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**UNIVERSITÀ DEGLI STUDI DI PADOVA  
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**DOTTORATO DI RICERCA INTERNAZIONALE IN IPERTENSIONE  
ARTERIOSA E BIOLOGIA VASCOLARE  
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**ISOLATION AND CHARACTERIZATION OF A PURE  
POPULATION OF ALDOSTERONE PRODUCING CELLS AS  
A NOVEL IN VITRO MODEL FOR THE STUDY OF THE  
ALDOSTERONE SECRETION**

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*Considerate la vostra semenza:  
fatti non foste a viver come bruti,  
ma per seguir virtute e canoscenza.*

*Dante, Divina Commedia, Inferno, XXVI, 118-120*

*Se un uomo parte con delle certezze finirà con dei dubbi; ma se si accontenta di iniziare con qualche dubbio, arriverà alla fine a qualche certezza.*

*F.Bacone*

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## RIASSUNTO

L'iperaldosteronismo primario (PA) colpisce l'11,2% degli ipertesi ed è quindi la più comune forma di ipertensione secondaria. E' caratterizzato da un'ipersecrezione, apparentemente autonoma, di aldosterone da parte della zona glomerulosa (ZG) del surrene. Le principali cause di iperaldosteronismo primario sono l'adenoma aldosterone-secerne (APA) e l'iperplasia surrenalica bilaterale idiopatica (IHA). Nonostante l'alta prevalenza di questa causa di ipertensione arteriosa (IA) i meccanismi fisiopatologici e molecolari che portano allo sviluppo dell'APA e che determinano l'ipersecrezione dell' aldosterone sono tuttora sconosciuti. Ciò dipende in larga misura dalla mancanza di modelli sperimentali ove investigare tali meccanismi.

Questo studio ha permesso di identificare l'antigene di membrana CD56 come marker specifico delle cellule di APA e ZG. Colorazioni di immunostochimica hanno mostrato che CD56 è abbondantemente espresso sia nella corteccia che nella midollare del surrene normale. Nella corteccia l'immunoreattività risulta intensamente positiva a livello della zona glomerulosa, mentre la zona fasciolata è solo debolmente positiva; CD56 risulta assente nella zona reticolare. Tutti gli APA esaminati esprimono alti livelli di CD56. In tutti i tipi di tessuto esaminati l'immunoreattività di CD56 è circoscritta alla membrana cellulare e in particolare a livello dei siti di contatto cellula-cellula.

Scopo dello studio è stato quello di sviluppare un metodo per l'isolamento di una popolazione pura di cellule da APA e da ZG sfruttando la presenza di questo antigene. E' stata, pertanto, sviluppata una metodica di immunoseparazione positiva per l'isolamento di questi tipi cellulari dal pool di cellule disperse ottenute in seguito a disgregazione del tessuto di partenza. Mediante immunoseparazione con biglie magnetiche

precondizionate con anticorpo anti CD56 sono state ottenute 2 popolazioni cellulari: cellule CD56 negative (CD56-) e cellule CD56 positive (CD56+) legate alle biglie magnetiche.

Le cellule CD56+, caratterizzate tramite microscopia elettronica immediatamente dopo immunoseparazione, mostrano mitocondri tipici di cellule di ZG con creste tubulo-lamellari, abbondante reticolo endoplasmatico liscio e la presenza di gocce lipidiche. In seguito a colorazione immunocitochimica le cellule CD56- risultano positive per CD90, antigene di superficie dei fibroblasti e per il Fattore di von Willebrand, antigene di superficie delle cellule endoteliali. Al contrario, la colorazione per il CD 90 e Fattore di von Willebrand risulta negativa nelle cellule CD56+. All'analisi morfologica, le cellule CD56+, coltivate fino a 6 giorni su matrigel mostrano una morfologia di tipo *epitelioide* con nucleo prominente e gocce lipidiche sparse nel citoplasma. Inoltre, le cellule CD56+ mostrano un pattern di crescita caratterizzato dalla formazione di cluster sulla superficie di matrigel, mentre le cellule CD56- crescono in monostrato, caratteristica già descritta per le cellule di ZF e ZR. All'RT-RTPCR le cellule CD56+ sono caratterizzate da un'elevata espressione genica non solo di CD56 ma anche dell'aldosterone sintetasi (CYP11B2), enzima che catalizza la trasformazione di corticosterone in aldosterone, espresso esclusivamente nella zona glomerulosa surrenalica. Le cellule CD56+ mantengono sino al sesto giorno di coltura un'elevata produzione di aldosterone confermando la loro origine dalla ZG.

La metodologia sviluppata nel nostro laboratorio consente di isolare una popolazione pura di cellule aldosterone-secernenti che mantengono nel tempo le caratteristiche morfologiche e funzionali proprie di cellule di ZG e APA ed è pertanto dotata di alta specificità.

## ABSTRACT

Primary aldosteronism (PA) is a common cause of secondary hypertension and is characterized by an excess “autonomous” secretion of aldosterone. In a percentage ranging from a half to two thirds of the cases it is due to a surgically curable aldosterone-producing adenoma (APA) and in the rest to bilateral adrenal hyperplasia. For both conditions the molecular mechanisms leading to aldosterone oversecretion are completely unknown. A major factor contributing to hindering investigation of these mechanisms entails the current unavailability of methods to isolate a pure population of normal adrenocortical zona glomerulosa (ZG) and APA cells.

We have recently identified CD56 (Neural cell adhesion molecule, NCAM) as a cell specific membrane antigen of the normal human zona glomerulosa and medulla of the adrenal gland. By immunohistochemistry we detected intense CD56 immunostaining in the normal adrenal gland that was strictly confined to ZG and medulla; at contrary, zona fasciculata (ZF) was only weakly stained and labeling was totally absent in zona reticularis. The APA and pheochromocytoma cells, which are histogenetically derived from the ZG and the medulla, respectively, also showed intense CD56 immunostaining.

Taken advantage of this unequivocal expression of CD56 we developed a novel cells isolating methodology based on CD56 immunobinding to magnetic beads. Human ZG and APA cells were obtained from adrenal gland and APA tissue after adrenalectomy and cells expressing CD56 antigen were selected by positive immunoseparation.

We compared the morphology by light, phase and electron microscopy, and the function, by real-time polymerase chain reaction and radioimmunoassay of APA and ZG CD56-positive (CD56+) *versus* CD56-negative (CD56-) cells.



Analysis of CD56 positive cells under light and phase contrast microscopy evidenced that these cells formed clumps, as the ZG cells; electron microscopy showed that they had multiple features typical of a steroidogenic phenotype ( such as mitochondria with tubulo-lamellar cristae, several lipid droplets, some lysosome, lipofuscin bodies). By immunocytochemistry CD56 antigen was detectable in all CD56+ cells examined. No appreciable immunoreactivities to CD90 and Factor Von Willebrand were detected in CD56+ cells, at contrary in CD56- cells immunolabeling for CD90 and Factor Von Willebrand was stained.

These results show that the mRNA expression of CD56 and CYP11B2 genes in CD56+ cells were markedly higher than CD56- cells (+1600% and 2100% increase, respectively). They also maintained an evident secretion of aldosterone in culture until day 6 (+1380% increase CD56+ cells versus CD56- cells).

Hence, this study clearly indicates that CD56+ cells in culture maintained differentiated phenotype of ZG and APA cells supporting the conclusion that this novel technique is suitable for an accurate isolation of a pure aldosterone producing cells.

# INTRODUCTION

## *Adrenal gland zonation*

The adrenal glands (also known as suprarenal glands) are the triangular-shaped endocrine glands, placed on the top of the kidneys, surrounded by an adipose capsule and renal fascia and, in humans, localized at the level of the 12th thoracic vertebra. Each adrenal gland is separated into two distinct structures: the adrenal cortex and medulla. Based on embryologic origin and hormones secreted, the cortex and medulla are best considered as separate endocrine organs. The medulla produces catecholamines and the cortex produces several steroid hormones.

The adult adrenal cortex is composed of three concentric layers: zona glomerulosa, zona fasciculata, and zona reticularis, originally described in 1866 by Arnold (Rainey, 1999). This *anatomic zonation* can be appreciated at the microscopic level, where each zone can be recognized and distinguished from one another based on structural and anatomic characteristics (Belloni et al., 1987). While this first description was based on the histological organization, it is now accepted that these zones have functionally distinct roles in steroid hormone production. The adrenal cortex exhibits *functional zonation* as well: by virtue of the characteristic enzymes present in each zone, the zones produce and secrete distinct hormones. Namely, the glomerulosa synthesizes mineralocorticoids, the fasciculata produces glucocorticoids, and in the human, the zona reticularis produces androgens.

The outermost zone of adrenal cortex is the zona glomerulosa. Cells within this zone tend to be columnar in shape and are arranged in irregular cords. In particular, zona glomerulosa contains outer undifferentiated small cells and inner well-differentiated larger elements, which display mitochondria with tubulo-laminar cristae, abundant smooth endoplasmic reticulum (SER)

and sparse lipid droplets (Belloni et al., 1987). The zona fasciculata is the middle and largest of the three zones in the cortex. Cells in the fasciculata are polyhedral and usually have a foamy appearance due to abundant lipid droplets, typical mitochondria with vesicular cristae, a well-developed SER and some lipofuscin-pigment granules, which increase in number in the cells of the inner portion of the zone (Belloni et al., 1987). They also are arranged in distinctively straight cords that radiate toward the medulla. The innermost zone of the cortex is the zona reticularis: cells within this zone are arranged in cords that project in many different directions and anastomose with one another. The zona reticularis cells possess the same morphological features as the zona fasciculata elements, but display a striking rise in the number of lipofuscin-pigment granules (Belloni et al., 1987).

The adrenal medulla is composed of neural crest cells called chromaffin cells or pheochromocytes or 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. The chromaffin cells, surrounded by a meshwork of blood vessels called venous sinusoids, when stimulated by the sympathetic nervous system, secrete noradrenaline and adrenaline into the sinusoids, which are delivered by the bloodstream to the rest of the body. Adrenomedullary chromaffin cells are found in all zones of the adult adrenal cortex, testified by specific immunostaining for the neuroendocrine protein chromogranin-A in all three zones of the human adrenal cortex. Some appear as rays, at times stretching from the adrenal medulla through the entire cortex (Bornstein et al., 1990; Bornstein and Ehrhart-Bornstein, 1992; Bornstein and Ehrhart-Bornstein, 1992) others appear as islets of many cells or as single cells surrounded by steroid-producing cells. Medullary cells, especially in the zona glomerulosa, frequently spread into the subcapsular region, forming large nests of chromaffin cells (Bornstein et al., 1990;

Bornstein and Ehrhart-Bornstein, 1992). Chromaffin cells produce a wide variety of autocrine/ paracrine regulatory factors, including neuropeptides, classic neurotransmitters, and cytokines that stimulate adrenocortical steroidogenesis (Ehrhart-Bornstein et al., 1998; Nussdorfer, 1996; Nussdorfer, 1996). In addition, a local corticotropin-releasing hormone (CRH)–corticotropin system in the adrenal medulla participates in the intra-adrenal regulation of steroidogenesis (Ehrhart-Bornstein et al., 1998).

### ***Steroidogenesis***

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

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

Within the human adrenal cortex, steroid synthesis involves the coordinated actions of five forms of cytochrome P450 and the enzyme 3  $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD); these enzymes are distributed between the

mitochondria and the endoplasmic reticulum. It is the differential expression of these enzymes within the three adrenocortical zones that allows for the wide array of steroid hormones secreted by this gland.

Once at the inner mitochondrial membrane cholesterol is converted to pregnenolone by P45011A1 (P450<sub>scc</sub>). P45011A1 catalyzes three separate reactions, 20  $\alpha$ -hydroxylation, 22  $\alpha$ -hydroxylation, and C20-22 bond scissions, converting the C27 cholesterol to the C21 pregnenolone. Once pregnenolone is formed, it can be 17  $\beta$ -hydroxylated by P450c17 to form 17  $\alpha$ -hydroxypregnenolone or converted to progesterone by 3  $\beta$ -hydroxysteroid dehydrogenase (3  $\beta$ -HSD), the only non-P450 enzyme in the pathways. P450c17 can also form 17  $\beta$ -hydroxyprogesterone from progesterone. P450 17 $\alpha$  also catalyzes a 17-20 lyase reaction on 17-hydroxypregnenolone and 17-hydroxyprogesterone to form the adrenal androgens dehydroepiandrosterone and androstenedione, respectively. Both progesterone and 17-hydroxyprogesterone can be 21-hydroxylated by P450 C21 to yield deoxycorticosterone and 11-deoxycortisol, respectively. The final reactions in adrenocortical steroid hormone biosynthesis involve the hydroxylation of 11-deoxycortisol and deoxycorticosterone by P45011B1 to form cortisol and corticosterone, respectively. Corticosterone, in ZG, can then be converted to aldosterone by P45011B2 (CYP11B2 or aldosterone synthase) in a series of hydroxylation steps.

### ***Regulation of aldosterone synthesis***

The zona glomerulosa (ZG) is the only one with the ability to produce aldosterone (Conley and Bird, 1997). Aldosterone secretion by the ZG is under multifactorial regulation: Angiotensin II (Ang II), potassium ion (K<sup>+</sup>), and adrenocorticotropin hormone (ACTH), as well as epinephrine, serotonin, endothelin,  $\alpha$ -MSH, and vasopressin are all known to be potent secretagogues (Ehrhart-Bornstein et al., 1998; Gallo-Payet et al., 1996).

The three most important extracellular physiological stimuli of aldosterone secretion, Ang II,  $K^+$  and ACTH, converge on two major intracellular signaling pathways: an increase in cAMP production and calcium ( $Ca^{2+}$ ) mobilization.

The octapeptide Ang II is formed from the plasma protein angiotensinogen by the sequential action of two proteases, renin and angiotensin-converting enzyme. Ang II stimulates aldosterone synthesis by the activation of multiple intracellular signaling pathways. The effects of Ang II in glomerulosa cells are mediated through type I Ang II (AT1) receptors, a G protein coupled receptor (Spat and Hunyady, 2004). Stimulation of the AT1 receptor initiates a cascade of signaling events that includes activation of phosphoinositide-specific phospholipase C and hydrolysis of phosphatidylinositol 4, 5-bisphosphate, yielding soluble inositol 1, 4, 5-trisphosphate (IP3), and diacylglycerol (DAG). While DAG activates protein kinase C (PKC), IP3 induces the release of  $Ca^{2+}$  from intracellular stores, followed by the activation of store-operated  $Ca^{2+}$  channels. (Foster et al., 1997) (Fig. 2). Ang II also activates both T- and L-type  $Ca^{2+}$  channels by inducing cell depolarization through the inhibition of  $K^+$  channels (Maturana et al., 1999).

$K^+$  exerts its effects on aldosterone secretion through generating cytoplasmic  $Ca^{2+}$  signal. Aldosterone secretion in vivo and production in vitro are stimulated by increases in  $K^+$  concentration as small as a few tenths of millimolar (Funder et al., 1969; Pralong et al., 1992; Spat and Hunyady, 2004; Varnai et al., 1998). Potassium signaling in glomerulosa cells involves a depolarization of the membrane leading to an influx of calcium through T and L-type channels. Glomerulosa cells express both low-threshold and high-threshold voltage-activated  $Ca^{2+}$  channels (Spat et al., 1991). While supraphysiological concentrations of  $K^+$  trigger  $Ca^{2+}$  influx through L-type  $Ca^{2+}$  channels, physiological concentrations act predominantly or exclusively

by activating T-type channels (Lotshaw, 2001). The activation threshold of T-type channels in rat glomerulosa cells is between  $-80$  and  $-70$ mV (Varnai et al., 1998). An increase in  $K^+$  concentration by 1mM or less induces a very tiny depolarization, there are, however, mechanisms in the glomerulosa cells that amplify the initial signal. It was observed on bovine glomerulosa cells by Barrett that the increase of cytoplasmic  $Ca^{2+}$  concentration activates CaMKII (Ca<sup>2+</sup>/Calmodulin-Dependent Protein Kinases) and reduces the activation threshold of T-type channels (Barrett et al., 2000). This mechanism serves as a positive feed-back mechanism of  $Ca^{2+}$  signaling. Potassium-induced cytoplasmic  $Ca^{2+}$  signal activates adenylyl cyclase, induces and activates StAR protein and also enhances the expression of aldosterone synthase (FIG 2) (Spat and Hunyady, 2004).

ACTH effects on aldosterone secretion are initiated by the binding to its melanocortin 2 receptor (MC2) (Liakos et al., 1998; Mountjoy et al., 1992), a Gs-protein-coupled receptor, leads to an increase in intracellular cAMP and an activation of protein kinase A (PKA) (Gallo-Payet and Payet, 2003). The stimulatory effect of ACTH can be divided into two phases. In its long term action, ACTH stimulates biosynthesis of the enzymes involved in steroidogenesis by increasing their respective mRNA levels (Hanukoglu et al., 1990). In its short term action, ACTH activates conversion of cholesterol to pregnenolone, and then to cortisol in fasciculata cells and to 18-hydroxycorticosterone and aldosterone in glomerulosa cells (Gallo-Payet et al., 1996; Hanukoglu et al., 1990). Furthermore, it promotes transcription-independent stimulation of adrenal steroid synthesis and release. Several studies have shown that ACTH action is mediated not only by cAMP but also by calcium ( $Ca^{2+}$ ). Briefly, cAMP, by activating PKA, enables the phosphorylation of L-type  $Ca^{2+}$  channel. This increases cytosolic  $Ca^{2+}$  concentration and promotes membrane depolarization;  $Ca^{2+}$  influx further stimulates adenylyl cyclase and consequently steroid secretion. A positive feedback loop between adenylyl

cyclase-PKA and  $\text{Ca}^{2+}$  channel ensures a slow but substantial  $\text{Ca}^{2+}$  intracellular increase. This concomitant increase in intracellular  $\text{Ca}^{2+}$  and cAMP acts in synergy to stimulate steroid secretion. Gallo-Payet clearly demonstrated that, in human ZG cells, ACTH-stimulated aldosterone secretion involves a tightly coupled interaction between cAMP and  $\text{Ca}^{2+}$  pathways (FIG 2) (Gallo-Payet and Payet, 1989; Gallo-Payet et al., 1996). One of the major signaling systems that is activated by elevated intracellular  $\text{Ca}^{2+}$  includes  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (CaMKs) that transduces elevated  $\text{Ca}^{2+}$  signals to a number of target proteins.

Moreover, ACTH,  $\text{K}^+$  and Ang II regulate the aldosterone secretion through the two main rate-limiting steps of steroidogenesis: the transport of cholesterol in mitochondria (fast regulation) and the transcription of aldosterone synthase gene (CYP11B2) (slow regulation). In particular, Ang II and  $\text{K}^+$  promote the synthesis of new transcription factors, such as the neuronal growth factor-induced clone B (NGFI-B) family members (NGFI-B, NURR1, and NOR1) that have been reported to regulate CYP11B2 expression. Several conserved cis-elements within the promoters of CYP11B2 gene were identified: a CRE element at -71/-64 (cAMP response element), an element termed Ad5 at -129/-114 and a cis-element termed NBRE-1 (-766/-759) (Nogueira et al., 2009; Romero et al., 2007). The NGFI-B response element (NBRE) has been shown to regulate transcription by its binding to members of the NGFI-B family. Ang II may also induce post-translational modifications of transcription factors already present in the cell, such as phosphorylation of cAMP response element (CRE)-binding proteins (CREBs). CYP11B2 CRE is able to bind members of the activating transcription factor, ATF-1 and ATF-2, and CRE-binding protein (CREB) families and CRE modulator (Nogueira and Rainey, 2010). Rainey et al. showed that Ang II induces phosphorylation of ATF1, ATF2, and CRE modulator in a time-dependent manner. These post-translation



modifications promote an increased association of these proteins with the CYP11B2 promoter after Ang II and K<sup>+</sup> treatment. Moreover, they demonstrated that knockdown of these transcription factors decreased Ang II- and K<sup>+</sup>-induced expression of CYP11B2 and aldosterone production. The combined knockdown of ATF/CREB family members and NURR1 also caused a further decrease in CYP11B2 expression. Instead, cotransfection of constitutively active ATF/CREB members with NURR1 (NGFI-B family member) had a synergistic effect on CYP11B2 promoter activity. The authors suggest that Ang II and K<sup>+</sup> regulate the CYP11B2 gene transcription by the induction of NGFI-B family expression and the activation of ATF/CREB members (Nogueira and Rainey, 2010).

### ***Primary Aldosteronism and Aldosterone Producing Adenomas***

#### ***Prevalence and diagnosis of Aldosterone Producing Adenomas***

Primary aldosteronism (PA) is considered one of the more common causes of secondary hypertension, defined as hypertension associated with low renin and increased aldosterone levels that are not suppressed by appropriate testing (saline infusion test, captopril test). Litynski reported the first cases (Litynski, 1953), but Conn was the first to well characterize the disorder in 1956 (Coon and Louis, 1956). Primary aldosteronism, described by Conn in a 34-year-old woman, was characterized by hypertension, intermittent paralysis, hypokalemia and metabolic alkalosis. Further biochemical analyses detected increased activity of urinary salt-retaining corticoid hormone. The patient was cured by removal of a benign adrenal adenoma (Coon and Louis, 1956).

In the Primary Aldosteronism Prevalence in Italy (PAPY) study, a prospective survey of 1180 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, respectively, thus leading to an overall prevalence of PA of 11% (Rossi et al., 2006 a). Clinically, the distinction between the 2 major causes of PA is crucial for the choice of treatment. While the treatment indicated for aldosteronomas is surgical removal, the treatment of choice for IAH is medical therapy with mineralocorticoid receptor antagonists (Rossi et al., 2005; Rossi et al., 2008).

The complete list of entities known to give a biochemical pattern of PA includes aldosterone-producing adenomas (APAs), aldosterone-producing renin-responsive adenomas (AP-RAs), bilateral adrenal hyperplasia or IAH, primary adrenal hyperplasia (PAH), and familial forms of PA. Two distinct genetic-familial varieties of PA exist: the type 1 variety of familial PA, glucocorticoid-remediable aldosteronism (GRA) and the type 2 variant of familial PH, which is not glucocorticoid sensitive (Rich et al., 1992). Normal regulation of aldosterone secretion is mediated to varying degrees by renin, serum potassium and sodium levels, intravascular volume status, and adrenocorticotrophic hormone (ACTH). While the various forms of PA differ in the altered role of these factors in regulation of aldosterone production, they share an autonomous and elevated secretion of aldosterone with plasma renin levels suppressed. As example, in patients with glucocorticoid-remediable aldosteronism, secretion of aldosterone (along with the other adrenal corticosteroids) is regulated completely by corticotropin, because of the presence of the abnormal steroidogenic gene (Lifton et al., 1992). In some case of aldosteronomas, aldosterone secretion is not influenced by ACTH (Ganguly and Donohue, 1983; Ganguly, 1992), in other case it is unresponsive to angiotensin II, as evident in an abnormal aldosterone response to postural testing (Ganguly et al., 1977). Other patients with aldosteronomas (Ganguly, 1992; Gordon, 1994; Irony et al., 1990) and those with idiopathic aldosteronism (Wisgerhof et al., 1981) are responsive to small increases in the plasma level of angiotensin II and have a normal plasma aldosterone

response on postural testing. Thus, there are at least two functionally and perhaps histologically different types of aldosteronomas: a corticotropin-responsive (and renin-unresponsive) type and a renin-responsive type.

The diagnosis of APA, the unique clinical form of PA identifiable with certainty, is established when the following “four corners” criteria are satisfied:

- (1) evidence of PA at the biochemical screening test (serum creatinine, serum and urine Na<sup>+</sup> and K<sup>+</sup> levels, PRA, aldosterone, cortisol, and glomerular filtration rate);
- (2) lateralization of aldosterone secretion at AVS or at <sup>131</sup>I-norcholesterol dexamethasone-suppressed adrenocortical scintigraphy;
- (3) evidence of adenoma at computed tomography, and/or magnetic resonance, and/or surgery, and/or pathology;
- (4) demonstration of normokalemia and HT cure, or improvement, at follow-up after adrenalectomy (Rossi et al., 2007).

### ***Pathophysiologic changes in aldosterone secretion***

The cardinal pathophysiologic anomaly causing primary aldosteronism (PA) syndrome is the autonomous aldosterone production. The steroid hormone aldosterone, secreted by the glomerulosa cells of the adrenal cortex, controls sodium and potassium balance and also influences acid-base homeostasis of the vertebrate organism. Its major physiological targets are the epithelial cells, mostly located in the distal nephron. It enhances Na<sup>+</sup> reabsorption as well as K<sup>+</sup> and H<sup>+</sup> excretion. Sodium retention promotes water retention, with expansion of the extracellular volume, hypertension, and suppression of renin production (Rossi et al., 2006 b). Excessive potassium loss causes hypokalemic alkalosis, which may be associated with various clinical features, varying from abnormal electrocardiographic findings up to muscular weakness and tetany. In past years, hypokalemia was thought to

be a mandatory finding in primary aldosteronism but, as just noted by Conn and colleagues in 1965 (Conn et al., 1965), the potassium levels in primary aldosteronism could also be normal. Moreover, more recent studies confirmed that most patients with primary aldosteronism are normokalemic (Calhoun et al., 2002; Mulatero et al., 2004; Rossi et al., 2002).

In addition to its epithelial actions, aldosterone has some effects on nonepithelial tissues. These include increased oxidative stress and collagen remodelling, resulting in endothelial dysfunction, left ventricular hypertrophy and fibrosis in the kidney, heart and blood vessels (Brown, 2005).

### ***Molecular studies on APA***

The genetic background of APA and the molecular mechanisms by which a normal adrenocortical zona glomerulosa evolves into an aldosteronoma causing autonomous hypersecretion of aldosterone remain poorly understood. For example about the genetic abnormalities, the most obvious candidate gene, CYP11B2, do not participate in tumorigenesis in most adrenocortical lesions: only in familial hyperaldosteronism type I there is an aberrant expression of the CYP11B2 gene (fusion between the corticotrophin-regulated promoter of 11-b-hydroxylase and the sequence that codes for aldosterone synthase) (Lifton et al., 1992).

It was suggested a transcriptional modulation of aldosterone production in the tumour leading to excess aldosterone production, based on the fact that the gene expression of CYP11B2 is higher in APA than in normal adrenocortical tissues (Bassett et al., 2005; Fallo et al., 2002). Assié et al. compared gene expression in APA and the adjacent ZG tissue, both differentiated for aldosterone production. The observed differences were independent of circulating angiotensin II levels and better reflect the steroidogenic alterations responsible for autonomous aldosterone

production. Differences in gene expression were characterized by two complementary approaches: SAGE and ISH. Analysis on mRNA levels of steroidogenic enzyme expressed in APA demonstrated overexpression of aldosterone synthase, 11 $\beta$ -hydroxylase and 21-hydroxylase. Interestingly they observed in APA a coordinated overexpression of several mRNAs encoding calcium-activating membrane receptors, calcium-signaling effectors, and proteins involved in endoplasmic reticulum calcium storage. For the authors this calcium activation could represent the starting point of the other gene expression changes observed in APA (Assie et al., 2005).

Bassett et al. compared, by microarray analysis and quantitative RT-PCR, the expression profiles of the steroidogenic enzymes in APA and normal adrenal cortex and demonstrated an overexpression of CYP11B2, 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (HSD11B2) and CYP21 (21-hydroxylase). They also examined, in adenoma samples, the presence/absence of SF-1 and DAX-1 that are known as two orphan receptors colocalized in the adrenal gland and are implicated in the transcriptional regulation of several steroidogenic genes, including StAR, CYP11A, CYP17, and CYP11B2. The study highlighted that mRNA levels for both SF-1 and DAX-1 were higher in APA than in normal adrenal cortex (Bassett et al., 2005).

Lenzini et al., by microarray analysis and quantitative RT-PCR, compared the whole transcriptome of APAs with a pool of histologically normal subcapsular adrenocortical tissues. They identified 2 APA subgroups: 1 featuring overexpression of CYP11B2, CAMK-I, 11- $\beta$ -hydroxylase, 3  $\beta$ -hydroxysteroid dehydrogenase, and 21-hydroxylase and the underexpression of CAMK-II $\beta$  and the other one with an opposite profile. The low CYP11B2 group exhibited a longer known duration of hypertension and a lower rate of long-term cure; instead, the high CYP11B2 patients had a significantly shorter known duration of hypertension. The authors suggested that aldosterone overproduction in APAs involves complex alterations of aldosterone

synthesis regulation rather than simply increased aldosterone synthase gene expression (Lenzini et al., 2007).

Williams et al. by oligonucleotide microarrays found 53 genes differentially expressed in APA compared with normal adrenals, of which 33 were upregulated and 20 were downregulated. They showed that teratocarcinoma-derived growth factor 1 (TDGF-1), also called Cripto-1, was upregulated in APA compared with normal adrenals. The authors investigated the function of TDGF-1 using as in vitro model H295 adrenocortical cell line. They demonstrated that 1) TDGF-1 increased aldosterone through the activation of Akt signaling pathway, and 2) that the inhibitors of phosphatidylinositol 3-kinase were able to block the increase TDGF-1-mediated aldosterone secretion. Moreover, TDGF-1 showed an antiapoptotic effect in H295 cells. The authors advanced that TDGF-1, may represent one of several changes involved in the pathogenesis of an APA since TDGF-1 is significantly upregulated in APA and mediates aldosterone hypersecretion and deregulated growth in adrenocortical cells in vitro (Williams et al., 2010).

The impossibility of using a homogeneous population of normal human zona glomerulosa cells as a referent could be considered as potential limitations of these studies: the comparison of the relatively homogeneous APA tissue with the heterogeneous subcapsular tissue/ZG, containing other cell phenotypes such as vascular cells, fibroblast cells, in the studies of Lenzini and Assié, or with the entire adrenal cortex in the studies of Bassett and Williams would be expected to cause, if any, an overassessment of the expression of genes involved in aldosterone production in APAs.

### ***Adrenocortical cell culture: state of the art***

The poor knowledge of the molecular mechanisms on the pathophysiology of APA requires a suitable model for investigating the regulation of

aldosterone secretion *in vitro*, such as a pure population of APA and normal adrenocortical zona glomerulosa (ZG) cells. Primary culture of adrenocortical cells is a widely used experimental model for the study of steroid-hormone secretion and its regulation. A number of *in vitro* systems have been developed, including: cell suspensions from acutely dispersed tissue, primary cultures from normal adrenal glands and adrenal tumours. The used methods for isolation of adrenocortical cells were based on either manual dissection of the different adrenal zones (Gallo-Payet et al., 1993; Gallo-Payet et al., 1996; Nussdorfer, 1986; Nussdorfer, 1996; Spinazzi et al., 2006) or density gradient centrifugation (Roskelley and Auersperg, 1990; Young et al., 2003).

In manual dissection zona fasciculata (ZF), zona reticularis (ZR) and medulla are cut away and the dispersed ZG cells are obtained by sequential enzymatic digestion and mechanical disaggregation.

Young et al. developed a method that separates human adrenocortical cells by density into two discrete fractions, identified as fraction I and fraction II. Cells cultured from fraction I had a high lipid content and a low nuclear to cytoplasm ratio and they produced high level of cortisol. In contrast, cells of fraction II were lipid-poor in appearance and had a high nuclear to cytoplasm ratio. Aldosterone production by fraction II cells was significantly greater than that by fraction I cells, basally and following stimulation with ACTH and AngII (Young et al., 2003).

However, ZG primary cultures established from adrenal glands with the methods previous described result composed of heterogeneous mixture of cell types: zona fasciculata cells, fibroblasts and vascular cells are recognized. Only laser capture microdissection provides separation of pure population of ZG cells, but is unfeasible for reason of costs and time and if a large number of cells is desired.

Furthermore, the limited life-span of adrenocortical cells in culture, due to their rapid de-differentiation into fibroblast-like cells (Nussdorfer, 1996), prevents the use of primary cultures in long-term studies. This is particularly true in the case of zona glomerulosa cells, which within 48-72 h of *in vitro* growth lose their capability to secrete aldosterone. Gallo-Payet et al. reported that one day in culture induces a decrease of 65% in the basal level of aldosterone secretions, compared to that observed in freshly isolated cells. Therefore, the authors suggested that cells can be maintained for 4 days at least in primary culture without change in their ultrastructural characteristic or their capacity to produce aldosterone under various hormonal stimulation (Gallo-Payet et al., 1993).

Spinazzi et al. compared the morphology and function of rat ZG cells grown on plastic and matrigel basement membrane matrix for up to 12 days. The authors showed that starting from day 6, ZG cells cultured on plastic lost their ultrastructural differentiated features, such as mitochondria with tubular cristae, smooth endoplasmic reticulum cisternae and lipid droplets, and they started to exhibit a fibroblast-like appearance. Moreover, the mRNA expression of the main steroidogenic enzymes, the baseline secretion of aldosterone and other post-pregnenolone hormones and the secretory response to ACTH, angiotensin-II and  $K^+$ , displayed a time-dependent decrease. Instead, matrigel was found to maintain unchanged both the ultrastructure and the expression of steroidogenic enzymes of ZG cells until day 12 of culture, even if, baseline and agonist-stimulated steroid-hormone secretion decreased with the duration of culture on matrigel, but was always higher than that observed in ZG cells grown on plastic. Hence, this study clearly indicates that the culture on matrigel favours the maintenance of rat ZG-cell differentiated phenotype, allowing the conclusion that the use of this matrix is suitable for long-term *in vitro* investigations (Spinazzi et al., 2006).



The use of stabilized and immortalized adrenocortical cell lines could overcome these difficulties (Rainey et al., 2004). Unfortunately, the presently available cell lines H295 and the HAC15 adrenocortical carcinoma cell lines do not appear to be a very suitable model for investigating adrenal gland physiology, inasmuch as their ultrastructural features do not reflect those of normal adrenocortical cells. The only human models available are. These cell lines, have been characterized to contain all the enzymes required for aldosterone biosynthesis and they are used by many endocrinologists as model system for studying the molecular and biochemical mechanism controlling adrenal steroidogenesis (Parmar et al., 2008; Rainey et al., 2004). However they do not allow the study of the mechanisms underlying the regulation of the normal adrenocortical zona glomerulosa neither do they provide information on benign aldosterone-producing adenoma pathophysiology.

Hence, the need of a primary culture technique allowing the isolation of a "pure" population ZG cells and the long-term preservation of the differentiated cell phenotype as aldosterone secreting cells is compelling.

### ***Specific cell surface antigen profile of adrenocortical cells***

The study of APA and PA pathophysiology has been hampered by the difficulty in obtaining purified human normal adrenocortical zona glomerulosa (ZG) and APA cells. Little is known about the cell surface antigen profile of adrenocortical cells and in particular antigens that are expressed differentially on specific cells of each zone.

Young et al. identified as specific cell surface markers to define distinct zones of the human adrenal cortex the tumour growth factor- $\beta$  receptor-1 (TGF $\beta$ -R1) and human leukocyte antigen (HLA-DR). TGF $\beta$ -R1 was expressed predominantly in the zona fasciculata cell layer with some positive-staining cells projecting into the zona reticularis. HLA-DR-positive

cells were found primarily within the zona reticularis (Young et al., 2003). Among some candidate cell surface antigens they investigated also the angiotensin receptor (AT-1) that was found to be expressed predominantly in the zona glomerulosa. Of interest, occasional focal nests of cells expressing AT-1 were observed in the zona fasciculata. AT-1 expression in human adrenal fasciculata cells has been previously described and angiotensin II has been found to stimulate cortisol expression and increase the expression of steroid enzyme mRNA (Lebrethon et al., 1994; Ouali et al., 1993).

Khorram-Manesh et al. evaluated the expression pattern of cell adhesion molecules (CAMs), such as N-cadherin (NCAD), E-cadherin (ECAD), neural cell adhesion molecule (NCAM), and CD44 in normal adrenal gland and in adrenal tumours (adrenocortical adenomas, adrenocortical carcinomas, and pheochromocytomas) by immunohistochemistry. The aim of the study was to evaluate the expression pattern of CAM in adrenal tumours regarding origin (cortex vs medulla) and biologic behaviour (benign vs malignant). NCAD was expressed in all layers of the adrenal cortex and in medulla. It was also detected in 12/27 benign pheochromocytomas (BPCs) (12 familial cases), 8/8 malignant pheochromocytomas (MPCs), 28/30 adrenocortical adenomas, and 9/22 adrenocortical carcinomas. No labeling was observed for ECAD in the normal adrenal cortex and medulla; it was expressed only in 2/22 adrenocortical carcinomas. NCAM was expressed in the normal adrenal cortex and medulla. The authors reported that strong labeling was present in the medulla, whereas labeling in all layers of the adrenal cortex was weaker. Immunohistochemically 26/27 BPCs, 7/8 MPCs, 21/30 adrenocortical adenomas, and 17/22 adrenocortical carcinomas were positive for NCAM. Focal and weak CD44 labeling was detected in cell membranes of zona glomerulosa cells and strong labeling in cell membranes of chromaffin cells of the medulla. CD44 was expressed in 23/27 BPCs, 6/8 MPCs, 7/30

adrenocortical adenomas, and 4/22 adrenocortical carcinomas (Khorram-Manesh et al., 2002).

Muench et al. and Erhart-Bornstein et al. investigated the expression of NCAM in fetal and adult adrenal gland. NCAM staining was restricted to the definitive zone (DZ) in the fetus (Muench et al., 2003), to the zona glomerulosa (ZG) and to adrenomedullary chromaffin cells in adults (Ehrhart-Bornstein and Hilbers, 1998). In particular, Muench et al. found that the cells of the definitive zone, localized in the fetal adrenal gland immediately beneath the capsule and considered as a progenitor (stem) population, capable of migrating into other zones, intensely express CD56 (Muench et al., 2003). Moreover, at late gestation, aldosterone synthase and other steroidogenic enzymes are expressed by DZ cells, like ZG cells in the adult adrenal (Haak and Fleuren, 1995; Mesiano et al., 1993). Although they did not examine the human adult adrenal gland, since they unequivocally obtained cells from the definitive zone by laser microdissection, their study strongly suggested the possibility that ZG expresses CD56 (Muench et al., 2003).

Moreover, Zeromski et al. investigated CD56 antigen and mRNA expression in normal adrenal cortex, in cortical hyperplasia and in adrenal adenoma: all tissue were positive with various intensity (Zeromski et al., 1998; Zeromski et al., 2001).

Taken together, these studies strongly suggest the possibility that NCAM/CD56 is a specific cell surface marker of zona glomerulosa or APA cells.

## **AIMS**

Aims of the study are:

- a) verify that CD56/NCAM is a specific membrane antigen of normal human ZG cells and APA cells;
- b) develop a novel methodology for isolating aldosterone-secreting cells from the normal human adrenocortical ZG and from surgically removed APA tissue based on use of CD56 binding to magnetic beads.

## MATERIALS AND METHODS

### *Subjects*

The study was approved by Ethics Committee and each patient consented to the study. Adrenocortical tissues from 10 patients with an APA and adrenomedullary tissues from 10 patients with pheochromocytoma were investigated. Histologically normal adrenocortical (NAC) tissue obtained at surgery from three patients with renal cancer undergoing unilateral nephrectomy and ipsilateral adrenalectomy were studied as controls for adrenocortical and APA tissues. Histologically normal medullary tissue (NAM) obtained from five patients undergoing adrenalectomy for non functioning incidentally discovered adrenal mass (incidentaloma) served as control for the pheochromocytoma (Pheo) tissues.

Tissues were obtained under sterile conditions at surgery, in the operating room and divided in two sections. One section was immediately fixed in neutral formalin and included in paraffin, the remaining tissue was reserved in medium M199, supplemented with 20 U/mL penicillin G, 20 µg/mL streptomycin sulfate and 50 ng/mL amphotericin B, until cell dispersion.

In the patients with APA the diagnosis was based on strict predefined criteria that comprised lateralization of aldosterone secretion at adrenal vein sampling (Rossi et al., 2001), surgery, pathology, and, more importantly, follow-up data. At follow-up, we required demonstration of normokalemia and cure or improvement of hypertension at least 120 days after adrenalectomy (Rossi et al., 2006a). Cure was defined as a systolic blood pressure <140 mmHg and diastolic blood pressure <90 mmHg 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, as described by the World Health Organization (Anonymous, 1999).

Pheo was diagnosed according to state-of-the-art criteria (Rossi et al., 2007), and confirmed at pathology, histology, and chromogranin A and synaptophysin immunostaining (Komminoth et al., 1995).

### *Immunohistochemistry*

Four  $\mu\text{m}$ -thick serial sections from paraffin blocks were stained with hematoxylin eosin (HE) for histological diagnosis. Serial 4  $\mu\text{m}$  sections were also used for immunohistochemistry (IHC) with an indirect immunoperoxidase-based technique (Bond polymer Refine detection, Vision Biosystem, Newcastle upon Tyne, UK) and a fully automated system (Bond-maX, Vision Biosystem, Newcastle upon Tyne, UK). The sections were dewaxed and rehydrated with Bond Dewax solution (Vision Biosystem, Newcastle upon Tyne, UK), ethanol and distilled water. Antigen was retrieved by heating the sections previously immersed in Bond Epitope Retrieval solution 1 (Vision Biosystem, Newcastle upon Tyne, UK) at 100°C for 30 minutes. Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide and then incubated with a mouse monoclonal antibody against human CD56 (clone 1B6, Novocastra, Newcastle upon Tyne, UK) for 5 minutes. The slides were washed three times with PBS and incubated with a secondary antibody labelled with horseradish peroxidase (HRP). SigmaFast 3-3'-diaminobenzidine substrate solution was applied to reveal the color of the antibody staining.

The sections were counterstained with hematoxylin, dehydrated, cleared and mounted.

## *Isolation of APA and ZG cells with CD56 precoated magnetic beads*

### *Cell dispersion and viability*

Normal adrenal glands, adjacent to APA, and APA tissues were immediately processed after surgery.

Aldosteronomas were well-circumscribed by an identifiable capsule, they were trimmed of fat and connective tissue and diced into small pieces.

Normal adrenal glands, adjacent to APA, were cleansed of fat, connective and adrenomedullary tissue and minced into small pieces.

Dispersed ZG or APA cells were obtained by sequential enzymatic digestion, in digestion mixture, and mechanical disaggregation. The digestion mixture consisted of 5 mL of Krebs Ringer solution containing 2 mg/mL collagenase-I, 0.1 mg/mL deoxyribonuclease-I, 4% bovine serum albumin. Digestion and mechanical dispersion were carried out twice for 40 minutes, each time at 37°C with gentle shaking, pipetting cells between each digestion. Then, the tissues were completely dissociated by gentle aspiration with a sterile 10 mL syringe and by filtering them through a 40 µm strainer (BD Biosciences, Buccinasco, Italy). The cells were concentrated by centrifugation for 10 min at 100xg at 4°C. The cells were then suspended in 5 mL in Krebs Ringer supplemented with 4% bovine serum albumin and centrifuged twice for 10 min at 100xg. Cell viability was assessed by trypan blue exclusion.

### *Cell isolation using Dynabeads*

Dynabeads are uniform spherical beads that have been made magnetisable and supermagnetic, meaning they are only magnetic in a magnetic field. The attachment of target-specific antibodies to the surface of the beads allows capture and isolation of intact cells directly from a complex suspension such as whole blood or tissue digests.

There are two main strategies for isolating a specific cell type, they are conventionally defined positive isolation or negative isolation.

Positive cell isolation is defined as the method whereby a single cell type is directly drawn out of a mixture of cells using cell-specific antibodies or ligands linked to magnetic beads. The antibodies or ligands can be covalently attached directly to the magnetic bead or via secondary antibodies. Positive cell isolation can be performed using two different approaches: the direct or indirect technique. Using the direct technique, the primary antibody or ligand is linked to the beads (primary coated beads) prior to cell isolation. The primary coated beads are mixed with the cell sample, and after a short incubation period, whereby the cells bind to the primary coated beads, cells are easily collected with the aid of a magnet (Fig. 3). In the indirect technique, usually, secondary coated Dynabeads are used. The cell sample is first mixed with the primary antibody (or other binding molecule, defining the target), and after a short incubation, excess antibody is removed by centrifugation (Fig. 3). Secondary coated Dynabeads (coated with an antibody or other molecules which can bind the primary antibody) are then mixed with the sample, and during a short incubation the cells of interest are bound to the beads, and can thereafter isolate by the aid of a magnet. Secondary coated Dynabeads can be used both in the direct and indirect technique.

Instead, negative cell isolation is defined as a method whereby the cell type of interest is isolated by removing all other cell type from the sample. Generally a cocktail of monoclonal antibodies against the unwanted cells is incubated with the sample for about 20 minutes. Excess antibodies are removed by a short centrifugation step, followed by addition of secondary coated magnetic beads. The unwanted antibody sensitized cells bind the secondary-coated magnetic beads and then are removed by aid of a magnet (Fig. 3). The advantage of negative cell isolation is that the cells of interest have not been attached to the antibodies on the magnetic beads at any time,



and thereby avoiding any possible antibody induced signaling through cell surface molecules.

Factors such as incubation time, temperature, and concentration of reactants have a measurable effect on the efficiency of cell isolation using magnetic beads. Furthermore, the process is also affected by specific parameters, such as the nature and state of the target cell, characteristic of the antigen/antibody binding, sample time, concentration, and ratio of beads and cells. Successful cell isolation with Dynabeads, which implies high yield and purity, is dependent on the concentration of the magnetic beads, the ratio of beads to target cells, and the choice of antibody. Monoclonal antibodies are generally used due to their high specificity towards the target antigen.

### ***Precoating of the magnetic beads with CD56 antibody***

In our experiments we used Dynabeads Goat anti-mouse IgG (secondary coated magnetic beads). They are uniform, supermagnetic polystyrene beads (4,5  $\mu\text{m}$  diameter) coated with polyclonal goat anti-mouse IgG antibodies.

The antibody coated onto Dynabeads recognizes the heavy chain of most mouse IgG subclasses and is Fc-reactive.

Briefly, in our protocol the primary mouse IgG antibody CD56 is pre-coated onto the beads prior to cell isolation. Then Dynabeads are mixed with the cell sample in a tube. The Dynabeads bind the target cells during a short incubation, and then the beads-bound cells are separated by a magnet (positive isolation, direct technique).

Magnetic beads (Dynabeads, Invitrogen, MI, IT) ( $4 \times 10^8$ ) conjugated with goat anti-mouse IgG were first washed three times with PBS/0,1% bovine serum albumin and then precoated with 8  $\mu\text{g}$  purified mouse anti-human CD56 IgG (Biolegend, San Diego, CA). Precoating was obtained by incubating the beads with the primary antibody overnight at 4°C under gentle tilting and rotation. Beads were washed for 3 times and then used for cell separation.

### ***Setting-up of the method***

To set-up our method we used as experimental model a mixed cell population of endothelial cells and H295 cells. Cell suspensions of experimentally mixed endothelial cells and H295 was prepared using 50% endothelial cells and 50% H295 cells. CD56 antigen is expressed only by H295 cells. To optimised the necessary ratio of cells to CD56 pre-coated magnetic beads for complete positive isolation of H295 cells from endothelial/H295 cell mixture, we used different ratios of cells:beads, such as 1:5, 1:10, 1:20. Single-cell suspension of experimentally mixed endothelial and H295 was washed with PBS/0,1% bovine serum albumin and then incubated with CD56 pre-coated magnetic beads by gentle shaking for 30 min at 4 °C. The H295 CD56 positive cells were separated with a magnet. The untouched cells were seeded on glass coverlips and were analysed for the presence of H295 cells by immunohistochemistry with CD56 antibody, as described below.

### ***Isolation and culture of ZG and APA cells***

ZG and APA single-cell suspension obtained after enzymatic and mechanic digestion was washed with PBS/0,1% bovine serum albumin and then incubated with CD56 pre-coated magnetic beads by gentle shaking for 30 min at 4 °C. Beads were used at a ratio of five beads per cell.

After separating the bead-bound CD56-positive cells with a magnet, the CD56-positive cells were harvested in PBS/0, 1% bovine serum albumin and seeded at a density of  $10^4$  cells/cm<sup>2</sup> into 24-well BD BioCoat Matrigel Invasion Chamber plates. The cells were cultured in M199 (Sigma, Milan, Italy) medium additionated with heat-inactivated 10% FCS, 20 U/mL penicillin G, 20 µg/mL streptomycin sulfate and 50 ng/mL amphotericin B, 1% ITS (insulin, transferrin and selenium) Premix (BD Biosciences, Buccinasco, Italy),

and maintained in a humidified 5% carbon dioxide/air atmosphere at 37 °C. CD56-negative cells were also cultured following the same procedure.

The culture medium was changed 24 h after seeding and every three days of culture.

### ***Electron microscopy***

Freshly immunoseparated APA CD56+ and CD56- cells were seeded in BD BioCoat Matrigel Invasion Chamber plates and formed a monolayer. After an over night incubation, the cells were fixed in 3% phosphate-buffered glutaraldehyde, and then post-fixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Epon-812 . Thin (60 nm) sections were cut with an LKB SuperNova ultramicrotome (Reichert-Jung, Vienna, Austria), counterstained with leadhydroxide and uranyl acetate, and observed in a Hitachi H-300 transmission electron microscope.

### ***Immunocytochemistry***

The CD56+ and CD56- cells were cultivated on glass coverlips, precoated with matrigel, and were analysed by immunocytochemistry at 6 day of culture. The cells were washed with PBS and fixed with 4% paraformaldehyde pH 7,4 for 30 minutes at 4 °C. The samples were rinsed twice times with ice cold PBS and then CD56+ and CD56- cells were permeabilized by incubation with PBS containing 0.5% Triton X-100 for 10 minutes at RT. Fixed and permeabilized cells were incubate in 3% H<sub>2</sub>O<sub>2</sub> solution for 10 minutes at room temperature to block endogenous peroxidase activity. Blocking was also performed with PBS additionated with 3% BSA (bovine serum albumin) and 10% fetal bovine serum to block unspecific binding of the antibodies. After glass coverlips were incubated overnight at 4 °C with primary antibodies diluted 1:100 in blocking buffer. The following antibodies were used: anti-human CD56 mouse monoclonal antibody (clone 1B6, Novocastra, Newcastle upon Tyne, UK), anti-human CD90 (Thy-

1)/fibroblast marker mouse monoclonal antibody (Dianova, Hamburg, DE) and polyclonal rabbit anti-human Von Willebrand Factor (DAKO, Glostrup, Denmark). The cells were washed three times with PBS, 15 min/wash. After washing, cells were exposed to secondary antibody for 1 hour at room temperature. In particular, peroxidase-labeled mouse IgG specific for human IgG (DAKO, Glostrup, Denmark) was used to visualize CD56 and CD90 bound antibodies and peroxidase-labeled rabbit IgG specific for human IgG (DAKO, Glostrup, Denmark) to visualize factor von willebrand bound antibody. The peroxidase activity was detected with H<sub>2</sub>O<sub>2</sub> and 3'-3'-5'-5'-tetramethyl benzidine.

Negative controls were identically processed, but with omission of the primary antibody.

### *Phenotypic characterization of CD56+ and CD56- cells*

The two distinct cell population separated by using precoated beads were cultured for 6 days to examine their phenotypical characteristics and growth patterns. To identify viable cells the CD56+ and CD56- cells were assessed with microscopy and also examined with trypan blue staining immediately after positive selection and at the end of the culture.

To determine if the CD56+ cells had an aldosterone-secreting phenotype the concentration of aldosterone was measured in the cell supernatant. At day 4 fresh medium was added, and after 48h it was collected and stored at -20°C until aldosterone was measured with a commercially available RIA kit (DiaSorin, Saluggia, Italy). Intra- and Inter-assay CV was <5.6%.

Moreover, RT-PCR was performed to detect the aldosterone synthase (CYP11B2) gene transcript, using the porphobilinogen deaminase (PBGD) gene as housekeeping gene. The CD56- cells were similarly investigated and compared to CD56+ cells for aldosterone production and CYP11B2 gene expression.

### *CYP11B2 and CD56 gene expression*

Total RNA was extracted from both cultured CD56-positive and CD56-negative cells using the RNAqueous Micro kit (Ambion, Texas, USA), following the manufacturer's protocol. Briefly, the cells were collected by trypsinization and disrupted in 100  $\mu$ L Lysis Solution. The lysate was then mixed with ethanol and applied to a silica-based filter that selectively bound RNA. Contaminants were washed away and RNA was eluted in 20  $\mu$ L RNase-free water.

The integrity and purity of the RNA were systematically checked with a laboratory-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano Assay (Agilent Technologies, Santa Clara, CA). The instrument uses capillary electrophoresis to analyze nucleic acid and results are visualized as electropherograms.

The quantity and quality were also determined by spectrophotometric readings at 260/280/230 nm.

Five hundred ng of total RNA was reverse-transcribed, in a final volume of 20  $\mu$ L, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Milan, Italy) following the manufacturer's recommendations and incubated at 25°C for 5 minutes, at 42°C for 30 minutes and at 85°C for 5 minutes. The RT-PCR reactions were performed in Delphi 1000™ Thermal Cycler (Oracle Biosystems).

The relative expression levels of CYP11B2 and CD56 mRNAs were measured with a real time RT-PCR in both CD56+ and CD56- cells. Primers and probes (Universal ProbeLibrary Probes, Roche, Monza, Italy) for the amplification of the genes of interest were designed using ProbeFinder Software (Roche, Monza, Italy, [www.lc480.it](http://www.lc480.it)). Sequences are shown in Table 1.

PCR reactions were performed using LightCycler 480 Instrument (Roche, Monza, Italy) in a total volume of 20  $\mu$ L per reaction in 96 multiwell plates following the reaction parameters recommended by the manufacturer. In

brief, for each gene, the 20- $\mu$ L total volume consisted of LightCycler® 480 Probes Master (Roche) 10  $\mu$ L, 200 nM of each primer, 200 nM of the probe, and 3  $\mu$ L of each first-strand cDNA sample. Cycle conditions were the same for all genes tested: initial denaturation at 95°C for 5 min, followed by 45 PCR cycles, each cycle consisting of 95°C for 10 sec, 60°C for 20 sec and 72°C for 1 sec. Porphobilinogen deaminase (PBGD) expression was investigated in all sample using the same parameters about volume reaction and cycle conditions. PBGD primers and probe are shown in Table 1.

Quantitative normalization of CD56 and CYP11B2 mRNAs in each cell-derived sample was performed using the expression of PBGD mRNA as an internal control. The generated Ct value of CD56 and CYP11B2 genes were normalized by its respected Ct value of PBGD mRNA ( $\Delta$ Ct). Then, CD56 and CYP11B2 gene expression in CD56+ cells was further normalized using the  $\Delta$ Ct value obtained for the same genes in CD56- cells ( $\Delta\Delta$ Ct). The final fold expression changes were calculated using the equation  $2^{-\Delta\Delta\text{Ct}}$  (Livak and Schmittgen, 2001).

## RESULTS

### *Immunohistochemistry*

Immunohistochemistry showed that CD56 is abundantly expressed in both the normal adrenal cortex and medulla (Fig. 4). In the cortex, labeling was strictly confined to zona glomerulosa (ZG), with a weak staining in zona fasciculata (ZF) and no appreciable immunolabeling in zona reticularis (ZR). The immunostaining was also clearly evident in all examined APA and pheochromocytoma tissue (Fig. 5, panel A and B). In both ZG and APA cells CD56 immunostaining was restricted to the cells surface and particularly intense at cell-cell contact sites. The specificity of CD56 labeling was confirmed by lack of staining when the primary antibody was omitted or after immunoprecipitation of the primary antibody with antigen excess.

### *Characterization of APA and ZG cells isolated with CD56 pre-coated magnetic beads*

#### *Setting-up of the method*

We used an immunoseparation approach based on magnetic beads precoated with an antibody specific for CD56 in order to obtain a pure CD56+ cell population.

To optimise the method we employed as experimental model a mixed endothelial cell/H295 population and CD56 precoated magnetic beads for the binding and separation of H295, that exclusively expressed CD56 antigen.

We optimised the necessary ratio of cells to CD56 magnetic bead that allows the complete positive isolation of the H295 cells from endothelial /H295 cellular pool. Ratios of cells:beads of 1:5, 1:10, 1:20 were chosen. After the separation we obtained two population: CD56+ cells and CD56- cells. We verified the the specificity of our method by immunocytochemistry for CD56 and factor von Willebrand in both cell population. In CD56- cells no

immunostaining was detectable for CD56 antigen at any of the tested ratios; all CD56<sup>-</sup> cells were positive for factor von Willebrand immunolabeling. On the other hand, no factor von Willebrand immunostaining was detectable in CD56<sup>+</sup> cells (data not shown). For the following experiments we decided to use five beads per cell ratio.

Staining with trypan blue did not show any loss of cell viability during the binding and separation steps, demonstrating that use of the CD56 pre-coated magnetic beads allowed separation of viable ZG and APA cells.

### *Morphological appearance of human ZG and APA cells*

Examination at light and phase microscopy showed that both cultured ZG and APA cells at day 6 maintained the epithelial-like morphology with a large prominent nucleus and sparse lipid droplets. At variance with the CD56<sup>-</sup> cells, which grew in a monolayer without any clustering, the CD56<sup>+</sup> cells formed clusters.

Electron microscopy demonstrated that dispersed CD56<sup>+</sup> APA cells were selectively bound to CD56 pre-coated beads (Fig 6, panel A and B). These cells displayed a striking abundance of lipid droplets. Mitochondria prevalently contained tubulo-lamellar or lamellar cristae, but some cells exhibited organelles with vesicular cristae. Smooth endoplasmic reticulum was abundant. Small lipofuscin-pigment bodies were observed in the cells (Fig. 6, panel A, B, C and D).

The CD56<sup>-</sup> APA cells resulted in a heterogeneous pool of cells: some exhibited a fibroblast-like elongated shape and were deprived of lipid droplets; other cells presented irregularly-shaped elongated mitochondria endowed with lamellar cristae or mitochondria with vesicular cristae. Several cells showed numerous dense bodies of probable lysosomal nature, abundant lipid droplets and lipofuscin bodies. In other cells, filaments,



which presumably corresponded to actin filaments, were clearly evident in a large portion of the examined field (Fig. 7, panel A, B and C).

CD56<sup>+</sup> and CD56<sup>-</sup> cells were also characterized by immunocytochemistry using CD56 antibody, CD90 antibody and Factor Von Willebrand at day 6 of culture. CD90 and Factor Von Willebrand antibodies were used to individuate fibroblasts and endothelial cells in our cell culture, respectively. Our immunocytochemical analysis of CD56 showed that the antigen was detectable in CD56<sup>+</sup> cells. Strong labelling was observed in all cells isolated with CD56 pre-coated beads (Fig. 8). No appreciable immunoreactivities to CD90 and Factor Von Willebrand were detected in CD56<sup>+</sup> cells (Fig. 8). At contrary, in CD56<sup>-</sup> cells the immunolabeling for CD90 and Factor Von Willebrand was detected, indicating that fibroblasts and endothelial cells were present among CD56<sup>-</sup> cells cultures (Fig. 9). A few CD56<sup>-</sup> cells were also positive for CD56 antibody (Fig. 9). The specificity of antibodies labeling was confirmed by lack of staining when the primary antibody was omitted or after immunoprecipitation of the primary antibody with antigen excess.

### ***CYP11B2 and CD56 gene expression and aldosterone secretion***

Real-time PCR evidenced that mRNA expression of CD56 was  $16.8 \pm 7.1$  fold higher in CD56<sup>+</sup> cells than in CD56<sup>-</sup> cells APA cells ( $p=0.002$ ) (Fig. 10, panel A). The CD56<sup>+</sup> APA cells showed higher CYP11B2 expression ( $21.0 \pm 3.8$  fold increase,  $n=6$ ;  $p=0.002$ ) (Fig. 10, panel B) than the CD56<sup>-</sup> cells. They also showed aldosterone production ( $79.0 \pm 36.75$  pg/APA cell,  $n=6$ ,  $p=0.046$  vs. CD56<sup>-</sup> cells) (Fig. 10, panel C) that was maintained in culture until day 6.

Aldosterone production was negligible ( $5.7 \pm 0.12$  pg/cell;  $n=2$ ) in CD56<sup>-</sup> cells.

Consistently with these findings the CD56+ cells from the normal adrenal cortex also showed a prominent expression of CYP11B2 (31.5±2.83 fold increase) and a sizeable secretion of aldosterone (47.0±23.06 pg/ZG cell); these values did not differ significantly from those found in CD56- cells, likely because of the small number (n=2) of experiments.

## DISCUSSION

In a systematic search for potential ZG-specific markers we identified some potential candidates. Among these CD56, previously known also as neural cell adhesion molecule (N-CAM), was of particular interest. CD56, is a member of the Ig-like supergene family and is composed of five-like domains and two fibronectin type III segments (Cunningham et al., 1987). N-CAM is a cell surface glycoprotein expressed on the membrane of neurons, glia, skeletal muscle and natural killer cells (Zeromski et al., 1998). NCAM is involved in cell-cell interactions and is thought to play a role in axonal growth and cell migration (Ronn et al., 1998; Walsh and Doherty, 1997). Although two large NCAM protein isoforms, with apparent molecular masses of 180- and 140-kDa, have been characterized, the 180-kDa isoform is more common in neuronal tissues and the 140-kDa isoform has been noted in endocrine tissues (Lahr et al., 1993; Zeromski et al., 2001). Hence, it is possible that NCAM may play a different role in neuronal and endocrine tissues.

N-CAM immunostaining in all zones of the rat adrenal cortex and medulla was first described by Lahr et al. (Lahr et al., 1993). Erhart-Bornstein et al. , also showed that N-CAM is selectively expressed in human adrenal gland, but, disagree with Lahr's findings in the rat, only ZG and medulla expressed NCAM on cell membrane (Ehrhart-Bornstein and Hilbers, 1998). Some years later, Muench et al. found that the cells of the definitive zone, which in the fetal adrenal gland is the zone immediately beneath the capsule intensely express CD56 at late gestation. They also demonstrated that only this zone, which is analogue to the zona glomerulosa in that it starts expressing steroidogenic enzymes, showed CD56 gene expression. Thus, collectively these findings suggested that ZG cell could express CD56.

Therefore, our findings that the normal human ZG and the histogenetically ZG-derived APA cells specifically express CD56, which can be used to isolate

aldosterone-producing cells, is novel and important for research in the field of aldosterone pathophysiology.

### *Evidences that immunoseparated cells are ZG cells*

By demonstrating that the CD56 is expressed at the cell membrane level in the normal human ZG and APAs the present IHC findings extend those of Erhart-Bornstein to a larger sample of human adult adrenal glands. We observed a prominent immunostaining in the subcapsular ZG, which progressively waned in the zona fasciculata and was absent in the zona reticularis. The immunostaining in ZF is possible, because morphologically the transition of ZG to the ZF in the normal adrenal gland has been thought to be rather gradual and even intermediate type cells and zone was just described in literature (CATER and LEVER, 1954). Of interest and practical implications, we could also confirmed that CD56 immunostaining was markedly expressed also in the normal adrenal medulla as well as in pheochromocytoma cells. Hence, we hypothesized that a strategy based on immunomagnetic beads pre-coated with CD56 could be used to isolate cells of the normal human ZG and APA. To test this hypothesis, we developed a methodology based on coating magnetic beads with an antibody specific for CD56 and used this technique on different specimen of human normal adrenal gland and human adrenal tumours. Results showed that this approach was effective in providing a viable ZG and APA cells, as demonstrated by no trypan blue-stained cells after immunoseparation.

Several lines of evidences support the conclusion that our method permitted the selection of a “pure” population of aldosterone-producing cells from the normal human ZG and from APA tissue.

Taken advantage of the study of Spinazzi, we seeded ours cells on Matrigel, that favours the maintenance of ZG-cell differentiated phenotype (Spinazzi et al., 2006). Analysis of the cultured CD56+ cells with light showed that they

had an epithelial-like morphology with a large prominent nucleus and sparse lipid droplets, indicating that both ZG and APA cells maintained their original phenotype. At electron microscopy, the CD56<sup>+</sup> APA cells, opposite to CD56<sup>-</sup> cells, exhibited the morphological features of actively steroid-secreting cells: mitochondria with tubulo-lamellar cristae, smooth endoplasmic reticulum (SER) profiles in the cytoplasm and lipid droplets. Our results are in agreement with the description reported by Belloni (Belloni et al., 1988).

Immunocytochemical analysis showed that fibroblasts and endothelial cells were observed in CD56<sup>-</sup> negative cells but not in CD56<sup>+</sup> cells that were all immunoreactive for CD56 antibody.

Moreover, CD56<sup>+</sup> cells showed growth pattern that was characterized by forming clumps on the surface of the matrigel cushion. This is consistent with the classic phenotype of ZG cells (Belloni et al., 1987; Spinazzi et al., 2006; Nussdorfer, 1986). Conversely, the monolayer growth pattern exhibited by CD56<sup>-</sup> cells was consistent with the pattern previously identifies as peculiar of the ZF cells (Young et al., 2003).

Compared with CD56<sup>-</sup> cells, the CD56<sup>+</sup> cells showed a much higher expression of not only the CD56 gene but also CYP11B2 transcript. In keeping with these results CD56<sup>+</sup> cells were found to produce aldosterone during culture until day 6. Conversely, aldosterone production was negligible in CD56<sup>-</sup> cells.

Thus, overall these findings support the conclusion that CD56<sup>+</sup> immunoseparated cells are aldosterone-producing ZG and APA cells.

### ***Novelty of the CD56 pre-coated magnetic bead-based method***

Previous methods for isolation of adrenocortical cells were based on either manual dissection of the different adrenal zones or density gradient centrifugation (Roskelley and Auersperg, 1990; Young et al., 2003). By itself

dissection cannot provide an accurate separation of the cells and carries contamination with ZF and ZR cells. Methods based on density cannot yield a pure population of ZG cells because a clear-cut density differences exist between the cells of the ZF and the other two zones (ZG and ZR), but not between the ZG and the ZR (Young et al., 2003).

Thus, the method herein described is not only novel as it applies available fragmented knowledge on NCAM from different sources to a specific aim, but also because it was shown to provide an accurate isolation of a cell population that showed multiple features of the aldosterone producing cells.

### *Caveats and limitations*

Since aldosterone levels and CYP11B2 gene expression were measurable, but not absent, in the cultured CD56- cells, it is likely that a small number of ZG cells was not bound to the CD56-precoated beads. We therefore analyzed the cultured unbound cells with IHC and found that a tiny percentage of cells were immunostained with CD56. This confirms that some ZG cells had been not bound to the beads during the separation process. However, since our aim was to obtain a pure cell population of ZG and APA cells starting from the adrenal tissue that contains a variety of cell types, we think that it is preferable to miss some cells rather than to recover ZG and APA cells mixed with ZF and/or ZR cells, and/or fibroblasts, and/or vascular cells, and/or other cell phenotypes.

The intense CD56 expression in the adrenal medulla is also important because it could allow purification of adrenomedullary and pheochromocytoma cells, free of mast cells, fibroblasts, smooth muscle and/or endothelial cells with the CD56 precoated beads-based method. However, it should be considered before undertaking the immunoisolation of ZG and APA cells, because the adrenal medulla must be carefully dissected out to minimize the chances for contamination. Nonetheless, a

small degree of contamination is probably inevitable since there are islets of chromaffin tissue intermingled in the normal human adrenal cortex (Nussdorfer, 1996).

Since medullary cells express at high levels CD56 and are intensely immunoreactive for CD56, the CD56 precoated beads-based method can be efficiently be applied to obtain a pure population of medullary cells, free of mastcells, fibroblasts, smooth muscle and/or endothelial cells.

### ***Conclusions and perspectives***

The present results testify the feasibility of using an immunomagnetic bead technique for isolating ZG cells and also APA cells, which are histogenetically derived from the ZG, that show an aldosterone-secreting phenotype. This technique can provide an important step forward in the investigation of cytophysiology of aldosterone-secreting cells and thereby for the understanding of the molecular mechanisms of the most common cause of human arterial hypertension.

Moreover, the possibility to obtained a pure population of medullary cells is also of interest to obtain a co-culture of medullary cells and ZG or APA cells to investigate the paracrine effects of chromaffine cells on aldosterone secretion.

Finally, we believe that the possibility of obtaining a purified population of ZG cells could be a major step forward in the field of whole transcriptome analysis. In fact, currently these types of studies have been carried out by comparing aldosterone producing adenoma with mRNA obtained from extract of the entire adrenal cortex (Williams et al., 2010; Assie, et al.2005; Bassett et al. 2005) with only one exception (Lenzini et al. 2007). The latter obviously carries the bias of “ diluting” the ZG-specific mRNA with transcripts pertaining to the other zones. Therefore, the relative expression data furnished by these studies were likely to provide results that are of

limited value and difficult interpretation. By contrast, the use of CD56+ ZG, and also of CD56+ adrenomedullary cells, will avoid these types of problems and can provided in the future a better identification of the transcriptome of APA and pheochromocytoma.



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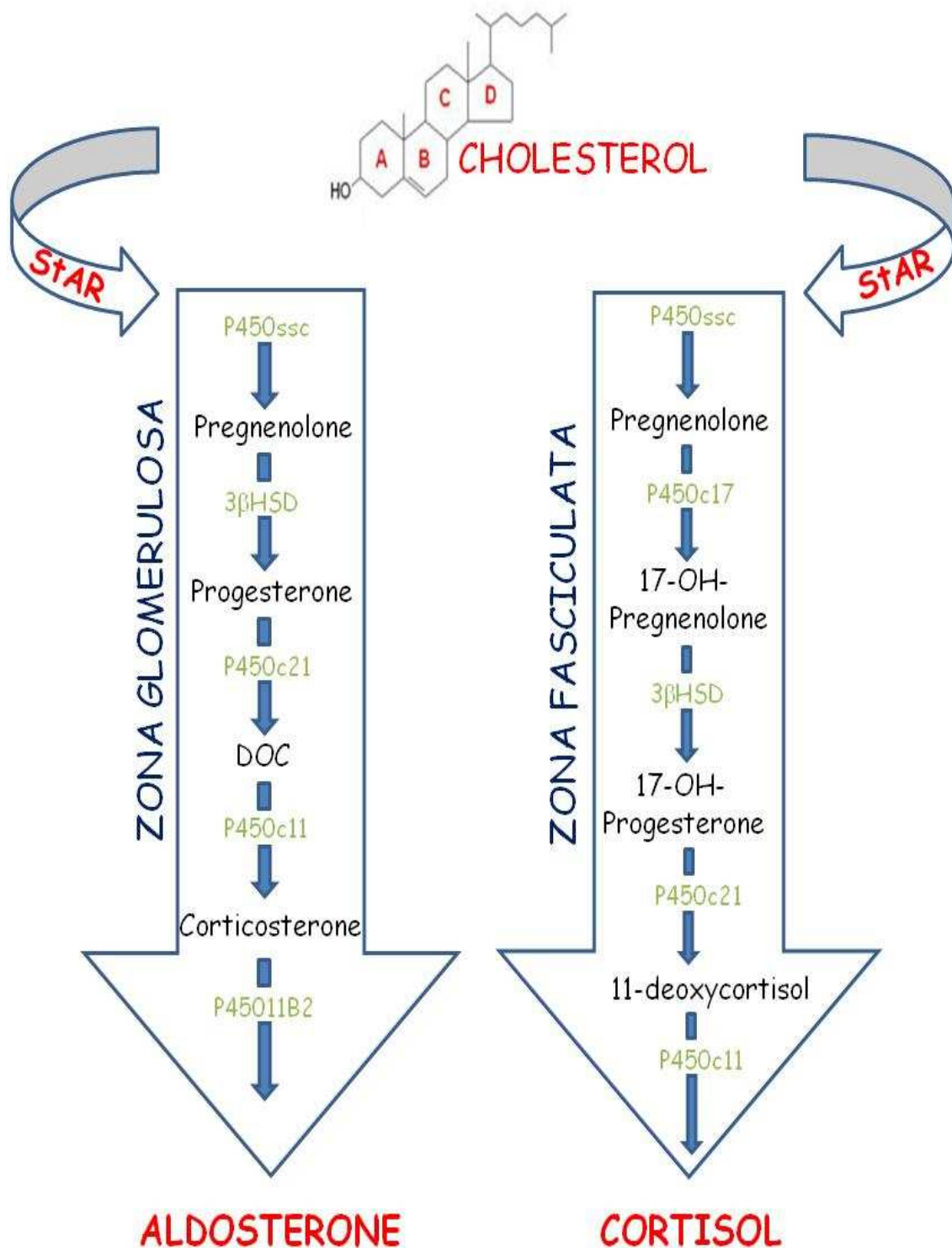


## APPENDIX

### TABLE AND FIGURES

TABLE 1. Primer sequences.

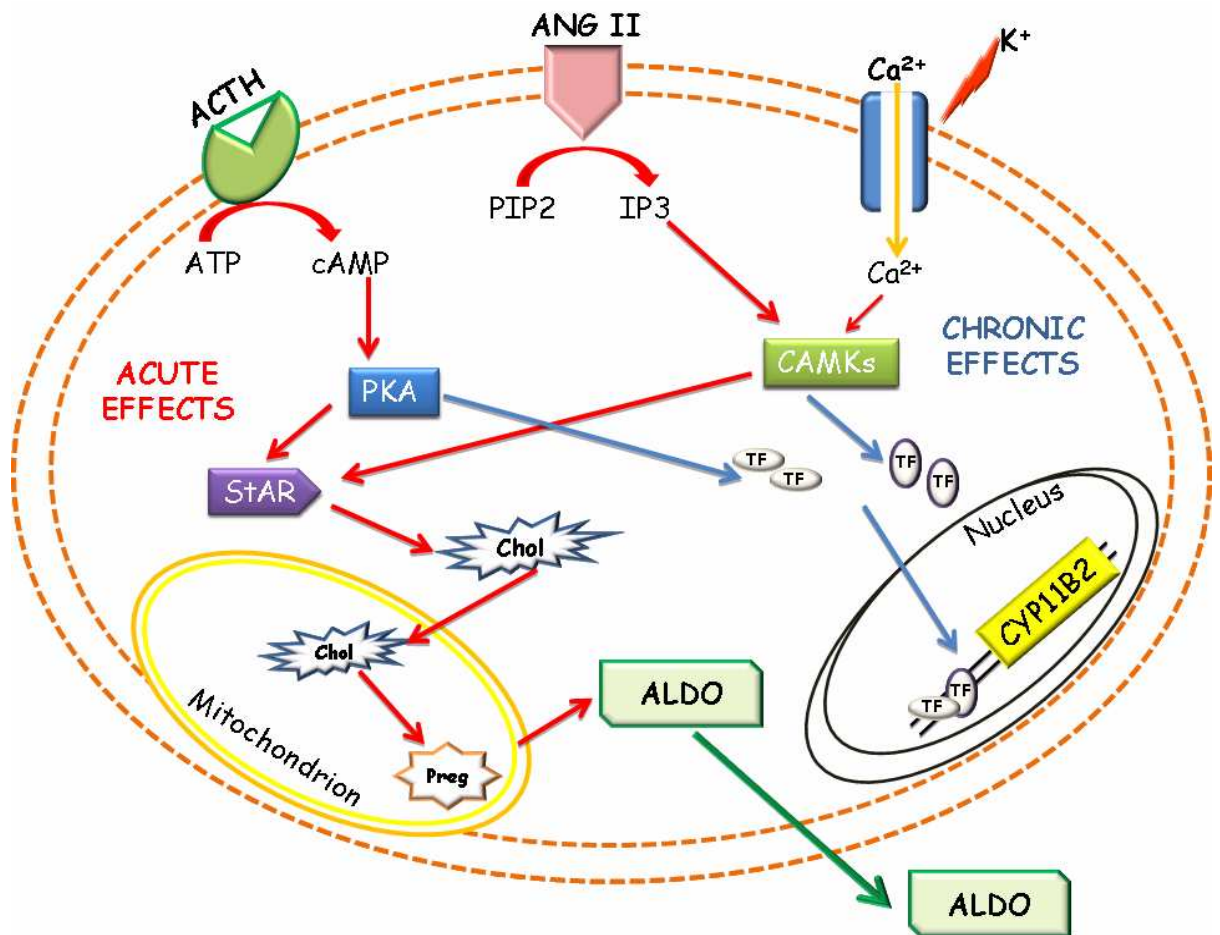
PRIMER	ACCESSION NUMBER	SEQUENCE
CD56 FORWARD	NM_181351.3	cggccaacctgtgtggaa
CD56 REVERSE		gctcctggactcatcttc
CYP11B2 FORWARD	NM_000498.3	gagcagggttatgagcac
CYP11B2 REVERSE		gtggtcctccaagtgtga
PBGD FORWARD	NM_000190.3	tgccctggagaagaatgaag
PBGD REVERSE		agatggctccgatggtga



**Figure 1:** Human adrenal steroid biosynthetic pathways for the production of aldosterone and cortisol. In adrenocortical cells, after the steroidogenic acute regulatory (StAR) protein-mediated uptake of cholesterol into the mitochondria, aldosterone and cortisol are synthesized through the

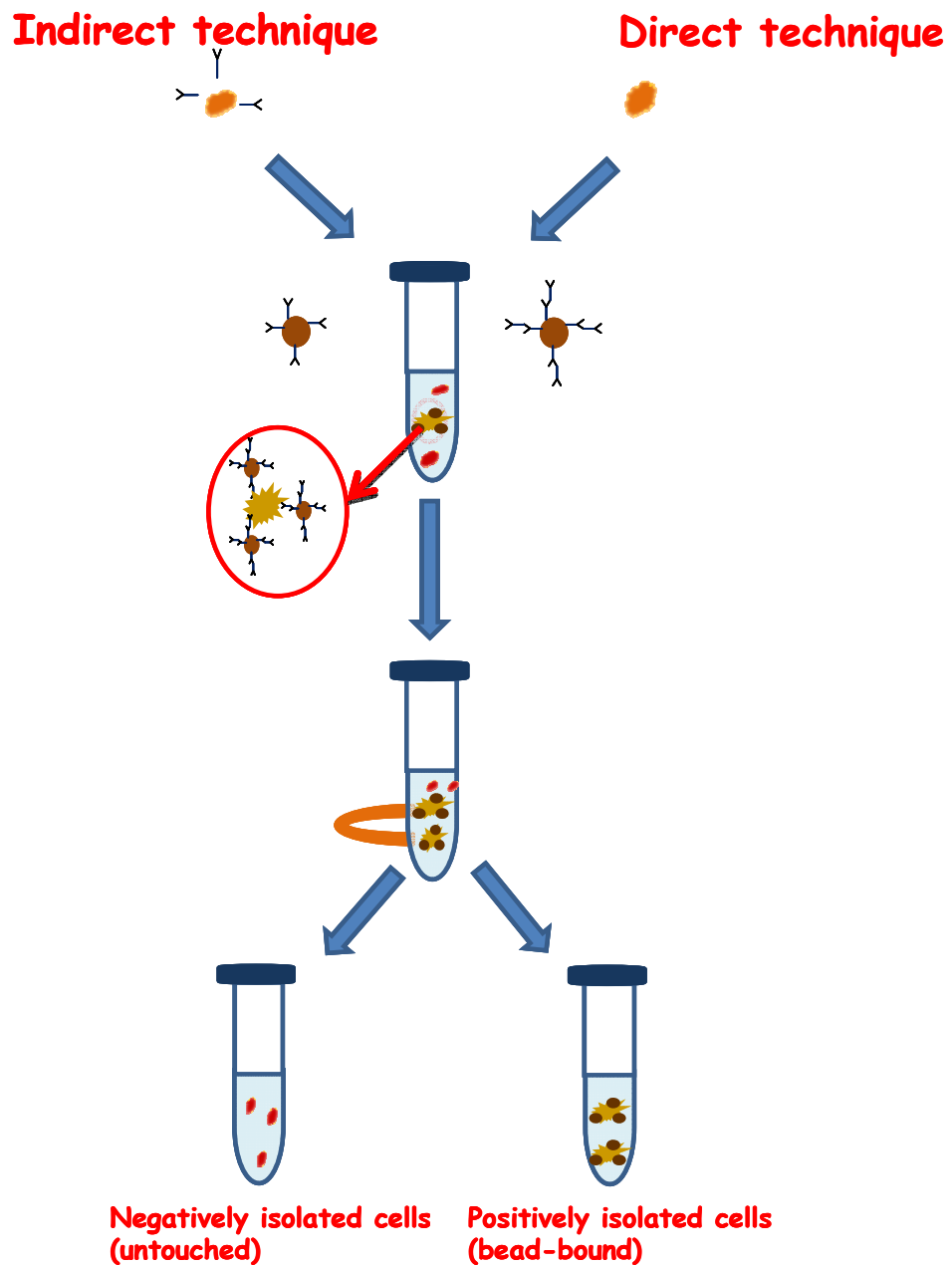
coordinate action of a series of steroidogenic enzyme in a zone-specific fashion.

P450ssc: P450 side-chain cleavage enzyme; 3bHSD: 3 $\beta$ -hydroxysteroid dehydrogenase; P450c21: 21 hydroxylase; P450c11: 11 $\beta$ -hydroxylase; P45011B2: aldosterone synthase; DOC: 11 deoxycorticosterone.

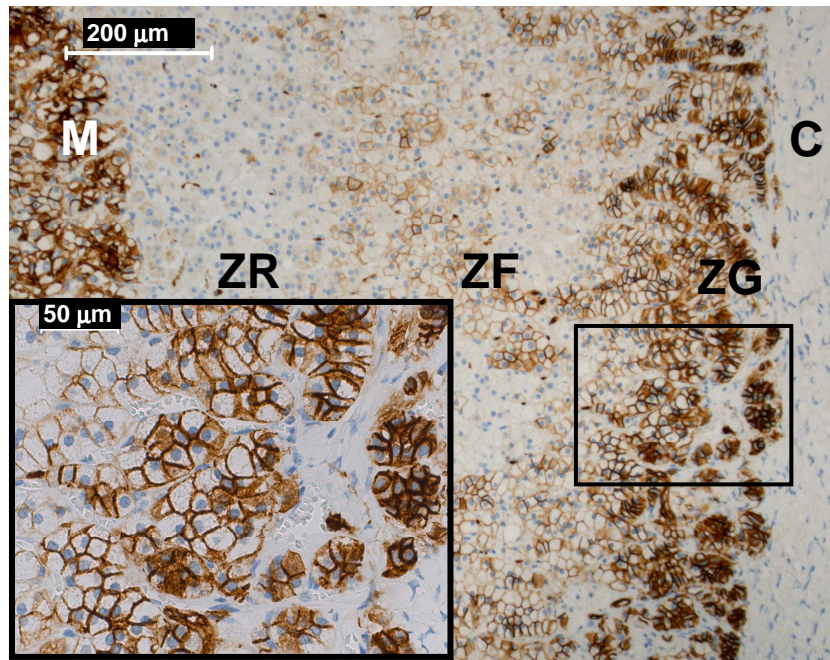


**Figure 2:** Signaling pathways that regulate aldosterone production in human zona glomerulosa cells. The most important regulators of aldosterone biosynthesis are ACTH, Angiotensin II and Potassium, which act at the level of the cell membran. Acute and chronic steroid hormone hormone production are both medaited by calmodulin-dependent protein kinases (CAMKs) and protein kinase A (PKA) pathways.

Chol: cholesterol; Preg: pregnenolone; StAR: steroidogenic acute regulatory protein; TF: transcription factors; ALDO: aldosterone; CYP11B2: aldosterone synthase; CAMKs: calmodulin-dependent protein kinases; PKA: protein kinase A

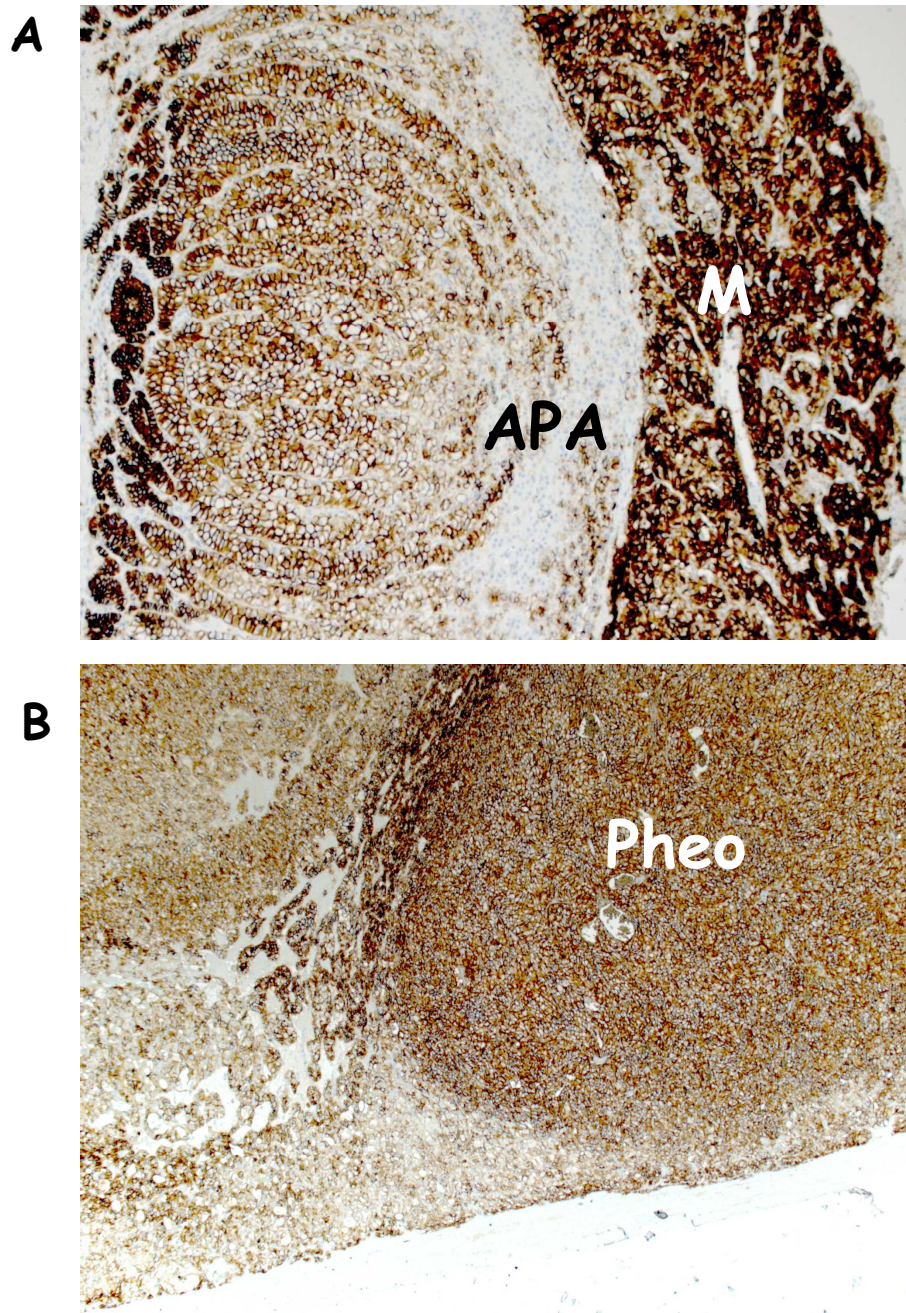


**Figure 3:** Schematic illustration of direct and indirect techniques for positively and negatively isolated cells. In indirect technique the cells are labelled with primary antibody; instead, in direct technique the Dynabeads are coated with primary antibody. Then, Dynabeads are incubated with the cells and the bead-bound cells are separated with a magnet.



**Figure 4:** Immunohistochemistry with an antibody against CD56 in a normal human adrenal gland shows prominent staining of the zona glomerulosa (ZG) and the medulla (M), while the Zona Fasciculata (ZF) cells reacted only weakly and no staining was seen in the Zona Reticularis. CD56 labeling was mainly located on cell membrane.

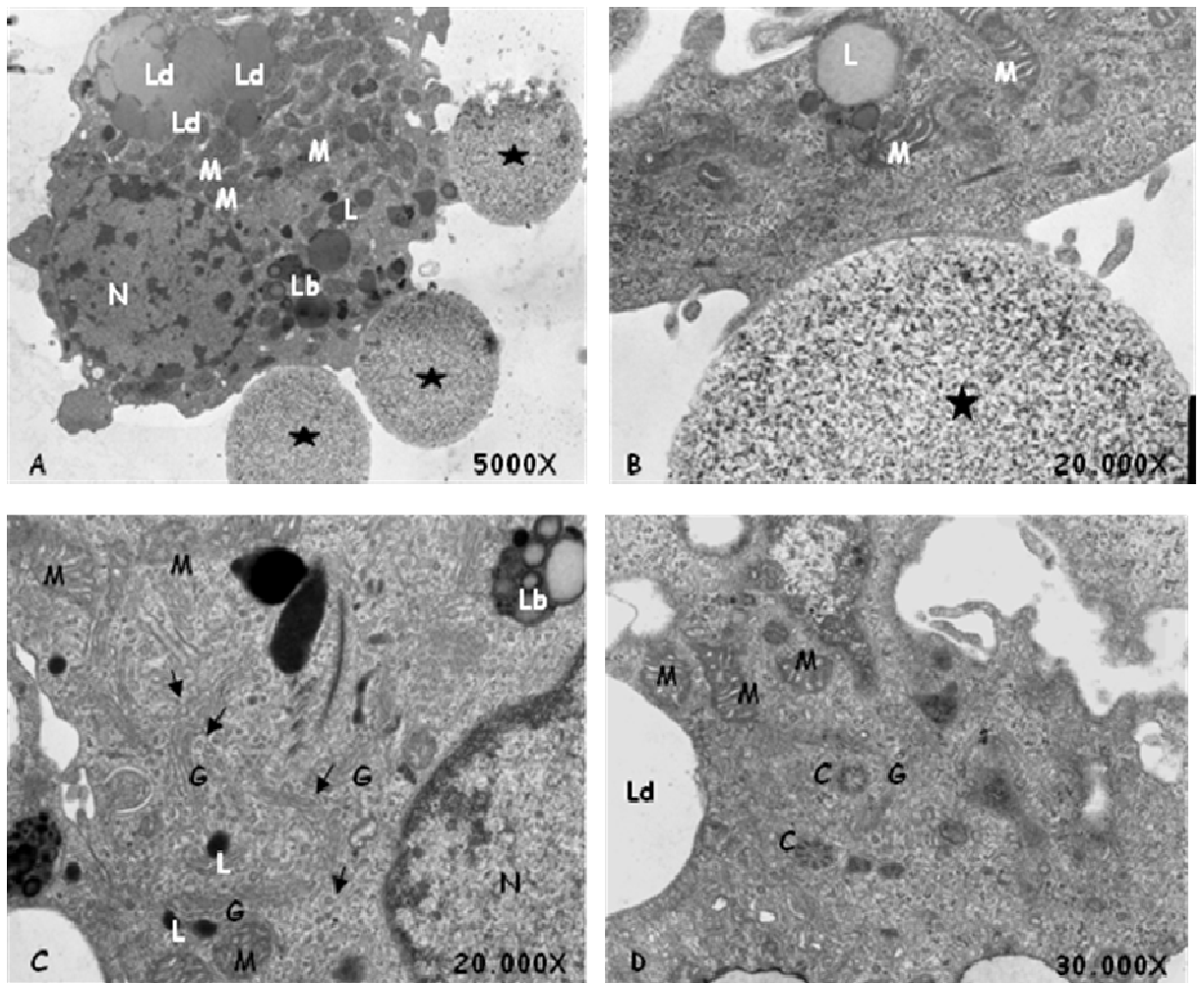
C, Capsula; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medulla.



**Figure 5:** Representative immunohistochemical staining for CD56 expression in aldosterone-producing adenoma (panel A) and pheochromocytoma (panel B) tissue.

Pheo: pheochromocytoma; APA: aldosterone-producing adenoma.

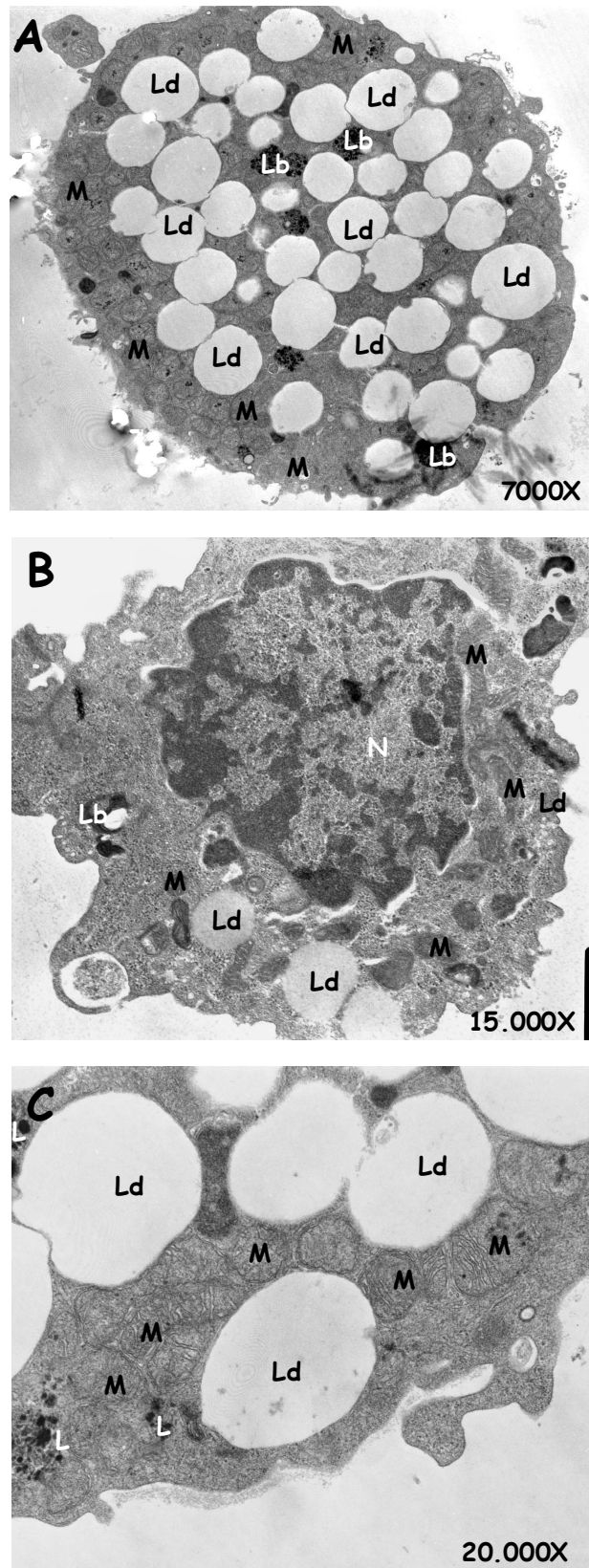




**Figure 6:** Electron micrographs illustrating the ultrastructural features of APA CD56+ cells immediately after immunoseparation. APA CD56+ cells showed a selective binding with CD56 precoated beads (stars) (panel A and B) and maintained the morphology of histogenetically ZG-derived cells: mitochondria (M) display tubulo-lamellar cristae and several lipid droplets (Ld), some lysosomes (L) and lipofuscin body (Lb).

At higher magnification (panel B) the Golgi apparatus (G) appeared prominent with associated coated vesicles (arrows). A well-developed smooth endoplasmic reticulum (SER) and round mitochondria (M) with tubular and lamellar cristae were also present.

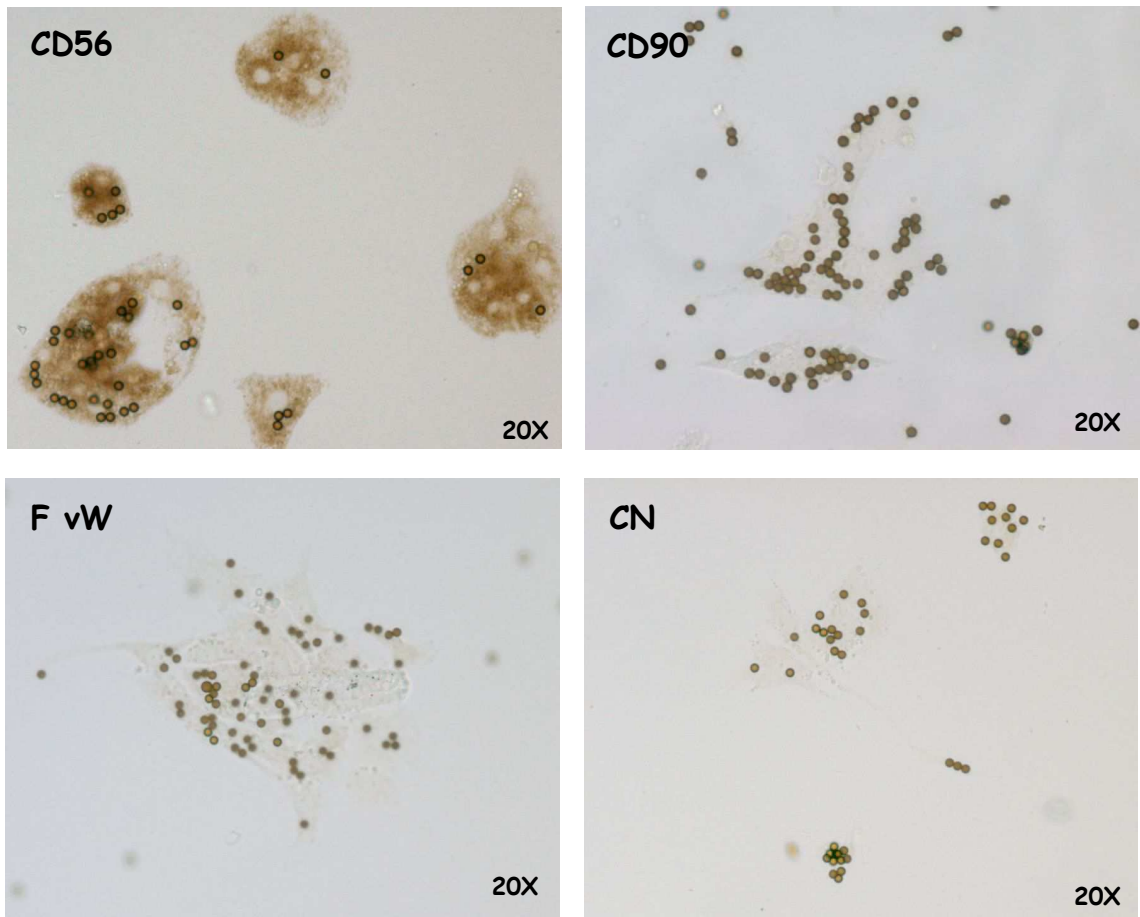
L: lysosomes; M: mitochondria; Ld: lipid droplets; N: nucleus; Lb: lipofuscin body; G: Golgi apparatus.



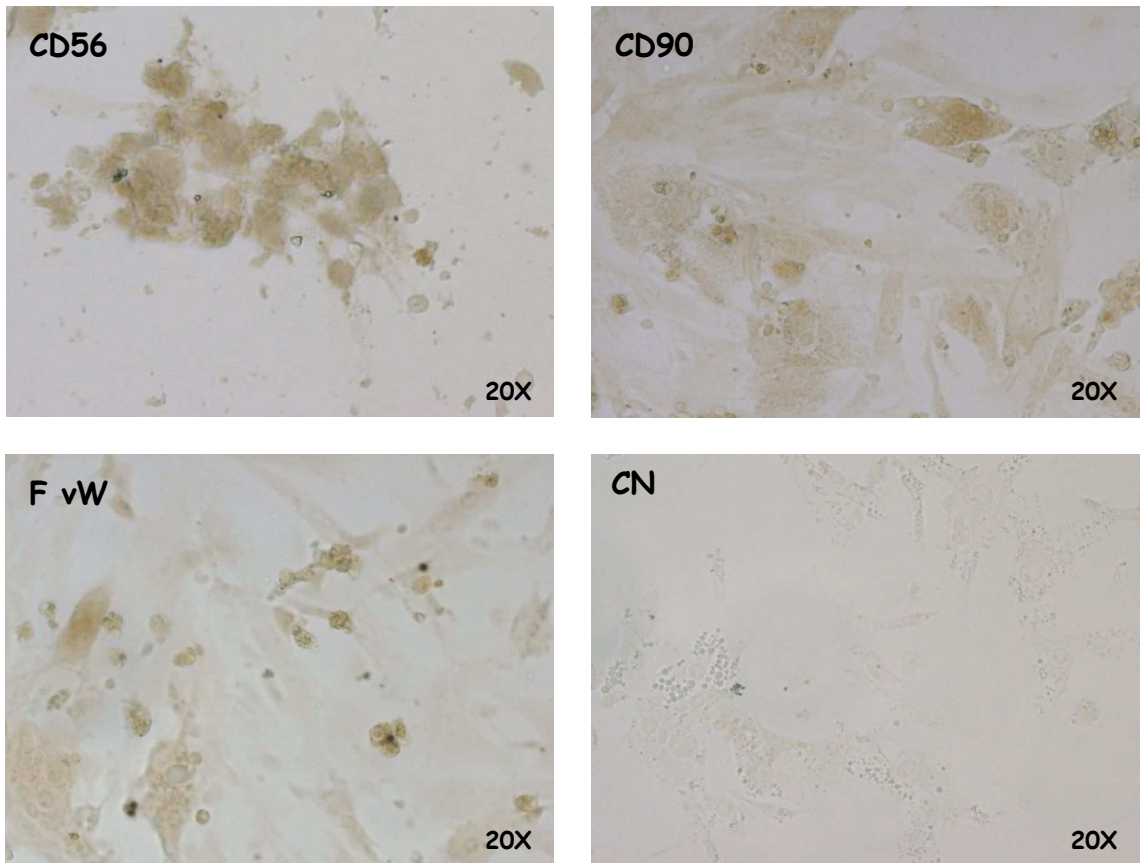
**Figure 7:** Electron micrographs illustrating the ultrastructural features of APA CD56- cells immediately after immunoseparation. APA CD56- cells results in a mixture of different cells. The cells show mitochondria with

tubulo-vesicular cristae or irregularly-shaped elongated mitochondria endowed with laminar cristae, numerous dense bodies of probable lysosomal nature (L), abundant lipid droplets (Ld) and lipofuscin bodies (Lb).

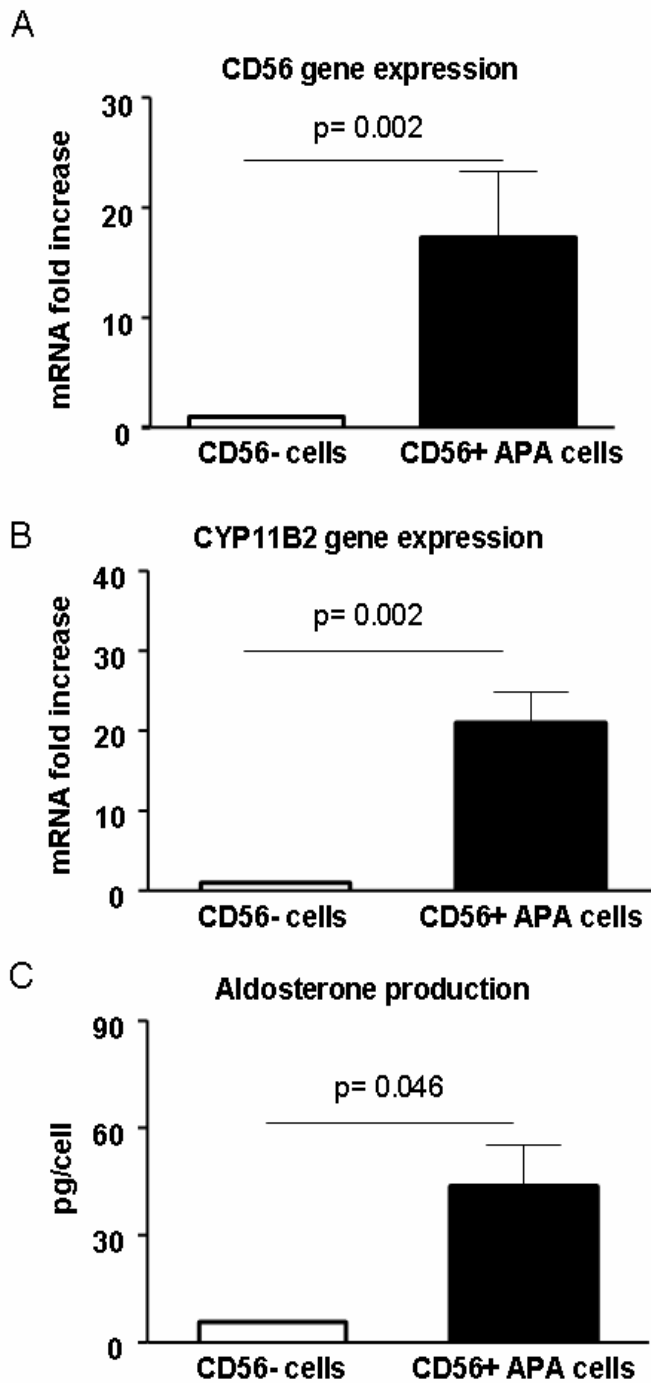
L: lysosomes; M: mitochondria; Ld: lipid droplets; N: nucleus; Lb: lipofuscin body; M: mitochondria.



**Figure 8:** Representative immunohistochemical staining for CD56, CD90 and Factor Von Willebrand antibody in CD56+ cells. CD56 antigen was detectable in all CD56+ cells examined. No appreciable immunoreactivities for CD90 and Factor Von Willebrand were detected in CD56+ cells.



**Figure 9:** Representative immunohistochemical staining for CD56, CD90 and Factor Von Willebrand expression in CD56- cells. Some nests of CD56- cells were positive for CD56 antibody. In CD56- cells immunostaining for CD90 and Factor Von Willebrand shows positive signals.



**Figure 10:** The bar graph shows the relative expression ( $\Delta\Delta Ct$ ) of the CD56 (panel A) and CYP11B2 (panel B) genes in C56+ and CD56- cells expressed as fold increase from the expression in CD56- (taken equal to 1). The aldosterone production of CD56+ and CD56- cells is also shown (panel C). Data are Bars are means  $\pm$  S.E.M ( N=6, CD56+ cells).



