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CICLO XX

EXPRESSION AND FUNCTIONAL ROLE OF UROTENSIN-II AND ITS RECEPTOR IN THE ADRENAL CORTEX AND MEDULLA

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RIASSUNTO

I meccanismi molecolari che determinano l'eccesso di catecolamine nei feocromocitomi (PHEO) e l'iper aldosteronismo negli adenomi producenti aldosterone (APA) sono complessi ed ancora sconosciuti, anche se è ipotizzabile l'intervento di numerose interazioni paracrine.

L'Urotensina II (UII) è un potente peptide vasoattivo che agisce legandosi al recettore specifico accoppiato alle proteine-G, denominato UT-R ed è ampiamente espresso nei tessuti, in particolar modo nel sistema cardiovascolare e nella ghiandola surrenalica umana e del ratto. L'individuazione dei trascritti genici di UII e UT-R in tumori istogeneticamenti differenti della ghiandola surrenale suggerisce un potenziale ruolo di UII nella fisiopatologia surrenalica e, di conseguenza, nella regolazione della pressione arteriosa. Tuttavia l'espressione quantitativa di UII e del suo recettore negli APA e nei PHEO, e gli effetti biologici di UII sulla ghiandola surrenalica, rimangono sconosciuti.

Nel lavoro di questa tesi ci si è proposto d'investigare l'intero trascrittoma mediante oligomicroarrays per individuare i pathways molecolari relativi a UII e UT-R nei tumori adrenocorticali e adreno-midollari.

Si sono quindi utilizzati la tecnica real-time Reverse Transcription Polymerase Chain Reaction per quantificare i trascritti dei due geni negli APA e nei PHEO, Western blot ed immunoistochimica per verificare l'espressione a livello proteico e la localizzazione di UII. Inoltre grazie al recente sviluppo del palosuran, un antagonista specifico per il recettore UT-R, si è investigato il ruolo dell'attivazione di UT-R sull'espressione adrenocorticale dell'aldosterone sintasi (CYP11B2) e di 11B-idrossilasi (CYP11B1) mediante infusione cronica di UII nella ghiandola surrenalica dei ratti.

SUMMARY

Numerous paracrine interactions between the adrenal cortex and medulla are likely to play a role in the pathogenesis of pheochromocytoma (PHEO) and aldosterone-producing adenoma (APA). Nonetheless, the mechanisms underlying excess production of catecholamines in PHEO and of aldosterone in APA are complex and largely unknown.

Urotensin II (UII) is a vasoactive peptide that acts through specific G protein-coupled receptor (GPR)-14, named UT-R, and is widely expressed in tissues, including the vasculature and the rat and human adrenal gland. Along with the detection of UII and UT-R gene transcripts in APA and PHEO, these findings led to propose a role of the UII in adrenal gland pathophysiology and, therefore, in the regulation of blood pressure and body fluid homeostasis. However, the quantitative expression of UII and UT-R in APA and PHEO and the biological effects of UII in the adrenal gland remained poorly known. Novel techniques may provide powerful investigative tools to shed new light on the molecular complexity of adrenal tumors. Hence, we used real-time RT-PCR and a whole transcriptome analysis to pinpoint UII and UT-R related pathways in adrenocortical and adreno-medullary tumors and to precisely quantify the UII and UT-R transcripts in APA and PHEO. We next sought for the UII peptide by Western blot analysis and immunohistochemistry. Recently, UT-R antagonists have been prepared and this can enable investigators to directly address the role played by UII and its receptors in different pathophysiological conditions.

Moreover, we took advantage of the recent development of palosuran, a specific UT-R antagonist, to investigate the role of UT-R activation on the adrenocortical expression of aldosterone synthase (CYP11B2) and 11β

hydroxylase (CYP11B1) by means of a chronic UII infusion on the rat adrenal gland.

INDEX

INTRODUCTION

ADRENAL GLAND

Adrenal zonation

The adrenal glands are the triangle-shaped, orange-coloured endocrine glands that sit on top of the kidneys. Each gland is separated into two distinct structures, both of which receive regulatory input from the nervous system: adrenal medulla and adrenal cortex. The adrenal medulla is the central core of the adrenal gland, being surrounded by the adrenal cortex. Composed mainly of hormone-producing chromaffin cells, the adrenal medulla is the principal site of the production of catecholamines (epinephrine and norepinephrine) and several other factors including vasopressin, oxytocin, galanin, neuromedine-N, neuropeptide-Y, chromogranin-A, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). Situated along the perimeter of the adrenal gland, the adrenal cortex is devoted to the synthesis of corticosteroid hormones from cholesterol. The mature mammalian adrenal cortex has three distinct zones, zona glomerulosa, zona fasciculata, and zona reticularis originally described in 1866 by Arnold, ([1]) with different cellular differentiation and specific steroids synthesized. The outermost layer of the adrenal cortex, close to the capsule, is the zona glomerulosa, which comprises clusters of cells that produce the mineralocorticoid aldosterone. The middle layer, the zona fasciculata, is composed of cords of cells, organized centripetally to the gland. The inner layer of cortex, the zona reticularis, consists of interlaced strands of cells. The zona fasciculata and the zona reticularis both synthesize the

glucocorticoid cortisol. Androgens are mainly produced by the zona reticularis.

In humans, functional zonation relies on the zone-specific expression of two cytochrome P450 isozymes termed CYP11B1 (11beta-hydroxylase) and CYP11B2 (aldosterone synthase) that catalyze the final steps in the biosynthesis of cortisol and aldosterone, respectively.

ADRENOCORTICAL TUMOR AND PRIMARY ALDOSTERONISM

Prevalence and diagnosis of Aldosterone Producing Adenomas

Primary aldosteronism (PA) resulting from an adrenocortical adenoma (aldosteronoma), as described by Conn,[2] is the most common cause of surgically correctable form of arterial hypertension.

Hypertension, hypokalemia, suppressed plasma renin activity (PRA), and increased aldosterone excretion are classical features of PA, which has emerged as the most common form of mineralcorticoid hypertension.[3,4]

Its two major subtypes are aldosterone producing adenoma (APA), which refers invariably to unilateral tumors of the adrenal cortex resulting in the autonomous secretion of aldosterone, and idiopathic hyperaldosteronism (IHA), which corresponds pathologically to bilateral adrenal hyperplasia (BAH). The differential diagnosis between these two forms is crucial for appropriate treatment because hypertension in APA can be cured by adrenalectomy, whereas individuals affected with BAH should receive targeted medical treatment with mineralocorticoid receptor antagonists.

A much less common form, unilateral hyperplasia or primary adrenal hyperplasia, is caused by zona glomerulosa hyperplasia of predominantly one adrenal gland.([5])

Numerous studies conducted in recent years have reported an increase in the prevalence of PA. In the prospective study of prevalence of PA in 1125 Italian hypertensive patients (PAPY study), APA and IHA were found in 4.8% and 6.4% of all patients, respectively, thus leading to an overall prevalence of PA of 11.2%.([3;6])

Diagnosis and treatment of PA are particularly important in light of recent data suggesting that individuals with aldosterone excess might be more prone to premature vascular disease including cerebrovascular disease, cardiac fibrosis and vascular inflammation.([7;8])

The diagnostic work-up for identifying patients with PA includes:([9]) (Fig. 1)

a) screening tests e.g. aldosterone renin ratio (ARR)([10]) and captopril tests aimed at recognizing patients with a high likelihood of PA;

b) exclusion tests aimed at identifying the false positive rates;

c) PA subtype identification tests, pursued at distinguish the surgically correctable forms of PA (caused by unilateral excess production of aldosterone) from not surgically correctable forms, induced by bilateral aldosterone over-secretion.

APA, however, requires histological and follow-up data after adrenalectomy to unequivocally confirm the diagnosis.

ADRENAL MEDULLARY TUMORS

Prevalence and diagnosis of Pheochromocytoma

Pheochromocytomas (PHEOs) are catecholamine-producing neuroendocrine tumors arising from chromaffin cells of the adrenal medulla or extra-adrenal paraganglia. Tumors from extra-adrenal chromaffin tissue are referred to as extra-adrenal PHEOs or paragangliomas. The term paraganglioma is also used for tumors derived from parasympathetic tissue in the head and neck, most of which do not produce catecholamines. Nearly 80–85% of this potentially curable form of endocrine hypertension arise from the adrenal medulla, whereas about 15–20% are from extra-adrenal chromaffin tissue.([11;12])

Catecholamine-producing extra-adrenal paragangliomas are usually found in the abdomen.([13;14])

Previous studies reported that the prevalence of PHEO in patients with hypertension is 0,1–0,6%.([15;16])

Despite PHEOs are discovered during life in one to six per million people/yr among the general population, the prevalence of PHEO in autopsy studies is relatively high (about 0.05%).([17-21]) Thus, many tumors may be missed, resulting in premature mortality.([22])

Most tumors are sporadic, but about 25% of cases are associated with germline mutation in one of five major susceptibility genes:([23;24])

1. the von Hippel-Lindau gene (*VHL*), which causes von Hippel-Lindau syndrome;

2. the *RET* gene leading to multiple endocrine neoplasia type 2;

3. the neurofibromatosis type 1 gene (*NF1*), which is associated with von Recklinghausen's disease;

4. the genes encoding the B and D subunits of mitochondrial succinate dehydrogenase (*SDHB* and *SDHD*), which are associated with familial paragangliomas and pheochromocytomas.([23;25;26])

Most but not all the clinical signs and symptoms of pheochromocytoma are due to the direct actions of secreted catecholamines. Hypertension, tachycardia, pallor, headache, and feelings of panic or anxiety, usually dominate the clinical presentation.([27])

Biochemical presentation of excessive production of catecholamines is an essential step for the diagnosis of PHEO.

However, many PHEO do not secrete consistently catecholamines and their presence can be incidentally discovered ([28]) or can be revealed by occasional use of drugs, as veralipride ([29]) or metaclopramide, and/or of foods.

Traditional biochemical tests include measurements of urinary and plasma catecholamines, urinary metanephrines (normetanephrine and metanephrine), and urinary vanillylmandelic acid (VMA). Measurements of

plasma-free metanephrines (normetanephrine and metanephrine) and chromogranin A represent more recently available tests. Because chromogranin A is not affected by drugs, its measurement should be included in the work-up for diagnosis of PHEO.([30-33])

Imaging techniques such as CT or MRI and functional ligands such as ^{123}I-MIBG are used to localise biochemically proven PHEOs. After the use of appropriate preoperative treatment to block the effects of secreted catecholamines, laparoscopic tumor removal is the preferred procedure.

CORTICO-MEDULLARY INTERACTIONS IN ADRENALS

Morphological and Functional Background of the Cortico-Medullary paracrine Interactions.

The intimate morphological interrelationships between cortex and medulla in the mammalian adrenal gland are well known to possess a great functional relevance.

Medullary chromaffin cells exert a control on the function of the cortex via:

1. nerve fibers that originate from medullary neurons and reach the cortex;([34])

2. islets or rays of medullary tissue that are closely linked with the cortical tissue.([35])

The nerve fibers reaching the cortex may have a two-fold origin,([36]) with a group of fibers originating from neurons located outside the adrenal gland and reaching it by following blood vessels or splanchnic nerves, and a second group, which probably includes all the peptidergic fibers, having its cell body in the adrenal medulla.([37])

The nerve fibers and the secretory products of chromaffin cells, locally released inside the cortex, may modulate steroid secretion by acting either directly on the adrenocortical cells, or on the gland vasculature by modifying cortical blood-flow rate, which is known to influence steroid-hormone and especially glucocorticoid release. Moreover, there is the possibility that adrenal medulla may control the function of the cortex by regulating the local release of endothelins and/or the activity of renin-angiotensin system (RAS) located in the zona glomerulosa.

The main secretory products of medullary chromaffin cells are epinephrine, norepinephrine and dopamine. Epinephrine, norepinephrine and adrenergic fibers stimulate steroid secretion, whereas dopamine exerts an opposite effect. Medullary chromaffin cells also secrete serotonin, a potent stimulator of steroid secretion. There is also evidence that the adrenal gland, at least in the rat, contains a corticotrophin-releasing hormone (CRH)-adrenocorticotropin (ACTH) system, duplicating the hypothalamo-pituitary one, which controls in a paracrine manner the secretion and growth of the cortex. In addition to catecholamines, serotonin and CRH-ACTH, adrenal chromaffin cells store and release numerous regulatory peptides, many of which to affect the function of the cortex in vivo and in vitro.([38])

The mechanisms by which molecules released by medullary chromaffin cells and nerve fibers control adrenocortical cells include:

1. Direct mechanisms.

   Regulatory molecules, released inside the cortex, bind specific receptors located on their plasma membrane and consequently activate

intracellular pathways. When such mechanisms are operative, the regulatory molecule is able to affect in vitro steroid secretion of dispersed adrenocortical cells.

2. Indirect mechanisms.

    a. Regulation of adrenal blood flow by neural and endocrine components (e.g. both splanchnic-nerve activation and ACTH);([39])

    b. Modulation of intra-adrenal RAS, mainly located in the capsule-zona glomerulosa, that plays an important role in the paracrine control of aldosterone secretion;([40])

    c. Modulation of intra-adrenal endothelin system because endothelins and their receptor subtypes A and B are expressed in the mammalian adrenals and plays a role in the stimulation of steroidogenesis, especially in aldosterone secretion.([41])

Control of Cortex Function by Medullary Monoamines.

Mammalian adrenocortical cells possess both α and β receptor subtypes for epinephrine and norepinephrine. Both catecholamines and their β-receptor agonists (e.g. isoprenaline and dobutamine) can enhance aldosterone secretion in vitro by zona glomerulosa preparations, and this effect can be blocked by β-adrenoreceptor antagonists (e.g. propanolol).([42])

Data indicate that β-adrenoreceptor activation is coupled with adenylate cyclase activation.([43]) Epinephrine and norepinephrine, acting via adenylate

cyclase-coupled β-receptors, directly stimulate mineralo- and probably glucocorticoid secretion of adrenocortical cells; they also appear to enhance gene expression of all the enzymes involved in steroid synthesis.([44])

Dopamine, acting via D_1 and D_2 receptors, exerts a direct concentration-dependent biphasic effect on the zona glomerulosa and aldosterone secretion: the activation of D_1 increases adenylate cyclase activity,([45]) while that of D_2 inhibits it.

Serotonin, acting via phospholipase C-coupled $5HT_2$ and adenylate cyclase-coupled 5-HT_4 receptors, directly stimulates aldosterone([46]) and glucocorticoid secretion of adrenal cortex; the glucocorticoid secretagogue action seems to be, at least in part, mediated by an increase in the adrenal blood flow.([47])

The Medullary Regulatory Peptides Affecting Adrenocortical Cell Function.

Since the discovery in the early 1980s that adrenal medulla contains and release, in addition to catecholamines, many other regulatory molecules, a large mass of data has been accumulating indicating that medullary chromaffin cells exert a paracrine control on the function and growth of the adrenal cortex. These regulatory molecules may exert stimulatory or inhibitory effects, act directly or indirectly on the cortex, and specifically affect the function of the zona glomerulosa or zona fasciculata-reticularis.

Stimulatory molecules include CRH-ACTH, arginine-vasopressin (AVP), oxytocin, enkephalins, pancreatic polypeptide, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). Inhibitory molecules include somatotropin release-inhibiting hormone (SRIH) and natriuretic peptides.

The main effect of intramedullary CRH is paracrine/autocrine ACTH release by chromaffin cells, the intramedullary CRH-ACTH system being mainly involved in the maintenance and stimulation of the secretion and growth of inner adrenocortical zones.

AVP, acting via phospholipase C-coupled receptors, plays an important role in maintenance and stimulation of the zona glomerulosa growth and mineralocorticoid secretion. AVP also stimulates glucocorticoid secretion and the growth of zona fasciculate, probably by activating intramedullary CRH-ACTH system.

Oxytocin directly stimulates basal steroid secretion of adrenocortical cells, but it inhibits ACTH-induced glucocorticoid secretion in rats.

SRIH, acting via specific receptors that interfere with the intracellular mechanisms transducing Angiotensin-II signals, exerts a direct and specific inhibitory action on the zona glomerulosa that has a notable physiological relevance, inasmuch as the prolonged administration of SRIH antagonists enhances zona glomerulosa growth and secretory capacity.

Enkephalins exert a direct stimulatory action on the secretion and growth of the adrenal cortex that may be mediated by μ opioid receptors.

Pancreatic polypeptide, at nM concentrations, directly enhances glucocorticoid secretion in rats and probably plays a major role in the adrenal response to hypoglicemic stress.

Intramedullary VIP is involved in the physiological regulation of zona glomerulosa functions. The acute aldosterone secretagogue effect is either direct or indirect and mediated by the stimulation of medullary

cathecolamine release, whereas the long-term trophic effect is only direct via specific receptors on zona glomeruolsa cells.

PACAP stimulates both mineralocorticoid and glucocorticoid secretion indirectly, by eliciting catecholamine release by medullary chromaffin cells and by activating the intramedullary CRH-ACTH system, respectively.

Intramedullary natriuretic peptides exert a physiologically relevant direct receptor-mediated inhibition of the secretion and growth of zona glomerulosa that appears to involve a selective impairment of voltage-dependent Ca^{2+} channels, as well as the activation of guanylate cyclase and the inhibition of adenylate cyclase.

Some data suggest that the cortico-medullary paracrine interactions can play a role in the pathogenesis of some diseases.

Human PHEOs synthesize and secrete, in addition to catecholamines, several regulatory peptides.

Radioimmunoassay (RIA) and immunohistochemistry studies demonstrated the presence in chromaffin cells of PHEO of SRIH-ir, PACAP-ir, enkephalin-ir, and natriuretic peptide-ir.

The presence in PHEO of the specific mRNAs of prepro-CRH has also been detected by both biochemical and in situ hybridization techniques.

Most of the regulatory molecules expressed by human Pheos are able to stimulate the secretion and growth of the cortex and especially of the zona glomerulosa. Hence, these findings may explain the pathophysiological

basis of some cases of Conn's adenomas or idiopathic aldosteronism associated with secreting Pheos.

UROTENSIN II AND UROTENSIN II RECEPTOR

Urotensin II and Urotensin II Receptor System

Urotensin II (UII) was first recognised some 30 years ago as an important teleost fish ormone, isolated from urophysis, a neurosecretory system in the fish caudal spinal cord that is functionally similar to the human hypothalamic-pituitary system.([48;49])

Its human homologue has subsequently been cloned and characterized.([50]) The predicted human UII (hUII) is a cyclic undecapeptide of 11 amino-acids containing a disulfide bridge between Cys^5 and Cys^{10}. (Fig. 2)

The C-terminal portion of UII contains a highly conserved cyclic hexapeptide sequence which is responsible for the majority of its biological activities, whereas the N-terminal portion shows low sequence homology between species.([51]) All known isoforms in mammalian, amphibian and fish species contain an identical cyclic motif. (Table 1) Conservation of this cyclic hexapeptide across isoforms spanning 550 million years of evolution suggests a significant physiological function for UII. This peptide derived from pre-pro UII, which consists of 124 and 139 amino-acids residues, by urotensin converting enzyme.([52])

In 1999 Ames et al ([53]) demonstrated UII to be the endogenous ligand of the G-protein coupled receptor, GPR14/ sensory epithelia neuropeptide-like

receptor (SENR) orphan receptor, with seven transmembrane domains, which was cloned recently and renamed UT receptor (UT-R).[54]

The human UT-R (hUT-R) comprises 386 residues and possesses two potential N-glycosylation sites in the N-terminal domain (Asn^{29} and Asn^{33}) and two cysteine residues in the first and second extracellular loops, which are held to participate in disulphide bonding. Intracellular portions contain the Glu/Asp-Arg-Tyr motif, which is well conserved amongst GPR14, and potential phosphorylation sites are found in the cytoplasmatic tail. (Fig. 2)

The UII and UT-R genes are located on human chromosome 1p36-p32 and 17q25.3, respectively.[55;56] In more recent years, UII has been identified as a vasoactive peptide in mammalian.

Several recent reports have revealed the powerful vasocontractile effect of UII (amounting to some eight- to 110-fold the potency of endothelin-1), which further testifies to the potential importance of this peptide in cardiovascular physiology and disease.[57]

In mammalian, UII was reported to have a vasoconstrictor action in isolated rat thoracic aorta denuded of endothelium.[58] However, the vasoconstrictor action of UII has not been found to be universal. The reported vasodepressor, and regionally selective vasodilator effect ([59]) of UII, suggests that the effect of this peptide on the vasculature is likely to be more complex than was first envisaged. In particular, attention has been focused on the fact that UII produces both endothelium-independent vasoconstriction and endothelium-dependent vasodilation.[60]

UII induced endothelium-dependent vasodilation in rat coronary arteries, and this effect was attenuated by removal of the endothelium.[61]

UII caused vasoconstriction in endothelium-denude coronary arteries from various species, including rats, dogs, and humans.([55;59;62]) This response is attributed to direct activation of UT-R ([53]) and is supported most compellingly by the absence of UII contractile activity in UT-R knockout mice.([63])

UII-mediated vasoconstriction is mediated via UT-R in vascular smooth muscle cells (VSMCs).([64]) (Fig. 3) Binding of UII to UT-R leads to Gq protein activation, which leads to activation of protein kinase C (PKC), protein tyrosine kinase, calmodulin, and phospholipase C (PLC), as evidenced by the inhibition of vasoconstriction by specific inhibitors of these enzymes.([65-67]) PLC leads to the production of arachidonate second messengers, inositol-1,4,5-triphosphate (IP_3)/diacylglycerol (DAG), which was demonstrated by an increase in [^{3}H]inositol-phosphate when aortic tissue was stimulated with UII. The activation of these messengers leads to release of Ca^{2+} from the sarcoplasmatic reticulum, which in turn stimulates extracellular Ca^{2+} influx leading to vasoconstriction, which can be blocked by extracellular Ca^{2+} channel blockers. In addition to Ca^{2+} -calmodulin-dependent myosin light chain kinase (MLCK), the vasoconstictive effects of UII are also mediated in part by the extracellular signal-regulated kinase (ERK) and RhoA/Rho kinase (ROCK)-related pathways.([54])

In contrast, UII can act as an endothelium-dependent vasodilator acting through release of nitric oxide (NO), prostacyclin (PGI_2), prostaglandin E_2 from endothelial cells in some arteries. Therefore, these findings suggest that UII contributes to the modulation of vascular tone and growth.

Spinazzi et al. ([68]) reported that UII stimulated angiogenesis in both *in vitro* and *in vivo* assays which was inhibited by UT-R antagonist palosuran([69]).

Plasma UII concentration measured by radioimmunoassay has been shown to be relatively low (in the range of a few fmol/ml).([70;71]). The low concentrations of circulating and tissue UII in conjunction with a wide tissue distribution of co-expression of UII and UT-R are quite analogous to other vasoactive peptide, such as endothelin and adrenomedullin, suggesting that UII can act predominantly as an autocrine/paracrine factor rathen than a hormone.

Localization of UII and its receptors

Localization of UII peptide and expression of pre-pro UII messenger RNA by northern blot, reverse transcriptase-polymerase chain reaction (RT-PCR), in situ hybridization and immunohistochemistry demonstrate that both the peptide and its receptor are most strongly expressed within the central nervous system (including the medulla oblongata and ventral horn motor neurones of the spinal cord). However, UII and UT-R are both present in a wide array of peripheral tissues. These include the cardiomyocyte, endothelium, vascular smooth muscle cells (including those in aorta and pulmonary artery), the kidney, pancreas, adrenal, thyroid and pituitary.([50;51;55;72;73]) This distribution of the peptide and its receptors, particularly in the heart, vasculature and kidney, suggested that UII may be a potential regulator of the cardiovascular function. However, despite an accumulating body of experimental evidence documenting the effects of UII in isolated cells, vessels, cardiac tissue, intact animals, and finally in conscious human, the true role of UII in the physiology and pathophysiology of the cardiovasculare system remains a mistery.

UII and human disease

UII has been implicated in a number of human cardiovascular diseases such as arterial hypertension, congestive heart failure (CHF), coronary artery disease and atherosclerosis, diabetes and metabolic syndrome. Plasma levels of UII were elevated in patients with heart failure ([74;75]) patients with chronic renal failure ([71]) and patients with type 2 diabetes mellitus ([70;76]). Urinary levels of UII were raised in patients with essential hypertension ([73]), and in patients with advanced diabetic nephropathy ([76])

The levels of expression of UII and UT-R are up-regulated by the inflammatory mediators such as interleukin-6 and 1β ([77]) and interferon-γ ([78]) and may be down-regulated by sympathetic nervous system, atrial natriuretic peptide (ANP), or brain natriuretic peptide (BNP). ([57;79]).

UII has been reported to exert mitogenic actions, probably as an autocrine/paracrine factor. Mitogenic action of UII was first described by Sauzeau et al.([54]) They showed that UII (10^{-7} M) stimulated rat aortic smooth muscle cells proliferation by both cell counting and DNA synthesis estimated by BrdU incorporation.

Moreover, as mentioned UII can play a role in neo angiogenesis, an effect that can be important in tumorigenesis and atherosclerosis.

UII increased ^{3}H-thymidine uptake in rabbit aortic vascular smooth muscle cells synergistically with mildly oxidized LDL via c-Src, PKC, and MAPK-dependent pathways. ([80]) These proliferative actions of UII were also reported to be mediated by RhoA and Rho-kinase ([54]) and phosphorylation of ERK.([81]).

Furthermore, UII was shown to promote proliferation of some cultured tumor cells e.g. a human renal carcinoma cell line VMRC-RCW, ([82]) a human adrenocortical carcinoma cell line SW-13 ([83]) and primary culture of human pheochromocytoma cells.([84]) Albertin et al. first reported the expression of UII and UT-R in the rat adrenal gland ([85]) and that UII suppressed basal secretion of corticosterone from in vitro cultured adrenocortical cells of Sprague-Dawley rats.

These reports raised the possibility that UII might have some regulatory roles in tumorigenesis and secretion of adrenal hormones in human adrenal tumors. In fact, even through paracrine interactions involving numerous peptides have been suggested, ([41;86]) the mechanisms underlying catecolamines excess in pheochromocytoma and hyper-aldosteronism in aldosterone-producing adenoma remain ([87]) unknown.

AIMS OF THE STUDY

The main goal of our study was to investigate the quantitative expression of UII, its specific receptor in APA and PHEO and to investigate the potential role of this peptide in the pathophysiology of the adrenal cortex.

Specific objectives were:

1. to quantify the UII and UT-R transcripts in APA and PHEO;
2. to identify the genes that may be involved in the UII and UT-R mediated signalling pathways in APA and PHEO;
3. to measure the protein expression of UII and UT-R;
4. to evaluate the gene expression of CYP11B2 and CYP11B1 in the rat adrenal gland after a chronic UII infusion with or without UT-R blockade by Palosuran.

METHODS

Adrenal specimens

Adrenocortical tissues from 22 patients with an APA and adrenomedullary tissues from 10 patients with PHEO were investigated. Histologically normal adrenocortical (NAC) and medullary (NAM) tissues obtained from 6 patients with renal cancer undergoing unilateral nephrectomy and ipsilateral adrenalectomy, and from 5 patients adrenalectomized for non functioning incidentally discovered adrenal mass 'incidentaloma', respectively, were studied in parallel as controls for the APA and PHEO tissues. All tissues were obtained at surgery under sterile conditions, immediately frozen in liquid nitrogen, and stored at -80°C until extraction, as described.([88]) In patients with APA the diagnosis was based on strict predefined criteria that comprised lateralization of aldosterone secretion at adrenal vein sampling,([89]) surgery, pathology, and, more importantly, follow-up data. At follow-up, we required demonstration of normokalemia and cure or improvement of hypertension at least 120 days after adrenalectomy, as described.([3]) Cure was defined as systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg without medications; improvement as systolic and diastolic blood pressure <140/90 mmHg, respectively, on the same or reduced number of medications and/or reduced recommended daily doses, as described by the World Health Organization.([90]) PHEO was diagnosed according to state-of-the-art criteria,([33]) and confirmed at pathology, histology, and immunohistochemistry for chromogranin A and synaptophysin.

RNA preparation

Total RNA was isolated from frozen tissue starting from about 20 mg of adrenal tissue. The tissues were disrupted using MagNA Lyser Instrument (Roche, Monza, Italy) in 600 µl of RLT buffer (Qiagen, Milan, Italy) added with β-mercaptoethanol. The next steps were performed at room temperature following the RNeasy Mini Kit protocol (Qiagen, Milan, Italy). The integrity and quality of the RNA were systematically checked with the use of the lab-on-chip technology in an Agilent Bioanalyzer 2100 (RNA6000 Nano Assay, Agilent Technologies, Palo Alto, CA). We started from 1 µl of total RNA and, after the run, proceeded with the analysis of the resulting electropherograms. Only samples with two distinct ribosomal peaks (18S and 28S) were used for the downstream analysis. Furthermore, the quantity and purity of samples were determined by spectrophotometric readings at 260/280/230 nm. The absorbance at 260 nm was used to get RNA concentration, using the following formula:

RNA concentration (µg/µl): (Absorbance 260 * 40 * Dilution factor)/1000

The absorbance at 280 nm reveals traces of protein contaminants, while the absorbance at 230 nm is specific for phenol or alcohol derivates. Only samples with a 260/280 and 260/230 ratios higher than 1.8 were included in the microarrays experiments.

Histologically normal adrenocortical tissue obtained from 5 consenting patients undergoing nephrectomy and ipsilateral adrenalectomy for renal cancer were collected and used to generate a pool of control RNA.

Two-Color Microarray-Based Gene Expression.

cRNA was synthesized from 500 ng of total RNA using the Low RNA Input Linear Amplification Kit and the Two-Color RNA Spike-In (Agilent Technologies, Palo Alto, CA). The Two-Color RNA Spike-In consists in a pre-mixed cocktails composed of 10 transcripts that serve as positive microarray controls to monitor microarray performance. The control pool and sample were labelled using Cyanine 3 (cy3) and Cyanine 5 (cy5) respectively. In order to control for gene specific dye biases and for dye intensity differences, dye swaps and biological replicates were included in the experimental design.

The Labelled/Amplified cRNA was purified using the Qiagen's RNeasy mini spin columns and hybridized on an oligomicroarray chip (Whole Human Genome Microarray Kit, 1 x 44K, G4112A Agilent Technologies), which contains about 44.000 60-mer *in situ* synthesized sequences that comprise the whole human genome. The chip was incubated in a rotor oven at 65°C for 17 hours.

Chip scanning and data analysis.

The chip was scanned using a dual-laser Microarray Scanner System (Agilent Technologies, Palo Alto, CA). After generating the microarray scan images, tiff images were extracted using the Feature Extraction 8.5 software (Agilent Technologies, Palo Alto, CA) and data from different microarray experiments were compared by using Rosetta Resolver (Rosetta Biosoftware, Seattle, WA).([87]) To determine similarities (or differences) across arrays and/or sequences cluster analysis was performed. We preliminary elected to use a cut-off value of 2.5-fold to identify over- and

under- expressed genes. Ontologies analysis of sequences differentially expressed was performed using the Gene Ontology annotation. ([91])

Measurement of UII and UT-R mRNAs

To investigate UII and UT-R mRNAs we used a novel real time reverse transcription (RT)-polymerase chain reaction (PCR) that utilizes Universal ProbeLibrary Probes (UPL) and Universal ProbeLibrary Assay Design by ProbeFinder Software (Roche, Monza, Italy) (www.LC480.it).

This new system uses a library of small TaqMan probes (8 bp) that match with conserved regions of the genome. The specificity of the system is determined by the primers design.

For RT-PCR we used the LightCycler® 480 Instrument (Roche, Monza, Italy). As to avoid overestimating the amount of RNA it is crucially important to obtain RNA free from contaminating DNA, each RNA sample (1 μg) was digested with Deoxyribonuclease I Amplification Grade (Invitrogen, Milan, Italy). After digestion, sample quality was re-assessed in an Agilent Bioanalyzer 2100 and re-quantified by spectrophotometry. Moreover, primers were chosen to span exon-exon boundaries to prevent co-amplification of genomic DNA. A SybrGreen assay was run to check the lack of genomic DNA amplification, amplifying a cDNA and a sample not reverse-transcribed. The melting curves analysis confirmed the absence of amplification in the latter. One μg of total RNA was reverse-transcribed with Iscript™ (Bio-Rad, Milan, Italy) in a final volume of 20 μL. Real-time quantitative PCR analysis was carried out on the LightCycler® 480 system by using LightCycler® 480 Probes Master (Roche) in 96 multiwell plates according to the manufacturer's instructions. 2 μL of each RT reaction was

amplified with specific primers in a final volume of 20 μL. Porphobilinogen deaminase (PBGD), an accepted housekeeping gene for adrenal gland gene expression studies, was similarly processed to normalize for RNA quality, quantity, and RT efficiency, in a separate one-step RT-PCR.([92])

Primers and probes are showed in Table 2. Cycle conditions were the same for all genes tested: initial denaturation at: 95°C for 5 min, followed by 55 PCR cycles, each consisting of 95°C for 10 sec, 60°C for 15 sec and 72°C for 3 sec. The Universal ProbeLibrary Probes fluorescence emission was monitored after each cycle. The specificity of the amplicons was verified by sequencing analysis. To this end the PCR products were cleaned by GenElute TM PCR Clean-up Kit (Sigma-Aldrich Corp., St. Louis, MO), dehydrated, re-amplified using the original primers, and sequenced by the BigDye Terminator cycle sequencing reaction and an ABI PRISM 3100 Genetic Analyzer at the CRIBI (Centro Ricerca Interdipartimentale Biotecnologie Innovative) of our University. Expression of UII, UT-R, and PBGD mRNA was quantified with the second derivative maximum method of the Light Cycler Software (Roche) by determining the crossing points of individual samples with an algorithm that identifies the first turning point of the fluorescence curve. UII and UT-R expression in APA and PHEO was calculated relative to PBGD, used as an internal control, and relative to the control pools of normal adrenocortical and medullary tissue, used as calibrators, for APA and PHEO, respectively. Quantification of gene expression was carried out by comparative Ct ($2^{-\Delta\Delta Ct}$) method. This mathematical model normalizes the gene target to a housekeeping gene (PBGD) with ΔCt calculation, and then compares each ΔCt value to the ΔCt of tissue type designated the calibrator with a ΔΔCt calculation.([93])

The application of this model requires the previous run of calibration curves for each gene, to check the efficiency of the polymerase reaction. The efficiency is calculated as $10^{-1/slope}$ of the calibration curve and should be equal to 1. This analysis was performed starting from 1ug of control pool RNA and diluting the samples 1:5 five times. A replicate was included for each dilution.

Measurement of UII and UT-R protein expression

The expression of UII and UT-R at the protein level was examined by Western blot analysis with a goat polyclonal antibody. APA, PHEO, NAC and NAM tissues were homogenized separately in 500 μl lysis buffer (20mM HEPES, 2 mM EGTA, 10 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 μM PMSF, 1 μM leupeptin, 5 μM aprotinin, 1 mM DTT) at 4°C with MagNA Lyser Instrument (Roche, Monza, Italy). The lysate was then sonicated with a Dounce sonicator, 3 times for 20 s at 4°C and then centrifuged at 10000 rpm for 10 min at 4°C. The protein concentration was determined with Lowry's method, using bovine serum albumin as standard.([94]) Lysate fraction (60 μg) was solubilized in Laemmli buffer and separated by electrophoresis through a polyacrylamide gel (15% for UII and 12% for UT-R). The proteins separated on the gel were electroblotted onto nitrocellulose membrane (Hybond ECL-Amersham Biosciences Europe, Freeburg, Germany) in blotting buffer containing Tris 48 mmol/l, glycine 192 mmol/l, SDS 0.1%, methanol 20% (v/v) per 3 h at 100 V in the cold. The membranes were blocked overnight at 4°C in T-PBS containing PBS, 0.05% (v/v) Tween, and 5% bovine serum albumin (BSA). They were thereafter incubated overnight at 4°C with a primary antibody against UII (1:500 dilution), or UT-R (1:500), both from Santa Cruz Biotechnology™ (Santa

Cruz, CA, USA). The membranes were washed (3× for 15 min) with the same buffer and then incubated with the horseradish peroxidase conjugated secondary anti-goat antibody (1:5000 Amersham Biosciences Europe). Detection was made with the Enhanced Chemiluminescence System (ECL) from Pierce (CELBIO, Milan, Italy). Blots were analyzed by the Quantity One Program of VersaDOC 1000 (Bio-Rad, Milan, Italy).

Immunohistochemistry

Immunohistochemistry was used to confirm the UII expression at the protein level and to visualize its distribution in the PHEO tissue. A Rabbit Polyclonal IgG for UII was used (Santa Cruz Biotechnology™). The reaction was detected with Sigma Fast 3',3'- diaminobenzidine, 0.7 mg-tablets (DAB Tablets Set, Sigma). Serial 7-μm slices of mounted PHEO were dehydrated three times in xylene and rehydrated in serial dilution of ethanol. Sections were incubated with hydrogen peroxidase (0.5%) for 30 min and with blocking serum solution (BSA 0.2% triton 0,2% NGS 1:50 in PBS) for 20 min. After incubation with primary antibody to UII Rabbit Polyclonal IgG (1:50) at 4°C overnight, the sections were incubated at room temperature for 1 h with a secondary Biotin SP-conjugated IgG Mouse anti Rabbit (Jackson ImmunoResarch Laboratories, Inc, West Grove, PA, USA) (1:500) and then with Peroxidase-conjugated Streptavidin (Jackson ImmunoResarch Laboratories™) at room temperature for 30 min (1:250). The reaction was developed for 5 min with DAB Tablets and stopped with water. Negative controls were performed by omission of the primary antibody and pre-incubation of the primary antibody with UII.

UT-R activation in rats

To investigate if UII affects the adrenocortical expression of the aldosterone synthase (CYP11B2) and 11β hydroxylase (CYP11B1) by activation the UT-R in the adrenal cortex we infused by osmotic mini-pumps (model 2ML2, Alzet, Palo Alto, CA) normotensive male Sprague-Dawley rats (of about 250 g b.w., n = 11) with UII (600 pmol/Kg/h), either alone or on top of the UT-R antagonist palosuran (300 mg/Kg)([69]) (Fig. 4). The effects of the known aldosterone secretagogue angiotensin II (AngII) (700 pmol/Kg/h) infusion were investigated in parallel.([95]) After 1 week infusion the rats were sacrificed, the adrenal gland was snap frozen in isopentane pre-cooled on dry ice and then stored under liquid nitrogen until used for the immunohistochemistry (IHC) and the gene expression experiments.

For IHC we used a specific antibody against the rat aldosterone synthase (CYP11B2) or 11β hydroxylase (CYP11B1).([96])

Tissue sections were processed as described for human PHEO. Binding was detected with primary antibody diluted 1:50 in BSS after an overnight incubation at 4°C. After washing sections were incubated with secondary antibody (Biotin-SP-Conjugated AffiniPure Goat Anti Mouse IgG+IgM (H+L), Jackson Immuno Research) diluted 1:500 in BSS for 1 h at room temperature. They were rinsed again and subsequently incubated for 30 min at room temperature with the Horseradish Peroxidase conjugated streptavidin (Peroxidase conjugated Streptavidin, Jackson Immuno Research) diluted 1:250 in BSS.

The reaction was developed for 5 min with DAB Tablets and stopped with water. Negative controls were performed by omission of the primary

antibody. Sections were mounted and examined at 5x magnification, using Leica DQ optical microscopy. The immunoreactive area was then calculated as percent of the total adrenal cortex.

Statistical analysis

Natural logarithm (ln) transformation of the expression of UII and UT-R genes was undertaken to achieve a normal distribution. Comparison of transcript levels between the normal adrenal tissue, the APA, and the pheochromocytoma tissue was carried out with ANOVA followed by Bonferroni post-hoc test. The statistical analysis was carried out with the SPSS for Windows software (Vers. 15.0, SPSS Italy Inc., Bologna, Italy).

RESULTS

Clinical features of the patients

The main anthropometric and clinical features of patients with APA and PHEO are shown in Table 3. The APA patients showed overtly elevated plasma aldosterone levels and aldosterone renin ratio (ARR), and low values of serum K^+ and plasma renin activity (PRA).([3]) The PHEO patients showed markedly increased urinary metanephrine and normetanephrine excretion. In all patients cure of the biochemical picture of PA or of the PHEO and a marked fall of blood pressure were seen post-adrenalectomy.

RNA preparation

The choice of the RNA extraction method was crucial to the microarrays application and to RT-PCR. Several tests were performed in order to get optimal results in the downstream steps of reverse transcription, amplification and labelling of RNA in Cy3-Cy5 cRNA. We found that residual traces of phenol or alcohol derivates and protein contaminants can dramatically drop down the specific activity of labelled cRNA, leading to poor quality arrays. This is probably caused by the inhibition of the polymerase enzymes involved in amplification and labelling reactions. Thus, we selected for the microarrays applications only samples with a 260/280 and 260/230 ratios higher than 1.8, limits chosen from the literature ([97]) and experimentally tested by our laboratory. In Fig. 5 the electropherograms resulting from the Agilent Bioanalyzer 2100 analysis of two samples (APA and PHEO) are reported, where the two ribosomal peaks are clearly distinguishable. The values of the spectrophotometric

readings ratios (260/280 and 260/230 ratio) and the concentrations of two samples are enlisted.

Gene expression profiling by microarrays

We could analyze by microarray the gene expression profiles of 12 APA vs a control RNA pooled from normal adrenocortical tissues and of 3 PHEO, as compared to a pool of medullary tissue. We found marked differences in the number of differentially expressed sequences between APAs and PHEOs. In APA 30 sequences were over-expressed and 435 under-expressed; in PHEOs 3,213 genes were over-expressed and 1,064 under-expressed, as compared to the respective controls pools.

To generate visual relationship across samples and genes, cluster analysis was performed by applying an agglomerative clustering algorithm. In the resulting dendrogram for APA profiles, UII expression resulted to be under-expressed (-2.1-fold; $p<0.0001$) compared to control pool, (Fig. 6 and 7) while the UT-R gene expression showed only a trend toward lower levels (-1.1-fold; $p=0.28$) (Fig. 8 and 9) By contrast, the PHEO dendrogram showed an over-expression of the UII gene, with a 4.5-fold increase ($p=0.0001$) as compared to control pool. (Fig. 10) The gene expression of UT-R was consistently lower than in controls (-3-fold, $p=0.04$). (Fig. 11)

Consistent over-expression of the putative mediators of UII signaling, including G-protein, calcium, mitogen-activated protein and inositol triphosphate signaling were found in PHEO.

Quantitative Real Time RT-PCR

By a novel real-time RT-PCR methodology based on Universal ProbeLibrary Technology we could detect UII and UT-R gene specific mRNAs in all human adrenal tissues, including specimens of APA and PHEO. The specificity of the amplicons was confirmed by sequencing (Fig. 12). We quantified the transcript levels of genes using the ΔΔCt method, where the gene expression is reported as percentage of change from control pool.

Standard curves were generated both for UII, UT-R and PBGD mRNA by serial dilutions of RT products by spanning five orders of magnitude, yielding a PCR efficiency close to one for each reaction. Each dilution was run in replicate. In Fig. 13 is reported the curve of investigated genes.

Results of quantification relative to the housekeeping gene PBGD and to the internal calibrator showed that UII expression was significantly higher in PHEO than in APA ($p=0.035$), while UT-R expression was lower in PHEO than in APA ($p=0.001$). The normal adrenal cortex and normal adreno-medullary tissues showed intermediate values (Fig. 14).

Western blotting and Immunohistochemistry

Western blot analysis demonstrated the UII peptide and UT-R protein in the normal adrenal cortex, and the APA and PHEO tissue (Fig. 15 panel A). Noteworthy, the UT-R, under the denaturing conditions, showed one band of the expected molecular weight (MW= 43 kDa) in myocardium, while, in all adrenal specimen, an additional band of 60 kDa was detected.

Immunohistochemistry confirmed the UII expression in PHEO (Fig. 15 panel B-D): prominent staining of groups of cells arranged in nodules and scattered throughout the tissue and sometimes located perivascularly was evident. The specificity of the UII detection was confirmed by, the lack of staining upon exposure of the tissue section to the secondary antibody and the detection reagents after omission of the primary antibody (Fig. 15 panel E) or after pre-absorption with UII.

Effect of chronic activation of the UT-R on the rat adrenal cortex

A one week infusion of UII in normotensive male Sprague-Dawley rats induced a marked increase of the CYP11B2 expression in the adrenocortical zona glomerulosa by IHC (Fig. 16 panels A-C). This increase was more prominent than that induced by a high dose of Ang II (Panel B) and was abolished by pretreatment with palosuran (Panel D).

Quantitative analysis confirmed that the effect of UII on the CYP11B2 occurring via UT-R was more pronounced than that on CYP11B1 (Fig. 17 top and mid panels). Both UII and Ang II induced an increase also of CYP11B1 expression, but this was unaffected by palosuran (Fig. 16 panels E-H). After correction for the effect on CYP11B1 the specific stimulation of the CYP11B2 by UII was even more evident (Fig 17 bottom panel).

DISCUSSION

The ubiquitous expression of UII and its receptor UT-R in vascular tissue,[98] along with the intimate relationship between the vasculature on one hand, and the adrenal cortex and medulla on the other, suggested the hypothesis that blood vessel wall-derived UII may modulate the release of adrenocortical hormones and catecholamines and thereby regulate blood pressure. In this study we used a whole transcriptome approach to investigate tumors that both cause arterial hypertension and cardiovascular damage. Among these tumors the APA are histogenetically derived from adrenocortical zona glomerulosa and the PHEO are held to originate from adrenal medullary chromaffin tissue.

With the oligomicroarray approach and using the normal human adrenal cortex and medulla as referent we found prominent differences in the gene expression of UII and related pathways between APA, and PHEO, thus supporting previous reports.[82;84] Takahashi et al first detected the transcripts of UII and UT-R in seven PHEO and four APA by using RT-PCR but they could not quantify their relative abundance. Of interest, they found increased UII-like immunoreactivity in one out of six PHEO. [82] In contrast with Takahashi's findings, Zeng et al described a lower level of UII and UT-R expression in PHEO tissue, as compared to the normal adrenal tissue by using semi-quantitative RT-PCR.[84] However, they did not substantiate this finding by peptide measurement, and used not normal adrenomedullary chromaffin tissue as referent. Thus, whether UII is differentially expressed in PHEO and APA, as compared to the corresponding normal adrenal tissue, remained contentious. More importantly, the functional role of UII in the adrenal gland was unknown.

Taking advantage of the availability of molecular techniques and novel pharmacological tool our present results fill this gap of knowledge.

With quantitative real time RT-PCR we could confirm the marked differences of UII mRNA across adrenal tumors found with oligomicroarray studies: the PHEO exhibited higher UII mRNA content than the APA (Fig. 14), which showed the lowest level among all adrenal tissues that we examined. Noteworthy, statistical significance for comparison was achieved notwithstanding a relatively large spread of the data, particularly for PHEO.

An opposite expression profile of UT-R transcripts was found across adrenal tumors: APA showed significantly higher mRNA levels than PHEO and the normal adrenal medulla tissue. These results suggest down-regulation of UT-R when UII expression is enhanced, and viceversa.

The finding that UII is over-expressed in PHEO lends support to the hypothesis that a quantitative derangement in the expression of this peptide plays a major role in the pathophysiology of PHEO and particularly in a subset of them that have been described to be associated with a biochemical picture of primary aldosteronism. The finding that UII and UT-R transcripts are translated into proteins in these tissues, as demonstrated by Western blotting and immunohistochemistry (Fig. 15), accords well with this contention. Western blot analysis demonstrated not only the UII peptide in the normal adrenal cortex, and in the APA and PHEO tissue but also the UT-R protein. Under denaturing conditions, Western blot analysis showed an additional band of 60 kDa in all adrenal specimen besides the expected 43 kDa band that was found in the human myocardium tested as a positive control tissue (Fig. 15 panel A). The additional band could be generated by

post-transcriptional processes, e.g. glycosilation. This contention may be supported by evidence of several glycosilation sites in UT-R protein and a glycosilation site has been demonstrated to have a molecular weight of 60 kDa. ([99])

Detection and localization of UII in pheochromocytoma tissue and related pathway.

Immunohistochemistry conclusively proved that UII gene is functionally expressed in the PHEO tissue. Moreover immunohistochemistry furnished information on UII localization, showing specific staining of clusters of pheochromocytoma cells, sometimes arranged in nests and occasionally located in perivascular regions (Fig. 15 panel B-C).

The detection by the whole transcriptome analysis of overexpression of putative mediators of UII signaling, ([54;65]) such as genes involved in G-protein, calcium- and mitogen-activated proteins, fits well with the hypothesis of a higher UII-mediated signal transduction in PHEO. Moreover, we found an overexpression of some members of Rho pathway in PHEO, an established UII signaling modulator and an overexpression of the Rhotekin gene, which encodes a scaffolding protein that interacts with GTP-bound Rho proteins in both APA and PHEO. The expression of the latter gene was reported to be dysregulated in some cancers, thus suggesting a functional role of this protein in carcinogenesis. Therefore, these findings implicate novel molecular mediators in the patho-physiology of adrenal tumors that need to be further studied.

Effect of chronic activation of the UT-R on the rat adrenal cortex

To investigate the potential functional role of UII in the adrenal gland we used a chronic infusion of UII in the presence or in the absence of an UT-R antagonist in normotensive male Sprague-Dawley rats. This allowed us to detect that chronic UT-R over-activation induces over-expression of both aldosterone synthase (CYP11B2) and 11β hydroxylase (CYP11B1). This experiment was made possible by the two recent accomplishments: 1) the development of specific antibodies for these enzymes,([96]) 2) the discovery of the UT-R antagonist palosuran.([69]) We could therefore evidence for the first time that UII specifically induced a marked increase of the CYP11B2 expression in the rat adrenocortical zona glomerulosa (Fig. 16). Noteworthy, this effect was specific, as it was abolished by concomitant UT-R induces, and appeared to be more potent than that of Ang II, the most widely known aldosterone secretagogue. Hence, these functional data are also consistent with the hypothesis that UII synthesized in the adrenal medulla and to a larger extent in PHEO, may exert a secretagogue effect on aldosterone by turning on the expression of the aldosterone synthase gene.

CONCLUSIONS

In summary, our results evidenced the gene expression of UII in the normal human adrenal cortex, in APA tissue and, at a higher level, in the adrenomedullary-derived PHEO.

The opposite trend of expression of UII receptor between APA and PHEO, along with the differences of genes implicated in UII signaling, supports a role of UII in the patho-physiology of PHEO.

The existence of profound paracrine interactions between the adrenal medulla and the cortex, ([41;86]) which are held to be relevant for the regulation of adrenal gland function, is in line with this contention.

Moreover, by showing that UII infusion in *vivo* determined a marked increase of the CYP11B2 expression in the rat adrenocortical zona glomerulosa, our results can provide pathophysiologic explanation for the rare concomitant occurrence of PHEO and primary aldosteronism.([100-105])

Table 1. Urotensin II isoform amino acid sequence in different species

Species	Amino acid sequence
HUMAN	E T P D **C F W K Y C** V
MOUSE	Q H G A A P E **C F W K Y C** I
RAT	Q H G T A P E **C F W K Y C** I
PIG (A)	G P T S E **C F W K Y C** V
PIG (B)	G P P S E **C F W K Y C** V
FROG	A G N L S E **C F W K Y C** V
GOBY	A G T A D **C F W K Y C** V
LAMPREY	N N F S D **C F W K Y C** V

Table 2. RT-PCR primers and PCR products

Primers	Sequence	Accession number	Amplicon
UII		NM_006786	105 bp
Sense	5′-CTCCTGCTGTTTGCTTTTCA-3′		
Antisense	5′-GCGTCTTCATGAGGTGCTG-3′		
Probe	5′-TCCTCTCC-3′		
UT-R		NM_018949	87 bp
Sense	5′-GCAACCCTCAACAGCTCCT-3′		
Antisense	5′-CAGCAGAGTCCCAATGGTG-3′		
Probe	5′-CAGCTCCC-3′		
PBGD		NM_000190	92 bp
Sense	5′- TGCCCTGGAGAAGAATGAAG-3′		
Antisense	5′- AGATGGCTCCGATGGTGA-3′		
Probe	5′- CTTCCTCC-3′		

Table 3. Anthropometric and biochemical features of the patients with APA and PHEO. Means ± SEM or median (and range) as appropriate. APA (n=22), PHEO (n=10); ranges in parentheses. Normal values are shown in square brackets.

	APA	**PHEO**
Age (year)	50 ±11	41±9.6
Gender (♂:♀)	7:15	6:4
Systolic blood pressure (mmHg)	170±16	149±7
Diastolic blood pressure (mmHg)	102±11	103±7
Serum K^+ concentration(mmol/L) [3.6-4.5]	3.3±0.6	3.8±0.2
Plasma renin activity (ng Ang-I/mL/hr) [0.65-2.65]	0.24 (0.13-0.37)	NA
Aldosterone plasma concentration (pg/mL) [<110]	501 (272-731)	NA
Aldosterone Renin Ratio (ng/dl)/(ng/ml/hr)	231±87	NA
Cortisol plasma concentration (ng/mL) [50-250]	162 (133-190)	438
Urinary metanephrine (μmol/24hr) [0.4-1.5]	NA	1.9±0.60
Urinary normetanephrine (μmol/24hr) [0.57-1.9]	NA	6.45±4.55

Reference

1. Rainey WE. Adrenal zonation: clues from 11beta-hydroxylase and aldosterone synthase. *Mol Cell Endocrinol*. 1999;151:151-160.

2. CONN JW. Presidential address. I. Painting background. II. Primary aldosteronism, a new clinical syndrome. *J Lab Clin Med*. 1955;45:3-17.

3. Rossi GP, Bernini G, Caliumi C et al. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J Am Coll Cardiol*. 2006;48:2293-2300.

4. Rossi GP. New concepts in adrenal vein sampling for aldosterone in the diagnosis of primary aldosteronism. *Curr Hypertens Rep*. 2007;9:90-97.

5. Omura M, Saito J, Yamaguchi K et al. Prospective study on the prevalence of secondary hypertension among hypertensive patients visiting a general outpatient clinic in Japan. *Hypertens Res*. 2004;27:193-202.

6. Rossi GP, Bernini G, Desideri G et al. Renal damage in primary aldosteronism: results of the PAPY Study. *Hypertension*. 2006;48:232-238.

7. Rossi GP, Di B, V, Ganzaroli C et al. Excess aldosterone is associated with alterations of myocardial texture in primary aldosteronism. *Hypertension*. 2002;40:23-27.

8. Rocha R, Funder JW. The pathophysiology of aldosterone in the cardiovascular system. *Ann N Y Acad Sci*. 2002;970:89-100.

9. Rossi GP, Pessina AC, Heagerty AM. Primary aldosteronism: an update on screening, diagnosis and treatment. *Journal of Hypertension*. 2008;26.

10. Schwartz GL, Turner ST. Screening for primary aldosteronism in essential hypertension: diagnostic accuracy of the ratio of plasma aldosterone concentration to plasma renin activity. *Clin Chem*. 2005;51:386-394.

11. Whalen RK, Althausen AF, Daniels GH. Extra-adrenal pheochromocytoma. *J Urol*. 1992;147:1-10.

12. Pacak K, Linehan WM, Eisenhofer G et al. Recent advances in genetics, diagnosis, localization, and treatment of pheochromocytoma. *Ann Intern Med*. 2001;134:315-329.

13. Coupland R. The natural history of the chromaffin cell. *Essex, UK: Longsman Green and Co*. 1965.

14. Erickson D, Kudva YC, Ebersold MJ et al. Benign paragangliomas: clinical presentation and treatment outcomes in 236 patients. *J Clin Endocrinol Metab*. 2001;86:5210-5216.

15. Anderson GH, Jr., Blakeman N, Streeten DH. The effect of age on prevalence of secondary forms of hypertension in 4429 consecutively referred patients. *J Hypertens*. 1994;12:609-615.

16. Sinclair AM, Isles CG, Brown I et al. Secondary hypertension in a blood pressure clinic. *Arch Intern Med*. 1987;147:1289-1293.

17. Stenstrom G, Svardsudd K. Pheochromocytoma in Sweden 1958-1981. An analysis of the National Cancer Registry Data. *Acta Med Scand*. 1986;220:225-232.

18. Beard CM, Sheps SG, Kurland LT et al. Occurrence of pheochromocytoma in Rochester, Minnesota, 1950 through 1979. *Mayo Clin Proc*. 1983;58:802-804.

19. Hartley L, Perry-Keene D. Phaeochromocytoma in Queensland--1970-83. *Aust N Z J Surg*. 1985;55:471-475.

20. Andersen GS, Toftdahl DB, Lund JO et al. The incidence rate of phaeochromocytoma and Conn's syndrome in Denmark, 1977-1981. *J Hum Hypertens*. 1988;2:187-189.

21. Fernandez-Calvet L, Garcia-Mayor RV. Incidence of pheochromocytoma in South Galicia, Spain. *J Intern Med*. 1994;236:675-677.

22. Lo CY, Lam KY, Wat MS et al. Adrenal pheochromocytoma remains a frequently overlooked diagnosis. *Am J Surg*. 2000;179:212-215.

23. Neumann HP, Bausch B, McWhinney SR et al. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med*. 2002;346:1459-1466.

24. Plouin PF, Gimenez-Roqueplo AP. Initial work-up and long-term follow-up in patients with phaeochromocytomas and paragangliomas. *Best Pract Res Clin Endocrinol Metab*. 2006;20:421-434.

25. Amar L, Bertherat J, Baudin E et al. Genetic testing in pheochromocytoma or functional paraganglioma. *J Clin Oncol*. 2005;23:8812-8818.

26. Gimm O, Koch CA, Januszewicz A et al. The genetic basis of phechromocytoma. *Front Horm Res*. 2004;31:45-60.

27. Bravo EL, Tagle R. Pheochromocytoma: state-of-the-art and future prospects. *Endocr Rev*. 2003;24:539-553.

28. Mantero F, Terzolo M, Arnaldi G et al. A survey on adrenal incidentaloma in Italy. Study Group on Adrenal Tumors of the Italian Society of Endocrinology. *J Clin Endocrinol Metab*. 2000;85:637-644.

29. Montemurro D, Rossi GP. Veralipride-induced acute coronary syndrome unmasking a non-secreting pheochromocytoma. *J Endocrinol Invest*. 2006;29:650-652.

30. Bernini GP, Moretti A, Ferdeghini M et al. A new human chromogranin 'A' immunoradiometric assay for the diagnosis of neuroendocrine tumours. *Br J Cancer*. 2001;84:636-642.

31. Boomsma F, Bhaggoe UM, Man in 't Veld AJ et al. Sensitivity and specificity of a new ELISA method for determination of chromogranin A in the diagnosis of pheochromocytoma and neuroblastoma. *Clin Chim Acta*. 1995;239:57-63.

32. Canale MP, Bravo EL. Diagnostic specificity of serum chromogranin-A for pheochromocytoma in patients with renal dysfunction. *J Clin Endocrinol Metab*. 1994;78:1139-1144.

33. Rossi GP, Seccia TM, Pessina AC. Clinical use of laboratory tests for the identification of secondary forms of arterial hypertension. *Crit Rev Clin Lab Sci*. 2007;44:1-85.

34. Kleitman N, Holzwarth MA. Catecholaminergic innervation of the rat adrenal cortex. *Cell Tissue Res*. 1985;241:139-147.

35. Palacios G, Lafarga M. Chromaffin cells in the glomerular zone of adult rat adrenal cortex. *Cell Tissue Res*. 1975;164:275-278.

36. Vinson GP, Hinson JP, Toth IE. The neuroendocrinology of the adrenal cortex. *J Neuroendocrinol*. 1994;6:235-246.

37. Vizi ES, Toth IE, Szalay KS et al. Catecholamines released from local adrenergic axon terminals are possibly involved in fine tuning of steroid secretion from zona glomerulosa cells: functional and morphological evidence. *J Endocrinol*. 1992;135:551-561.

38. Toth IE, Hinson JP. Neuropeptides in the adrenal gland: distribution, localization of receptors, and effects on steroid hormone synthesis. *Endocr Res*. 1995;21:39-51.

39. Vinson GP, Hinson JP. Blood flow and hormone secretion in the adrenal gland. Ravenn Press, New York , 71-86. 1992. Vinson GP, 71-86,1992. 1992.

Ref Type: Magazine Article

40. Mulrow PJ. Adrenal renin: regulation and function. *Front Neuroendocrinol*. 1992;13:47-60.

41. Nussdorfer GG, Rossi GP, Belloni AS. The role of endothelins in the paracrine control of the secretion and growth of the adrenal cortex. *Int Rev Cytol*. 1997;171:267-308.

42. Neri G, Andreis PG, Prayer-Galetti T et al. Pituitary adenylate-cyclase activating peptide enhances aldosterone secretion of human adrenal gland: evidence for an indirect mechanism, probably involving the local release of catecholamines. *J Clin Endocrinol Metab*. 1996;81:169-173.

43. Kawamura M, Nakamichi N, Imagawa N et al. Effect of adrenaline on steroidogenesis in primary cultured bovine adrenocortical cells. *Jpn J Pharmacol*. 1984;36:35-41.

44. Ehrhart-Bornstein M, Bornstein S.R., Trzeciak W.H. et al. Adrenaline stimulates cholesterol side-chain cleavage cytochrome P450 mRNA accumulation in bovine adrenocortical cells. *J Endocrinol*. 1991;131:R5-R8(Ehrhart-bornstein m, 1991b, 131-r5-r8).

45. Gallo-Payet N, Chouinard L, Balestre MN et al. Dual effects of dopamine in rat adrenal glomerulosa cells. *Biochem Biophys Res Commun*. 1990;172:1100-1108.

46. Matsuoka H, Ishii M, Goto A et al. Role of serotonin type 2 receptors in regulation of aldosterone production. *Am J Physiol*. 1985;249:E234-E238.

47. Holzwarth MA, Brownfield MS. Serotonin coexists with epinephrine in rat adrenal medullary cells. *Neuroendocrinology*. 1985;41:230-236.

48. Bern HA, Pearson D, Larson BA et al. Neurohormones from fish tails: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory system of fishes. *Recent Prog Horm Res*. 1985;41:533-552.

49. Pearson D, Shively JE, Clark BR et al. Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc Natl Acad Sci U S A*. 1980;77:5021-5024.

50. Coulouarn Y, Lihrmann I, Jegou S et al. Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc Natl Acad Sci U S A*. 1998;95:15803-15808.

51. Douglas SA, Ohlstein EH. Human urotensin-II, the most potent mammalian vasoconstrictor identified to date, as a therapeutic target for the management of cardiovascular disease. *Trends Cardiovasc Med*. 2000;10(6):229-237.

52. Russell FD, Kearns P, Toth I et al. Urotensin-II-converting enzyme activity of furin and trypsin in human cells in vitro. *J Pharmacol Exp Ther*. 2004;310:209-214.

53. Ames RS, Sarau HM, Chambers JK et al. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature*. 1999;401:282-286.

54. Sauzeau V, Le Mellionnec E, Bertoglio J et al. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res*. 2001;88:1102-1104.

55. Maguire JJ, Kuc RE, Davenport AP. Orphan-receptor ligand human urotensin II: receptor localization in human tissues and comparison

of vasoconstrictor responses with endothelin-1. *Br J Pharmacol*. 2000;131:441-446.

56. Stirrat A, Gallagher M, Douglas SA et al. Potent vasodilator responses to human urotensin-II in human pulmonary and abdominal resistance arteries. *Am J Physiol Heart Circ Physiol*. 2001;280:H925-H928.

57. Douglas SA, Tayara L, Ohlstein EH et al. Congestive heart failure and expression of myocardial urotensin II. *Lancet*. 2002;359:1990-1997.

58. Gibson A. Complex effects of Gillichthys urotensin II on rat aortic strips. *Br J Pharmacol*. 1987;91:205-212.

59. Bottrill FE, Douglas SA, Hiley CR et al. Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br J Pharmacol*. 2000;130:1865-1870.

60. Maguire JJ, Davenport AP. Is urotensin-II the new endothelin? *Br J Pharmacol*. 2002;137:579-588.

61. Ishihata A, Ogaki T, Aita T et al. Role of prostaglandins in urotensin II-induced vasodilatation in the coronary arteries of aged rats. *Eur J Pharmacol*. 2005;523:119-126.

62. Douglas SA, Sulpizio AC, Piercy V et al. Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br J Pharmacol*. 2000;131:1262-1274.

63. Behm DJ, Harrison SM, Ao Z et al. Deletion of the UT receptor gene results in the selective loss of urotensin-II contractile activity in aortae isolated from UT receptor knockout mice. *Br J Pharmacol*. 2003;139:464-472.

64. Giebing G, Tolle M, Jurgensen J et al. Arrestin-independent internalization and recycling of the urotensin receptor contribute to long-lasting urotensin II-mediated vasoconstriction. *Circ Res*. 2005;97:707-715.

65. Saetrum OO, Nothacker H, Ehlert FJ et al. Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. *Eur J Pharmacol*. 2000;406:265-271.

66. Tasaki K, Hori M, Ozaki H et al. Mechanism of human urotensin II-induced contraction in rat aorta. *J Pharmacol Sci*. 2004;94:376-383.

67. Rossowski WJ, Cheng BL, Taylor JE et al. Human urotensin II-induced aorta ring contractions are mediated by protein kinase C, tyrosine kinases and Rho-kinase: inhibition by somatostatin receptor antagonists. *Eur J Pharmacol*. 2002;438:159-170.

68. Spinazzi R, Albertin G, Nico B et al. Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial cells, and urotensin-II via UT-R stimulates angiogenesis in vivo and in vitro. *Int J Mol Med*. 2006;18:1107-1112.

69. Clozel M, Binkert C, Birker-Robaczewska M et al. Pharmacology of the urotensin-II receptor antagonist palosuran (ACT-058362; 1-[2-(4-benzyl-4-hydroxy-piperidin-1-yl)-ethyl]-3-(2-methyl-quinolin-4-yl) - urea sulfate salt): first demonstration of a pathophysiological role of the urotensin System. *J Pharmacol Exp Ther*. 2004;311:204-212.

70. Totsune K, Takahashi K, Arihara Z et al. Increased plasma urotensin II levels in patients with diabetes mellitus. *Clin Sci (Lond)*. 2003;104:1-5.

71. Totsune K, Takahashi K, Arihara Z et al. Role of urotensin II in patients on dialysis. *Lancet*. 2001;358:810-811.

72. Dschietzig T, Bartsch C, Pregla R et al. Plasma levels and cardiovascular gene expression of urotensin-II in human heart failure. *Regul Pept*. 2002;110:33-38.

73. Matsushita M, Shichiri M, Imai T et al. Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J Hypertens*. 2001;19:2185-2190.

74. Ng LL, Loke I, O'Brien RJ et al. Plasma urotensin in human systolic heart failure. *Circulation*. 2002;106:2877-2880.

75. Richards AM, Nicholls MG, Lainchbury JG et al. Plasma urotensin II in heart failure. *Lancet*. 2002;360:545-546.

76. Totsune K, Takahashi K, Arihara Z et al. Elevated plasma levels of immunoreactive urotensin II and its increased urinary excretion in patients with Type 2 diabetes mellitus: association with progress of diabetic nephropathy. *Peptides*. 2004;25:1809-1814.

77. Johns DG, Ao Z, Naselsky D et al. Urotensin-II-mediated cardiomyocyte hypertrophy: effect of receptor antagonism and role of inflammatory mediators. *Naunyn Schmiedebergs Arch Pharmacol*. 2004;370:238-250.

78. Birker-Robaczewska M, Boukhadra C, Studer R et al. The expression of urotensin II receptor (U2R) is up-regulated by interferon gamma. *J Recept Signal Transduct Res*. 2003;23:289-305.

79. Mallamaci F, Cutrupi S, Pizzini P et al. Urotensin II in end-stage renal disease: an inverse correlate of sympathetic function and cardiac natriuretic peptides. *J Nephrol*. 2005;18:727-732.

80. Watanabe T, Pakala R, Katagiri T et al. Synergistic effect of urotensin II with mildly oxidized LDL on DNA synthesis in vascular smooth muscle cells. *Circulation*. 2001;104:16-18.

81. Tamura K, Okazaki M, tamura m et al. Urotensin II-induced activation of extracellular signal-regulated kinase in cultured vascular amooth muscle cells: involvement of cell adhesion-mediated integrin signaling. *Life Sci*. 2003;72:1049-1060.

82. Takahashi K, Totsune K, Murakami O et al. Expression of urotensin II and its receptor in adrenal tumors and stimulation of proliferation of cultured tumor cells by urotensin II. *Peptides*. 2003;24:301-306.

83. Takahashi K, Totsune K, Murakami O et al. Expression of urotensin II and urotensin II receptor mRNAs in various human tumor cell lines and secretion of urotensin II-like immunoreactivity by SW-13 adrenocortical carcinoma cells. *Peptides*. 2001;22:1175-1179.

84. Zeng ZP, Liu GQ, Li HZ et al. The effects of urotensin-II on proliferation of pheochromocytoma cells and mRNA expression of urotensin-II and its receptor in pheochromocytoma tissues. *Ann N Y Acad Sci*. 2006;1073:284-289.

85. Albertin G, Casale V, Ziolkowska A et al. Urotensin-II and UII-receptor expression and function in the rat adrenal cortex. *Int J Mol Med*. 2006;17:1111-1115.

86. Nussdorfer GG. Paracrine control of adrenal cortical function by medullary chromaffin cells. *Pharmacol Rev*. 1996;48:495-530.

87. Lenzini L, Seccia TM, Aldighieri E et al. Heterogeneity of aldosterone-producing adenomas revealed by a whole transcriptome analysis. *Hypertension*. 2007;50:1106-1113.

88. Rossi G, Albertin G, Belloni A et al. Gene expression, localization, and characterization of endothelin A and B receptors in the human adrenal cortex. *J Clin Invest*. 1994;94:1226-1234.

89. Rossi GP, Sacchetto A, Chiesura-Corona M et al. Identification of the etiology of primary aldosteronism with adrenal vein sampling in patients with equivocal computed tomography and magnetic resonance findings: results in 104 consecutive cases. *J Clin Endocrinol Metab*. 2001;86:1083-1090.

90. 1999 World Health Organization. International Society of Hypertension Guidelines for the management of Hypertension. Guidelines Subcommittee. *J Hypertens*. 1999;17:151-183.

91. Ashburner M, Ball CA, Blake JA et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 2000;25:25-29.

92. Rossi GP, Sticchi D, Giuliani L et al. Adiponectin receptor expression in the human adrenal cortex and aldosterone-producing adenomas. *Int J Mol Med*. 2006;17:975-980.

93. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods*. 2001;25:402-408.

94. Lowry OH, Rosebrough TD, Farr AL et al. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:165-175.

95. Antonello M, Montemurro D, Bolognesi M et al. Prevention of hypertension, cardiovascular damage and endothelial dysfunction with green tea extracts. *Am J Hypertens*. 2007;20:1321-1328.

96. MacKenzie SM, Clark CJ, Fraser R et al. Expression of 11beta-hydroxylase and aldosterone synthase genes in the rat brain. *J Mol Endocrinol*. 2000;24:321-328.

97. Imbeaud S, Graudens E, Boulanger V et al. Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Res*. 2005;33:e56.

98. Watson AM, May CN. Urotensin II, a novel peptide in central and peripheral cardiovascular control. *Peptides*. 2004;25:1759-1766.

99. Boucard AA, Sauve SS. Photolabelling the rat urotensin II/GPR14 receptor identifies a ligand-binding site in the fourth transmembrane domain. *Biochem J*. 2003;370:829-838.

100. Gordon RD, Bachmann AW, Klemm SA et al. An association of primary aldosteronism and adrenaline-secreting phaeochromocytoma. *Clin Exp Pharmacol Physiol*. 1994;21:219-222.

101. Kitajima W, Saruta T, Kondo K et al. A case of secondary aldosteronism induced by pheochromocytoma. *J Urol*. 1975;114:141-143.

102. Mannhart M, Ludin H, Veyrat R et al. Secondary hyperaldosteronism caused by cervical phaeochromocytoma cured by ablation of the tumor. *Helv Med Acta*. 1971;35:479-483.

103. Tan GH, Carney JA, Grant CS et al. Coexistence of bilateral adrenal phaeochromocytoma and idiopathic hyperaldosteronism. *Clin Endocrinol (Oxf)*. 1996;44:603-609.

104. Wajiki M, Ogawa A, Fukui J et al. Coexistence of aldosteronoma and pheochromocytoma in an adrenal gland. *J Surg Oncol*. 1985;28:75-78.

105. Kamba S, Yukawa, Kato D et al. A case report of primary aldosteronism combined with pheochromocytoma, struma nodosa and aneurysm of the abdominal aorta. *Geka Chiryo*. 1978;38:491-497.

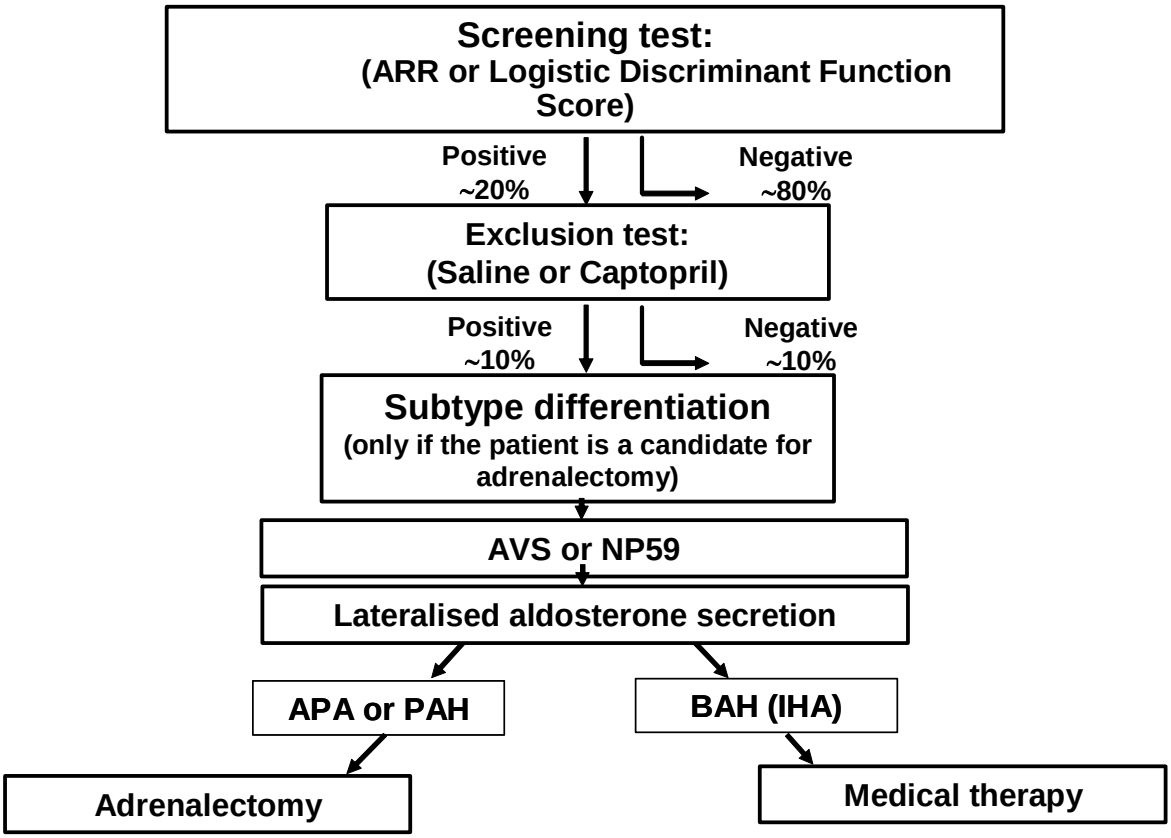
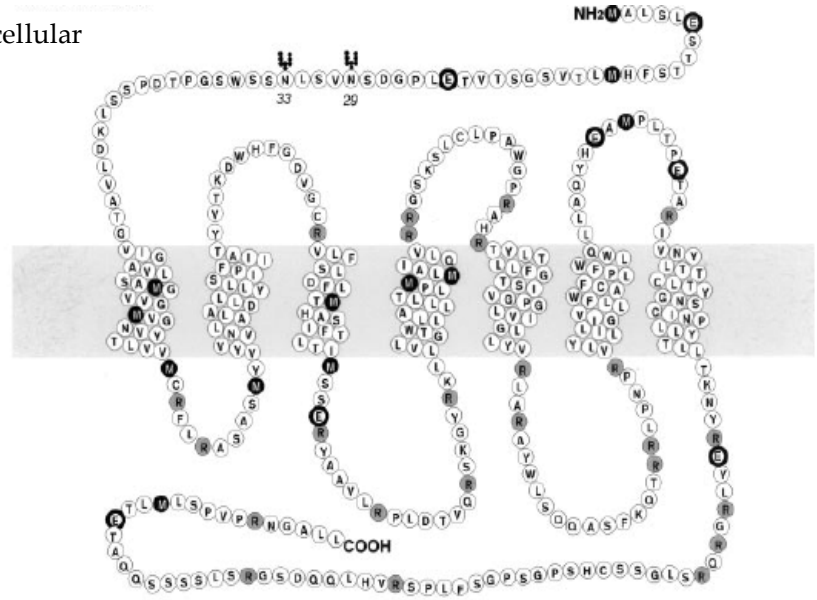


Fig. 1 Flow chart for the diagnostic work-up of Primary Aldosteronism.
Aldosterone/renin ratio (ARR), plasma aldosterone concentration (PAC)/plasma renin activity (PRA) ratio; AVS, adrenal vein sampling.

A hU-II Glu[1]-Thr[2]-Pro[3]-Asp[4]-Cys[5]-Phe[6]-Trp[7]-Lys[8]-Tyr[9]-Cys[10]-Val[11]

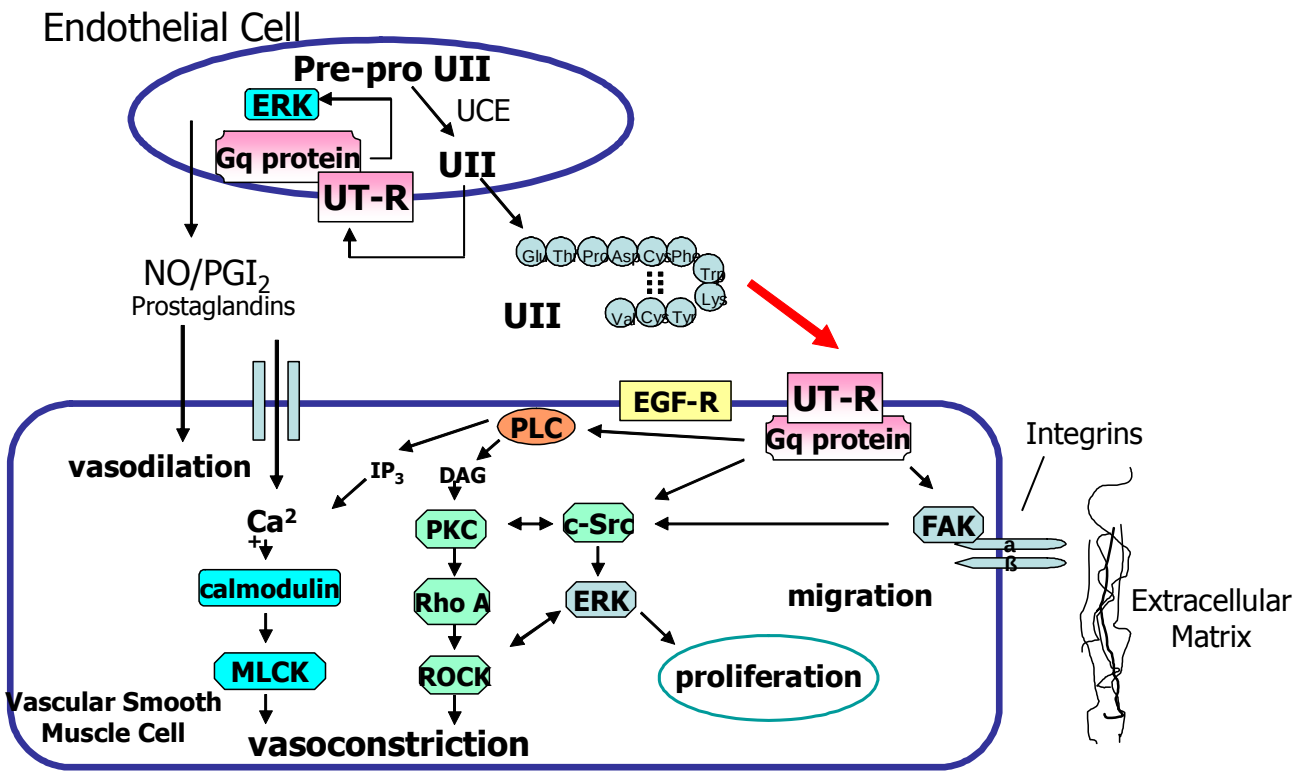
S S

B Extracellular



Modified from Boucard AA et al, Biochem J 2003

Fig. 2 (A) Amino acid sequence of human U-II. (B) Two-dimensional representation of the primary structure of the rat UT-R.



Mod. from Watanabe T. Hyp Res 2006

Fig. 3 Intracellular signal tranduction pathways mediated by Urotensin II (UII) and Urotensin II Receptor (UT-R) System.

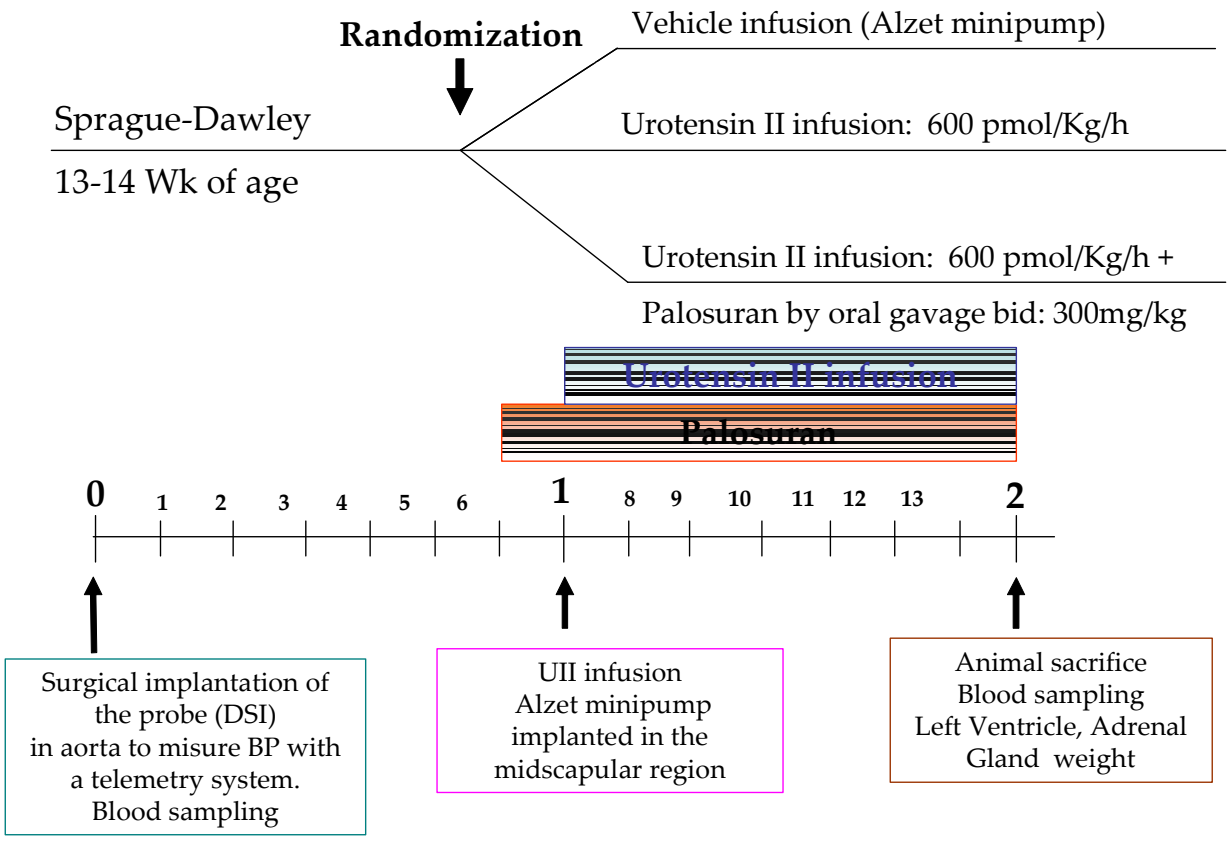
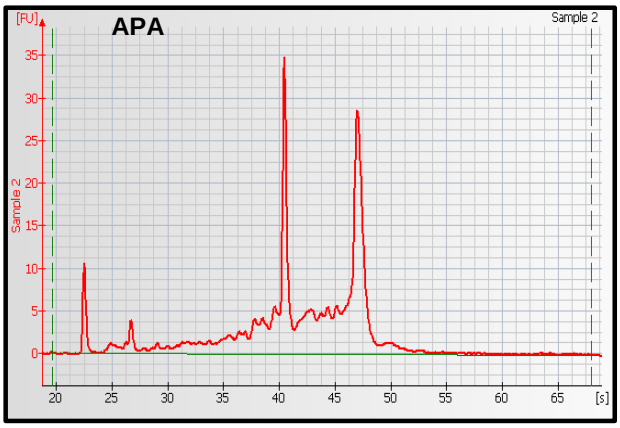
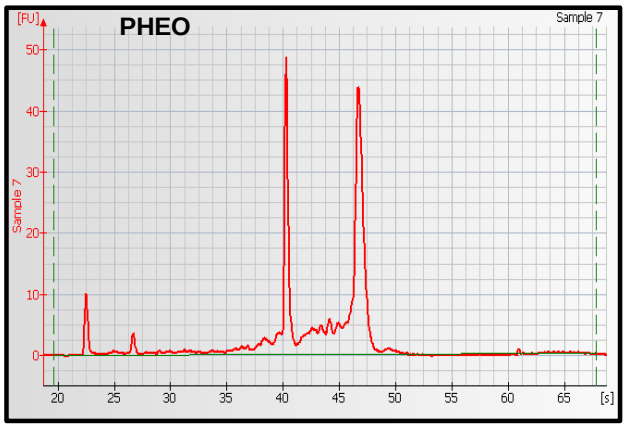


Fig. 4 Study Protocol. In vivo assessement of UT-R mediated effects on CYP11B1 and CYP11B2 expressions

a)





b)

260/280 ratio	260/230 ratio	CONC. (ng/µl)
1,8	2,7	643

260/280 ratio	260/230 ratio	CONC. (ng/ µ l)
1,8	2,4	624

Fig. 5 a) Electropherograms resulting from the Agilent Bioanalyzer 2100 analysis of APA and PHEO RNA. b) Spectrophotometric readings ratios (260/280 and 260/230 ratio) and concentrations of the samples (ng/ µ l).

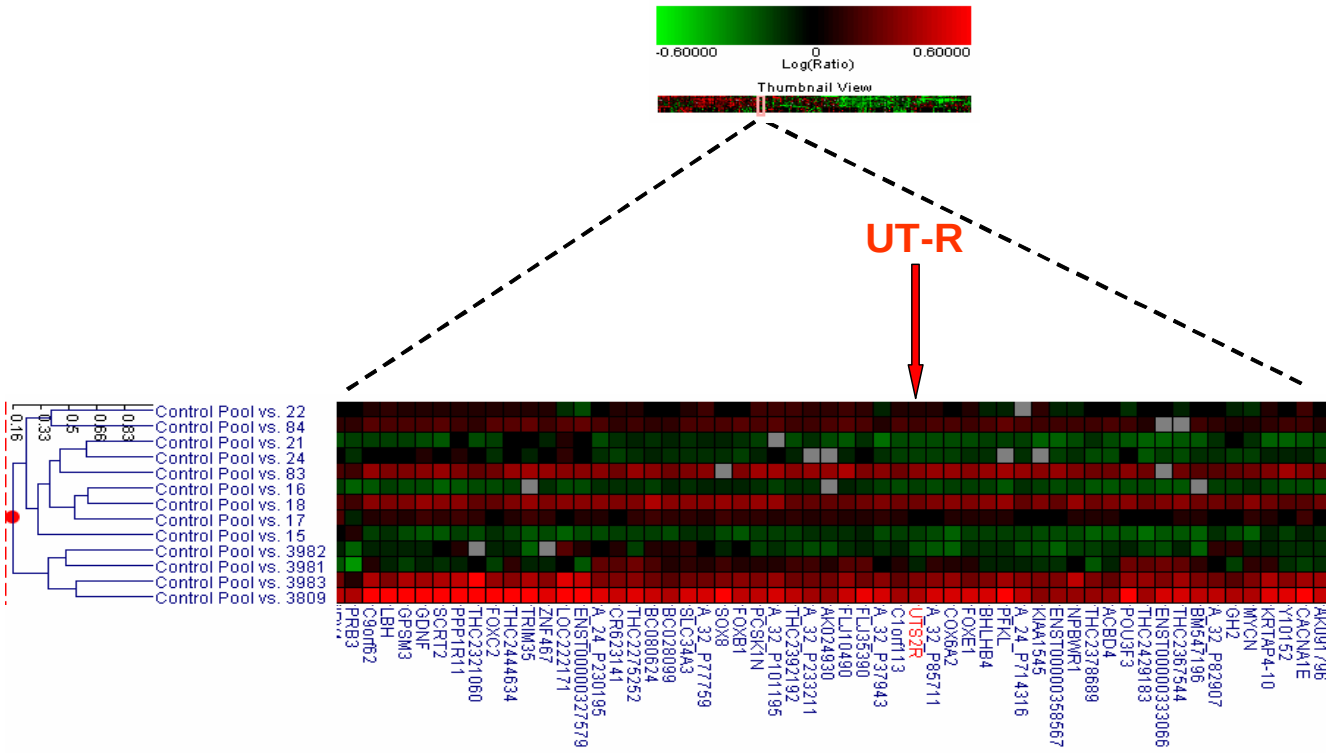


Fig. 8 Cluster analysis of differentially expressed genes in APAs. The results of a portion of 2D cluster are displayed in a heat-map, in which each row represents one sample of APA and each column one sequence in the chip. Each cell in the heat-map corresponds to a log (ratio) and is represented by one color: the genes over-expressed are in red and those under-expressed in green. The default saturation threshold is -2 to +2.

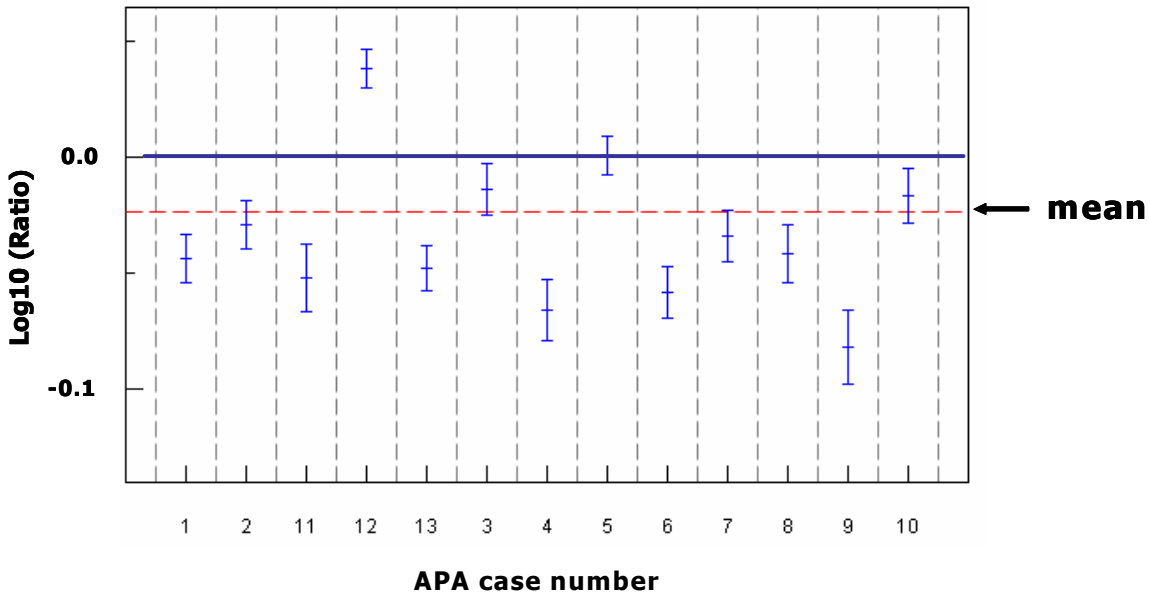


Fig. 7 UII variance plot in APAs. The expression level of the gene is reported as log ratio of the intensity of the green channel (APA RNA) to the red channel (Control pool). An Anova and Benjamini-Hochberg post-hoc test were performed ($P < 0.0001$). The red line defines the mean value. The expression profile of UII gene in the APAs has revelead a low expression in the majority of APAs.

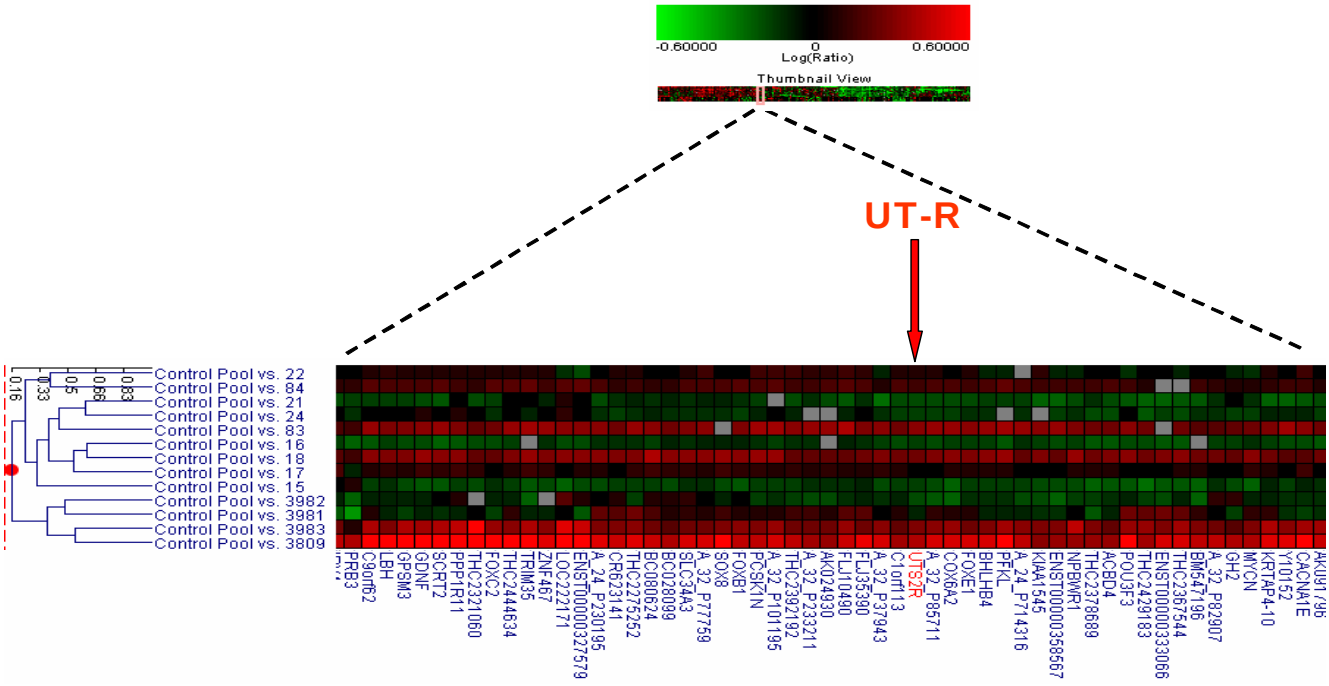


Fig. 8 Cluster analysis of differentially expressed genes in APAs. The results of a portion of 2D cluster are displayed in a heat-map, in which each row represents one sample of APA and each column one sequence in the chip. Each cell in the heat-map corresponds to a log (ratio) and is represented by one color: the genes over-expressed are in red and those under-expressed in green. The default saturation threshold is -2 to +2.

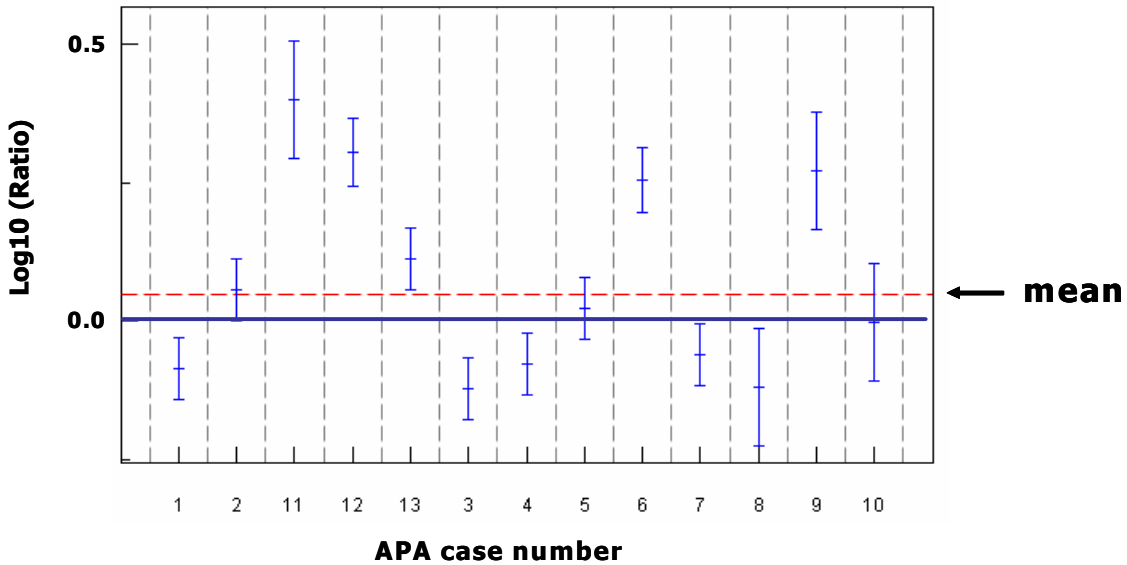


Fig. 9 UT-R variance plot in APAs. The expression level of the gene is reported as log ratio of the intensity of the green channel (APA RNA) to the red channel (Control pool). An Anova and Benjamini-Hochberg post-hoc test were performed ($P < 0.0001$). The red line defines the mean value. The expression profile revealed high heterogeneity across the samples, with 6 APA featured by an higher UT-R expression , 2 with a level similar to the control tissue and 5 with a lower expression than the control tissue.

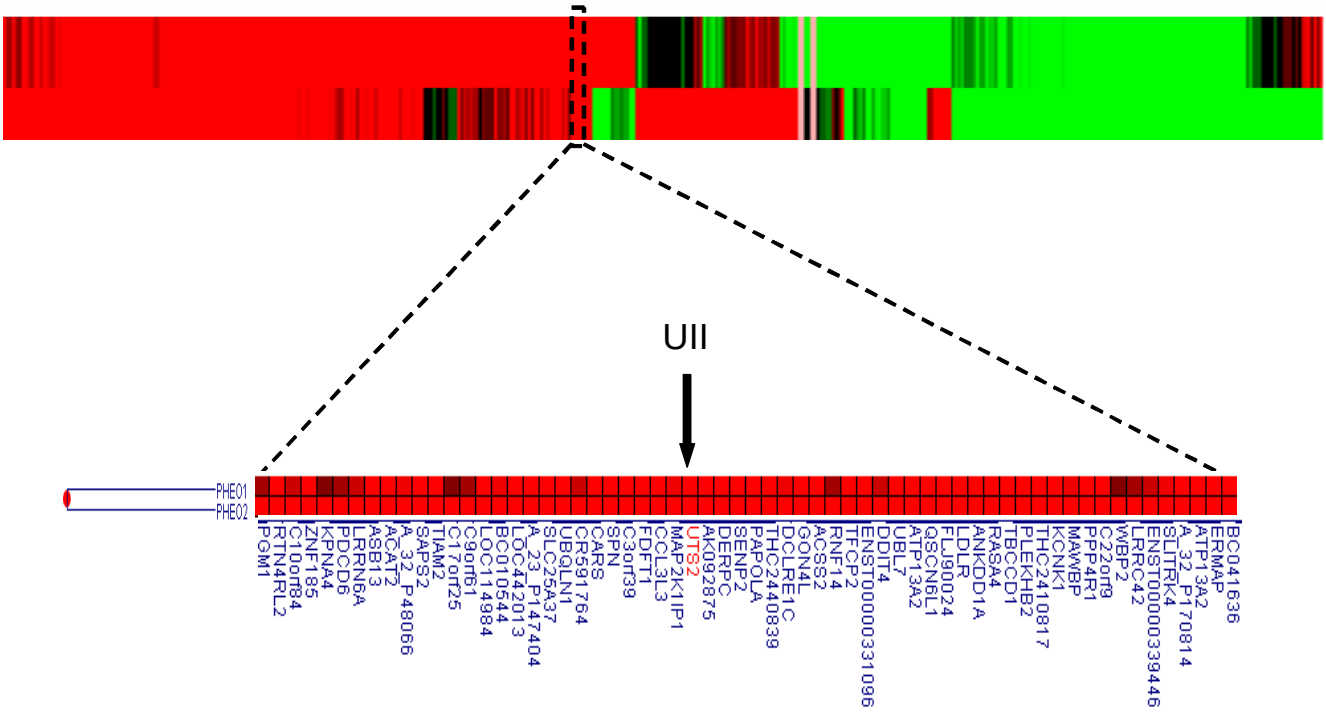


Fig. 10 Cluster analysis of differentially expressed genes in PHEOs. The results of a portion of 2D cluster are displayed in a heat-map, in which each row represents one sample of PHEO and each column one sequence in the chip. Each cell in the heat-map corresponds to a log (ratio) and is represented by one color: the genes over-expressed are in red and those under-expressed in green. The default saturation threshold is -2 to +2.

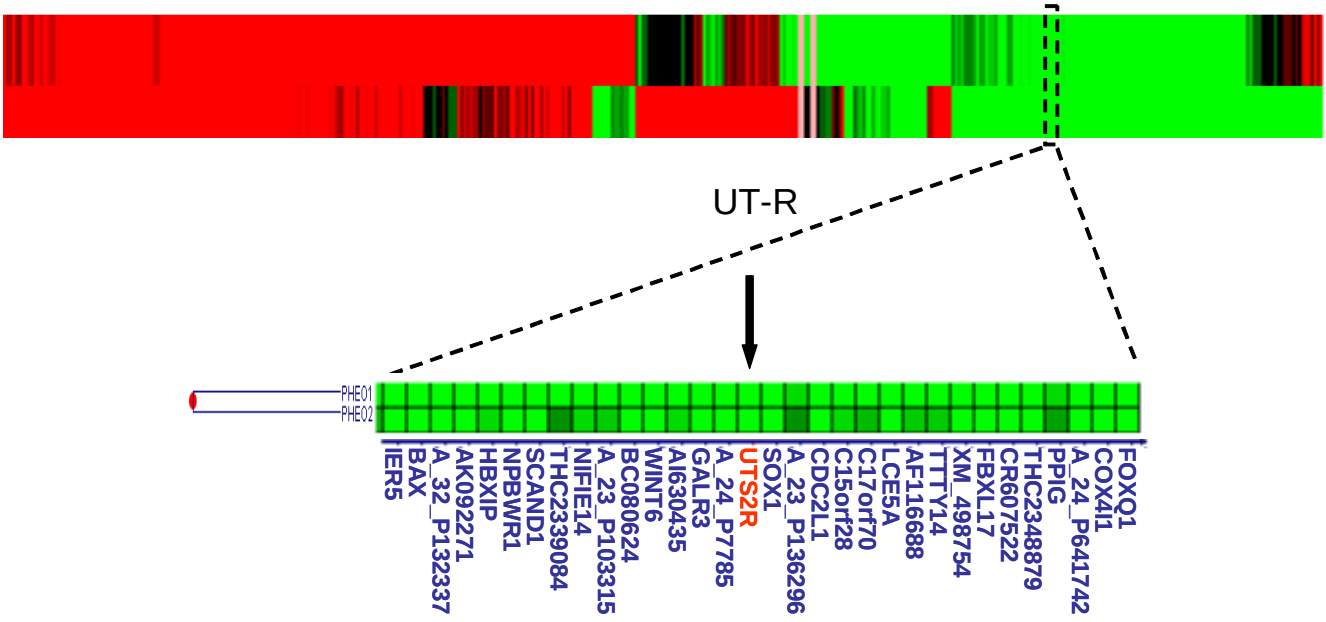


Fig. 11 Cluster analysis of differentially expressed genes in PHEOs. The results of a portion of 2D cluster are displayed in a heat-map, in which each row represents sample of PHEO and each column a sequence in the chip. Each cell in the heat-map corresponds to a log (ratio) and is represented by one color: the genes over-expressed are in red and those under-expressed in green. The default saturation threshold is -2 to +2.

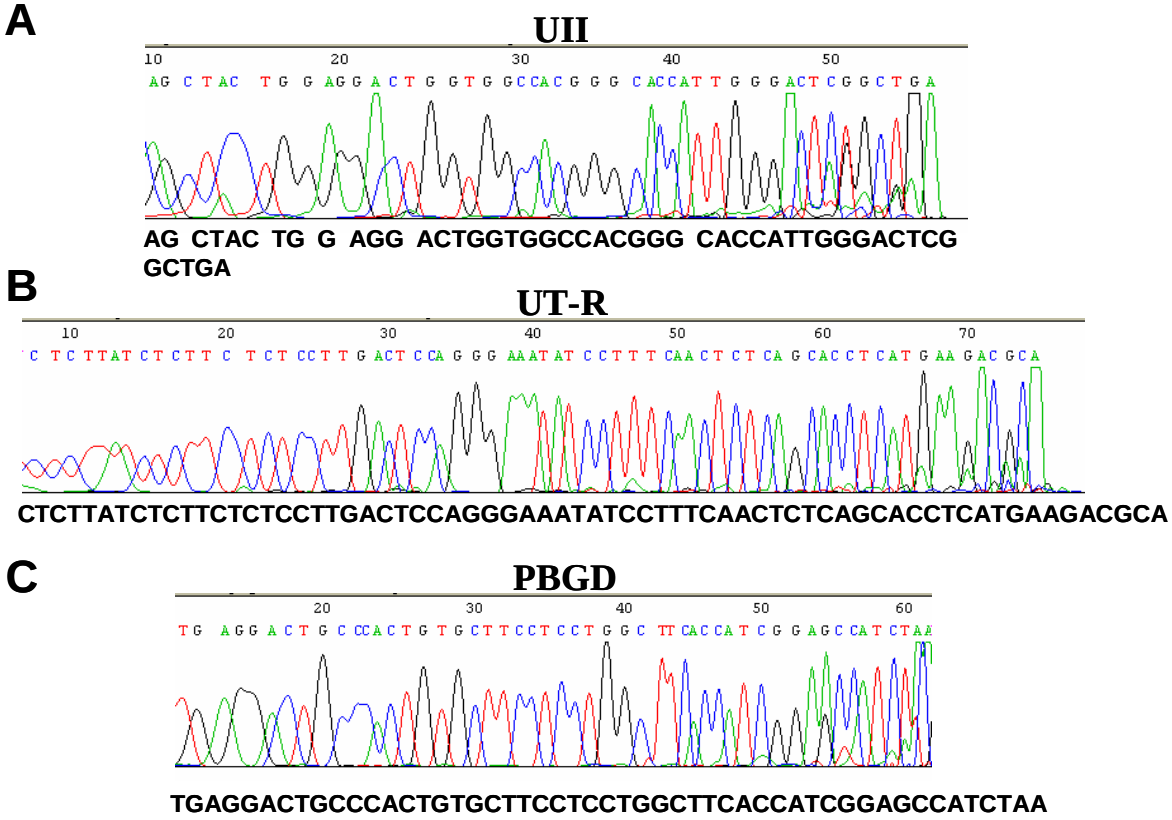


Fig. 12 Results of sequencing of Real Time PCR amplification products of UII (panel A), UT-R (panel B) and PBGD (panel C) genes

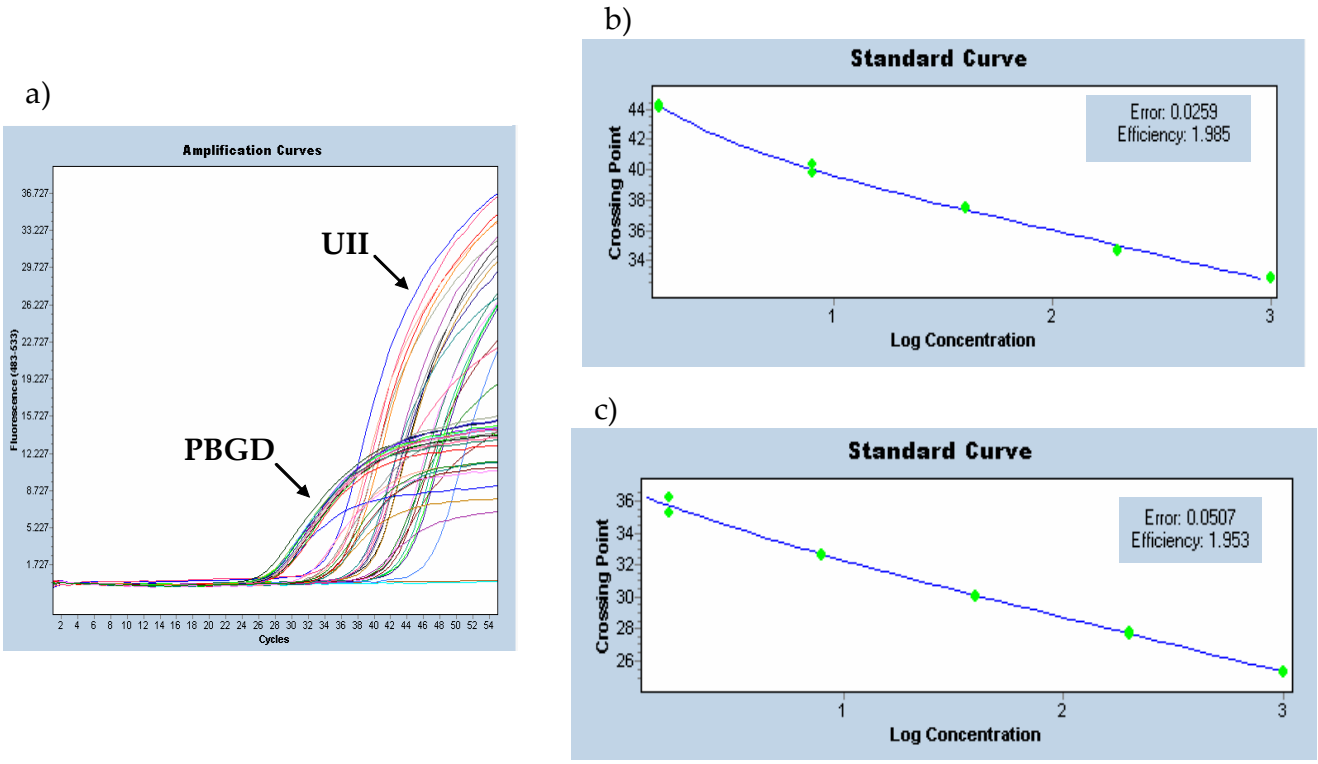


Fig. 13 Amplification (a) and Calibration curves of UII (b) and PBGD (c) genes. This analysis was performed starting from 1 µg of control pool RNA and diluting the samples 1:5 five times. A replicate was included for each dilution.

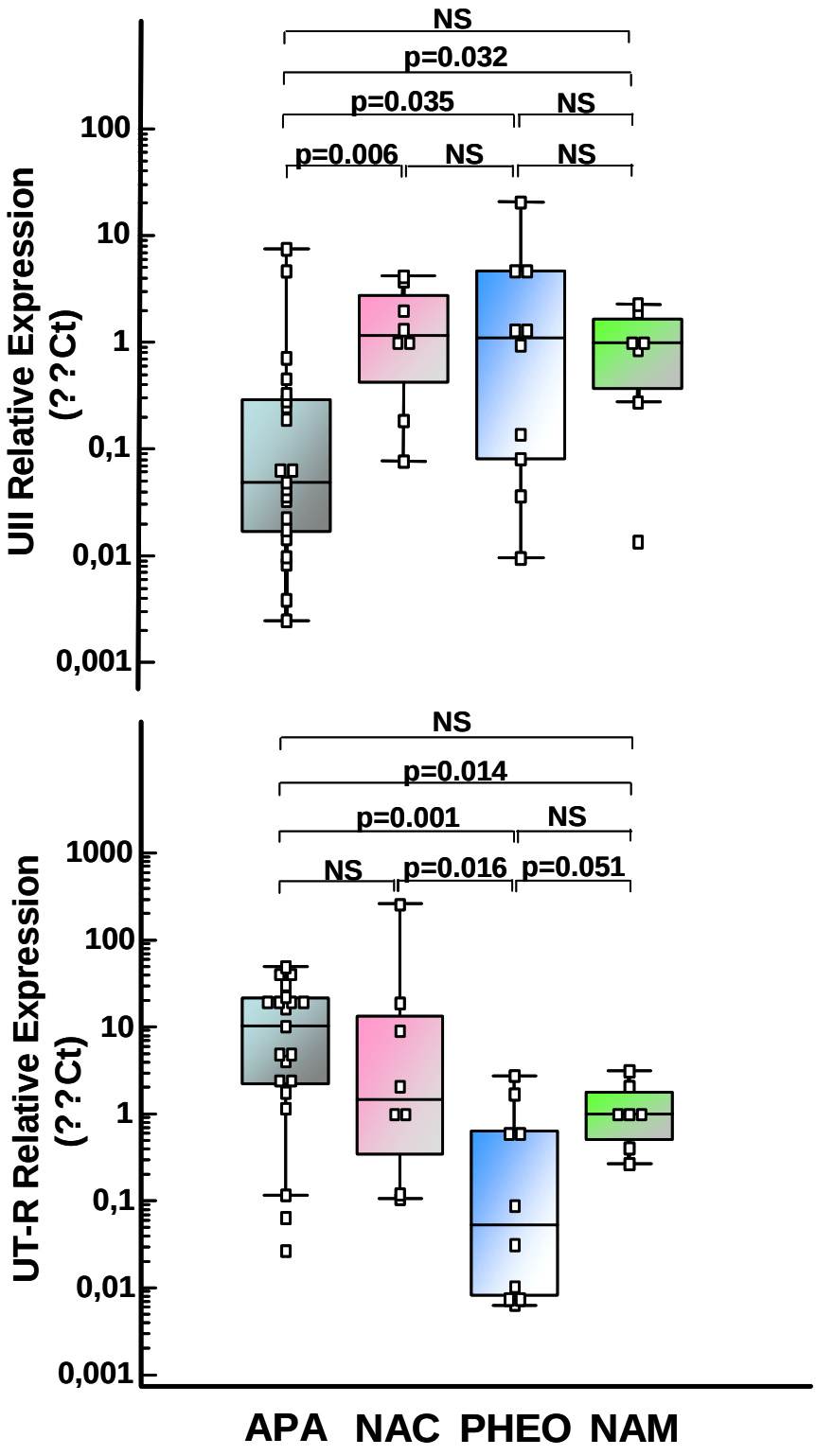


Fig. 14 Relative Expression of UII (upper panel) and UT-R (lower panel) in Aldosterone-Producing Adenoma (APA), Normal Adrenal Cortex (NAC), Pheochromocytoma (PHEO) and Normal Adrenomedullary Tissue (NAM) by the Box & Whisker (median and interquartile range) plot.

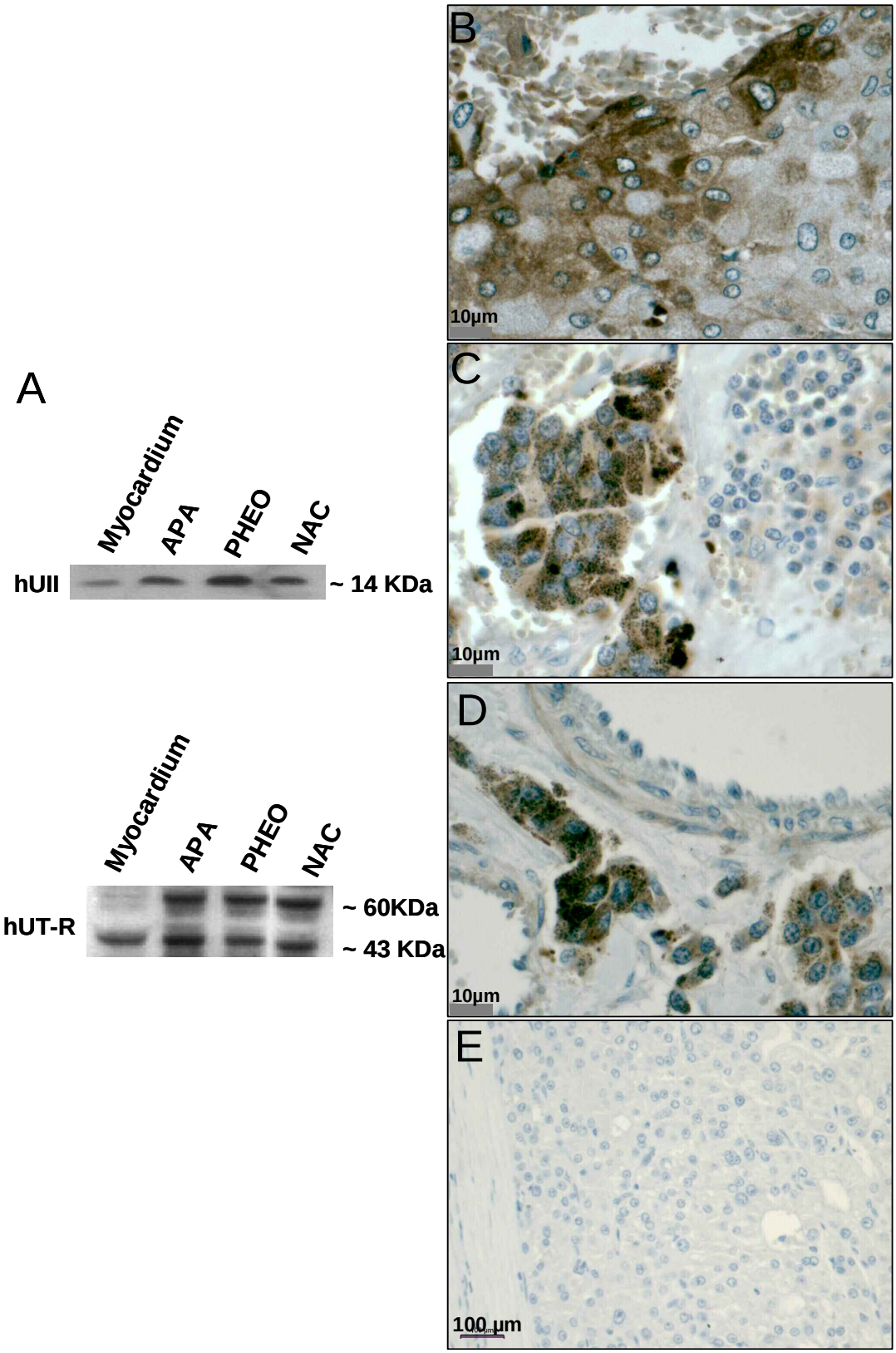


Fig. 15 Protein expression of UII and UT-R. Panel A: Western Blot analysis of human UII (upper panel) and UT-R (lower panel) protein expression. Panel B-D: Immunohistochemistry of UII protein expression in PHEO. Panel E: negative control by omission of primary antibody.

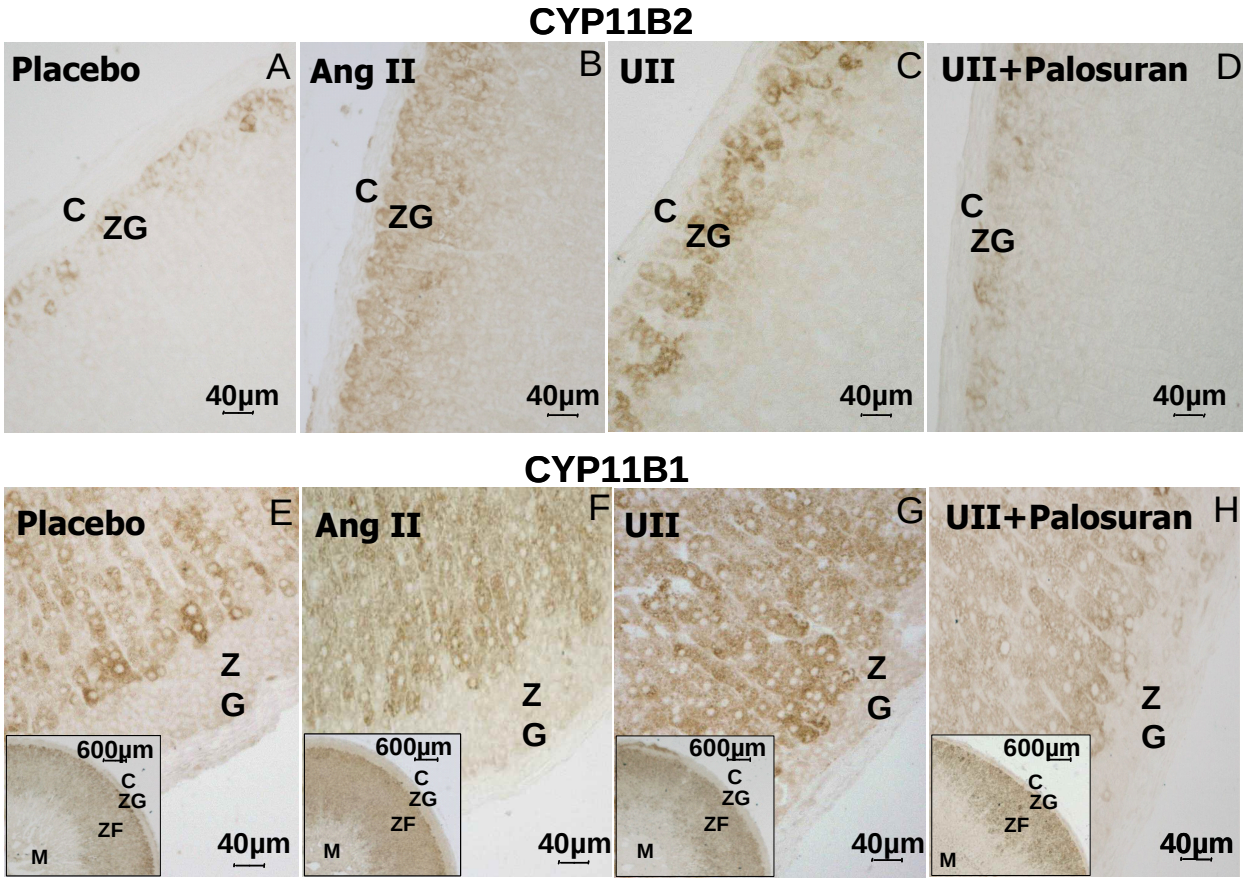


Fig. 16: Immunohistochemistry in the rat adrenal gland. Results showing staining in the rat adrenocortical zona glomerulosa with a specific antibody for the aldosterone synthase (CYP11B2, top panels) and zona fasciculata-reticularis for the 11ß hydroxylase (CYP11B1, bottom panels), after one-week infusion of vehicle (Placebo), Ang II, and UII alone or on top of the UT-R antagonist palosuran. C, capsula; ZG, zona glomerulosa; ZF, zona fasciculata-reticularis; M, medulla. The insets in the bottom panels show at low power magnification the staining of the adrenal cortex and medulla.

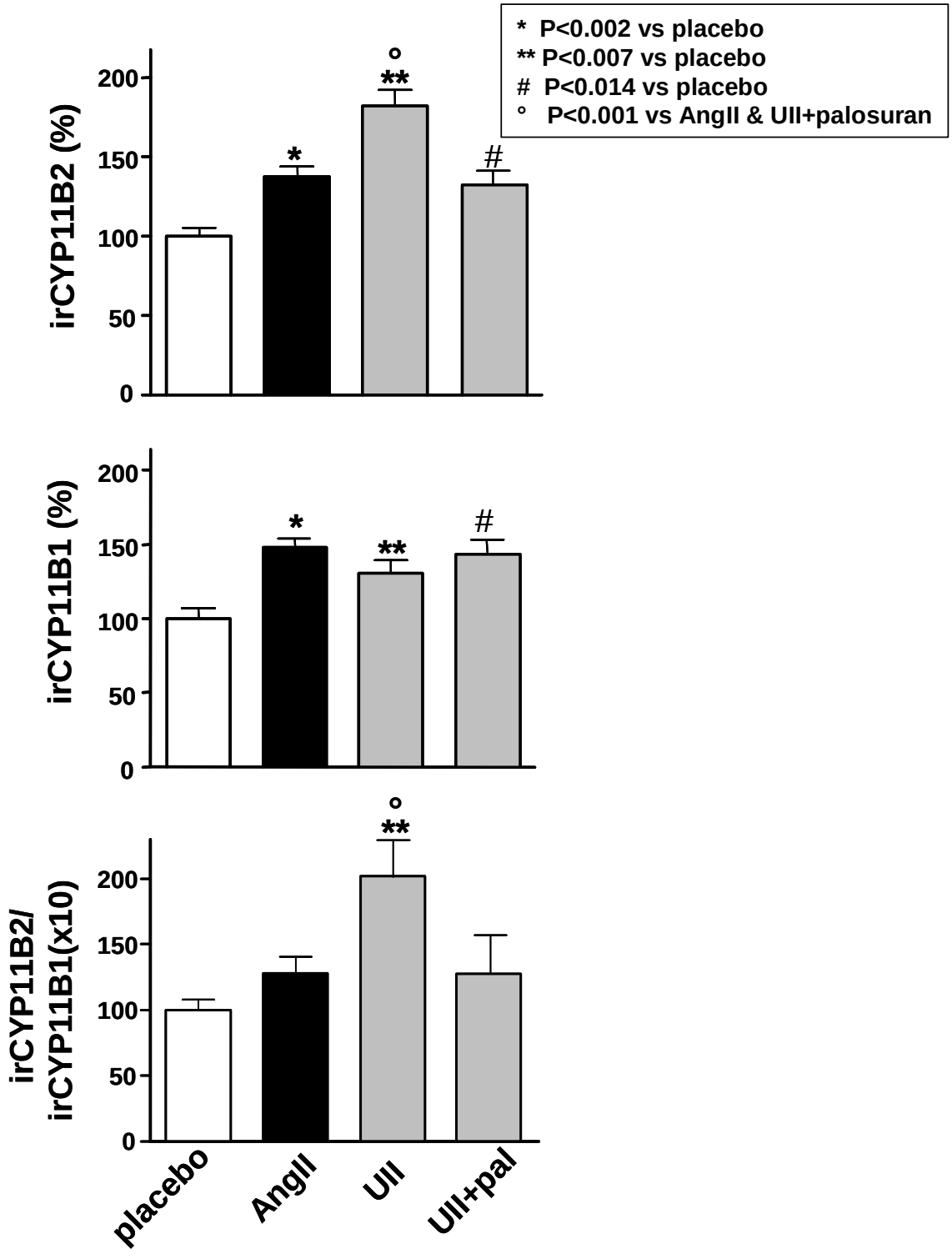


Fig. 17 Quantitative analysis of chronic activation of CYP11B1 and CYP11B2 in the rat adrenal cortex. The bar graphs show the effect of a one-week infusion of vehicle (Placebo), Ang II, and UII alone or on top of the UT-R antagonist palosuran. Staining of the zona glomerulosa with a specific antibody for CYP11B2 is shown in the top panel; staining of the zona fasciculata-reticularis for CYP11B1 is shown in the mid panel whereas the ratio of the two is shown in the bottom panel. Results are expressed by arbitrary attributing 100% to the staining observed with placebo.