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VASCULAR BIOLOGY

CICLO XXI

HUMAN PRIMARY ALDOSTERONISM; INVESTIGATION OF THE MECHANISMS LEADING TO “AUTONOMOUS” ALDOSTERONE EXCESS

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Riassunto

Obiettivo: Alcuni studi hanno suggerito che l'espressione genica CYP11B2 è più elevata in pazienti con APA rispetto ai soggetti normali, tuttavia non esistono anticorpi in grado di identificare questa proteina. Quindi abbiamo deciso di effettuare studi di ibridazione in situ (ISH) per localizzare CYP11B2 a livello tissutale. Un altro obiettivo del nostro studio è quello di capire se la presenza di micronoduli possa influire sulla fisiopatologia dell'adenoma. La nostra ipotesi è che un fattore umorale stimolante potrebbe svolgere un ruolo nella genesi di APA.

Metodo: Abbiamo sviluppato un metodo non radioattivo basato sull'uso di una specifica sonda oligo per localizzare CYP11B2. Per la rilevazione dell'housekeeping gene, abbiamo utilizzato una sonda a doppio filamento per il gene PBGD, (NM_000190). Entrambe le sonde sono state marcate con Digoxigenin. Abbiamo inoltre misurato i livelli di anticorpi anti-AT1 nel siero di pazienti con APA e la loro localizzazione tissutale tramite IHC nella ghiandola surrenalica.

Risultati: all'istopatologia si sono evidenziati noduli satelliti che presentavano l'espressione del CYP11B2. L'ISH ha mostrato una colorazione specifica di tutti i noduli a livello prevalentemente nucleare. La presenza di Auto anticorpi anti AT1 è stata dimostrata con test ELISA nel siero, ma la localizzazione nel tessuto non ha ancora mostrato risultati apprezzabili.

Conclusione: questa tecnica non radioattiva di ISH dimostra in modo inequivocabile la presenza di micro satelliti produttori aldosterone. Elevati livelli di autoanticorpi nel siero dei pazienti con APA ci permettono di ipotizzare che questo disordine della ghiandola surrenale possa essere di origine autoimmune.

Summary

Objective: Due to its unique expression of aldosterone synthase cytochrome P450 (CYP11B2), the enzyme required for the final steps of aldosterone biosynthesis aldosterone is exclusively expressed in the adrenocortical zona glomerulosa cell. Some studies suggested that the CYP11B2 gene expression can be higher in APAs than in normal adrenocortical tissues, leading to postulate a transcriptional modulation of aldosterone overproduction in these tumors but antibodies capable to identify this protein do not exist. Thus we performed studies of in-situ hybridization (ISH) to analyze CYP11B2 localization. Another aim of our study is to understand whether the occurrence of micronodularity peripheral to the main adenomatous mass in some of our patient could be involved in the pathophysiology of the adenoma. Our hypothesis is that a similar humoral stimulating factor could play a role in the genesis of APA.

Method: We developed a novel non radioactive in-situ hybridization technique based on use of a CYP11B2 specific oligo probe to localize the specific transcripts of the CYP11B2. Adrenocortical tissue from patients was studied. As housekeeping gene, we used PBGD, (NM_000190) probe labeled with Digoxigenin. The oligonucleotide probe for the CYP11B2 was 5' conjugated with DIG. Negative controls was detected with probe not labeled. The second approach was measuring of serum auto-antibodies anti AT1 in patients with APA and their localization on adrenal gland tissue by IHC.

Results: At histopathology intensely labeled areas called "nodules producing aldosterone" and negative areas were detected in the APA analyzed. ISH showed specific staining of all nodules. The staining was predominantly nuclear, a finding consistent with a good preservation of the transcript at this level. The AAbodies' anti AT1 presence has been demonstrated with ELISA test but the tissue localization still did not show appreciable results.

Conclusion: this novel non radioactive ISH technique unequivocally demonstrated the presence of aldosterone-producing microsatellites. High levels of AutoAntibodies in the serum of patients with APA allow us to hypothesize that this adrenal gland disorder is autoimmune.

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INTRODUCTION

Anatomy of the Adrenal Gland

The two adrenal glands are located immediately anterior to the kidneys, encased in a capsule of connective tissue and are usually found under a layer of peri-adrenal fat. Like the kidneys, the adrenal glands lie beneath the peritoneum, e.g. they are retroperitoneal. However, the exact location compared to the kidney and the shape of the adrenal gland can vary across species. The weight of normal glands is 4-6 grams each, after the dissection of the fat, and concerning the size, the normal adrenal gland is about 5 x 3 x 1 cm.

The adrenal arteries are multiple and result from many different ramifications, which are mainly ramifications of the inferior diaphragmatic arteries. This is a network of vessels that are located above the renal vessels. Three main groups can be identified:

1. The superior adrenal artery
2. The media adrenal artery
3. The inferior adrenal artery

The adrenal veins, have a simpler organization. The right adrenal vein is very short, with an almost horizontal course, and drains directly into the inferior vena cava. The left adrenal vein is longer and has a nearly vertical course. Often, the inferior phrenic vein joins the left adrenal vein forming a common trunk, termed the pheric-adrenal that runs toward the left renal vein. Other times, the left adrenal vein receives renal venous ramifications. The gonad vein and lumbar veins may have connections with the left adrenal vein as well this anatomic pattern has profound

implications from the clinical standpoint for performing adrenal vein sampling, which is the “gold” standard for identifying the surgically curable subtypes of primary aldosteronisms.

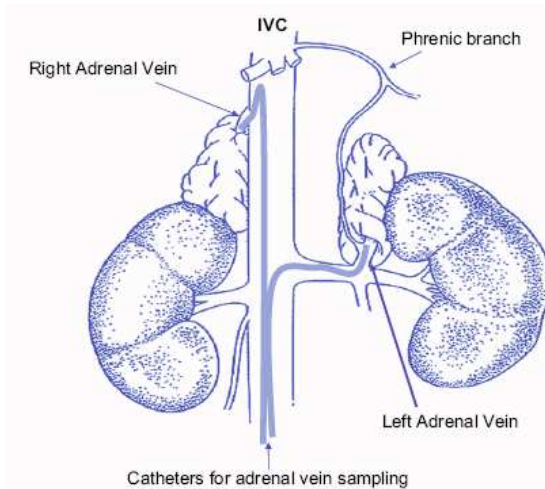


Fig. 1
Schematic representation of vein of human adrenal gland. In this scheme is possible observable the selectivity of catheterisation.
IVC = inferior vena cava

The mature mammalian adrenal cortex has three distinct zones, termed zona glomerulosa, zona fasciculata, and zona reticularis. They were originally described in 1866 by Arnold, (1) and have different cellular differentiation and specific steroids synthesized:

Zona glomerulosa: The zona glomerulosa lies just beneath the adrenal capsule and it is composed of small clusters containing less cytoplasm than other cortical cells. **Zona fasciculata:** The zona fasciculata forms broad band of large cells with distinct membranes arranged in cords of about two cells wide. Their cytoplasm has numerous small lipid vacuoles which may indent the central nucleus and resemble lipoblasts; 70-80% of cortical volume and lipid stores are depleted by ACTH. **Zona reticularis:** The zona reticularis is composed of organized cells, which are smaller than zona fasciculata cells. These cells have granular, eosinophilic cytoplasm and lipofuscin but minimal lipid and are thinner than zona glomerulosa or fasciculata.

Adrenal Medulla

The adrenal medulla is composed of neural crest cells called chromaffin cells (also called pheochromocytes, medullary cells). The chromaffin cells are arranged in small nests and cords separated by prominent vasculature. They comprise large polygonal cells with poorly outlined borders, abundant granular and usually basophilic cytoplasm. They show mild variation in cell size.

The adrenal medulla is a source of the catecholamines epinephrine and norepinephrine and of several other factors including vasopressin, oxytocin, galanin, neuromedine-N, neuropeptide-Y, chromogranin-A, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) and urotensin II . The chromaffin cell is the principle cell type. The medulla is richly innervated by preganglionic sympathetic fibers and is, in essence, an extension of the sympathetic nervous system. An outer cortex, which secretes several classes of steroid hormones (glucocorticoids and mineralocorticoids, plus a few others).(2)

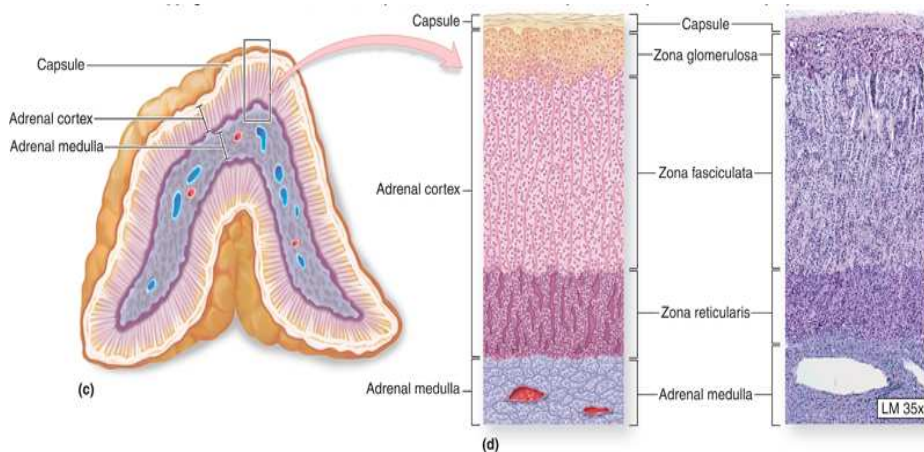


Fig.2 human adrenal gland zonation

Steroidogenesis

The secretion of the adrenal cortical hormones is controlled by a region of the brain called the hypothalamus, which releases a corticotropin-releasing hormone and, for the ZG, by the RAS. This hormone stimulates the release from the anterior pituitary of adreno-corticotropin (ACTH), which, in turn, enters the blood and targets the adrenal cortex. There, it binds to receptors on the surface of the gland's cells and stimulates them to produce the steroid hormones. (3) The adrenal steroids have either 19 carbon atoms (androgens), or 21 carbon atoms (glucocorticoids and mineralocorticoids). These hormones can be classified into three main classes, glucocorticoids, mineralocorticoids, and corticosterone.

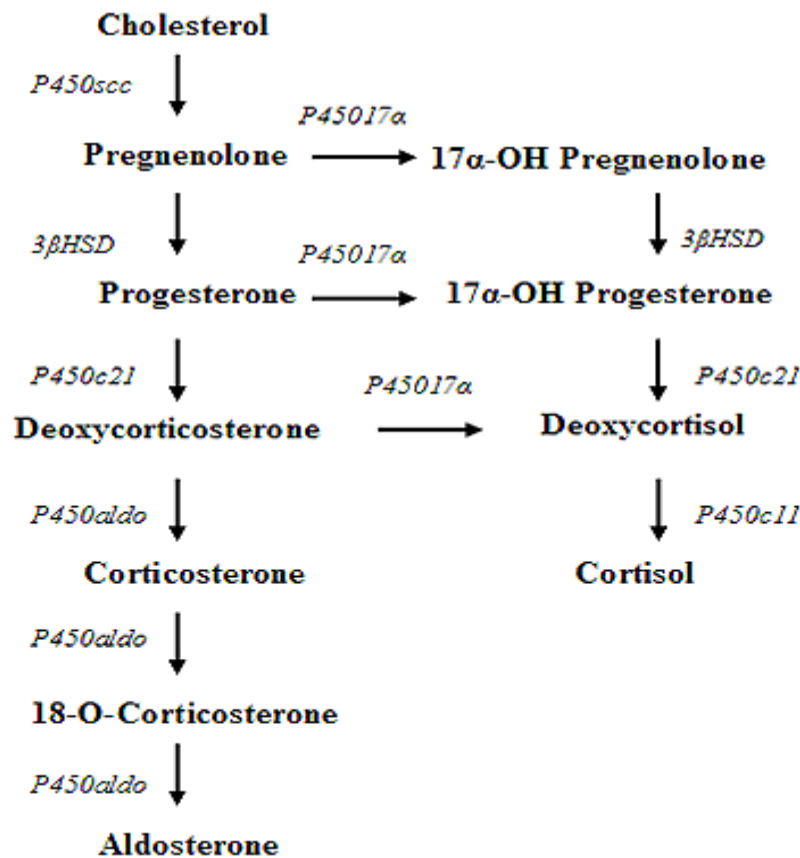


Fig. 3 Steroidogenesis (4)

The mineralocorticoids are essential for maintaining the balance of sodium in the blood and body tissues and the volume of the extracellular fluid in the body. Aldosterone, the principal mineralocorticoid produced by the zona glomerulosa, enhances the uptake and retention of sodium in cells, as well as the cells' release of potassium. This steroid also causes the duct collectors cortical of the kidneys to retain sodium in exchange for potassium of hydrogen, thus maintaining levels of this ion in the blood, while increasing the excretion of potassium into the urine. Simultaneously, aldosterone increases reabsorption of bicarbonate by the kidney, thereby decreasing the acidity of body fluids and causing metabolic alkalosis, which is measured by loss of hydrogen. A deficiency of adrenal cortical hormone secretion causes Addison's disease.

Mineralocorticoid release is also influenced by factors circulating in the blood. The most important of these factors is angiotensin II, the end product of a series of steps starting in the kidney. When the body's blood pressure declines, this change is sensed by a special structure in the kidney called the juxtaglomerular apparatus. In response to decreased pressure in kidney arterioles the juxtaglomerular apparatus releases an enzyme called renin into the kidney's blood vessels. There, the renin turns angiotensinogen to angiotensin I, which undergoes a further enzymatic change in the bloodstream outside the kidney to angiotensin II.

Ang II stimulates the adrenal cortex to release aldosterone, the increased concentration of sodium in the blood-filtering tubules of the kidney causes an osmotic movement of water into the blood, thereby increasing the blood pressure.

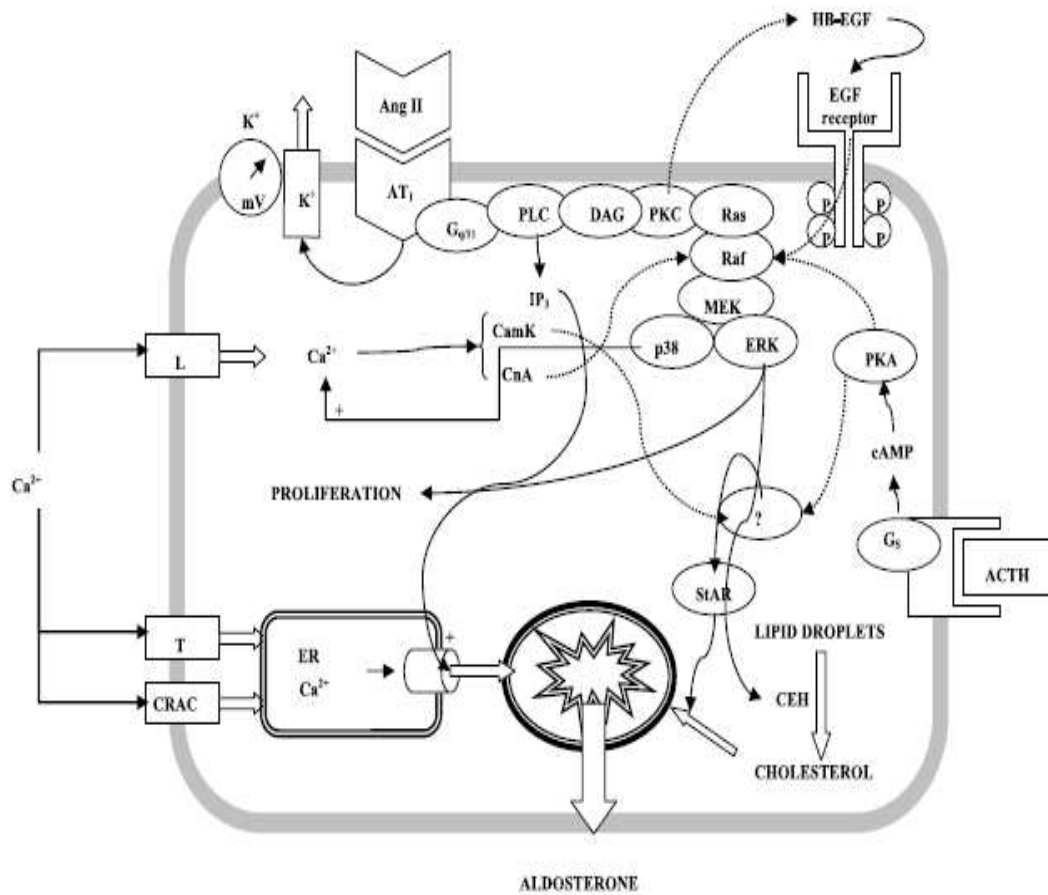


Fig. 4 Model for signalling pathways, interactions and regulators in adrenal ZG cells

Molecular Regulation of Steroidogenesis

In humans, functional zonation relies on the zona-specific expression of two cytochrome P450 isozymes:

- CYP11B1 (11beta-hydroxylase) which catalyzes the final steps in the biosynthesis of cortisol
- CYP11B2 (aldosterone synthase) which catalyzes the final steps of aldosterone

These genes are located on chromosome 8q22 and composed of 9 exons.

(5)

chromosome: 8; Location: 8q21-q22 [CYP11B2 and CYP11B1](#)

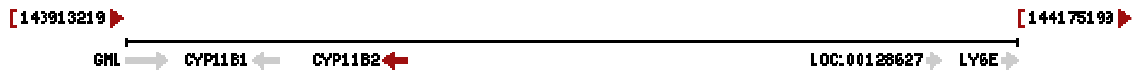


Fig. 5 Cyp11b1 and Cyp11b2 chromosome location NCBI

The nucleotide sequence is identical in 95 % of the coding regions and in 90 % of the introns and the proteins are of 479 aminoacids.(6)

In the glomerulosa the 11 β -hydroxylation of 11-deoxycorticosterone to corticosterone and its subsequent 18-hydroxylation and 18-oxidation are accomplished by aldosterone synthase. The human aldosterone synthase, CYP11B2, is found in adrenal glomerulosa cells and expressed within the zona glomerulosa of the adrenal cortex. (7) The expression level of aldosterone synthase is controlled at the level of gene transcription. In the region 5'-flanking of CYP11B2 is present a portion of consensus for cAMP

response element (CRE) (position -74/-64), it plays a critical role in transcriptional regulation of CYP11B2.

Angiotensin II is the major regulator of aldosterone synthesis in the adrenal gland it can stimulate increase aldosterone synthase mRNA in human primary adrenal zona glomerulosa cells. Study in-vitro demonstrated Ang II stimulation of H295 cells results in an increase in aldosterone production and aldosterone synthase mRNA levels. The effect of Ang II on the synthesis of aldosterone has been shown to be through the activation of CYP11B2 transcription common cis-elements and the CRE site requires major induction in CYP11B2 promoter (8).

Ormones like Angiotensin II (Ang II), adrenocorticotrophin (ACTH) and ione potassium (K^+) stimulates aldosterone production by binding to the melanocortin 2 receptor, a Gs-protein-coupled receptor that stimulates adenylate cyclase and cAMP formation, and leads to the cAMP-dependent protein kinase (PKA) activation. (9)

Variance of Ang II and increases in K^+ concentration regulate the aldosterone secretion by modulating the intracellular concentration of calcium (Ca^{2+}). (10) The binding of Ang II to AT1 receptors activates an serial cascade of enzyme:

- Phospholipase C-dependent hydrolysis of plasma membrane phosphatidylinositol 4,5- biphosphate, which results in the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (11)
- IP3 binds causes an increase in Ca^{2+} cytosolic concentration by the rapid release of Ca^{2+} from endoplasmatic stores,
- DAG activates protein kinase C (PKC).

The increase in intracellular Ca^{2+} , opening of the voltage-dependent (T- and L-types) Ca^{2+} channels and the sustained entry of Ca^{2+} from the external space.

Studies *in-vitro* demonstrated that these pathways are interrelated, ACTH inhibits Ca^{2+} and Ang II stimulated aldosterone secretion via cAMP formation in zona glomerulosa cells. (12,13)

Arterial Hypertension from Adrenal hyper function

The adrenal cortex plays a crucial role in the regulation of Na^{+} and water homeostasis and of total peripheral resistance through the generic and non generic actions of aldosterone. The two principal causes for the arterial hypertension from adrenal disorder are:

a) Cushing's syndrome

Corticosteroids are critically involved in blood pressure regulation and glucocorticoid excess in Cushing's syndrome invariably results in arterial hypertension. Hypertension is one of the most distinguishing features of endogenous Cushing's Syndrome, as it is present in about 80% of adult patients. (14,15)

Causes

(a) Exogenous glucocorticoids

(b) Small ACTH-producing pituitary adenoma or hyperplasia. Adrenals usually exhibit nodular or diffuse hyperplasia, nodules are often multiple, associated with hyperplastic cortex. Zona glomerulosa is difficult to identify in adults, fasciculata has lipid-depleted cells, reticularis cells are vacuolated.

- (c) bilateral adrenal hyperplasia, adrenal adenoma or adrenal carcinoma
- (d) Ectopic ACTH production by non-adrenal neoplasm. Tumors secrete ACTH-like substance. In adults, usually due to small cell carcinoma of lung or carcinoid tumors of lung or thymus; also medullary thyroid carcinoma, pancreatic endocrine neoplasms, pheochromocytomas, ovarian tumors
- (e) Rarely caused by tumors producing cortisol releasing factor

b) Hyperaldosteronism

The primary hyperaldosteronism (PA) is an endocrine disorder characterized by excessive production of aldosterone, with the appearance of arterial hypertension often resistant to therapy medical and, in a proportion of cases of hypokalaemia and metabolic alkalosis. This disorder can manifest itself with fatigue, muscle cramps, cardiac arrhythmias also dangerous to the life and polyuria due to resistance tubular antidiuretic hormone resulting in reduced ability to concentrate urine.

Dr. Jerome Conn, who first described the syndrome in 1954, recognized this about ten years later, that not all individuals were affected by PA ipokaliemici but for many decades in clinical practice is rooted in the use and subjected to further diagnostic only those with hypertension who presented ipokaliemia spontaneous or induced by diuretics. This has led to claim that the PA was considered a rare cause of hypertension, comprising less than 0.5-1% of all hypertensive subjects (16,17).

With the introduction of the screening test which considers the ARR (aldosterone: renin ratio) which is the ratio of plasma aldosterone (PAC, plasma aldosterone concentration) and plasma renin activity (PRA, plasma renin activity) (18), was could identify cases in which the secretion of

aldosterone is inappropriate compared to that of renin. Thus leading to an increase in the frequency with which the PA is diagnosed, revealing, among other things, that only 20-40% of patients is ipokaliemico (19).

Clinic cases of CONN

The most common forms of PA are aldosterone-producing adenoma (APA) and bilateral adrenocortical hyperplasia (BAH), also referred to as idiopathic hyperaldosteronism (IHA). It is possible to distinguish in the two different cases (20):

1. Surgically curable

- Aldosterone-producing adenoma (aldosteronoma, APA)
- Multinodular unilateral adrenocortical hyperplasia (MUAN)
- Pheochromocytoma causing Primary aldosteronism

2. Surgically not curable

- Bilateral adrenal hyperplasia (BAH)
- Unilateral APA with BAH
- Glucocorticoid-remediable aldosteronism (GRA): genetic disease with autosomal transmission, is a crossing-over leading to the formation of a chimeric gene where the promoter of CYP11B1, regulates the expression of CYP11B2. This leads to an aldosterone throughout the adrenal cortex
- Familial hyperaldosteronism type II (FH-II).

Bilateral adrenal hyperplasia versus CONN

APA and BHA are considered separate entities, but there is no border between the two adrenal disorders, because there are not sharp criteria to distinguish them. The only investigation that allows you to better define the presence or absence of a hormonal hypersecretion, to discriminate surgically curable forms (APA and UAH) from not surgically curable forms (IHA) is the Adrenal Vein Sample (AVS). It consists in the measure of aldosterone and cortisol in a sample of blood collected from the adrenal veins and from the inferior vena cava.

However, there is no consensus on the cut-off used to define the selectivity of the sample and the lateralization as yet.

The values we have used to prove for the lateralization are a ratio between the dominant aldosterone and the controlateral aldosterone, each normalized for the corresponding value of cortisol (21):

$$\frac{\text{aldosterone}_{\text{dominant}}/\text{cortisol}_{\text{dominant}}}{\text{aldosterone}_{\text{controlateral}}/\text{cortisol}_{\text{controlateral}}} > 2$$

A problem for AVS is that it is an invasive test, it requires technical skill and a "devoted" radiologist specific personnel and not always it is possible to obtain a selective sampling due to the anatomy of the right adrenal vein, which leads directly into the inferior vena cava. In spite of this, many authors believe that this is an essential test for the diagnosis of arterial hypertension from adrenal disorders (22,23,24).

At a pathological level the distinction between the two forms, APA and BHA, is difficult because the Bilateral adrenal hyperplasia are not surgically removed and therefore there aren't enough case histories.

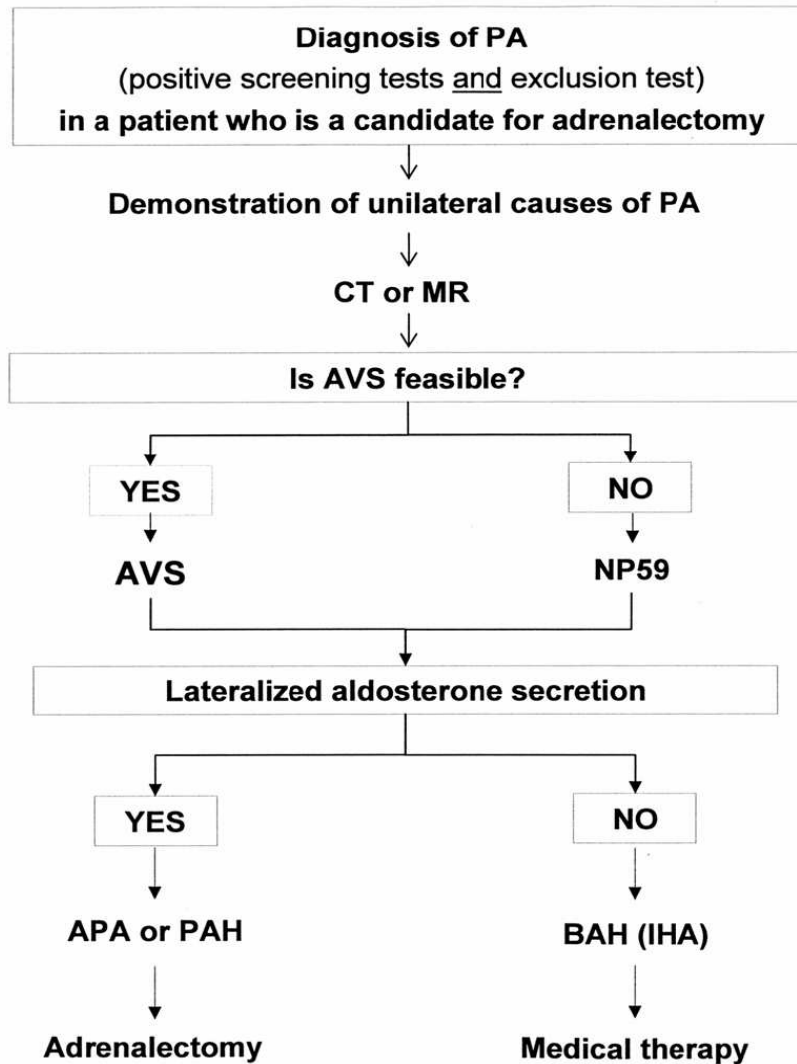


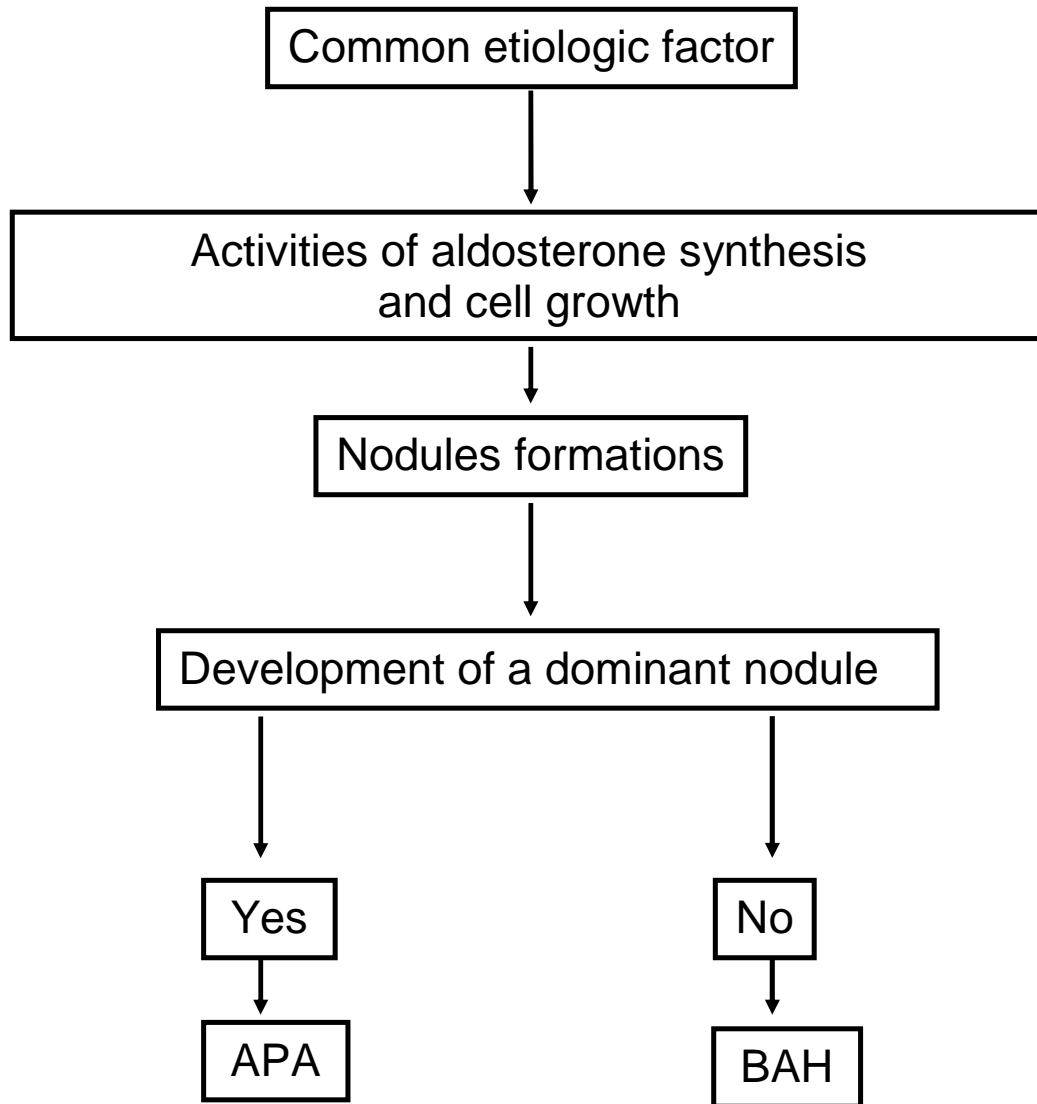
Fig.6 Flow chart for the diagnostic work-up of subtype identification of primary aldosteronism (PA). APA = aldosterone-producing adenoma; AVS = adrenal vein sampling; CT = computed tomography; IHA = idiopathic hyperaldosteronism; MR = magnetic resonance; PAC = plasma aldosterone concentration; PAH = primary adrenal hyperplasia; PCC = plasma cortisol concentration; NP59 = 6b-[131I] methyl-19-norcholesterol.

In the Primary Aldosteronism (PA) Prevalence in Italy (PAPY) study, a prospective survey of 1125 consecutive newly diagnosed hypertensive patients referred to specialized hypertension centers, aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism (IHA) were found in 4.8% and 6.4% of all patients(25,26), respectively, thus leading to an overall prevalence of PA of 11.2% (20).

Notwithstanding were than 50 years of results the etiologic factor(s) leading to primary hyperaldosteronism remain unknown. According to the most credited hypothesis one or more factors have been contented to chronically stimulate Aldosterone synthesis thereby leading to hyperplasic changes in the adrenocortical ZG and thus to node formation. According to one view, many nodules would be initially present, after which one would take over therefore becoming the " dominant" nodule and leading, because of hyperaldosteronism. Body fluid expression and suppression of the RAS, and therefore of Aldoserone synthesis in the other nodules. This view is supported by earlier experience with tumorectomy for Conn's adenoma, which was abandoned because of early recurrence of PA and nodule formation because of enlargement of one of the satellite nodules. Although plausible, this hypothesis, which raises the contention that PA can be an autonomon disorder like Graves' disease has never been formally investigated thus far.

AIM OF THE STUDY

Flow chart for testing the General Hypothesis



1. retrospective assessment of the APA tumors bank to determine the presence of multiple "satellite" nodules besides the dominant nodule.
2. ascertainment of the Aldosterone production in the dominant nodule and satellite nodules by In-Situ Hybridization
3. development of alternative markers of aldosterone production with antibody for CD56

Specific Hypothesis

Considering the difficulty of discriminating APA from BAH and the likely existence of a "continuum" between the two extreme of the spectrum we hypothesize that a common etiologic factor through common mechanisms can lead to either APA or BAH (IHA).

1. Is primary aldosteronism an autoimmune disorder (similar to Graves' disease?)

Study Approach

The identification of aldosterone producing cells in surgically available specimen from patients with primary aldosteronism (PA) has been precluded thus mainly because of the steroid nature of aldosterone. Thus , because this liposolubility of the hormone is lost during the fixation-dehydration steps are required for immunocytochemistry. Moreover, while antibodies specific for CYP11B1 and CYP11B2 exist for rat enzymes but not for human enzymes. Thus, the lack of antibodies specifically directed against the human aldosterone synthase, which is largely due to large degree of homology of the protein with the 11beta-hydroxylase, has precluded the development of reliable immuno histochemistry techniques for assessing aldosterone productions in tissue sections. In fact, the CYP11B2 gene encoding this enzyme shows a 95% homology with the CYP11B1 gene (Mornet E Journal of Biological Chemistry 1989) encoding the 11-beta-hydroxylase, therefore explaining the aforementioned difficulties in obtaining specific antibodies and molecular tools.

Preliminary studies have suggested the possibility of circumventing these problems by designing molecular probes that can be used for in-situ hybridization (ISH) (Sasano).

However, these techniques did not gain widespread use, because they require radioactive labeling of the probe.

Hence, for this study we have decided to attempt to develop a novel non radioactive ISH that could be applied to detect aldosterone synthase in tissue sections and therefore to identify the sites and cells producing aldosterone in aldosterone-producing adenoma and in adrenocortical hyperplasia.

The second approach used in the identification of nodules in APA was to investigate the presence and distribution of other markers of ZG, aldosterone synthesizing cells. In this regard, we have undertaken a search using a pool of different monoclonal antibodies which mostly gave unspecific results. However, we came across a partial result with an antibody for another protein the CD56.

The expression of CD56 is extensive in developing neuronal and endocrine tissues, including the rat adrenal and also is expressed by NK cells and some T cells. CD56 belongs to the Ig superfamily of adhesion molecules and plays a role in the morphogenesis of several organs. Expression of CD56 has been noted in both developing fetal organ systems and physiologic and regenerative processes in adults.

Its role in these processes has been suggested to range from altering migration to initiating differentiation and stimulation of signaling cascades.

(27) Thus, being a specific marker of ZG and adrenal medullary cells we hypothesize that an IHC technique using CD56 antibody will be used to

identify aldosterone producing cells and nodules in PA and adrenal gland patients with APA.

MATERIALS AND METHODS

Adrenal specimens.

Adrenal gland tissues from ten patients with APA were studied. The diagnosis of APA was based on strict predefined criteria that entail on lateralization of aldosterone secretion at adrenal vein sampling, surgery, pathology and, more importantly, follow-up data. For the latter, demonstration of normokalemia and cure or improvement of hypertension at least 120 days after adrenalectomy were required (PAPY). Cure was defined as normotension without medications; improvement as a systolic and diastolic blood pressure <140/90 mmHg, respectively, on the same or reduced number of medications, and/or reduced defined daily doses (GP Rossi J Am Coll Cardiol 2006).

Tissue were immediately fixed in neutral formalin and included in paraffin. All gave an informed consent to the study, which had been approved by the local Ethical Committee.

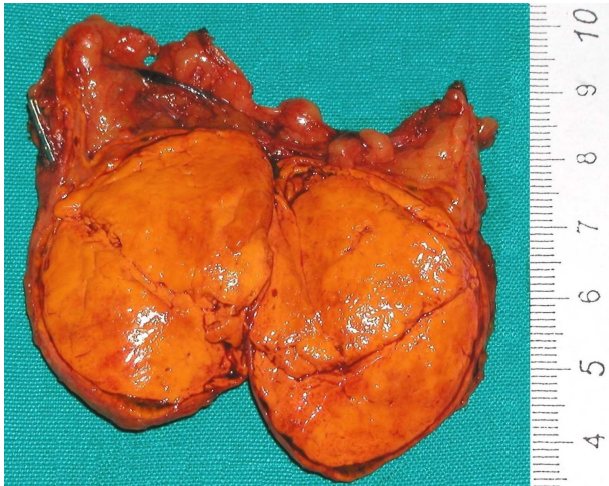


Fig. 7
Human Adrenal Gland
with Adenoma Producing
Aldosterone

Choice of the technique for identification of aldosterone synthase

We decided to use a technique of in situ hybridization, because the use of an antibody against aldosterone is not possible as this hormone has a lipid nature, and it is extracted during the fixation of the tissue.

Therefore, the only way to identify cells producing aldosterone is to study the distribution of the enzyme which regulates its production (CYP11B2).

We chose to mark the probes with digoxigenine (DIG) because this is a vegetable steroid, absent in animal cells.

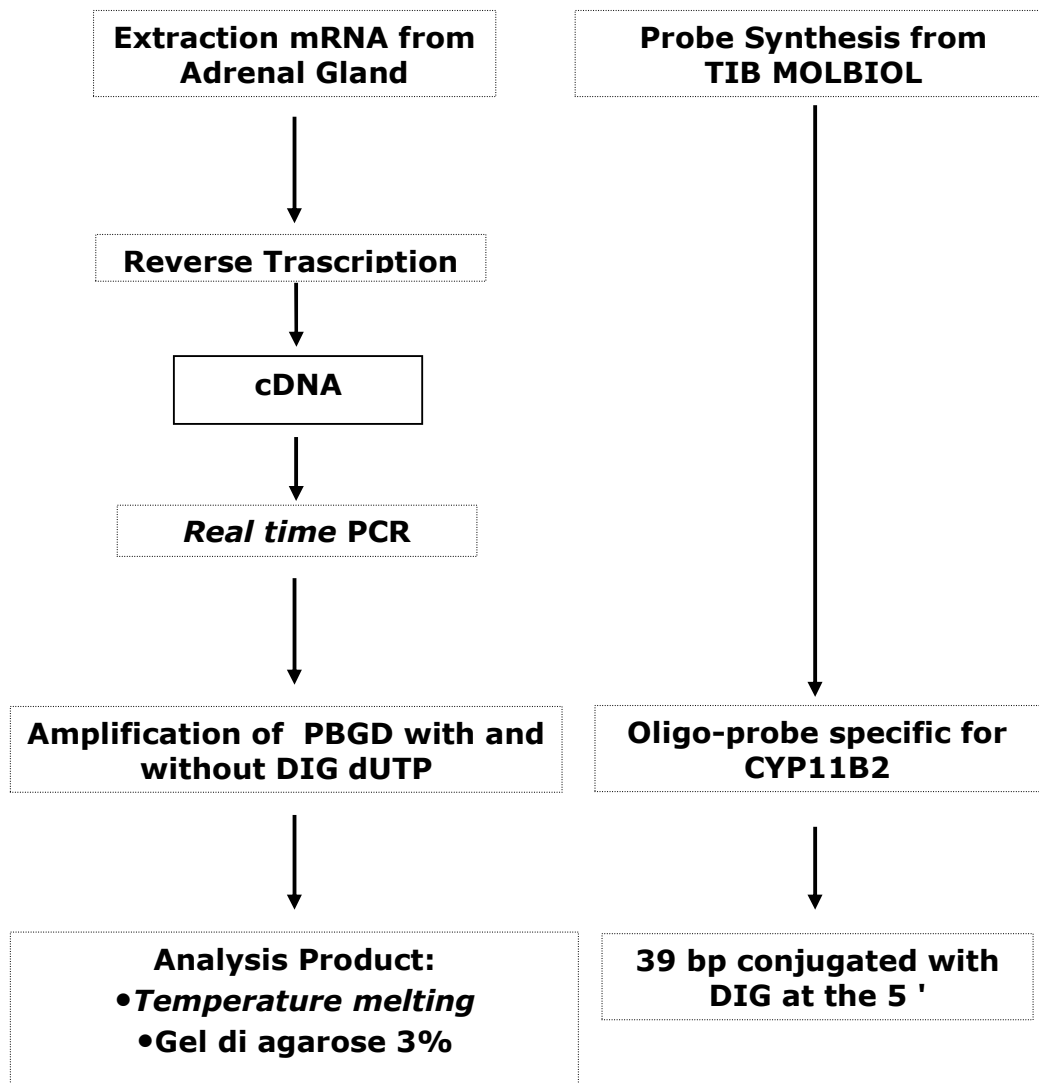
This detection system has the advantage of being relatively simple, sensitive and specific without the use of radioactivity.

Furthermore, We adopted a highly specific oligo-nucleotide probe for the CYP11B2 after blasting of the two enzymes and identification of not homologous areas.

The probe was analyzed by a second blast on the human genome to control cross-reactions with other genes. The specificity was demonstrated by a selective staining of the zone glomerulosa of a normal human's

adrenal gland. This oligo-nucleotide probe is resistant to RNase and do not require denaturation before use.

**Work flow for the synthesis of probes for the housekeeping gene
Porphobilinogen Deaminase and for aldosterone synthase**



Production probes for hybridization probes

For the hybridization have been used various types of probes:

- Oligonucleotide probe for the gene Cyp11b2.

The oligonucleotide probe specific for the CYP11B2 (28) have been synthesized by TIB MOLBIOL on our design and is 39 bp long. It was conjugated with DIG at the 5'. Its sequence is:

5'-DIG-GCCTTGCTATTTGACAGCCTGGCAAGCCCCAGTCCTGG

For hybridization of the housekeeping gene (PBGD) transcript, we designed a 92 bp probe labeled with Digoxigenin, corresponding to position 406-498 of the mRNA sequence.

- Probe of double-strand DNA (dsDNA) for a housekeeping gene (PBGD NM_000190) variant 2 is selectively expressed by erythroid cells, whereas variant 1 is a housekeeping gene ideal, since it is expressed in number of copies low and relatively constant in all cells of the body (29)

The dsDNA probes must be denatured and are less sensitive; however, they can be easily built by RT-PCR, and also they do not required purification before using them (30).

The amplified stretch is 92 bp long. The following primers were used:

fw primer 5'-TGCCCTGGAGAAGAATGAAG

rev primer 3'-AGATGGCTCCGATGGTGA.

The mRNA extracted from normal adrenal tissue was reverse transcribed with iScript™ cDNA Synthesis Kit (Biorad): 6 µl of mRNA were

mixed with 4 μ l of 5X iScript Reaction Mix, 1 μ l of iScript reverse transcriptase and 9 μ l of nuclease-free water to a final volume of 20 μ l, the reaction was done on a thermocycler. The thermal cycle profile comprised 5 minutes at 25 ° C, 30 minutes at 42 ° C and 5 minutes at 85° C.

The sequences of interest were amplified in with Real-Time thermocycler (Light cycler, Roche) using SybrGreen (Roche) to check through the melting temperature of the reaction product, both in presence and in absence of DIG-dUTP (Roche): 2 μ l of master mix (Roche), 1.6 μ l of MgCl₂, 0.4 μ l of dimethylsulfoxide (DMSO), 0.5 μ l of forward primer, 0.5 μ l of primer and reverse 2 μ l of cDNA, 3 μ l of DIG -dUTP and 1 μ l of Sybr Green and water until the final volume of 20 μ l. The protocol used was denaturation at 95 ° C for 2 minutes, 45 cycles consisting of 10 seconds denaturation at 95 ° C, 10 seconds at 60 ° C annealing of primers and 2 minutes extension at 72 ° C, and a cooling at 37 ° C for 30 seconds. To verify the success and the selective amplification, and the successful incorporation of DIG-dUTP, the analysis of melting temperature (T_m) and 3% agarose gel electrophoresis were both used.

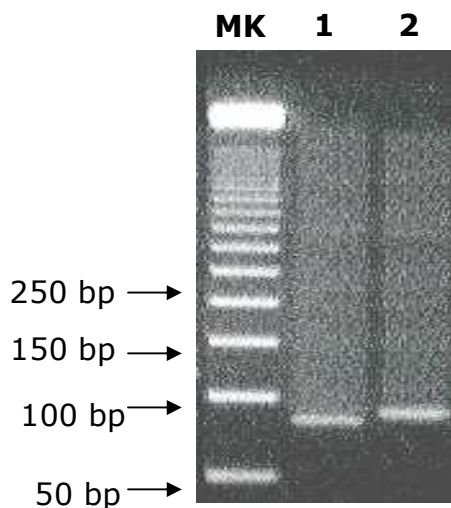
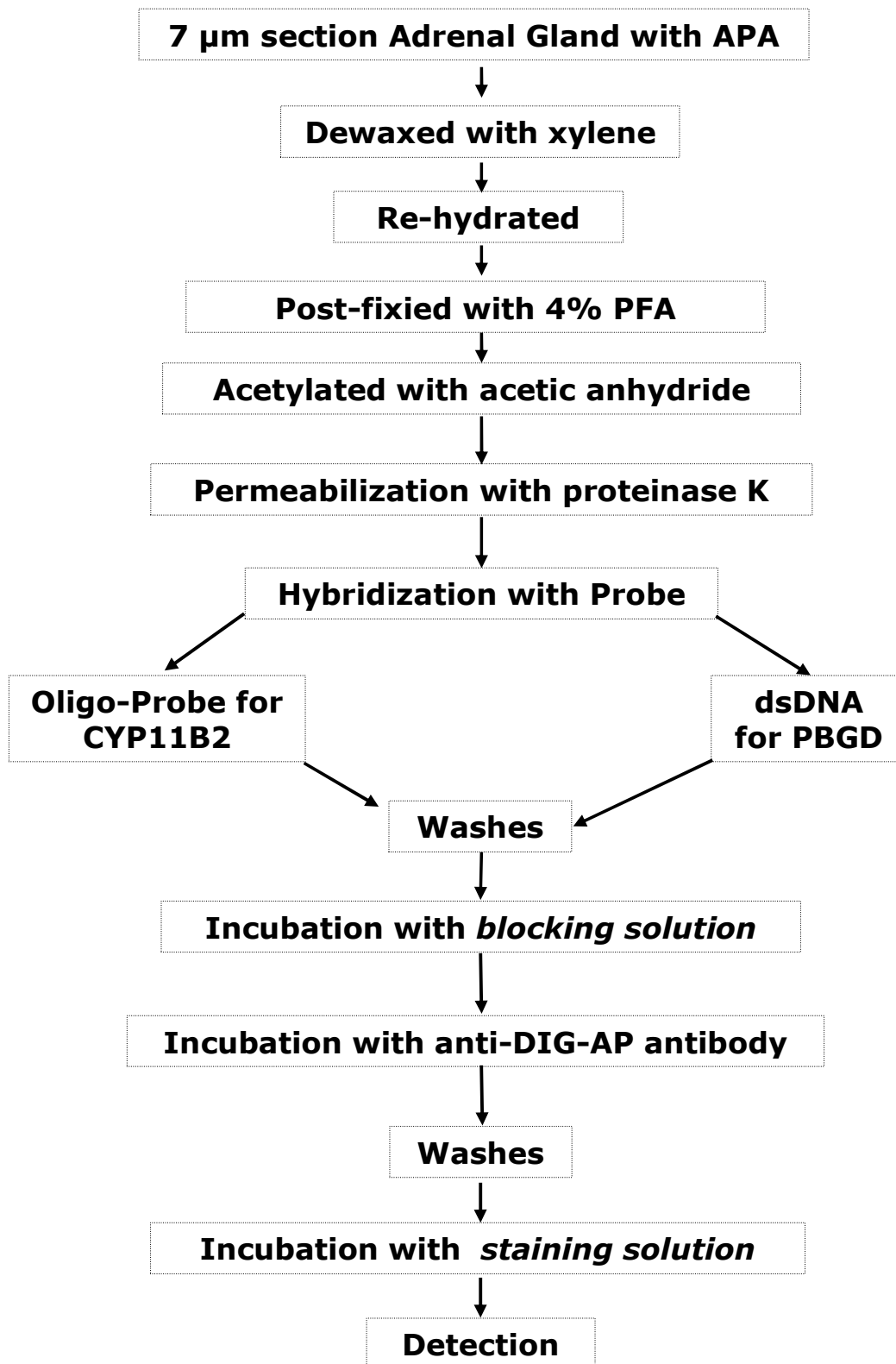


Fig. 8

3% Agarose Gel-electrophoresis
real time RT-PCR.

1 Probe PBGD (92 bp) without DIG
2 Probe PBGD (92 bp) with DIG
MK marker

Work Flow for the In situ Hybridization for CYP11B2 and the Housekeeping gene PBGD



In situ Hybridization.

As preliminary studies showed that formalin and paraphormaldheid fixed adrenocortical tissue sections were unsuitable for ISH because of inadequate preservation of the transcripts we had no choice other than used frozen (cryostat sections). This required the collection of all the APA tissues directly in the operating room immediately after laparoscopic adrenalectomy.

The tissues were cut in 7 μ m-thick slices and sections were mounted on glass slide. All hybridization steps were conducted under RNase-free conditions.

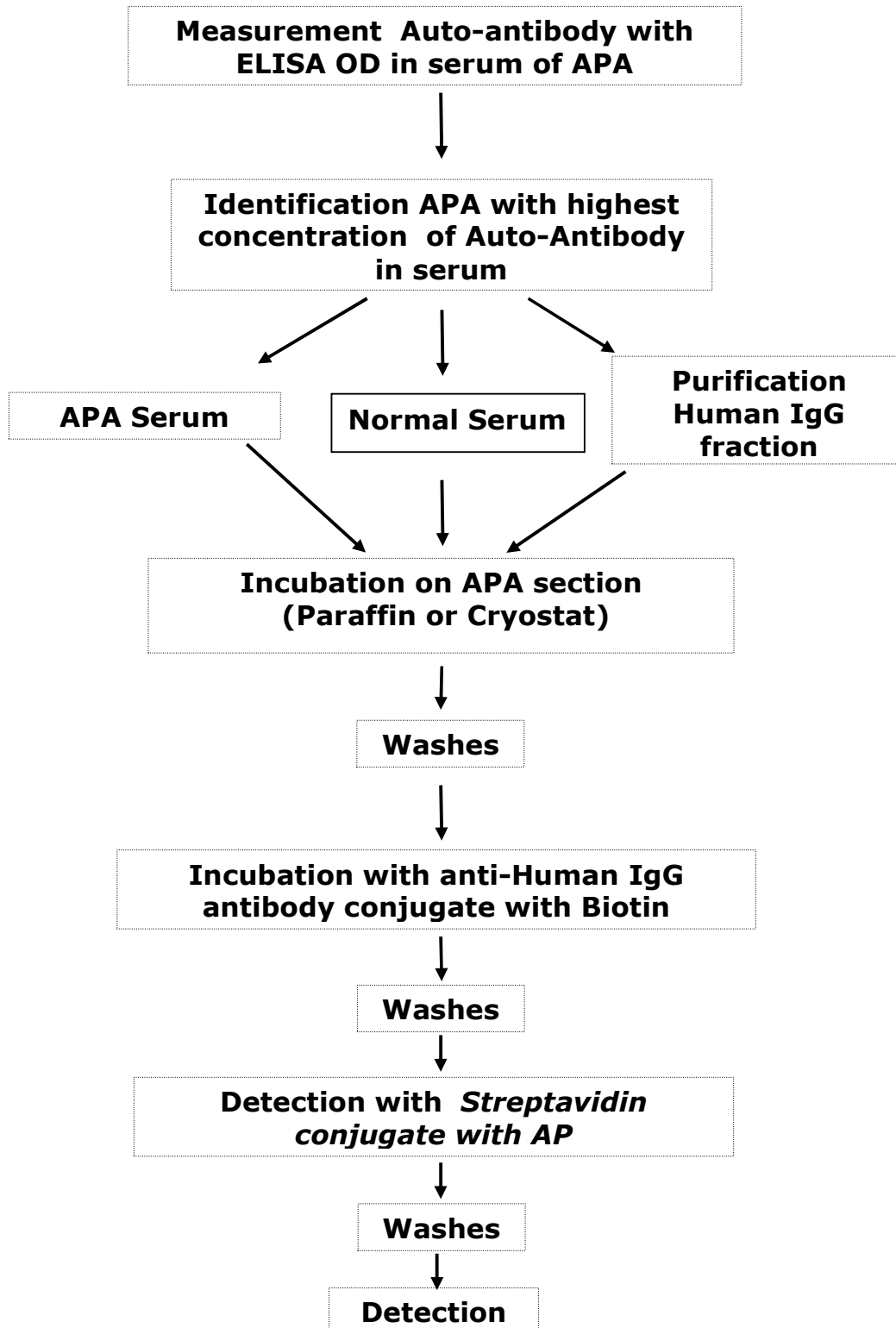
Sections were dewaxed with xylene (5min for 2 times), and re-hydrated in different ethanol concentrations (5 min for 2 times), quickly washed in DEPC-dH₂O and phosphate buffered saline (PBS, Oxoid) (5 min for 2 times). After post-fixing the sections for 10 min with 4% PFA in (Phosphate Buffer Saline)PBS and washing in PBS (5 min for 2 times), they were acetylated for 5 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (TEA, Sigma) pH 8.0 and subsequently with acetic anhydride (Fluka, Italy) 0.25% (v/v) in TEA and in SSC 2X (Sigma, Italy) (3 min). The sections were treated with proteinase K (Sigma), 10 μ g/ml in PBS, at 37°C for 20 min and then immersed in glycine (Fluka), 2 mg/ml in PBS, at 4°C for 1 min 30 seconds and PBS (5 min for 2 times). The sections were incubated for 2 h at 37°C with pre-hybridization buffer and washed in SSC 2X (5 min), then incubated with hybridization solution containing DIG-labeled probes in hybridization buffer (50% deionized formamide (Sigma), 20% (w/v) dextran sulfate, 1% Denhardt's 50X

solution (Sigma), 20% SSC 2X, 1% Dithiothreitol (DTT, Fluka) and 1% Salmon sperm DNA (19mg/ml, Invitrogen, Italy) at 37°C overnight. Slides were washed with DTT (1.5mg/ml)/SSC 1X at room temperature (5 min), 45°C (2 times for 15 min) and DTT (1,5mg/ml)/SSC 0.5X at 45°C (10 times for 2min), at room temperature (5min) and then transferred to Tris-buffered saline (TBS) (100 mM Tris HCl, 150 mM NaCl, pH 7,5) and washed 5 min for 3 times. The sections were covered with blocking solution (TBS, 0.1% tritonX-100 and 1% sheep serum, Sigma) at room temperature for 30 min and subsequently incubated with anti-DIG antibody (Roche, Italy) diluted 1:2000 overnight at 4°C. After washing the sections 3 times for 5 min in TBS, they were incubated with staining solution (Fast™ NBT/BCIP in 10 ml of dH₂O add 1% levamisol 1M, Sigma) overnight at room temperature.

Immunohistochemistry CD56

For IHC the best results, often a long set of preliminary work, were auctioned with formalin fixed and paraffin embedded. Specimen 4 µm sections of the routinely processed paraffin blocks were stained with hematoxylineosin (HE) for histopathological diagnosis. IHC staining was performed using an indirect immunoperoxidase technique (Bond Polymer Refine Detection; Vision BioSystems, UK) with a fully automated system (Bond-maX; Vision BioSystems, UK). Four-micron-thick sections from paraffin blocks were dewaxed and rehydrated by successive incubation in Bond Dewax Solution (Vision BioSystems, UK), ethanol, and distilled water. Antigen retrieval was performed by heating sections (100°C, 30 min) in Bond Epitope Retrieval Solution 1 (Vision BioSystems, UK). Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide before a 5 min incubation with a mouse monoclonal anti-CD56, clone 1B6 (diluted 1:100, Novocastra, Newcastle upon Tyne, UK). Antigen was detected by incubation with labelled polymer (HRP) and diaminobenzidine. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

Work Flow for the identification of Auto-Antibody on tissue sections



Measurement of Auto Antibody against AT-1 receptor serum APA

Currently, there is no communally technique for the measurement of antibodies AT-1 receptor in human plasma or tissue. This methods heve been in house developed. The ELISA for the measurement of circulating auto antibodies was performed in the laboratory of Prof. Ermanno Rossi, at the Azienda Ospedaliera di reggio Emilia with an in-house developed kit. Briefly, AT1 receptor peptide were coated on plates and left overnight. The walls were then saturated with phosphate buffered saline. After washing with PBS-Tween 20, the serum were added to the coated plates for 1 h at 37°C. After three additional washings, horseradish peroxidase-conjugated anti-human IgG antibodies were added for 1 h at 37°C. The plates were then washed three more times, the substrate (with H₂O₂ and 3'-3'-5'-5'-tetramethyl benzidine) was added for 5 min, and the reaction was ended. Optical density (OD) was measured at 450 nm in a microplate reader, using the ratio of the OD of positive serum to the OD of negative serum.

Immunohistochemistry Auto Antibody

We used an immunohistochemistry technique to analyze sections from APA. 7 µm-thick sections of tissues fixed in formalin and included in paraffin were mounted on glass slide. For the detection of auto-antibody sections were de-waxed three times in xylol, re-hydrated in a graded series of ethanol and unmasked with Antigen unmasking buffer (DIAPATH, Bergamo, Italy) at 96 °C for 30 min. Sections were then exposed to serum or fraction IgG human purify from APA diluted 1:1000 in Blocking Serum Solution (BSA 0,2% Triton 0,2% normal goat serum 1:50 in PBS) for 1 hour at 37°C. Endogenous peroxide was blocked and non-specific background staining was minimized with blocking serum solution. Slides were washed with PBS at room temperature (3 times for 5 min).

After incubation with normal serum, we have exposed the section with Avidin D solution for 15 minutes. Rinse briefly with buffer, then incubate for 15 minutes with the biotin solution.

Sections were then exposed to primary antibody conjugate with Biotin (Anti Human IgG antibody Santa Cruz Biotechnology) diluted 1:1000 or 1:2000 in BSS and incubated overnight at 4°C. After incubation, we have used the streptavidin enzyme conjugate with HRP (30 min at room temperature) then detected with with H₂O₂ and 3'-3'-5'-5'-tetramethyl benzidine. Negative controls were identically processed, but with omission of the primary antibody or the serum/IgG fraction of patients for the control of background for the presence of IgG in the APA tissue .

RESULTS

In situ hybridization

The APA analyzed showed intensely labeled areas called “nodules producing aldosterone” and negative areas. In some cases, the positive cells were localized under the capsule, while the central part of the mass was not clearly marking. This experimental data has revealed an expression of the gene Cyp11b2 reflecting considerable biological variability of samples analyzed. It is therefore conceivable that some cells have a quantity of mRNA for the gene Cyp11b2 and thus transcriptional activity at the level of baseline adrenal gland that the methodology was not sensitive enough to detect.

The presence of a nuclear marking with this method of hybridization is readily explain when one considers that the transcription of DNA to mRNA occurs in the nucleus and thus it is that the compartment when the transcript is more better preserved. In the surrounding cytoplasm, the staining is not appreciably different from that of cells. The nuclear staining was obtained through specific bond oligo-probe to mRNA of Cyp11b2, and not as an aspecific binding, since the probe was found only cells corticosurrene, leaving those of the negative bone marrow or 'vascular endothelium (Fig. 9 A, B). Moreover, samples treated with probes labeled with Digosigenin or those in which the probe was omitted were completely negative (Fig.9 D).

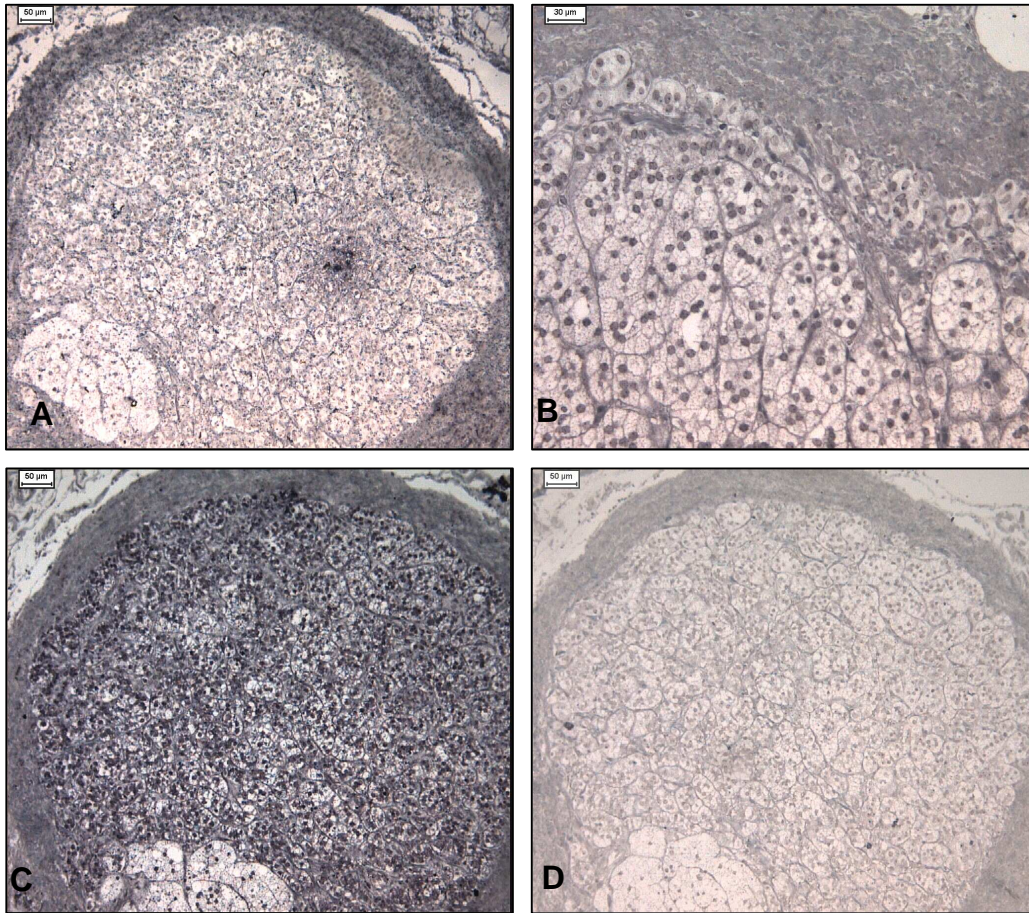


Fig. 9 In situ Hybridization: (A and B) nuclear staining for mRNA Cyp11b2 of nodule producing aldosterone, (C) nuclear staining for housekeeping gene PBGD with dsProbe, (D) negative control

CD56 Immunohistochemistry

Immunohistochemistry using a specific antibody showed an intensive staining of the human ZG which was not seen in the ZF and ZR. Moreover, an intensive staining of the AM was detected. We could detect staining of the APA, which was more prominent at the periphery of the discriminant nodule. Of interest the staining overall showed a parallel distribution to that observed with ISH but while the IHC staining was at the cell

membrane the ISH was mainly in the nodules (Fig A B C). Based on these results we investigated the of using CD56 to develop a method for the isolation of ZG and APA cells using magnetic beads (Fig D).

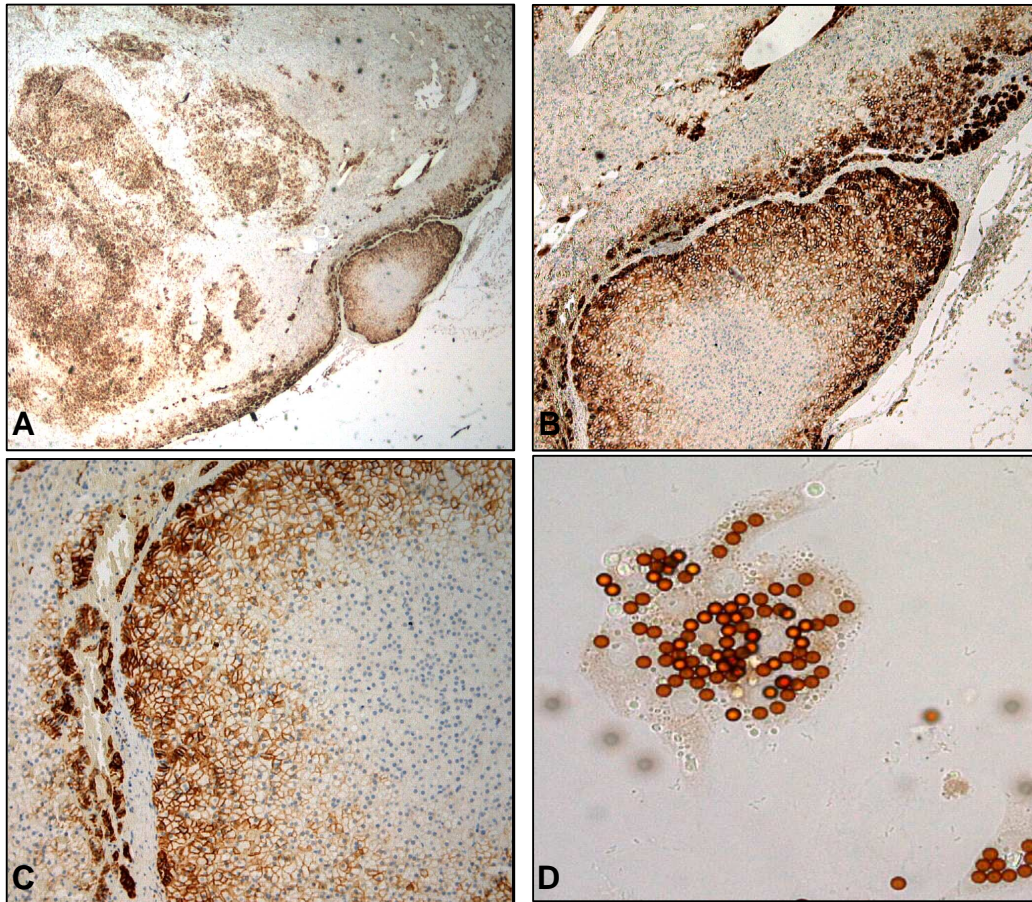
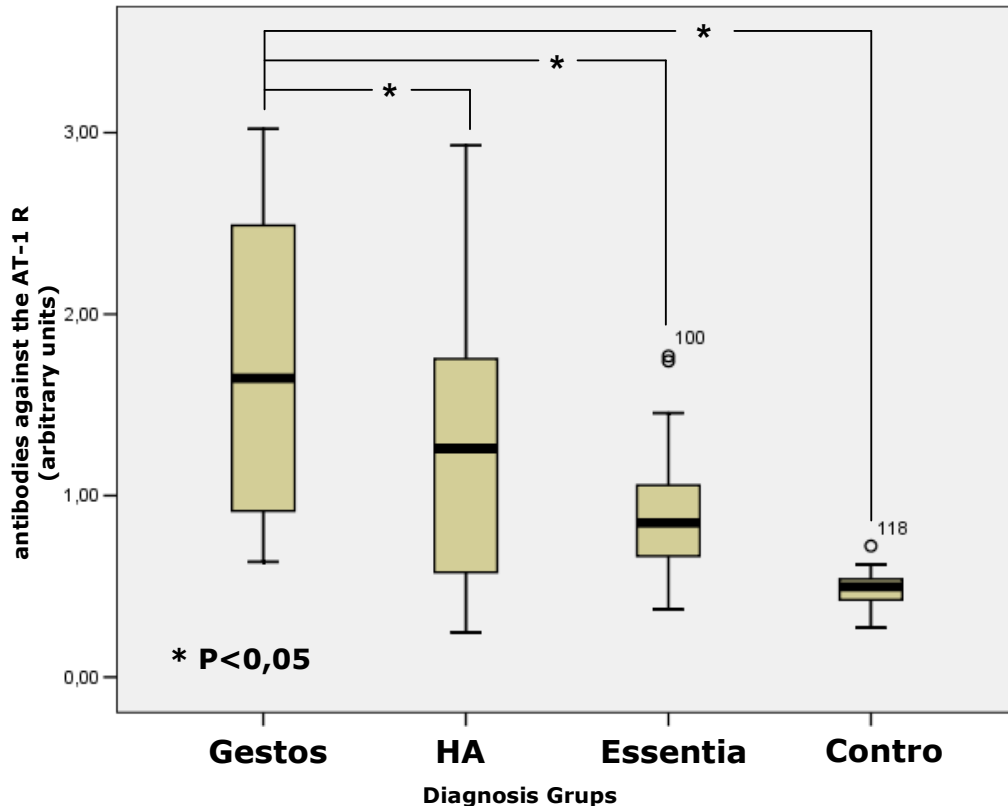


Fig. 10 immunohistochemistry for CD56: (A, B and C) peripheral staining of nodule producing aldosterone, (D) isolation of zona glomerulosa cells with magnetic beads

Auto Antibody Immunohistochemistry

In patients affected by primary hyperaldosteronism with used a semiquantitative ELISA method, we were able to detect antibody against the AT-1 receptor not only in plasma from woman with eclampsie, which are human to show the highest levels in patient with PA. Those with an APA showed a fund to higher levels them patients with IHA. Both PA grups showed titer significantly higher then essential hypertension patients.

| APA | Optical Density |
|----------------------|------------------------|
| 1 | 1,243 |
| 2 | 1,258 |
| 3 | 1,271 |
| 4 | 1,573 |
| 5 | 1,704 |
| 6 | 1,705 |
| 7 | 1,728 |
| 8 | 1,747 |
| 9 | 1,852 |
| 10 | 1,910 |
| 11 | 2,061 |
| 12 | 2,122 |
| 13 | 2,220 |
| 14 | 2,265 |
| 15 | 2,839 |
| 16 | 2,929 |
| Background | 0,05 |
| Normotensive Control | 0.48 +/- 0.09 |



Our immunohistochemical study on adrenal tissue was designed in order to fulfill the second major criteria (later explained).

Unaware of the key to initially disclose the subtle equilibrium among AAb affinity, serum titer, proportion of AAb bound to adrenal tissue (but conscious that often in autoimmune conditions the AAb titer do not correlate well neither with the severity of tissue-lesions, nor with the strength of a supposed agonistic – or inhibitory, as in Hashimoto thyroiditis - activity), we performed two kinds of experiments: a DIRECT and an INDIRECT Immunohistochemistry.

In Direct IHC we used paraffin-included and cryostat sections of APA from selected patients incubated with a biotinylated anti IgG primary antibody.

In Indirect IHC, sections from “healthy” human adrenal gland or plates of

cultured H295 cells were incubated with patients' serum and the same primary antibody described above.

Patients selection was performed on the basis of a semiquantitative detection of anti angiotensin II AT1 receptor AAb in their serum.

DISCUSSION

In situ hybridization

By using a novel nonradioactive methodology for detection aldosterone syntheses mRNA, which we developed we could show that Aldosterone synthesis occurs only in the ZG in the normal human adrenal cortex.

This experimental evidence is consistent with other findings that reveal a cytological heterogeneity of the APA, which may be made of phenotypes-like cell clusters, fascicule-like and mixed in different proportions (32). Moreover, given that the samples used were stored in paraffin, it may be assumed that the mRNA content in the nucleus was better preserved because it protected from the degradation than cytoplasmic.

It is also necessary to remember that the work so far published in which it was studied the distribution to mRNA of Cyp11b2 through hybridization, the subcellular localization is not indicated (33, 28, 34).

Auto Antibody Immunohistochemistry

In contrast with other tumorous condition, the exact pathway, mutations and triggering factors leading to the development of aldosterone producing adenomas have not been clarified yet.

The occurrence of micronodularity peripheral to the main adenomatous mass in some of our patient and the frequent recurrence of APA in the controlateral adrenal gland after therapeutic adrenalectomy aroused the suspicion that a chronic proliferogenic stimulus could be involved in the pathophysiology of the PA. Thus a continuum between hyperplasia, micronodularity and adenomas could be hypothesized.

None of the actually known adrenotropic stimulating factors seems to be able to play such a trophic role, and the possible culprit is unknown.

Nevertheless, we noted a similarity between the histopathological behaviour of these APA with satellite micronodularity and a well known clinical entity: Grave's disease. This thyreotoxic condition associated with hyperfunction of Thyroid gland is often referred to as a "diffuse hyperplasia " of the gland, but the natural history of the disease leads often to a micronodular pattern in which the single nodes can grow autonomously and a "dominant node" can develop. In this pathologic hyperplasia, the *primum movens* consist in a peculiar autoimmune reaction against the TSH-receptor as the autoantigen: what differentiates this autoimmune disease from many others is the agonistic activity expressed by the autoantibodies on the receptor. This provides the necessary trophic and hormone release-inducing stimulus.

Our hypothesis is that a similar humoral stimulating factor could play a role in the genesis of APA, with an implementation of the mitogenic potential of adrenal cells and a consequent increased risk of adenoma degeneration of a diffuse or nodular hyperplasia.

Stating an agonistic autoantibody-nature of this factor would consequently make us assume APAGenesis as an autoimmune process: to validate this suspicion, the criteria proposed by Witebsky and Rose to define a condition as an autoimmune in nature should be fulfilled. Briefly, these criteria are summarized as the following:

➤ Major criteria (Witebsky and Rose 1957)

1. Lympho-plasmocytic infiltration of the target organ or systemic involvement.
2. Demonstration of circulating and/or tissue-localization of autoantibodies (AAb) and/or autoreactive lymphocytic clones.
3. Identification of autoantigens involved in the autoimmune reaction, induction of the disease in animals by injection of these autoantigens and passive transfer of the disease by serum or lymphocytes.

➤ Minor criteria (Rose and Bona 1993)

1. Correlation with the MHC genes.
2. Response to immunosuppressive therapy.
3. Association with other autoimmune diseases.

Angiotensin II (Ang II), the main active peptide of the renin-angiotensin system (RAS), is a potent vasoconstrictor hormone that is cleaved from

angiotensinogen by renina and the angiotensin-converting enzyme (ACE). This octapeptide mediates its effects via two G-protein coupled receptors, the angiotensin II type 1 (AT1) and type 2 (AT2) receptors. In particular, the interaction of Ang II with the AT1 receptor stimulates pathophysiological processes including hypertension.

Anti-AT1-receptor antibodies were first discovered by Fu et al. in the serum of patients with malignant hypertension (35) They were then found to exist with high titer in pre-eclampsia patients by Wallukat et al (36) and in refractory hypertensive patients by Liao et al. (37), whereas only a small percentage of patients with nonrefractory hypertension were positive for AT1-AA. One may speculate that the presence of AT1-AA contributes to a worse therapeutic response in the patients with refractory hypertension. Interestingly, the authors identified even 7.5% of normotensive controls as antibody-positive.

Many studies (38,39) have shown that these antibodies exhibit an agonist-like activity similar to Angiotensin II, such as a stimulatory positive chronotropic effect on neonatal rat cardiomyocytes; however an agonistic activity also on aldosterone producing cells of adrenal zona glomerulosa (mediated by the same receptor) has not been demonstrated yet. The prosecution of our studies could clarify this point in future.

Our present knowledge about the role of these AAb in patients affected by primary hyperaldosteronism is the detection, by a semiquantitative ELISA method, of an Optical Density (OD) significantly higher than the same parameter measured in the serum of control normotensive patients described above (see table).

This measurements induced us to focus our attention on those patients who revealed an higher OD, and we performed all the described IHCs on sections of their APAs (for direct IHC) or using their serum or IgG-fraction from serum (for indirect IHC).

To validate the third and last major criteria, we are planning to produce an animal model of autoimmunity against the AT1 receptor, by immunizing rats with the synthetic peptide corresponding to the second extracellular peptide of human AT1 as the antigen, identified in literature as the binding site for antiAT1-AAb detected in preeclamptic women and malignant-hypertension affected patients, and used in the ELISA assays to detect the AAb. By raising them for two to six months, we'll study Blood pressure, Heart rate, biochemical profile including PRA and PAC, vascular and cardiac remodeling and adrenal gland histopathology, in order to reveal the *in vivo* effect of these AAb. Fu et al. (39) and Wang et al. (40) already immunized rats with the synthetic peptide corresponding to the second loop of human AT1receptor (residues 165–191) as antigen, and then produced antibodies from the immunized serum: they demonstrated a persistent agonistic activity of AAb and a vascular remodelling, respectively.

Contemporarily to this study, a passive transfer of the possible alterations enlisted above could be attempted by injecting other rats with the serum of those previously immunized

Reference

1. Rainey,W.E. Adrenal zonation: clues from 11beta-hydroxylase and aldosterone synthase. *Mol. Cell Endocrinol.* **151**, 151-160 (1999)
2. Dr. Chris Doumen Lecture 4 2402 : Anatomy/Physiology Major Endocrine Organs
3. William F. Ganong Book
4. Chapter 11. Adrenal Cortex, Development, Anatomy, Physiology I. Vrezas Department of Endocrinology, Diabetes and Rheumatology, University Hospital, , Duesseldorf, Germany H.S. Willenberg Department of Endocrinology, Diabetes and Rheumatology, University Hospital, , Duesseldorf, Germany S.R. Bornstein Technical University of Dresden, Medical Department, , Fetscherstraße, Dresden, Germany
5. (Mornet,E., Dupont,J., Vitek,A. & White,P.C. Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11 beta)). *J. Biol. Chem.* **264**, 20961-20967 (1989))
6. (Bassett,M.H., White,P.C. & Rainey,W.E. The regulation of aldosterone synthase expression. *Mol. Cell Endocrinol.* **217**, 67-74 (2004))
7. Curnow KM, Tusie-Luns M, Pascoe L, Natajara R, Gu J, Nadler JL, White PC 1991 The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. *Mol Endocrinol* 5:1513–1522.

8. JIALI GU, YESHAO WEN, ANGELES MISON, AND JERRY L. NADLER
12-Lipoxygenase Pathway Increases Aldosterone Production,
3 β ,5 β -Cyclic Adenosine Monophosphate Response Element-
Binding Protein Phosphorylation, and p38 Mitogen-Activated Protein
Kinase Activation in H295R Human Adrenocortical Cells.
Endocrinology 144(2):534–543
9. Fakunding,J.L., Chow,R. & Catt,K.J. The role of calcium in the
stimulation of aldosterone production by adrenocorticotropin,
angiotensin II, and potassium in isolated glomerulosa cells.
Endocrinology 105, 327-333 (1979).
- 10.Foster,R.H. Reciprocal influences between the signalling pathways
regulating proliferation and steroidogenesis in adrenal glomerulosa
cells. *J. Mol. Endocrinol.* 32, 893-902 (2004).
- 11.Hajnoczky,G., Csordas,G., Bago,A., Chiu,A.T. & Spat,A. Angiotensin
II exerts its effect on aldosterone production and potassium
permeability through receptor subtype AT1 in rat adrenal
glomerulosa cells. *Biochem. Pharmacol.* 43, 1009-1012 (1992).
- 12.Yoshida,A., Nishikawa,T., Tamura,Y. & Yoshida,S. ACTH-induced
inhibition of the action of angiotensin II in bovine zona glomerulosa
cells. A modulatory effect of cyclic AMP on the angiotensin II
receptor. *J. Biol. Chem.* 266, 4288-4294 (1991).
- 13.Balla,T., Hollo,Z., Varnai,P. & Spat,A. Angiotensin II inhibits K(+)-
induced Ca²⁺ signal generation in rat adrenal glomerulosa cells.
Biochem. J. 273(Pt 2), 399-404 (1991).
- 14.Nakamura Y, Yano H, Nakashima T. False intranuclear inclusions in
adrenal cytomegaly *Arch Pathol Lab Med.* 1981 Jul;105(7):358-60

15. Deborah P. Merke. Approach to the adult with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Endocrinol Metab.* 2008, 93: 653-660.
16. Gordon RD, Ziesak MD, Tunny TJ, Stowasser M, Klemm SA. Evidence that primary aldosteronism may not be uncommon: 12% incidence among antihypertensive drug trial volunteers. *Clin Exp Pharmacol Physiol.* 1993;20:296-298.
17. Young WF, Jr. Minireview: primary aldosteronism--changing concepts in diagnosis and treatment. *Endocrinology.* 2003;144:2208-2213.
18. Hiramatsu K, Yamada T, Yukimura Y, Komiya I, Ichikawa K, Ishihara M, Nagata H, Izumiyama T. A screening test to identify aldosterone-producing adenoma by measuring plasma renin activity. Results in hypertensive patients. *Arch Intern Med.* 1981;141:1589-1593.
19. Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, Gomez-Sanchez CE, Veglio F, Young WF, Jr. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J Clin Endocrinol Metab.* 2004;89:1045-1050.
20. a mazza c pessina et al. Endocrine arterial hypertension: diagnostic approach in clinical practice. *Minerva Endocrinol* 2008 33, 127-43
21. Rossi GP, Sacchetto A, Chiesura-Corona M, De Toni R, Gallina M, Feltrin GP, Pessina AC. 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.
22. Stowasser M, Gordon RD. Primary aldosteronism. *Best Pract Res Clin Endocrinol Metab.* 2003;17:591-605.
23. Young WF, Stanson AW, Thompson GB, Grant CS, Farley DR, van Heerden JA. Role for adrenal venous sampling in primary aldosteronism. *Surgery.* 2004;136:1227-1235.
24. Rossi GP, Ganzaroli C, Miotto D, De Toni R, Palumbo G, Feltrin GP, Mantero F, Pessina AC. Dynamic testing with high-dose adrenocorticotrophic hormone does not improve lateralization of aldosterone oversecretion in primary aldosteronism patients. *J Hypertens.* 2006;24:371-379.
25. Rossi, G.P. et al. Renal damage in primary aldosteronism: results of the PAPY Study. *Hypertension* 48, 232-238 (2006).
26. Rossi, G.P. et al. A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J. Am. Coll. Cardiol.* 48, 2293-2300 (2006).
27. MARCUS O. *The Journal of Clinical Endocrinology & Metabolism* 88(8):3921-3930
28. Pascoe L, Jeunemaitre X, Lebrethon MC, Curnow KM, Gomez-Sanchez CE, Gasc JM, Saez JM, Corvol P. Glucocorticoid-suppressible hyperaldosteronism and adrenal tumors occurring in a single French pedigree. *J Clin Invest.* 1995;96:2236-2246.
29. Grandchamp B, De Verneuil H, Beaumont C, Chretien S, Walter O, Nordmann Y. Tissue-specific expression of porphobilinogen

- deaminase. Two isoenzymes from a single gene. *Eur J Biochem.* 1987;162:105-110.
30. Alabi W, Cerghet M, Skoff RP, Ghandour MS. Detection of oligodendrocytes in tissue sections using PCR synthesis of digoxigenin-labeled probes. *J Histochem Cytochem.* 2003;51:913-919.
31. Sasano H, Mason JI, Sasano N. Immunohistochemical study of cytochrome P-45017 alpha in human adrenocortical disorders. *Hum Pathol.* 1989;20:113-117
32. Neville AM, Symington T. Pathology of primary aldosteronism. *Cancer.* 1966;19:1854-1868.
33. Enberg U, Volpe C, Hoog A, Wedell A, Farnebo LO, Thoren M, Hamberger B. Postoperative differentiation between unilateral adrenal adenoma and bilateral adrenal hyperplasia in primary aldosteronism by mRNA expression of the gene CYP11B2. *Eur J Endocrinol.* 2004;151:73-85.
34. Shigematsu K, Kawai K, Irie J, Sakai H, Nakashima O, Iguchi A, Shimamatsu J, Shimamatsu K, Kusaba Y, Takahara O. Analysis of unilateral adrenal hyperplasia with primary aldosteronism from the aspect of messenger ribonucleic acid expression for steroidogenic enzymes: a comparative study with adrenal cortices adhering to aldosterone-producing adenoma. *Endocrinology.* 2006;147:999-1006.
35. Fu ML, Herlitz H, Wallukat G, Hilme E, Hedner T, Hoebeke J, Hjalmarson A (1996) Non-desensitized positive chronotropic effect

- of anti-angiotension II receptor autoantibodies in a patient with malignant hypertension (abstract). *Circulation* 94:4046a
36. Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, Jüpner A, Baur E, Nissen E, Vetter K, Neichel D, Dudenhausen JW, Haller H, Luft FC (1999) Patients with preeclampsia develop agonistic antibodies against the angiotension AT1 receptor. *J Clin Invest* 103:945–952
37. Liao YH, Wei YM, Wang M, Wang ZH, Yuan HT, Cheng LX (2002) Autoantibodies against AT1-receptor and alpha1- adrenergic receptor in patients with hypertension. *Hypertens Res* 25:641–646
38. Fu ML, Leung PS, Wallukat G, Bergstrom G, Fu H, Schulze W, Herlitz H (1999) Agonist-like activity of antibodies to angiotensin II receptor subtype 1 (AT1) from rats immunized with AT1 receptor peptide. *Blood Press* 8:317–324
39. Fu ML, Herlitz H, Schulze W, Wallukat G, Micke P, Eftekhari P, Sjogren KG, Hjalmarson A, Muller-Esterl W, Hoebeke J (2000) Autoantibodies against the angiotensin receptor (AT1) in patients with hypertension. *J Hypertens* 18:945–953
40. Bin Wang, Yu-Hua Liao, Zihua Zhou, Liudong Li, Fen Wei, Ming Wang, Yumiao Wei (2004) Arterial structural changes in rats immunized by AT1-receptor peptide. *Heart Vessels* (2005) 20:153–158