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HUMAN PRIMARY ALDOSTERONISM: FROM CLINICAL OBSERVATIONS TO *IN-VIVO* AND *EX-VIVO* STUDIES ON THE PRESSOR EFFECTS OF UROTENSIN II

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Riassunto

In una paziente affetta da iperaldosteronismo primario abbiamo identificato la presenza di un feocromocitoma non secernente catecolamine. L'analisi del transcrittoma nel feocromocitoma ha rivelato un'elevata espressione di Urotensina II (UII).

L'UII è un peptide ciclico di 11 aminoacidi, identificato inizialmente nel sistema neurosecretorio dei pesci teleostei e successivamente nell'uomo, che oggi è ritenuto un potentissimo vasocostrittore coinvolto nella fisiologia e nella fisiopatologia del sistema cardiovascolare i cui meccanismi di azione sono tuttora largamente sconosciuti. Molti studi su diversi modelli animali, sia *in-vitro* che *in-vivo*, hanno documentato che l'UII può indurre ipertrofia cardiaca, aumento della matrice extracellulare e riduzione della contrattilità miocardica, suggerendo una sua possibile implicazione nel rimodellamento cardiaco post-ischemico o nell'insufficienza cardiaca. Nonostante i suoi effetti sul cuore e sull'omeostasi pressoria siano tuttora controversi si è generato un notevole interesse nell'identificazione del ruolo fisiologico dell'UII e dei suoi meccanismi d'azione.

Abbiamo studiato l'espressione dell'UII e del suo recettore nel contesto delle ghiandole surrenaliche e in diversi tumori surrenalici constatando un andamento pressochè opposto circa l'espressione di UII e del suo recettore tra feocromocitoma ed adenoma secernente aldosterone: nel feocromocitoma si è documentato un più alto contenuto di di UII rispetto all'adenoma secernente aldosterone che, viceversa, ha mostrato una maggiore espressione del recettore, suggerendo una down-regulation recettoriale secondaria alla produzione di UII. Queste evidenze suggeriscono un ruolo importante dell'UII nelle interazioni paracrine tra midollare e corticale surrenalica e nella fisiopatologia del pheocromocitoma e dell'adenoma secernente aldosterone, dando peraltro una spiegazione meccanicistica per quei feocromocitomi che si presentano clinicamente con iperaldosteronismo.

Abbiamo inoltre testato in vivo l'effetto dell'UII sulla pressione arteriosa mediante la sua infusione cronica nel ratto documentando un andamento pressorio molto simile a quello che si ottiene nell'infusione cronica di aldosterone e caratterizzato da un iniziale effetto ipertensivo seguito da un adattamento pressorio verso i valori di partenza. Questo supporta ulteriormente l'ipotesi di stretta interazione tra UII e sistema renina-angiotensina-aldosterone o addirittura di un'azione dell'UII mediata dalla produzione di aldosterone. A questo proposito, partendo dal presupposto che il peculiare andamento pressorio negli stati di iperaldosteronismo è pesantemente influenzato dagli adattamenti di volume che si verificano grazie al fenomeno renale di "escape" abbiamo voluto verificare se vi fossero cambiamenti significativi nell'andamento pressorio durante l'infusione cronica di UII e la concomitante soppressione del meccanismo di escape. I risultati sono stati sorprendenti visto che per la prima volta si è documentato in vivo un effetto ipertensivo, non solo transitorio ma continuativo, dell'UII.

Le evidenze ottenute, sia per quel che riguarda gli effetti dell'UII sulla pressione arteriosa che sullo sviluppo dei tumori surrenalici, sicuramente consentono di fare un passo in avanti circa l'identificazione del meccanismo d'azione a sostegno dell'ipotesi che l'UII possa agire come mediatore tra midollare e corticale surrenalica o mediante la produzione stessa di aldosterone. Gli studi in cui prevediamo di utilizzare ratti transgenici per l'espressione di UII nella midollare surrenalica potranno contribuire a confermare o confutare la nostra ipotesi.

Abstract

In a patient affected by primary aldosteronism we have identified the presence of a pheochromocytoma not secreting catecholamines. The analysis of the transcriptome in the pheochromocytoma revealed a high expression of Urotensin II (UII).

UII is a somatostatin-like cyclic 11-aminoacid vasoconstrictor peptide, first identified in the teleost fish caudal-neuro-secretory system and then in humans, that has been demonstrated to be a potent vasoactive peptide involved in the physiology and pathophysiology of the cardiovascular system through mechanisms still largely unknown. Its action is now the subject of intensive studies on the effects it produces in different animal models and in different vascular beds. The direct effects on the myocardium such as ventricular hypertrophy, increased extracellular matrix and reduction of myocardial contractility suggest a role of UII in cardiac remodelling after myocardial infarction and heart failure. Even if the effects on cardiovascular system are still controversial UII has attracted considerable scientific interest aimed to investigate on its pathophysiology and on its the mechanism of action.

We investigated among the expression of UII and its receptor in human adrenal glands and in different adrenal tumors. The opposite trend of expression of UII receptor obtained between aldosterone-producing adenoma and pheocromocitoma, along with the differences of genes implicated in UII signaling, supported a role of UII in the paracrine interactions between the adrenal medulla and cortex. Being relevant for the regulation of adrenal gland function and for the pathophysiology of pheocromocitoma and aldosterone-producing adenomas, these interactions might provide a mechanistic explanation for the pheocromocitomas that presented clinically as aldosteronism.

We tested the “in vivo” effects of UII on blood pressure in rats, showing a delayed transient hypertensive effect that resembles the pressure trend in chronic infusion of

aldosterone. Thus further supporting the hypothesis of a close interaction between UII and the renin-angiotensin-aldosterone system or even that UII acts through production of aldosterone as well as assess its hypertensive activity, its effect of cardiac hypertrophy and fibrosis and its role in regulating the growth of adrenocortical cells in rats. Considering that the mechanisms involved in the so called "escape phenomenon" seem responsible of the peculiar blood pressure trend in hyperaldosteronism we tested the blood pressure modifications in chronic infusion of UII counteracting the "escape phenomenon" through unilateral nephrectomy, high salt diet and concomitant infusion of spironolactone. The results obtained were promising: for the first time was shown an in-vivo not transient but continuous hypertensive effect of UII.

The evidences obtained in blood pressure modification during chronic infusion of UII combined with the ones regarding UII and development of cortical tumors, has taken a step forward in identifying the mechanism of action of UII supporting the hypothesis of a possible close communication between medulla and adrenal cortex and a possible action of UII mediated by aldosterone. Additional investigations on the transgenic rat overexpressing UII in adrenal medulla will certainly contribute to verify our hypothesis.

1. Urotensin II

Urotensin II (UII) is a somatostatin-like 11-aminoacid (ETPDCFWKTCV) vasoconstrictor peptide that was initially isolated from the urophysis of the goby fish (Gillichthys mirabilis)[1] and subsequently from the white suckerfish and the carp. Although there were variations in the amino acid sequence near the N-terminus, in both species the UII molecule retained the COOH-terminal cyclic sequence: -Cys6-Phe7-Trp8-Lys9-Tyr10-Cys11 (Figure 1). It is this sequence, likewise for somatostatin, that is thought to confer biological activity to the peptide.[2] The in vitro demonstration of the vasoactive effects of fish UII in rats and in human vessels[3,4] led to identify a mammalian homolog. As the fish UII activated the orphan G protein coupled receptor GPR14, a human GenBank search was performed using the fish UII sequence. This led to the discovery of a human-expressed sequence tag with 25% identity to the fish sequence yielding a 688-base pair cDNA from which the human UII propeptide amino acid sequence was deduced.[5] Recent evidences support the notion that UII belongs to the same gene superfamily of somatostatin. The remarkable conservation of somatostatin-like peptides among the species underscores the vital role of UII as a basic physiological mediator.[6]

After its discovery and isolation UII was revealed to be a very potent vasoconstrictor sharing many functional similarities with other vasoactive peptides. UII elicits strong vascular smooth muscle cell (VSMC)-dependent vasoconstriction and weak endothelium-dependent vasodilation as endothelin 1 (ET-1). Like ET-1 and adrenomedullin, the circulating concentrations of UII in humans are found to be very low (picomolar range), which suggests that UII acts in an autocrine and paracrine fashion.[7] This theory is supported also by the fact that both the body distribution and the plasma concentration of UII and its receptor (UT-R) are very similar to those of

other known powerful autocrine-paracrine endothelial-derived vasoconstrictors.[8] Moreover the UT-R, like the ET-1 and angiotensin II receptors, is coupled to a Gq11 subtype of heterotrimeric G proteins. Like angiotensin II, UII is thought to elicit RhoA membrane translocation and activation via receptor-Gq11 coupling.[8]

Although the sources of UII production in the body remain to be fully determinated, arteriovenous gradients have been identified across the heart, liver, kidney, pancreas and adrenals indicating that these organs may be primary sources for UII.[9] Moreover altered plasma concentrations of UII have been detected in several pathological conditions, such as heart failure, essential hypertension, renal disease, diabetes, and liver cirrhosis[10] suggesting the potential usefulness of UII as a biomarker. This has stimulated a great deal of research on its possible implications in the pathophysiology of many cardiovascular diseases.

1.1 UII receptor

The UII receptor (UT-R) was initially discovered in rat using PCR and genomic DNA library screening; an orphan G protein-coupled receptor was discovered and termed GPR14. GPR14 showed a 27% overall sequence homology with the somatostatin receptor SSTR4. Rat GPR14 was used to probe the human genomic library which allowed the detection of a human 389 amino acid GPCR with 75% identity to rat GPR14. Subsequently, the functional role of the orphan GPR14 was discovered proving the human GPR14 receptor to be highly selective for the fish and human UII. This led to the identification of GPR14 as the UT-R.[11,12]

The studies carried out on the interactions between UII and UT-R provided evidence that the COOH-terminal region is largely responsible for the biological activity of UII.

Moreover investigations on the structure and function of UT receptor suggest that transmembrane domains IV, VI, and VII are involved in the formation of the ligand-receptor binding pocket.[13-15]

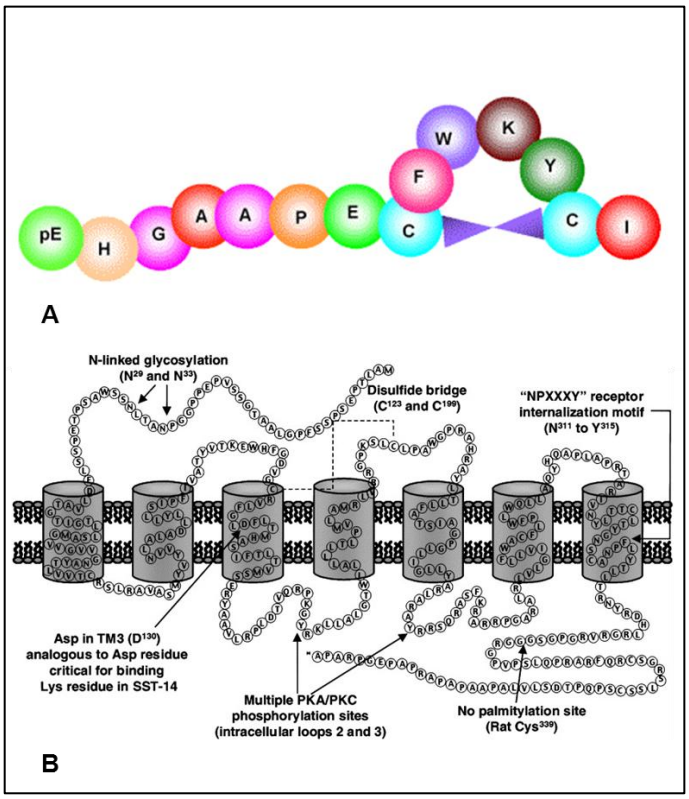

Figure 1. Urotensin II amino-acid configuration (A) and its G protein copulated receptor (B)

This receptor has been identified in varying amounts in cardiac myocytes, VSMCs, endothelial cells, spinal cord, central nervous system, and kidneys. On the other hand, UII has been detected by immunohistochemistry in blood vessels of the heart, pancreas, kidney, placenta, thyroid, adrenal gland, and umbilical cord, as well as in human epithelial cells of the kidneys. Both ligand and receptor seem to be ubiquitously expressed in human tissues though lymphocytes and macrophages, respectively, are the largest producers of UII and UT-R in sites of atherosclerotic lesions.

1.2 Vasoactive activity of UII

In vitro studies showed that UII elicited contractile effects on most smooth muscle of fishes and in mammalian tissue[16]. These in vitro demonstrations proved that the contractile potency of UII (-log[EC50] of 9.09) was greater than that of ET-1 (-log[EC50] of 7.9), noradrenaline (-log[EC50] of 7.58), and serotonin (-log[EC50] of 6.27), making UII the most potent human vasoconstrictor identified to date.[17,18]

Many studies have examined the effects of UII on the vasculature and have reported large differences in responses to UII among species, different vascular beds and even within the same vascular bed of the same species. An example of this is the isolated rat aorta, where potent vasoconstriction is observed in the thoracic segments with lower effect in the abdominal portion (estimated approximately about 27% of that expressed on the thoracic segments). This differential responsiveness may be due to differences in the expression level of UT-Rs, or to the ability of the receptor to couple to its signaling pathway.[16] The action of UII was found to be independent of endothelial cells and to work via mobilization of intracellular calcium as well as through stimulation of extracellular calcium influx (Ca^{2+} release via PLC/dependent IP3 and calmodulin-dependent myosin light-chain kinase pathways).[17] Contrary, subsequent studies showed a possible mediation of endothelial cells (by NO production) determining relaxation of rat aortic tissue (precontracted with noradrenaline) in presence of lower concentration of UII with the maintenance of the contractile effect at higher concentration.[18]

Further experiments where performed also with human UII showing an extremely powerful contractile effect (greater than ET-1) on isolated rat aorta,[5] completely independent by the endothelial cells and without evidence of relaxation of precontracted aorta.

1.2 UII and cardiovascular system

Regarding cardiac tissue, recently the presence of UII and its receptor in both myocytes and cardiac fibroblasts has been demonstrated; moreover has been suggested a possible role of UII in cardiac fibrosis.[19] Furthermore, in a rat model of heart failure following myocardial infarction, UII and UT-R expression were increased in both infarct and non-infarct regions of the left ventricle (LV) compared with sham-operated controls,[20] suggesting an important role for UII in myocardial ischemia. Additionally, in vitro stimulation of rat neonatal cardiac fibroblasts with UII has been shown to result in increased collagen synthesis and increased expression of the extracellular matrix proteins α1(I)- procollagen, α1 (III)-procollagen and fibronectin genes similar to, or greater than, that produced by the profibrotic stimulus, endothelin. Adenoviral-mediated overexpression of the rat UT-R in neonatal fibroblasts results in a two-fold increase in collagen synthesis following UII stimulation, suggesting that the up regulation of UT-R expression observed during pathophysiological conditions such as myocardial infarction, may be responsible for increase in collagen synthesis observed under these conditions.[20] Such pro-fibrotic effects lead to cellular hypertrophy, protein synthesis, and expression of natriuretic factors, all induced by UII.[20-22]

UII stimulation of rat neonatal cardiomyocytes, either transiently transfected with recombinant rat UT receptor or infected with adenoviruses expressing the rat UT-R, results in a hypertrophic phenotype inducing atrial natriuretic peptide (ANP), myosin light chain 2v-CAT and α skeletal actin activity. In addition, UII increases protein synthesis, myocyte size and myofibril organization pathways.[20]

UII has been shown to have a direct inotropic effects on the myocardium, increasing contractility in human right atrial trabeculae in a dose-dependent manner. A small increase in contractility was observed at a maximal UII concentration in right

ventricular trabeculae from an explanted heart with idiopathic dilated cardiomyopathy. This responsiveness of UII is consistent with the expression of UT-Rs previously determined in all four chambers of the heart.[21]

In heart failure UII plasma concentrations are not only increased but also correlate with Big ET-1 and brain natriuretic peptide levels as well as with NYHA functional classes[22,23] further supporting the hypothesis that UII plays a predominant role in the pathophysiology of the cardiovascular system.

1.4 UII and atherogenesis

UII has been shown to act as a mitogenic and hypertrophic agent resulting, in part, in the enlargement of cells and in the reorganization of sarcomeres. These cellular responses result from UT-R-binding which promote tyrosine phosphorylation of epidermal growth factor receptor and in turn activate mitogen-activating protein kinases, extracellular signal-regulated kinase 1/2 (ERK 1/2), and p38.[24,25] UII seems also to increase cell proliferation in VSMCs by acting synergistically with oxidized low-density lipoproteins (LDL), amongst several other mitogens.[24] UII has also been shown to increase expression of NADPH oxidase and plasminogen activator inhibitor-1 (PAI-1) in VSMCs. These observations, in association with the evidences emerged using transgenic and crossbred mouse strains showing that selective induction of UT-R expression in mice VSMCs displayed far greater aortic lesions, led to hypothesize a role of UII in the progressing of atherosclerosis.[26]

In addiction among the inflammatory cell population, lymphocytes appear to be the largest producers of UII, whereas monocytes and macrophages appear to express the

UT-R to support that UII is an important mediator in inflammatory processes such as atherosclerosis.[27]

1.5 UII, insulin resistance and metabolic syndrome

UII seems also to have a role in insulin secretion and the pathogenesis of type II diabetes mellitus (DMII). Both UII and UT-R are present in the human pancreas and seem to directly inhibit beta cell function, thus inhibiting insulin release.[28,29] In fact, UII levels have been reported to be 1.7-fold higher in diabetic patients than in healthy controls. However, UII levels did not correlate with fasting blood sugar levels demonstrating that hyperglycemia is not responsible for UII increase. It has been suggested that elevated UII plasma concentrations in metabolic syndrome may be a result of damaged endothelial cells, as is the case in cardiovascular diseases.[30] The metabolic syndrome, which includes DMII, is associated with increased inflammatory cytokines and elevated free fatty acids across its spectrum. In fact, UII can up-regulate IL-6 which plays a role in atherogenesis and hypertrophy, as well as in insulin resistance.[31] Insulin resistance may further impair vasodilation, as plasma insulin levels and blood pressure are correlated in patients with hypertension. Interestingly, several single nucleotide polymorphism identified on the UII gene have been associated with insulin resistance and therefore with a susceptibility to DMII. In addition, DMII patients with renal dysfunction exhibited plasma and urine UII levels higher than in patients with normal function.[30] These elevated levels are probably due to an increased production of UII in renal tubular cells as a result of renal damage. In effect, insulin resistance causes increased blood pressure and consequently results in atherosclerosis and renal damage. These observations may have important implications in the

understanding of micro and macro-vascular disease, which is a consequence of this condition.

The discovery of CT-058362, commonly known as *palosuran*, the most known potent, selective, and competitive antagonist of the UT-R,[32-34] allowed several investigation resulting in an increase of knowledge on the possible systemic effects of UII.

In an experimental study, it has been shown that long term treatment of streptozotocin-induced diabetic rats with palosuran improved survival, increased insulin, and slowed the increase in glycemia, glycosylated hemoglobin, and serum lipids. Furthermore, palosuran increased renal blood flow and delayed the development of proteinuria and renal damage.[33] These evidences suggest that UT-R antagonism might be a new therapeutic approach for the treatment and/or prevention of diabetic nephropathy. UII is also thought to play a role in kidney physiology, as increased plasma UII levels have been reported in chronic renal failure patients on hemodialysis as well as in those not yet requiring dialysis.[35] This increase can, however, merely represent impaired clearance of UII from the circulation, rather than increased production in these patients. Palosuran was shown to be effective in macroalbuminuric diabetic patients who are prone to the development of renal disease with an overall clinically significant reduction of 24.3% in the 24-hour urinary albumin excretion rate.[36]

1.6 UII and blood pressure

On the basis of available evidence on the vasoconstricting properties of UII it is altogether likely that UII can directly or indirectly be involved in the homeostasis of blood pressure (BP). Acute systemic effects of UII have been assessed in some *in vivo* animal models with controversial results. Infusion of UII in cynomologus monkey

demonstrated reductions in mean BP together with reduction in carotid blood flow and reduced cardiac output as well as increase in LV end-diastolic pressure and total peripheral resistance. However the animals thereafter went into a state of cardiovascular collapse which was thought to be related to either reduced cardiac function and/or impaired coronary perfusion due to intense coronary vasoconstriction.[5]

Some other studies reported different effects of UII, resulting in myocardial depression and fatal circulatory collapse. UII reduced heart rate, mean blood pressure, and carotid and coronary blood flow, but pulmonary pressure was increased[25]. This increase in pulmonary pressure was consistent with the *in vitro* evidences that UII is a potent vasoconstrictor of primate pulmonary arteries[37] and may be important in the development of right ventricular (RV) heart failure. Similar hemodynamic effects of UII have been noted in anesthetized rats causing systemic vasodepression.[38,39]

More recent studies assessing the acute effects of UII infusion on hemodynamics and cardiac function in rats found that UII dose-dependently decreased mean arterial pressure, LV systolic pressure and dP/dt in the first 5 min following administration.[39] In conscious rats UII caused a vasodilation accompanied by an increased heart rate, probably due to a direct action of UII as well as baroreceptor reflex activation.

In the first study investigating the effects of chronic infusion of UII in rats it was shown that, although the dose used of 300µmol UII /Kg/h had no effect on systemic pressures, it markedly decreased myocardial contractility (40% increase of left ventricular end diastolic pressure) probably secondary to an increase of the proportion of collagen I:III in the LV.[40]

In vivo studies on the role of UII in the homeostasis of BP are nowadays few and their results, sometimes conflicting, do not provide reliable information on the BP response of the organism exposed to high levels of UII, leaving many questions unresolved. In fact it would be natural to assume that an endocrine-peptide with such an intense

vasoconstrictor activity has a systemic hypertensive effect contrary to what emerged since now. Can UII increase BP? And if so, when and how does it happen? Could be possible a transient hypertensive effect of UII subsequently damped by a series of systemic responses of the organism?

If this exist, probably a continuous and invasive blood pressure monitoring could help on identifying all the BP variations since the beginning of a chronic infusion of high levels of UII, thus that may have not been identified in *in vivo* studies already conducted. Beside remain completely unknown mechanisms by which the UII may acts on BP homeostasis and the hypothesis that the activity of UII is closely related with some other endocrine system seems to be more and more consistent.

2. Is UII involved in secretion of aldosterone?

The research mark in the field of hypertension especially regarding the renin-angiotensin-aldosterone system (RAAS) led us to hypothesize a synergistic action between UII and RAAS or even by means of the direct stimulation of production of other mediators such as aldosterone.

The concomitant studies we have carried out on adrenal glands in patients affected by hyperaldosteronism showed an increased expression of UII and its receptor in different adrenal tumors, suggesting a role of UII in regulating the growth of adrenocortical cells.[41] On this basis we hypothesized a role of UII in hyperaldosteronism aimed to clarify its mechanism of action.

2.1 Notes on primary aldosteronism

Primary aldosteronism (PA) is an endocrine disorder characterized by an excessive production of aldosterone totally or partially independent by the RAAS. The inappropriate mineralocorticoid activity resulting determine the suppression of renin, salt retention and excessive urinary excretion of potassium. Clinically this corresponds to the appearance of arterial hypertension of varying degrees, sometimes resistant to medical therapy, and in a certain percentage of cases, of hypokalemia and metabolic alkalosis. The cause of PA lies in the excessive secretion of aldosterone by the adrenal cortical tissue: different classifications that result can therefore emphasize the neoplastic or hyperplastic origin of the disease, or the mono/bilateral nature of the phenomenon. For the purpose of choosing the best treatment, however, the classification that is more effective and functional is that which distinguishes forms surgically treatable (monolateral disease) by forms not surgical treatable (bilateral disease)[42] (Table 1).

Classically, forms recognized as the most representative of the two categories, are respectively the aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA), which corresponds anatomo-pathologically to the bilateral adrenal hyperplasia (BAH) that usually occurs with a milder clinical presentation.[43] Other subtypes include:

1) *The adrenal cortical carcinoma* (APC, aldosterone-producing adrenal carcinoma), which generally is distinguished by the adenoma because of the bigger size, the ability to produce other steroids over aldosterone (ex. androgens), the tendency to local invasiveness and metastasis, the presence of areas of necrosis and a high number of mitoses.
2) *The unilateral adrenal hyperplasia* (UAH, unilateral adrenal hyperplasia) where the aldosterone excess originates from hyperplasia of only one of the two adrenal glands and which is correctable by surgical removal of the adrenal affected, and the condition known as "MUAN" (unilateral multiple adrenocortical micronodules), of which only 5 cases have been reported up to day[44,45] which clinical relevance is considered so high to be sometimes classified as independent category.
3) *Familial hyperaldosteronism type I (FH type I) correctable with glucocorticoids, also known as GRA (glucocorticoid remediable aldosteronism)* which is an autosomal dominant genetic disorder characterized by high production of aldosterone ACTH-dependent and high levels of hybrid steroids (18OHcortisol and 18oxo-cortisol) hypertension and renin suppression.

   In *FH type II*, in which is observed in the same family the presence of BAH or of APA[46], the transmission seems autosomal dominant although the small number of affected cases in the families studied and reported in the literature do not allow to establish it with certainty. The FH type II is also indistinguishable

clinically, biochemically and morphologically from forms of PA apparently "sporadic". It is therefore considered that on the basis of such phenotypic variability is genetic heterogeneity, with possible involvement of several genes active in the regulation of steroidogenesis or cellular proliferation.[47]

Recently it has been described a case that constitute a new form of PA family (*FH type III*) characterized by severe hypertension, hyperaldosteronism and hypokalemia with significant organ damage already in pediatric age, resistant to aggressive antihypertensive therapy, with levels of hybrids steroids up to 1000 times higher than patients with glucocorticoid remediable aldosteronism (GRA), paradoxical response to dexamethasone suppression test (increased aldosterone and lack of suppression of cortisol) and very marked unilateral adrenal hyperplasia.[48]

Surgically treatable
- Aldosterone-secreting adenoma (APA)
- unilateral
- bilateral
- Unilateral adrenal hyperplasia (UAH)
- Unilateral micronodular adrenal hyperplasia (Muan)
- Ovarian Cancer aldosterone-secreting
- APA or bilateral hyperplasia (BAH) with concomitant pheochromocytoma
- Adrenal cortical carcinoma
Not surgically treatable
- Bilateral adrenal hyperplasia (BAH)
- Unilateral APA and BAH
- Family hyperaldosteronism type I (FHI), also known as hyperaldosteronism correctable with glucocorticoids (GRA)
- Hyperaldosteronism familial type II (FHII)
- Apparent mineralocorticoid excess (AME)
- Chronic consumption of liquorice
- Use of carbenoxolone

Table 1. Causes of primary aldosteronysm

(What today really discriminate between the choice of a surgical or a pharmacological approach is not in fact the "morphological data" of unilaterality of the lesion, but the

"functional data" deriving from the adrenal venous catheterization (AVS, adrenal venous sampling). To underscore how this investigation is decisive, the results of the study PAPY [49] have recently shown that the proportion of the two PA subtypes, object in the past of conflicting assessments, is actually intimately conditioned by the availability of the AVS. In centers where this diagnostic test is used, the APA include about 2/3 of cases of PA, while in other places the IHA is the subtype more frequent).

Despite the existence of these rare forms, it is clear that by far the most common subtypes of PA are APA and BAH. However, this dichotomy between adenoma and hyperplasia, apparently simple and solid, in reality sometimes leaves space to differentiative challenges because of the lack of well-defined criteria concerning their functional and morphologic characterization. The tissue surrounding the adenoma usually shows paradoxical hyperplasic changes and contains multiple nodules ranging from microscopic to macroscopic measures. Moreover, cases of multinodular adrenal hyperplasia with evidence of lateralization of aldosterone secretion are widely documented. Therefore, bilateral hyperplasia and aldosterone-producing adenoma could be regarded as steps of a common pathological *continuum*, which could feature a gradual transition from hyperplasia to a nodular phase. However the underlying biological mechanisms driving this evolution are unknown. Since the multinodularity of adrenal glands from PA patients is reminiscent of multinodular goiters and Graves' disease, it can be hypothesized the involvement of immunological phenomena as a common pathogenic background. Recent studies from our group provide support to this interpretation.[50] Overall, even though a conclusive proof for an autoimmune basis of PA pathogenesis remains to be given, a strong implication of the immune system in PA genesis could be contended. It would seem reasonable to argue that humoral immunity could trigger an hyperplastic and eventually tumorous expansion of adrenal cells, and

cell-mediated mechanisms could take part in this process by controlling its development.

If in PA are established clinical conditions such as high BP, reduction in plasma levels of potassium and angiotensin II, which should break down the values of aldosterone, it's not clear why the levels of aldosterone remain high. It is reasonable to assume that the humoral autoimmune phenomena above described could contribute in a small percentage of case to the maintenance of aldosterone production. Recently mutations in KCNJ5 gene which encodes the inward rectifying K^+ channel Kir3 have been shown to be involved in the pathogenesis of both FH-III and sporadic APA.[50] Although the role of the wild-type KCNJ5 protein in the regulation of aldosterone biosynthesis remains unclear, two recurring mutations in the *KCNJ5* gene are commonly found in APA tumors. Transcriptome and real-time PCR analyses demonstrate that APA with *KCNJ5* mutations exhibit enhanced *CYP11B2* expression and that overexpression of KCNJ5 mutations in adrenal cells increases aldosterone production by augmenting the transcription of *CYP11B2* and of its regulatory transcription factors.[52] Besides these findings certainly the overproduction of aldosterone may led to speculate among other mechanism such as humoral stimulation by other peptides: the parathyroid hormone (PTH) for example stimulates aldosterone secretion and cell proliferation in human adrenocortical cells; moreover, in rats hyperaldosteronism has been shown to be associated with hyperparathyroidism. Hence, PTH could drive aldosterone excess in human primary aldosteronism.[53]

In this regard may be reasonable to speculate if also UII may contribute to the hyper-secretion of aldosterone and to the onset of PA.

3. Is there any evidence that UII can induce high blood pressure and primary aldosteronism in humans?

Many evidences show that the functional regulation of the adrenal gland involves paracrine interactions of numerous peptides[54,55] and UII and its receptors (UT-Rs)[56-59] is widely expressed in many tissues[56-63] including the vasculature and the adrenal gland.[66-66] The finding of UII and UT-R gene transcripts in aldosterone-producing adenoma (APA) and pheochromocytoma (Pheo)[66,67] suggested the participation of UII in the regulation of blood pressure and body fluid homeostasis. Despite the quantitative expression of UII and UT-R in APA and Pheo and the biological effects of UII in the adrenal gland are unknown, the suspicion of a paracrine effect in the context of the gland on the basis of these diseases is increasingly founded.

In rare cases PA and Pheo are found simultaneously[68-74]. These cases have been the object of a great scientific speculation which led to the hypothesis that the PA can sometimes be "secondary" to Pheo by stimulation of the zona glomerulosa through unknown mediators.

3.1 Clinical case

A lady of 44 year-old was elsewhere diagnosed, about 10 years before with idiopathic hyperaldosteronism because of the detection of persisting hypertension and hypokalemia. During the follow-up (ultrasound, CT scan and arteriography) a left adrenal mass compatible with a left adrenal adenoma was identified. However, the adrenal vein sampling (AVS) highlighted an increased aldosterone production by the right kidney (Tables 2,3), and for this reason the patient was submitted to right adrenalectomy.

	Baseline (ng/ml)	Post ACTH (ng/ml)
Cort IVC	259	204
Cort RAV	286	239
Cort LAV	704	594
Aldo IVC	594	457
Aldo RAV	413	528
Aldo LAV	825	704

Table 2. Cortisol and aldosterone levels in selective sampling of inferior vena cava (IVC), right adrenal vein (RAV) and left adrenal vein (LAV)

	Baseline (ng/ml)	Post ACTH (ng/ml)
Cort RAV/IVC	1.1	1.17
Cort LAV/IVC	2.72	2.91
Aldo RAV/Cort RAV/Aldo LAV/cort LAV	1.26	1.83

Table 3. Cortisol and aldosterone levels in selective sampling of inferior vena cava (IVC), right adrenal vein (RAV) and left adrenal vein (LAV) (ratio)

The histological examination documented hypertrophy of the zona reticularis and the presence of paracortical micronodules and islands of medullary tissue in the cortex as shown in Figure 2.

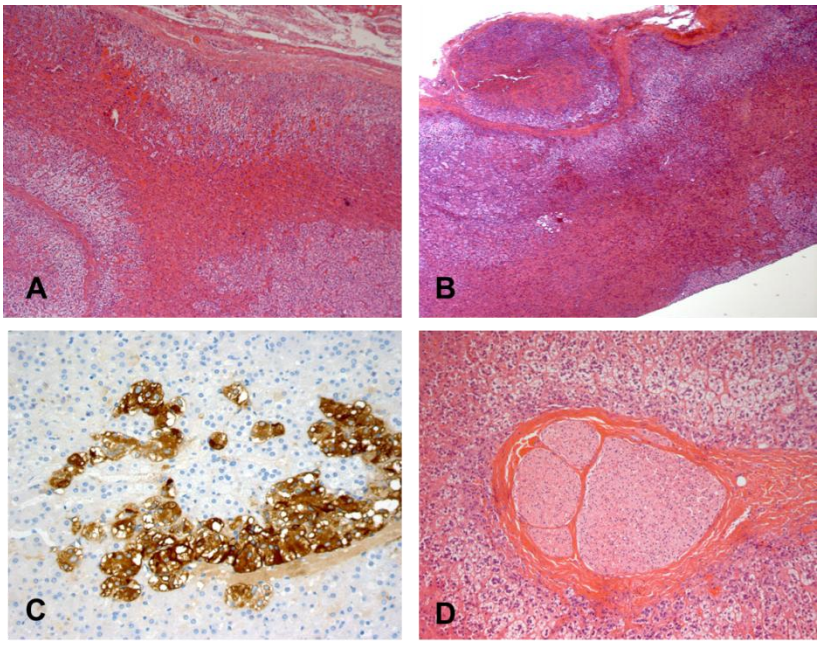

Figure 2. Right adrenal gland. A: Hyperplasia reticularis, B: paracortical micronodule; C: islands of medullary tissue in the cortex; D: neuronal tissue in the cortex

The follow-up was characterized by the maintenance of good BP values, but persisting mild hypokalaemia. Five years later, the patient was hospitalized again for an episode of severe tachycardia secondary to hypokalemia. The MR of the abdomen showed a further enlargement of the known left adrenal mass with radiological characteristics that were not typical for adenoma. The patient was therefore subjected to adrenocortical sparing surgery of the left adrenal mass. The histological diagnosis of the excised node was adrenal Pheo (positivity for Cromogranin A, sinaptofisina, eNSA, MIB-1, S-100) (Figure 3).

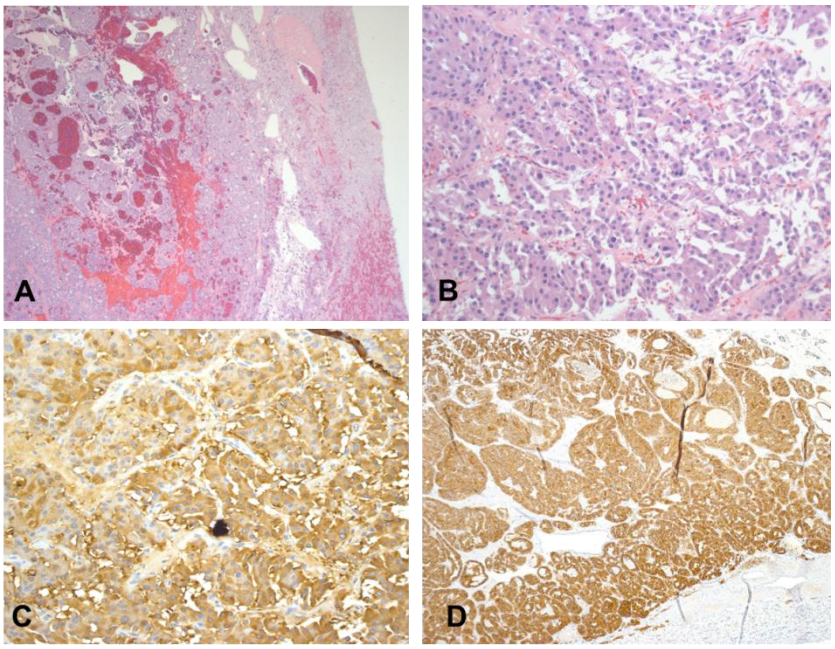

Figure 3: Left adrenal gland. A: Vascular gaps and areas of recent hemorrhage; B proliferating cells in cord arrangement with compression of the cortex; C:positivity for cormagranine A; D: positivity for synaptofisine

Comments: the probability of a casual coincidence of Pheo and PA in the same patient is extremely low. It could, therefore, be that the Pheo was the cause of hyperaldosteronism. In this regard, there are two possible ways to explain the pathophysiology: A) the production of catecholamines by the Pheo induces the secretion of renin (by the stimulation of B2 receptors) and therefore cause secondary aldosteronism or B) unknown mediators induced proliferation and hypersecretion of the zona glomerulosa resulting in "primary hyperaldosteronism". A whole transcriptome analysis of the Pheo tissue of this lady revealed a marked overexpression of UII as compared to normal human adrenomedullary tissue.

(Biochemical and molecular analysis are fully explained in chapter 4.4).

4. Can the secretagogue effect of UII on aldosterone be proven in vivo?

There are many evidences in the literature that support the hypothesis of a cross-talk between adrenal medulla and cortex through paracrine mediators;[54] UII can entail such mediators as documented by many authors. The transcripts of UII and its receptor have been identified in four APA and seven Pheo and an increased UII-like immunoreactivity in one of six Pheo.[64] Immunostaining for both UII and UT-R was confirmed in in Pheo, and for UII in the adrenal medulla and to a lesser extent in the cortex and in adrenocortical tumors.[75] At variance, has been described also a lower UII and UT-R expression in Pheo tissue, compared with the normal adrenal tissue.[67] Therefore, it is not only contentious whether UII is differentially expressed between the normal adrenal cortex and medulla and between Pheo and APA, but also, more importantly, whether UII played any functional role in the adrenal gland. The finding of UII and UT-R gene transcripts in APA and Pheo[64] suggested the participation of UII in the regulation of blood pressure and body fluid homeostasis.

4.1 Expression and functional role of UII and its receptor in the adrenal cortex and medulla

Based on the evidences reported above, we have conducted a study in 22 patients affected by APA and in 10 patients affected by Pheo aimed to identify the expression of UII and UT-R in adrenocortical and adrenomedullary tumors and the functional effects of UT-R activation.

Therefore, molecular techniques were used to quantify precisely the UII and UT-R transcripts in APA and Pheo and to pinpoint UII and UT-R related pathways in adrenocortical and adrenomedullary tumors. The presence of the UII peptide was

investigated by immunoblotting and immunohistochemistry in APA and Pheo. Finally, the role of UT-R activation on the adrenocortical expression of aldosterone synthase (CYP11B2) and 11_ hydroxylase (CYP11B1) was investigated by means of an *in vivo* UII infusion in rats taking advantage of palosuran. Adrenocortical tissues from 22 patients with APA and adrenomedullary tissues from 10 patients with Pheo were investigated. Histologically normal adrenocortical (NAC) and normal adrenomedullary (NAM) tissue obtained from six patients with renal cancer carcinoma undergoing unilateral nephrectomy and ipsilateral adrenalectomy and from five patients adrenalectomized for non functioning incidentally discovered adrenal mass ("incidentaloma") were studied as controls for the APA and Pheo tissues, respectively. The tissues obtained under sterile conditions at surgery were immediately frozen in liquid nitrogen and stored at -80 C until they were used as described.[76]

UII and UT-R mRNAs were measured with a real time RT-PCR; UII and UT-R expression in APA and Pheo was calculated relative to porphobilinogen deaminase, used as an internal control, and to the control pools of NAC and NAM tissue, used as calibrators, for APA and Pheo, respectively.

Two-color microarray was used to evaluate gene expression**;** immunoblot analysis with a goat polyclonal antibody was used to investigate the expression of UII and UT-R at the protein level.

To investigate whether the UT-R activation up-regulates the adrenocortical expression of the CYP11B2 and CYP11B1, UII (600 pmol/kg/h)[77] was infused by osmotic minipumps (model2ML2;Alzet, Palo Alto, CA) either alone or on top of the UT-R antagonist palosuran (300mg/kg) into normotensive male Sprague Dawley rats (of about 250 g body weight; n:11). Angiotensin II (Ang II) (700 µg/kg/d) was infused in parallel as positive control. After 1-week infusion, the rats were killed, the adrenal gland was snap-frozen in isopentane, precooled on dry ice, and then stored under liquid nitrogen

until used for immunohistochemistry and gene expression studies. Specific antibodies against the rat CYP11B2 or CYP11B1[78] were used for immunohistochemistry. Negative controls were performed by omission of the primary antibody. Sections were mounted and examined at 5X magnification, using a Leica DQ optical microscope. The immunoreactive area was then calculated with a specific routine developed to detect in an operator-independent fashion the brownish staining as percentage of the total adrenal cortex. Plasma aldosterone concentrations were measured before and after the UII infusion as described.[77]

The Pheo exhibited higher UII mRNA content than the APA, which showed the lowest level among all adrenal tissues examined. It is noteworthy that an opposite expression profile of UT-R transcripts across adrenal tumors was detected: the APA showed higher mRNA levels than the Pheo and the normal adrenal medulla, suggesting down-regulation of UT-R when UII expression is enhanced and vice versa. These differences suggest a pathophysiological role of UII in the adrenal gland.

Our studies also showed the UII peptide and the UT-R protein in the normal adrenal cortex and in the APA and Pheo tissue. At immunoblotting, besides the expected 43-kDa band (detected in the human myocardium), we found two additional bands of 60 and 80 kDa in all adrenal specimens. The disappearance of the 60-kDa band after protein deglycosylation indicates the occurrence of a glycosylated form of UT-R in the adrenal. The persistence of the 80 kDa after this treatment in APA suggests the presence of UT-R dimerization, a finding that deserves further specific research.

Overall, the UII overexpression in Pheo suggests a major role of UII in the pathophysiology of these tumors and particularly in a subset of them that have been causally associated with primary aldosteronism.[79-83]

4.2 Detection and localization of UII in pheocromocitoma tissue and related pathway

Immunohistochemistry confirmed that UII peptide is synthesized in Pheo and furnished novel information on its localization. It showed a specific staining of clusters of Pheo cells, sometimes arranged in nests and located in perivascular regions. Moreover, we identified 18 sequences with an expression profile similar to that of UT-R, e.g. high in APA and low in Pheo. Of these, two pertain to G protein-coupled receptors: the galanin receptor 3 (GALR3) and G protein-coupled receptor 153 (GPR153). GALR3 binds galanin, a neuropeptide that is synthesized in the adrenal medulla, and Pheo and is involved in the regulation of adrenocortical function.[84] GPR153 encodes an orphan receptor not characterized as yet. Of the other sequences, SOX1 and SOX17 entail transcription factors belonging to a class of genes known to be expressed during adrenal fetal development.[85] Therefore, these findings emphasize the importance of the interactions between the medulla and cortex and implicate these novel mediators in the UII-related molecular mechanisms.

4.3 Effects of UT-R activation in the rat adrenal cortex

We explored the functional effect of a chronic UT-R over activation on CYP11B2 or CYP11B1 expression with a 1-week infusion of UII in the presence or absence of an UT-R antagonist in normotensive rats. This experiment was made possible by the development of specific antibodies for these enzymes[78] and the discovery of the UT-R antagonist palosuran.[33]

UII markedly increased the plasma concentration of aldosterone and the CYP11B2 expression in the zona glomerulosa; this latter effect was blunted by palosuran, thus indicating that it occurred via UT-R activation. Moreover, the UII-induced stimulation

of CYP11B2 is likely to be physiologically relevant because the result was more potent than that elicited by a high dose of Ang II, the most widely known secretagogue for aldosterone. Hence, our data support the hypothesis that UII synthesized in the adrenal medulla, and to a larger extent in Pheo, stimulates aldosterone secretion by specifically turning on the expression of the CYP11B2 gene in the zona glomerulosa (Figure 4, 5).

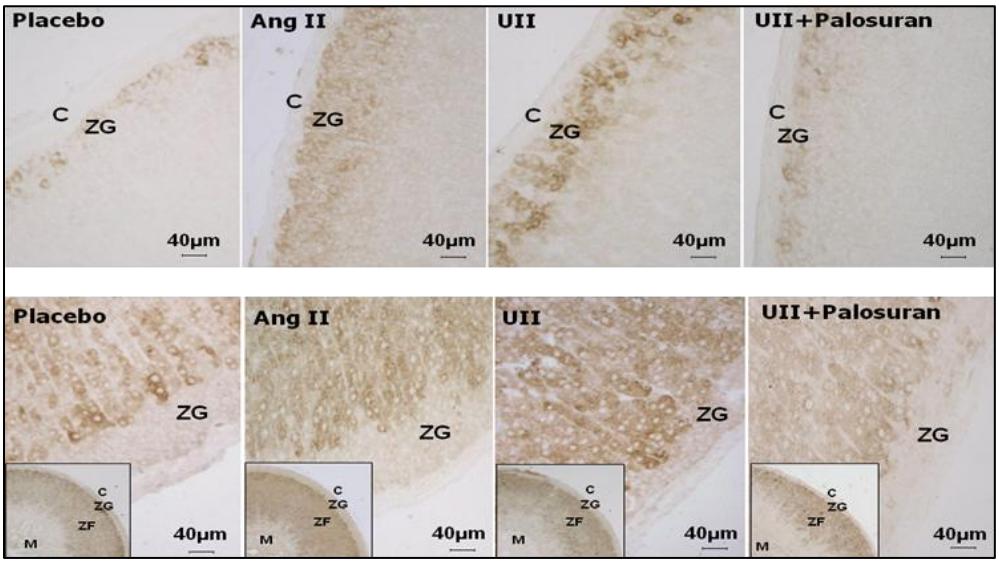

Figure 4: Immunohistochemistry shows staining of the rat adrenocortical zona glomerulosa (ZG) with a specific antibody for the CYP11B2

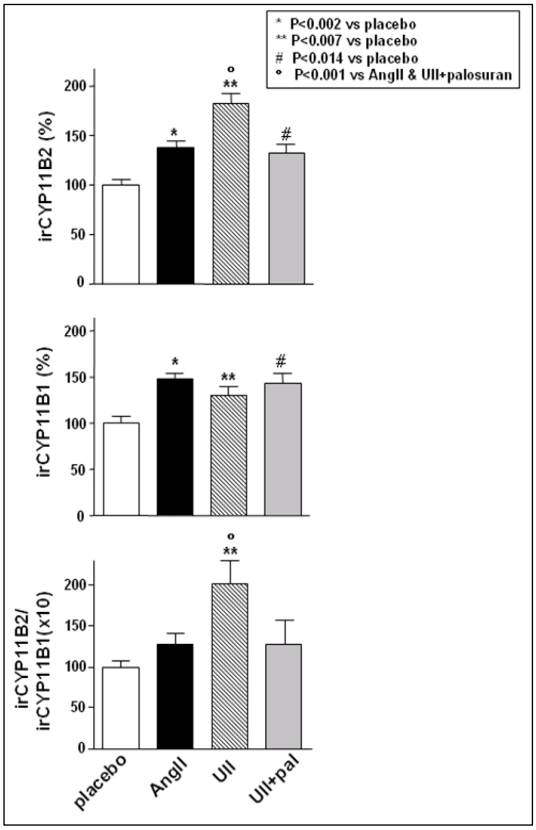

Figure 5. The histograms show results of quantification of the percentage immunostaining performed in an operator-independent way. B. The ratio of CYP11B2/CYP11B1 immunostaining showed a significant (*, $P < 0.05$) increase only for the group infused with UII

The results of this experiments showed the expression of UII in the normal human adrenal gland, in APA and, at a higher level, in Pheo tissue. The opposite trend of expression of UT-R between APA and Pheo, along with the differences of genes implicated in UII signaling, supports a role of UII in the paracrine interactions between the adrenal medulla and cortex. Being relevant for the regulation of adrenal gland function and for the pathophysiology of Pheo and APA, these interactions might provide a mechanistic explanation for the Pheo that presented clinically as aldosteronism.[79-83]

4.4 Biochemical and molecular assessments

4.4.1 Measurement of UII and UT-R mRNAs

We measured UII and UT-R mRNAs with 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) in the LightCycler® 480 Instrument (Roche, Monza, Italy). 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. 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. 2μL of each RT reaction was amplified with specific primers in a final volume of 20 μL. Porphobilinogen deaminase (PBGD), an accepted housekeeping gene for both adrenocortical and adrenomedullary gland gene expression studies, was similarly processed to normalize for RNA quality, quantity, and RT efficiency.[86]

The specificity of the amplicons was verified by sequencing analysis. The PCR products were cleaned by GenElute TM PCR Clean-up Kit (Sigma-Aldrich Corp., St. Louis, MO), and sequenced 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 samples. 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-ΔΔCt) method.[87] Standard curves were generated 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.

4.4.2 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. 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 in the soluble supernatant with Lowry's method, using bovine serum albumin as standard.[88] 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 a 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) and thereafter. The membranes were 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) and then

After washing three times for 15 min the membranes were 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).

4.4.3 Two-color microarray-based gene expression

Total RNA was isolated from frozen tissue using RNeasy Mini Kit (Qiagen, Milan, Italy). The integrity of RNA was systematically checked using the lab-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Furthermore the purity was determined by spectrophotometric readings at 260/280/230 nm. 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).[89] 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) dyes 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.

4.4.4 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). 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-fold to identify over- and under-expressed genes. Ontology analysis of sequences differentially expressed was performed using the Gene Ontology annotation.[90]

4.4.5 Immunohistochemistry

Immunohistochemistry was used to confirm the UII expression at the protein level and to visualize its distribution in the pheochromocytoma tissue serial 7-µm slices of mounted Pheo were dehydrated three times in xylene and rehydrated in serial dilution of ethanol. 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), as described.[89] 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.

4.4.6 UT-R activation in rats

After 1 week infusion (UII (600 pmol/Kg/h), either alone or on top of the UT-R antagonist palosuran (300 mg/Kg) by osmotic Alzet mini-pumps and Ang II (700 pmol/Kg/h) in parallel as positive control [91]) 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).[92]

Tissue sections were processed as described for human Pheo. Binding was detected with primary antibody diluted 1:50 in BSS was performed overnight 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 with a specific routine that was developed to detect the brownish staining in an operator-independent fashion as percent of the total adrenal cortex.

5. Does UII determine transient increase of blood pressure by stimulating aldosterone secretion?

5.1 Analogies between blood pressure trend in chronic infusion of UII and chronic infusion of aldosterone

What emerged in our studies regarding the expression of UII and its receptor (UT-R) in adrenal tumors suggests a key role UII as regulator of adrenal secretion and on development of cortical tumors. This strengthened the hypothesis that the mechanism of action of UII is closely connected with RAAS.

We have investigate the effects on BP in rats chronically infused with UII. UII was infused via osmotic Alzet minipumps at a dose of 600 µmol/Kg/h. BP was continuously monitored by an invasive telemetric system through intra-abdominal aortic blood probes as detailed described on chapter 5.2.2. With this system it was possible to have a continuous and not intermittent monitoring of BP obtaining a high quantity of values without losing any, albeit transient, BP variation.

Figure 6 shows one typical plot that we obtained regarding the BP trend since the beginning of UII infusion.

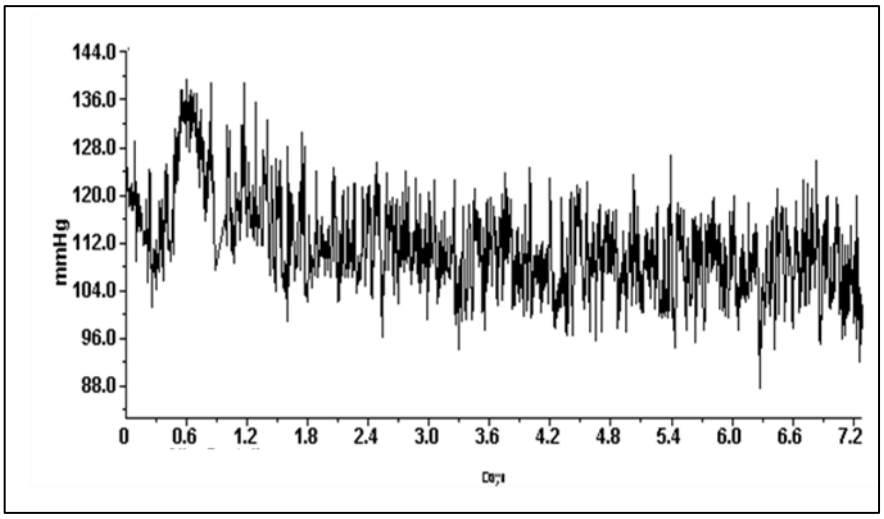

Figure 6. Blood pressure trend in chronic infusion of UII in rats

This BP plot was very interesting in our opinion since it shows a rise in BP that was detected approximately 24 hours after the beginning of administration of UII followed by a subsequent adaptation to the baseline BP values. This was in contrast with the other "*in vivo*" studies since for the first time led to suspect a even transient systemic pressor effect of UII.

We observed that the BP trend in rats chronically infused with UII looks similar to the BP trend in chronic infusion of aldosterone[93] (Figure 7) and this further supports the hypothesis of interaction between UII and RAAS or even that UII acts through production of aldosterone as well as assess its hypertensive activity, its effect of cardiac hypertrophy and fibrosis and its role in regulating the growth of adrenocortical cells in rats.

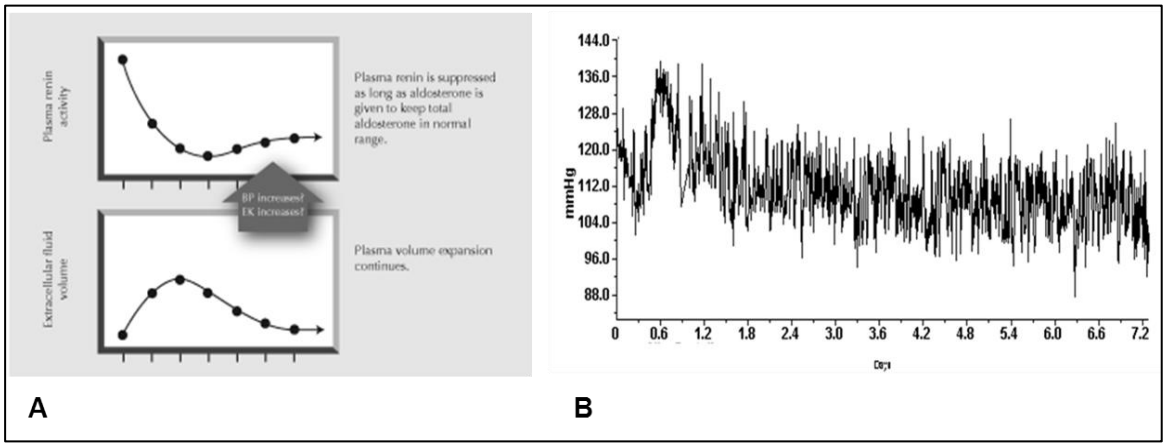

Figure 7. The figure highlights the analogy between the development pressure in the chronic infusion of aldosterone (A) as assess by Grim CE [93] and in chronic infusion of Urotensin II (B) Both trends are characterized by an initial hypertensive peak followed by a phase of adaptation returning to the baseline blood pressure values. This trend in the case of chronic infusion of aldosterone is attributable to the renal "escape phenomenon".

The BP curve in both chronic infusion of aldosterone and UII tends to settle on the baseline values after an initial increase of BP, and this in cases of hyperaldosteronism is attributed to the onset of the mechanisms that regulate the renal phenomenon of "escape": after the initial hypertensive state secondary to the reabsorption of sodium and water, the subsequent natriuresis induces a reduction in volume resulting in return to

normal blood pressure values. Thus, if our hypothesis is true, we expect that eliminating the effect of escape phenomenon the pressure curve in the chronic infusion of UII will present the disappearance of the adjustment of BP after the initial pressor effect.

5.2 Evaluation of interactions between UII and the renin-angiotensin aldosterone system by chronic infusion of UII in rats

The results just shoen along with those from studies on adrenal tumors, reinforced the hypothesis that UII is a putative mediator of the effects of the adrenal medulla on the adrenocortical zona glomerulosa, playing a key role in regulating the growth of adrenocortical cells. Moreover, this pathophysiological link might account for the reported causal relationship between pheochromocytoma and primary aldosteronism.[41]

The contention that UII acts through the RAAS or that UII might increase via unknown mechanisms the production of aldosterone has been also supported by our previous evidences in which it was shown that the BP trend in rats chronically infused with UII was similar to that of chronic infusion of aldosterone (figure 7B).

Based on these considerations we aimed to investigate among the mechanism of action of UII evaluating any possible change in BP trend once counteracted the mechanisms that affect the physiological development of blood volume and pressure in states of hyperaldosteronism.

In fact if in states of hyperaldosteronism (such as chronic infusion of aldosterone) is observed a progressive increase in BP related to the reabsorption of sodium and water followed by a gradual return to the baseline pressure values due to the mechanisms of renal escape (figure 7A,B), assuming an interaction between UII and RAAS, we expect a change in blood pressure trend in chronic infusion of UII by removing the renal phenomenon of escape. We tested this hypothesis on four groups of rats, each one consisting in chronic infusion of UII in association to a concomitant system to antagonize the RAAS and the "escape" assessments.

5.2.1 Study design

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), 15 weeks of age (main weight 427g ± 59.8) were divided into four groups of twelve animals each consisting in 6 cases and 6 controls; each case was treated by infusion of UII (600pmol/kg/h) each control by infusion of vehicle. In each group was set up a system aimed to antagonize the renal adaptation to the hyperaldosteronism (escape phenomenon) or to abolish the aldosterone production. The effect of renal escape was counteract through unilateral nephrectomy, thus reducing by 50% the natriuretic renal capacity in association with the administration of diet high in sodium to counteract the effect of the natriuresis. Administration of spironolactone was also used to counteract the natriuresis, while bilateral adrenalectomy was performed in order to abolish the aldosterone production. Four groups of rats were identified:

- Group 1 unilateral nephrectomy (left kidney) and very high sodium diet (4% NaCl)
- Group 2 unilateral nephrectomy (left kidney) and high sodium diet (2% NaCl)
- Group 3 bilateral adrenalectomy
- Group 4 concomitant administration of spironolactone

Originally it was thought that only a group with unilateral nephrectomy and hypersodic diet (NaCl 4%) could allow to solve the question. However, considering the high mortality of the rats probably secondary to the high salt concentration and the consequent dehydration we found it necessary to prepare another group to be submitted to a milder hypersodic diet (2% NaCl, group 2). The hypersodic intake was in fact achieved by giving a normal sodium diet and adding salt to drinking water prepared as distilled water plus NaCl. With 4% NaCl the rats did not dink enough and therefore died of dehydration. The lower saline concentration (2% NaCl in group 2) was better tolerated. BP was continuously monitored through intra-aortic blood probe via DSI

telemetry system (as described in details in chapter 5.2.2). At the time of intra-abdominal probe implantation was also performed left nefrectomy or bilateral adrenalectomy according to the different group of treatment. UII (600 pmol/Kg/h) and spironolactone (20mg/Kg/die), were administered subcutaneously by osmotic mini-pumps (model 2ML1, 7-day infusion, Alzet, Palo Alto, CA) starting seven days after surgery; a skin incision at the interscapular region was performed on the animals under gas general anesthesia and the minipump was there implanted within the subcutaneous tissue. Alzet minipumps with saline solutions were used for controls. Plasma aldosterone, sodium and potassium were measured in blood samples collected from the tail vein at the end of the first week and the end of the experiment. Urinary collection and sampling were obtained by metabolic cages (blood pressure mesurements were not obtained in those rats). On day 14 the rats were anesthetized, weighed and killed. Hearts, adrenals and kidneys were removed, weighted and collected for histology. Left ventricle-to body weight (LV mass index) was calculated. Figure 8 resumes the study design.

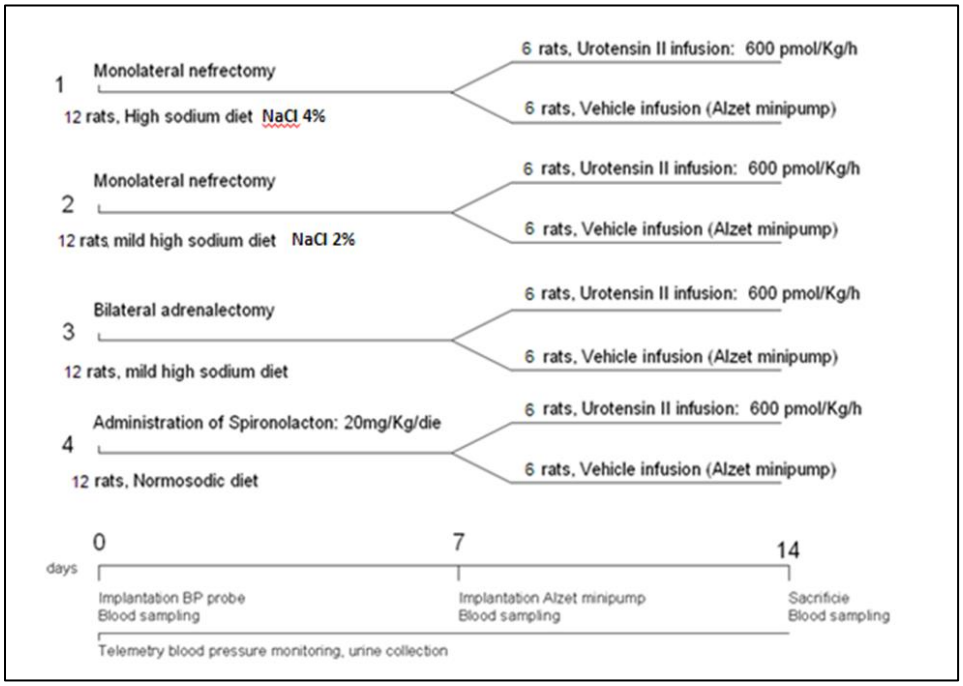

Figure 8. Study design

The protocol followed current guidelines (NIH publication no. 93-23, revised 1985) and was approved by the Institutional Animal Care and Use Committee.

5.2.2 Telemetry system for blood pressure monitoring

The Dataquest IV telemetry system (Data Sciences International, Arden Hills, MN) was used for the direct measurement of systolic, diastolic and mean blood pressure (MBP), through intra-abdominal aortic BP probes (Figure 9). The implantation of the probes was obtained in general gas anesthesia (isofurane) through a midline laparotomy and isolation of the abdominal aorta. After its clamping with a silk thread suspension, the aorta was cannulated above the carrefour with a 21G needle and then running through to introduce the tip of the probe inside the aorta. The small entry hole was sealed with 3M vetbond tissue adhesive (DSI) and a patch of cellulose (DSI). The probe body was fixed to the abdominal wall within the abdominal cavity by means of suture thread with flexidene. The rats were then placed individually in their cages, and were unrestrained and untethered. Systolic and diastolic blood pressure were monitored continuously at a sampling rate of 5 minutes during 24 h. After exclusion of outliers the mean daytime (rest time: 7 AM to 6:59 PM) and night-time values were calculated.

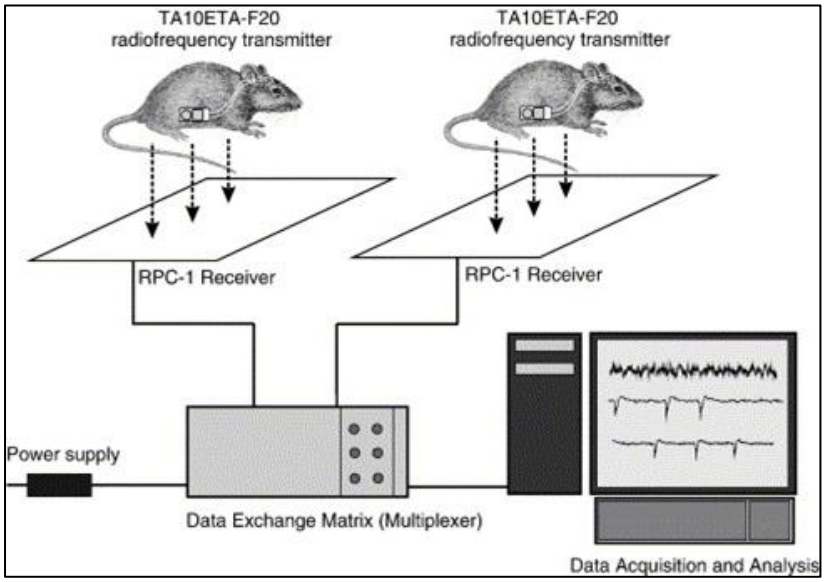

Figure 9. After the cannulation of abdominal aorta and the storage of the probe in the abdominal cavity the rats were placed on their cages and a telemetry system detected blood pressure through radio frequencies and ploted the values.

5.3.2 Blood Pressure trends and systemic response

Group 1 (UII + unilateral nephrectomy and diet added of 4% NaCl)

In this group the escape phenomenon was contrasted by a 50% reduction in renal function (unilateral (left) nephrectomy) in combination with hypersodic diet (water added of 4% NaCl). In this first group the rats showed bad general conditions from the beginning of the observation, the 4% saline solution administered proved to be poorly tolerated by the animals and the amount of water consumed per day was extremely little. which suggested that this amount of sodium in water was not appropriate and decreased markedly water intake. This group was characterized by high mortality (4 rats, 33%): one rat died because of intraoperative hemorrhagic shock during the implantation of the BP probe; 3 rats died during the observation, one of them had paraplegia of posterior extremities and the remained 2 died for unknown causes. Those that completed the 15 days treatment (8 rats (67%), 5 cases and 3 controls) showed very poor general conditions characterized by extremely low reactivity and very clear clinical signs of dehydration.

Mean baseline weight (measured at the day 7, corresponding to the beginning of UII infusion) was 410g while at the time of sacrifice it was reduced at 310g with a mean weight loss of 91g; both cases and controls showed weight loss at the time of sacrifice (mean weight loss for cases: -80g, mean weight loss for controls: -110g). Table 4 resumes weights among groups.

BP measurement was obtained in a total of 4 rats, 3 cases and 1 control. In cases treated with UII mean weekly BP during the day (MBP-day) was 115mmHg, and mean weekly BP during the night (MBP night) was 112mmHg. The only control treated showed surprisingly higher BP values than the cases (MBP-day: 140mmHg, MBP-night: 138 mmHg). Table 5 resume BP values among groups.

Despite the discrepancy relative to the average pressure values among cases and controls the time course of the pressure in the cases was exciting considering the tendency towards progressive increase and the disappearance of the chronic phenomenon of adaptation towards the initial values, as showed in Figure 10.

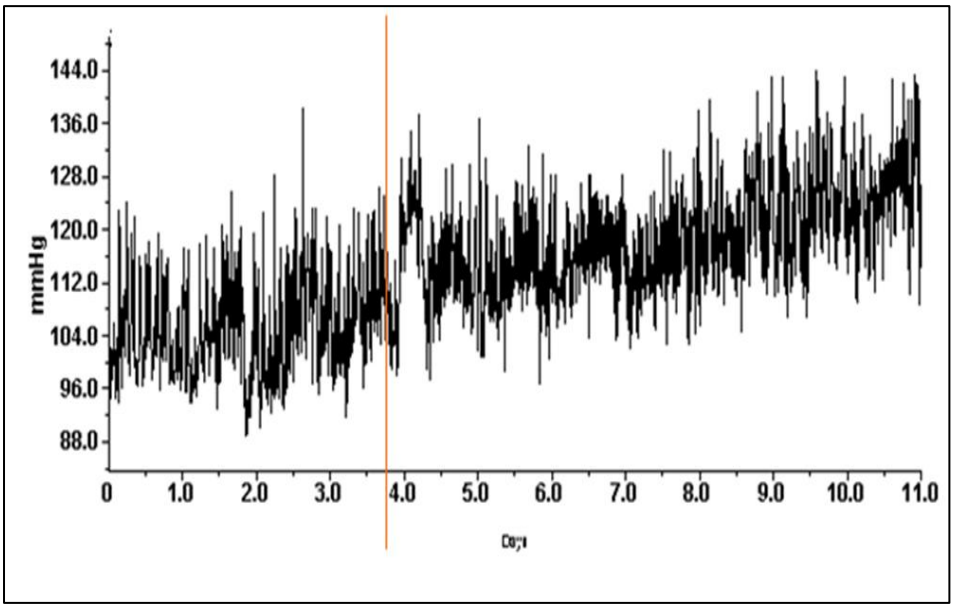

Figure 10. Blood pressure trend in a case treated by UII infusion + left nephrectomy and 4% NaCl diet (group 1). Red line point the beginning of UII infusion. Blood pressure values tend to increase over the time with the disappearance of the chronic adaptation to the initial level seen in it the UII infusion alone.

BP trend in the cases was characterized by a progressively increase of values with the disappearance of the initial 24h peak and the chronic adaptation to baseline values observed in the infusion of UII alone (figure 10). Thus, this result appeared to be consistent with the hypothesis of a possible interaction between aldosterone and UII seemed to be confirmed: the abolition of the escape effect led to a continuous chronic increase in blood pressure. However the comparison with the only control survived was disappointing since the mean blood pressure values were higher than cases even in absence of infusion of UII . Probably the baseline values in that rat was higher than the controls, or its renal function was still impaired and not excludible any problem

regarding the transducer. Despite the average higher pressure values the tendency to gradual pressure increase over time in this rat was less evident.

LV mean weight in group 1 was 0.73g, (0.72g for cases and 0.75 for controls) while the ratio LV over body weight (LV/BW) ratio was 2.3 for cases and 2.6 for controls.

Table 6 shows the left ventricle weights comparing the groups.

Group 2. (UII + unilateral nephrectomy and diet added of 2% NaCl)

In view of the results obtained in group 1 (especially with regard to mortality) it was decided to change the protocol and to introduce group 2 characterized by unilateral left nephrectomy in addition to mild hypersodic diet, giving water containing 1% NaCl in the first week after nephrectomy and implantation of the probe, and 2% in the last seven days.

The general conditions of rats in this group were globally better than those of rats in Group 1. The lower concentration of sodium chloride (2%) allowed the rats to drink a daily amount of water sufficient to avoid the heavy dehydration that characterized group 1. The mortality was 17% (2 rats) one case because of intra-operative complications during probe implantation, one during the treatment probably because of uroperitoneum. Final general conditions of rats were at the end the observation globally good, and this was confirmed also by the weight and blood pressure values analysis.

Mean baseline body weight of group 2 was 454g, and the final was 395g (with a total decrease approximately of 60g); the weight decrease was more evident in cases (less 80g from day 7 to day 14) but was globally lower than group 1. (Table 4)

BP monitoring was obtained in 3 cases and 3 controls with a MBP-day of 132mmHg and MBP-night of 124 mmHg for cases and respectively of 122 mmHg and 117 for controls (Table 5). The BP plots in cases receiving UII were very satisfying since the

BP trend was confirmed to be progressively increasing during the UII infusion, with the disappearance of the chronic adaptation to the baseline values. (Figure 11)

This confirmed that the infusion of UII in association with counteracting the phenomenon of escape (by unilateral left nephrectomy and high sodium diet) causes a progressive increase in BP with the disappearance of the phenomenon of chronic regression toward baseline values.

Also in this group controls documented a BP trend less homogeneous albeit with high mean values despite the absence of treatment and probably influenced by the increased concentration of NaCl (2%) in the diet.

Mean LV weight in this group was 0.76g (0.85g in cases, 0.65 in controls) and LV/BW ratio was respectively 2.03 and 2.01. (Table 6)

The survival rate and the general conditions of the rats completing the observation were significantly better than the group 1.

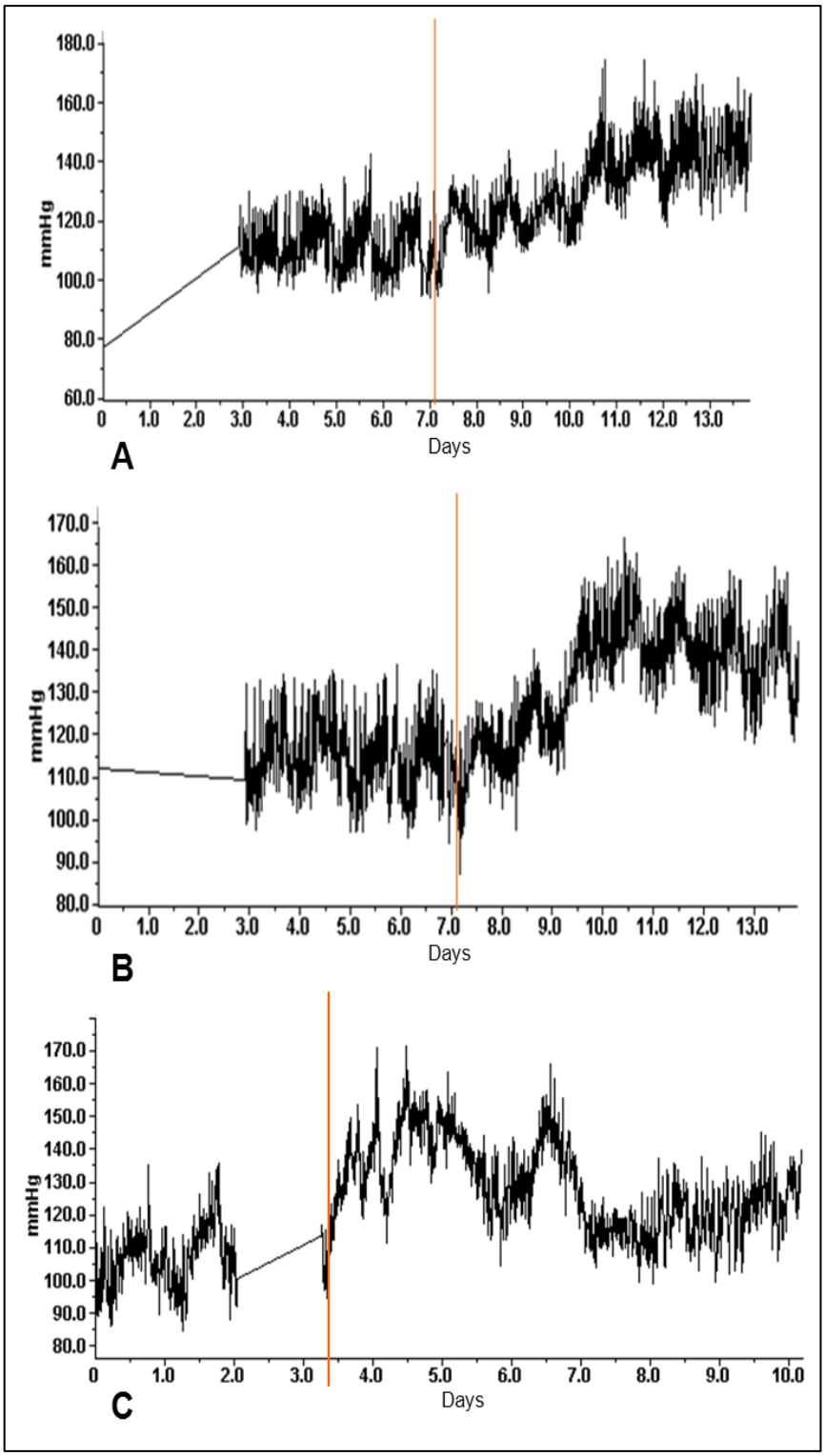

Figure 11. Blood pressure trend in two cases (A, B) and one control (C) of group 2 (left nephrectomy and 2% NaCl diet). The red line shows the beginning of UII infusion. In A and B the plots show a trend of continuous increasing of blood pressure values with the disappearance of the chronic adaptation to baseline values seen in UII infusion alone. The plot C (vehicle) documents a less regular pattern of blood pressure, although higher values than those of baseline, much less prone to the progressive increase

Group 3 (UII + bilateral adrenalectomy)

This group was planned to investigate among the effects of UII in absence of aldosterone production. Unfortunately, this group had the worst outcome. The mortality was extremely high (rising 83%, 10 rats on 12) and the majority of animals died two dyes after the adrenalectomy. Only two cases survived until the end of the experiment. No BP analysis was obtained in these two cases. Their final general condition were very poor showing an extremely reduced reactivity.

The mean body weight showed a little decrease (from 399g to 385g) and the LV mean weight was 0.74g with a LV/BW of 1.93.

Group 4 (UII + spironolactone)

In this group, the escape phenomenon was antagonized by co-administration of spironolactone at a dose of 20 mg/kg/die at the same time of UII via Alzet minipump.

The mortality in this group was 25% (3 over 12 rats enrolled) with a total of 6 cases and 3 controls survived till the end of the experiment.

General conditions of rats, in term of reactivity and blood pressure values, were good during all the days of observation, and this was the only group in which we could document a global increase of the final body weight (plus 10g from the baseline), due to an increase of about 30g in cases against a similar weight loss of controls (Table 4).

BP monitoring was obtained in 4 cases showing a global maintenance of baseline values, with a MBP-day of 100mmHg and MBP-night of 100mmg. The plots showed the absence of the chronic increase in blood pressure as observed in the previous groups while an initial hypertensive peak (similar to the one observed in the infusion of UII alone) was detectable in all the four plots obtained. (Figure 12)

LV mean weight was 0.86g (0.86g in cases and 0.74 in controls, with a LV/BW respectively of 1.78 and 2.24).

The general conditions of the animals at the end of observation were good and the performance pressure proved to be stable, without the propensity to increase over time and maintaining that initial hypertensive peak observed at approximately 24 hours after administration of single continuous UII (figure 12).

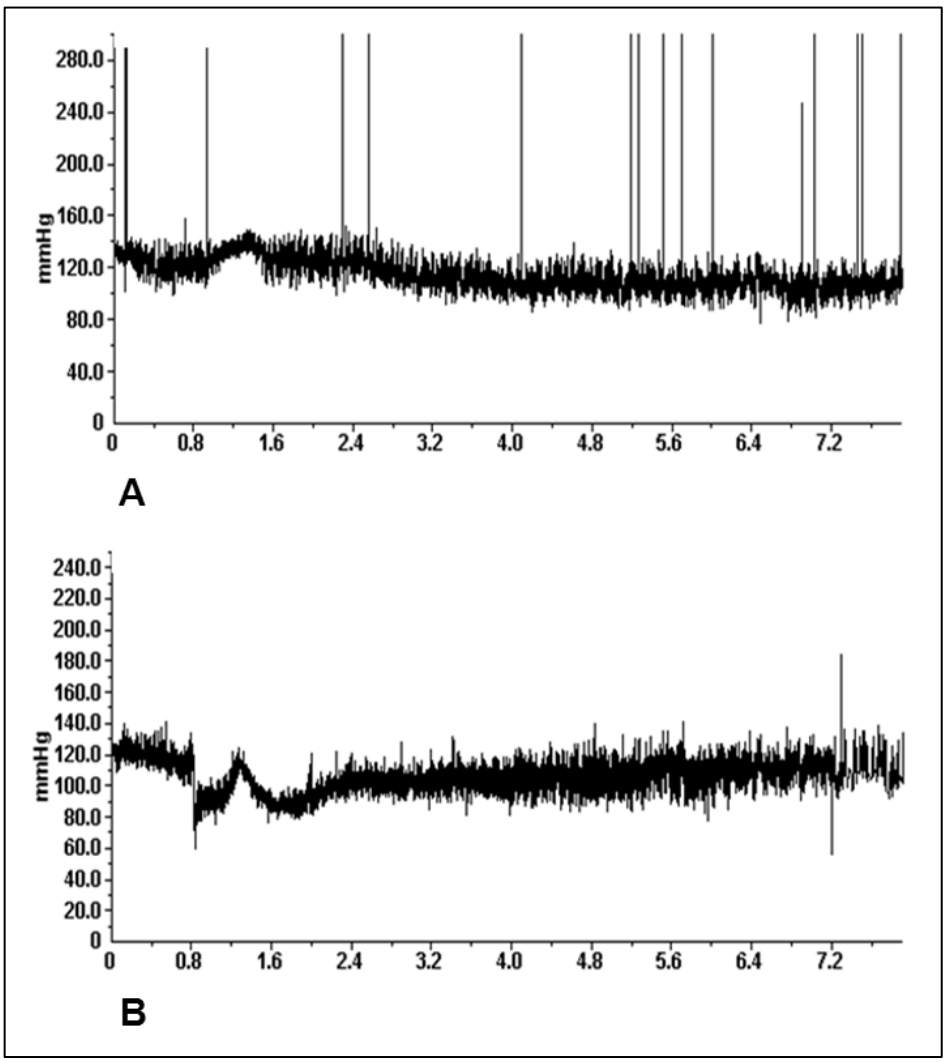

Figure 12. Blood pressure trend in concomitant infusion of UII and spironolactone (group 4). The plots denote a global maintenance of baseline values over the time, without tending to increase. It is evident an initial hypertensive peak as observed in the UII infusion alone

Groups	Global		Cases		Controls	
	Baseline weight	Final weight	Baseline weight	Final weight	Baseline weight	Baseline weight
1	401	310 (-91)	399	321 (-80)	403	291 (-110)
2	454	395 (-60)	501	421 (-80)	407	365 (-40)
3	399	385 (-16)	399	385 (-15)		
4	435	445 (+10)	446	476 (+30)	414	385 (-30)

Table 4. Mean body weight (expressed in grams) for each group and for cases and controls. In brackets the main variation among the beginning and the end of treatment.

		Global		Cases		Controls	
		Mean (mmHg)	**Std. Dev**	**Mean (mmHg)**	**Std. Dev**	**Mean (mmHg)**	**Std. Dev**
MBP-Day	**Group 1**	121	11.1	115	2.32	140	1.9
	Group 2	127	6.92	132	2.7	122	6.07
	Group 4	100	6.96	100	6.96	-	-
MBP-Night	**Group 1**	119	11.67	112	3.07	138	1.76
	Group 2	121	5.69	124	2.97	117	5.88
	Group 4	100	6.31	100	6.31	-	-

Table 5. Mean blood pressure during day (MBP-Day) and night (MBP-Night) among groups.

Groups	LV			LV / BW		
	Global	Cases	Controls	Global	Cases	Controls
1. (UII + NaCl 4%)	.73	.72	.75	2.4	2.3	2.67
2. (UII + NaCl 2%)	.76	.85	.65	2.03	2.03	2.01
3. (Adrenalectomy)	.74	.74	-	1.93	1.93	-
4. (UII + spironolactone)	.86	.86	.74	1.95	1.78	2.24

Table 6. Left ventricle (LV) mean weight expressed in grams for each group and for cases and controls. Left ventricle over body weight (LV/BW) expressed in absolute value for each group and for cases and controls.

5.2.4 Considerations on BP trends and statistical analysis

On a first analysis of the graphs is observed a trend of progressive increase of the MBP pressure in rats of groups 1 and 2, while in group 4, the pressure seems to adapt substantially to the values of baseline. The pressure peak that was observed within 24 hours from the beginning of single UII infusion seems less evident in groups 1 and 2 than in group 4.

Statistical analysis of BP has confirmed these observations, documenting the average values pressure (analyzed for day and night) significantly higher for groups 1 and 2 compared to group 4. (Table 5)

We made a statistical analysis of covariance normalizing the results for the BP values of the baseline and of the day of implantations that has confirmed the progressive increase in BP for groups 1 and 2 than in group 4. (Figure 13)

Among the unexpected data is the fact that have been obtained higher BP values have in rats belonging to the group 2 (2% NaCl diet) compared to rats belonging to the group 1 (4% NaCl data). It is likely that the 4%NaCl diet has played an important role in the dehydration of rats thus resulting in a volume depletion and hypotension.

The effect of spironolactone in influencing the development of BP in rats of group 4 was evident, however, in group 4 remains still evident the peak pressure to 24 hours after the start of administration of UII.

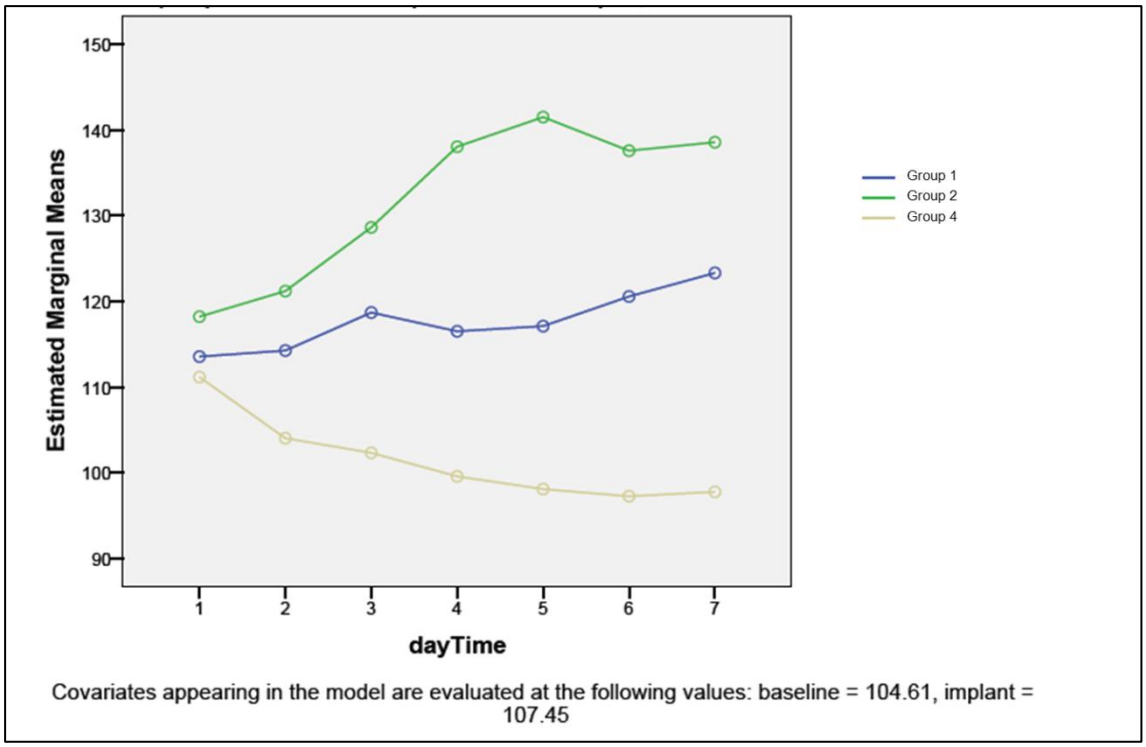

Figure 13. Covariate analysis of blod pressure trends in cases of group1, 2 and 4 normalized for baseline and implantation day values.

As regards the LV hypertrophy Table 6 shows that both in group 2 and in group 4 cases have a LV weight greater than the controls. In group 1 controls appear to have greater cardiac hypertrophy, probably secondary to the fact that controls in group 1 had higher blood pressure values. Looking at LV/BW ratio it was evident that the group with higher values was group 1: however, this can be interpreted as a "false hypertrophy" in consideration of the fact that these rats at the end of the experiment had a significant decrease in weight as excessively dehydrated (data confirmed also by controls that have a normalized ratio even higher despite the absence of UII).

5.2.5 Limitations of the study

Despite the results obtained regarding in particular BP trends this study still maintains many limitations. The main weakness of this study concerns the control groups, which are too few and sometimes inappropriate. This affected especially group 1 where the only control plotted probably had baseline characteristics so peculiar to confuse the results obtained. Despite the idea of suppressing the endogenous production of aldosterone by the bilateral adrenalectomy in group 3 seemed initially good we should have considered to supply the animals with exogenous corticosteroids (ex. pellets in continuous release of DOC) in addition to a mild hypersodic diet in order to avoid the acute hypoadrenalism that probably led to death the majority of the rats belonging to group 3. Moreover, it will be necessary to perform a further control group characterized by nephrectomy plus hypersodic diet and concomitant administration of both UII and spironolactone in order to prove the aldosterone mediated role in the pressure action of UII.

Notwithstanding these limitations this study documented for the first time the *in-vivo* pressure effect of UII. The fact that the trend of BP changes in situation of abolition of the "escape phenomenon" suggests that UII acts through the RAAS or at least that there is close communication between UII and RAAS.

These study, in combination with the evidences regarding the role of UII in the development of cortical tumors, has taken a step forward in identifying the mechanism of action of UII in supporting to the hypothesis of a possible close communication between medulla and adrenal cortex and a possible action of UII mediated by aldosterone.

6. Transgenic rat overexpressing UII

To further prove the concept of a paracrine interaction between the adrenal medulla and the zona glomerulosa involving UII, after two years of unsuccessful attempts , in collaboration with Prof. M. Bader at MDC (Max Delbrück Center for Molecular Medicine) in Berlin we finally generated a transgenic rat overexpressing UII in adrenal medulla. This animal model will provide us more information about the physiological role and mechanism of action of UII, particularly, we believe it will certainly clarify the real existence of a paracrine effect of UII within the adrenal gland and its close correlation with aldosterone secretion.

To obtain the specific expression of UII in the adrenal medulla, we generated a knock-in rat model with UII gene under the control of the promoter of phenylethanolamine N-Methyltransferase (PNMT), a gene held to be exclusively expressed in the adrenal medulla. The tissue-specific UII knock-in rat was obtained by the conventional transgenic method in which a DNA fragment containing the PNMT promoter linked to the UII coding sequence is microinjected into the pronuclei of fertilized rat oocytes. After the transfer the oocytes into foster mothers, the progeny expressing the UII coding sequence in the desired pattern was identified.

Currently we are going to characterize the first offspring of UII knock-in rat. The expression of UII gene will be assessed in adrenal medulla of the UII knock-in rat using quantitative real time PCR (qRT-PCR). To verify that UII is exclusively overexpressed in adrenal medulla immunohistochemistry analysis will be performed on adrenal gland sections.

After the assessment that the UII knock-in rat overexpress UII specifically in adrenal medulla, several experiments will be carry on in order to deepen the investigations about the effects of UII on blood pressure and aldosterone secretion.

We intend to use again the telemetry system for monitoring blood pressure in our animal model and real time PCR to investigate on the expression of steroidogenic enzymes in the adrenal gland.

We are confident that we will solidly confirm what we already demonstrated so far.

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