UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Mediche e Chirurgiche

Scuola di dottorato di ricerca in Scienze Mediche, Cliniche e Sperimentali

Indirizzo: Metodologia Clinica e Scienze Endocrinologiche

Ciclo: Ventiquattresimo Ciclo

Mineralocorticoid receptor activation, insulin sensitivity and adipose tissue: analysis of their inter-relationship in human and experimental models

Direttore della Scuola: Ch.mo Prof. Gaetano Thiene

Coordinatore d'indirizzo: Ch.mo Prof. Roberto Vettor

Supervisori: Ch.mo Prof. Francesco Fallo e Dr. Catia Pilon

Dottorando: Riccardo Urbanet

TABLE OF CONTENTS

“..lodate sèsto che te ghè un bel manego, ma ricordate sempre tesoro che chi se loda se imbroda..”Me lo ricorderò sempre, nonna.

SUMMARY

STUDY IN PADOVA

Analysis of insulin sensitivity in adipose tissue of patients with primary aldosteronism

Objective: To assess a possible role of visceral adipose tissue in the pathogenesis of insulin resistance in primary aldosteronism.

Methods: Visceral adipose tissue was obtained from patients with aldosterone-producing adenoma (APA; n=14) and, as controls, non-functioning adenoma (NFA; n=14) undergoing laparoscopic adrenalectomy. Homeostasis model assessment index was higher and potassium was lower in APA than in NFA patients (P<0.05). Immunohistochemistry, Western blotting and real-time PCR were used to detect and quantify mineralocorticoid receptor (MR) expression. Transcript levels of peroxisome proliferative-activated receptor-g, insulin receptor, glucose transporter 4, insulin receptor substrate (IRS)-1, IRS-2, leptin, adiponectin, interleukin-6, monocyte chemoattractant protein-1, glucocorticoid receptor (GR)a, 11b-hydroxysteroid dehydrogenase type 1 (HSD11B1) and 2 were quantified. The effects of increasing aldosterone concentrations on 2-deoxy-[^{3}H]*d*-glucose uptake and expression of insulin signaling intermediates were tested in human subcutaneous abdominal adipocytes.

Results: Expression of MR was demonstrated in VAT, with no difference between APA and NFA as to mRNA levels of MR, GRa, HSD11B1, as well as glucose metabolism and inflammation factors. In cultured adipocytes, basal and insulin-stimulated glucose uptake were unaffected by 1-100 nM (normal/hyperaldosteronism) and impaired only by much higher, up to 10 mM, aldosterone concentrations. The impairment was prevented by RU486 but not by eplerenone in insulin-stimulated glucose uptake.

Conclusions: No alteration of gene expression of insulin signaling or inflammatory molecules was present in VAT of APA patients. Only at pharmacological concentrations and through GR activation did aldosterone reduce glucose uptake in adipocytes. Systemic insulin resistance in primary aldosteronism might occur in compartments other than fat and/or depend on concurrent environmental factors.

STUDY IN PARIS.

Phenotypic and metabolic characterization of a new double transgenic mouse model, conditionally overexpressing the human mineralocorticoid receptor in adipocytes

Objective: to explore the specific effects of mineralocorticoid over activation in the adipose tissue, through the phenotypic and metabolic characterization of a new double transgenic mouse model doxycycline-inducible, conditionally overexpressing the human mineralocorticoid (hMR) receptor in adipose tissues.

Methods: Subcutaneous inguinal adipose tissue (SAT), brown adipose tissue (BAT), visceral epididymal adipose tissue (EVAT) and visceral retroperitoneal adipose tissue (PVAT), were obtained from double transgenic mice (DT; n=8) and their wild type control littermates (wt; n=9) after hMR transgene induction with doxycycline. Adipocytes primary cultures were set up from EVAT and SAT of DT and wt mice. Transcript levels of hMR, endogenous mouse MR (mMR), neutrophil gelatinase-associated lipocalin (NGAL), Plasminogen activator inhibitor-1 (Pai1), MCP1, tumor necrosis factor α (TNFα) and PPARγ, were quantified by qRT-PCR. Analysis of mouse transcriptome in PVAT was performed by microarray.

Results: HMR was found specifically expressed in all adipose tissues, but it was significantly overexpressed, as compared to mMR, only in PVAT, SAT and BAT. A preliminary qRT-PCR analysis revealed an hMR-overexpression-associated increase of NGAL mRNA. Transcriptome analysis reported that MR overexpression in PVAT allowed 101 genes to be up-regulated and 246 to be down-regulated in DT mice. The geneontology analysis showed that an extra dose of MR in lean mice promotes adipogenesis and hinders inflammation by up regulating genes involved in fatty acid and PPAR pathways and down regulating those involved in immune system activation. Cultured DT adipocytes showed an efficient tet-On adipo-MR system and preliminary data confirm a significantly higher expression of NGAL in DT adipocytes.

Conclusions: Preliminary results are open to further investigations on DT-MR model. The next steps will be: 1) to evaluate the changes in the metabolic phenotype of DT mice overfed with a high fat diet, e.g. glycaemia, insulinaemia, serum free fatty acids and blood pressure; 2) to continue with *in vitro* experiments in DT adipocytes, in describing regulation of adipokines expression upon stimulation with mineralocorticoid/glucocorticoids agonists and antagonists.

SUMMARY, ITALIAN VERSION

STUDIO A PADOVA

Valutazione della sensibilità insulinica nel tessuto adiposo dei pazienti affetti da iperaldosteronismo primitivo

Obbiettivo: valutare un possibile ruolo del tessuto adiposo viscerale nella patogenesi dell'insulinoresistenza nell'iperaldosteronismo primitivo.

Metodi: Il tessuto adiposo viscerale è stato ottenuto da pazienti con adenoma-producente aldosterone (APA, n = 14) e da pazienti con adenoma non funzionante (NFA, n = 14) sottoposti a surrenectomia laparoscopica. L'indice HOMA era più alto e la concentrazione di potassio era più bassa negli APA rispetto ai pazienti NFA ($p < 0,05$). L'analisi immunoistochimica, il western blotting e la real-time PCR sono stati utilizzati per rilevare e quantificare l'espressione di MR. Tramite qPCR sono stati quantificati anche i livelli trascrizionali di PPAR-γ, IR, GLUT4, IRS-1, IRS-2, leptina, adiponectina, IL6, MCP-1, GRα, HSD11B1 e 2. Su colture di adipociti umani sottocutanei, sono stati testati gli effetti di dosi crescenti di aldosterone sulla captazione del 2-deossi-[3H]-D-glucosio e sull'espressione dei trasduttori intermedi del segnale insulinico.

Risultati: L'espressione di MR è stata dimostrata nel VAT. Nessuna differenza è stata rilevata tra i pazienti APA e NFA, nei livelli di MR, GRα, HSD11B1, dei geni del metabolismo del glucosio e dei geni codificanti per i fattori di infiammazione. Negli adipociti in coltura, la captazione del glucosio, basale o insulino-stimolata, è risultata inalterata dalla stimolazione con aldosterone alle concentrazione più basse, 1-100 nM (normale/iperaldosteronismo), mentre è risultata ridotta alla concentrazione più alta di 10 μM. Negli esperimenti, questa riduzione è risultata prevenuta da pretrattamento con RU486, ma non con eplerenone.

Conclusioni: Nel VAT dei pazienti APA non è stata trovata alcuna alterazione dell'espressione delle molecole regolatrici del segnale insulinico o dell'infiammazione. L'aldosterone riduce l'assorbimento del glucosio negli adipociti solo a concentrazioni farmacologiche e probabilmente attraverso l'attivazione di GR. L'insulino-resistenza sistemica osservata nell'iperaldosteronismo primitivo potrebbe verificarsi a causa del coinvolgimento di altri organi (oltre che al grasso) e/o dipendere da altri fattori ambientali concomitanti.

STUDIO A PARIGI

Conseguenze funzionali e molecolari dell'attivazione del recettore umano dei mineralcorticoidi: sovra-espressione nel tessuto adiposo di topo

Obiettivo: esplorare gli effetti specifici di una super attivazione del sistema mineralcorticoide nel tessuto adiposo attraverso la caratterizzazione fenotipica e metabolica di un nuovo modello condizionale di topo transgenico (inducibile con doxiciclina), sovraesprimente hMR negli adipociti.

Metodi: i tessuti SAT, BAT, EVAT e PVAT, sono stati ottenuti da topi doppio-transgenici (DT; n = 8) e da topi di controllo (wt; n = 9) dopo l'induzione di hMR con doxiciclina. Culture primarie di adipociti sono state allestite dai tessuti EVAT e SAT di topi DT e wt. Tramite qPCR sono stati quantificati i livelli di trascrizione di hMR, mMR, NGAL, Pai1, MCP1, TNFα e PPARγ in tutti i tessuti adiposi. Nel PVAT del topo DT è stata eseguita l'analisi del trascrittoma tramite microarray.

Risultati: hMR è risultato specificamente espresso in tutti i tessuti adiposi, ma significativamente sovraespresso, rispetto a MR endogeno, solo nel PVAT, nel SAT e nel BAT. Un primo studio tramite qPCR ha rivelato come a un aumento di espressione di hMR sia sempre associato un aumento di espressione di NGAL nei tessuti adiposi. L'analisi del trascrittoma ha evidenziato inoltre come l'iperespressione di MR nel PVAT del topo DT comporti contemporaneamente l'aumento e la riduzione di espressione di rispettivamente 101 geni e 246 geni. L'analisi genontologica di questa lista ha rivelato come una dose supplementare di MR nei topi DT promuova l'adipogenesi e ostacoli l'infiammazione, in particolare, aumentando l'espressione di geni coinvolti nella regolazione degli acidi grassi e della via del PPARγ e diminuendo l'espressione dei geni coinvolti nell'attivazione del sistema immunitario. Gli adipociti DT in coltura primaria hanno confermato l'efficienza del nostro sistema doppio transgenico anche in vitro e l'analisi preliminare in qPCR conferma anche negli adipociti DT l'aumento di espressione di NGAL.

Conclusioni: I risultati preliminari incoraggiano ulteriori indagini sul modello DT. I prossimi passi saranno: 1) valutare le variazioni del fenotipo metabolico dei topi DT sovralimentati con una dieta ricca di grassi, 2) continuare gli esperimenti in vitro negli adipociti DT, valutando le conseguenze della stimolazione con agonisti/antagonisti di MR e GR sull'espressione genica.

ABBREVIATION LIST

17OHP	17 hydroxy progeterone
ACE	angiotensin converting enzyme
ACTH	adrenocorticotropic hormone
Aldo/aldo	aldosterone
aP2	adipocyte protein 2 / fatty acid bindin protein 4
APA	aldosterone producing adenoma
BAT	browun adipose tissue
BMI	body mass index
CT	computer tomography
ctrl	control
db	diabetic
DT/dt	double transgenic
EH	essential hypertensive
Enac	ephitlial sodium channel
EVAT	epididymal adipose tissue
GLUT4	glucose transporter 4
GR	glucorticoid receptor
hMR	human mineralocorticoid receptor
HOMA	homeostasis model sssessment index
HSD11B1-2	11 beta hydroxysteroid dehydrogenas type 1 and 2
IgG	immunoglobulin g
IL-6	interleukin-6
IR	insulin receptor
IRS	insulin receptor substrate
Kg/mg	kilograms/milligrams
L	litre
m2	square metre
MCP1	monocyte chemoattractant protein-1
mmol/nmol/pmol	milli moles / nano moles / pico moles
mMR	enogenous mouse mr
MNR	mgnetic resonace
MR/MCR	mineralocorticoid receptor
mRNA	messenger rna
n	number
NA	not available
NFA	non-functioning adrrenal adenoma

NGAL/Lcn2	neutrophil gelatinase-associated lipocalin or lipocalin 2
NT	normotensive
ob	obese
OGTT	oral glucose tolerance test
PA	primary aldosteronism
PAI1/Pai1	plasminogen activator inhibitor-1
PCR	polymerase chain reaction
PET	positron emission tomography-computed tomography
PPARγ	peroxisome proliferative-activated receptor gamma
PRA	plasma renin activity
PVAT	retroperitoneal adipose tissue
qRT-PCR/qPCR	quantative real time pcr
RAAS	renon angiotensin aldosterone system
SAT	subcutaneous adipose tissue
TNFα	tumor necrosis factor α ,
UCP1	uncoupled protein 1
VAT	visceral adipose tissue
WAT	white adipose tissue
wt	wild type control mouse
μM/nM/mM	micro/nano/milli molar

INTRODUCTION

Aldosterone and mineralocorticoid receptor overview

Aldosterone (Aldo/aldo) is a steroid hormone produced by the cortex of the adrenal gland (Figure 1), and is mainly known for being one of the most important regulators of blood pressure and salt/water balance in humans. The alteration in its primary production (i.e., the presences of adrenal aldosterone-producing adenoma) and the altered regulation of its secretion (i.e., dysfunction in the renin-angiotensin system) are recognized to account for at least 20% of diagnosed cases of secondary hypertension.

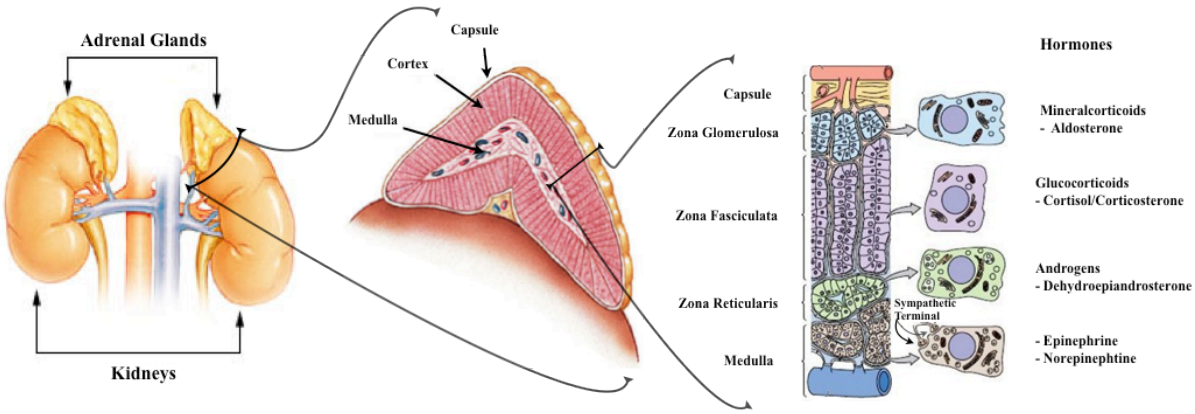

Figure 1. Schematic representation of adrenal glands in human.
The adrenal glands are endocrine glands which sit atop the kidneys. In humans, the right gland is triangular shaped, while the left one is semilunar shaped. Each adrenal gland has two distinct structures: the outer adrenal cortex and the inner medulla. Adrenal cortex comprises three different anatomical zones, that can be recognized at microscopic level by characteristic histological structures. Each zone express also specific enzymes devoted to production of distinct hormones, i.e., aldosterone, in zona glomerulosa, cortisol, in zona fasciculata, and androgens in zona reticularis. The medulla chiefly produces epinephrine and norepinephrine.

Aldosterone is the final effector of a more complex hormonal network in charge of regulating blood pressure and fluid balance, i.e., the renin-angiotensin-Aldosterone system (RAAS) (Figure 2).

When blood volume is low, juxtaglomerular cells in the kidneys secrete renin directly within the blood circle. Plasma renin then carries out the enzymatic conversion of angiotensinogen, a protein synthetized and released by the liver, to Angiotensin I which is subsequently converted to angiotensin II by the enzyme Angiotensin Converting Enzyme (ACE) expressed in the lungs. The Angiotensin II, in itself a potent vaso-active peptide able of causing blood vessels to constrict, is also the necessary stimulus for the secretion of Aldosterone from the adrenal cortex. This stimulation eventually leads the convoluted distal tubules and the collecting ducts of the nephron in kidneys to increase the reabsorption of sodium and water into the blood. The overall effect of these two mechanisms, the increase in fluid volume together with the constriction of blood vessels, ends with an increase of blood pressure.

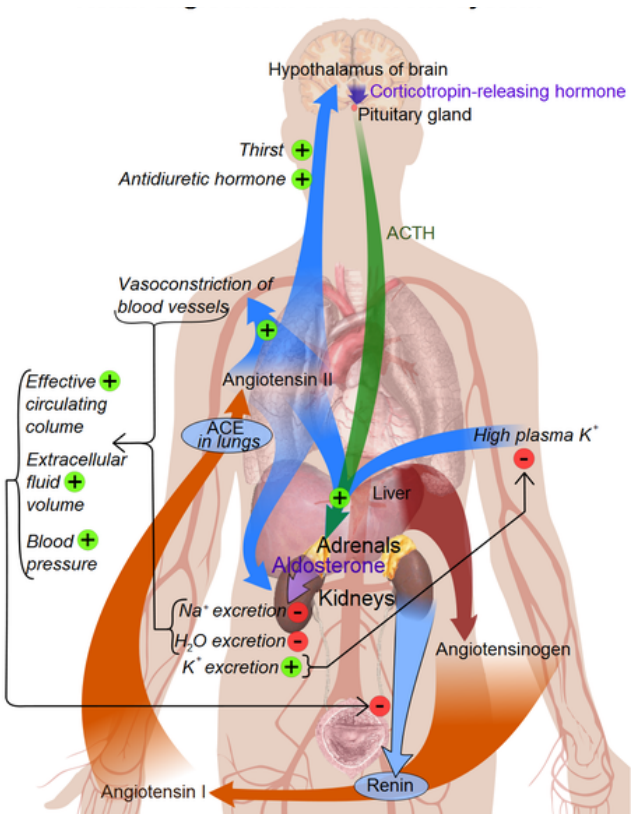

Figure 2. The renin angiotensin aldosterone system

Aldosterone synthesis occurs in the cortex of the adrenal gland, a region of the gland specialized in the synthesis of many steroid hormones i.e., mineralocorticoids, glucocorticoids and sex steroids (Figure 2). The cortex is subdivided in 3 major regions depending on their histological aspect, the “zona glomerulosa”, the “zona fasciculata” and the “zona reticularis”.

The cellular subtype of each zone is mainly dedicated to production of a particular type of hormone, mineralocorticoids, e.g. Aldosterone, in the zona glomerulosa, glucocorticoids, e.g. cortisol, in the zona fasciculata and sex steroid in zona reticularis. All of these hormones are synthetized starting from the point at which a molecule of cholesterol gets involved in a series of specific enzymatic transformations in each zone (Figure 3).

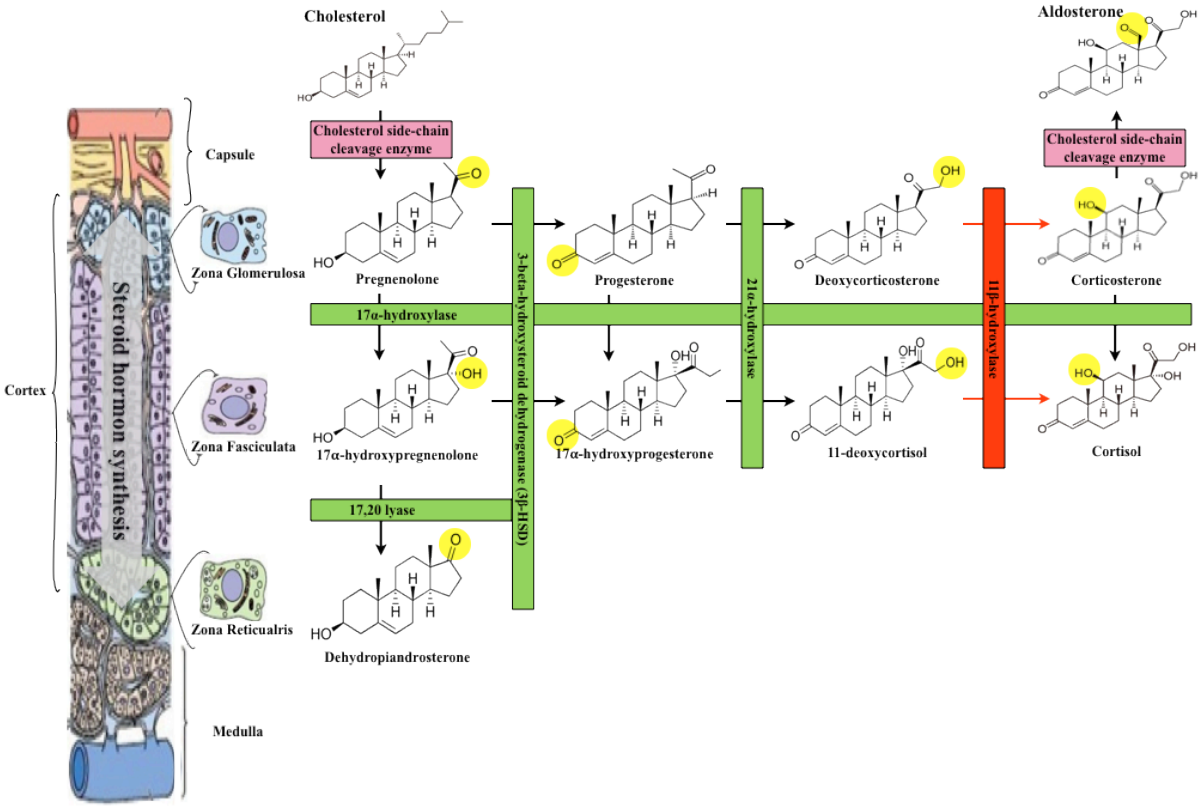

Figure 3. Schematic representation of enzymatic steps occurring in each zone of the adrenal cortex.
The cortex of the adrenal gland is responsible for the production of many steroid hormones. Specific enzymes (green and red bars) are expressed in each anatomical zone of the cortex and allow the production of characteristic hormones. In the scheme are reported the most important enzymatic reactions transforming a molecule of cholesterol into aldosterone, cortisol and dehydroepiandrosterone.

Aldosterone exerts its actions by binding to its own specific receptor, i.e., the mineralocorticoid receptor (MR). MR is a cytosolic receptor belonging to the nuclear receptor super family characterized by the ability of inducing gene transcription once activated, in that, the presence of Aldosterone allows the formation in the cytoplasm of the active Aldo-MR

complex which is targeted to the nucleus. Once in the nucleous, Aldo-MR complex homodimerizes and recognizes the HRE DNA sequences (hormone-responsive-elements) present in the promoter region of the induced genes. This results in the recruitment of transcriptional machinery on the activated genes causing their transcription into mRNA (Figure 4).

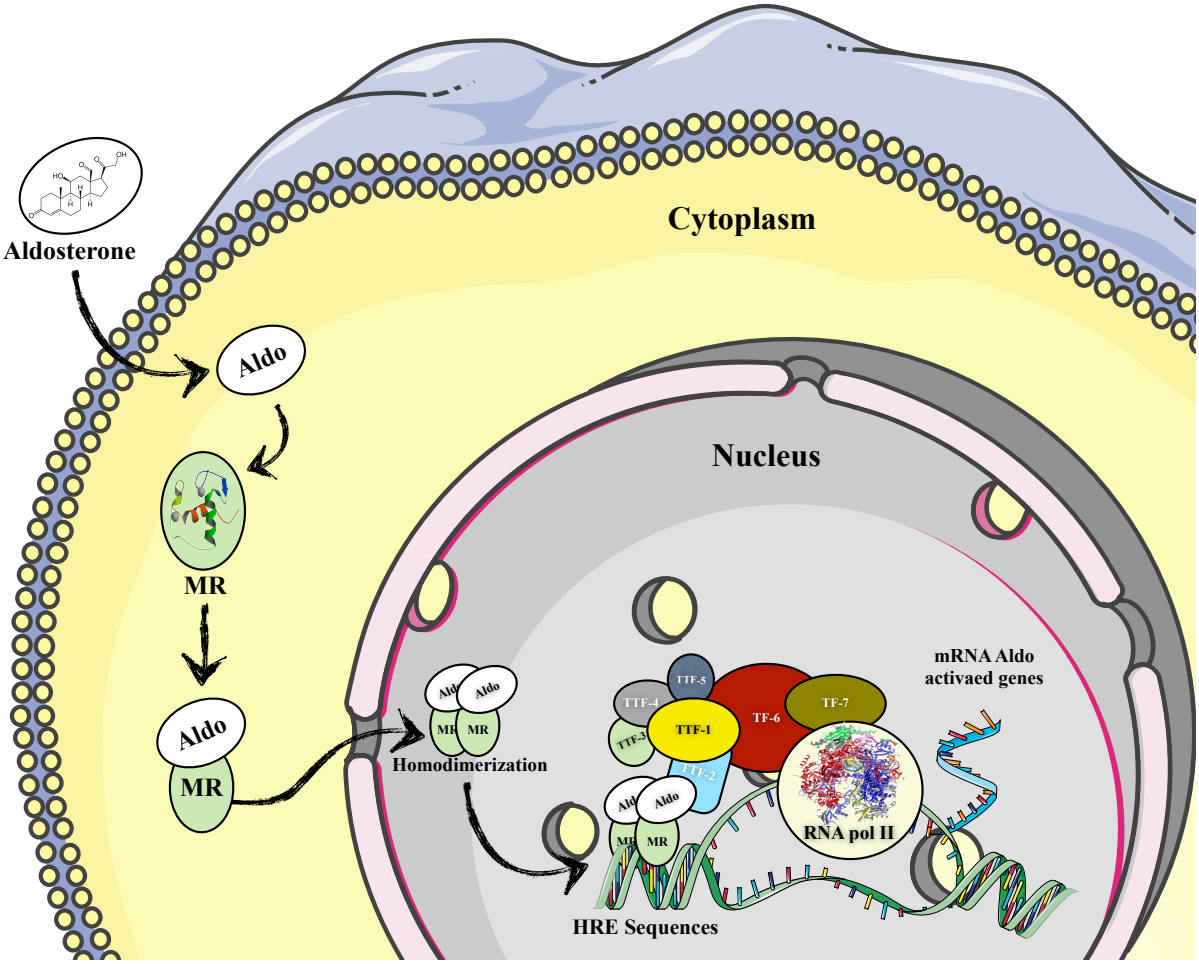

Figure 4. Schematic representation of aldosterone and mineralocorticoid receptor mechanism of action. Aldosterone is lipophilic molecule able to freely diffuse across the cellular membrane. Once within the cytoplasm, aldosterone can bind to mineralocorticoid receptor and form the activated complex which is subsequently targeted to the nucleus. Inside the nucleus, the aldo-MR complex heterodimerizes and recognizes specific hormone response elements on DNA, ultimately triggering the recruitment of aldo-specific tissue transcription factor (TF) and starting gene transcription of aldo related genes.

MR possesses similar receptor affinity for Aldosterone and for the physiological glucocorticoid, cortisol (corticosterone in rodents). The binding capacity of MR for these

molecules is 10-fold higher than that of the specific glucocorticoid receptor itself (1); and, since glucocorticoids circulate at 100- to 1000-fold higher concentrations than those of Aldosterone (0.1–1nM), MR selectivity for Aldo is granted by the intracellular enzymatic action of 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), which transforms glucocorticoids into inactive compounds cortisone (11-dehydrocorticosterone in rodents) (2, 3) (Figure 5).

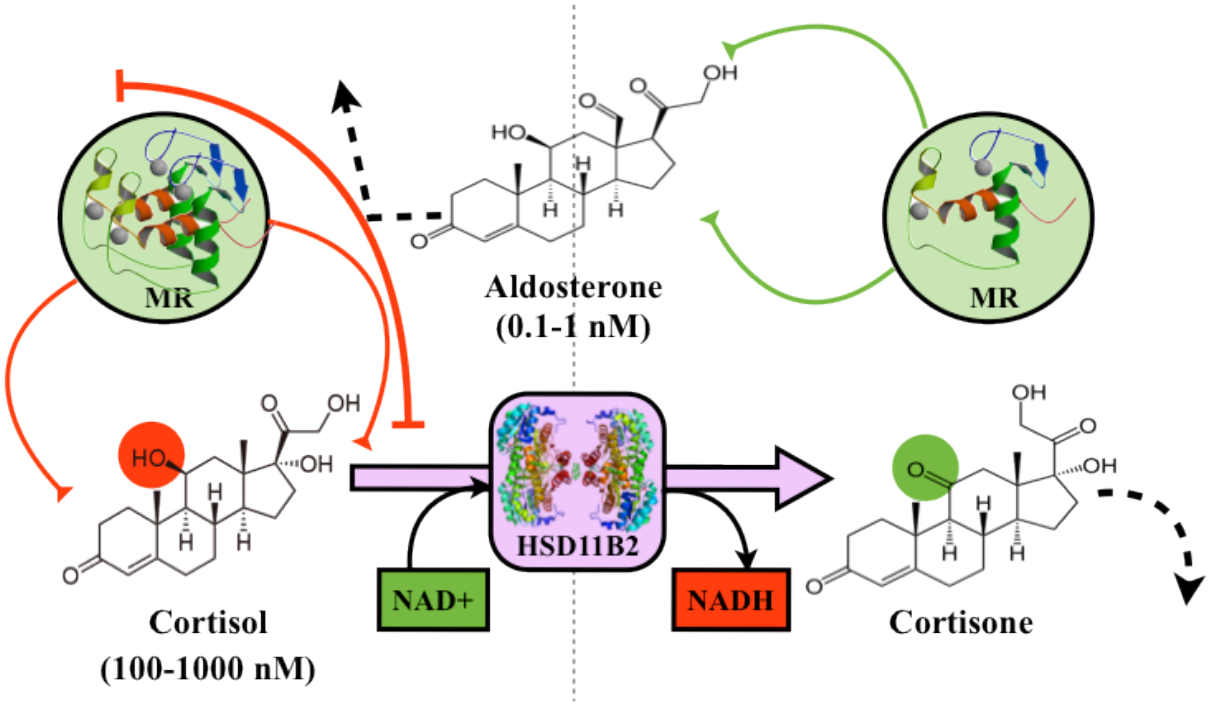

Figure 5. Schematic representation of HSD11B2 protection reaction allowing the binding of aldo to MR. Cortisol is 100 to 1000 folds more concentrated than aldosterone and, given the high affinity of MR for both of the ligands, in aldosterone responding tissue aldo specific MR activation is allowed by the co-expression of the enzyme HSD11B2 that catalyzes cortisol oxidation to MR-inactive cortisone compound by reducing a molecule of NAD+.

Since its first identification in the early 1970's and the following 30 years, MR expression was considered to be restricted to polarized epithelia (4) such as those of renal distal convoluted tubules and cortical collecting ducts (5, 6), distal colon (7), salivary (8) and sweat glands (9). In these tissues the classical scheme of activation of MR results in transcription of sodium transport related genes (the epithelial sodium channel, ENaC, Na+/K+

pump, serum and glucocorticoid induced kinase, SGK1) with the well known effect on body sodium reabsorption and, therefore, on water retention and blood pressure (10, 11).

More recently, MR expression and Aldosterone response have been proved in other tissues not previously taken into account, starting with classical, epithelial type, to non-classical, non-epithelial/non-expressing HSD11B2 types (12). This effectively extends the physiological role and pathological potential of the Aldo-MR system in many different pathophysiological and clinical issues, from inflammatory response to skin physiology as well as vasculopathies, metabolic syndrome and cardiovascular diseases.

Active MR, together with expression of HSD11B2, has been demonstrated to operate in the epithelia of the lung and eyes (13-16) and in the smooth muscle cells of the vessels (17) where, supposedly, it regulates respectively, the lung's ionic fluid concentration, the eyes' retina hydration and the vasoconstrictor tone of the blood vessels.

Instead, MR without 11BHD2 expression, has been detected in the brain, particularly in the hippocampus and hypothalamus (18, 19); in the heart and vessels, specifically cardiomyocytes and endothelial cells (20), in macrophages/monocytes (21), in adipocytes (22-24) and in keratinocytes (25, 26). In all these sites, the mechanism of action and effects of aldosterone mediated by MR is still poorly understood. In contrast to what occurs in epithelial tissues, where HSD11B2 provides the most potent MR/Aldo-protective system, in other tissues MR selectivity for aldosterone may be granted by other arrangements rather than enzymatic ones or perhaps glucocorticoids themselves may contribute to MR-specific effects in these tissues. Although plasma glucocorticoids are 100 to 1000 fold more concentrated than aldosterone, they are bound to plasma albumin and globulin which causes an important reduction in percentage of free glucocorticoids (10%) available to the mineralocorticoids receptor (27-29). Alternatively, even if aldosterone circulates mainly in a free form (not bound to plasma proteins), increasing evidence supports the hypothesis that change in the

redox state of the cell could allow and increase MR signaling mediated by glucocorticoids (30-32). From a general point of view, it is plausible, that cellular context and intervention of still unknown tissue-specific cofactors is probably necessary to ensure either Aldo- or glucocorticoids- specific effects via mineralocorticoid receptors.

Role of aldosterone and mineralocorticoid receptor in pathophysiology

Aldosterone was first identified in 1954 as "electrocortin" and at that time recognized as an unknown molecule able to regulate the Na+/K+ electrolytes concentration in plasma. The following year, twenty years before even the discovery of the Aldo specific receptor, J.W. Conn described the first human Aldo-related diseases, i.e., the primary aldosteronism (PA) or hyperaldosteronism (33). Patients affected by PA are characterized by an autonomous overproduction of aldosterone that causes excessive sodium retention, excessive potassium excretion, suppressed renin activity, hypokalemia and, finally, hypertension (34). For fifty years clinicians and researchers have worked diligently in characterizing the disease, beginning with descriptions of the clinical signs to the setting up of diagnostic methods for screening PA patients.

However, during the last decade, as a result of a better knowledge on MR/Aldo system, a new and stronger interest has been raised within the scientific community regarding PA. In fact this disease in humans is a natural model for studying the physio/pathological role of aldosterone and MR in the condition, as that of aldosterone excess, allowing the system to be stressed. Indeed, many studies have found that excess of Aldo, and likely activation of MR, is associated with many pathological conditions in humans, (e.g. cardiovascular diseases,

insulin resistance, vasculopaties) independently of its well known effect on blood pressure (35). PA patients are found with impaired glucose homeostasis, insulin resistance and lower level of plasma adiponectin compared to patients with essential hypertension, Table 1. Aldosterone excess alone has been shown to increase arterial wall stiffness by causing fibrosis (36-38). Ultimately PA patients have higher rates of cardiovascular events (39) and renal damage (40) compared to patients with essential hypertension.

On the other hand, patients with diabetes and metabolic syndrome show enhanced clinical signs correlated to higher, but non-excessive, levels of plasma aldosterone, as is the case where high plasma Aldo has been associated to more severe insulin resistance (41-43).

Table 1: Studies on insulin resistance in primary aldosteronism (PA)

Reference [n°]	Patients (No.)	Insulin Resistance PA vs. NT Controls	Insulin Resistance PA vs. EH Controls	Method
Shamiss et al. 1992 (44)	5	Yes	No	Clamp
Shimamoto et al. 1994 (45)	7	Yes	NA	Clamp
Ishimori et al. 1994 (46)	15	No	NA	OGTT
Šindelka et al. 200 (47)	9	Yes	NA	Clamp
Widimský et al. 2000 (48)	12	Yes	NA	Clamp
Widimský et al. 2001 (49)	36	NA	No	OGTT
Haluzik et al. 2002 (50)	11	Yes	NA	Clamp
Strauch et al.2003 (51)	24	NA	No	OGTT
Skrha et al. 2004 (52)	16	Yes	NA	Clamp
Catena et al. 2006 (53)	47 (20)	Yes	No	OGTT/HOMA (clamp)
Giacchetti et al. 2007 (54)	61	Yes	NA	OGTT/HOMA βHOMA
Mosso et al. 2007 (55)	30	NA	No/Yes	HOMA/βHOMA
Fallo et al. 2007 (56)	40	Yes	Yes	HOMA

PA, primary aldosteronism;
NT, normotensive;
EH, essential hypertensive;
OGTT, oral glucose tolerance test;
HOMA, Homeostasis model sssessment index [fasting glucose (mmol/L) x fasting insulin (mU/mL)/22.5];
NA, not available.

Alternatively important clinical trials, RALES (1998) and EPHESUS (2003), have assessed taht a reduced Aldo activity by indirectly treating patients with MR antagonist added to conventional treatment, markedly reduced the overall and cardiovascular mortality in patients with heart failure or in acute myocardial infarct complicated by left ventricular dysfunction (57 , 58, 59). MR antagonism has been also shown to be beneficial in diabetic nephropathy, as well as in progression of chronic renal diseases (60, 61). Finally, use of MR antagonists improves glucose tolerance, decreases insulin resistance, triglycerides and pro-inflammatory cytokines, in experimental models of obesity and diabetes (mice ob/ob, db/db, high fat diet fed) (62 , 63 , 64). The beneficial effects of MR antagonists are mainly explained by a decrease of cardiac fibrosis, improvement of peripheral vascular function and reduction of an aldosterone induced inflammation process (65, 66).

Adipose tissue: effect of aldosterone and MR activation

In the last twenty years the idea around adipose tissue has deeply evolved and the simplistic picture of adipose tissue as merely a fat depot is no longer the case. To date, body fat is almost considered as new endocrine organ well characterized by its own innervations, vascularization and distinct cellular sub types, i.e., white and brown adipocytes, preadipocytes, fibroblasts, endothelial cells, macrophages and stem cells. The function of the adipose organ is to regulate the energy homeostasis of the body and this is mainly accomplished in two ways; first, by physical storage/release of energy in/from adipocytes in form of triglycerides and fatty acids (67) and, secondly, by endocrine signaling through the secretion of adipocyte hormones. Adipocyte hormones include the adipokines, such as leptin,

adiponectin, IL-6, MCP1 and TNFα, that refer to the overall status of fat mass and influence the central need of energy supply, the general level of inflammation and oxidative stress, the vascular function and finally, glucose and lipid metabolism (68). The storage of fat in the adipose tissues occurs primarily through its ability to respond to insulin, which is the principal signal into adipocytes regulating both glucose uptake and lipogenesis. Typically, a healthy tissue is able to respond to insulin and therefore is able to uptake glucose and expand its volume by stocking lipids. On the contrary, an ill tissue is characterized by an impairment in insulin signaling, e.g. a dietary fuel overload, and that causes adipose organ to fail in its primary function, which leads initially to glucose toxicity, dyslipidemia and insulin resistance, and ultimately to obesity and type 2 diabetes (68).

Adipose organ should be considered as an extensive organ, like skin, with function and morphology of the fat depots that can vary extensively depending on spatial distribution and neighbored organs. However, according to the aspect of adipocyte fraction, it can be said that there are at least two different types of adipose tissues in mammals: the white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the most represented adipose tissue in human adults and - can be further divided into two main subtypes, the subcutaneous adipose tissue (SAT) in continuity with the dermal tissue forming a continuous layer under the skin and the visceral adipose tissue (VAT) that is located around the internal organs within the abdominal cavity and the thorax.

White adipocytes are morphologically characterized by the presence of single big lipid vacuole which almost fully occupies the cytosol and compress the nucleus and the other cellular organelles to the membrane. White adipose tissue is the part of adipose organ mainly dedicated to the store/release of energy in body and it is one of the major targets, together with liver and muscle, of insulin signaling, accounting for, in particular in visceral fat, the whole-body insulin sensitivity (69, 70) (Figure 6, A and B).

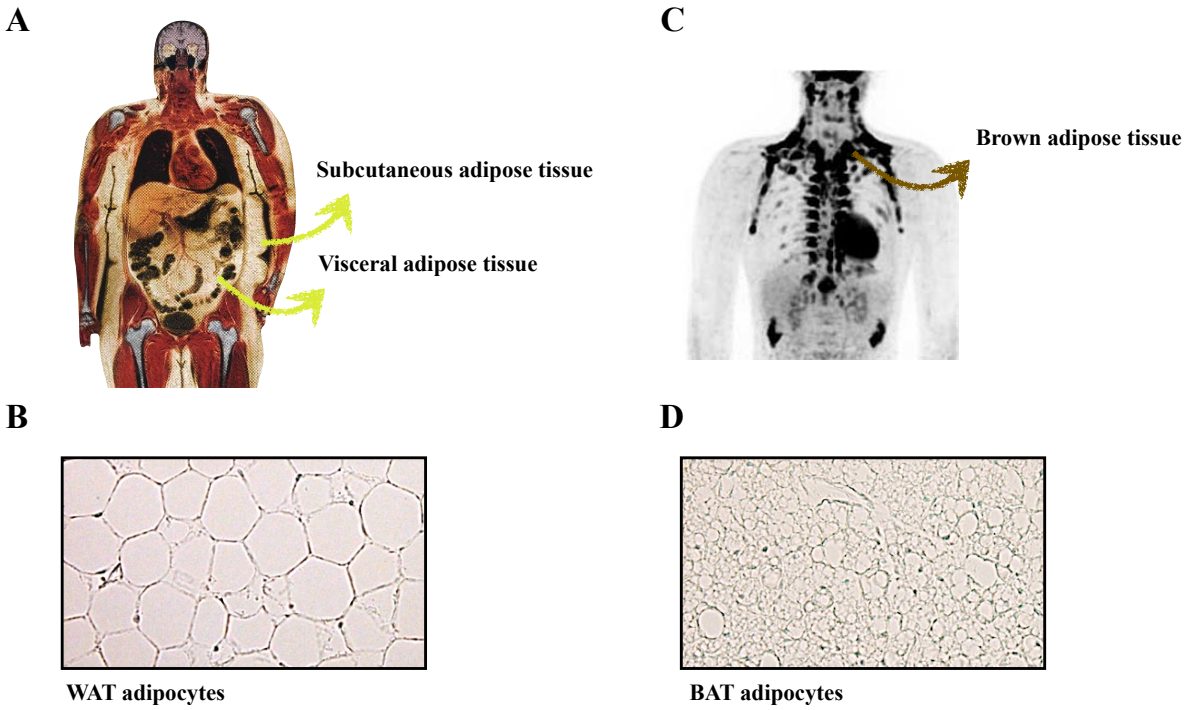

Figure 6. Representation of adipose organ distribution and histological aspects.
(A) Total white adipose tissue distribution (photo courtesy of Dr. David Heber). Subcutaneous and visceral adipose tissues are located respectively in continuity with derma and within the abdominal cavity. Histological cross-section of a white adipose pad (B): white cells are characteristically by the presence of a single large lipid droplet surrounded by a thin layer of cytoplasm. (C) PET-CT scan revealing brown adipose tissue deposit (black) in human thorax. (D) Histological cross-section of a brown adipose pad: compared to white adipocytes, brown cells are smaller and contain hundred of small lipid droplets.

At variance, BAT is especially abundant in new-born mammals (5% of the body mass), mainly located in the upper half of the spine and toward the shoulders (71) while its extension is increasingly reduced in adults (Figure 6, C and D). Brown adipocytes are characterized by the presence of hundreds of small lipid droplets and mitochondria rather than a unique fat lobule. The high numbers of mitochondria not only confer the characteristic brown coloration to the tissue, but also imply its function, i.e., thermogenesis (72, 73). Therefore it becomes clear the importance of BAT immediately after birth when a great amount of this tissue is necessary for avoiding the risk of lethal cold. However, despite its decrease during growth, recent studies have highlighted how BAT still conserves an important metabolic activity in human adults (74-77), where, above its small depots in upper

chest and neck, it also coexists at the level of the other white tissues in which its relative amount and functiondepend upon several factors, such as environmental temperature, nutritional status, age and gender (78, 79).

The first evidence describing an important role of MR in adipose organ was demonstrated in BAT by Zennaro, et al. in 1998 (80). The authors had developed a transgenic mouse model expressing a viral protein (SV40 large T antigen) under the control of the two promoters (P1 and P2) of human MR (hMR). In this model the intent was to use the viral protein (able to cause a tumor when expressed in a tissue) as a reporter of activation of hMR-P1 promoter. In that model, the malignant hybernoma (brown fat tumor) developed by animals, clearly reveals the transcriptionally activation of MR in BAT. Later, other studies proved how *in vitro* aldosterone treatment in brown adipocytes induced a significant increase in triglyceride accumulation, together with increased expression of adipogenic genes, LPL, PPARγ and aP2 (81) and inhibited expression and function of UCP-1 (65, 82), a mitochondrial protein that plays critical role in the regulation of thermogenesis. Interestingly, MR signaling in BAT seems to act by enhancing lipid storage rather than heat production, perhaps promoting in brown adipocytes the specific function of white adipocytes. These evidences together with the observations in the early 1990's of the adipogenic effect of aldosterone in 3T3-L1 mouse preadipocytes (24, 83) have pointed out the potential involvement of Aldo/MR system in adipose organ physiology and move researchers' attention towards WAT.

MR has been found expressed during adipose differentiation of white 3T3-L1 mouse adipocytes (84) where it is responsible for inducing adipocyte differentiation key markers (leptin, adiponectin, PPARγ) via specific aldosterone stimulation (22). However, due to very low HSD11B2 expression in adipocytes and considering both the high concentration of glucocorticoids in plasma *in vivo* and the importance of glucocorticoid stimulus in cell

adipogenic media, the role of MR in adipogenesis activation may be dependent on glucocorticoid rather than aldosterone. Consistent with this hypothesis, experiments of transient knock-down of MR or GR, have proven that a significant reduction of MR expression, rather than of GR expression, can inhibit glucorticoid-induced 3T3-L1 adipose conversion (22).

All these data show a first central role of MR in adipose tissue physiology, i.e., the adipocytes differentiation and lipid accumulation/release, but they further imply a greater pathological potential in case of aldo-MR system dysregulation. As has been previously described, adipose tissues also have an endocrine function and therefore impairing its physiology means changing its endocrine signaling which results in affecting the whole-body energy homeostasis.

Recently Guo et al. (62), have shown how aldosterone stimulation of 3T3-L1 fully differentiated adipocytes, is sufficient to modulate the expression of the major adipokines, such as the down regulation of adiponectin, and up regulation of MCP1, IL-6 and TNFα, therefore suggesting an aldosterone pro inflammatory effect in adipose tissue.

In obesity and diabetes, the failing of adipose tissue lies in energy storage, i.e., due to an overload of energetic substrates or inability to respond to insulin, it is always connected with chronic low-grade inflammation of the tissue that is set up by in situ recruitment and activation of macrophages (85, 86).

This process is accompanied by a change of adipose tissue morphology and expression pattern. In particular, if a healthy tissue is able to expand whether increasing adipocytes volume or number, in that characterized by a normal insulin sensitivity and regular expression of PPARγ and adipokines, a dysregulated adipose tissue is instead more hypertrophic than hyperplastic, and is characterized by a measurable increase in adipocytes size with down

regulation of adiponectin and PPARγ, as well as up regulation of inflammatory adipokines, such as MCP1, IL-6, TNFα and PAI-1 (87-90) (Figure 7).

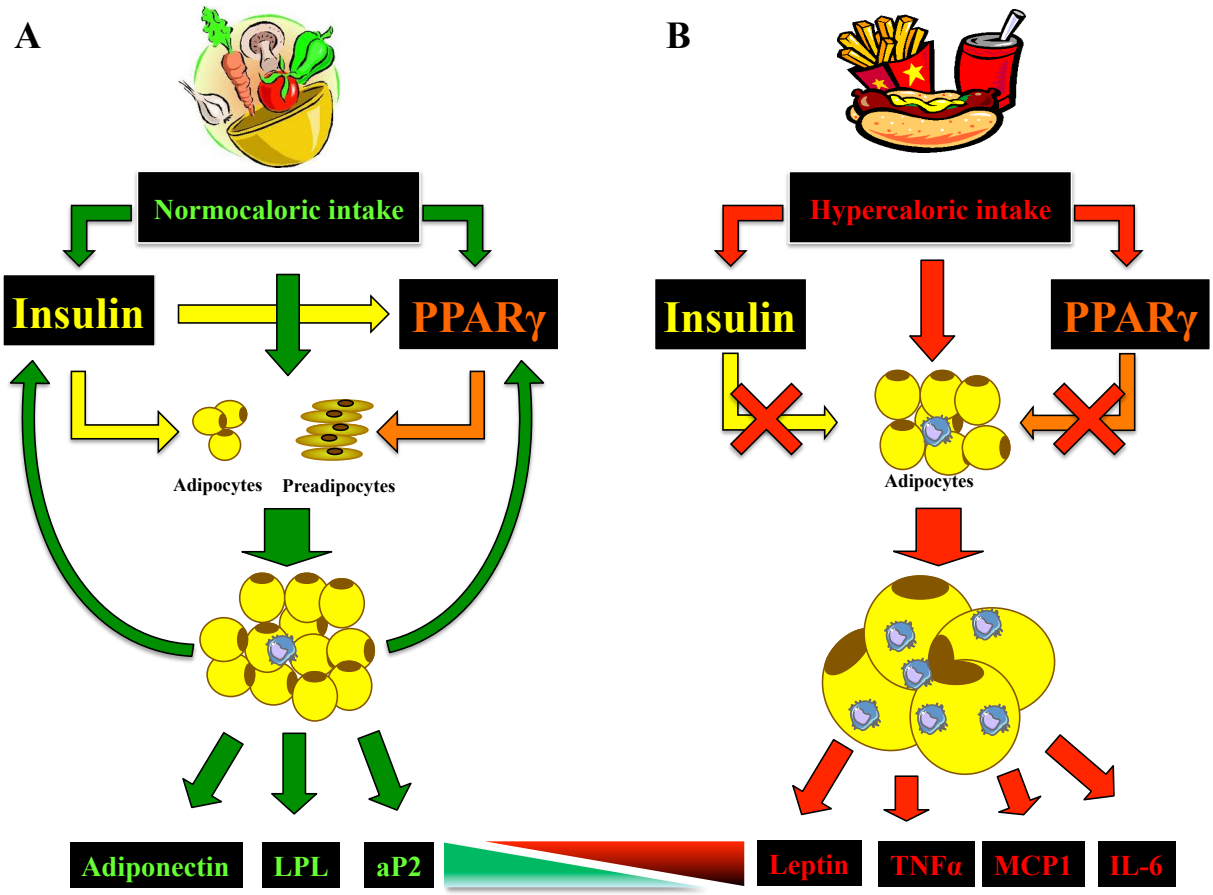

Figure 7. Schematic representation of mechanisms involving insulin and PPARγ in developmental of adipose tissue.
In physiological condition (A), the presences of a mild surplus of metabolic fuels stimulates adipose tissue expansion that is accomplished by coordinated action between insulin, that stimulates energy intake and lipid synthesis (hypertrophic process) and PPARγ, which induces adipocytes differentiation and recruitment of new adipocytes from preadipocytes pool (hyperplastic process). In pathological condition (B), i.e., obesity, the overload of energy leads to overload of adipocytes too, causing them to down regulate adiponectin and PPARγ and to up regulate the proinflammatory adipokines, such as TNFα, MCP1 and IL-6. Theses event start the process of recruitment and activation of macrophages in adipose tissue, which ultimately ends with a low-grade chronic inflammation and the inhibition of insulin response.

This expression pattern is very similar to that shown by Guo et al., in aldosterone stimulated 3T3-L1 adipocytes. Moreover, further support to the involvement of MR activation in adipose tissue inflammation comes from the studies on the effects of MR antagonism in the more common animal models for obesity and diabetes. Once again, Guo et al. observed that treatment with eplerenone (a specific MR antagonist) for 17 weeks in genetically obese db/db

mice, was able to restore the same expression profile of the adipose tissue of lean mice (Figure 8) (62).

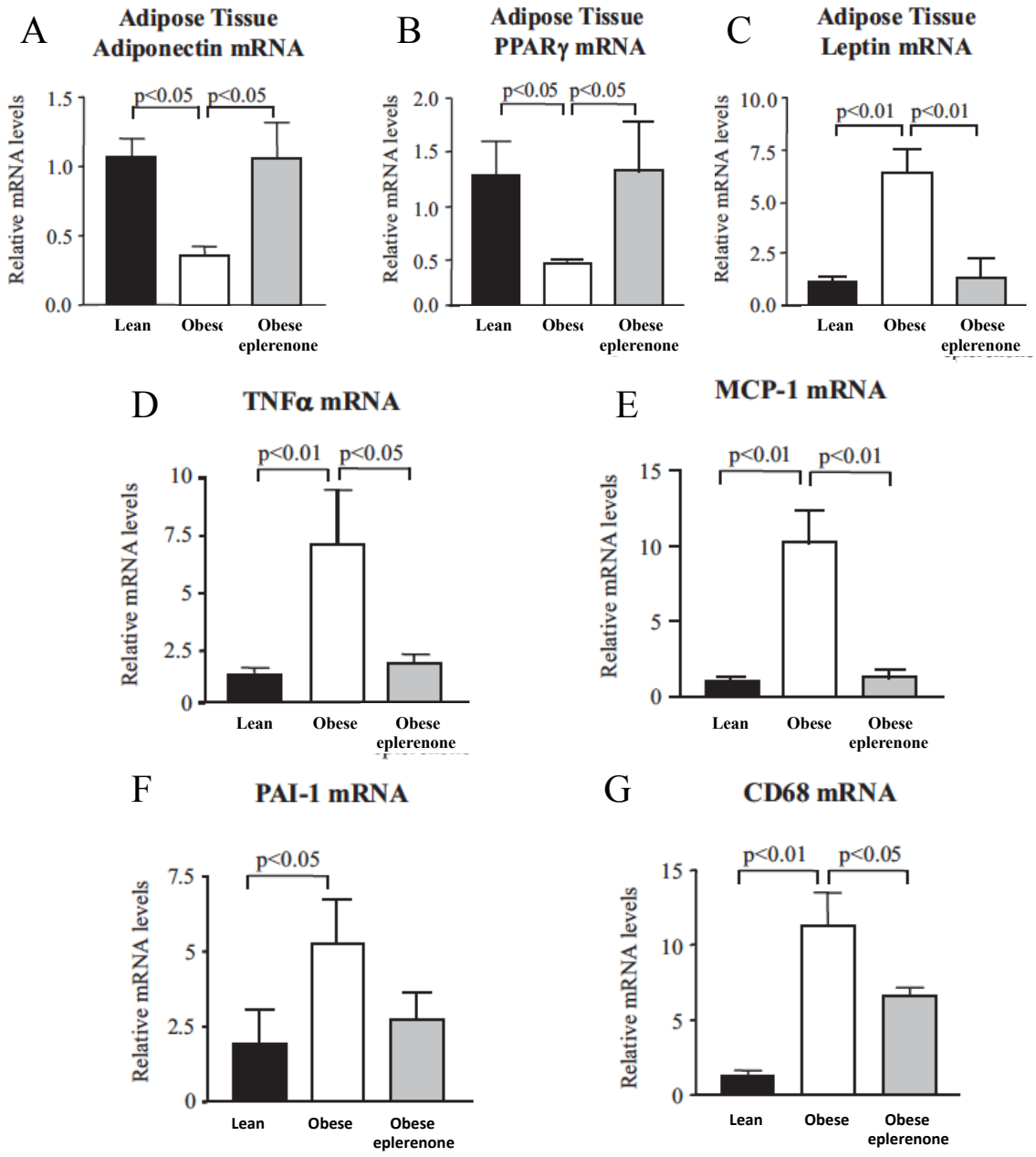

Figure 8. Expression profile in retroperitoneal adipose tissue of obese (db/db) mice, adapted from Guo et al. 2008 (62).
Adiponectin (A), PPARγ (B), leptin (C), TNFα (D), MCP-1 (E), PAI-1 (F), and CD68 (G) mRNA levels of 25 weeks old, lean (*db/-*) mice, obese (*db/db*) mice, and obese (*db/db*) mice treated with the MR antagonist eplerenone (100 mg/kg per day from age 8 to 25 weeks). mRNA levels are expressed relative to 18S rRNA. n=8 per group. Data are mean ±SE.

Similar data was also shown also by Hirata et al. (64), in that acute effects (3 weeks) of eplerenone administration in db/db and ob/ob mice showed an improvement of insulin sensitivity through the reduction of ROS and the suppression of infiltration of adipose tissue macrophages.

All these data are ultimately depicting an important role of aldosterone and MR in both, adipose tissue physiology, i.e., the adipocytes differentiation and lipid storage activity, and pathology, i.e., adipose tissue inflammation and insulin resistance.

However, signaling activation, i.e., relative contribution of glucocorticoid vs. aldosterone, as well as signaling pathway, e.g., the direct target genes transcribed via activated MR, or events shifting MR role from pro-adipogenic in healthy tissue to pro-inflammatory in pathological environment, still remain to be elucidated.

AIMS OF THE THESIS

The work during the three years of my PhD essentially has focused on the evaluation and examination of functions of the aldo/MR system in adipose tissue from two distinct points of view: my studies included assessing the effects of aldosterone excess in the adipose tissue as well as estimating the phonotypical consequences of MR overexpression in adipocytes.

For the first two years, I worked in Italy at the laboratory of Prof. RobertoVettor and Prof. Francesco Fallo in Padova, where I started a collaboration with a team of clinicians in order to assess the effect of aldosterone on insulin sensitivity patients with primary aldosteronism. My specific role was: 1) to study molecular characteristics of adipose tissue collected from patients with primary aldosteronism; 2) to set up experiments on the effect of aldosterone on primary cultures of human adipocytes.

In the last year, I have been working in the French laboratory directed by Dr. Frederic Jaisser in Paris, where I started the phenotypic characterization of a new transgenic mouse model conditionally overexpressing MR in adipocytes.

STUDY IN PADOVA:

ANALYSIS OF INSULIN SENSITIVITY IN ADIPOSE TISSUE OF PATIENTS WITH PRIMARY ALDOSTERONISM

INTRODUCTION

Aldosterone is a major regulator of blood pressure and salt/water balance, activating the mineralocorticoid receptor in sodium-transporting epithelia of distal renal tubules, distal colon, sweat and salivary glands (10). Primary aldosteronism is characterized by autonomous production of aldosterone causing excessive sodium retention, excessive potassium excretion, suppressed renin activity, hypokalemia and hypertension (34). Recent observations indicate aldosterone as a direct cardiovascular risk factor, acting on the mineralocorticoid receptor (MR) in non-epithelial cardiovascular tissues, including vascular smooth muscle cells, cardiomyocytes, and fibroblasts (91, 92). Aldosterone excess may lead in fact to cardiovascular damage independently from its effect on blood pressure (91, 93, 94). Studies in humans have shown an association between increased plasma aldosterone levels and insulin resistance independent of other components of the metabolic syndrome (42, 43). We and others (table 1) showed that aldosterone excess in patients with primary aldosteronism is related to impaired glucose homeostasis and to insulin resistance, suggesting that the reported high rates of cardiovascular events in primary aldosteronism might be due to increased prevalence of the metabolic syndrome in the former condition (53, 56, 95, 96). Evidence suggests that aldosterone exerts these metabolic detrimental effects through a pro-

inflammatory action (65, 66). Adipose tissue, in particular visceral fat, is one of the major insulin-target tissues accounting for whole-body insulin sensitivity (97, 98). This occurs also with the participation of several fat-derived hormones, i.e., adipokines, regulating inflammation, oxidative stress, energy balance, vascular function and, in turn, glucose metabolism (68). Therefore, adipose tissue may have a key role in the pathogenesis of the impaired glucose utilization seen in primary aldosteronism.

The aims of this study were twofold: 1) to examine the expression of MR and of the genes involved in adipogenesis, glucose transport, insulin signaling and inflammation, into the ex-vivo visceral (omental) fat biopsies of a series of patients with primary aldosteronism due to an aldosterone-producing adenoma (APA); 2) to assess in vitro the effect of aldosterone on glucose uptake and insulin signaling cascade on fully differentiated human adipocytes obtained from starting with human subcutaneous abdominal adipose tissue.

MATERIALS AND METHODS

Patients

Fourteen patients with an aldosterone-producing adenoma (APA) and fourteen patients with incidentally discovered non-functioning adrenal adenoma (NFA) were studied. The two patient subgroups were selected from a much larger patient population consecutively referred to our Institutions over the last 5 years, and were purposely matched for sex-, age and body mass index (BMI). Patients with clinical and/or laboratory evidence of associated clinical conditions such as cerebrovascular, coronary or peripheral artery disease, cardiac insufficiency, renal and/or hepatic disease, and patients with history of cardiovascular and cerebrovascular events, were excluded. Renal disease was defined as the presence of serum creatinine greater than 133 μmol/L in men and greater than 120 μmol/L in women and/or albuminuria greater than 300 mg/d. Patients with type 2 diabetes, i.e., those with fasting glucose levels above 7.0 mmol/L on two separate occasions (Expert Committee),(99) or with obesity (i.e., BMI > 30 kg/m2) were also excluded. During evaluation and at time of surgery, all subjects were consuming a diet containing daily amount of 120–150 mmol of sodium and 60 mmol of potassium and followed a normocaloric diet. All medications were withdrawn for 3 weeks (at least 6 weeks for spironolactone). In hypertensive patients in whom treatment could not be withdrawn for ethical reasons, a calcium-channel blocker and/or an alpha-receptor blocker were allowed at the minimal doses required to achieve blood pressure control. These agents are known to have a neutral effect on renin and aldosterone levels (100) and not to impair glucose and lipid parameters (101). In patients taking lipid-lowering drugs, treatment was withdrawn at least 3 weeks before biochemical evaluation. All blood pressure measurements were performed according to the European Society of Hypertension-European

Society of Cardiology guidelines (99). The surgical procedure was unilateral laparoscopic adrenalectomy for all patients.

Differential diagnosis criteria for the different forms of primary aldosteronism and for essential hypertension were previously described (96). Briefly, a cut off upright plasma aldosterone (ng/dL)/PRA (ng/mL/h) ratio greater than 40 in the presence of aldosterone greater than 15 ng/dl and suppressed PRA was used as screening test for primary aldosteronism. In the case of an aldosterone/PRA ratio greater than 40, patients underwent saline infusion (0.9% NaCl 500 ml/h for 4 h) as a confirmatory test, and only those with plasma aldosterone levels that failed to fall below 5 ng/dl after the saline infusion were diagnosed as having primary aldosteronism. In all patients a computed tomography scan with fine cuts of the adrenal and an adrenal venous sampling were performed to differentiate between aldosterone-producing adenoma (APA) and bilateral hyperplasia, i.e., idiopathic hyperaldosteronism (IHA). Other secondary causes of hypertension were excluded. Hypokalemia (serum K <3.5 M) was present in 10 out of 14 patients. The diagnosis of adrenocortical adenoma was histologically confirmed after surgical resection, and the adrenal cells from APA were classified microscopically into the four types described by Neville and (102). Adrenal tumors at histology ranged from 1.5 to 3.0 cm.

Adrenal NFA were incidentally discovered in patients by non-invasive abdominal imaging techniques (CT scan and/or MNR) performed for reasons other than suspected adrenal disease. Inclusion criteria were: 1) absence of specific signs and/or symptoms of hormone excess; 2) normal tests of hypothalamic-pituitary-adrenal axis; 3) morphological aspect of adrenal mass suggesting the presence of a cortical adenoma (round shape with smooth edges, homogeneous with relatively low density; Ref.(103)). All subjects received an extensive endocrine evaluation to exclude a functioning adrenal tumor. Baseline data included determination of urinary free cortisol, plasma ACTH, serum dehydroepiandrosterone sulfate,

serum 17-OH progesterone (17OHP), serum testosterone, upright plasma aldosterone/PRA, urinary catecholamines excretion, plasma cortisol rhythm. Dynamic tests included overnight 1 mg dexamethasone test in 9 cases, with suppression of serum cortisol below 1.8 mmol/L). All had normal ACTH levels. The decision to perform surgery was made by the size of adrenal adenoma (≥3.0 cm in diameter, 7 cases), increase in size over time (2 cases), or patients' preference (5 cases). The adrenal tumor size at histology ranged from 2.5 to 6 cm. Twelve resected tumors were diagnosed histologically as adrenocortical adenoma and another two tumors were diagnosed as of non-adrenocortical origin, i.e. a myelolipoma.

During laparoscopic surgery, visceral adipose tissue (omental) was obtained by biopsy for each patient. Biopsies were snap-frozen in liquid nitrogen and then stored at -80°C until bio-molecular analysis. Institutional review board and local ethical committee approvals were obtained. All participants gave written consent.

Hormone and biochemical assays

PRA and aldosterone were determined by radioimmunoassay as previously described (56). Normal range for upright PRA was 1.5-5.2 ng/mL/h. Normal range for upright plasma aldosterone was 5-35 ng/dL. Twenty-four-hour urinary cortisol was measured by radioimmunoassay using a kit from Diagnostic Products Co. (CA, USA), The intra-assay coefficient of variation (CV) was 6%, and the inter-assay coefficient of variation was 8.2%. The normal range was 55 to 330 nmol/day. Plasma ACTH was measured by chemiluminescence (Immulite 2000, Diagnostic Products Co., CA, USA); intra-assay CV 6.2% and inter-assay CV 4.8%; normal range at 08:00 h, 2-11 pmol/L. Serum cortisol was measured by chemiluminescence (Immulite 2000, Diagnostic Products Co., CA, USA); intra-

assay CV 7.4% and inter-assay CV 5.2%; normal range at 08:00 h, 138-690 nmol/L. Insulin sensitivity was calculated according to the formula of the homeostasis model assessment (HOMA) index method of insulin resistance = fasting plasma insulin (mUI/mL) x fasting plasma glucose (mmol/L)/22.5. (104). Plasma insulin concentration was measured by a chemiluminescence immunoassay using a commercially available kit (Immulite 1 analyzer, DPC, Los Angeles, CA, USA): normal range, 6-24 mUI/mL. All other biochemical variables were assayed in plasma or serum using standard methods. For hormone measurements, intra- and inter-assay coefficients of variation were less than 10%.

Isolation of human preadipocytes and adipose differentiation in vitro

Subcutaneous visceral fat was obtained from 5 subjects (3 females and 2 males, 24-47 years) who requested abdominoplasty after weight loss. At time of surgery (i.e., circumferential abdominoplasty), patients had normal lipid profile, normal glucose tolerance, as measured by homeostasis model assessment index (1.8 ± 0.5), and BMI <30 kg/m2. All patients were on an unrestricted dietary regimen, and body weight was stable during the three months preceding surgery. No patient had previous bariatric surgery. One set of cell culture experiments was performed from each subject. The stromal vascular fraction was isolated from adipose tissue by collagenase type II digestion (1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) in DMEM at 37°C for 1 h. Cell suspension was centrifuged (350g, 8 min) and pellet containing stromal cells was resuspended in erythrocyte-lysing buffer, washed and seeded in DMEM/F12 supplemented with 10% FBS (0.7×106 cells/well in 24-well plates). After 16 to 20 hours for cell attachment, cultures were re-fed with a serum-free adipogenic medium containing serum free DMEM/F12, 33 μM biotin, 17 μM pantothenate, 10 μg/mL human transferrin (Sigma-

Aldrich, St. Louis, MO, USA), 66 nM insulin (Lilly Research, Indiana, IN, USA), 100 nmol/L dexamethasone, 1 nM triiodothyronine, 0.25 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO, USA) and with 10 µM rosiglitazone. 3-isobutyl-1-metylxanthine and rosiglitazone were removed after 3 days; medium was changed three times per week. All experiments were performed in fully differentiated adipocytes (i.e., after 6 days of differentiation). -

RNA extraction and quantitative RT-PCR

Total RNA was extracted from biopsies of visceral adipose tissue or from adipocytes with RNeasy mini kit (Qiagen GmBh, Hilden, Germany). One µg RNA was treated with DNase Treatment and Removal Reagents (Ambion, Inc. Austin, TX, USA) and reverse-transcribed for 1 h at 37°C in a 50µl reaction volume containing 1X RT buffer, 150 ng random hexamers, 0.5 mM deoxynucleotide triphosphates, 20 U RNAsin ribonuclease inhibitor and 200 U M-MLV RT (Promega Corporation, Madison, WI, USA). Quantitative PCR was carried out using a DNA Engine (Opticon 2 Continuous Fluorescence Detection System; Biorad Laboratories Inc., Hercules, CA, USA).

Reactions were performed in duplicate for each sample in a total volume of 12µl: 5µl of cDNA (1ng/µl) 1µl of forward/reverse primer mix (3.6µmol/L) and 6µl of SYBR Green (Platinum® SYBR® Green qPCR SuperMix-UDG; Invitrogen Corporation, Carlsbad, CA, USA). The thermal cycling parameters were: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, at annealing temperature (Table. 1) for 15sec and at 60°C for 1 min. Relative expression of the mRNA for each sample was quantified the software and

results were normalized by dividing the amount of the candidate mRNA by the amount of the reference rRNA 18S (18S).

The genes were analyzed and their specific primers sequences and annealing temperature are listed in table 1. For MR, the amplified MR cDNA fragments were also separated in 2% agarose (Sigma-Aldrich, St. Louis, MO, USA) in Tris-borate buffer and visualized with ethidium bromide staining in order of taking pictures.

Table 2: Quantitative RT-qPCR: Genes, primers sequences and conditions

Chart Label	Gene official symbol and full name	Oligonucleotide primers	$[C]_f$ nM	Annealing temperature	Amplicon
18S	RN18S1, 18S ribosomal RNA	Forward: 5'- CG GCT ACC ACA TCC AAG GAA -3' Reverse: 5'- GCT GGA ATT ACC GCG GCT -3'	300 50	60°	187 bp
MR	NR3C2, nuclear receptor subfamily 3, group C, member 2, also known as mineralocorticoid receptor	Forward: 5'- CTG GTT CCT CAG CTC TCC AC -3' Reverse: 5'- GGA TCA TCT GTT TGC CTG CT -3'	300 300	59°	169 bp
GRα	NR3C1, nuclear receptor subfamily 3, group C, member 1, also known as glucocorticoid receprot	Forward: 5'- TGG ATT CTA TGC ATG AAG TGG -3' Reverse: 5'- TCG ACT TTC TTT AAG GCA ACC -3'	300 300	56°	207 bp
HSD11B1	HSD11B1, hydroxysteroid (11-beta) dehydrogenase 1	Forward: 5- AGC TCC CCC TTT GAT GAT CT -3' Reverse: 5'- TTG CTT TGG ATG GGT TCT TC -3'	300 300	55°	188 bp
HSD11B2	HSD11B2, hydroxysteroid (11-beta) dehydrogenase 2	Forward: 5- GAC CTG ACC AAA CCA GGA GA -3' Reverse: 5'- GCC AAA GAA ATT CAC CTC CA -3'	300 300	55°	174 bp
Leptin	LEP, leptin	Forward: 5'-GTG CGG ATT CTT GTG GCT TT-3' Reverse: 5'-GGA ATG AAG TCC AAA CCG GTG-3'	100 100	63°	174 bp
Adiponectin	ADIPOQ, adiponectin, C1Q and collagen domain containing	Forward: 5'-CCT CAT GAA TTA AAA CCT CC-3' Reverse: 5'-GAT ATC TCA TCA GAG AAG CTC C-3'	300 300	55°	225 bp
PPARγ	PPARG, peroxisome proliferator-activated receptor gamma	Forward: 5'-ACC CAG AAA GCG ATT CCT TCA-3' Reverse: 5'-AGT GGT CTT CCA TTA CGG AGA GAT C-3'	900 900	60°	87 bp
IL-6	IL6, interleukin 6 (interferon, beta 2)	Forward: 5- TTC GGT ACA TCC TCG ACG -3' Reverse: 5'- AAG CAT CCA TCT TTT TCA GC -3'	300 300	54°	136 bp
MCP-1	CCL2, chemokine (C-C motif) ligand 2	Forward: 5- CCC CAG TCA CCT GCT GTT AT -3' Reverse: 5'- TGG AAT CCT GAA CCC ACT TC -3'	300 300	55°	171 bp
IR	INSR, insulin receptor	Forward: 5'- ACG TCC CGT CAA ATA TTG C -3' Reverse: 5'- GGT TTG AAG AAG CGT AAA GC -3'	300 300	54°	144 bp
IRS-1	IRS1, insulin receptor substrate 1	Forward: 5'- GTT TCC AGA AGC AGC CAG AG -3' Reverse: 5'- TGA AAT GGA TGC ATC GTA CC -3'	300 300	55°	182 bp
IRS-2	IRS2, insulin receptor substrate 2	Forward: 5'- CTT GTC CCA CCA CTT GAA GG -3' Reverse: 5'- CAC AGT CAT TGC TCA GAT CCA -3'	300 300	55°	166 bp
GLUT4	SLC2A4, solute carrier family 2, member 4, also known as glucose trasnsporter 4	Forward: 5'- AGC TTC TTC TAA GAC GAG ATG C -3' Reverse: 5'- CCG TTT CTC AGG CTC CCT CTC -3'	300 300	58°	200 bp

Immunohistochemistry

Serial 5-micron-thick paraffin sections of adipose surgical biopsy were processed by immunohistochemistry. Sections were incubated with a mouse monoclonal anti-MR clone 1D5, provided by Dr. Gomez-Sanchez. This primary antibody was diluted 1:50 and incubated overnight in a buffer 50 mM Tris pH 7.4. Sections were then exposed to a secondary biotinylated IgG (ScyTek Lab., Logan, UT, USA) and visualized by incubation for 3 minutes with a peroxidase substrate solution containing the chromogen DAB. Slides were then washed, counterstained with hematoxylin, shed in water and ethanol and mounted in synthetic resin. Positive control was a normal kidney, while specificity was validated in parallel negative control sections by omitting the primary antibody.

Immunofluorescence

For immunofluorescence, fully differentiated adipocytes were fixed with 4% paraformaldehyde in for 20 min, washed with PBS and incubated with 0.50% Triton X100 in PBS for 10 min at room temperature. A goat polyclonal primary anti-human MR antibody [MCR (C-19):sc-6861, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA] diluted 1:50 in PBS was used. After incubation, binding of primary antibodies was detected with ALEXA-488 conjugated secondary antibodies, diluted 1:200 in PBS. The cells were then examined for localization of fluorescence with a Leica confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Glucose uptake in primary human fat cells

After differentiation, adipocytes were left with serum free DMEM F12 for 24 hours. Adipocytes were then treated with aldosterone 1nM-10μM (Sigma-Aldrich, St. Louis, MO, USA), eplerenone 10μM (Tocris Bioscience, Ellisville, MO, USA), hydrocortisone 1μM (Sigma-Aldrich, St. Louis, MO, USA), Mifepristone (RU486) 10μM (Tocris Bioscience, Ellisville, MO, USA), alone or in combination, for 24 hours. Adipocytes were washed in 0,49 mM Ca2+ , 0,90mM Mg2+ PBS (PBS-Ca2+-Mg2+) medium and incubated with or without insulin 2μM for 30 min at 37°C in a 5% CO2 incubator. The cells were again washed twice with PBS-Ca2+-Mg2+. Glucose uptake was initiated by the addition of 2-deoxy-[3H]d-glucose (1,5μCi/ml, final assay concentration, GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 15 min at 37°C. Glucose uptake was terminated by two washes with ice-cold PBS-Ca2+-Mg2+ and cells were lysed with 0,5M NaOH. Radioactivity was determined by scintillation counting radioactivity (Wallac, PerkinElmer, Boston, MA).

Protein extraction.

Samples of stored VAT from each APA and NFA patient were rapidly thawed and lysed in RIPA buffer [(20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin)] completed with phosphatase and protease inhibitor (PhosSTOP and Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany), and extracts were stored at -80°C until western blot analysis.

Differentiated adipocytes (2 $x10^6$ cells for each plate well) were left with serum free DMEM F12 for 24 hours and then were treated with vehicle, with 1 nM aldosterone or with

10 μM aldosterone for other 24 hours. After treatment, adipocytes were washed twice with PBS-Ca2+-Mg2+ and stimulated with or without 2 μM insulin for 5 min at 37°C in a 5% CO2 incubator. After insulin stimulation, cells were snap lysed at 4°C with the protein the same buffer used for issues and then conserved at -80°C until analysis.

Western blotting and immunodetection

Protein samples (30-80 μg) from patients' biopsies or from adipocytes were subjected to SDS-PAGE. Resolved proteins were dry transferred to PVDF membrane and subsequently hybridized with the primary antibody against the protein of interest. For detection of MR the mouse monoclonal antibody 1D5 was used, kindly provided from the team lead by Professor Celso E. Gomez-Sanchez (Division of Endocrinology, G.V. (Sonny) Montgomery VA Medical Center, 1500 East Woodrow Wilson Drive, Jackson, MS 39110) (105). Total Akt and phospho-Akt Ser473 were detected by antibody #9272 and antibody #4060 (Cell Signaling Technology, Beverly, USA), respectively. For detection of total ERK 1/2 and phospho-ERK 1/2, anti-p44/42 #13-6200 antibody (Invitrogen Co.) and anti-phospho-p44/42 antibody (thr202/tyr204) #9101 (Cell Signaling Technology) were used, respectively. Beta-actin was detected by #A5441 antibody (Sigma-Aldrich). For detection of primary antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Laboratory, Bar Harbor, USA) were used. For all the antibodies the best conditions of hybridization are listed in table 3.

Probed blots were incubated with Immobilon® Western HRP substrate (Millipore Corporate, Billerica, MA, USA) and exposed to Hyperfilm ECL film (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The films were developed with GBX Kodak developmental solutions (Sigma-Aldrich). Densitometric analysis of the immunoreactive protein bands were performed using Molecular Analyst software (Bio-Rad Laboratories, Inc., Marnes La Coquette, France).

Table 3: list and working dilution of western blot antibodies

Antibody	Type	Dilution
Mouse monoclonal antibody 1D5	Primary	1:100 in PBS-T*
Rabbit monoclonal antibody anti Akt #4060 (Cell Signaling Technology, Beverly MA)	Primary	1:1000 in PBS-T
Rabbit polyclonal antibody anti phospho-Akt Ser^{473} #9272 (Cell Signaling Technology, Beverly MA)	Primary	1:1000 in PBS-T
Monoclonal antibody anti-p44/42 MAPK ERK1/2 #13-6200 (Invitrogen Co.)	Primary	1:1000 in PBS-T
Rabbit polyclonal antibody anti-phospho-p44/42 MAPK ERK1/2 (thr202/tyr204) #9101 (Cell Signaling Technology)	Primary	1:1000 in PBS-T
Mouse monoclonal anti-β-actin antibody #A5441 (Sigma-Aldrich)	Primary	1:1000 in PBS-T
Anti-rabbit horseradish peroxidase-conjugated (HRP) linked-antibodies (Jackson Laboratory, Bar Harbor, ME)	Secondary	1:20000 in PBS-T
Anti-mouse HRP-linked-antibodies (Jackson Laboratory, Bar Harbor, ME)	Secondary	1:20000 in PBS-T

* PBS-T: 1% non-fat milk powder in 0.1% Tween PBS.

Statistical analysis

Results are expressed as mean ± SE. Variables data were tested for normal distribution using the Kolmogorov-Smirnov test, and different groups were compared by ANOVA with Bonferroni correction. Statistical analysis was carried out using the MDAS 2.0 (Medical Data Analysis System) software package (EsKay Software, Pittsburgh, PA). *P* values < 0.05 were considered significant.

RESULTS

Patients: clinical characteristic

Characteristics of the subjects are summarized in Table 4. The two groups were pre-selected as being matched for sex-, age and body mass index (BMI). In spite of similar BMI, insulin levels and HOMA index were higher in PA than in controls NFA. Also, blood pressure, plasma aldosterone/PRA ratio were higher and serum potassium was lower in PA than in controls NFA ($P<0.05$).

Table 4: Clinical characteristics of patients with aldosterone producing adenoma (APA) and with adrenal non-functioning adenoma (NFA)

	APA (*n* = 14)	NFA (*n* = 14)	*P* value
Age (yr)	60 ± 4	59 ± 3	NS
Sex (F/M)	7 / 7	7 / 7	NS
Body mass index (kg/m^2)	25.9 ± 0.7	25.5 ± 0.8	NS
Waist circumference F/M (cm)	85.7 ± 0.9 / 94.7 ± 1.2	87.3 ± 1.3 / 96.6 ± 1.4	NS
Systolic BP (mm Hg)	173 ± 4	129 ± 2	<0.0001
Diastolic BP (mm Hg)	98 ± 3	83 ± 2	<0.0001
Total cholesterol (mg/dl)	208.9 ± 9.1	234.1 ± 11.2	NS
Triglycerides (mg/dl)	147.5 ± 9.9	132.1 ± 14.6	NS
Fasting glucose (mg/dl)	96.8 ± 3.8	91.8 ± 2.1	NS
Insulin (μU/ml)	19.1 ± 1.6	15.5 ± 0.6	0.03
HOMA index	4.6 ± 0.4	3.4 ± 0.1	0.01
Serum potassium (mEq/liter)	3.1 ± 0.2	4.2 ± 0.1	<0.0001
Aldosterone (ng/dl)	38.9 ± 4.3	22.5 ± 1.6	<0.001
PRA (ng/ml.h)	0.1 ± 0.02	3.2 ± 0.3	<0.0001
Aldosterone/PRA ratio	295 ± 60	7 ± 1	<0.0001
Urinary cortisol (μg/24 h)	66 ± 6	63 ± 5	NS

All data are expressed as means ± SE. F, Females; M, males; BP, blood pressure; HOMA, homeostasis model assessment; PRA, plasma renin activity

Patients: MR expression in human adipose tissue and adipocytes

The presence of MR in human adipose tissues and in vitro cultured subcutaneous fat-derived adipocytes was first demonstrated by qRT-PCR analysis and, in order to better show this result, the amplified cDNA fragments were visualized in agarose gel with ethidium bromide staining (Figure 9, A).

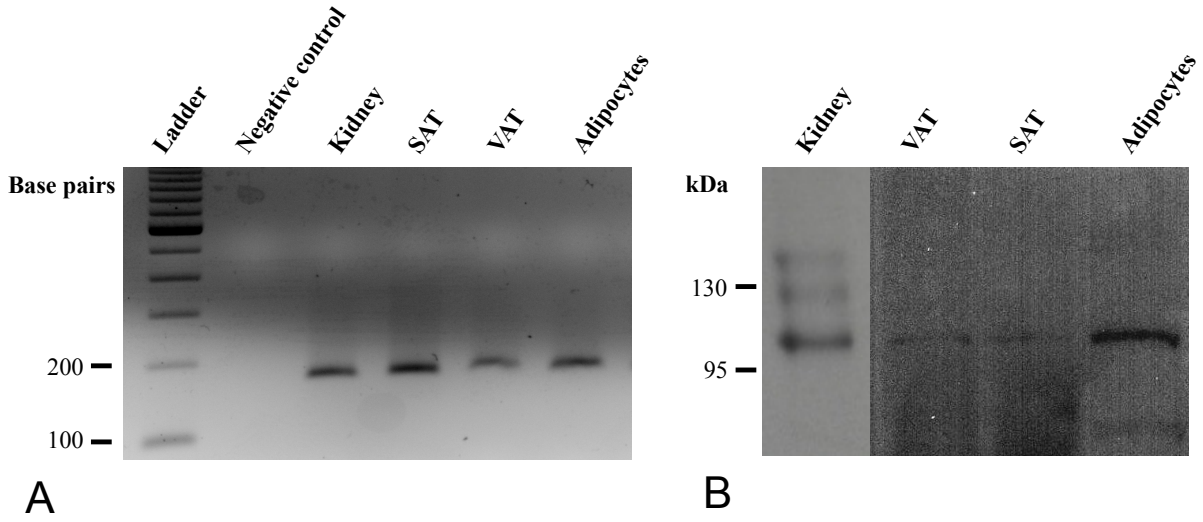

Figure 9. Mineralocorticoid receptor (MR) expression in adipose tissue. (A) Agarose gel electrophoresis of amplification products of MR obtained by quantitative real-time PCR from visceral adipose tissue (VAT), abdominal subcutaneous adipose tissue (SAT) of one non-functioning adenoma patient and differentiated subcutaneous adipocytes of a subject undergoing abdominoplasty; (B) Western blot analysis for MR protein obtained from the same samples, i.e., SAT, VAT and adipocytes: the specific MR band is localized at ~107 kDa. In both experiments kidney was used as positive control.

MR mRNA was found expressed in visceral and subcutaneous adipose tissue, as well as in cultured adipocytes (kidney tubular tissue was used as positive control). Western blotting confirmed the presence of MR related protein in the two adipose tissues and in adipocytes (Figure 9, B).

Further support to these data was also come from histological analysis of tissues and from immunofluorescence tagging of cells (Figure 10, A and B).

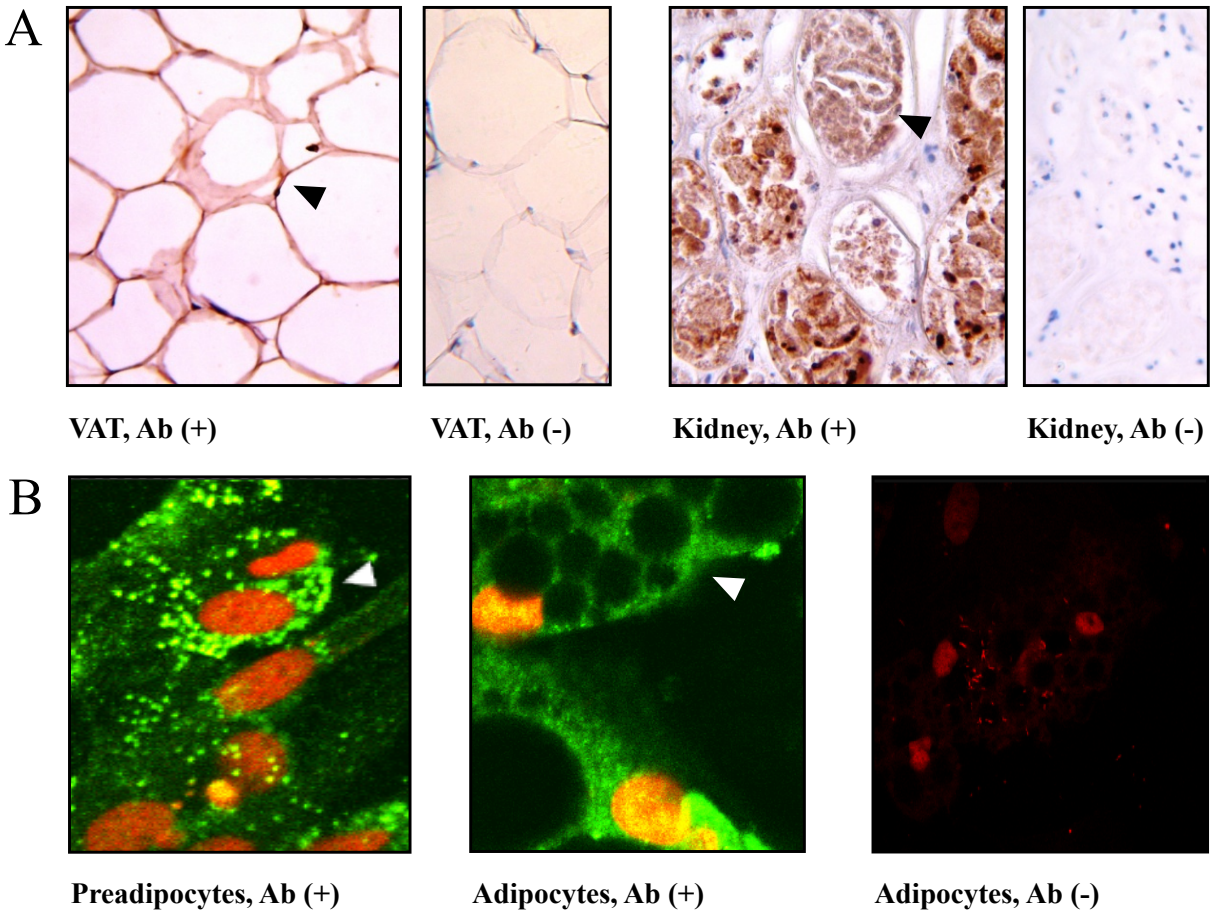

Figure 10. Mineralocorticoid receptor (MR) immune-staining in adipose tissue.
(A) Immune-histochemical staining (40x) of MR in VAT, a section from normal human kidney is also shown as positive control. (B) Immunofluorescence tagging (63x) of MR, in preadipocytes and differentiated adipocytes. The green signal of MR is mostly localized around the nuclei highlighted with the red staining obtained with propidium iodide.

Patients: VAT expression profile and insulin signaling analysis

The expression profile in VAT of patients with APA and with NFA was analyzed by qRT-PCR and it reveals how transcript levels of MR, GRα, HSD11B1 and HSD11B2, this one barely detectable, were similar in the two groups. mRNA expression levels of PPARγ and of some of the major adipokines, like leptin, adiponectin, IL-6 and MCP-1, as well as of some of the main insulin signaling related gens, as GLUT4, IR, IRS-1, IRS-2, were also found similar in both groups (Figure 11).

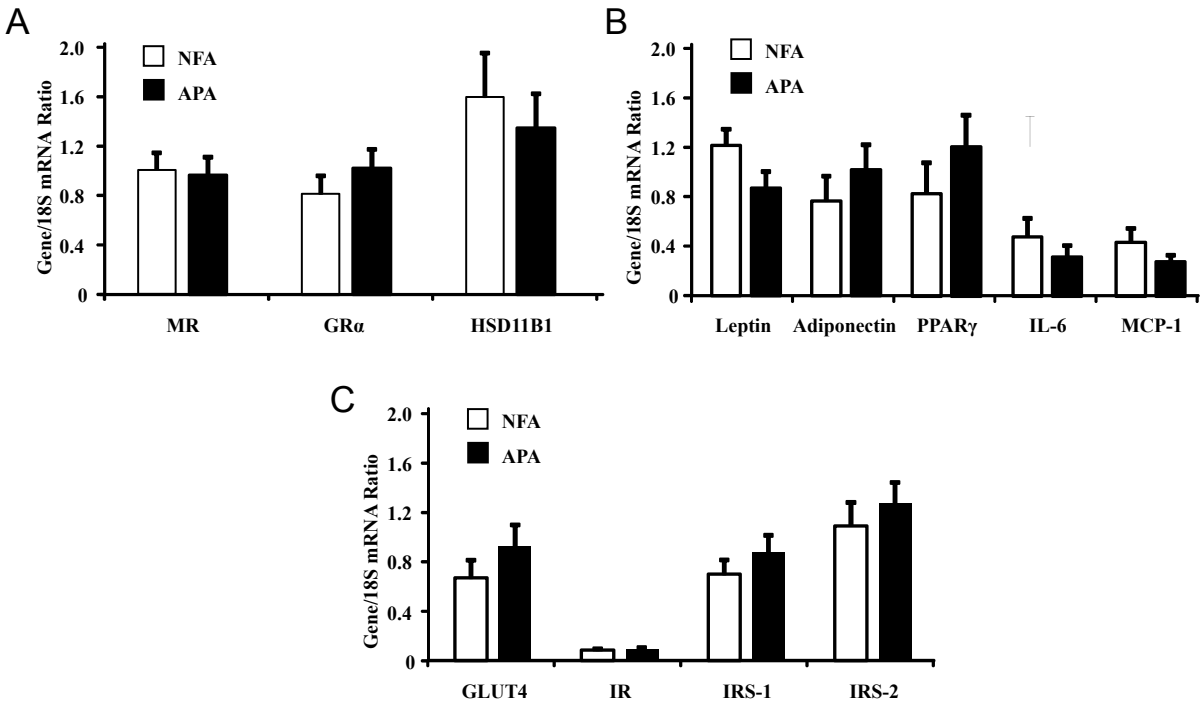

Figure 11. VAT expression profile of patients with APA and with NFA Quantification of mRNA expression by qRT-PCR of (A) MR, GRα and HSD11B1, (B) leptin, adiponectin, PPARγ, IL-6 and MCP-1, (C) IR, GLUT4, IRS-1, IRS-2 in visceral adipose tissue biopsies from patients with adrenal non-functioning adenomas (NFA) (white bar) and aldosterone-producing adenomas (APA) (black bar). Each bar represents the means ± SE of 14 samples for each group.

We next analysed whether aldosterone was able to affect the activation of major insulin signaling intermediates, such as Akt and MAPK (106) by measuring their phosphorylation status, and no differences were found between the two groups (Figure 12).

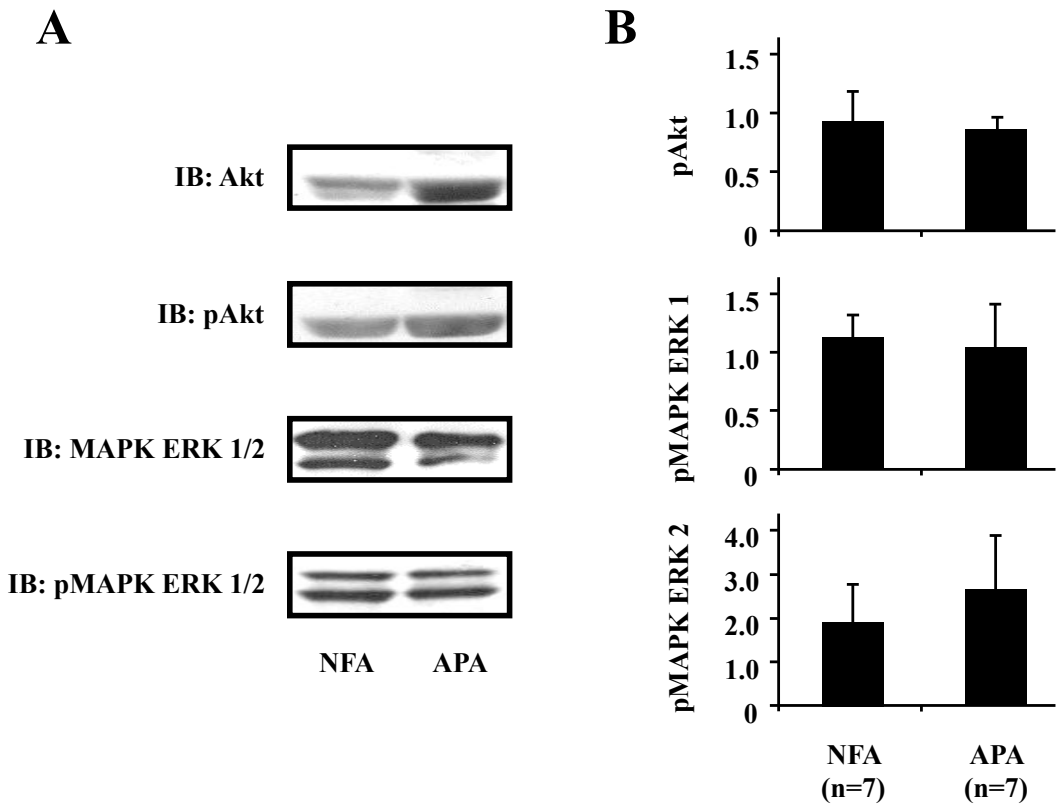

Figure 12. Western blot analysis of insulin signaling in VAT of patients with APA and with NFA
(A) Representative immune-blots showing total Akt, phosphorylated (p) Akt at Ser473, total MAPK ERK 1/2 and pMAPK ERK 1/2 at Thr202/Tyr204 in visceral adipose tissues (VAT) of patients with aldosterone-producing adenoma (APA) and non-functioning adrenal adenoma (NFA). (B) bars represent the means ± SE arbitrary units expressing the phosphorylated to total Akt and to total MAPK ERK 1/2 ratios in VAT of APA (n=7) and NFA (n=7).

In vitro adipocytes Glucose uptake and insulin signaling analysis

Both basal (i.e., non-insulin stimulated) and insulin-stimulated glucose uptake were assessed on fully differentiated human subcutaneous fat cells after 24 hours pre-treatment with different stimuli. Basal glucose uptake was similar to control when adipocytes were pre-treated with aldosterone at physiological/supraphysiological doses, i.e., 1-100 nM, whereas it was significantly lower ($P<0.05$) after pre-treatment with 10 μM aldosterone or with 1 μM hydrocortisone; neither the addition 10 μM eplerenone or of 10 μM RU486 influenced the effect of 10 μM aldosterone as well as the effect of hydrocortisone pre-treatment (Figure 13, A). Similarly, insulin-stimulated glucose uptake in the adipocytes did not differ when cells were pre-treated with vehicle or with 1-100 nM aldosterone. Aldosterone at pharmacological dose only (10 mM) and 1 μM hydrocortisone were able to decrease insulin-stimulated glucose uptake in comparison with vehicle; this effect was prevented by RU486, but not by eplerenone. (Figure 13, B).

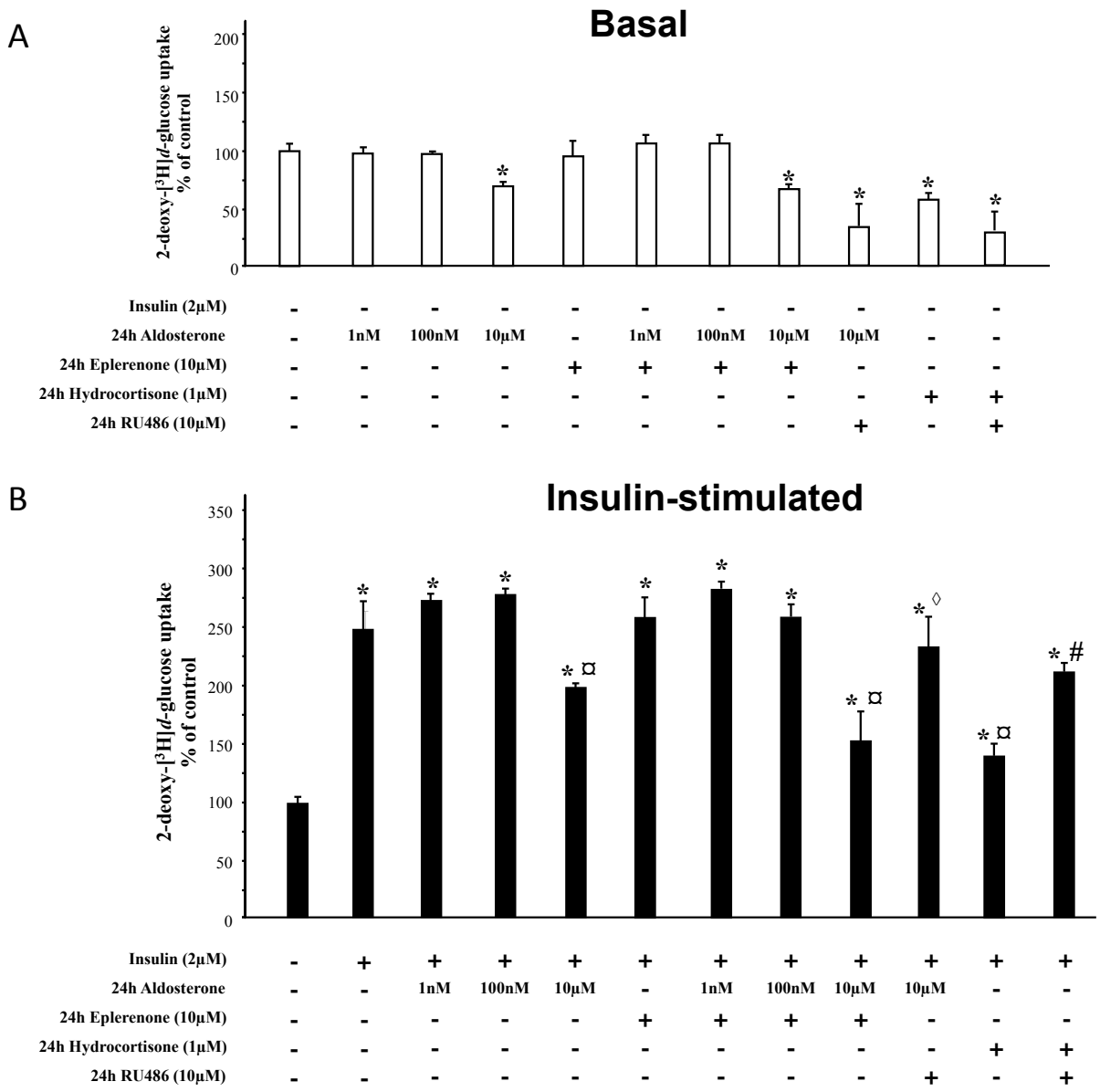

Figure 13. Uptake of 2-deoxy-[3H]d-glucose in fully differentiated human adipocytes.
Control was defined as glucose uptake by cells in the absence of insulin. Cells were studied in basal conditions (A) or were incubated for 30 min. to 2 µM insulin (B), after 24 hours pre-treatment with vehicle, aldosterone, eplerenone, aldosterone+eplerenone, aldosterone+RU486, hydrocortisone, or hydrocortisone+ RU486. Each bar represents the means ± SE of five separate experiments. *P<0.05 vs. control; ¤P<0.05 vs. vehicle + insulin alone; ◊P<0.05 10 µM aldosterone + RU486 vs. 10 µM aldosterone alone; #P<0.05 1 µM hydrocortisone + RU486 vs. 1 µM hydrocortisone alone.

We next analyzed whether aldosterone was able to affect activation of major insulin signaling intermediates, such as Akt and MAPK. The aldosterone dose previously shown to decrease glucose uptake in adipocytes was also assessed. Pre-treatment with physiological (1 nM) or pharmacological doses of aldosterone (10 mM) did not affect basal or insulin-induced phosphorylation of Akt at Ser473 nor that of MAPK ERK 1/2 (Figure 14).

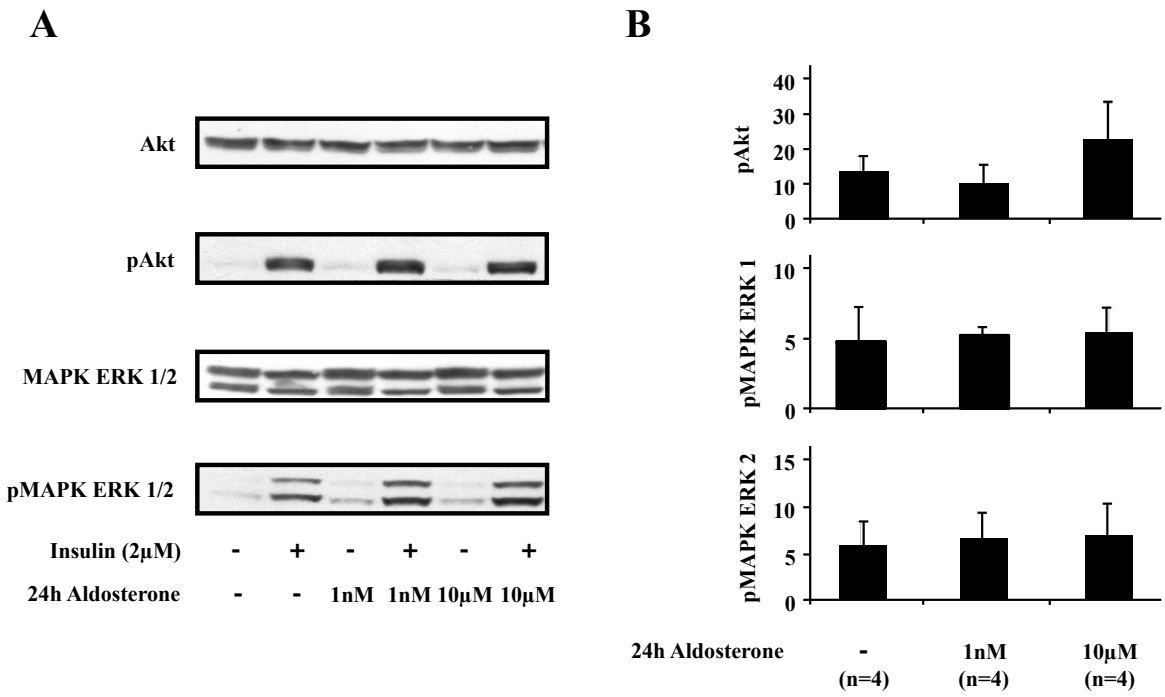

Figure 14. Western blot analysis of insulin signaling in fully differentiated human adipocytes
(A) Representative immunoblots showing total Akt, phosphorylated (p) Akt at Ser473, total MMAPK ERK 1/2 and pMAPK ERK 1/2 at Thr202/Tyr204 in human subcutaneous (sc) adipocytes incubated for 30 min with 2 µM insulin, after 24 hours pre-treatment with vehicle, aldosterone 1 nM or 10 µM aldosterone. (B) Bars represent the means ± SE arbitrary units (n = 4 experiments) expressing the phosphorylated to total Akt and to total MAPK ERK 1/2 ratios in sc adipocytes in the same study conditions.

DISCUSSION

To the best of our knowledge, this study was the first to explore: 1) the expression of MR gene and of genes involved in adipogenesis, glucose handling, insulin signaling, and inflammation in the visceral adipose tissues of patients with primary aldosteronism; 2) the

effect of aldosterone on glucose uptake and insulin signaling in cultured subcutaneous human adipocytes.

Both MR mRNA and protein, as well as GRα and HSD11B1 gene expression, were clearly present in a series of human visceral adipose tissues, with no difference between APA and NFA patients. Moreover, immunofluorescence revealed that MR was localized mostly in the perinuclear region of fully differentiated adipocytes obtained from the stromal vascular fraction of adipose tissue of subjects undergoing abdominoplasty. Subcellular localization of MR, in the absence of ligand, is consistent with that of other studies localizing fluorescent-tagged MR in living non-epithelial human cells (107). However little is known about the potential involvement of MR in white adipose tissue biology. Following early studies (24, 84), suggesting an involvement of MR on differentiation of 3T3-L1 cells to mature adipocytes, Caprio et al. showed that, whether stimulated by glucocorticoids or by aldosterone, MR represents an important pro-adipogenic transcription factor mediating adipocytes differentiation(22). Although we did not specifically address this question in our fat cell culture, the similar expression of PPARγ, a master regulator of adipogenesis, in the omental tissue of our APA and NFA patients, seems not to support a role of aldosterone as pro-adipogenic hormone at least at level of mature tissue composing the patients' biopsies. Moreover, even if previous works by Carranza et al. (108) showed a reduction in number and affinity of insulin receptors in SAT of one patient with primary aldosteronism, our more extended analysis (13 patients with APA and 13 with NFA) of expression pattern in VAT, does not highlight any differences in genes involved in insulin signaling, i.e., GLUT4, IR, IRS-1 and IRS-2, or inflammatory process, i.e., MCP1 and IL-6, between the two groups. So our first assessment of the effect of aldosterone excess in omental adipose tissue of patients with APA compared to those with NFA, turned out to show no differences in mRNA

expression level for all tested genes, suggesting that aldosterone excess in vivo may not lead to altered fat insulin sensitivity or inflammation.

However, insulin signal is a complex network of interacting proteins (106) and its regulation is more a matter of protein structure modification, i.e., by phosphorylation, rather than protein quantity accounted by related mRNA expression level. Indeed aldosterone could interference with insulin signal by affecting the phosphorylation status of signal transducers, like IRS1/2, Akt or ERK1/2, involved in the "classic" insulin pathway, in that, linking activation of insulin receptor to the final step of translocation to plasma membrane of GLUT4, a specific insulin-related glucose transporter which enables glucose uptake (106). Therefore, aldosterone could impair this mechanism by decreasing insulin sensitivity within adipocytes causing them to reduce their glucose uptake.

Previous studies, in mice, have already shown how aldosterone was able to alter insulin signal in adipose tissue. In addition, the work of Kraus et al. showed that aldosterone dose-dependently impaired the insulin-induced glucose uptake in cultured murine brown adipocytes (65) and the paper Wada et al. (109) showed that non-physiological doses of aldosterone deteriorated metabolic action of insulin in 3T3-L1 adipocytes by production of reactive oxygen species (ROS), facilitating the degradation of IRS-1 and IRS-2 via GR. In our experiments, neither basal nor insulin-mediated glucose uptake by adipocytes was affected by 1-100 nM aldosterone, i.e., a dose range encompassing aldosterone plasma levels in patients with APA, but glucose uptake was significantly lower after pre-treatment with 10 μM aldosterone (pharmacological concentration). In basal conditions, probably due to great inter-experimental variability, i.e., large SE of the mean, the inhibitory effect of aldosterone 10 μM on glucose uptake was not significantly prevented by either RU486 or eplerenone. On the contrary, the impairment of insulin-stimulated glucose uptake caused by a pharmacological dose of aldosterone, as that induced by hydrocortisone, was prevented by the GR antagonist

RU486 but not by the MR antagonist eplerenone. This lends support to the concept that very high aldosterone levels attenuate insulin signaling via GR. Our findings are in accordance with of Hellal-Levy et al. (110), who showed in COS-7 cells transfected with human MR and GR that only 10 μM aldosterone concentration (pharmacological) was able to induce transcription via GR or MR, whereas lower concentrations induced a selective MR activation. The absence of significant HSD11B2 expression in visceral adipose tissue of both our patient groups, reflecting the inability of this enzyme to inactivate cortisol, seems to further indicate glucocorticoids as the predominant endogenous ligands of MR, as in other non-epithelial tissues (111). Mechanisms of impaired glucose handling in human adipocytes caused by very high aldosterone concentrations are possibly similar to those activated by glucocorticoids (112). In this regard, it should be noted that in our experimental model of human adipocytes, insulin-stimulated Akt and MAPK ERK1/2 phosphorylation were unaffected by pharmacological doses of aldosterone, indicating that insulin signaling proximal to Akt was unaltered. Inhibition of GLUT1 function and/or of GLUT4 translocation to the adipocyte plasma membrane by high dose aldosterone, as suggested for glucocorticoids (113, 114), might rather be considered as an alternative mechanism. Our results suggest indeed that aldosterone is able to inhibit the insulin-mediated effect on glucose uptake in adipocytes only at pharmacological concentrations, and likely through GR activation.

Some potential limitations of these findings need to be discussed. First, our in vitro experimental conditions may not have reproduced some changes of the in vivo microenvironment found in APA patients, i.e., hypokalemia, possibly contributing to alter insulin sensitivity of adipose cells (115).

Second, mineralocorticoids can also exert acute actions in non-epithelial tissues independently of gene transcription (116). These rapid (<15 min)/non-genomic actions seem to be particularly relevant in cardiovascular disease, as they may result in cardiac hypertrophy and

endothelial dysfunction (117, 118). Although the lack of aldosterone effect on insulin-related metabolic function and genes in our long-term experiments suggests the absence of an aldosterone-related genomic action, this issue was not specifically addressed in our study model.

Third, due to technical difficulty in obtaining a sufficient amount of tissue, we did not use human visceral adipose cells, either from NFA or APA subjects, to test aldosterone effect on glucose uptake and insulin signaling. Although we cannot exclude different results using visceral adipocytes, our cells which were obtained from the most inner depot of subcutaneous abdominal fat, have been already shown to have a strong correlation to insulin resistance (119) and an adipokines pattern expression (120) similar to that of visceral fat.

Fourth, aldosterone has been reported to affect energy expenditure, by regulating expression and function of uncoupling proteins in murine brown adipocytes (112, 121). This observation could be relevant considering the potential role of brown adipose tissue in adult human metabolism (122). Although limited, there are observations concerning a potential direct effect of aldosterone on other conventionally insulin-sensitive tissues accounting for whole-body glucose disposal, i.e., vasculature, liver and skeletal muscle. In vascular smooth muscle cells, insulin resistance related to aldosterone has been shown to result in c-Src and ROS-mediated increase in proteasomal degradation of IRS-1 (123). An aldosterone-modulated insulin effects has been shown in primary cultured mouse hepatocytes and human hepatoma HepG2 cell line, a cell model proposed for testing insulin sensitivity on the liver (124, 125). Lastra et al. (126) showed that at a non-blood pressure lowering dose, MR blockade improved insulin sensitivity in the skeletal muscle of a rodent model of renin-angiotensin-aldosterone activation and insulin resistance. Recently, it has been observed that inducers of heme-oxygenase system, decreasing oxidative stress, led to an improvement of insulin sensitivity in the gastrocnemius muscle of rats with deoxycorticosterone-acetate

hypertension, an animal model of primary aldosteronism (127). Thus, it is tempting to speculate that aldosterone could impair whole-body insulin sensitivity by acting on tissues different from fat. Restoration of normal insulin sensitivity reported in vivo in primary aldosteronism after MR antagonists or surgery (53, 54) may simply reflect the recovery of insulin action on these peripheral tissues. Recent studies also suggest that intrahepatic fat is better correlated with the metabolic consequences of obesity than visceral fat and might be a more important contributor to insulin sensitivity (128). Also, we recently found a high prevalence of nonalcoholic fatty liver disease in primary aldosteronism (129). In conclusion, no alteration of gene expression of insulin signaling or inflammatory molecules was present in VAT of patients with primary aldosteronism due to APA. Only at pharmacological concentrations, and likely through GR activation, aldosterone reduced glucose uptake in human adipocytes.

Systemic insulin resistance in primary aldosteronism might occur in compartments other than fat, i.e., liver and/or skeletal muscle, and/or depend on concurrent environmental factors (Figure 15).

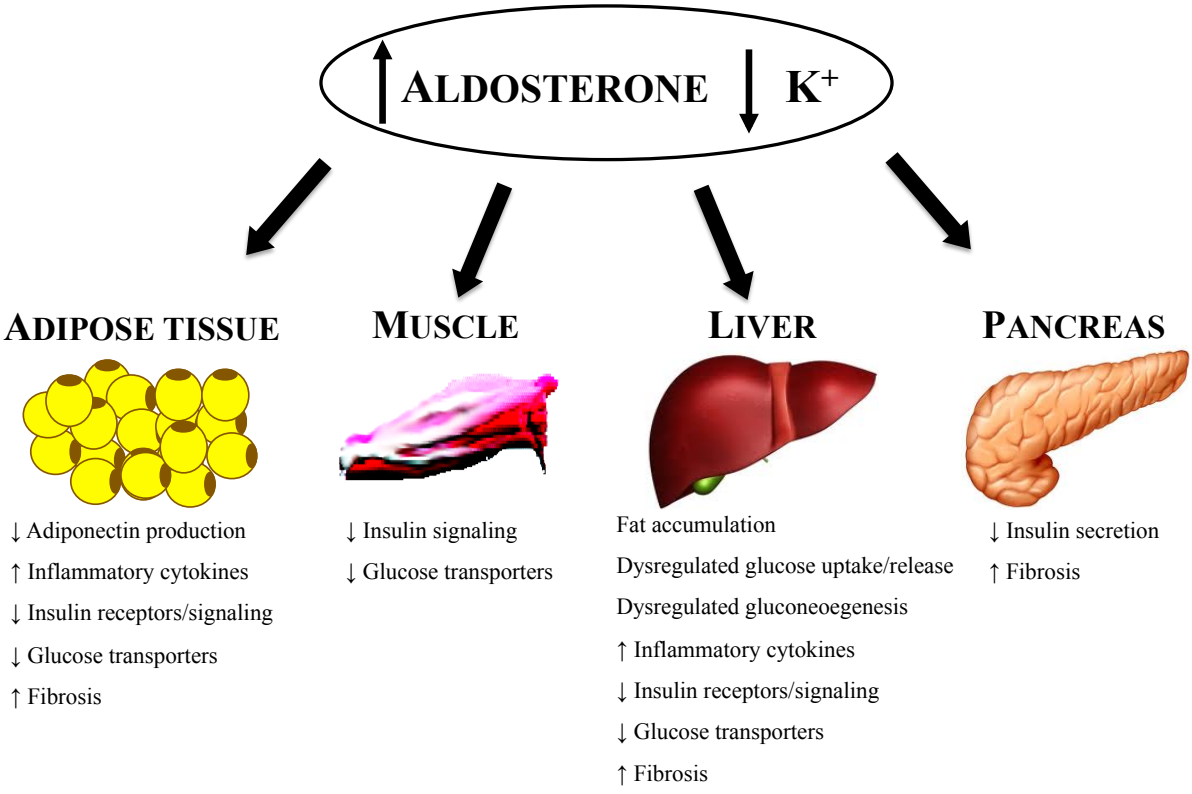

Figure 15. Schematic representation of mechanisms involving aldosterone excess and/or concurrent hypokalemia, which may lead to impairment of insulin action as part of the metabolic syndrome.
Aldosterone may induce insulin resistance acting on insulin-target tissues through alteration of insulin receptors and insulin signaling, adipokine production, increased fibrosis, fat accumulation or glucose uptake/release and gluconeogenesis dysregulation. A reduced insulin secretion by pancreatic β-cells might also occur.

STUDY IN PARIS

PHENOTYPIC AND METABOLIC CHARACTERIZATION OF A NEW DOUBLE TRANSGENIC MOUSE MODEL, WITH CONDITIONAL OVEREXPRESSION OF HUMAN MINERALOCORTICOID RECEPTOR IN ADIPOCYTES

INTRODUCTION

The laboratory of Dr. F. Jaisser, INSERM U872, Cordeliers Research Center in Paris, has been involved for more than fifteen years in the analysis of the pathophysiological roles of MR activation in cardiovascular and renal diseases (17, 130-145). The lab is interested in delineating the role of the MR activation in metabolic disorders and the related consequences in cardiovascular diseases, paying particular attention to the identification of possible early biomarkers of MR dysregulation. As has been previously shown in this dissertation, there is a great amount of published data describing the consequences of aldo-MR system activation/dysregulation, however, still no biomarkers are available for accounting to the whole-body MR activation status. Such biomarkers could be of great therapeutic interest in helping the identification of patients prone to MR antagonism. In fact until today, the only available biomarker still remains the aldosterone circulating level, which is not of great use, since MR could be activated either by glucocorticoids or ligand-independent mechanisms (57 , 58).

In the last few years, the team of Dr. Jaisser, has identified some new molecular targets of MR in the heart (146) and vessels (unpublished data) and one of them, the lipocalin-

2 protein LCN2, also known as NGAL (for neutrophil gelatinase-associated lipocalin), appeared to be particularly interesting. NGAL is a small soluble protein of 25-kDa encoded in humans by LCN2 gene located in chromosome nine and it belongs to a family of small proteins, lipocalins, engaged in the transmembrane transportation of lipophilic substances. Originally isolated from specific granules of neutrophils (147), NGAL was later found expressed in bone marrow cells, in lung bronchial and colon epithelial cells (148, 149) as well as in adipocytes (150). Little current data is available describing the mechanisms of action of NGAL. Of the available data, some evidences report its ability to tightly bind the bacterial ferric siderophores resulting in a potent bacteriostatic action (151), and other studies reveal its property to stabilize the active form of the matrix metallo-peptidase 9 (MMP9) (152) a protein member of the family of the matrix metallo-proteinases (MMP), thus postulating a role of NGAL in MMP9 related activities, i.e., activation of innate defense through proteolytic shedding of the lipopolysaccharide (LPS) CD14 receptor in macrophages, and breakdown of extracellular matrix in tissues remodeling during bone healing after fracture or in neoangiogenesis following tumor metastasis processes (153).

Although its biological meaning still remains obscure, many studies report a significant correlation between an early increase of NGAL plasma level and the establishment of many different pathological situations, from bacterial inflammations to cancer (154, 155), from cardiac and renal failure to atherosclerosis and metabolic syndrome (156 , 157 , 158).

Most interestingly, NGAL has been recently proposed as a novel adipokine produced by adipocytes (159 , 160) and secreted within the blood circle where it is found increased in animal models for obesity as well as in obese patients (159 , 161). Indirect evidences, coming from a NGAL knock out mouse model, show that the inactivation of NGAL improves insulin resistance and glucose tolerance in mouse fed with a high fat diet (162). Concerning the relationship between aldo-MR system and NGAL, Jaisser et al. have shown that NGAL

expression is significant increased in heart and vessels of MR overexpressing mice as well as in the most common model of mouse and rat hyperaldosteronism, i.e., aldo-salt treated animals. Most interestingly, they also showed in all these models, as well as in obese db/db mouse, that plasma levels of NGAL result significant decrease upon in vivo MR antagonism (unpublished data, Figure 16 and 17).

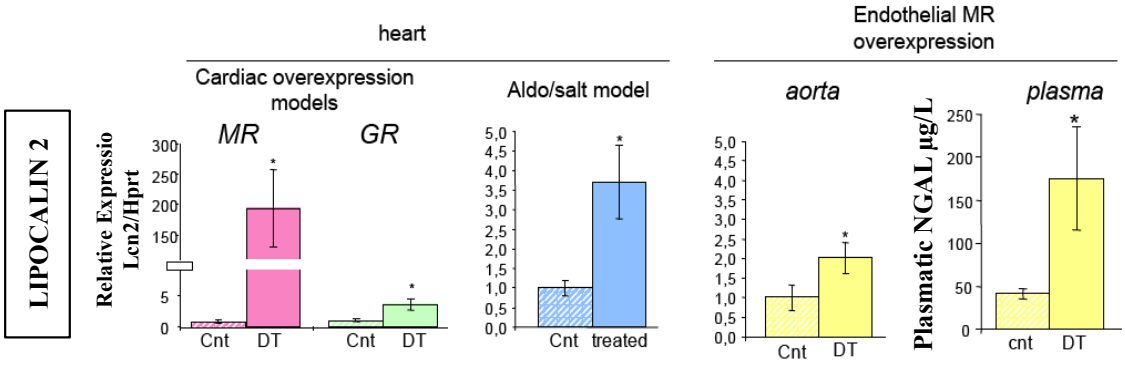

Figure 16. NGAL gene (Lcn2) expression is up-regulated within heart of DT mouse with a conditional overexpression of MR in cardiomyocytes (x200), as well as in heart of aldo-salt treated mice (x3.5) and in aorta of DT mice overexpressing MR in the endothelium (x2). Plasmatic level of NGAL is also increased in the latter model.

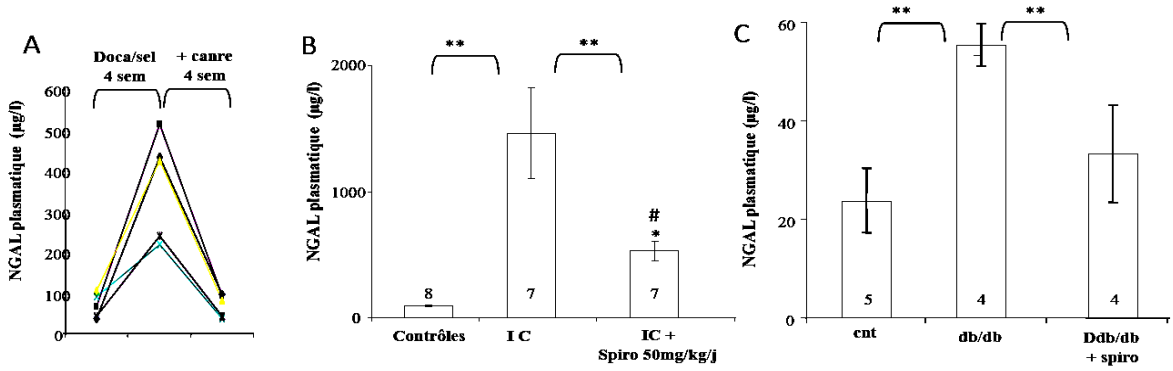

Figure 17. Pharmacological antagonism of MR is able to reverse the rise of plasmatic level of NGAL in (A) DOCA-salt mouse model, (B) in rat with cardiac infarction (CI) and (C) in the db/db obese mice.

All these data imply a strong relationship between MR activation and NGAL increase, postulating a direct role of aldo/MR system on regulation of NGAL expression and secretion. When I arrived in Paris in January 2011, the lab had just started a new project working with a new double transgenic mouse model (DT), conditionally overexpressing MR in adipocytes

(adipo-hMR mouse). The intent was three fold: 1) to describe the metabolic phenotype of the mouse overexpressing MR in the adipose tissue; 2) to evaluate the possible consequences on the cardiovascular function of the hyperactivation of MR in adipose tissue; 3) to assess the modulation of NGAL expression and secretion by adipocytes overexpressing MR.

My personal objective during the last part of my or internship in France was essentially focused on the first step, which was to characterize phenotypically the new adipo-hMR DT mouse model.

Primarily we have evaluated three aspects: 1) The reliability of the parental lines necessary for obtaining the DT mouse model, i.e., the adipo-mouse and hMR-mouse lines; 2) The expression profile in the omental fat of the adipo-hMR mouse compared to the wild type (wt) mouse; 3) The reliability of the transgenic system adipo-hMR in vitro through an analysis of the expression profile in the primary culture of adipocytes derived from the adipose tissues of adipo-hMR mouse.

MATERIALS AND METHODS

Adipose tissue lacZ or MR conditional mouse models

The previously characterized tetO-hMR (hMR-mouse) and tetO-lacZ (lacZ-mouse) mouse strains (163, 164) were crossed with the aP2-rtTA (adipo-mouse) transactivator mouse strain in order to obtain the aP2-rtTA/tetO-hMR (adipo-hMR mouse) or aP2-rtTA/tetO-LacZ (adipo-lacZ) double transgenic (DT) mouse strains with conditional adipocytes-specific hMR or lacZ overexpression (Figure 18). In order to induce the transgene expression in DT mice, the animals were treated for at least for three weeks with doxycycline (Dox) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in drinking water at the concentration of 2g/L.

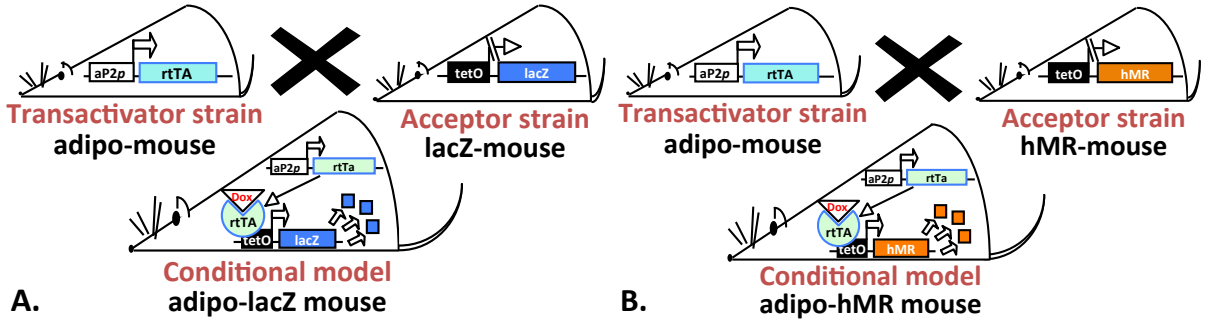

Figure 18. **Schematic representation of conditional adipose tissue-specific mouse models: adipo-lacZ (A) e adipo-hMR (B).**
The two models derived from a crossbreed of two murine transgenic strains, one that is defined as "acceptor strain" and it is specific for each DT model and another one named "transactivation strain", that is common to both. Acceptor strain carries in its DNA a genetic construct made of two genetic sequences fused together, "tetO", that is a bacterial operator (like a human promoter) sequence, and lacZ (A) or hMR (B), which are the sequences coding respectively for β-galactosidase enzyme and the human mineralocorticoid receptor protein. On the other hand, the transactivation strain was genetically engineered for carrying the promoter sequence of "aP2" gene, a gene specifically expressed in adipose tissue, fuses together with rtTA gene, coding for a bacterial transcription factor able to activate tetO. In other words, aP2 promoter is, for definition, only active in adipose tissue and therefore allows the adipocytes specific expression of its linked transgene rtTA. In turn, in the presence of Dox, rtTA binds to and activates the operator tetO, causing the tetO-linked transgene (lacZ or hMR) to be expressed constitutively. Therefore treating, or not treating, the mice with Dox allows when to choose to activate, or inactivate, the transgene expression.

At the end of Dox treatment, mice were sacrificed in order to recover organs and tissues used for the subsequent manipulations of cell culture, histology or bio-molecular analysis. In particular, attention was focused on four different type of adipose tissue: inguinal subcutaneous adipose tissue (SAT), epididymal visceral adipose tissue (EVAT), retroperitoneal visceral adipose tissue (PVAT) and brown adipose tissue (BAT). The use of animals was in accordance with the guidelines of the European Community and approved by our Institutional Animal Care and Use Committee.

Histological analysis of the DT adipo-lacZ mouse model

After three weeks of Dox (2g/L) transgene induction, 5 DT adipo-lacZ and 5 wt littermate control mice were used for histological analysis. Following the sacrifice, adipose tissue (SAT,

BAT, EVAT and PVAT) biopsies were incubated for ten hours in an X-Gal (Sigma-Aldrich, St. Louis, MO, USA) staining solution. Adipo-lacZ animals are characterized by the expression in adipocytes of the β-galactosidase enzyme, an enzyme able to convert the substrate X-Gal into an intensely blue product which is insoluble and stains the tissue. This allows for the easy identification of the tissue where the β-gal is active. Stained tissues were then fixed in 4% paraformaldehyde overnight and finally paraffin-embedded. For taking pictures, a series of 6μm sections were cut from the paraffinized blocks of every adipose biopsy.

Mouse adipocytes culture and *in vitro* induction of hMR transgene

For each experiment, SAT or EVAT biopsies were obtained from 2 to 3 Dox-untreated DT-hMR mice in order to pool them together and allow a sufficient amount of starting material for culture. The isolation of murine preadipocytes from adipose pads was then accomplished as previously described in this dissertation for human adipocytes.

At the end of differentiation, adipocytes were left for 72 hours in DMEM/F12 at 5% FBS w/o steroids (Charcoal Stripped FBS Life Technologies Corporation, Carlsbad, CA, USA) stimulated with vehicle, as negative control, or with Dox (1μg/mL) for inducing the expression of hMR transgene. Adipocytes were then also treated with aldosterone 10nM (Sigma-Aldrich, St. Louis, MO, USA), for 24 hours before lysis and RNA extraction.

RNA extraction and quantitative RT- PCR

According to respective manufacturer protocol, total RNA from mouse cultured adipocytes, 5 wells per condition (vehicle or Dox 1μg/mL), were extracted using TRIZOL® reagent (Life Technologies Corporation, Carlsbad, CA, USA), while total RNA from frozen adipose tissues (SAT, BAT, EVAT and PVAT), 5 DT adipo-hMR mice and 5 wt control littermate mice, was obtained with the RNeasy Mini Kit (Qiagen GmBh, Hilden, Germany). The extraction was optimized by a farther step of RNA DNase treatment (Qiagen GmBh, Hilden, Germany). Reverse transcription of RNA to cDNA was performed with Superscript II reverse transcriptase KIT (Life Technologies Corporation, Carlsbad, CA, USA) using 500 ng, for cells, or 2 μg, for tissues, of the total RNA. Transcripts levels of considered genes were analyzed by real time (SYBR Green detecting) PCR in an iCycler iQ apparatus (Biorad Laboratories Inc., Hercules, CA, USA). Reactions were performed in duplicate for each sample in a total volume of 12μl: 5μl of cDNA (1ng/μl) 1μl of forward/reverse primer mix (3.6μmol/L) and 6μl of SYBR GREEN SuperMix (Biorad Laboratories Inc., Hercules, CA, USA). The thermal cycling parameters were: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Relative expression of the mRNA was quantified using the equation described by M.W. Pfaffl (165): ratio=(E_{target})^Ct_{target}(mean control-sample)/(E_{ref})^Ct_{ref}(mean control-sample). For each sample, mRNA levels were normalized by dividing the amount of the candidate gene by the geometric mean of the amount of a series of housekeeping genes, 18S, β-2-microglobulina (β2μ), ubiquitin C (UBC) and hypoxanthine guanine phosphoribosyl transferase (HPRT). The genes were analyzed and their specific primer sequences are listed in Table 5.

Table 2: Sequence of the primers and reaction conditions used for quantitative RT-PCR in mouse

Chart Label	Gene official symbol and full name	Oligonucleotide primers	$[C]_f$ nM	Annealing temperature	Amplicon
18S	Rn18S, 18S ribosomal RNA	Forward: 5'- CG CCG CTA GAG GTG AAA TTC -3' Reverse: 5'- T CTT GGC AAA TGC TTT CGC -3'	300 300	60°	63 bp
β2μ	B2m, beta-2 microglobulin	Forward: 5'- TC TAT ATC CTG GCT CAC ACT GAA -3' Reverse: 5'- ACA TGT CTC GAT CCC AGT AGA -3'	300 300	60°	113 bp
HPRT	Hprt, hypoxanthine guanine phosphoribosyl transferase	Forward: 5'- T CTA ACT TTA ACT GGA AAG AAT GTC -3' Reverse: 5'- T CCT TTT CAC CAG CAA GCT -3'	300 300	60°	133 bp
UBC	Ubc, ubiquitin C	Forward: 5'- AGG TCA AAC AGG AAG ACA GAC GTA -3' Reverse: 5'- TCA CAC CCA AGA ACA AGC ACA -3'	300 300	60°	80 bp
hMR	NR3C2, nuclear receptor subfamily 3, group C, member 2, also known as mineralocorticoid receptor	Forward: 5'- G AGG CTT CAG GATG CCA TTA -3' Reverse: 5'- TCT GCA AGC AGG ACA ATT CTT -3'	300 300	60°	153 bp
mMR	Nr3c2, nuclear receptor subfamily 3, group C, member 2, also known as mineralocorticoid receptor	Forward: 5'- CC AGA AGA GGG GAC CAC ATA -3' Reverse: 5'- GG AAT TGT CGT AGC CTG CAT -3'	300 300	60°	162 bp
Total MR*	NR3C2+Nr3c2	Forward: 5'- GG CTA CCA CAG TCT CCC TGA -3' Reverse: 5'- CGT TGA CAA TCT CCA TGT -3'	300 300	60°	129 bp
NGAL	Lcn2, lipocalin 2, also known as NGAL	Forward: 5'- GG ACC AGG GCT GTC GCT ACT -3' Reverse: 5'- GG TGG CCA CTT GCA CAT TGT -3'	300 300	60°	120 bp
GRα	Nr3c1, nuclear receptor subfamily 3, group C, member 1, also known as glucocorticoid receptor	Forward: 5'- GG CAA AGG CGA TAC CAG GAT -3' Reverse: 5'- TGT ATG ATC TCC AAC CCA GGG -3'	300 300	60°	110 bp
PPARγ	Pparg, peroxisome proliferator-activated receptor gamma	Forward: 5'- TT TTC AAG GGT GCC AGT TTC -3' Reverse: 5'- AA TCC TTG GCC CTC TGA GAT -3'	300 300	60°	198 bp
Adiponectin	Adipoq, adiponectin, C1Q and collagen domain containing	Forward: 5'- T GTT GGA ATG ACA GGA GCT GAA -3' Reverse: 5'- TGA ACG CTG AGC GAT ACA CAT -3'	300 300	60°	100 bp
PAI1	Serpine1, serine (or cysteine) peptidase inhibitor, clade E, member 1	Forward: 5'- A GCC AAC CAC AGC TGA GCG -3' Reverse: 5'- GGG CTG AAG ACA TCT GCA TCC -3'	300 300	60°	81 bp
MCP-1	Ccl2, chemokine (C-C motif) ligand 2, also known as monocyte chemotactic protein-1	Forward: 5'- CA GCA CCA GCA CCA GCC AAC -3' Reverse: 5'- GC CGG CAA CTG TGA ACA GCA -3'	300 300	60°	103 bp
TNFα	Tnf, tumor necrosis factor	Forward: 5'- GG GAC AGT GAC CTG GAC TGT -3' Reverse: 5'- AGT GAA TTC GGA AAG CCC ATT -3'	300 300	60°	52 bp
CD68	Cd68, CD68 antigen	Forward: 5'- AC AAG GGA CAC TTC GGG CCA -3' Reverse: 5'- GT CGT CTG CGG GTG ATG CAG -3'	300 300	60°	132 bp
VNN3	Vnn1, vanin 1	Forward: 5'- C CTT TGA TGG GCT GCA CAC CGT -3' Reverse: 5'- CG GGT TCC AAA AGT GCC GCT -3'	300 300	60°	157 bp
VNN3	Vnn3, Vanin 3	Forward: 5'- G TGG GGA ACC CGT GGG GTC AGC -3' Reverse: 5'- AG GCA AAG GGG CTC CAC CTC GAC -3'	300 300	60°	179 bo
PTGDS	Ptgds, prostaglandin D2 synthase	Forward: 5'- TA CAG CGC GGG CCT CGC CTC -3' Reverse: 5'- GC CCC CGC AGG CTG CAG TAC -3'	300 300	60°	173 bp
CYP4A10	Cyp4a10, cytochrome P450, family 4, subfamily a, polypeptide 10	Forward: 5- GA TGG TTC TGG GGA AGC AAG GCC -3' Reverse: 5'- AA GGC TGG GGT TAG CAT CCT CCT -3'	300 300	60°	167 bp
IL1β	Il1b, interleukin 1 beta	Forward: 5- GC CTC GTG CTG TCG GAC CCA TAT -3' Reverse: 5'- T CCT TTG AGG CCC AAG GCC ACA -3'	300 300	60°	143 bp
UNC5	unc5a, unc-5 homolog A (C. elegans)	Forward: 5'- TC GTT CTC TGC CCC GCG GTA -3' Reverse: 5'- GG GAT GGC GTC CGG GGG TAT -3'	300 300	60°	117 bp

* Total MR, designs a primers pairs able to amplify a common sequence between hMR and mMR, therefore accounting for the total level of MR gene mRNA

Microarray analysis

After three weeks of Dox (2g/L) transgene induction, 5 DT adipo-hMR and 5 wt control littermate mice were used for microarray experiments. Total RNA was prepared from PVAT samples as previously described and its quality was assessed with Bioanalyzer 2100 (Agilent Technologies, Inc., Wilmington, DE, USA). Microarray experiments were carried out using mouse GE 4x44K V2 microarray kit (Agilent Technologies Inc., Wilmington, DE, USA). Labeling of the RNAs, hybridization (1 animal/array) and scanning of the microarrays were performed according to manufacturer's instructions. Data were analyzed using Genespring software (Agilent Technologies Inc, Wilmington, DE, USA). The false discovery rate and fold change threshold retained were 5% and >1.5 respectively.

Gene ontology analysis was performed by using the funnet free on-line tool developed by France INSERM in collaboration with the Cordeliers Research Centre in Paris (http://www.funnet.info/). Specifically, we based our analysis on KEGG collection (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/) in order to identify the possibly interesting enzymatic pathways perturbed by MR overexpression.

RESULTS

Histological and qRT-PCR validation of adipose-specific transgene expression

X-Gal exposure of adipose tissues, SAT, BAT, EVAT and PVAT, from mice adipo-lacZ Dox-treated, adipo-lacZ non-Dox treated and wt-control, results evident in blue adipose tissues staining only in DT Dox-treated Mice (Figure 19).

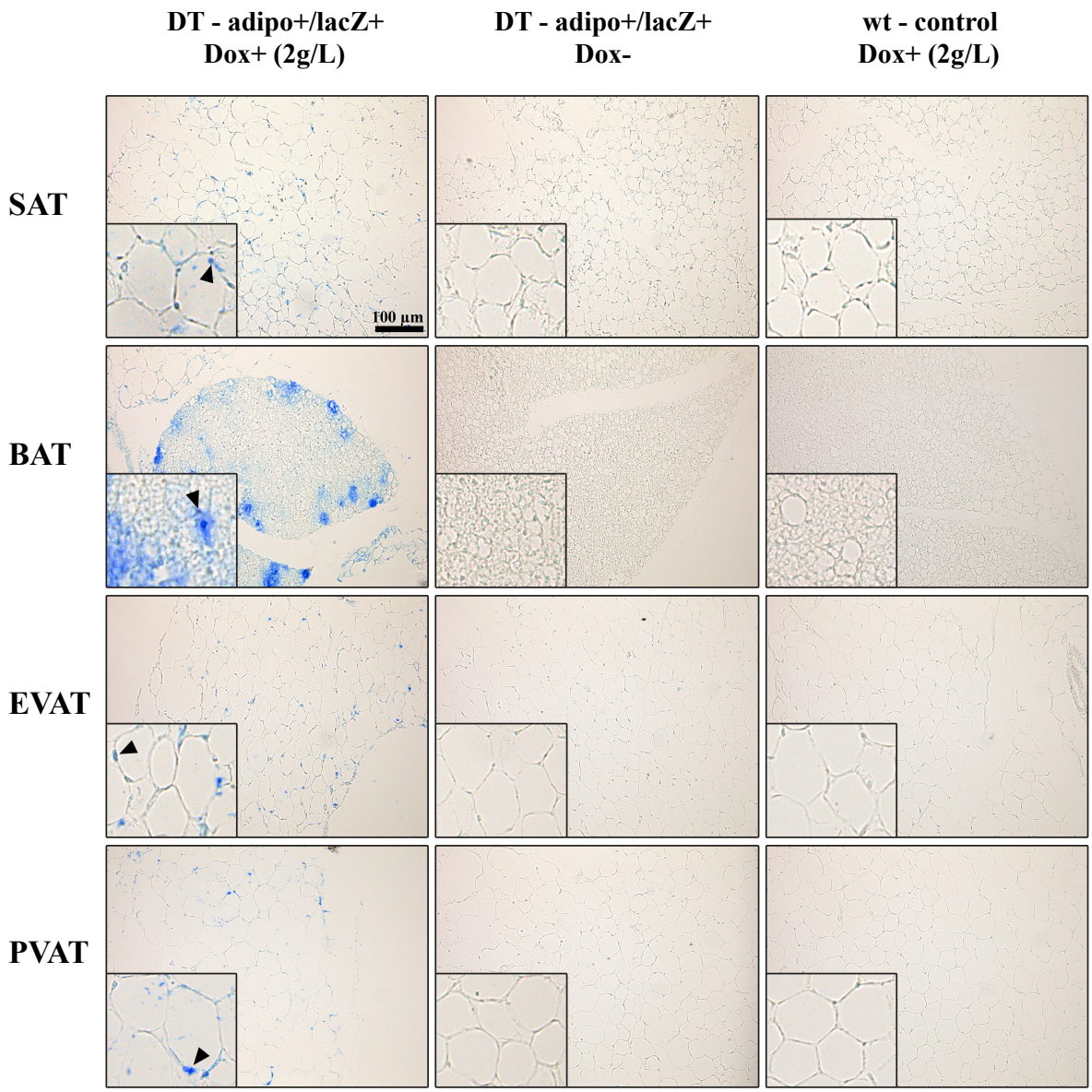

Figure 19. Representative images obtained with X-Gal staining of adipose tissues, i.e., SAT, BAT, EVAT and PVAT, obtained from double transgenic mice adipo-lacZ treated or not with doxycycline (Dox +/-), for 3 weeks at the concentration of 2g/L (n=3) and from wild type control litter mate mice (n=3). A blue staining of the nucleolus (black arrow) is observable in every adipose tissue of only Dox-treated DT mouse.

QRT-PCR analysis for hMR gene expression in adipo-hMR and wt-control littermate mice, confirms the expression of hMR transgene only in adipose tissues of DT Dox-treated mice. Additionally, given the fact that expression of the endogen mouse MR results are similar between the two group (Figure 20), the significant ($p<0.05$) increase in total MR level observed in SAT, PVAT and BAT of DT mice is necessarily due to hMR transgene overexpression.

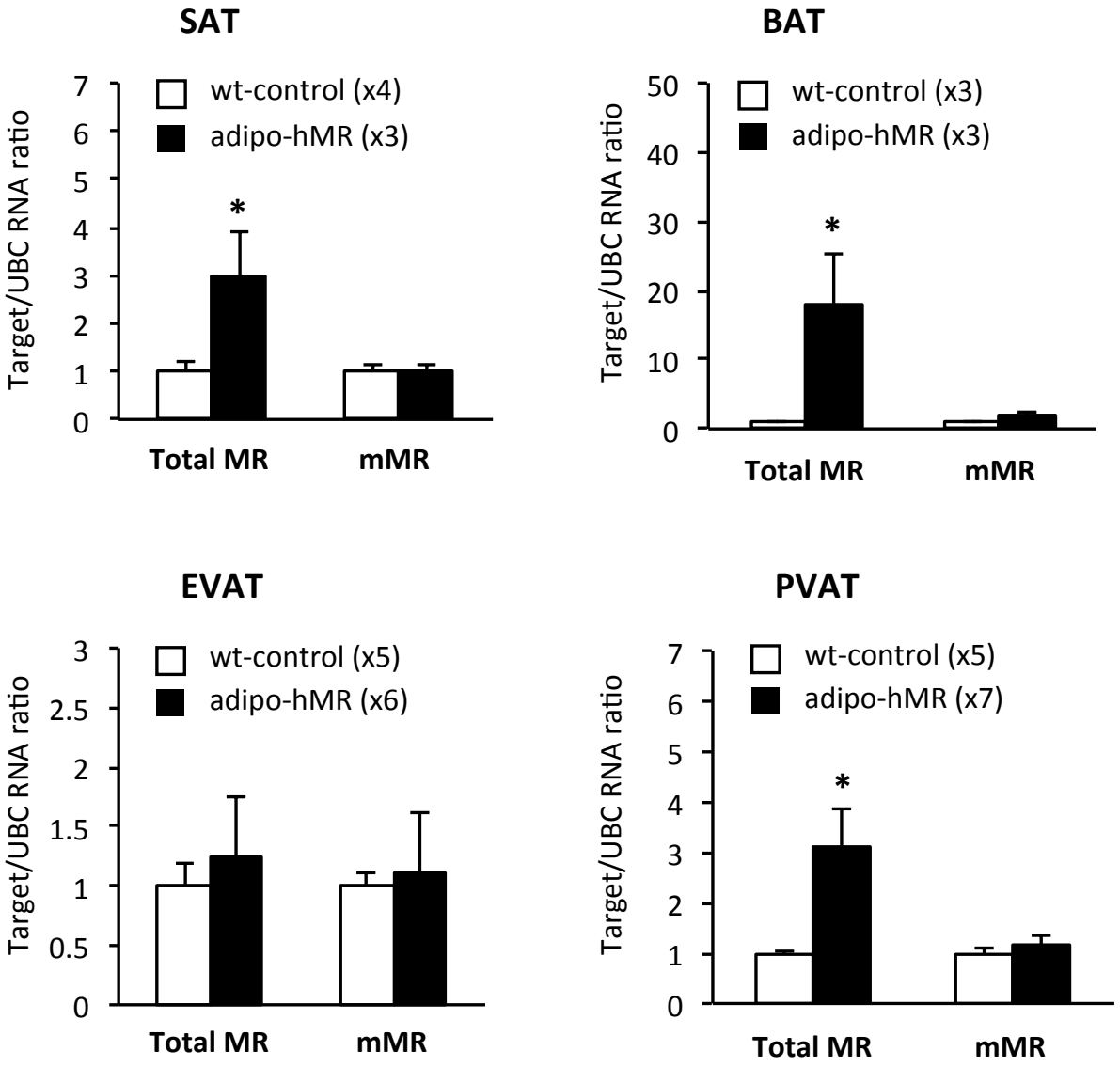

Figure 20. Quantification of mRNA expression by qRT-PCR of mMR and total MR in subcutaneous, brown, epididymal and retroperitoneal adipose tissue biopsies from wt-control mice (white bar) and DT adipo-hMR mice (black bar). Each bar represents the means ± SE of 3 to 7 samples for each group, *, means a significant difference between the two groups ($p<0.05$).

Transcriptome analysis in PVAT adipo-hMR DT mice

The expression profile in PVAT of DT and wt-control littermate mice was analyzed by a preliminary qRT-PCR assay, followed by an extensive total mRNA transcripts microarray analysis.

QRT-PCR reveals how transcript levels of mMR, GRα, PPARγ, adiponectin, Pai1, MCP1, TNFα e CD68 are similarly expressed in the two groups, whereas total MR and

NGAL are significantly ($p<0.05$) higher, 2.5 and 8 folds respectively, in DT than in control mice. (Figure 21).

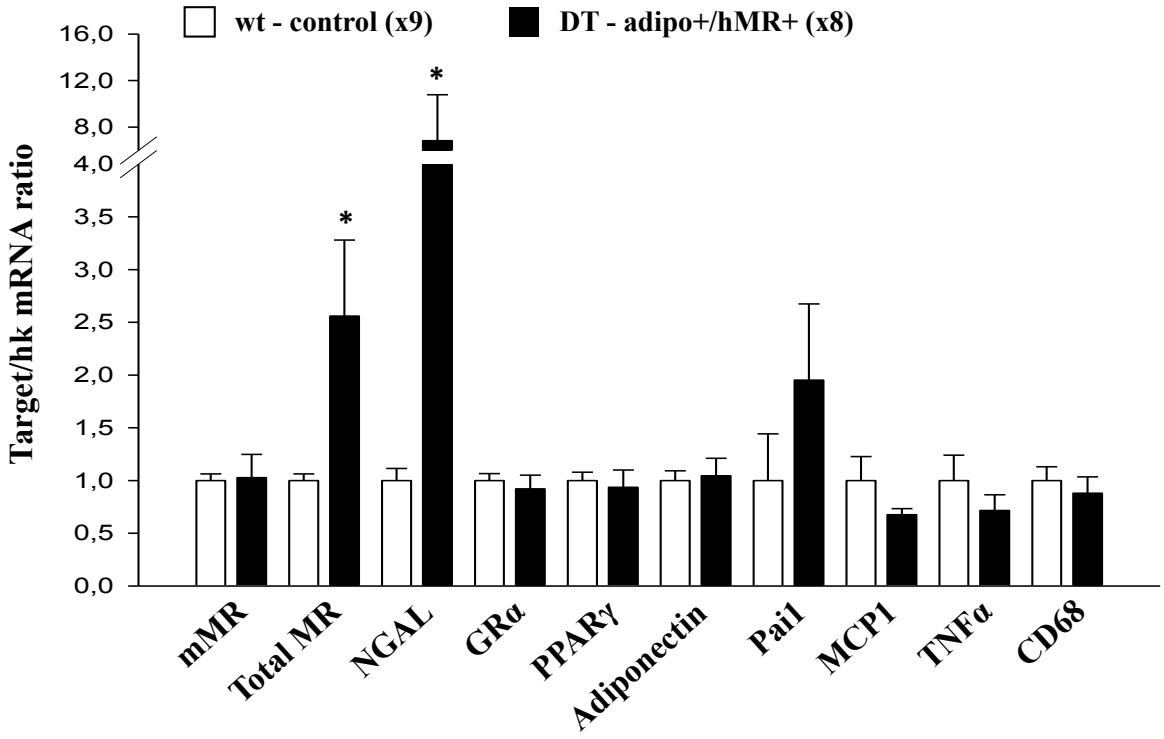

Figure 21. Quantification of mRNA expression by qRT-PCR of mMR, total MR, GRα, PPARγ, adiponectin, Pai1, MCP-1, TNFα e CD68 in retroperitoneal visceral adipose tissue biopsies from wt mice (white bar) and DT adipo-hMR mice (black bar). Each bar represents the means ± SE of 9 samples for each group, *, means a significant ($p<0.05$) different between the two groups.

Microarray analysis comparing total mRNA transcripts levels in DT and control mice shows farther differences between the two groups, particularly that there are 101 up-regulated and 246 down-regulated genes (fold change >1.5, $p<0.05$) in PVAT of DT-MR mice compared to their control littermates (Figure 22).

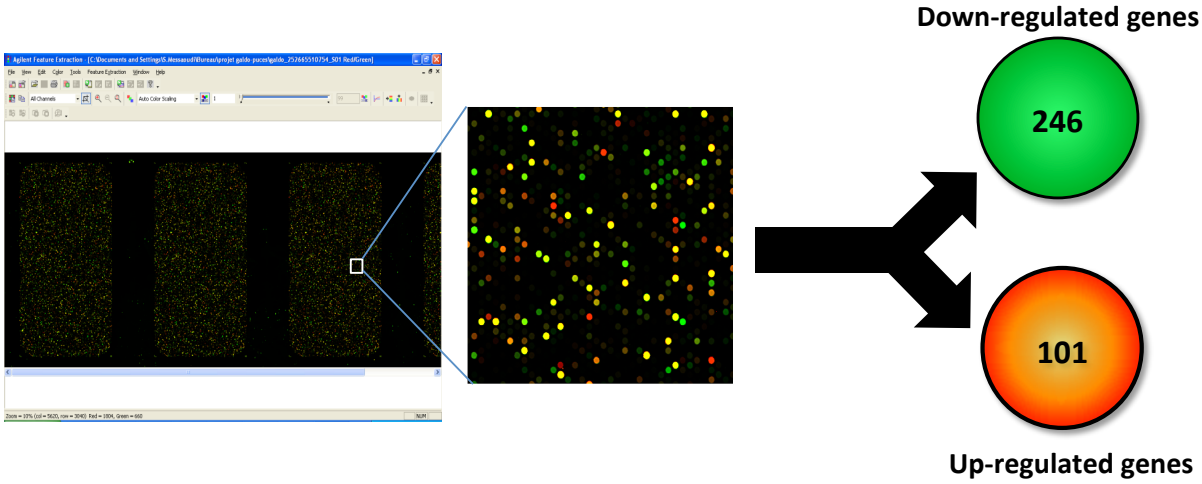

Figure 22. Representative image of a probed GE-4x44K-V2 chip from the microarray analysis performed on total mRNA transcript from DT adipo-hMR and relative wt-control littermate mice (n=5 for each group). In the red and green spheres are shown the number of the genes resulted significantly up- and down-regulated in PVAT of DT mice after Dox induction of hMR.

The genonthology analysis performed with the funnet algorithm, reveals that these genes correspond mainly to proteins involved in metabolic pathways of fatty acid synthesis (up-regulation) and immune response, e.g. cytokines receptor (down-regulation) (Figure 23).

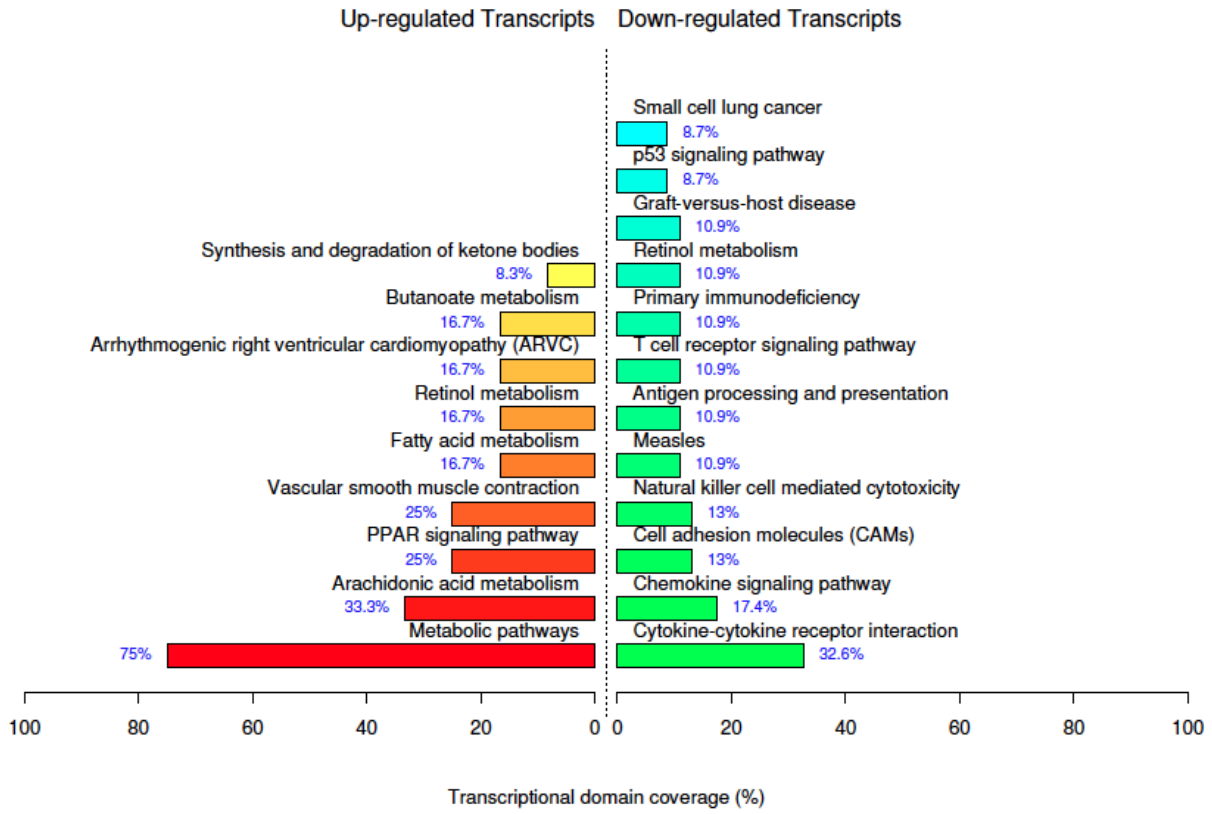

Figure 23. Schematic representation of funnet analysis based on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway collection. Bars represent the percentage of total up-(red)/down- green) regulated genes, involved in every category found significant by the funnet algorithm.

In order to validate the RNA chip, we selected some of MR-specific up-/down-regulated genes to be measured by real time PCR in other series of DT/control mice. Specifically we took into account the genes resulted in more modulated by MR overexpression, i.e., vanin 1 (VNN1), vanin 3 (VNN3), prostaglandin D2 synthase (PTGDS) and cytochrome P450, family 4, subfamily a, polypeptide 10 (CYP4A10), between the up-regulated genes, and interleukin 1 beta (IL1β) and C. elegans unc-5 homolog A C. elegans

(UNC5) between the down-regulated ones. The analysis by real time PCR confirms the same trend observed in the microarray analysis (Figure 24).

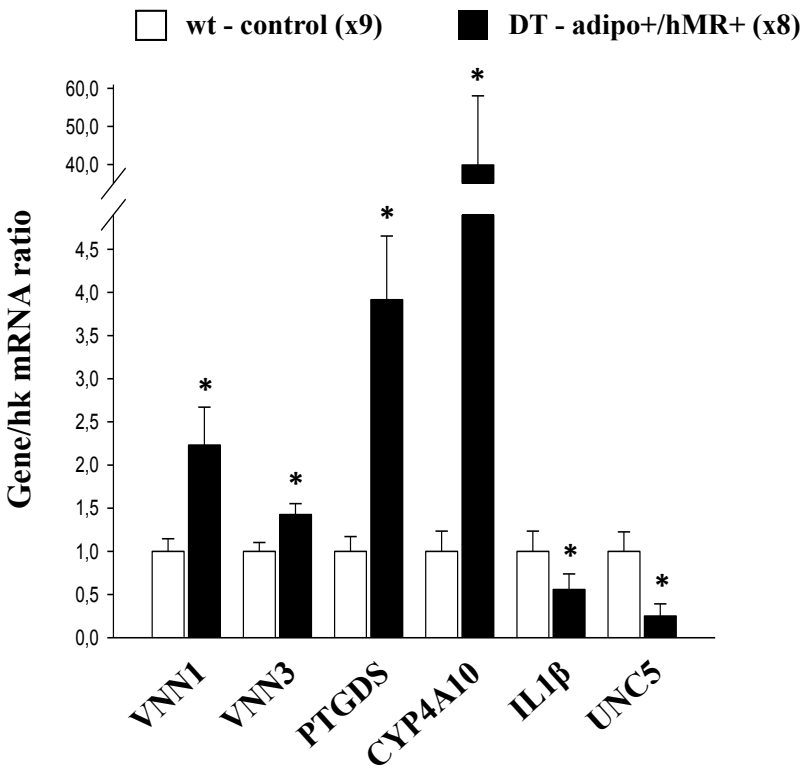

Figure 24. Validation by qRT-PCR of some of the differentially-expressed genes, vanin 1 (Vnn1), vanin 3 (Vnn3), prostaglandin d2 synthase (ptgds), cytochrome P450, family 4, subfamily a, polypeptide 10 (CYP4a10), interleukin 1 beta (Il1b) and unc-5 homolog A, C. elegans (UNC5), identified in microarray analysis in the PVAT biopsies from wt mice (white bar) and DT adipo-hMR mice (black bar). Each bar represents the means ± SE of 9 samples for each group, *, means a significant ($p<0.05$) different between the two groups.

In vitro adipo-hMR model evaluation.

Overexpression of hMR was assessed on *in vitro* fully differentiated mouse subcutaneous and epididymal primary cultured adipocytes after 72 hours pre-induction with Dox (1 µg/ml) and 24h treatment with Aldo (10nM). DT adipocytes not only express hMR gene transcript but

also present a significant increased level of total MR and NGAL mRNA ($p<0.05$) when compared to wt control adipocytes (Figure 25).

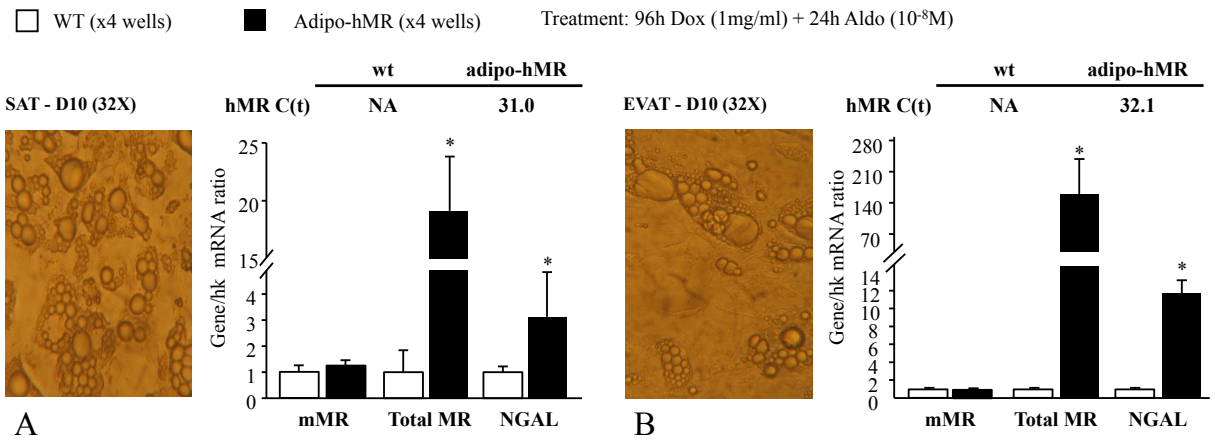

Figure 25. Cultured murine SAT (A) and EVAT (B) DT adipo-hMR adipocytes.
Quantification of mRNA expression by qRT-PCR, after 96h of Dox transgene induction and 24h Aldo treatment, of hMR, mMR, total MR and NGAL, in SAT and EVAT adipocytes derived from wt (white bar) and DT adipo-hMR (black bar) mice. Each bar represents the means ± SE of 4 sample for each group.

DISCUSSION

During my third year of PhD study in Paris, I demonstrated that the double transgenic offspring obtained by crossbreeding the murine line adipo-mouse with the line lacZ-mouse or hMR-mouse, was found to specifically express the transgene, respectively lacZ or hMR, in adipose tissue when subjected to Dox treatment (2g/L). In particlular, all adipose deposits analyzed,e.g. subcutaneous adipose tissue, brown adipose tissue, retroperitoneal adipose tissue and epididymal adipose tissue, were found positive for hMR and lacZ expression induced by Dox. For the MR-DT line, *in vitro* experiments on fully differentiated adipocytes obtained by isolating and differentiating the vascular cellular fraction from subcutaneous or epididymal adipose tissue of DT animals indicated that the system was still conditional ex vivo, allowing Dox-dependent induction of hMR expression. These preliminary studies were conducted in order to verify the reliability of our tet-On system.

After this necessarily validation of the MR-DT model, we could start its phonotypical and metabolic characterization in order to explore the direct effects of an enhanced genetic dose of MR in fat physiology and the related functional consequences on other organs such as the cardiovascular system.

We began our study by evaluating the potential effects of MR overexpression in the visceral adipose tissue and the related consequences on its activity of regulating whole-body glucose homeostasis and insulin sensitivity (69). Using a preliminary analysis by real time PCR, we evaluated the expression level of some of the major adipose genes and adipokines. DT mice showed a significant increase in NGAL expression but, interestingly, no differences in GRα, PPARγ, adiponectin, Pai1, MCP1, TNFα and CD68. On the contrary, these were previously found to be well-modulated by MR activation in adipose tissue by other authors using *in vivo* pharmacological antagonism and *ex vivo* adipocyte stimulation with aldosterone (62, 64, 65). Subsequently, we performed an entire transcriptome microarray analysis in order to further identify other modulated genes and discovered that MR overexpression in PVAT allowed 101 genes to be up-regulated and 246 to be down-regulated in DT mice as compared to their control littermates. The gene ontology analysis of this gene list reveals a quite surprising outcome. In fact, the presence of an extra dose of MR, *in vivo*, seems to promote adipogenesis and hinder inflammation by respectively up regulating a series of genes involved in fatty acid and PPAR pathways and down regulating others involved in immune cells activation and cytokines interaction. This implies a potential protective role for mineralocorticoid receptors in adipose tissue acting out of the usual scheme. Of note this microarray analysis was done in basal condition. It would be necessary to react this experiment in a stressed conditionsuch as that of high a fat diet.

Although Caprio et al. (22) already proposed MR to have an essential role in 3T3-L1 adipocytes physiological differentiation, as a pro-adipogenic factor, MR activation *in vivo*

strongly contributes negatively to pathological conditions rather than beneficial effects. RALES and EPHESUS clinical trials, as well as many works in human and animal models, highlighted how MR antagonism could ameliorate the pathological outcome in cardiovascular and metabolic disorders. Taking as example the studies on obese mice conducted by Guo et al. (62) and Hirata et al. (64), it appears clear that MR activation in adipose tissue , promotes inflammation and contributes to worsening the pathological status of the tissue by decreasing insulin response and enhancing hypertrophic process in adipocytes. This was demonstrated by aldosterone direct stimulation on adipocyte cultures as well as indirectly by treating obese mice with specific MR antagonism. The apparent discrepancy between these data and ours, can be overcome by taking into account the fact that our mice are not obese nor fed with a high fat diet, and so their adipose tissues have to be considered as normal/healthy tissues.

Therefore, I believe it is important to start thinking about the role of MR in adipose tissue, as profoundly linked to a specific fat physio-/pathological status, rather than to believe in a single rigid pathway of MR in this tissue. From this point of view, the overactivation of MR in the adipose tissue should not be taken as necessarily negative, but more probably should be considered as a parallel system that contributes to define a given status of the tissue. Additional support to this idea, comes also from my previous studies published with the team in Padova, where we reported on visceral adipose tissue of patients with primary aldosteronism the absence of a negative influence on insulin signaling and adipokines expression. It should be mentioned, however, that the patients were selected with normal, non-obese/non-over weight, BMI and waist circumferences (23, 166).

Coming back to the transcriptome analysis, it is important to point out the fact that the simple overexpression of MR seems to be sufficient to induce the related up regulation of the NGAL gene. NGAL already has been proposed as a novel adipokine (159 , 160) and its concentration is increased in blood of two mouse models for obesity (db/db and ob/ob) as

well as in obese patients (159 , 161). Furthermore Guo et al (162) showed that NGAL-KO prevented insulin resistance and glucose intolerance when NGALK KO mice were fed with high fat diet. These data together with those of the team of F. Jaisser referring to NGAL as new molecular target of MR in the heart and vessels (unpublished data) appears particularly interesting for connecting Aldo/MR adipose dysfunction with metabolic syndrome and cardiovascular disease.

Even if the role of NGAL in the metabolic-cardiovascular relationship as well as factors modulating its expression still remain to be better identified, NGAL seem to be a good candidate as a reporter molecule for Aldo/hMR system activation. Further evidence supporting this concept arose from our first experiments on DT adipocyte culture, where we confirmed a drastic increase of NGAL expression after few days of Dox treatment for inducing hMR in differentiated adipocytes.

Expected results

This first group of data on the adipo-hMR mouse has disclosed a new powerful tool for deeply investigating the role of MR activation in adipose tissue and its possible implications in cardiovascular diseases. Preliminary data coming from metabolic analysis of DT mice, which will not be addressed in this dissertation, apparently do not reveal any significant phenotypic differences in fat body composition, blood pressure or insulin/glucose tolerance between DT mice and their control littermates when they are fed with a normocaloric diet, thus confirming what we demonstrated on activated MR in healthy adipose tissue.

It must be also pointed out that adult and healthy mice do not have a great amount of adipose tissue in any deposits and this could probably contribute to minimize the potential phenotype related to adipose MR excess.

In order to stress the environmental and miming pathological process, like those that establish in obesity or type II diabetes, we have already started to feed DT animals with a high fat diet providing the animals with 4397 kcal/kg composed by 22.5% of lipids, 42.3 % of carbohydrates and 17% of proteins. On these animals, we planned to perform extensive phenotypic and metabolic analyses, such as blood pressure measurement, insulin and glucose tolerance tests, dosage of blood concentration of fatty acid, glucose and NGAL protein, analysis of body fat composition by magnetic resonance imaging and analysis of cardiac function by echocardiography. Parallel to these experiments in living animals, we will also continue with *in vitro* experiments in primary DT adipocytes, our objective being to describe in differentiated cells the regulation of expression of NGAL and of the other adipokines upon stimulation with mineralocorticoid/glucocorticoids agonists and antagonists

REFERENCES

1. **Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM** 1987 Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268-275
2. **Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER, Monder C** 1988 Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. Lancet 2:986-989
3. **Funder JW, Pearce PT, Smith R, Smith AI** 1988 Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 242:583-585
4. **Marver D, Stewart J, Funder JW, Feldman D, Edelman IS** 1974 Renal aldosterone receptors: studies with (3H)aldosterone and the anti-mineralocorticoid (3H)spirolactone (SC-26304). Proc Natl Acad Sci U S A 71:1431-1435
5. **Lombes M, Farman N, Oblin ME, Baulieu EE, Bonvalet JP, Erlanger BF, Gasc JM** 1990 Immunohistochemical localization of renal mineralocorticoid receptor by using an anti-idiotypic antibody that is an internal image of aldosterone. Proc Natl Acad Sci U S A 87:1086-1088
6. **Krozowski ZS, Rundle SE, Wallace C, Castell MJ, Shen JH, Dowling J, Funder JW, Smith AI** 1989 Immunolocalization of renal mineralocorticoid receptors with an antiserum against a peptide deduced from the complementary deoxyribonucleic acid sequence. Endocrinology 125:192-198
7. **Pressley L, Funder JW** 1975 Glucocorticoid and mineralocorticoid receptors in gut mucosa. Endocrinology 97:588-596
8. **Funder JW, Feldman D, Edelman IS** 1972 Specific aldosterone binding in rat kidney and parotid. J Steroid Biochem 3:209-218
9. **Kenouch S, Lombes M, Delahaye F, Eugene E, Bonvalet JP, Farman N** 1994 Human skin as target for aldosterone: coexpression of mineralocorticoid receptors and 11 beta-hydroxysteroid dehydrogenase. J Clin Endocrinol Metab 79:1334-1341
10. **Fuller PJ, Lim-Tio SS, Brennan FE** 2000 Specificity in mineralocorticoid versus glucocorticoid action. Kidney Int 57:1256-1264
11. **Farman N, Rafestin-Oblin ME** 2001 Multiple aspects of mineralocorticoid selectivity. Am J Physiol Renal Physiol 280:F181-192
12. **Funder JW** 2005 Mineralocorticoid receptors: distribution and activation. Heart Fail Rev 10:15-22
13. **Krozowski Z, Funder JW** 1981 Mineralocorticoid receptors in the rat lung. Endocrinology 109:1811-1813
14. **Keller-Wood M, Wood CE, McCartney J, Jesse NM, Perrone D** 2011 A role for mineralocorticoid receptors in the physiology of the ovine fetus: effects on ACTH and lung liquid composition. Pediatr Res 69:491-496
15. **Touyz RM, Callera GE** 2009 A new look at the eye: aldosterone and mineralocorticoid receptors as novel targets in retinal vasculopathy. Circ Res 104:9-11
16. **Mirshahi M, Nicolas C, Mirshahi A, Hecquet C, d'Hermies F, Faure JP, Agarwal MK** 1996 The mineralocorticoid hormone receptor and action in the eye. Biochem Biophys Res Commun 219:150-156
17. **Nguyen Dinh Cat A, Griol-Charhbili V, Loufrani L, Labat C, Benjamin L, Farman N, Lacolley P, Henrion D, Jaisser F** 2010 The endothelial mineralocorticoid receptor regulates vasoconstrictor tone and blood pressure. FASEB J 24:2454-2463
18. **Han F, Ozawa H, Matsuda K, Nishi M, Kawata M** 2005 Colocalization of mineralocorticoid receptor and glucocorticoid receptor in the hippocampus and hypothalamus. Neurosci Res 51:371-381
19. **Moguilewsky M, Raynaud JP** 1980 Evidence for a specific mineralocorticoid receptor in rat pituitary and brain. J Steroid Biochem 12:309-314
20. **Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP** 1992 Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. Circ Res 71:503-510
21. **Armanini D, Strasser T, Weber PC** 1985 Characterization of aldosterone binding sites in circulating human mononuclear leukocytes. Am J Physiol 248:E388-390

22. **Caprio M, Feve B, Claes A, Viengchareun S, Lombes M, Zennaro MC** 2007 Pivotal role of the mineralocorticoid receptor in corticosteroid-induced adipogenesis. FASEB J 21:2185-2194
23. **Urbanet R, Pilon C, Calcagno A, Peschechera A, Hubert EL, Giacchetti G, Gomez-Sanchez C, Mulatero P, Toffanin M, Sonino N, Zennaro MC, Giorgino F, Vettor R, Fallo F** 2010 Analysis of insulin sensitivity in adipose tissue of patients with primary aldosteronism. J Clin Endocrinol Metab 95:4037-4042
24. **Rondinone CM, Rodbard D, Baker ME** 1993 Aldosterone stimulated differentiation of mouse 3T3-L1 cells into adipocytes. Endocrinology 132:2421-2426
25. **Mitts TF, Bunda S, Wang Y, Hinek A** 2010 Aldosterone and mineralocorticoid receptor antagonists modulate elastin and collagen deposition in human skin. J Invest Dermatol 130:2396-2406
26. **Farman N, Maubec E, Poeggeler B, Klatte JE, Jaisser F, Paus R** 2010 The mineralocorticoid receptor as a novel player in skin biology: beyond the renal horizon? Exp Dermatol 19:100-107
27. **Brien TG** 1981 Human corticosteroid binding globulin. Clin Endocrinol (Oxf) 14:193-212
28. **Pearson J, Keane PM, Walker WH** 1967 Binding of cortisol to human albumin. Nature 216:1334-1335
29. **Smith JB, Nolan G, Jubiz W** 1980 The relationship between unbound and total cortisol: its usefulness in detecting CBG abnormalities. Clin Chim Acta 108:435-445
30. **Wang H, Shimosawa T, Matsui H, Kaneko T, Ogura S, Uetake Y, Takenaka K, Yatomi Y, Fujita T** 2008 Paradoxical mineralocorticoid receptor activation and left ventricular diastolic dysfunction under high oxidative stress conditions. J Hypertens 26:1453-1462
31. **Young MJ** 2008 Mechanisms of mineralocorticoid receptor-mediated cardiac fibrosis and vascular inflammation. Curr Opin Nephrol Hypertens 17:174-180
32. **Nagase M, Matsui H, Shibata S, Gotoda T, Fujita T** 2007 Salt-induced nephropathy in obese spontaneously hypertensive rats via paradoxical activation of the mineralocorticoid receptor: role of oxidative stress. Hypertension 50:877-883
33. **Conn JW** 1955 Primary aldosteronism. J Lab Clin Med 45:661-664
34. **Conn JW** 1965 Hypertension, the potassium ion and impaired carbohydrate tolerance. N Engl J Med 273:1135-1143
35. **Shargorodsky M, Zimlichman R** 2002 Primary aldosteronism: the most frequent form of secondary hypertension? Isr Med Assoc J 4:32-33
36. **Strauch B, Petrak O, Wichterle D, Zelinka T, Holaj R, Widimsky J, Jr.** 2006 Increased arterial wall stiffness in primary aldosteronism in comparison with essential hypertension. Am J Hypertens 19:909-914
37. **Holaj R, Zelinka T, Wichterle D, Petrak O, Strauch B, Widimsky J, Jr.** 2007 Increased intima-media thickness of the common carotid artery in primary aldosteronism in comparison with essential hypertension. J Hypertens 25:1451-1457
38. **Bernini G, Galetta F, Franzoni F, Bardini M, Taurino C, Bernardini M, Ghiadoni L, Bernini M, Santoro G, Salvetti A** 2008 Arterial stiffness, intima-media thickness and carotid artery fibrosis in patients with primary aldosteronism. J Hypertens 26:2399-2405
39. **Catena C, Colussi G, Nadalini E, Chiuch A, Baroselli S, Lapenna R, Sechi LA** 2008 Cardiovascular outcomes in patients with primary aldosteronism after treatment. Arch Intern Med 168:80-85
40. **Tuck ML, Corry DB** 2007 Renal damage in primary aldosteronism: results of the PAPY study. Curr Hypertens Rep 9:87-89
41. **Jefic D, Mohiuddin N, Alsabbagh R, Fadanelli M, Steigerwalt S** 2006 The prevalence of primary aldosteronism in diabetic patients. J Clin Hypertens (Greenwich) 8:253-256
42. **Bochud M, Nussberger J, Bovet P, Maillard MR, Elston RC, Paccaud F, Shamlaye C, Burnier M** 2006 Plasma aldosterone is independently associated with the metabolic syndrome. Hypertension 48:239-245
43. **Lastra-Lastra G, Sowers JR, Restrepo-Erazo K, Manrique-Acevedo C, Lastra-Gonzalez G** 2009 Role of aldosterone and angiotensin II in insulin resistance: an update. Clin Endocrinol (Oxf) 71:1-6
44. **Shamiss A, Carroll J, Rosenthal T** 1992 Insulin resistance in secondary hypertension. Am J Hypertens 5:26-28
45. **Shimamoto K, Shiiki M, Ise T, Miyazaki Y, Higashiura K, Fukuoka M, Hirata A, Masuda A, Nakagawa M, Iimura O** 1994 Does insulin resistance participate in an impaired glucose tolerance in primary aldosteronism? J Hum Hypertens 8:755-759
46. **Ishimori M, Takeda N, Okumura S, Murai T, Inouye H, Yasuda K** 1994 Increased insulin sensitivity in patients with aldosterone producing adenoma. Clin Endocrinol (Oxf) 41:433-438

47. **Sindelka G, Widimsky J, Haas T, Prazny M, Hilgertova J, Skrha J** 2000 Insulin action in primary hyperaldosteronism before and after surgical or pharmacological treatment. Exp Clin Endocrinol Diabetes 108:21-25
48. **Widimsky J, Jr., Sindelka G, Haas T, Prazny M, Hilgertova J, Skrha J** 2000 Impaired insulin action in primary hyperaldosteronism. Physiol Res 49:241-244
49. **Widimsky J, Jr., Strauch B, Sindelka G, Skrha J** 2001 Can primary hyperaldosteronism be considered as a specific form of diabetes mellitus? Physiol Res 50:603-607
50. **Haluzik M, Sindelka G, Widimsky J, Jr., Prazny M, Zelinka T, Skrha J** 2002 Serum leptin levels in patients with primary hyperaldosteronism before and after treatment: relationships to insulin sensitivity. J Hum Hypertens 16:41-45
51. **Strauch B, Widimsky J, Sindelka G, Skrha J** 2003 Does the treatment of primary hyperaldosteronism influence glucose tolerance? Physiol Res 52:503-506
52. **Skrha J, Haas T, Sindelka G, Prazny M, Widimsky J, Cibula D, Svacina S** 2004 Comparison of the insulin action parameters from hyperinsulinemic clamps with homeostasis model assessment and QUICKI indexes in subjects with different endocrine disorders. J Clin Endocrinol Metab 89:135-141
53. **Catena C, Lapenna R, Baroselli S, Nadalini E, Colussi G, Novello M, Favret G, Melis A, Cavarape A, Sechi LA** 2006 Insulin sensitivity in patients with primary aldosteronism: a follow-up study. J Clin Endocrinol Metab 91:3457-3463
54. **Giacchetti G, Ronconi V, Turchi F, Agostinelli L, Mantero F, Rilli S, Boscaro M** 2007 Aldosterone as a key mediator of the cardiometabolic syndrome in primary aldosteronism: an observational study. J Hypertens 25:177-186
55. **Mosso LM, Carvajal CA, Maiz A, Ortiz EH, Castillo CR, Artigas RA, Fardella CE** 2007 A possible association between primary aldosteronism and a lower beta-cell function. J Hypertens 25:2125-2130
56. **Fallo F, Della Mea P, Sonino N, Bertello C, Ermani M, Vettor R, Veglio F, Mulatero P** 2007 Adiponectin and insulin sensitivity in primary aldosteronism. Am J Hypertens 20:855-861
57. **Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M** 2003 Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med 348:1309-1321
58. **Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J** 1999 The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. N Engl J Med 341:709-717
59. **Zannad F, McMurray JJ, Krum H, van Veldhuisen DJ, Swedberg K, Shi H, Vincent J, Pocock SJ, Pitt B** 2011 Eplerenone in patients with systolic heart failure and mild symptoms. N Engl J Med 364:11-21
60. **Graf K, Hucko T, Stawowy P** 2008 Cardiac benefits of mineralocorticoid receptor inhibition in renal failure. Hypertension 52:209-210
61. **Mehdi UF, Adams-Huet B, Raskin P, Vega GL, Toto RD** 2009 Addition of angiotensin receptor blockade or mineralocorticoid antagonism to maximal angiotensin-converting enzyme inhibition in diabetic nephropathy. J Am Soc Nephrol 20:2641-2650
62. **Guo C, Ricchiuti V, Lian BQ, Yao TM, Coutinho P, Romero JR, Li J, Williams GH, Adler GK** 2008 Mineralocorticoid receptor blockade reverses obesity-related changes in expression of adiponectin, peroxisome proliferator-activated receptor-gamma, and proinflammatory adipokines. Circulation 117:2253-2261
63. **Wada T, Kenmochi H, Miyashita Y, Sasaki M, Ojima M, Sasahara M, Koya D, Tsuneki H, Sasaoka T** 2010 Spironolactone improves glucose and lipid metabolism by ameliorating hepatic steatosis and inflammation and suppressing enhanced gluconeogenesis induced by high-fat and high-fructose diet. Endocrinology 151:2040-2049
64. **Hirata A, Maeda N, Hiuge A, Hibuse T, Fujita K, Okada T, Kihara S, Funahashi T, Shimomura I** 2009 Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice. Cardiovasc Res 84:164-172
65. **Kraus D, Jager J, Meier B, Fasshauer M, Klein J** 2005 Aldosterone inhibits uncoupling protein-1, induces insulin resistance, and stimulates proinflammatory adipokines in adipocytes. Horm Metab Res 37:455-459
66. **Cooper SA, Whaley-Connell A, Habibi J, Wei Y, Lastra G, Manrique C, Stas S, Sowers JR** 2007 Renin-angiotensin-aldosterone system and oxidative stress in cardiovascular insulin resistance. Am J Physiol Heart Circ Physiol 293:H2009-2023
67. **Ahima RS, Flier JS** 2000 Adipose tissue as an endocrine organ. Trends Endocrinol Metab 11:327-332

68. **Vettor R, Milan G, Rossato M, Federspil G** 2005 Review article: adipocytokines and insulin resistance. Aliment Pharmacol Ther 22 Suppl 2:3-10
69. **MacLaren R, Cui W, Simard S, Cianflone K** 2008 Influence of obesity and insulin sensitivity on insulin signaling genes in human omental and subcutaneous adipose tissue. J Lipid Res 49:308-323
70. **Macor C, Ruggeri A, Mazzonetto P, Federspil G, Cobelli C, Vettor R** 1997 Visceral adipose tissue impairs insulin secretion and insulin sensitivity but not energy expenditure in obesity. Metabolism 46:123-129
71. **Gesta S, Tseng YH, Kahn CR** 2007 Developmental origin of fat: tracking obesity to its source. Cell 131:242-256
72. **Nicholls DG, Locke RM** 1984 Thermogenic mechanisms in brown fat. Physiological reviews 64:1-64
73. **Champigny O, Ricquier D** 1996 Evidence from in vitro differentiating cells that adrenoceptor agonists can increase uncoupling protein mRNA level in adipocytes of adult humans: an RT-PCR study. J Lipid Res 37:1907-1914
74. **Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P** 2009 Functional brown adipose tissue in healthy adults. N Engl J Med 360:1518-1525
75. **Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, Iwanaga T, Miyagawa M, Kameya T, Nakada K, Kawai Y, Tsujisaki M** 2009 High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. Diabetes 58:1526-1531
76. **Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR** 2009 Identification and importance of brown adipose tissue in adult humans. N Engl J Med 360:1509-1517
77. **Zingaretti MC, Crosta F, Vitali A, Guerrieri M, Frontini A, Cannon B, Nedergaard J, Cinti S** 2009 The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. FASEB J 23:3113-3120
78. **Cinti S** 2009 Transdifferentiation properties of adipocytes in the Adipose Organ. Am J Physiol Endocrinol Metab
79. **Frontini A, Cinti S** 2010 Distribution and development of brown adipocytes in the murine and human adipose organ. Cell Metab 11:253-256
80. **Zennaro MC, Le Menuet D, Viengchareun S, Walker F, Ricquier D, Lombes M** 1998 Hibernoma development in transgenic mice identifies brown adipose tissue as a novel target of aldosterone action. J Clin Invest 101:1254-1260
81. **Penfornis P, Viengchareun S, Le Menuet D, Cluzeaud F, Zennaro MC, Lombes M** 2000 The mineralocorticoid receptor mediates aldosterone-induced differentiation of T37i cells into brown adipocytes. Am J Physiol Endocrinol Metab 279:E386-394
82. **Viengchareun S, Penfornis P, Zennaro MC, Lombes M** 2001 Mineralocorticoid and glucocorticoid receptors inhibit UCP expression and function in brown adipocytes. Am J Physiol Endocrinol Metab 280:E640-649
83. **Hauner H, Entenmann G, Wabitsch M, Gaillard D, Ailhaud G, Negrel R, Pfeiffer EF** 1989 Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J Clin Invest 84:1663-1670
84. **Fu M, Sun T, Bookout AL, Downes M, Yu RT, Evans RM, Mangelsdorf DJ** 2005 A Nuclear Receptor Atlas: 3T3-L1 adipogenesis. Mol Endocrinol 19:2437-2450
85. **Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M, Cinti S** 2008 Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J Lipid Res 49:1562-1568
86. **Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H** 2003 Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 112:1821-1830
87. **Berg AH, Scherer PE** 2005 Adipose tissue, inflammation, and cardiovascular disease. Circ Res 96:939-949
88. **Skurk T, Alberti-Huber C, Herder C, Hauner H** 2007 Relationship between adipocyte size and adipokine expression and secretion. J Clin Endocrinol Metab 92:1023-1033
89. **Wellen KE, Hotamisligil GS** 2005 Inflammation, stress, and diabetes. J Clin Invest 115:1111-1119
90. **Neels JG, Olefsky JM** 2006 Inflamed fat: what starts the fire? J Clin Invest 116:33-35
91. **Connell JM, MacKenzie SM, Freel EM, Fraser R, Davies E** 2008 A lifetime of aldosterone excess: long-term consequences of altered regulation of aldosterone production for cardiovascular function. Endocr Rev 29:133-154

92. **Rossi G, Boscaro M, Ronconi V, Funder JW** 2005 Aldosterone as a cardiovascular risk factor. Trends Endocrinol Metab 16:104-107
93. **Cachofeiro V, Miana M, de Las Heras N, Martin-Fernandez B, Ballesteros S, Fernandez-Tresguerres J, Lahera V** 2008 Aldosterone and the vascular system. J Steroid Biochem Mol Biol 109:331-335
94. **Struthers AD** 2004 Aldosterone-induced vasculopathy. Mol Cell Endocrinol 217:239-241
95. **Giacchetti G, Sechi LA, Rilli S, Carey RM** 2005 The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. Trends Endocrinol Metab 16:120-126
96. **Fallo F, Veglio F, Bertello C, Sonino N, Della Mea P, Ermani M, Rabbia F, Federspil G, Mulatero P** 2006 Prevalence and characteristics of the metabolic syndrome in primary aldosteronism. J Clin Endocrinol Metab 91:454-459
97. **Eriksson JW, Smith U, Waagstein F, Wysocki M, Jansson PA** 1999 Glucose turnover and adipose tissue lipolysis are insulin-resistant in healthy relatives of type 2 diabetes patients: is cellular insulin resistance a secondary phenomenon? Diabetes 48:1572-1578
98. **Giorgino F, Laviola L, Eriksson JW** 2005 Regional differences of insulin action in adipose tissue: insights from in vivo and in vitro studies. Acta Physiol Scand 183:13-30
99. 2003 Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care 26 Suppl 1:S5-20
100. **Mulatero P, Rabbia F, Milan A, Paglieri C, Morello F, Chiandussi L, Veglio F** 2002 Drug effects on aldosterone/plasma renin activity ratio in primary aldosteronism. Hypertension 40:897-902
101. **Stas S, Appesh L, Sowers J** 2006 Metabolic safety of antihypertensive drugs: myth versus reality. Curr Hypertens Rep 8:403-408
102. **Neville AM, O'Hare MJ** 1985 Histopathology of the human adrenal cortex. Clin Endocrinol Metab 14:791-820
103. **Mancini T, Kola B, Mantero F, Arnaldi G** 2003 Functional and nonfunctional adrenocortical tumors demonstrate a high responsiveness to low-dose adrenocorticotropin. J Clin Endocrinol Metab 88:1994-1998
104. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC** 1985 Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412-419
105. **Gomez-Sanchez CE, de Rodriguez AF, Romero DG, Estess J, Warden MP, Gomez-Sanchez MT, Gomez-Sanchez EP** 2006 Development of a panel of monoclonal antibodies against the mineralocorticoid receptor. Endocrinology 147:1343-1348
106. **Leney SE, Tavare JM** 2009 The molecular basis of insulin-stimulated glucose uptake: signalling, trafficking and potential drug targets. J Endocrinol 203:1-18
107. **Tanaka M, Nishi M, Morimoto M, Sugimoto T, Kawata M** 2005 Imaging analysis of mineralocorticoid receptor and importins in single living cells by using GFP color variants. Cell Tissue Res 320:447-453
108. **Carranza MC, Torres A, Calle C** 1991 [Decreased insulin receptor number and affinity in subcutaneous adipose tissue in a patient with primary hyperaldosteronism]. Rev Clin Esp 188:414-417
109. **Wada T, Ohshima S, Fujisawa E, Koya D, Tsuneki H, Sasaoka T** 2009 Aldosterone inhibits insulin-induced glucose uptake by degradation of insulin receptor substrate (IRS) 1 and IRS2 via a reactive oxygen species-mediated pathway in 3T3-L1 adipocytes. Endocrinology 150:1662-1669
110. **Hellal-Levy C, Couette B, Fagart J, Souque A, Gomez-Sanchez C, Rafestin-Oblin M** 1999 Specific hydroxylations determine selective corticosteroid recognition by human glucocorticoid and mineralocorticoid receptors. FEBS Lett 464:9-13
111. **Funder JW** 2009 Reconsidering the roles of the mineralocorticoid receptor. Hypertension 53:286-290
112. **Gathercole LL, Bujalska IJ, Stewart PM, Tomlinson JW** 2007 Glucocorticoid modulation of insulin signaling in human subcutaneous adipose tissue. J Clin Endocrinol Metab 92:4332-4339
113. **Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, Fukushima Y, Onishi Y, Ono H, Fujishiro M, Kikuchi M, Oka Y, Asano T** 2000 Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. Diabetes 49:1700-1708
114. **Ngo S, Barry JB, Nisbet JC, Prins JB, Whitehead JP** 2009 Reduced phosphorylation of AS160 contributes to glucocorticoid-mediated inhibition of glucose uptake in human and murine adipocytes. Mol Cell Endocrinol 302:33-40
115. **Zierler K, Rogus EM, Scherer RW, Wu FS** 1985 Insulin action on membrane potential and glucose uptake: effects of high potassium. Am J Physiol 249:E17-25
116. **Funder JW** 2005 The nongenomic actions of aldosterone. Endocr Rev 26:313-321

117. **Schiffrin EL** 2006 Effects of aldosterone on the vasculature. Hypertension 47:312-318
118. **Johnson FK, Johnson RA, Durante W** 2005 Aldosterone promotes endothelial dysfunction via prostacyclin independent of hypertension. Hypertension 46:29-30
119. **Laviola L, Perrini S, Cignarelli A, Natalicchio A, Leonardini A, De Stefano F, Cuscito M, De Fazio M, Memeo V, Neri V, Cignarelli M, Giorgino R, Giorgino F** 2006 Insulin signaling in human visceral and subcutaneous adipose tissue in vivo. Diabetes 55:952-961
120. **Lundgren M, Buren J, Ruge T, Myrnas T, Eriksson JW** 2004 Glucocorticoids down-regulate glucose uptake capacity and insulin-signaling proteins in omental but not subcutaneous human adipocytes. J Clin Endocrinol Metab 89:2989-2997
121. **Zennaro MC, Caprio M, Feve B** 2009 Mineralocorticoid receptors in the metabolic syndrome. Trends Endocrinol Metab 20:444-451
122. **Walker GE, Verti B, Marzullo P, Savia G, Mencarelli M, Zurleni F, Liuzzi A, Di Blasio AM** 2007 Deep subcutaneous adipose tissue: a distinct abdominal adipose depot. Obesity (Silver Spring) 15:1933-1943
123. **Hitomi H, Kiyomoto H, Nishiyama A, Hara T, Moriwaki K, Kaifu K, Ihara G, Fujita Y, Ugawa T, Kohno M** 2007 Aldosterone suppresses insulin signaling via the downregulation of insulin receptor substrate-1 in vascular smooth muscle cells. Hypertension 50:750-755
124. **Yamashita R, Kikuchi T, Mori Y, Aoki K, Kaburagi Y, Yasuda K, Sekihara H** 2004 Aldosterone stimulates gene expression of hepatic gluconeogenic enzymes through the glucocorticoid receptor in a manner independent of the protein kinase B cascade. Endocr J 51:243-251
125. **Liu G, Grifman M, Keily B, Chatterton JE, Staal FW, Li QX** 2006 Mineralocorticoid receptor is involved in the regulation of genes responsible for hepatic glucose production. Biochem Biophys Res Commun 342:1291-1296
126. **Lastra G, Whaley-Connell A, Manrique C, Habibi J, Gutweiler AA, Appesh L, Hayden MR, Wei Y, Ferrario C, Sowers JR** 2008 Low-dose spironolactone reduces reactive oxygen species generation and improves insulin-stimulated glucose transport in skeletal muscle in the TG(mRen2)27 rat. Am J Physiol Endocrinol Metab 295:E110-116
127. **Ndisang JF, Jadhav A** 2010 The heme oxygenase system attenuates pancreatic lesions and improves insulin sensitivity and glucose metabolism in deoxycorticosterone acetate hypertension. Am J Physiol Regul Integr Comp Physiol 298:R211-223
128. **Fabbrini E, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW, Okunade A, Klein S** 2009 Intrahepatic fat, not visceral fat, is linked with metabolic complications of obesity. Proc Natl Acad Sci U S A 106:15430-15435
129. **Fallo F, Dalla Pozza A, Tecchio M, Tona F, Sonino N, Ermani M, Catena C, Bertello C, Mulatero P, Sabato N, Fabris B, Sechi LA** 2010 Nonalcoholic fatty liver disease in primary aldosteronism: a pilot study. Am J Hypertens 23:2-5
130. **Favre J, Gao J, Zhang AD, Remy-Jouet I, Ouvrard-Pascaud A, Dautreaux B, Escoubet B, Thuillez C, Jaisser F, Richard V** 2011 Coronary endothelial dysfunction after cardiomyocyte-specific mineralocorticoid receptor overexpression. Am J Physiol Heart Circ Physiol 300:H2035-2043
131. **Griol-Charhbili V, Fassot C, Messaoudi S, Perret C, Agrapart V, Jaisser F** 2011 Epidermal growth factor receptor mediates the vascular dysfunction but not the remodeling induced by aldosterone/salt. Hypertension 57:238-244
132. **Hernandez-Diaz I, Giraldez T, Arnau MR, Smits VA, Jaisser F, Farman N, Alvarez de la Rosa D** 2010 The mineralocorticoid receptor is a constitutive nuclear factor in cardiomyocytes due to hyperactive nuclear localization signals. Endocrinology 151:3888-3899
133. **Zhao M, Valamanesh F, Celerier I, Savoldelli M, Jonet L, Jeanny JC, Jaisser F, Farman N, Behar-Cohen F** 2010 The neuroretina is a novel mineralocorticoid target: aldosterone up-regulates ion and water channels in Muller glial cells. FASEB J 24:3405-3415
134. **Khouzami L, Bourin MC, Christov C, Damy T, Escoubet B, Caramelle P, Perier M, Wahbi K, Meune C, Pavoine C, Pecker F** 2010 Delayed cardiomyopathy in dystrophin deficient mdx mice relies on intrinsic glutathione resource. Am J Pathol 177:1356-1364
135. **Sacre K, Brihaye B, Hyafil F, Serfaty JM, Escoubet B, Zennaro MC, Lidove O, Laissy JP, Papo T** 2010 Asymptomatic myocardial ischemic disease in antiphospholipid syndrome: a controlled cardiac magnetic resonance imaging study. Arthritis Rheum 62:2093-2100
136. **Andersson KB, Winer LH, Mork HK, Molkentin JD, Jaisser F** 2010 Tamoxifen administration routes and dosage for inducible Cre-mediated gene disruption in mouse hearts. Transgenic research 19:715-725

137. **Ziera T, Irlbacher H, Fromm A, Latouche C, Krug SM, Fromm M, Jaisser F, Borden SA** 2009 Cnksr3 is a direct mineralocorticoid receptor target gene and plays a key role in the regulation of the epithelial sodium channel. FASEB J 23:3936-3946
138. **Palais G, Nguyen Dinh Cat A, Friedman H, Panek-Huet N, Millet A, Tronche F, Gellen B, Mercadier JJ, Peterson A, Jaisser F** 2009 Targeted transgenesis at the HPRT locus: an efficient strategy to achieve tightly controlled in vivo conditional expression with the tet system. Physiol Genomics 37:140-146
139. **Nguyen Dinh Cat A, Ouvrard-Pascaud A, Tronche F, Clemessy M, Gonzalez-Nunez D, Farman N, Jaisser F** 2009 Conditional transgenic mice for studying the role of the glucocorticoid receptor in the renal collecting duct. Endocrinology 150:2202-2210
140. **Gomez AM, Rueda A, Sainte-Marie Y, Pereira L, Zissimopoulos S, Zhu X, Schaub R, Perrier E, Perrier R, Latouche C, Richard S, Picot MC, Jaisser F, Lai FA, Valdivia HH, Benitah JP** 2009 Mineralocorticoid modulation of cardiac ryanodine receptor activity is associated with downregulation of FK506-binding proteins. Circulation 119:2179-2187
141. **Defer N, Wan J, Souktani R, Escoubet B, Perier M, Caramelle P, Manin S, Deveaux V, Bourin MC, Zimmer A, Lotersztajn S, Pecker F, Pavoine C** 2009 The cannabinoid receptor type 2 promotes cardiac myocyte and fibroblast survival and protects against ischemia/reperfusion-induced cardiomyopathy. FASEB J 23:2120-2130
142. **Di Zhang A, Nguyen Dinh Cat A, Soukaseum C, Escoubet B, Cherfa A, Messaoudi S, Delcayre C, Samuel JL, Jaisser F** 2008 Cross-talk between mineralocorticoid and angiotensin II signaling for cardiac remodeling. Hypertension 52:1060-1067
143. **Loyer X, Gomez AM, Milliez P, Fernandez-Velasco M, Vangheluwe P, Vinet L, Charue D, Vaudin E, Zhang W, Sainte-Marie Y, Robidel E, Marty I, Mayer B, Jaisser F, Mercadier JJ, Richard S, Shah AM, Benitah JP, Samuel JL, Heymes C** 2008 Cardiomyocyte overexpression of neuronal nitric oxide synthase delays transition toward heart failure in response to pressure overload by preserving calcium cycling. Circulation 117:3187-3198
144. **Duverger O, Lee D, Hassan MQ, Chen SX, Jaisser F, Lian JB, Morasso MI** 2008 Molecular consequences of a frameshifted DLX3 mutant leading to Tricho-Dento-Osseous syndrome. J Biol Chem 283:20198-20208
145. **Gellen B, Fernandez-Velasco M, Briec F, Vinet L, LeQuang K, Rouet-Benzineb P, Benitah JP, Pezet M, Palais G, Pellegrin N, Zhang A, Perrier R, Escoubet B, Marniquet X, Richard S, Jaisser F, Gomez AM, Charpentier F, Mercadier JJ** 2008 Conditional FKBP12.6 overexpression in mouse cardiac myocytes prevents triggered ventricular tachycardia through specific alterations in excitation-contraction coupling. Circulation 117:1778-1786
146. **Latouche C, Sainte-Marie Y, Steenman M, Castro Chaves P, Naray-Fejes-Toth A, Fejes-Toth G, Farman N, Jaisser F** 2010 Molecular Signature of Mineralocorticoid Receptor Signaling in Cardiomyocytes: From Cultured Cells to Mouse Heart. Endocrinology
147. **Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N** 1993 Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 268:10425-10432
148. **Seveus L, Amin K, Peterson CG, Roomans GM, Venge P** 1997 Human neutrophil lipocalin (HNL) is a specific granule constituent of the neutrophil granulocyte. Studies in bronchial and lung parenchymal tissue and peripheral blood cells. Histochemistry and cell biology 107:423-432
149. **Bartsch S, Tschesche H** 1995 Cloning and expression of human neutrophil lipocalin cDNA derived from bone marrow and ovarian cancer cells. FEBS Lett 357:255-259
150. **Yan QW, Yang Q, Mody N, Graham TE, Hsu CH, Xu Z, Houstis NE, Kahn BB, Rosen ED** 2007 The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance. Diabetes 56:2533-2540
151. **Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK** 2002 The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell 10:1033-1043
152. **Yan L, Borregaard N, Kjeldsen L, Moses MA** 2001 The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL). Modulation of MMP-9 activity by NGAL. J Biol Chem 276:37258-37265
153. **Page-McCaw A, Ewald AJ, Werb Z** 2007 Matrix metalloproteinases and the regulation of tissue remodelling. Nature reviews Molecular cell biology 8:221-233
154. **Shi H, Gu Y, Yang J, Xu L, Mi W, Yu W** 2008 Lipocalin 2 promotes lung metastasis of murine breast cancer cells. Journal of experimental & clinical cancer research : CR 27:83
155. **Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Silva C, Rotellar F, Hernandez-Lizoain JL, Baixauli J, Valenti V, Pardo F, Salvador J, Fruhbeck G** 2011 Up-regulation of the novel

proinflammatory adipokines lipocalin-2, chitinase-3 like-1 and osteopontin as well as angiogenic-related factors in visceral adipose tissue of patients with colon cancer. J Nutr Biochem 22:634-641

156. **Yndestad A, Landro L, Ueland T, Dahl CP, Flo TH, Vinge LE, Espevik T, Froland SS, Husberg C, Christensen G, Dickstein K, Kjekshus J, Oie E, Gullestad L, Aukrust P** 2009 Increased systemic and myocardial expression of neutrophil gelatinase-associated lipocalin in clinical and experimental heart failure. European heart journal 30:1229-1236
157. **Hemdahl AL, Gabrielsen A, Zhu C, Eriksson P, Hedin U, Kastrup J, Thoren P, Hansson GK** 2006 Expression of neutrophil gelatinase-associated lipocalin in atherosclerosis and myocardial infarction. Arterioscler Thromb Vasc Biol 26:136-142
158. **Bolignano D, Basile G, Parisi P, Coppolino G, Nicocia G, Buemi M** 2009 Increased Plasma Neutrophil Gelatinase-Associated Lipocalin Levels Predict Mortality in Elderly Patients with Chronic Heart Failure. Rejuvenation Res
159. **Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Silva C, Rotellar F, Gil MJ, Cienfuegos JA, Salvador J, Fruhbeck G** 2009 Increased adipose tissue expression of lipocalin-2 in obesity is related to inflammation and matrix metalloproteinase-2 and metalloproteinase-9 activities in humans. J Mol Med 87:803-813
160. **Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, Bickel PE, Andersen JS, Fernandez MM, Bunkenborg J, Roepstorff P, Kristiansen K, Lodish HF, Mann M, Pandey A** 2002 A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. Mol Cell Proteomics 1:213-222
161. **Wang Y, Lam KS, Kraegen EW, Sweeney G, Zhang J, Tso AW, Chow WS, Wat NM, Xu JY, Hoo RL, Xu A** 2007 Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans. Clin Chem 53:34-41
162. **Guo H, Jin D, Zhang Y, Wright W, Bazuine M, Brockman DA, Bernlohr DA, Chen X** 2010 Lipocalin-2 deficiency impairs thermogenesis and potentiates diet-induced insulin resistance in mice. Diabetes 59:1376-1385
163. **Ouvrard-Pascaud A, Sainte-Marie Y, Benitah JP, Perrier R, Soukaseum C, Cat AN, Royer A, Le Quang K, Charpentier F, Demolombe S, Mechta-Grigoriou F, Beggah AT, Maison-Blanche P, Oblin ME, Delcayre C, Fishman GI, Farman N, Escoubet B, Jaisser F** 2005 Conditional mineralocorticoid receptor expression in the heart leads to life-threatening arrhythmias. Circulation 111:3025-3033
164. **Puttini S, Beggah AT, Ouvrard-Pascaud A, Legris C, Blot-Chabaud M, Farman N, Jaisser F** 2001 Tetracycline-inducible gene expression in cultured rat renal CD cells and in intact CD from transgenic mice. Am J Physiol Renal Physiol 281:F1164-1172
165. **Pfaffl MW** 2001 A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 29:e45
166. **Urbanet R, Pilon C, Giorgino F, Vettor R, Fallo F** 2011 Insulin signaling in adipose tissue of patients with primary aldosteronism. J Endocrinol Invest 34:86-89

PUBLICATIONS

During the course of this work, the following articles have been published or submitted for publication:

Fallo F, Pilon C, Urbanet R. 2011. Primary aldosteronism and metabolic syndrome. Horm Metab Res.; [Epub ahead of print]

Urbanet R, Pilon C, Calcagno A, Peschechera A, Hubert EL, Giacchetti G, Gomez-Sanchez C, Mulatero P, Toffanin M, Sonino N, Zennaro MC, Giorgino F, Vettor R, Fallo F. 2010. Analysis of insulin sensitivity in adipose tissue of patients with primary aldosteronism J Clin Endocrinol Metab.; 95(8):4037-42.

Urbanet R, Pilon C, Giorgino F, Vettor R, Fallo F. 2011. Insulin signaling in adipose tissue of patients with primary aldosteronism.J Endocrinol Invest.;34 (2):86-9.

ACKNOWLEDGMENTS

When at dawn in Paris, I dart by Velib' headed to the lab and I cross the Pont d'Austerlitz, I always take a look at the sunrise lighting up La Seine, Notre Dame and la Tower Eiffel. I can't help but pause in amazement at how far fate and my impatience toward the understanding of me and the world have led me.
I would like to dedicate this work to everyone who, in these past three years, have helped me with affection, esteem and "mental" support.

To Prof. Fallo, Catia, Fred, Nicolette and Vinz, my gratitude for teaching me how to work and move in this world of research, while sharing with me expectations, anxieties, frustrations and successes.
To my colleagues, Marnie, Sonialeandriiii, Guillaume, Basile and Antoine, thanks for making me feel at home working together (loved and cuddled).
To my French friends, Cedric and Cath, Vin, Biéres et Fromages Fondus, thanks for the hospitality, availability and lovely time we spent together.
To my dearest and oldest friends, San and Betty, thanks for having always supported me and remaining close. With you I feel like time and space distances do not exist.
To Micol, thanks for facing with me, all my crises, my troubles and my weirdness. Thank you for always being by my side, despite everything.

To Mom and Dad, Zia Anna, Stefy, Tati and Gio, Niky, Cesco, Tommy and Co, thanks for being my family. I hope to make you proud of me and what I've done these years.

P.S. I wish all of you, with me, will also thank Susan DeBruhl, my English teacher!! Without her this text would not even exist. Thank you so much Susan.

CURRICULUM VITAE

Date of birth:	04.04.1981
Place of birth:	Asolo (Italy)

1987-1992	Scuola Statale Generale Giardino, Bassano del Grappa (Italy)
1992-1995	Scuola media statale Vitorelli, Bassano del Grappa
1995-2000	Liceo Scientifico G.B. Brocch", Bassano del Grappa
2000-2006	University of Padova, Corso di Luarea in Medical Biotechnology
2006-2008	Italian PRIN (Research Project of National Interest) in the laboratory of Prof. Roberto Vettor and Prof. Francesco Fallo, Department of Medical and Surgical Sciences, University of Padova. Title: "Aldosterone and Insulin: Assessment of a Pathogenic Role of Adipose Tissue"
2009-2012	PhD in the Department of Medical and Surgical Sciences of the University of Padova. From 2010 to 2011 in the laboratory of Prof. Roberto Vettor and Francesco Fallo (University of Padova). In 2011 in the laboratory of Dr. Frédéric Jaisser, (University Pierre et Marie Curie and University Paris Descartes, Paris, France).

ERRATA CORRIGE

Page	Text line	Correction
9	2	*progeterone,* **progesterone**
9	5	*bindin,* **binding**
9	8	*browun,* **brown**
9	15	*ephitlial,* **epithelial**
9	20	*sssessment,* **assessment**
9	21	*dehydrogenas,* **dehydrogenase**
9	31	*enogenous* **endogenous**
9	31	*mr,* **MR**
9	32	*resonace,* **resonance**
9	34	*rna,* **RNA**
9	37	*adrrenal,* **adrenal**
10	12	*pcr,* **PCR**
10	13	*renon,* **renin**
16	1	*nucleolus,* **nucleus**
21	19	*sssessment,* **assessment**
66	19	**insert page break**