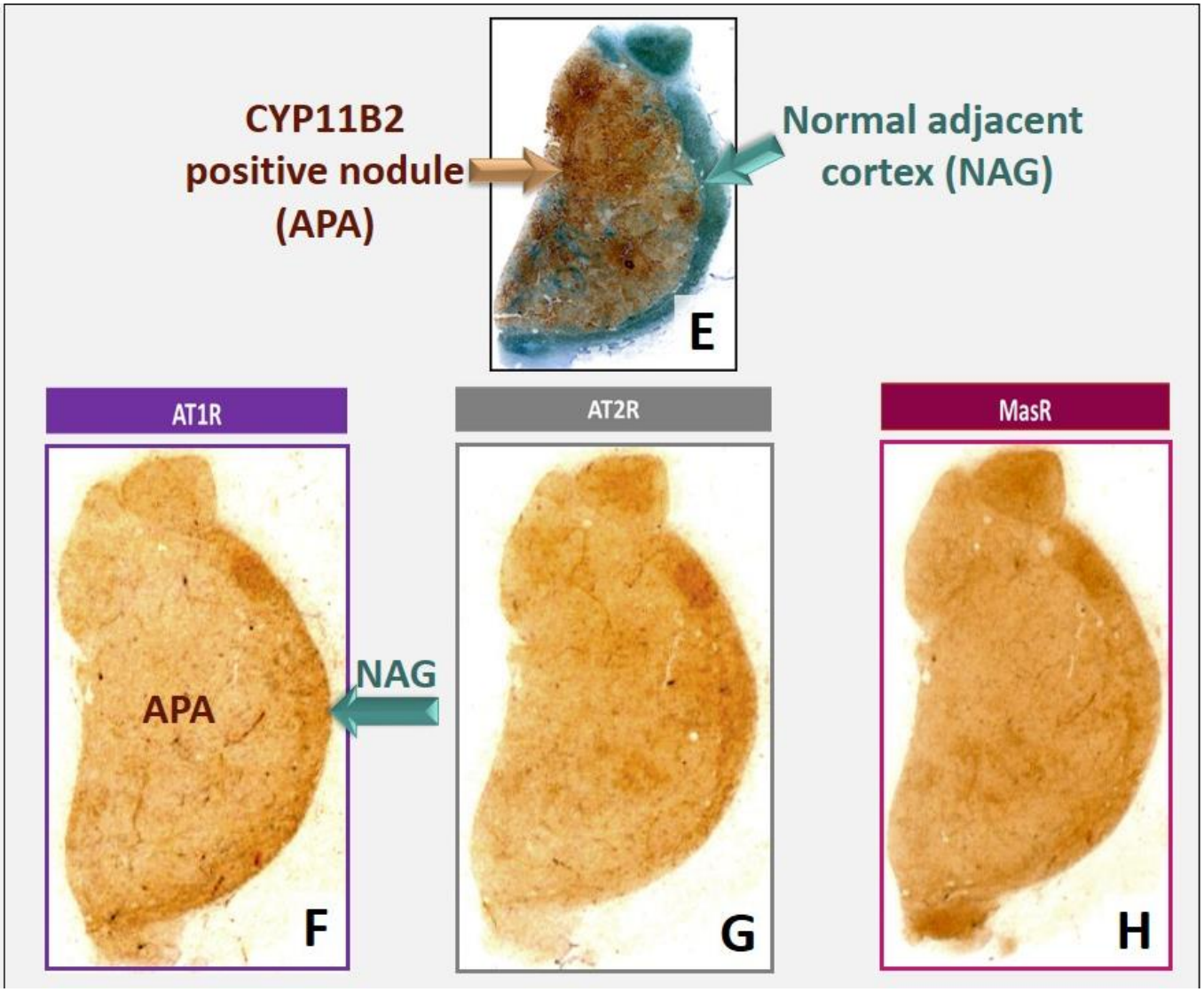


Figure 7: Representative immunohistochemical staining for angiotensin receptors expression in normal adrenal tissue and in aldosterone-producing adenoma

Panel E: tissue immunostained with CYP11B2 antibody. Panels F,G,H: angiotensin receptors immunostaining

AT1R= angiotensin II-type 1 receptor; AT2R= angiotensin II-type 2 receptor; MasR= angiotensin-(1-7) receptor; NAG= normal adrenal gland; APA = aldosterone-producing adenoma

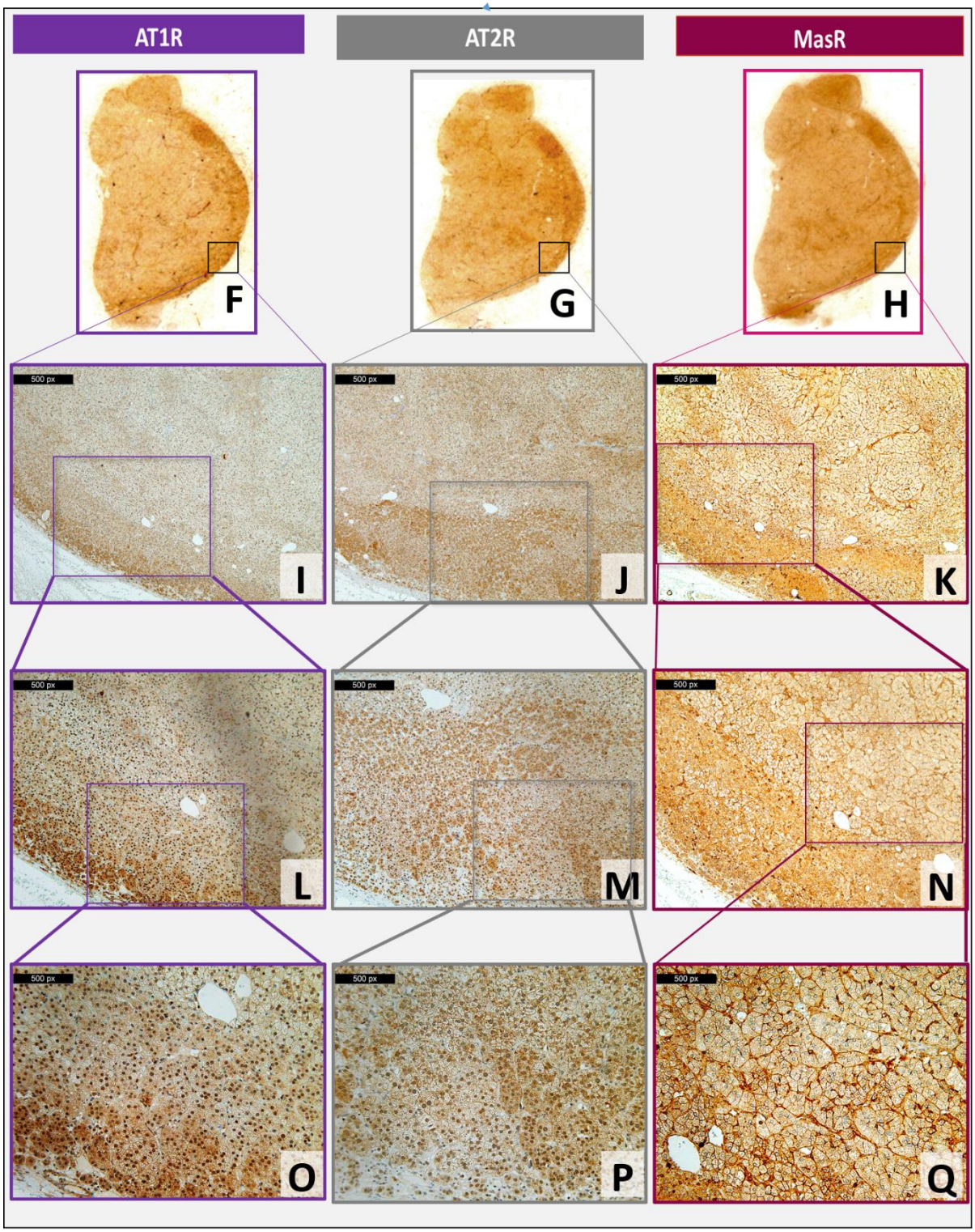


Figure 8: Representative immunohistochemical staining for angiotensin receptors expression in normal adrenal tissue and in aldosterone-producing adenoma

Panels I, J ,K: angiotensin receptors immunostaining at a 5-fold magnification in NAG.
Panels L, M, N: angiotensin receptors immunostaing at a 10-fold magnification in NAG.
Panels O, P, Q: angiotensin receptors immunostaining at a 20-fold magnification in NAG.
AT1R= angiotensin II-type 1 receptor; AT2R= angiotensin II-type 2 receptor;
MasR= angiotensin-(1-7) receptor; NAG= normal adrenal gland;
APA = aldosterone-producing adenoma

II- Effects of angiotensin-(1-7) on *CYP11B1* and *CYP11B2* gene expression, and in aldosterone production in human adrenocortical cells (H295R and HAC15).

Before stimulation we checked gene and protein expression level of *AT1R*, *AT2R*, *MasR*, and *ACE2* in the two human adrenocortical cell lines (Figure 5). Moreover, we compared the *CYP11B1* and *CYP11B2* gene expression in both cells (Figure 9). HAC15 significantly expressed more *CYP11B2* gene than H295R.

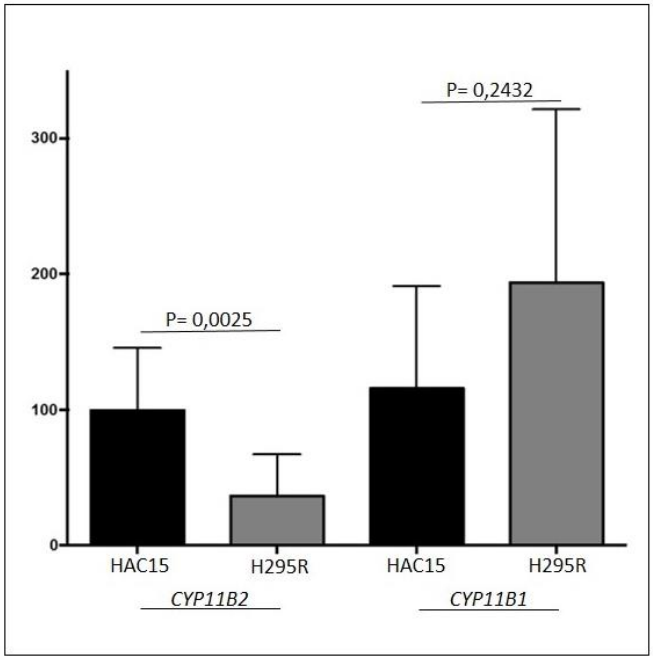


Figure 9: Relative *CYP11B2* and *CYP11B1*gene in human adrenocortical cell lines.

To clarify the effects of angiotensin-(1-7) on aldosterone and cortisol production, we performed, a set of stimulation with angiotensin-(1-7) in H295R cells with a range of concentrations from 100 nM to 600 nM (Figure 10). At these low concentrations no effect was detected in *CYP11B1* and *CYP11B2* gene expression.

Because of the lack of effect of angiotensin-(1-7) at low concentrations, we next decided to increase concentration of angiotensin-(1-7) up to 100 µM.

On both cells we observed a significant increase in *CYP11B1* and *CYP11B2* gene expression, albeit with differences between the cell lines (Figure 11): in the H295R cells there was a concentration-dependent stimulation with a significant increase in gene expression level from 10 µM (Figure 11). In the HAC15 cells we noted a decrease at this concentration (Figure 11).

Of note, HAC15 cells producing more aldosterone at baseline, weakly responds to angiotensin stimuli. That is why, we decided to continue the study with H295R cells only.

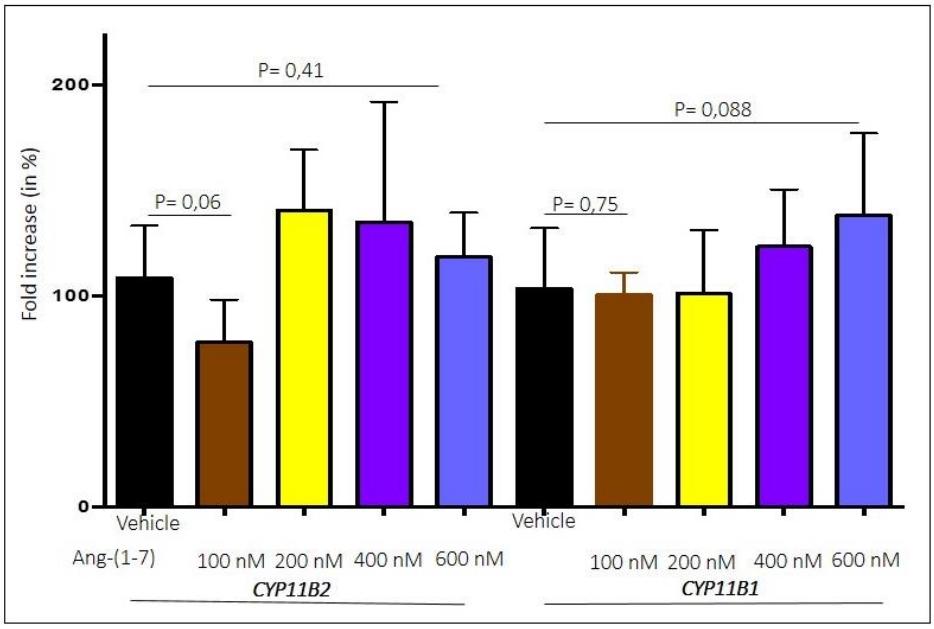


Figure 10: *CYP11B2* and *CYP11B1* gene expression in H295R cells after stimulation with angiotensin-(1-7) (n= 2)

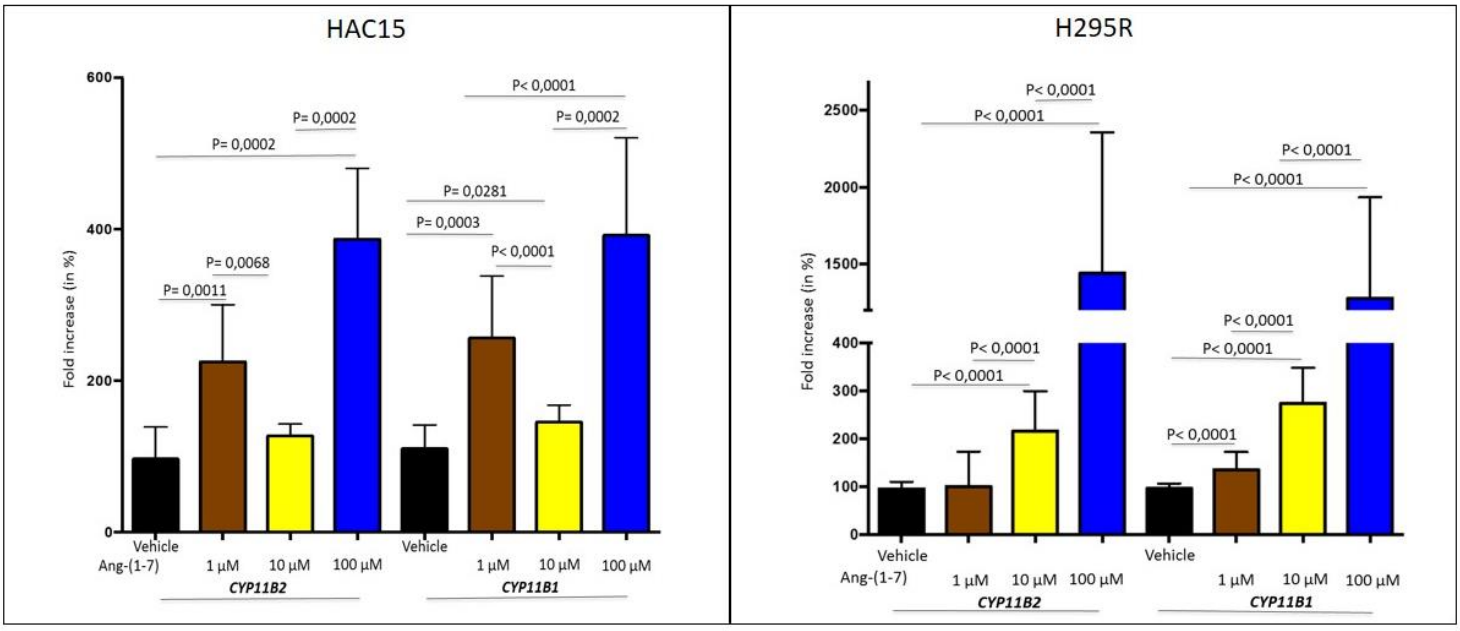


Figure 11: *CYP11B2* and *CYP11B1* expression in HAC15 and H295R cells after stimulation with angiotensin-(1-7) (respectively n= 3 and n= 5)

Angiotensin-(1-7) only at a concentration of 100 µM significantly affected the effects of angiotensin II (10 µM) on *CYP11B2* gene expression (Figure 12).

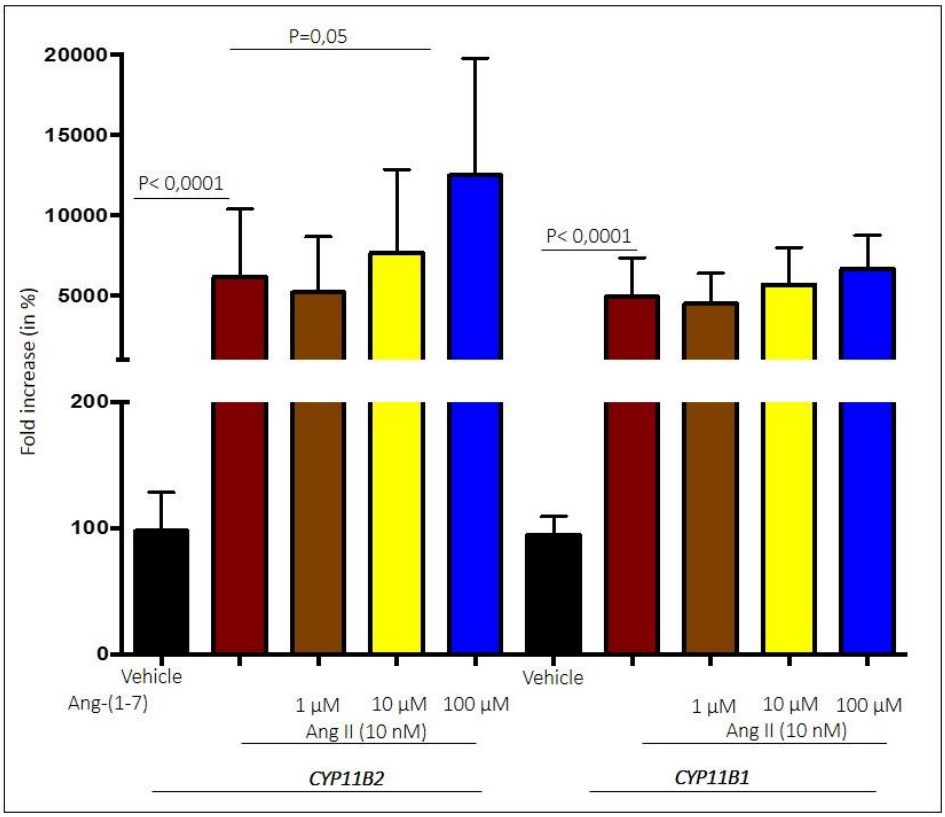


Figure 12: *CYP11B2* and *CYP11B1* expression in H295R cells after stimulation with angiotensin-(1-7) and angiotensin II (n= 3)

In regard to these first results, we hypothesized a binding of angiotensin-(1-7) on AT1R.

To challenge the hypothesis that angiotensin-(1-7) effects at 100 µM on *CYP11B2* gene expression involved an off target acting via the AT1R, we used irbesartan, a strong AT1R antagonist. We also used A779 a potent MasR antagonist. We incubated cells with antagonists 30 minutes before to add angiotensin-(1-7) as previously reported (oki K *et al*., 2013). Irbesartan was used at 10 µM concentration and A779 at 100 nM.

Irbesartan blunted the effect of angiotensin-(1-7), whereas A779 has not a significant effect (Figure 13).

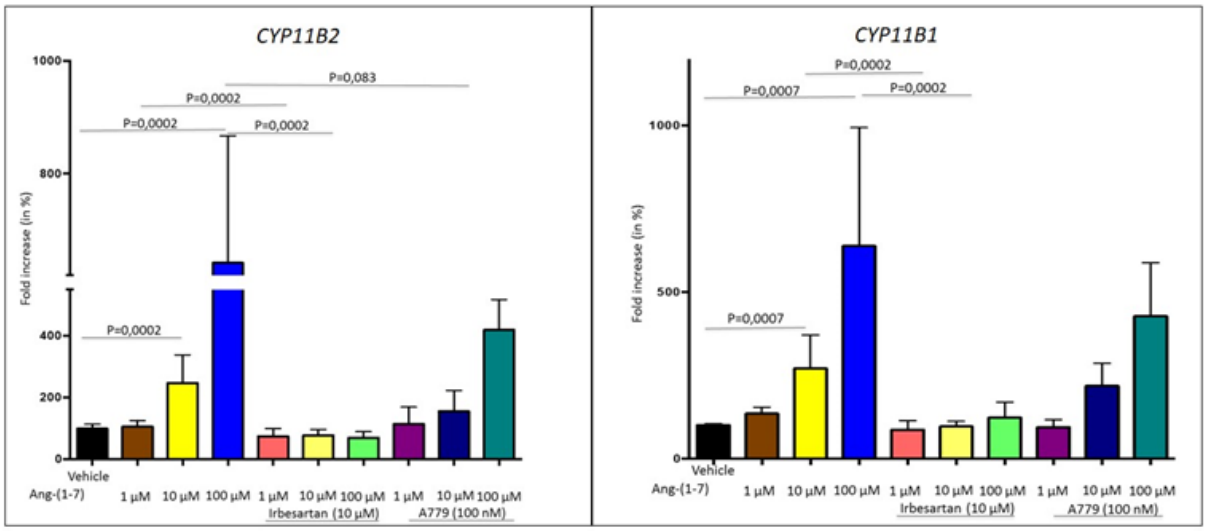


Figure 13: *CYP11B2* and *CYP11B1* expression in H295R cells after stimulation with angiotensin-(1-7) and angiotensin receptors inhibitors (n=3)

Irbesartan= angiotensin II type 1 receptor antagonist; A779= angiotensin-(1-7) receptor anatagonist

Furthermore, the effect of angiotensin-(1-7) in *CYP11B2* gene expression has been searched in aldosterone secretion. As shown in figure 14, a trend is observed. However, the absence of statistical significance can be due to the short time of stimulation and to aldosterone degradation *in vitro*.

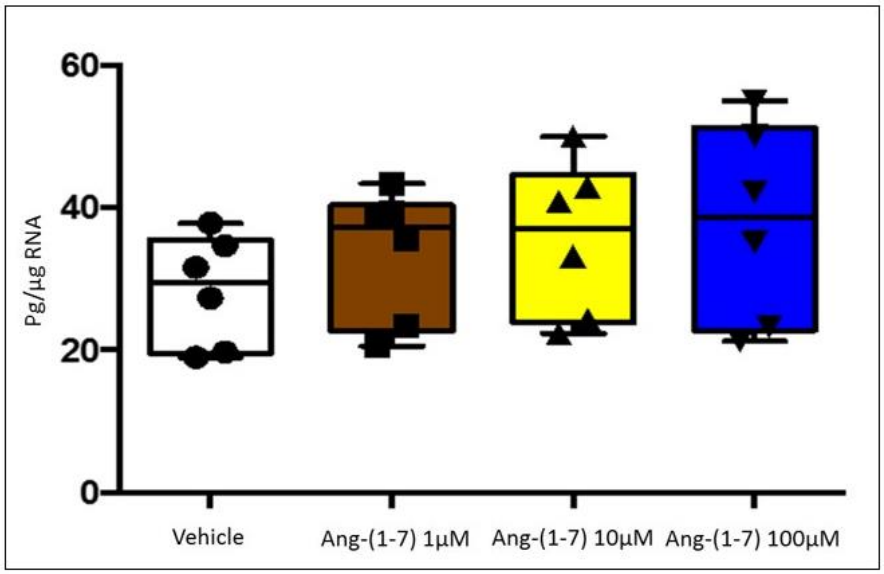


Figure 14: Offset of stimulation with angiotensin-(1-7) on aldosterone production in H295R cells (n= 3)

One way ANOVA: P= 0,48.

Finally, to determine the effect of angiotensin-(1-7) on *ACE2* gene expression, we checked the *ACE2* gene expression after stimulation. In H295R cells this peptide has not significant effect on *ACE2* gene expression (Figure 15).

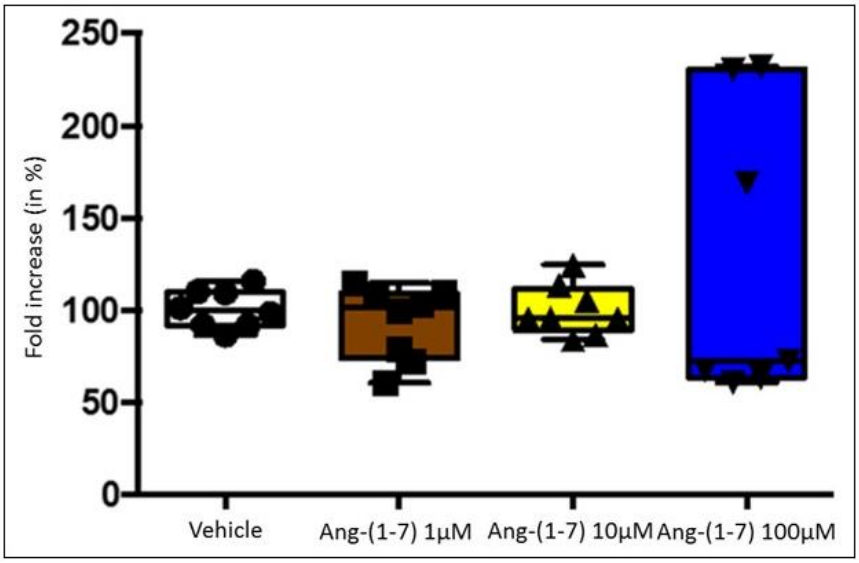


Figure 15: Relative angiotensin converting enzyme gene expression after angiotensin-(1-7) stimulation in H295R cells (n= 3)

III- Effects of compound 21 (C21) on *CYP11B1* and *CYP11B2* gene expression, and in aldosterone production in human adrenocortical cells (H295R and HAC15).

To study the role of AT2R in aldosterone and cortisol secretion we used compound 21 (C21). To achieve this, we stimulated human adrenocortical cell lines with a range of concentration of C21 from 10 nM to 10 µM according to previous study (Menk M *et al*., 2015).

In both cell lines, C21 had not effect on *CYP11B1* and *CYP11B2* gene expression at low concentration but has a significant effect at 10 µM. Angiotensin II (100 nM) was used as a positive control (Figure 16).

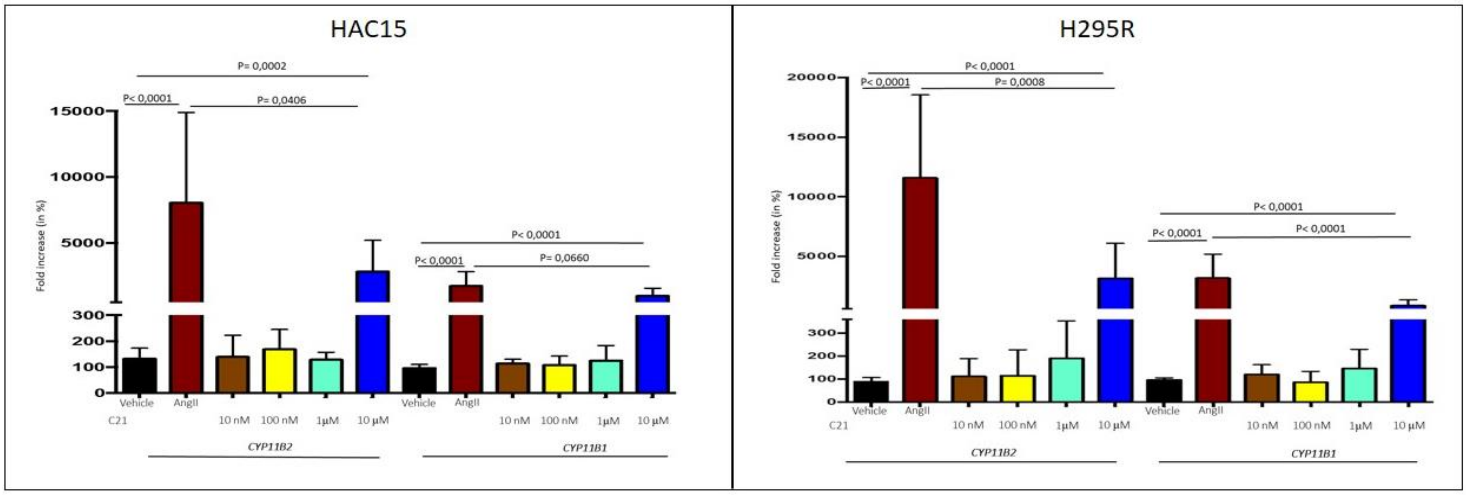


Figure 16: *CYP11B1* and *CYP11B2* gene expression in HAC15 and NCI-H295R after stimulation with C21 (n= 3)

In order to determine the effects of AT2R under high aldosterone production, we used angiotensin II at 100 nM. C21 significantly increased the effect of angiotensin II in *CYP11B1* and *CYP11B2* gene expression at high concentration (10 µM) in HAC15 (Figure 17), but not in H295R.

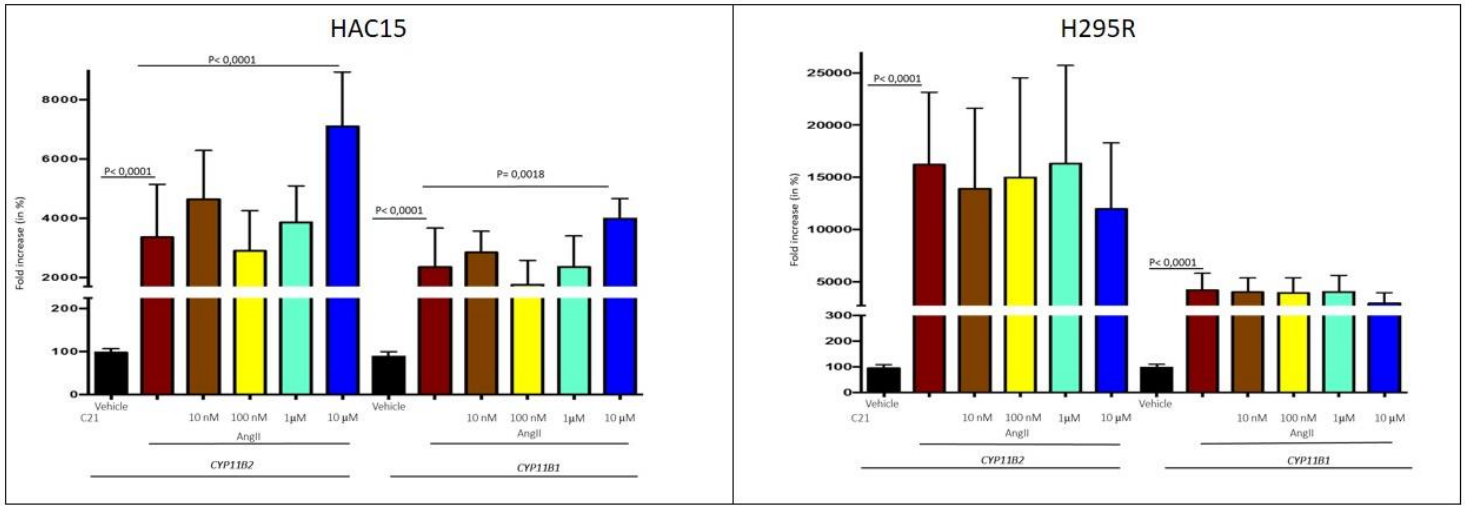


Figure 17: *CYP11B1* and *CYP11B2* gene expression in HAC15 and in H295R cells after stimulation with C21 and Ang II (100 nM) (n= 3)

C21 had increased the *CYP11B*1 and *CYP11B2* gene expression at 10 µM. As for angiotensin-(1-7), we hypothesized that at this high concentration C21 can lose its specificity for AT2R and bind to AT1R.

To test this hypothesis we used irbesartan, like we did for angiotensin-(1-7).

In both HAC15 and in H295R cells, irbesartan completely blunted the effect of C21 (Figure 18). Angiotensin II (100 nM) is used as a positive control.

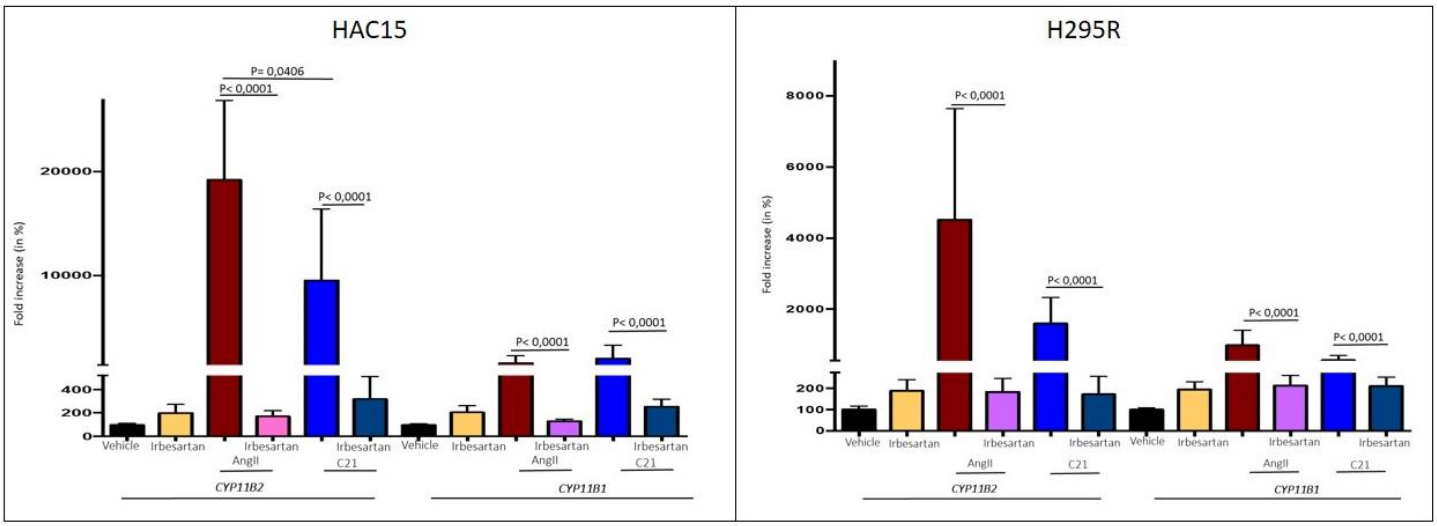


Figure 18: *CYP11B2* and *CYP11B1* expression in HAC15 and in H295R cells after stimulation with C21 and irbesartan at 10 µM (n= 3)

After stimulation with C21, we checked the aldosterone secretion in both cells (Figure 19). In HAC159 C21 increased aldosterone secretion at 10 µM.

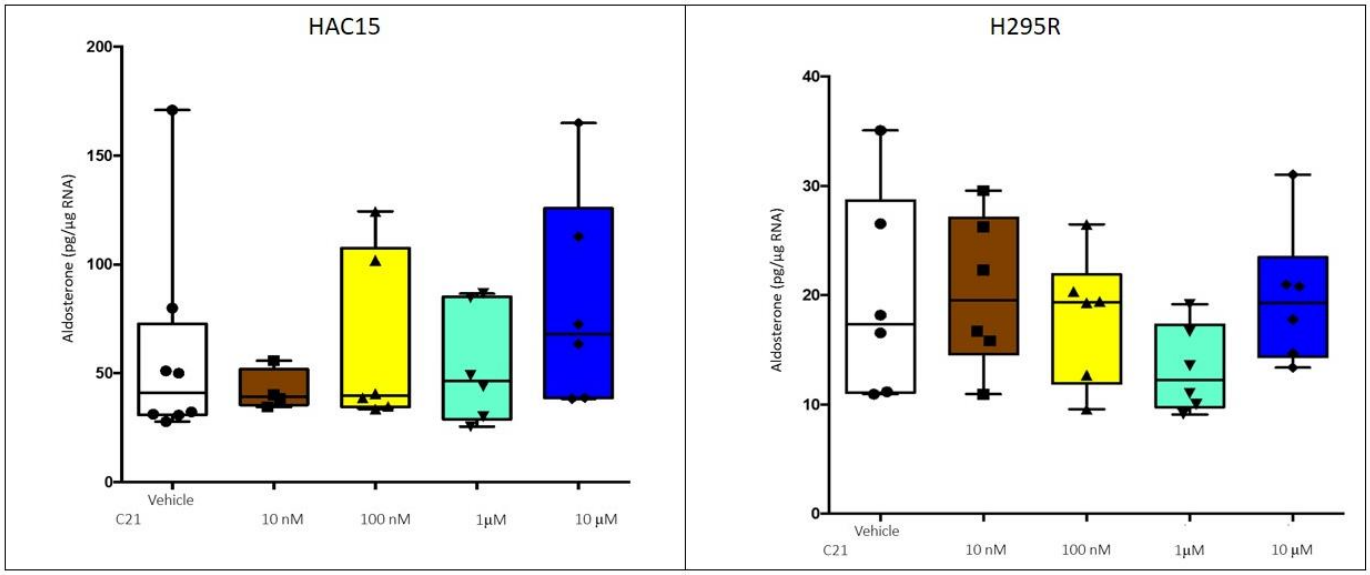


Figure 19: Offset of stimulation with C21 on aldosterone production in HAC15 and H295R (n=3).

One way ANOVA P= 0,23 for HAC15 cells and P= 0,38 for H295R cells.

As aldosterone secretion physiologically results from diverse stimulus: angiotensin II, potassium and ACTH, we wished to determine the effect of C21 on aldosterone and cortisol

secretion driven by potassium. Hence, we stimulated H295R cells, which are known to be sensitive to potassium unlike HAC15, with potassium at 10 mM and 20 mM (Figure 20).

C21 did not regulates the potassium effect on *CYP11B1* and *CYP11B2* gene expression (Figure 20).

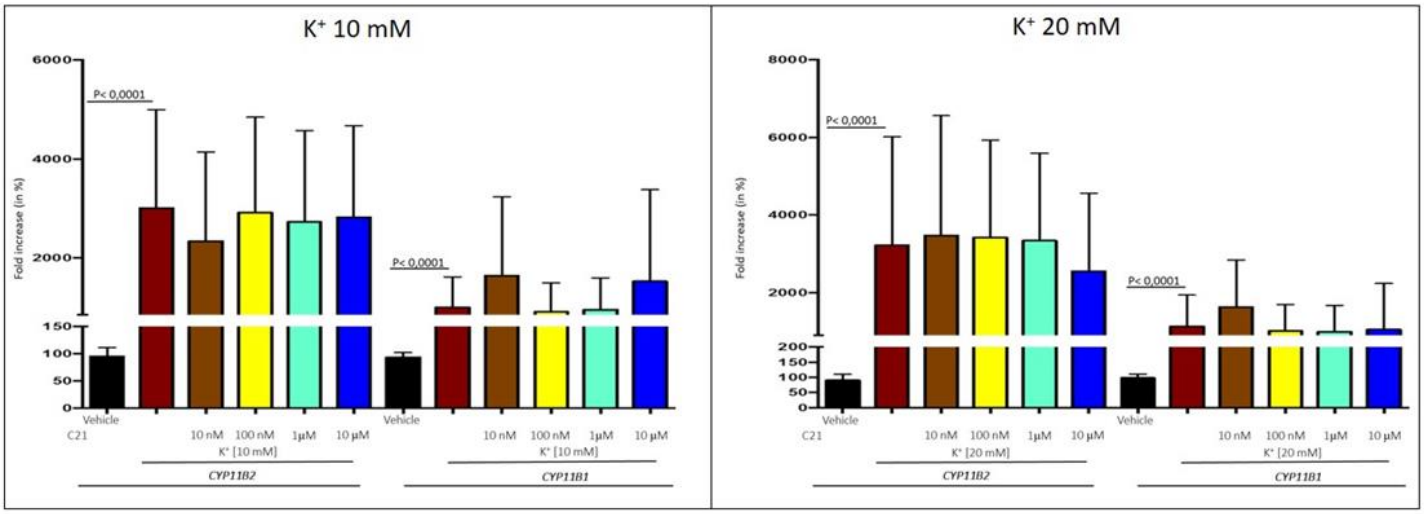


Figure 20: *CYP11B2* and *CYP11B1* expression in H295R after stimulation with C21 and K^+ (n= 4)

It has been demonstrated in some models that C21 sometimes presented regulatory effects only when administrated with low dose of AT1 receptor antagonist (for brief review see Foulquier S *et al*,. 2012). To determine the effects of AT2R on aldosterone and cortisol production under AT1 receptor blockade we stimulated H295R cells with low doses of C21 coupled with irbesartan (Figure 21).

C21 significantly increased *CYP11B2* gene expression at 1nM. This increase is blunted by irbesartan. Moreover, no differences were found in *CYP11B2* gene expression when C21 was administrated with irbesartan (Figure 21). However, at 100 nM C21 increased *CYP11B1* gene expression under a blockade of AT1 receptor. To evaluate if this effect is AT2R-dependent, the AT2 receptor antagonist PD123319 will be used.

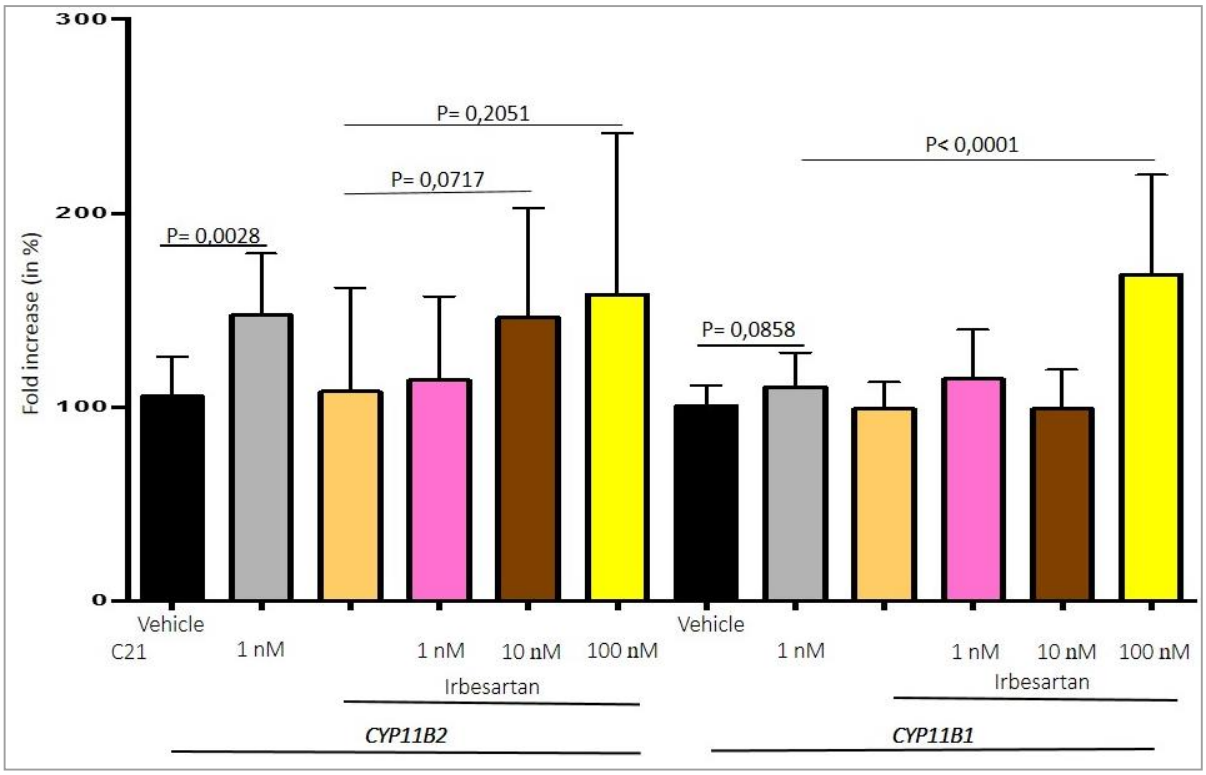


Figure 21: *CYP11B2* and *CYP11B1* expression in H295R cells after stimulation with C21 and irbesartan (n= 3).

IV- Effects of compound 21 (C21) on *CYP11B1* and *CYP11B2* gene expression, and in aldosterone-producing adenoma (APA) and in normal adrenal gland (NAG).

Stimulation of aldosterone and cortisol production can be studied *ex vivo* with adrenal tissue from patient. Nevertheless, contrary to cells, these tissues heterogeneously expressed angiotensin receptors and cytochromes. These differences account for high heterogeneous response to stimuli.

In APA, C21 did not significantly affect *CYP11B1* and *CYP11B2* gene expression (Figure 22).

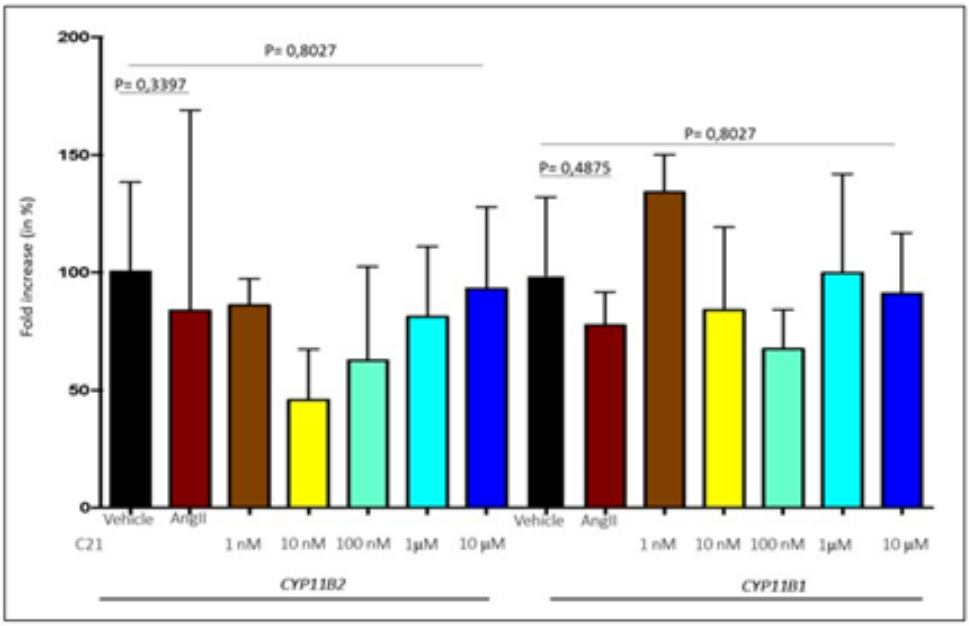


Figure 22: *CYP11B2* and *CYP11B1* expression in aldosterone-producing adenoma after stimulation with C21 (n= 3).

Of note, whereas C21 did not affect gene expression in APA, in NAG it significantly decreased *CYP11B1* gene expression at 10 µM (Figure 23).

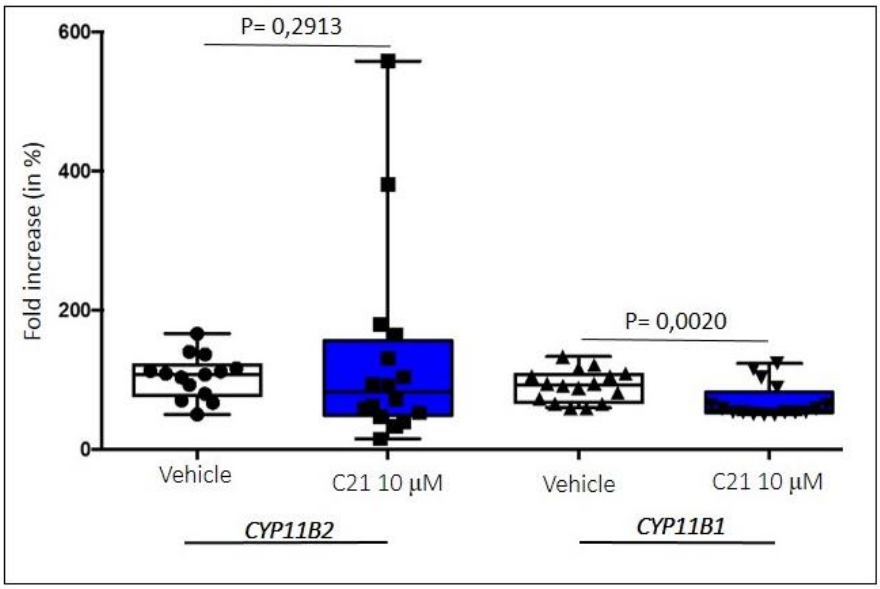


Figure 23: *CYP11B2* and *CYP11B1* expression in normal adrenal gland after stimulation with C21 at 10 µM (n= 4)

Moreover, preliminaries data, showed an increase of aldosterone production in normal adrenal tissue after a stimulation with C21 at 10 µM (Figure 24).

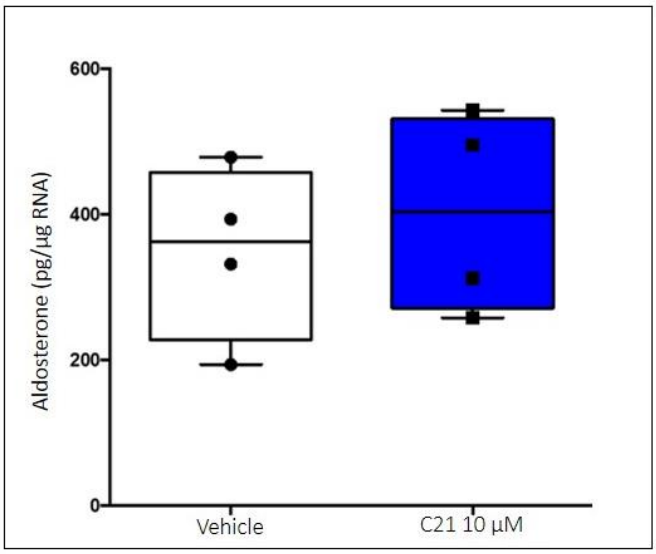


Figure 24: Offset of stimulation with C21 on aldosterone production in NAG (n= 2)

As we did in cells, we used angiotensin II to stimulate aldosterone and cortisol production. We did not observed any significant effect of C21 when co-incubated with angiotensin II (100 nM) in *CYP11B1* and *CYP11B2* gene expression in both tissues (Figure 25).

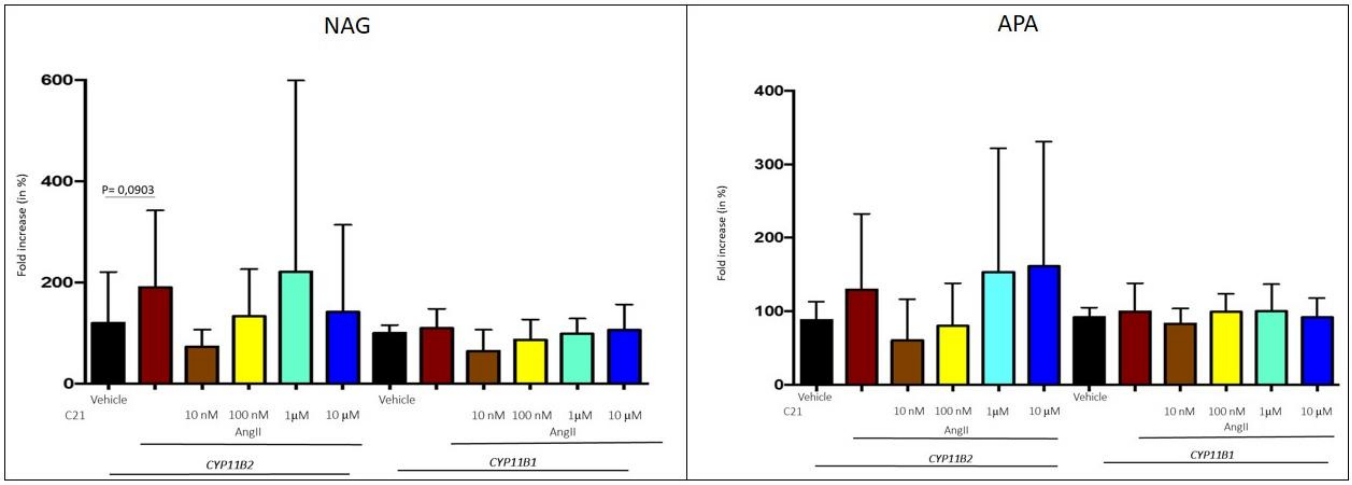


Figure 25: *CYP11B2* and *CYP11B1* expression in NAG (n= 4) and APA (n= 3) after stimulation with C21 and Ang II

NAG = normal adrenal tissue, APA = Aldosterone-producing adenoma

Of note, in APA and NAG tissues from these patients, *AT2R* gene expression is significantly lower in APA compared to NAG (Figure 26).

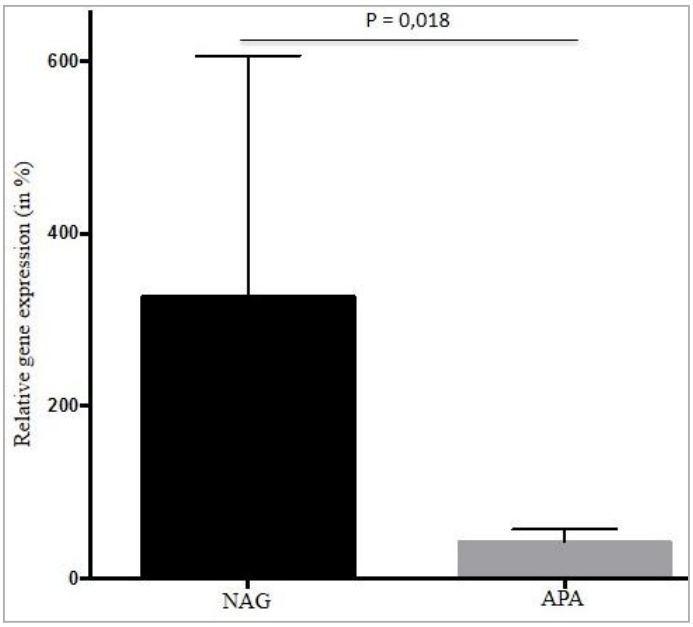


Figure 26: Relative angiotensin II type 2 gene expression in NAG (n= 3) and in APA (n= 4)

NAG= normal adrenal gland; APA= aldosterone-producing adenoma

V- Effects of ACE2 activation on *CYP11B1* and *CYP11B2* gene expression in human adrenocortical cell lines (H295R).

As previously described in introduction, the angiotensin converting enzyme 2 (ACE2) converts angiotensin II to angiotensin-(1-7). We already demonstrated the presence of ACE2 in NAG and in APA, and we liked to determine whether DIZE, an ACE2 activator, may be involved in aldosterone and cortisol production regulation.

Following previous *in vitro* study (Tao L *et al*., 2016), we used DIZE at a range of concentrations from 100 nM to 100 µM. Angiotensin II is used, in one hand, to stimulate aldosterone and cortisol production, and, in a second hand, as a substrate for DIZE.

We first verified if DIZE can increased *ACE2* gene expression in human adrenocortical cells at high concentrations.

Angiotensin II increased *ACE2* gene expression (Figure 27). Moreover, when co-incubated with angiotensin II, at 1 µM DIZE increased *ACE2* gene expression (Figure 27). However, to determine if this increase is due to a potentialisation of angiotensin II effect or to a direct effect of DIZE, we will performed stimulations with DIZE alone.

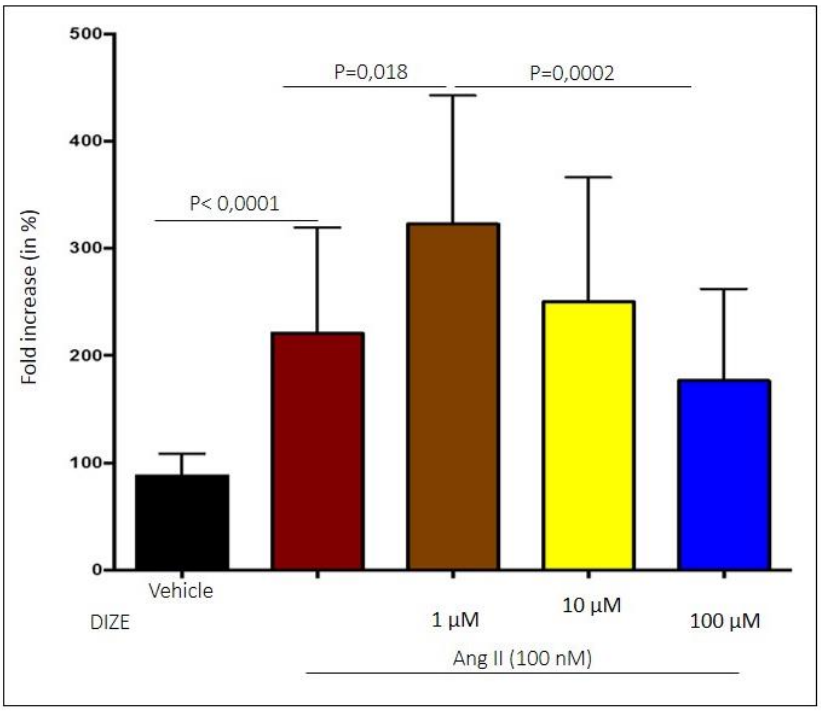


Figure 27: Relative gene expression of angiotensin converting enzyme 2 after stimulation with diminazene aceturate and angiotensin II in H295R cells (n= 4)

We hypothesized a decrease of cell viability to explain the absence of effect at 10 µM and 100 µM. To test this hypothesis we evaluated the toxicity of DIZE at high concentrations on H295 cells (Figure 28). DIZE was found to be not toxic for human adrenocortical cells.

Treatment	Cell viability (in %)
vehicle	91 ± 0
DIZE 10 µM	85 ± 1
DIZE 100 µM	83 ± 5

Figure 28: Effect of diminazene aceturate on H295 cells viability (n= 2)

Moreover, DIZE had not effect on *CYP11B1* and *CYP11B2* gene expression after a stimulation with angiotensin II (Figure 29).

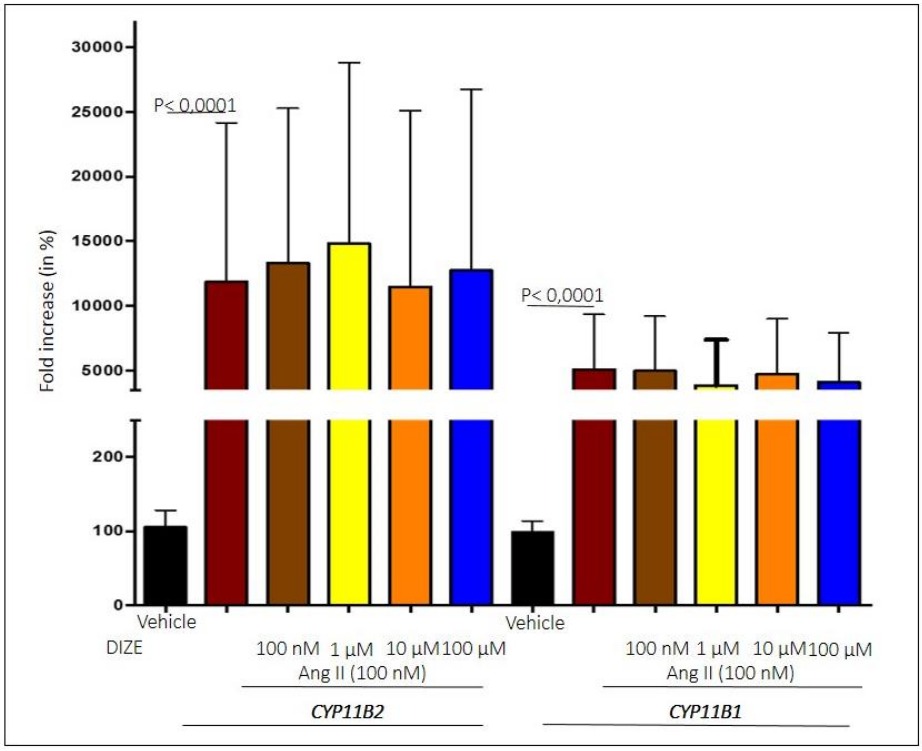


Figure 29: *CYP11B2* and *CYP11B1* expression in H295R cells after stimulation with diminazene aceturate and angiotensin II (n= 4)

Discussion

At a stage when the non-canonical pathway of the RAS is emerging as a protective arm of the system and targets for pharmacological interventions are being considered and actually developed, it seemed appropriate and timely to investigate the consequences of these novel strategies on the function of the human adrenal cortex. The latter is the main site of production of key hormones for the regulation of sodium, water, and blood pressure balance as cortisol and aldosterone. The latter, when produced in excess, is known to contribute substantially to cardiovascular pathologies and events, notably arterial hypertension and heart failure, but also in other conditions (Cesari M *et al*,. 2016). Hence, in these conditions the probability of developing strategies to blunt aldosterone secretion would seem much desirable. Likewise desirable is to known the effects on aldosterone and cortisol of agents that stimulate the receptors/pathways involved in the protective arm of the RAS as angiotensin II type 2 receptor (AT2R), angiotensin-(1-7) receptor (MasR), and alamandine receptor (MrgD).

First and foremost, we investigated gene expression of AT1R, AT2R, MasR, MrgD, ACE, and ACE2 in human adrenal gland and in aldosterone-producing adenoma (APA). We found the mRNA of the above mentioned components of the RAS. In line with earlier studies (Pawlikowski M *et al*,. 2008; Herada K *et al*,. 2010; Wu Z *et al*,. 2010), we found a prevalence of *AT1R* gene expression compared to *AT2R* gene expression in both tissue. Furthermore, compared to *AT2R* gene expression, *MrgD* gene is expressed at the same level, whereas *MasR* gene is more than 15-fold lower expressed. Moreover, we observed a significant decrease of *AT2R* gene expression in APA compared to NAG. In addition, *ACE* and *ACE2* gene are equally expressed in healthy adrenal gland and in APA.

Since this does not prove that the protein are made, we investigated the protein with immunoblot and immunohistochemistry. For immunohistochemistry we used APA and the direct adjacent-tissue. Although not clearly normal, this APA-adjacent tissue is composed of the three cortex layers, whereas APA is solely made of a zona fasciculata a zona glomerulosa (Nakamura Y *et al*,. 2014). With immunoblots and immunohistochemistries we strongly demonstrated the presence of AT1R, AT2R, MasR, MrgD, ACE, and ACE2 in both tissues. Additionally, we observed a stronger positive staining of AT1R in zona glomerulosa compared to the two others layers of the adrenal cortex. AT1R can be used as a marker for zona glomerulosa.

To the best of our knowledge this study is the first to demonstrate the presence of MasR, MrgD and ACE2 in human adrenal cortex. Indeed, it was known since years that AT1R and AT2R were expressed in human adrenal tissue and in APA (Tanabe A *et al*,. 1998; Allen AM *et al*,.

2000; Herada K *et al*,. 2010; Wu Z *et al*,. 2010), whereas the presence of MasR has been demonstrated, but only recently, in rats (Shefer G *et al*. 2016).

Having found the protein of the above mentioned components of the RAS, we investigated their functions using a pharmacological approach.

In compliance with preceding studies, and notably some performed in HAC15 cells, (Yatabe J *et al*,. 2011; Oki K *et al*,. 2013), aldosterone and cortisol secretion were not impacted by acute stimulation with low doses of Ang-(1-7) (< 1 µM), not even when the aldosterone production is upregulated. However, at a concentration higher than 1 µM, Ang-(1-7) bound to AT1R and acted as an agonist. To our knowledge, agonist effect of Ang-(1-7) through AT1R was only observed once (Caruso-Neves C *et al*,. 2000). Indeed, even if Ang-(1-7) is affine for AT1R at high concentration (Table 2) (Bosnyak S *et al*,. 2011), it has been reported that at high dose, Ang-(1-7) acts most often as an AT1R-antagonist (Mahon JM *et al*,. 1994). To explain ours results only two hypothesis can be proposed. First, as observed in other tissues (Lyngso C *et al*,. 2009), AT1R and MasR can heterodimerized in human adrenal gland, and it is not yet clear how it can affect the signal transduction. To note, we did not explored this hypothesis. The second one relies on a difference in the intracellular pathway activated, despite a binding of Ang-(1-7) on the Ang II-binding site. In this hypothesis, Ang-(1-7) and Ang II would be in competition for the same AT1R-binding site. One of the methodology to demonstrate this hypothesis is the crystallography technique.

What's more, as mentioned above, MasR was found in rat adrenal tissue. In fructose-fed rat model, used to study the metabolic syndrome, Yonit Marcus *et al* observed a chronic regulatory effect of Ang-(1-7) treatment on circulating aldosterone and plasma renin (Marcus Y *et al*,. 2013). Later, to determine whether MasR can be involved in this regulation, they demonstrated its expression in zona glomerulosa, and stimulated these cells with increasing concentrations of Ang II, potassium, and ACTH concomitantly with Ang-(1-7) at 1 µM (Shefer G *et al*,. 2016). Despite an absence of effect on baseline aldosterone secretion, Ang-(1-7) significantly decreased aldosterone production due to Ang II and ACTH. However, it did not affect potassium effect. Using the MasR antagonist A779 at high concentration (1 µM), which blunted Ang-(1-7) effect, they concluded that MasR counter-regulates Ang II and ACTH aldosterone secretion in rat. This observation is opposite to ours. These differences can be explain with different comments. First of all, the species are different. And it is known since decades that angiotensin II receptors are not the same in rodent and in human. Whereas AT1R

exists under two isoforms on rodents (AT1Ra and AT1Rb), only one isoform is expressed in human. Furthermore, they did not determine the relative quantity of the three main angiotensin receptors: AT1R, AT2R, and MasR. Of course, nothing is still known about MasR quantity in rat adrenal gland, but a prior study (Herada K *et al*,. 2010) proved, via RT-PCR and immunoblot, that AT2R is ten-fold more expressed in rat adrenal cortex than in adrenal medulla, whereas in human AT2R is two-fold more present in adrenal medulla than in adrenal cortex. Finally, they did not investigate the possible AT2R/MasR heterodimerization. Thus, we concluded that the effects observed in rats could be not relevant in human pathophysiology. Furthermore, considering the relative quantity of AT2R in rat adrenal cortex compared with adrenal medulla, the regulatory role of Ang-(1-7) could be an effect of its binding to AT2R. Indeed, they did not use AT2R antagonist, so beyond AT1R effects, AT2R activation remains a possible explanation.

Regarding C21, we found the same agonist effects than Ang-(1-7) in our *in vitro* models. Indeed, in human adrenocortical cell lines, HAC15 and NCI-H295R, at high doses (from 10 µM) C21 binds to AT1R and acutely acts as a secretagogue for aldosterone and cortisol. As for Ang-(1-7), it has been shown that C21 is affine for AT1R with an IC_{50} of 10 µM (Bosnyak S *et al*,. 2011). However, unlike Ang-(1-7), it has already been observed, *in vivo*, that C21 can play a role as an AT1R agonist at high dose (Danyel LA *et al*,. 2013; Verdonk K *et al*,. 2012). To note, in our experiments, C21 did not counter-regulate the secretagogue effect of Ang II and potassium. This last observation is interesting because it allows us to conclude that the effect of C21 is aldosterone and cortisol concentration-independent.

As discussed above, acute stimulation with Ang-(1-7) and C21 increased aldosterone and cortisol secretion, because MasR and AT2R were significantly less expressed than AT1R. And in our *in vitro* models, i.e human adrenocortical cell lines, the respective proportion of the three angiotensin receptors is respected. Thus, it is a useful model to study the role of the non-canonical RAS in human adrenal cortex.

By the way, in addition to cell lines, we also used tissues directly from patients to study C21 effect. Even if it is known that APA has a low sensitivity to Ang II (Nakamura Y *et al*,. 2014), this model is efficient to study the effect of several compounds on aldosterone and cortisol secretion in human. To be sure of the availability of AT1R, tissue-slices were starved in serum-free medium for 6 hours. Another method could consist to wash tissue with a Krebs solution.

Nonetheless, the heterogeneity of response is a limitation of this model. To have clear results an important number of experiments has to be performed.

Besides, we have to take in consideration the significant down-regulation of *AT2R* gene in APA compared to NAG. Indeed, it has been strongly demonstrated that the AT1R-opposed actions of AT2R are not only determined by specific signaling pathways but also by the levels of AT2R expression within a given tissue (Foulquier S *et al*,. 2012). Thus, AT2R could have a physiological effect in healthy adrenal tissue, but probably not in APA.

Furthermore, we stimulated human adrenocortical cells with an ACE2 activator, DIZE which has been co-incubated with Ang II. Firstly, we observed an increase of *ACE2* gene expression under stimulation with Ang II. Moreover, this gene expression was significantly increased when Ang II was coupled with DIZE at 1 µM. To determine if this effect is only due to DIZE or to a potentialisation of Ang II effect, stimulations with DIZE alone will be performed. Nevertheless, DIZE was not effective to decrease aldosterone and cortisol production in human adrenocortical cells.

ACE2 plays a critical role in maintaining the balance between the canonical and the non-canonical pathways of the RAS. In our models, Ang-(1-7) had no effect on *ACE2* gene expression, whereas Ang II increased it. Thus it may exist a feed-back loop which can be overstimulated with DIZE. However, we did not know if ACE2 is functional in human adrenal cortex. Moreover, we did not quantify Ang-(1-7) concentration in human adrenal cortex.

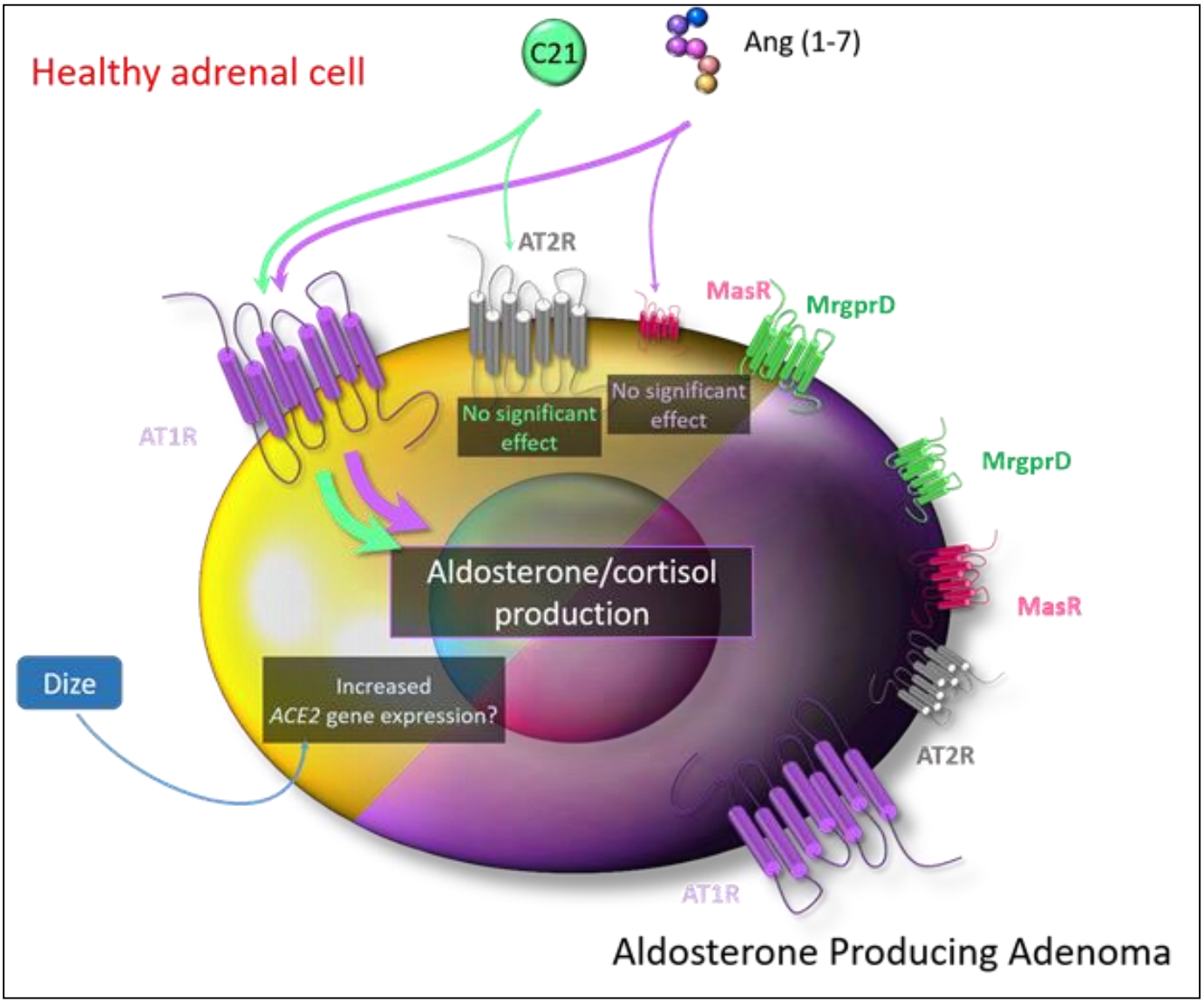
Finally, we clarified the role of Ang II on cortisol production. This effect is still controversial (Seyedi N *et al*,. 1995; Carey RM *et al*,. 2005). We strongly demonstrated that Ang II and potassium are mediators for cortisol secretion *in vitro*.

Conclusion

Since two decades, and the first observation of AT2R-counter-regulatory effect, the non-canonical RAS pathway is extensively studied. However, nothing was investigated in human adrenal gland and in aldosterone-producing adenoma (APA).

We clearly demonstrated the presence of the main protective RAS pathway components in human adrenal and in APA. The pharmacological approach used showed that Ang-(1-7) did not affect aldosterone and cortisol production at low concentration not even in case of aldosterone upregulation. C21 had similar effect. Both acted only at high concentrations when they lose their specificity. We concluded that AT2R and MasR are too lowly expressed in human adrenal cortex and could not be considered as therapeutic targets to decrease aldosterone and cortisol secretion. However, as we determined the loss of Ang-(1-7) and C21 specificity at high concentrations, our results are useful for pharmaceutical industry, particularly for Vicore Pharma which kindly provides us C21. More interestingly, we showed the effect of Ang II and DIZE in *ACE2* gene expression. The stimulation of this enzyme will be study more extensively.

Finally, in this work, we did not study the possible role of alamandine in aldosterone and cortisol production. Furthermore, it remains to investigate a possible AT1R/MasR, and AT2R/MasR heterodimerization, that could be an interesting possibility to decrease cell proliferation in APA.



Conclusion figure: Proposed model.

The angiotensin receptors studied were found in healthy adrenal cells and in aldosterone-producing adenoma. Furthermore, Angiotensin-(1-7) binds to MasR and AT1R, but is effective on aldosterone and cortisol secretion only via AT1R. The same effect was shown with C21. DIZE probably increased *ACE2* gene expression.

AT1R= angiotensin II type 1 receptor; AT2R = angiotensin II type 2 receptor; MasR= angiotensin-(1-7) receptor; MrgprD= alamandine receptor; ACE2= angiotensin converting enzyme 2; DIZE = diminazene aceturate; Ang-(1-7)= angiotensin-(1-7)

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