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THE NOVEL ADIPOKINE CTRP-1(C1qTNFα RELATED PROTEIN-1) AS A POSSIBLE LINK BETWEEN OVERWEIGHT – OBESITY AND HYPERTENSION

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Abstract

CTRPs (Complement-C1q tumour necrosis factor-α-related protein) entail a family of recently identified adipokines. Since CTRP-1 was found to stimulate aldosterone production in the adrenocarcinoma-derived cell line H295R, we hypothesized that this adipokine represents a pathophysiologic link between aldosteronism and hypertension in overweight obese patients. Hence, our aim was to investigate the role of the CTRPs in particular CTRP-1 in the hypertension.

CTRP-1 gene expression was measured with quantitative real-time RT-PCR in human normal and pathologic adrenal tissue, adipose tissue, fibroblasts, vascular smooth muscle cells (VSMCs), endothelial and H295 cell line. CTRP-1 protein expression was investigated with immunohistochemistry (IHC) and confocal microscopy in sections from a wide array of tissues, including adrenal gland and fat. An enzyme-linked immunosorbent assay (ELISA) was developed to measure CTRP-1 levels in human plasma, and the plasma levels of CTRP-1 were measured in lean and obese subjects.

CTRP-1 gene was found to be expressed in all examined tissues. Higher expression levels were found in phaeochromocytoma than in APA and incidentaloma. All the examined cell types expressed CTRP-1, with the highest levels in VSMCs. IHC confirmed the expression of CTRP-1 protein in the adrenal gland and the adipose tissue. A marked immunoreactivity was also found in the media layer of the small peri-adrenal arteries.

The high CTRP-1 expression levels in the APA suggested that CTRP-1 might modulate the aldosterone synthesis in the zona glomerulosa by autocrine/paracrine effects. The marked CTRP-1 expression in the media layer of the small arteries and in the VSMCs may also suggest a role of CTRP-1 in the vascular remodelling associated with aldosteronism.

Riassunto

Background: CTRP1 (Complement-C1q tumour necrosis factor-α-related protein) è un'adipokina recentemente identificata, che stimola la produzione di aldosterone in una linea cellulare di adrenocarcinoma (H295R). Pertanto CTRP-1 potrebbe rappresentare un link fisiopatologico tra iperaldosteronismo e ipertensione arteriosa dei pazienti con sovrappeso-obesità.

Obiettivo: Indagare il ruolo delle CTRPs in particolare la CTRP-1 nel ipertensione

Metodi: L'espressione del gene CTRP-1 è stata misurata mediante real-time RT-PCR quantitativa. A tal fine, l'RNA è stato estratto da tessuto surrenalico umano (incidentalomi, feocromocitomi, adenomi producenti aldosterone (APA), da tessuto adiposo omentale e perisurrenalico, e da linee cellulari di fibroblasti, cellule muscolari lisce, cellule endoteliali e H295. L'espressione di CTRP-1 a livello proteico è stata indagata mediante immunoistochimica (IHC) in sezioni paraffinate di surrene, tessuto adiposo, aorta, arteria polmonare, tibiale e mammaria e verificata mediante la microscopia confocale.

Risultati: CTRP-1 è risultata espressa nel tessuto adiposo (espressione media: 181±105) e in tutti i tessuti surrenalici esaminati. I feocromocitomi (50±22) hanno mostrato livelli di espressione maggiori rispetto a APA (30±10) e a incidentalomi (33±23). Tutti le linee cellulari esprimono CTRP-1; le VSMCs hanno più alti livelli di espressione rispetto alle cellule endoteliali e ai fibroblasti. L'IHC ha confermato l'espressione di CTRP-1 a livello proteico nel surrene e nel tessuto adiposo e evidenziato che CTRP-1 è espressa prevalentemente nella zona glomerulosa. Il segnale IHC è risultato particolarmente intenso nella tonaca media delle arteriole perisurrenaliche, meno intenso nelle arterie mammarie, polmonare e tibiale ed appena rilevabile nell'aorta.

Conclusioni. Questi risultati dimostrano che nell'uomo l'espressione di CTRP-1 non è confinata al tessuto adiposo, ma si verifica anche nel surrene, cuore e nella arteria. Gli elevati livelli di espressione di CTPR-1 nell'APA e nel feocromocitoma suggeriscono

un'attività autocrino/paracrina di CTRP-1 nella modulazione della sintesi di aldosterone e nella regolazione del tessuto cromaffine, un effetto sulla proliferazione cellulare, similmente ad altre citokine. La localizzazione di CTRP-1 nella tonaca media arteriolare e gli alti livelli di espressione genica nelle VSMC suggeriscono un ruolo di CTRP-1 nel rimodellamento vascolare associato all'iperaldosteronismo.

Introduction

Definition and epidemiology of obesity

In the United States and elsewhere in the world obesity has increased dramatically since 1980. Between 1980 and 2004 the prevalence of obesity increased from 15% to 33% among adults and the prevalence of overweight in children increased from more than 6% to 19% in the United States. (1, 2) Overweight children often become obese adults, and obesity in adults is associated with an increased risk of morbidity and mortality.(3)

Obesity generally is defined as excess body fat. The human body contains essential lipids and also nonessential lipids in the form of triglycerides stored in the adipose tissue cells. The definition of excess, however, is not clear-cut. Adiposity is a continuous trait not marked by a clear division into normal and abnormal. Moreover, it is difficult to measure body fat directly. Consequently, obesity often is defined as excess body weight rather than as excess fat. In epidemiologic studies, body mass index (BMI) calculated as weight in kilograms divided by height in meters squared is used to express weight adjusted for height. The WHO classification defined overweight as a BMI of 25 or greater and obesity as a BMI of 30 or greater, along with some additional subdivisions (grade I, II and III for BMI ranges 30-34.9, 35-39.9 and > 40, respectively). Similar definitions were recommended by a National Heart, Lung, and Blood Institute (NHLBI) expert committee in the NHLBI Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults and by the US Dietary Guidelines.(4)

Prevalence estimates of obesity usually are derived from surveys or population studies because systematic data on obesity generally cannot be gathered from medical records. Virtually all data on prevalence and trends are based on measurements of weight and height using the classifications described earlier rather than on body fat because of the logistical difficulties involved in measuring body fat in population studies. A series of cross-sectional, nationally representative examination surveys conducted by the National Health and Nutrition Examination Survey (NHANES) provided national estimates of

overweight and obesity for adults, adolescents, and children in the United States from 1960.(2) Because the NHANES program before 1988 only included individuals up through 74 years of age, trends are shown for adults 20–74 years of age. The prevalence changed relatively little over the time period from 1960 to 1980. In the early 1960s, the prevalence of obesity was 11% among men and 16% among women. Between 1976 and 1980 and 1988 and 1994, the prevalence of increased considerably, to about 21% in men and to about 26% in women. By 2003–2004 the prevalence had increased to almost 32% in men and 34% in women. (Tab.1)

Table 1. Prevalence and Trends of Overweight and Obesity Among Adults Ages 20–74 Years in the United States: 1960–2004

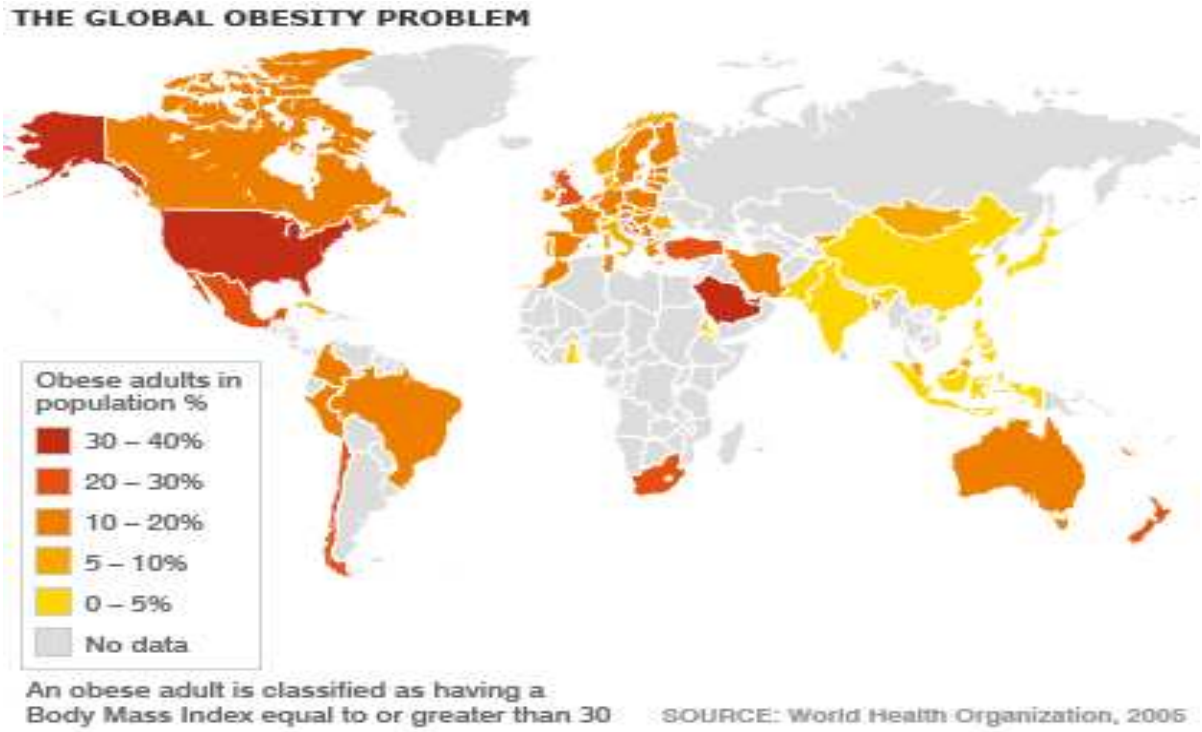
Sex	Survey year[a]	Overweight or obese[b]		Overweight but not obese		Obese[b]		Extremely obese[b]	
		%	SE	%	SE	%	SE	%	SE
All	1960–1962	44.9	.8	31.5	.5	13.3	.6	.9	.1
	1971–1974	47.2	.8	32.7	.6	14.5	.4	1.3	.2
	1976–1980	47.1	.8	32.1	.6	15.0	.4	1.4	.1
	1988–1994	55.9	.9	32.6	.6	23.2	.7	3.0	.3
	1999–2000	64.5	1.6	33.6	1.0	30.9	1.6	5.0	.6
	2001–2002	65.7	.7	34.4	1.1	31.3	1.2	5.4	.5
	2003–2004	66.2	1.1	33.4	1.2	32.9	1.3	5.1	.6
Men	1960–1962	49.4	1.1	38.7	.7	10.7	.7	.3[c]	.1
	1971–1974	53.8	1.1	41.7	1.1	12.1	.6	.6[c]	.2
	1976–1980	52.6	1.0	39.9	.8	12.7	.6	.4	.1
	1988–1994	60.9	1.0	40.3	.8	20.5	.7	1.8	.3
	1999–2000	67.0	1.8	39.2	1.5	27.7	1.6	3.3	.7
	2001–2002	69.9	1.0	41.5	1.5	28.4	1.1	3.9	.7
	2003–2004	71.1	1.5	39.4	1.5	31.7	1.4	3.0	.4
Women	1960–1962	40.5	1.0	24.7	.8	15.8	.6	1.4	.2
	1971–1974	40.9	.8	24.3	.7	16.6	.6	2.0	.3
	1976–1980	41.9	1.0	24.9	.8	17.0	.6	2.2	.3
	1988–1994	51.0	1.1	25.1	.8	25.9	1.0	4.1	.3
	1999–2000	62.0	2.3	28.0	1.7	34.0	1.8	6.6	.8
	2001–2002	61.4	1.0	27.3	1.6	34.1	1.6	6.8	.6
	2003–2004	61.4	1.9	27.4	1.3	34.0	1.9	7.3	1.0

NOTE. Overweight, BMI 25 to _30; obesity, BMI _ 30; extreme obesity, BMI _ 40 (in kg/m2). SE, standard error.
[a]National Health Examination Survey (1960–1962); National Health and Nutrition Examination Survey (I, 1971–1974; II, 1976–1980; III, 1988–1994; 1999–2000; 2001–2002; 2003–2004).
[b]Significant increasing trend for all, men, and women (*P* _ .05).
[c]Does not meet standard of statistical reliability and precision. Relative SE was _30% but _40%.

Table 1: Adapted from Ogden et al., GASTROENTEROLOGY 2007;132:2087–2102

The gradual increase in the prevalence of obesity has not been observed in the United States alone. Similar increases have been reported from a number of other countries and regions of the world. (Fig.1) For example, in England the prevalence of obesity among women 25–34 years of age increased from 12% to 24% in only 9 years between 1993 and 2002.(5) Less-developed countries also have seen increases in obesity. Differences in the prevalence of obesity between countries in Europe or between race-ethnic groups in the United States tend

to be more pronounced for women than for men.(6) For example, in Europe, the WHO Multinational Monitoring of trends and determinants in Cardiovascular Disease Study, which gathered data from 39 sites in 18 countries, found the prevalence of obesity was similar for men across all sites. For women, however, there were marked differences in prevalence between sites, with higher values for women from Eastern Europe. Similarly, in the United States, there are marked differences in the prevalence of obesity by race-ethnic group for women but not for men.



Excess cardiovascular risk associated with obesity

Obesity is considered a major risk factor for cardiovascular (CV) disease. The modernized lifestyle in Western societies dramatically increased the prevalence of obesity and overweight in the past years, mainly due to reduced physical activity, and extensive consumption of unfavorable dietary fats in low-cost food. Consequently, obesity increases various risk factors that contribute to early morbidity and mortality. Several studies identified obesity, particularly truncal obesity in men, to be an independent risk factor for coronary artery disease, which is the main cause of death in the Western population.(7) However, several other metabolic disorders such as hypertriglyceridemia, increase in small

dense LDLs, low HDL levels, insulin resistance and diabetes mellitus are commonly associated with obesity and all of them appear to be important risk factors for coronary artery disease as well.

Obesity is also associated with increased risk of heart failure. This association is deemed to be mediated by increased blood volume, elevated cardiac output, left ventricular hypertrophy, left ventricular diastolic dysfunction, and adipositas cordis that are caused by obesity. However, frequently coexisting conditions like diabetes, hypertension and coronary artery disease contibute to enhance the risk. In the Framingham heart study, after 14 years of follow-up for 5881 patients heart failure developed in 496. After adjustment for established risk factors there was a calculated increase in the risk of developing heart failure by 5% in men and 7% in women for each increment of 1 above 30 on the BMI.(8)

An increase in the incidence of sudden cardiac death and arrhythmias in obesity was also reported.(9) Fatal arrhythmias may be the most frequent cause of death among obese patients and according to the Framingham data, sudden cardiac death was 40 times higher in obese men and women.(9) (Moreover, increased BMI and waist – hip ratio are independent risk factors for stroke, even after adjusting for hypertension, hypercholesterolemia, and diabetes (Fig.2). In the prospective Physician's Health study cohort of 21,414 men, overweight/obese patients had a multiple adjusted relative risk of total stroke of 1.32 (95% CI, 1.14 –1.54) and 1.91 (95% CI, 1.45–2.52), respectively, compared to normal weight subjects.(10)

The association between overweight/obesity is mediated only in part by the traditional risk factors, such as hypertension, dyslipidemia (particularly low levels of high-density lipoprotein and increased levels of triglycerides), and insulin resistance/impaired glucose tolerance. In fact, evidences show that inflammation can be an independent factor linking obesity with CVD. In particular, the metabolically active fat associated with visceral obesity is thought to play a role in the inflammatory processes underlying atherosclerosis, which, in turn, may cause CV disease, particularly coronary artery disease and stroke.

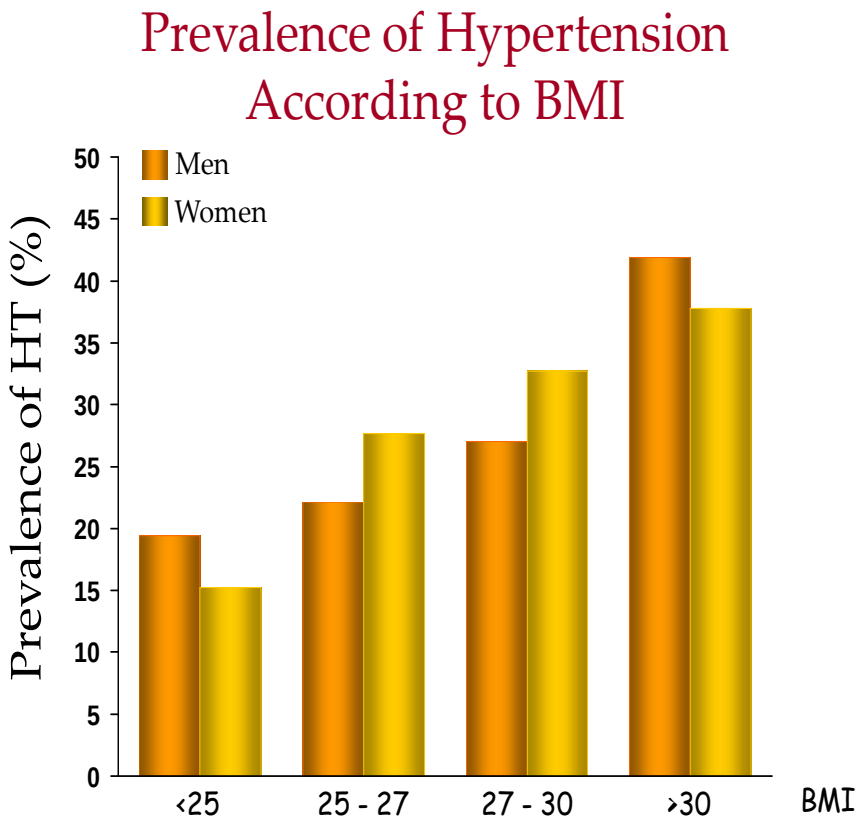


Figure 2: Mod. from Brown C.D. et al. Obes Res 2000; 8: 605

The coexistence of overweight/obesity and HT in the metabolic syndrome

The metabolic syndrome (MS) entails a group of modifiable risk factors occurring in the same individual and associated with an increased risk of developing cardiovascular disease and type 2 diabetes mellitus. Reports of clustering of metabolic risk factors date back to the early 1920s.(11) However, there was little interest in this phenomenon until 1988, when Gerard M. Reaven coined the term syndrome X to describe a disorder consisting of insulin resistance, glucose intolerance, increased triglyceride and decreased high-density lipoprotein (HDL) cholesterol levels, and hypertension.(12) He postulated that the common feature was insulin resistance and that all the other changes were likely to be secondary to this basic abnormality (Tab. 2).

A year later, Norman Kaplan added another factor, i.e. central adiposity (increase of the fat depots in the abdominal region) to the disorders described by Reaven, and since then

obesity was considered one of the typical components of the syndrome.(13) Kaplan summarized the main characteristics of the syndrome as four components (central adiposity, impaired glucose tolerance, hypertriglyceridaemia and hypertension), which were clustered as '*the deadly quartet'* to point out its great importance for the development of cardiovascular disease. Because during the following years the results of several studies suggested a central role of the insulin resistance for the development of the remaining disorders of the syndrome, DeFronzo and Ferrannini used the term 'insulin resistance syndrome' to describe this entity.(14)

IDF	NCEP	WHO	AACE
Diagnosed if glycemia is abnormal and 2 further criteria are present	Diagnosed if 3 out of 5 criteria are present	Diagnosed if glycemia is abnormal and 2 further criteria are present	Indicates risk factors
Fasting glycemia 100-125 mg/dL or DM2	Glycemia 110-125 mg/dL	Glucose intolerance, DM2 or insulin-resistance due to HOMA-IR	Fasting glycemia 110-125 mg/dL or > 140 mg/dL 2 hours after oral GTT
WC ≥ 94 cm MWC ≥ 80 cm W	WC > 102 cm MWC > 88 cm W	BMI > 30 and HWR > 0.9 M and > 0.85 W	BMI ≥ 25 and WC >102 cm M and WC > 88 cm W
Tg ≥ 150 mg/dL or HDL< 40 M and < 50 W	Tg ≥ 150 mg/dL or HDL< 40 M and < 50 W	Tg ≥ 150 mg/dL or HDL< 35 M and < 39 W	Tg ≥ 150 mg/dL or HDL< 40 M and < 50 W
On treatment for SAH or BP ≥ 130x85 mmHg	BP ≥ 130x85 mmHg	On treatment for SAH or BP ≥ 160x90 mmHg	BP ≥ 130x85 mmHg
		Microalbuminuria ≥ 20 mcg/min	

AACE = American College of Endocrinology/American Association of Clinical Endocrinologists; BMI = Body mass index; BP = arterial blood pressure; DM2 = diabetes mellitus type 2; GTT = oral glucose tolerance test; HOMA = homeostasis model assessment; HWR = hip:waist ratio; IDF = International Diabetes Federation; M = men; NCEP = US National Cholesterol Education Program; SAH = systemic arterial hypertension; Tg = triglycerides; W = women; WC = waist circumference; WHO = World Health Organization.

Table 2: Definitions of metabolic syndrome

Currently there are three definitions more commonly recognized, as the one suggested by the International Diabetes Federation (IDF), National Cholesterol Education Program Adult Treatment Panel (NCEP), World Health Organization (WHO), and American College of Endocrinology/American Association of Clinical Endocrinologists (AACE). In all definitions hypertension has a central role: beyond the small differences in the definition and the emergency of the so-called new risk factors, all of metabolic, pro-inflammatory and pro-thrombotic nature, hypertension is one constant in each definition.

The prevalence of the metabolic syndrome increases with the age and is present in almost half of the over 60 population (Fig. 3).

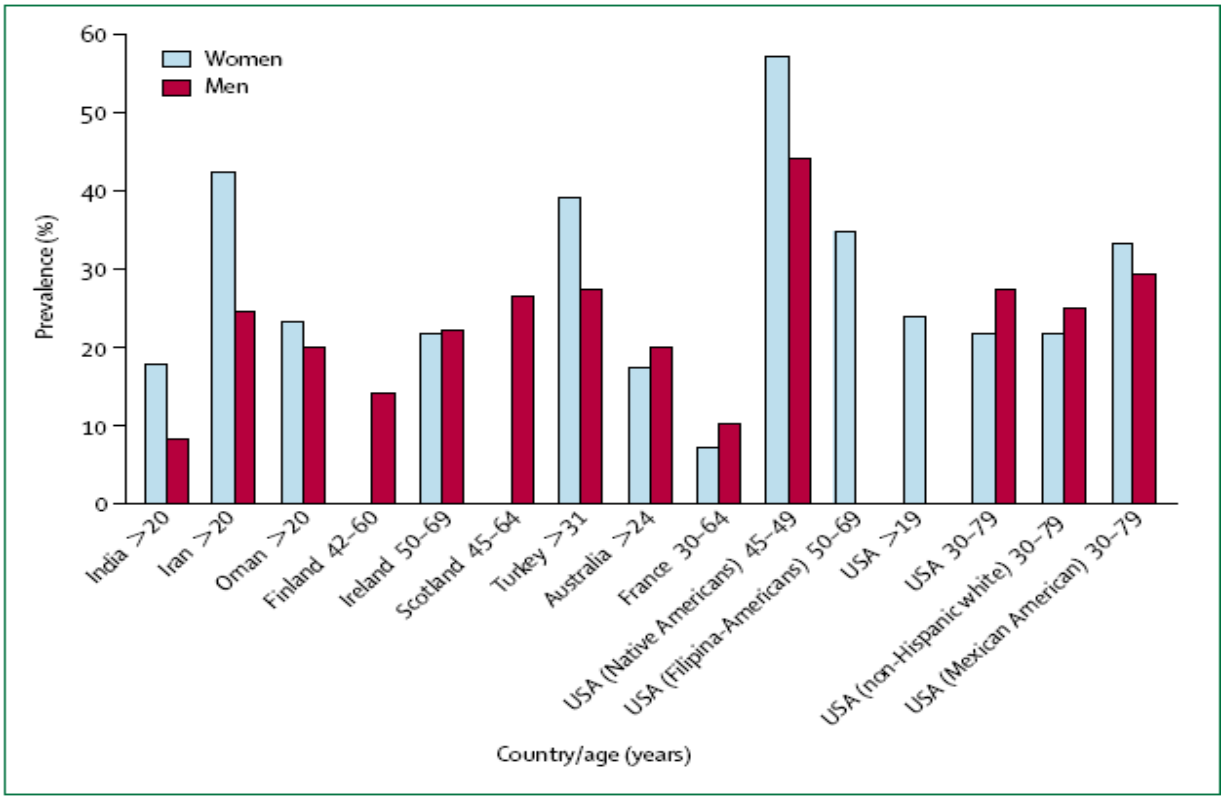


Figure 3: Prevalence of the metabolic syndrome from NCEP - ATPIII definition. Adapted from Cameron et al. Endocrinol Metab Clin North Am 2004; 33: 351–75.

Moreover, its prevalence increases with aging from 6.7% in patients with 20-29 years to 43% in the over 70. In both genders the prevalence quickly increases after the third decade of life, reaching a peak between the 50 and 70 years in the men and the 60-80 years in the women.

Notwithstanding the key role of hypertension in the MS the underlying mechanism remains poorly known.

The prevailing theory is that obesity leads to insulin resistance and compensatory hyperinsulinemia.(15) Most insulin-resistant individuals are able to maintain the degree of hyperinsulinemia required to prevent decompensation of glucose homeostasis. If pancreatic insulin secretion fails to increase adequately, impaired glucose tolerance or diabetes mellitus develops. The metabolic syndrome is a proinflammatory and prothrombotic state contributing to the metabolic and vascular abnormalities. The adipose tissue, in fact, not only stores fat, but also acts an active endocrine organ that respond to signals from many sources, including the central nervous system. Adipocytes are a source of metabolically active substances, as more described below in detail.

Moreover, hyperinsulinemia secondary to insulin-resistance may play an important role in raising blood pressure via stimulation of the sympathetic nervous system, with consequent vasoconstriction, and/or retention of sodium ion (Na^+) responsible for increasing plasma volume. In fact, because insulin regulates Na^+ re-absorption at the tubular level by affecting Na^+ / H^+ pump chronic, chronic hyperinsulinemia associated with the metabolic syndrome translates into reduced renal sodium excretion and increased volemia. (Fig.4)

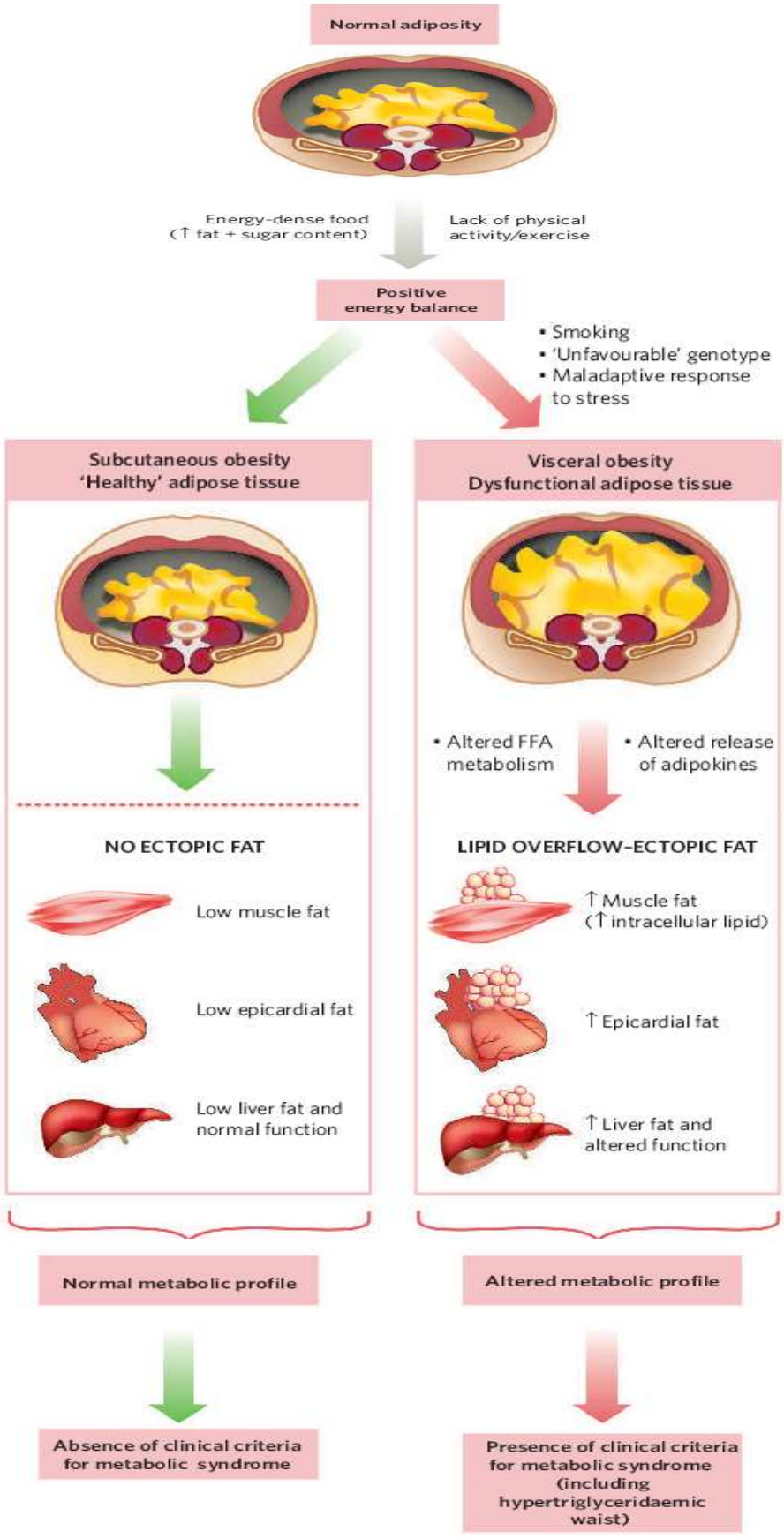


Figure 4: Adapted from Despres JP, Nature. 2006;444:881-887

Figure 4 : Excess visceral fat accumulation might be causally related to the features of insulin resistance, but might also be a marker of a dysfunctional adipose tissue being unable to appropriately store the energy excess. According to this model, the body's ability to cope with the surplus of calories (resulting from excess caloric consumption, a sedentary lifestyle or, as is often the case, a combination of both factors) might, ultimately, determine the individual's susceptibility to developing metabolic syndrome. There is evidence suggesting that if the extra energy is channelled into insulin-sensitive subcutaneous adipose tissue, the individual, although in positive energy balance, will be protected against the development of the metabolic syndrome. However, in cases in which adipose tissue is absent, deficient or insulin resistant with a limited ability to store the energy excess, the triacylglycerol surplus will be deposited at undesirable sites such as the liver, the heart, the skeletal muscle and in visceral adipose tissue.
Factors associated with a preferential accumulation of visceral fat and with
Features of insulin resistance include, among others, smoking, the well documented genetic susceptibility to visceral obesity and a neuroendocrine profile related to a maladaptive response to stress. The resulting metabolic consequences of this 'defect' in energy partitioning include visceral obesity, insulin resistance, an atherogenic dyslipidaemia and a pro-thrombotic, inflammatory profile. These are defining features of metabolic syndrome. This constellation of abnormalities can be detected by the clinical criteria for metabolic syndrome, the two simplest being the simultaneous presence of increased waist girth and fasting triacylglycerol levels, a condition that has been described as 'hypertriglyceridaemic waist'

Pathophysiology

Pathophysiology of visceral obesity

There is ample evidence that an impaired non-esterified fatty acid (NEFA) metabolism could contribute to the insulin-resistant state observed among individuals with visceral obesity. Hypertrophied intra-abdominal adipocytes are characterized by a hyperlipolytic state that is resistant to the anti-lipolytic effect of insulin. The resulting NEFA flux to the liver may impair liver metabolism, leading to increased hepatic gluconeogenesis. Hepatic insulin resistance is also associated with decreased apolipoprotein B degradation and increased production of triacylglycerol-rich lipoproteins. A high-fat diet promoting visceral adiposity in a canine model of diet-induced visceral obesity can induce hepatic insulin resistance with respect to glucose production, whereas the sensitivity of peripheral tissues seems to be less affected by the diet induced increase in visceral adiposity. In humans, although there is a correlation between visceral fat accumulation and portal delivery of NEFAs to the liver, most portal NEFAs originate from the systemic circulation. This suggests that other factors might explain the altered metabolic profile of viscerally obese patients. There is evidence that adipose tissue is not only specialized in the storage and mobilization of lipids, but that it is also a remarkable endocrine organ releasing numerous cytokines including pro-inflammatory molecules such as interleukin (IL)-6 and tumour necrosis factor-α (TNF-α). In obesity, there is evidence of macrophage infiltration in adipose tissue, which could contribute to the inflammatory profile that has been reported in abdominally obese patients. In fact, plasma levels of C-reactive protein (CRP), an inflammatory marker that is predictive of a risk of myocardial infarction possibly greater than that estimated by traditional risk factors, are increased in patients with visceral obesity.

The protein adiponectin is specifically derived from the adipose tissue. However, as opposed to many other pro-inflammatory adipokines, adiponectin plasma levels are reduced in obese individuals, particularly among patients with excess visceral adiposity. Thus is important to since adiponectin has been found to have many effects in vitro that are compatible with improved insulin signaling and potential protection against atherosclerosis.

The reduced adiponectin levels observed in viscerally obese patients could therefore be one of the key factors responsible for their atherogenic and diabetogenic metabolic risk factor profile. Abdominally obese patients with an excess of visceral adipose tissue have elevated plasma CRP concentrations accompanied by elevated IL-6 and TNF-α levels and by reduced adiponectin concentrations. However, although low adiponectin levels are a salient feature of visceral obesity, whether this adipokine has a central role in the altered metabolic risk profile of patients with visceral obesity remains uncertain. In fact, in a study by our group where the propensity score was used to adjust for the unbalanced distribution of risk factors between patients with adiponectin above and below the median value, no relationship with cardiovascular events was found.

Overall, these results are consistent with an important endocrine function of the expanded visceral adipose depot not only via altered NEFA metabolism but also through a proinflammatory state(16) that might contribute to the insulin resistance and altered glucose homeostasis of viscerally obese patients. Both the altered NEFA metabolism and the endocrine function hypotheses imply that visceral adipose tissue is causally involved in the pathophysiology of the metabolic syndrome that is often found in patients with visceral obesity. However, another possibility (which does not exclude a contribution from the two mechanisms described above) is that excess intra-abdominal fat accumulation represents a marker of the relative inability of subcutaneous adipose tissue to act as an 'energy sink' when an individual has to handle a calorie surplus due to excess energy intake and/or reduced energy expenditure (Fig. 4). Such a relative deficit in the capacity of subcutaneous fat to store excess energy would result in increased accumulation of fat at undesired sites such as the liver, the skeletal muscle, the heart and even in pancreatic β-cells, a phenomenon that has been described as "ectopic fat deposition" (17). In humans, the severe insulin-resistant state found in patients with lipodystrophic conditions is also consistent with the role of subcutaneous adipose tissue as a depot buffering the energy excess. In accordance with this hypothesis, treatment with glitazones increases subcutaneous fat deposition, which might help to explain the beneficial effects of this class of drug on muscle and liver insulin sensitivity. Thus, the insulin-resistant dyslipidaemic state found in patients with the

metabolic syndrome might be only partly explained by the peculiar metabolic and endocrine properties of the expanded visceral adipose tissue. Visceral obesity might also be a marker of defective fat partitioning between the adipose tissue, the skeletal muscle, the liver and the heart. On the basis of the association between abdominal, especially visceral, adiposity and the presence of the features of metabolic syndrome, the measurement of waist circumference has been proposed as a crude anthropometric correlate of abdominal and visceral adiposity(18).

Adipokines

Adipokines are proteins produced mainly by adipocytes. Although the adipose tissue secretes a variety of factors, only leptin and adiponectin (and possibly resistin, adipsin, and visfatin) are primarily produced by adipocytes and can therefore be properly classified as adipokines (19).

The first description of the cDNA encoding Acrp30 was reported in 1995.(20) The transcript encoded a hydrophilic protein with a predicted size of 29 kDa and contained a putative N-terminal signal sequence. The protein was called adipocyte complement-related protein of 30 kDa (Acrp30) based on its closest homolog, complement factor C1q, and on size by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). This cDNA was isolated by a subtractive hybridization screen comparing 3T3-L1 adipocytes with undifferentiated pre-adipocytes. The mRNA was induced over 100-fold during differentiation and was expressed exclusively in adipocytes. Developmentally, expression of the mRNA for Acrp30 is detected at late stages of embryogenesis, on day 17 of gestation (21). This expression pattern is consistent with that seen in the mouse for other adipocyte markers during embryogenesis (22).

The ACRP30 protein was secreted and detected in plasma. Subsequently, several groups independently reported the isolation of both mouse and human forms of Acrp30: AdipoQ , apM1, adiponectin and gelatin-binding protein 28 (GBP28). All these names (Acrp30,

AdipoQ, apM1, GBP28 and adiponectin) are currently used interchangeably in the literature(23).

Adiponectin

Adiponectin is the adipokine that circulates at the highest levels (in the microgram per milliliter range versus nanograms per milliliter for leptin) and is known for its role in the regulation of insulin sensitivity (24). The adiponectin molecule is composed of a globular and a collagenous domain. Once synthesized, adiponectin forms trimers, which then oligomerize to form polymers composed of 4 to 6 trimers (Fig 5). Both trimers and oligomers, but not monomers, of adiponectin are present in the circulation. The globular domain of adiponectin presents close structural, though not sequence, similarities with TNF-α (25). Both the full-length and the globular fractions have been used to evaluate the biologic activity of adiponectin, and a debate exists as to whether the 2 forms have the same activity. Leukocyte elastase cleaves adiponectin and generates the globular domain, which can then trimerize but does not further polymerize (26). Thus activated leukocytes might modulate adiponectin bioactivity in ways that remain unclear. A further level of complexity is added by the glycosylation pattern of adiponectin, which appears to be necessary for full biologic activity. Although adipocytes are the most important source of adiponectin, serum adiponectin levels do not increase with obesity as leptin levels do, but rather there is a tendency for reduced adiponectin levels in obese subjects and increased levels in lean subjects as patients with anorexia nervosa(27). The finding that adiponectin levels are significantly reduced in patients with type 2 diabetes, suggested that adiponectin can act as an insulin sensitizer. However, the mechanism by which the insulin-resistant state is associated with low levels of adiponectin is not clear. TNF-α, which is increased in the visceral adipose tissue of obese subjects, might down-regulate adiponectin production and, interestingly enough, adiponectin reduces the production and activity of TNF-α. Adiponectin has also been contended to play an anti-inflammatory role that can be

protective for the vascular wall. The anti-inflammatory activities of adiponectin extend to inhibition of IL-6 production and induction of the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist. Inhibition of nuclear factor kB (NF-kB) by adiponectin might explain at least part of these effects. Furthermore, adiponectin reduces induction of the endothelial adhesion molecules ICAM-1 and vascular cell adhesion molecule 1 by either TNF-α or resistin. In vivo overexpression of adiponectin in apolipoprotein E– deficient mice suppresses atherosclerosis, in part by down regulating adhesion molecules. On the basis of all the above-mentioned effects, adiponectin appears to act as an anti-inflammatory molecule (Fig 5).(19)

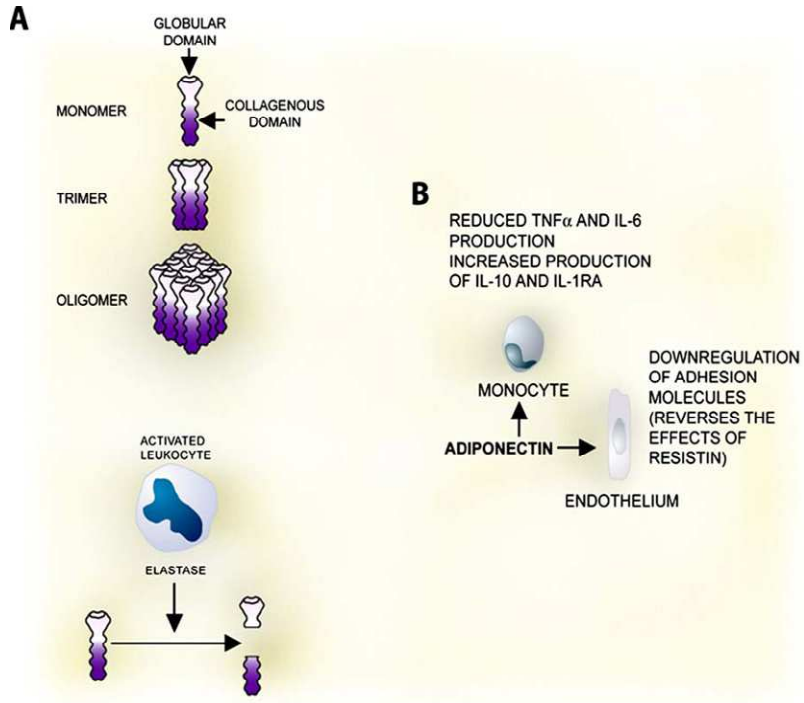


Figure 5: Adiponectin: structure and anti-inflammatory effects. Left, Adiponectin is composed of a collagenous and a globular domain. Adiponectin monomers trimerize through tight interactions in the collagenous domain. Trimers can then oligomerize. Both trimers and oligomers are present in the circulation and might have different effects on insulin sensitivity. Leukocyte elastase released by activated immune cells cleaves the globular domain of adiponectin, which might have activities distinct from those of the full-length molecule. Right, Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist in monocytes-macrophages, while inhibiting IL-6 and TNF-a levels. Adiponectin also inhibits the biologic activity of TNF-a. In endothelial cells adiponectin down-regulates the expression of adhesion molecules, thus contrasting the effect of resistin.

Leptin

Leptin is a 16-kd protein encoded by the ob gene (28). Adipocytes are the most important source of leptin, and circulating leptin levels directly correlate with adipose tissue mass. (29). Control of appetite is the primary role of leptin. In fact, mice with a mutation in the leptin (ob/ob mice) or leptin receptor (db/db mice) gene, as well as human subjects with

mutations in the same genes, are massively obese. Excellent reviews have been published on the effects of leptin in regulating appetite and other physiologic functions. (28, 30) Leptin's role in regulating immunity has been fueled by early observations of thymus atrophy in db/db mice. Since its cloning in 1994, many details of leptin's effects on the immune and inflammatory response have been clarified. Below, we briefly summarize these effects (31). Leptin protects T lymphocytes from apoptosis and regulates T-cell proliferation and activation. Leptin also influences cytokine production from T lymphocytes, generally switching the phenotype toward a TH1 response. Of note, cytokine production from T lymphocytes is suppressed in leptin deficient children and restored by leptin administration. In addition to its effects on T lymphocytes, leptin also influences monocyte activation, phagocytosis, and cytokine production. Signal transduction pathways activated by leptin in immune cells include the Janus kinase–signal transducer and activator of transcription system (particularly signal transducer and activator of transcription 3), as well as phosphatidylinositol 3-kinase and mitogen-activated protein kinase.

In endothelial cells leptin induces oxidative stress and up-regulation of adhesion molecules. In experimental animals inflammatory stimuli acutely induce leptin mRNA and increase serum leptin levels; however, this is not always true in human subjects. Most of the *in vivo* studies on the immune-modulating effects of leptin have been generated by using leptin-deficient ob/ob mice. In this setting leptin deficiency is associated with reduced inflammation in models of autoimmune disease but also with increased susceptibility to bacterial and viral infections. These are expected consequences of given the immune-activating effects of leptin. However, leptin deficiency is also associated with increased susceptibility to the toxicity of proinflammatory stimuli, such as endotoxin and TNF-α, an effect that might be mediated by leptin's activity on the kidney (32). Nevertheless, the general consensus is that leptin exerts a proinflammatory role, while at the same time protecting against infections. Figure 6 summarizes the role of leptin in modulating immunity and inflammation. (19).

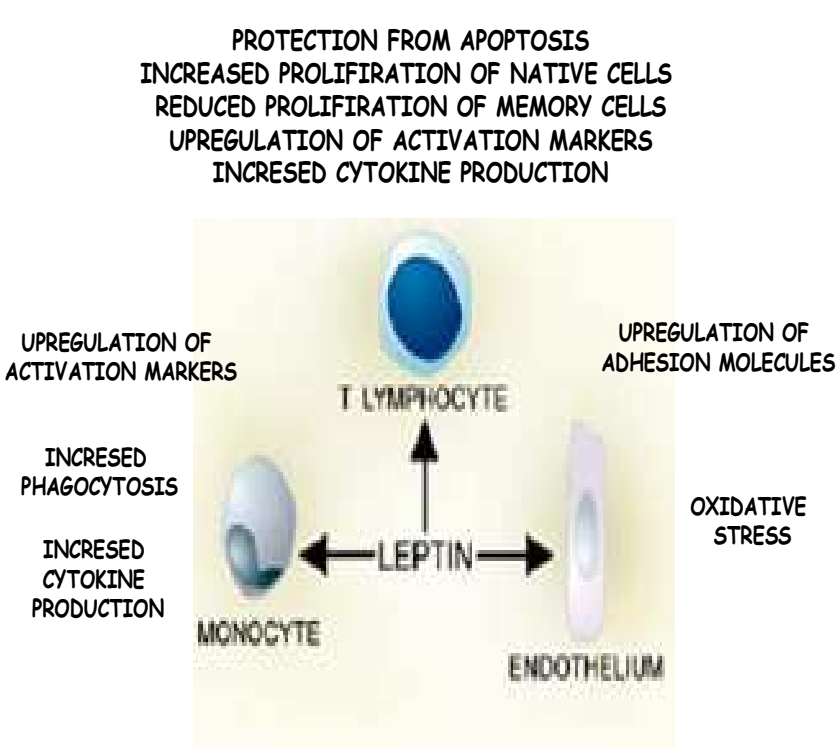


Figure 6: Effects of leptin on immunity and inflammation. Leptin is produced by adipocytes and acts on the long isoform of its receptor, which is expressed by many cell types, including lymphocytes, monocytes, and endothelial cells. Leptin protects T lymphocytes from apoptosis and modulates T-cell proliferation, increasing the proliferation of naive T cells while reducing the proliferation of memory T cells. Leptin modulates T cell–derived cytokine production and increases expression of the activation markers CD25 and CD71 in CD41 and CD81 cells. In monocytes leptin increases the expression of various activation markers and upregulates phagocytosis and cytokine production. In endothelial cells leptin upregulates the expression of adhesion molecules and induces oxidative stress. Many of the modulating activities of leptin in immune and inflammatory responses are observed only when a costimulus is present.

Adiponectin: Detection of the R1 and R2 receptor in the human adrenocortical zona glomerulosa (ZG) and in APA by our laboratory

The cloning and sequencing of the genes coding these receptor subtypes have been recently accomplished, thus providing novel opportunities for the investigation of their tissue distribution at the mRNA level.(33) As the adrenal gland is not only exposed to circulating adiponectin but is deeply embedded in adipose tissue, endocrine-paracrine interactions might exist between the adrenal cortex and adipocytes. Two adiponectin receptor subtypes, referred to as adipo-R1 and adipo-R2, mediate the biological effects of adiponectin in the normal adrenocortical ZG and in benign tumours. Hence, we detected that adipo-R1 and adipo-R2 genes are expressed not only in adipose tissue but also in adrenocortical tissue specimens. Overall even though no clear-cut effect of adiponectin an aldosterone production could be found, these finding cannot support to the hypothesis that visceral adipose tissue could affect the physiology and pathophysiology of aldosterone producing cells. This contention finds further support by the observation that

in the PAPY study BMI was directly significantly correlated with plasma aldosterone levels and that this relation was particularly significant in the hypertensive patients who were OO.(33) Histologically normal adrenocortical tissue, APA specimens and control adipose tissue were found to consistently express both adiponectin receptor subtypes. (34). Therefore several cytokines produced by visceral adipose tissue could be involved in causing arterial hypertension and related damage in target organs. Among these cytokines, the family of CTRP proteins could play an important role.

Biochemistry of CTRP

The members of the CTRP family show a similar biochemical structure, as illustrated in Figure 7.

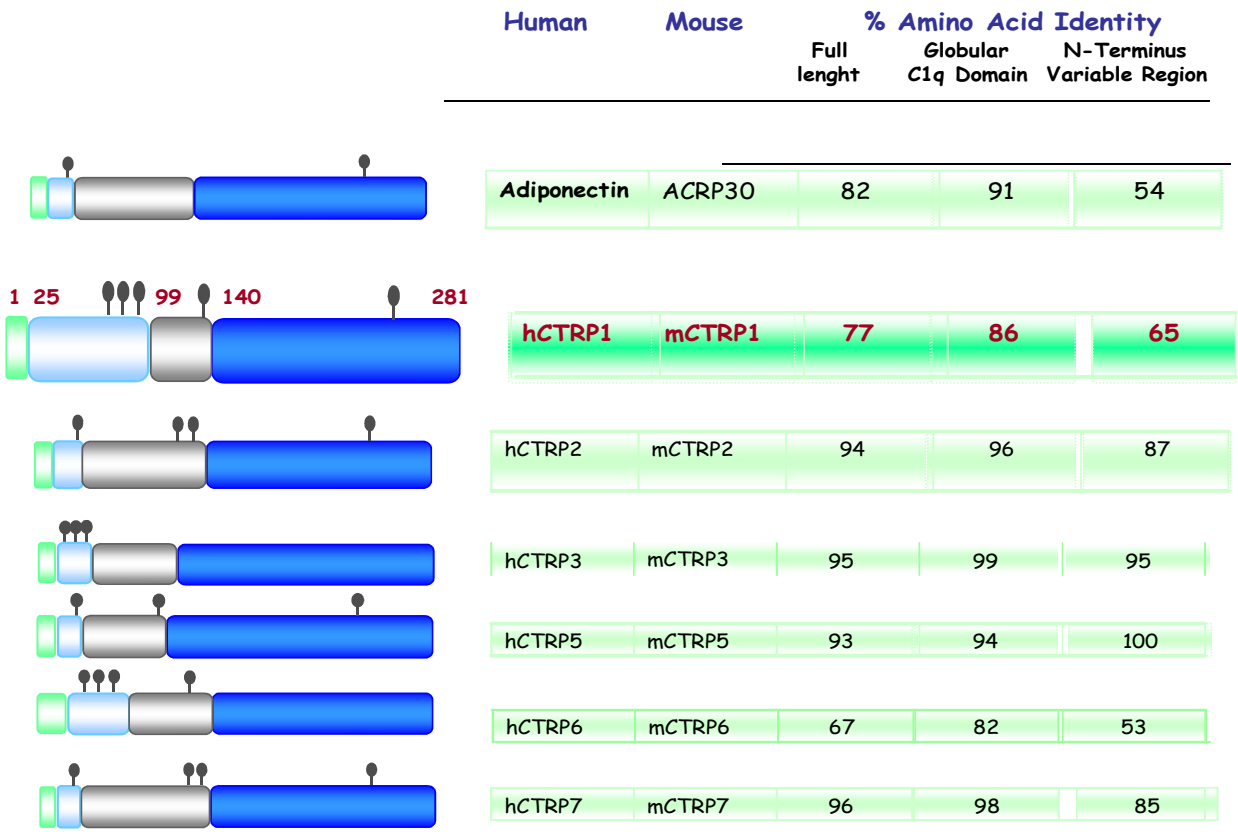


Figure 7: Structure of adiponectin/ACRP30 paralogs CTRPs 1–7. The predicted amino acid sequences of all of the CTRPs share a similar modular organization to adiponectin and consist of four distinct domains; a signal peptide (green), a short variable region (azure), a collagenous domain with various length of Gly-X-Y repeats (grey), and a C-terminal globular domain homologous to complement C1q (blue). Indicates cysteine residues; cysteine residues in the signal peptides are not shown because they are not part of the mature proteins. hCTRPs and their corresponding mouse orthologs are highly conserved. The numbers on the right refer to the percent amino acid identity between human and mouse orthologs when comparing the full-length protein (first column), the C-terminal globular domain (second column), or the N-terminal variable region (third column). Adapted from: *A family of Acrp30/adiponectin structural and functional paralogs.* G. W. Wong, et al.; PNAS **101,** 10302 (2004).

CTRPs were highly conserved during evolution. Messanger RNAs for CTRPs (mCTRP) and their corresponding human protein orthologs share 53–100% amino acid identity in their short N-terminal variable regions and 82–99% amino acid identity in their C-terminal globular domains (Fig. 7). The C-terminal globular domains of CTRP *paralogous* are also conserved, ranging from 27% to 73% amino acid identity between each other and among other proteins of the C1q/TNF-α superfamily (Table 3).

	Acrp30	mCTRP1	mCTRP2	mCTRP3	mCTRP4	mCTRP5	mCTRP6	mCTRP7
Acrp30	100							
mCTRP1	30	100						
mCTRP2	42	33	100					
mCTRP3	31	27	27	100				
mCTRP4	30	35	31	31	100			
mCTRP5	41	29	38	28	35	100		
mCTRP6	34	64	31	32	38	32	100	
mCTRP7	43	31	73	29	33	40	32	100

Numbers indicate percent amino acid identities.

Table 3: Comparison of the amino acid sequence of the C-terminal globular domains of adiponectin and CTRPs; published as supporting information on the PNAS (www.pnas.org cgi doi 10.1073 pnas.0403760101)

Of the seven CTRPs, the globular domains of mCTRP2 and mCTRP7 share the highest degree of amino acid identity (42–43%) to adiponectin. Structure-based alignment between adiponectin, complement C1q, and TNF family members (TNF-α, TNF-β, and CD40L) reveals four highly conserved residues (Tyr-161, Gly-159, Phe-237, and Leu-242 in adiponectin) in all CTRPs,(35) which are head to be important in the packing of the protomer's hydrophobic core.

The structural characterization of adiponectin and its homotrimeric globular C1 domain by Shapiro et al. in 1998 allowed to demonstrate that the TNF-ligand family proteins and the C1q complement family proteins originated by divergence from a precursor recognition

molecule of the innate immune system (36). The adiponectin protein and its gene structure share common characteristics with both the C1q family members and the TNF-ligand family members. Each of the 10 β-strands of the globular head domain of adiponectin can be superimposed simultaneously with the strands of TNF-α, TNF-β and CD40L. The relative positions and lengths of the β-strands are almost identical among adiponectin and the TNF ligands (36). The 3D organization of the globular head domain of globular C1q resembles that of a flower leading to the term 'bouquet of flowers' structure. The bouquet of C1q consists of six heterotrimers (18 polypeptide chains); the bouquet of adiponectin consists of four homotrimers (12 polypeptide chains). The C1q and TNF-ligand family proteins also share a similar gene structure (37). Both C1q and adiponectin have a collagenous stalk region with 22 perfect Gly–X–Y collagen triplets. The exact structural organization and the structure – function relationships of C1q, TNF ligands and adiponectin were reviewed extensively by Kishore et al. Interestingly, there is also evidence for a dichotomy of the globular C1q–receptor interaction because there are two high- and low-affinity receptors for globular C1q and full-length adiponectin: AdipoR1 and AdipoR2 (adiponectin receptors type 1 and type 2). These receptors can mediate both adiponectin - and globular C1q-induced activation of adenosine mono-phosphate activated kinase (AMPK) and p38 mitogen-activated protein kinase (p38 MAPK) and phosphorylation of ACC (acetyl-CoA-carboxylase) (38). Similar to certain TNF receptors, AdipoR1 and AdipoR2 can also form homo- and hetero-multimers (38). Taken together, proteins with a globular C1q domain similar to that of TNF, C1q and adiponectin have been classified as members of the newly described C1q/TNF molecular superfamily.

Family of CTRP

The identification of novel metabolic mediators produced by adipose tissue led to the discovery of a highly conserved family of secreted proteins, designated as C1q/TNF (Complement-C1q Tumour Necrosis Factor-*α* Related Protein) related proteins (CTRP-1 to -10).

Most CTRPs are expressed by adipose tissue and circulate in plasma. Information on their plasma levels in humans is scant. In animals their circulating levels vary according to sex, genetic background, and metabolic state. All CTRPs form trimers as their basic structural unit; CTRP-3, CTRP-5, CTRP-6 and CTRP-10 trimers are further assembled into higher-order oligomeric complexes via disulfide bonding mediated by their N-terminal cysteine residues. Besides forming homo-oligomers, CTRP-1/CTRP-6, CTRP-2/CTRP-7 and adiponectin/CTRP2 are secreted as heterotrimers, thus providing a mechanism to potentially generate functionally distinct ligands. Functional characterization of one such family member, CTRP-1, showed that it specifically activates Akt and p44/42- MAPK (mitogen-activated protein kinase) signalling pathways in differentiated mouse myotubes. Moreover, injection of recombinant CTRP-1 into mice significantly reduced their serum glucose levels. Thus at least CTRP-1 may be considered a novel adipokine. Additionally, CTRPs form combinatorial associations, representing a potential mechanism to generate functionally distinct ligands with altered receptor specificity and/or function (39). Recombinant protein administration or adenovirus-mediated over-expression of some of these CTRPs reduces blood glucose in mice. Apart from these studies, little is known about the biological significance and modes of action of CTRPs. (40, 41) However, at variance with the metabolic functions of adiponectin which are well characterized, the physiological processes regulated by CTRPs are only beginning to be appreciated.

CTRP-1

C1qTNF-related protein-1 (CTRP-1; GenBank accession: NM_198594) belongs to a family of proteins characterized by a common TNF alpha–like globular domain. (35, 36) A less conserved structural element in this family is the N-terminal collagen-like region with typical glycine-X-Y repeats. The basic structure of proteins in the CTRP family appears to be a trimer that, in turn, can form higher order structures.(42) While structurally related, members of this protein family are functionally diverse and include the plasma protein C1q, which is involved in immune functions and possibly platelet hemostasis (43), and

adiponectin (44, 45), and the hibernation proteins 20, 25 and 27, which are thought to be regulators of metabolism (46).

CTRP-1 was characterized originally as a vascular wall protein that can bind to fibrillar collagen and inhibits collagen-induced platelet aggregation by blocking von Willebrand factor binding to collagen (47). CTRP-1 contains a putative signal peptide and represents a secreted protein that forms monomers, dimers, trimers and multimeric complexes. CTRP-1 is expressed in preadipocytes and upregulated dramatically in rat adipose tissue on LPS stimulation and in adipose tissue of murine models with genetically determined obesity (48). This LPS effect was mediated by TNF and IL-1β (48) and both of these pro-inflammatory mediators can induce CTRP-1 expression in adipocytes. Thus, CTRP-1 can be regarded as an LPS-responsive and cytokine-responsive secreting product of adipose tissue that links inflammation, adipose tissue and platelet aggregation.

CTRP-1 was specifically expressed in the zona glomerulosa of the adrenal cortex, where aldosterone is produced. Increased aldosterone production by CTRP-1 in cells of the human adrenal cortical cell line H295R was dose-dependent and is expressed at high levels in the adipose tissue of obese Zucker diabetic fatty (fa/fa) rats.(49) CTRP-1 expression is induced by proinflammatory cytokines, including TNF-α and IL-1β. Moreover, CTRP-1 inhibits collagen-induced platelet aggregation by specifically blocking binding of the von Willebrand factor to collagen (47).

CTRP-2

C1qTNF-related protein-2 (GenBank accession no: NM_031908) consists of 285 aminoacids. It is held to increase glycogen accumulation and fatty acid oxidation by activating AMP-activated protein kinase (35).

CTRP-3

C1qTNF-related protein-3 (Cartducin/Cors-26; GenBank accession no: NM_181435) consists of 246 aminoacids. It is a growth plate cartilage-derived secretory protein first identified

during a search for the genes underlying the induction of chondrocyte differentiation. More recent studies confirmed that CTRP-3 indeed plays an important role in regulating chondrogenesis and cartilage development (50, 51). In fact, it stimulates chondrogenic precursor cell proliferation(51) and promotes angiogenesis(52). Consistently, CTRP-3 is overexpressed in the osteosarcoma cell line.(53)

CTRP-3, being detected in human monocytic and dendritic cells, was deemed to have a role in the inflammation.(54) Recently, Li et al.(55) examined the temporal patterns of gene expression in a balloon-injured rat carotid artery model using microarray analysis coupled with real time RT-PCR and found transient up-regulation of CTRP-3 gene in injured artery tissue during a period characterized by neointima formation. This latter involves multiple processes, including vascular smooth muscle cell proliferation, angiogenesis, vascular remodeling and extracellular matrix deposition.(56) The angiogenic process is also a multi-step process that requires proliferation and migration of endothelial cells, and is modulated by pro-angiogenetic factors and inhibitors(52). It is still unknown which steps of the angiogenic process can be modulated by CTRP-3. Moreover, it is unclear whether CTRP-3 plays a pro- or anti-inflammatory role in the neointima formation.

In primary monocytes derived from healthy, recombinant human CTRP-3 reduced proinflammatory cytokine IL-6 and TNF-α secretion in response to lipopolysaccharide stimulation, thereby suggesting an anti-inflammatory role (57).

Other *in vivo* studies showed that circulating levels of CTRP-3 are inversely correlated with leptin levels; CTRP-3 increases with fasting, while decreases in diet-induced obese mice with high leptin levels, and increases in leptin-deficient ob/ob mice. A modest 3-fold elevation of plasma CTRP-3 levels by recombinant protein administration is sufficient to lower glucose levels in normal and insulin- resistant ob/ob mice, without altering insulin or adiponectin levels. The glucose-lowering effect in mice is linked to activation of the Akt signalling pathway in liver and a marked suppression of hepatic gluconeogenic gene expression. Consistent with its effects in mice, CTRP-3 acts directly and independently of insulin to regulate gluconeogenesis in cultured hepatocytes. In humans, alternative splicing generates

two circulating CTRP-3 isoforms differing in size and glycosylation pattern. The two human proteins form heteroligomers, an association that does not require inter-disulfide bond formation and appears to protect the longer isoform from proteolytic cleavage. Recombinant human CTRP-3 also reduces glucose output in hepatocytes by suppressing gluconeogenic enzyme expression (58). Beyond these in vitro studies, the biological relevance and function of CTRP-3 in vivo remain unknown.

Human CTRP-3 are expressed in mature adipocytes (but not in preadipocytes) (59), and CTRP-3 acts as an adipocyte-derived immunomodulatory and anti-inflammatory secretory protein(57, 60). CTRP-3 is a secreted protein, which can be detected in human serum and forms stable trimers(61). CTRP-3 can reduce LPS-induced IL-6 and TNF secretion from monocytic cells by suppressing NFkB signaling (57). Moreover, PPARγ activation exerts anti-inflammatory effects and PPARγ can bind specifically to the CTRP-3 promoter and inhibit CTRP-3 gene expression when activated by troglitazone, an exogenous PPARγ activator. Furthermore, recombinant CTRP-3 stimulates anti-inflammatory adiponectin secretion and proinflammatory resistin secretion from mature adipocytes (60). Thus, CTRP-3 can be regarded as a potent anti-inflammatory adipokine secreted by the adipose tissue and it further regulates the adipocytic secretion of immunomodulatory adipokines.

CTRP-5

C1qTNF-related protein-5 (GenBank accession no: NM_015645) is a 243-amino acid protein that consists of an N-terminal signal peptide, a collagen repeat, and a C-terminal globular domain. It was initially identified as a gene responsible for late-onset macular degeneration and long anterior lens zonules. Mutation of CTRP-5 leads to insufficient levels of secreted CTRP-5, causing late-onset retinal macular degeneration. The recent study showed that CTRP-5 mediates translocation of glucose transporter 4 (GLUT4) and fatty acid oxidation via the activation of AMP-activated protein kinase (AMPK). Serum CTRP-5 levels are significantly higher in obese/diabetic animals (OLETF rats, ob/ob mice, and db/db mice). Although the expression pattern of CTRP-5 is still not completely clear, several studies

showed that CTRP-5 is widely expressed in various tissues, including adipose tissues, liver, spleen, lung, eye, and testis. CTRP-5 is involved in glucose and lipid metabolism, the mechanism by which the CTRP-5 gene is activated in the liver at a transcriptional level. However, the basis for transcriptional regulation of CTRP-5 expression is largely unknown (62).

CTRP-6

C1qTNF-related protein-6 (GenBank accession no: NM_031910) is a 240-amino acid protein that consists of a signal peptide, a short variable region, a collagen- like region, and a C-terminal globular domain. CTRP-6 can stimulate fatty acid oxidation via the activation of AMP-activated protein kinase (AMPK) (62).

CTRP-8

C1qTNF-related protein-8 (GenBank accession no: NM_207419) is expressed predominantly in lung and testis. In addition to forming homotrimers, CTRP-8 also forms heteromeric complexes with C1q-related factor (CRF). CRF is a secreted multimeric protein that forms heteromeric complexes with CTRP-1, CTRP-9, and CTRP-10.(39) CTRP-8 is highly conserved throughout evolution, with amino acid identity ranging from 57% to 98% between full-length human and other vertebrate CTRP-8. Structure based alignment between adiponectin, complement C1q, and TNF family members (TNF-α, TNF-β, and CD40L) reveals four highly conserved residues (Gly-159, Tyr- 161, Phe-237, and Leu-241 in adiponectin) important for the packing of the protomer's hydrophobic core.(35)

CTRP-9

C1qTNF-related protein-9 (The GenBank accession no: NM_178540) shows the highest degree of amino acid identity to adiponectin in its globular C1q domain and forms a heterodimer with adiponectin.(41) CTRP-9 is expressed predominantly in the adipose tissue and females expresses higher levels of the transcript than males. Moreover, its expression levels in ob/ob mice changed in an age-dependent manner, with significant up-regulation in

younger mice. CTRP-9 is a secreted glycoprotein with multiple post-translational modifications in its collagen domain that includes hydroxylated prolines and hydroxylated and glycosylated lysines. It is secreted as multimers (predominantly trimers) from transfected cells and circulates in the mouse serum with levels varying according to sex and metabolic state of mice. Furthermore, CTRP-9 and adiponectin can be secreted as hetero-oligomers when co-transfected into mammalian cells and, *in vivo*, adiponectin/CTRP-9 complexes can be reciprocally co-immunoprecipitated from the serum of adiponectin and CTRP-9 transgenic mice.(41) Two isoforms of human CTRP-9 were detected, CTRP-9A and CTRP-9B, which share 98% amino acid identity and are encoded by distinct genes. While CTRP-9A is robustly secreted as a multimeric protein, CTRP-9B requires physical association with CTRP-9A or adiponectin for its secretion (39).

Hypothesis

Aldosteronism is a keynote of "essential" hypertension and particularly of hypertension associated with OO. Although some data suggest a direct relationship of the adipose tissue with plasma aldosterone concentrations in patients with OO and hypertension,(33) knowledge of the mechanisms underlying the association between hypertension, OO and aldosteronism remains scant. Since there is evidence of abundant synthesis of CTRP-1 in the adipose tissue and recent data suggest the CTRP-1 may stimulate aldosterone synthesis in adrenocortical cell line,(49) we hypothesize that CTRP-1 may modulate aldosterone synthesis thereby promoting development of hypertension.

Aim of the study

The aim of our study was to investigate whether CTRPs, and more specifically CTRP-1, play a role in the pathogenesis of hypertension in the patients with overweight-obesity.

Specific objectives

Task 1: To investigate the gene expression of CTRP-1 in a wide array of human tissues.

Task 2: To investigate the protein expression of CTRP-1 in the adrenal gland, adipose tissue, arteries and in the smooth muscle cells.

Task 3: To identify the cell types that can produce CTRP-1 in the adrenal gland and locate CTRP-1 at the sub-cellular level.

Task 4: To identify the cell types that can produce CTRP-1 in an adrenal gland harbouring a myelolipoma and an APA.

Task 5: To investigate whether CTRP-1 may affect the aldosterone synthesis.

Task 6: To develop an *in-house* ELISA assay to measure the plasma levels of CTRP-1 in humans.

Task 7: To evaluate the gene expression of CTRP-3, -5, -6, -7 in a wide array of human tissues.

Materials and methods

Task 1: To investigate the gene expression of CTRP-1 in a wide array of human tissues.

Subjects. The study was approved by Ethics Committee and each patient gave the consent to the study. Adrenocortical tissues from 10 patients with an APA and adrenomedullary tissue from 10 patients with pheochromocytoma were investigated. Histologically normal adrenocortical tissue obtained at surgery from patients with renal cancer undergoing unilateral nephrectomy and ipsilateral adrenalectomy were used as controls for adrenocortical and APA tissues. Histologically normal medullary tissue obtained from 5 patients undergoing adrenalectomy for non functioning incidentally discovered adrenal mass (incidentaloma) served as control for the pheochromocytoma tissue.

Tissues were obtained under sterile conditions at surgery in the operating room and divided in two sections. One section was immediately fixed in neutral formalin and included in paraffin; the remaining tissue was reserved in RNA later (cat AM7021 Ambion).

In the patients with APA the diagnosis was based on strict predefined criteria that comprised lateralization of aldosterone secretion at adrenal vein sampling(63),(63) surgery, pathology, and, more importantly, follow-up data. At follow-up, we required demonstration of normokalemia and cure or improvement of hypertension at least 120 days

after adrenalectomy (63). Cure was defined as a systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg without medications or with the same or reduced number of medications and/or reduced defined daily doses, as described by World Health Organization.(64)

Pheochromocytoma was diagnosed according to state-of-the-art criteria(65), and confirmed at pathology, histology, and positive immunostaining for chromogranin A and synaptophysin.(66)

Cell Culture

Human endothelial cells were isolated from giugular veins using collagenase and cultured with Medium 199 supplemented with 20% fetal calf serum. Briefly, the vein was obtained under sterile conditions and perfused with 200-400 ml of lactated Ringer's solution to remove blood. The vein lumen was filled with Dulbecco's phosphate-buffered saline (PBS, with Ca^{2+} and Mg^{2+}) containing 1 mg/ml collagenase. After a 10-20-min incubation at 37°C, the contents of the vein were gently flushed out with an equal volume of Hanks' Balanced Salt Solution (BSS, calcium- and magnesium-free) and collected in a siliconized glass, conical centrifuge tube. The small white pellet obtained from centrifugation (200xg for 5 min) was resuspended in the culture medium.

RNA isolation

Total RNA was isolated from frozen tissue starting from about 20 mg of adrenal tissue. The tissues were disrupted using MagNA Lyser Instrument (Roche, Monza, Italy) in 600 μl of RLT buffer (Qiagen, Milan, Italy) added with β-mercaptoethanol. The next steps were performed at room temperature following the RNeasy Mini Kit protocol (Qiagen, Milan, Italy). The integrity and quality of the RNA were systematically checked with the use of the

lab-on-chip technology in an Agilent Bioanalyzer 2100 (RNA6000 Nano Assay, Agilent Technologies, Palo Alto, CA). We started from 1 μl of total RNA and, after the run, proceeded with the analysis of the resulting electropherograms. Only samples with two distinct ribosomal peaks (18S and 28S) were used for the downstream analysis

RNA isolation from human adipose tissue

Approximately 100 mg of subcutaneous, omental and peri-adrenal fat were obtained at surgery and immediately frozen in liquid nitrogen and stored at -80 °C for subsequent RNA extraction. Initial attempts to isolate high quality RNA from subcutaneous adipose tissue using TRIZOL (Invitrogen, Town, CA) procedure provided low 260/280 ratios and yields (10 ug/g). Therefore, we developed a method that would yield high quality RNA in appropriate amounts for RT-PCR from subcutaneous adipose tissue. Snap frozen adipose samples (< 100 mg) were homogenized in 1 ml of QIAZOL® Lysis Reagent (Qiagen Sciences, Maryland 20874, USA) centrifuged for 5 min at 30 oscillating /second (MagNA Lyser, Roche) to remove cellular debris and excess lipid. Chloroform (0.2 ml/ml of QIAZOL used) was added, mixed, and centrifuged at 1400 rpm, 24 °C for 2 min. After homogenation we obtained two phases (fat and aqueous) containing fat and RNA, respectively. Isopropanol (0.57x vol of aqueous layer) was added to the clear aqueous layer, and this phase was added to PureYield™ RNA Midiprep columns (Promega, Madison, WI). RNA was then purified following the manufacturer's directions (RNeasy® Mini kit Qiagen).

Total RNA was extracted by the RNeasy Mini Kit (QIAGEN, Hilden, Germany). One μg of RNA was treated with DNase and specific reagents to remove excess salts (RNAqueous® - Micro, Cat 1931, Ambion, Inc., Austin, TX)

Measurement of RNA quantity and assessment of its quality

RNA was quantified with BECKMAN DU® 530 (Life Science UV/Vis, Gaithersburg, MD, USA) spectrophotometer. The quality of RNA was evaluated with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using RNA 6000 Nano chips, RNA ladder and Agilent analysis software (Agilent Technologies).

Real-time RT-PCR analysis

Total RNA from peri-adrenal fat, subcutaneous fat, kidney, brain, artery wall, endothelial cells, lung, normal adrenal gland, pheocromocytoma, non functional adrenocortical adenoma (incidentaloma) and APA was isolated using Qiazol. One μg of total RNA was reverse transcribed using iScriptTMcDNA Synthesis kit (BioRad, Milan, Italy) at 25°C for 5 min, 30°C for 30 min, 85°C for 5 min and hold at 4°C (Delphi 1000TM Thermal Cycler, Oracle, BioSystemsTM, Italy). PCR for the amplification of various target genes was then performed. The following primers were used for real time PCR (human C1qTNF1 (NM_030968.2) forward: 5'ggC ggg AgA tgC TcT Ag-3', reverse 5'gCA CAC gAC TCA ggA CCA; human PBGD (NM_000190.3) forward 5'-tgc cct gga gaa gaa tga ag-3', reverse 5'-agatggctccgatggtga-3'). PCR was performed in a real-time LightCycler®480 Software (Roche, Monza, Italy), with amplification cycles of 10 s of denaturation at 95°C, 30 s of annealing at 60°C, and 5 s of extension at 72°C. Total number of cycles was 45. The amplification efficiency of the target was equal to that of the internal control. PBGD was used as the endogenous reference in the comparative comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Task 2: **To investigate the protein expression of CTRP-1 in the adrenal gland, adipose tissue, arteries and in the smooth muscle cells.**

Cell culture

Smooth muscle cells (SMC) were isolated from the thoracic aorta obtained from patients undergoing surgery for aneurysm. Human SMCs were grown in DMEM (Dulbecco's Modified Eagl's Medium, Code: D6429, Sigma) with 4500 mg glucose/L, sodium pyruvate 110 mg/L and L-glutamine; containing 10% FCS, 2% HEPES, 1% glutamine and 1% penicillin.

Contaminating fibroblasts were separated from human SMC using magnetic beads and anti-human CD90 (Invitrogen, Dynabeads® Goat anti-Mouse IgG; Cat no. 110.33) grown in DME containing 10% calf serum.

Immunohistochemistry

Adrenal gland tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Five μm-thick sections were deparaffinized and incubated in 0.3% H_2O_2 in Tris buffered saline (TBS) containing 50 mM Tris HCl and 0.9% NaCl, pH 7.4 to block endogenous peroxidase activity, followed by 2% normal goat serum (NGS) and 0.2% Triton X-100 in PBS for 1 h at room temperature. Sections were then incubated overnight in a humid atmosphere at 4°C with a specific monoclonal antibody against CTRP-1 (MAB 3145; R& D, Space Import-Export, Milan, Italy) at several dilution of 1:50, 1:100, 1:200 and 1:300 in PBS containing 2% NGS and 2% Human Serum (HS). After three washes in PBS, sections were incubated with biotinylated Goat Anti-Mouse IgG (Code number: 115-065-068, Jackson-Immuno Research, UK) at a concentration of 1.2 g. L^{-1} for 30 min at room temperature. After washing, the sections were incubated in streptavidin peroxidase complex (0.5 $\mu g.mL^{-1}$, dilution 1:250) for 30 min at room temperature and the final product was revealed by chromogen 3.3'-diaminobenzidine Tablets (DAB)(Sigma, Milan, Italy) and urea hydrogen peroxide dissolved in 5 mL water, yielding a brown reaction product. After 5 min the reaction was stopped with H_2O and the sections were incubated with hematoxylin for 1 min. After subsequent washes in water for 20 min, the sections were mounted onto slides. Photographs were taken with a Leica microscope equipped with a DC 200 camera. Controls used to validate the

specificity of the immunoreactivity included use of preimmune serum in place of primary antibody and also omission of the primary antibody.

Task 3: **To identify the cell types that can produce CTRP-1 in the adrenal gland and investigate whether CTRP-1 is expressed at the cell membrane or at the intra-cellular level.**

Culture of H295 cell line

H295R and HAC15 human adrenocortical carcinoma cells line were cultured in DMEM/F12 medium (Sigma, Italy), containing 1% insulin-transferrin sodium selenite plus Premix (ITS) (BD Bioscience, Bedford, USA) and 2.5% Nu-Serum (BD Bioscience) at 37°C in a 5% CO_2 atmosphere.

Isolation of the adrenocortical cells and immunoseparation of CD56 positive cells

Single-cell suspension obtained after enzymatic and mechanic digestion was washed with PBS/0.1% BSA and then incubated with CD56 precoated magnetic beads by gentle shaking for 30 min at 4°C. Beads were used at a ratio of five beads per cell. After separating the bead-bound CD56-positive (CD56+) cells with a magnet, the CD56+ cells were harvested in PBS/0.1% BSA and seeded at a density of 104 cells/cm2 into 24-well BD BioCoat Matrigel invasion chamber plates. The CD56+ and CD56- cells were cultured in M199 (Sigma, Milan, Italy) medium enriched with heat-inactivated 10% fetal calf serum, 20 U/ml penicillin G, 20 µg/ml streptomycin sulfate, 50 ng/ml amphotericin B, and 1% insulin, transferrin, and selenium premix (BD Biosciences) and maintained in a humidified 5% carbon dioxide/air atmosphere at 37°C. At d 4, fresh medium was added.

Confocal microscopy

After growing H295R, HAC15 and CD56+ adrenocortical cells at a density of $3x10^4$ cells/well, they were incubated for 1 hour at 37°C. Cells were washed 3 times in PBS, fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 in PBS for 4 minutes at 4°C. After washing and blocking with 2% BSA for 1 hour at room temperature, cells were incubated for 1 hour with anti-C1qTNF1 mAb, anti-CD56 rabbit polyclonal, diluted 1:300 in blocking buffer.

After washing, bound antibodies were detected by using Alexa Fluor 488-conjugated goat anti-mouse IgG secondary Ab (1:200; Invitrogen S.R.L., San Giuliano Milanese, Italy) and anti-rabbit Alexa Fluor 594 (Invitrogen, 1:200) for 30 minutes at room temperature in the dark. Samples were analyzed with confocal microscopy Leica TCS SP5 by using laser excitation at 488 nm and LAS AF software.

Task 4: **To identify the cell types that can produce CTRP-1 in an adrenal gland harbouring both a myelolipoma and an APA.**

Double Immunohistochemistry

Double staining method was performed on paraffin-embedded sections using BOND-maX Vision Biosystems (Leica, New Castel, UK). Briefly, 5 μm-thick sections were deparaffinized with Bond Dewax Solution at 72°C, washed three times with alcohol, and three with Bond wash Solution, incubation with Peroxide Block for 5 min, incubation with anti-human CD61 (monoclonal mouse anti-human, clone Y2/51, Code F080, reed to use) to detect monocytes and inflammatory infiltrates, anti-human glycophorin C (Monoclonal Mouse Anti-Human, Code N. M 0820, dilution 1:200; DAKO) to detect cells of erythroid origin and anti-human myeloperoxidase (MPO) (Monoclonal Mouse Anti-Human, 1:4000; DAKO) to detect cells of the myeloid lineage. The final product was revealed with BondTMPolymer AP Red Detection (Cat No: DS9305). A second blocking step was omitted because blocking is due to

the first stain, and the second incubation was made with C1qTNF1 (Monoclonal Anti-Mouse, R&D). Finally the product was revealed by DAB (Sigma).

Five μm-thick sections from AML were prepared as described for immunohistochemistry. Before staining, non-specific protein binding was blocked by incubating slides overnight in 2% BSA. Samples were then stained 1h at room temperature with the same monoclonal antibody diluted 1:300 followed by the goat anti-mouse IgG2B Alexa Fluor 488 (Invitrogen, 1:300). For co-localization experiments with C1qTNF1 a rabbit polyclonal anti-MPO (1:4000; DAKO) and a mouse monoclonal anti-CD61 (1:300, DAKO) were used, followed respectively by a goat anti-rabbit Alexa Fluor 594 (1:200; Invitrogen) and a goat anti-mouse IgG1 PE-conjugated (1:300; Invitrogen). The slides were then mounted with a cover slip and fluorescence was detected using the Leica TCS SP5 confocal system equipped with a fluorescence filter set for excitation at 488 nm and LAS AF software.

Task 5: **To investigate whether CTRP-1 may affect the aldosterone synthesis.**

Cloning of CTRP-1

mRNA extracted from human adipocytes was retro-transcribed in cDNA with SuperScript II reverse transcriptase (Invitrogen, San Diego CA) and amplified using the following primers: forward 5′-CCCGGATCCATGGGCTCCCGTGGA-3′ and reverse 5′-CCCCTCGAGGGGGGGCTCGGTGGCGT-3′. The PCR product was cloned into a pET21b vector to provide the protein of a 6His tag at the C-terminus. The construct pET21b-CTRP-1 was used to transform *E. coli* BL21 (DE3) strain for protein expression.

Expression and purification of the human recombinant CTRP-1

Five hundred ml of *E. coli* BL21 (DE3) transformed with the plasmid pET21b-CTRP-1 were grown until a value of OD_{600} of 0.6. The His-tagged CTRP-1 expression was then induced by applying 1 mM of isopropyl β-d-1-thiogalactopyranoside (IPTG) to the culture for 2 h.

Bacteria were pelletted, resuspended in 10 ml of lysis buffer (30 mM Na_2HPO_4, 500 mM NaCl, pH 7,8) and lysed using a French Pressure Cell Press (Thermo Scientific, Waltham MA). Lysate was clarified by centrifugation and the supernatant was concentrated to a final volume of 5 ml using a 10 kDa cut-off concentrator (Sartorius-Stedim, Aubagne France).

The His-tagged-CTRP-1 was purified with a 5 ml HiTrap chelating HP (GE healthcare, Uppsala Sweden) in an Akta Basic 100 system (Amersham Pharmacia Biotech, Amersham, UK). Protein was eluted from the column by applying a solution of 30 mM Na_2HPO_4, 500 mM NaCl, pH 7.8 containing 200 mM of imidazole. Two fractions (Fraction A and Fraction B) containing CTRP-1 were collected and pooled.

Treatment of H295 cells with CTRP-1

H295R cells were cultured in RPMI medium (Sigma) as above described. The cells were sub-cultured onto 24-well dishes at a density of 3×10^5 cells/well for subsequent treatment with commercially available protein CTRP-1 (400 ng/mL, Biovendor) and Fractions A and B obtained from CTRP-1 cloning in *E. coli* (800 ng/mL for 12 and 24 h). Treatment with 10^{-7} M AngII for 12h as positive control. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen,) following the protocol reported above and expression of CYP11B2 was measured with quantitative RT-PCR analysis using universal ProbeLibrary probes (LightCycler 480 Instrument, Roche, Monza, Italy). CYP11B2 expression was calculated relative to porphobilinogen deaminase (PBGD), used as an internal control, by comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Task 6: To develop an *in-house* ELISA assay to measure the plasma levels of CTRP-1 in humans.

ELISA assay

ELISA sandwich assays are designed for detecting and measuring substances such as proteins and hormones. In an ELISA an antigen have to be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme (Fig. 5). Detection is accomplished by incubating this enzyme-complex with a substrate that produces a detectable product. The most crucial element of the detection strategy is highly specific antibody-antigen interaction.

Reagents: Phosphate Buffered Saline (P4417-100TAB; Sigma Aldrich), Tween®20 (P8416-100ML; Sigma Aldrich), carbonate buffer (Na_2CO_3 pH 9.5), milk powder (Roth®), Tris – buffered saline pH 7.3 (20 mM Trizma base, 150mM NaCl) containing 0.1% bovine serum albumin (BSA), 0.1M acetate buffer pH4; monoclonal and biotinylated anti-human C1qTNF1 antibody were purchased from R&D SYSTEMS® (Minneapolis, MN, USA). Protein CTRP-1 was purchased from BioVendor Laboratories, Inc. (Germany). Streptavidin-Peroxidase Polymer, Ultrasensitive (S2438; Sigma-Aldrich, Saint Louis, Missouri, USA), ABTS® Peroxidase Substrate (50-66-18; KPL, USA). Unless otherwise stated, all chemicals used in this study were of analytical grade, and all solutions were prepared with water purified with a MilliQ UV Plus system.

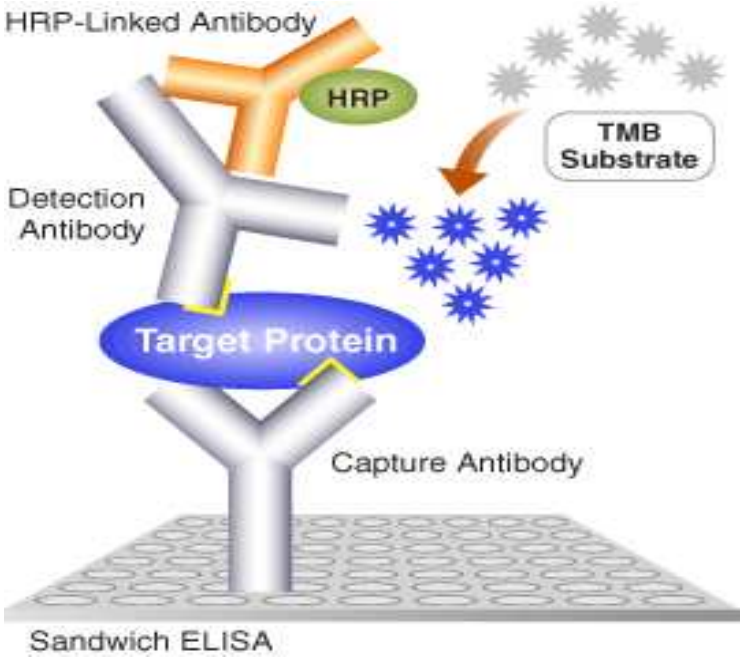


Figure 5: Schematic representation of the position of targets in a single well.

Protocol

Plates in PVC (code number: 353072, Becton Dickinson, France) were coated with anti-C1qTNF1 monoclonal antibody diluted in buffer bicarbonate / carbonate sodium (pH= 9.55) at a concentration of 20 ug / ml and incubated over night at 4°C. After washing 4 times with PBS and 0.05% Tween, the plates were then blocked to prevent non-specific binding of antibodies by adding 100 µl of 5% milk powder for 2 h at room temperature. They were then washed 4 times with 300 µl PBS, soaking for 1 min between each wash. Serum or standard (100 µl in duplicate) was added to test wells and 100 µl of PBS was added to the blank control wells. The plates were incubated for 1 h at room temperature and washed 4 times with PBS as previously described. The plate was then incubated overnight at 4°C with polyclonal antibody (R&D, Cat No: BAF 3145) at concentration 10 µg /mL (diluted in PBS/BSA) and washed four times.

Two hundred µl of streptavidin-Peroxidase Polymer, Ultrasensitive (diluted 1:200 in PBS with 0.05% Tween20, Code Numbe: S2438, Sigma) was added to each well and the plate was incubated for 1 hour at room temperature. The final step was incubation for 15 minute with 200 µl peroxidase substrate ABTS®, (Code: 50-66-18, Gaithersburg, MD, USA. www.kpl.com) and measurement of the optical density at 405 – 450 nm with Elx 808 (Bio-TEX Instruments, Inc., Milan, Italy).

The standard curve was performed by serial dilutions (1:2) using C1qTNF1 BioVendor protein (Cat No: RD 172153100). The protein diluted in PBS + 1% milk powder was incubated for 2 hours at room temperature.

Task 7: To evaluate the gene expression of CTRP-3, -5, -6, -7 in a wide array of human tissues.

Real-time RT-PCR analysis

Total RNA from normal adrenal gland, APA, pheocromocytoma, subcutaneous and peri-adrenal fat, was isolated using RNeasy Mini Kit protocol following the protocol above described. The primers used for this analysis are shown in Tab. 4. PBGD and β-actin were used as endogenous references for the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

PRIMER	SEQUENCE FOR	SEQUENCE REV
CTRP-3	5′- ACA gCT TTC gAg gCT ACC AA	5′- CCA TTg TTT CCA Tgg TTT CC
CTRP-5	5′- Agg gAg CgA ACC Agg ACT	5′- CTA CTC C gg ACC CTC CAg T
CTRP-6	5′- gAg gCC ACA ggA CAC Agg	5′- CAT Agg gAT CTC ACA CAT CAg g
CTRP-7	5′- gAA Agg TgA AAA ggg AAC TgC	5′- TCT CTC CCT CTg gTC CTA Tgg

Table 4: Sequences of forwarde and reverse primes

Statistical analysis

The Statistical Package for the Social Science (SPSS for Windows version 18.0) was employed for the analysis. Continuous variables are expressed as mean ± SEM. Repeated measures ANOVA test or Student's t-test was used to evaluate the differences between groups. Statistical significant was defined at a P value of < 0.05.

Results

CTRP-1 mRNA Expression

CTRP-1 expression in the human adrenal gland, pheochromocytoma, non functioning adrenocortical adenoma incidentaloma, APA (Fig. 6A) was investigated using RT-PCR analysis. CTRP-1 was found to be more markedly expressed in the normal adrenal gland and incidentaloma as compared to the secreting tumours pheochromocytoma and APA ($p < 0.01$). CTRP-1 was also found to be markedly expressed in the human adrenocortical carcinoma cell line H295R (data non showed).

CTRP-1 was greatly expressed in the human adipose tissue, either peri-adrenal or subcutaneous fat. When examining the gene expression pattern in a wide array of tissues, we found that also heart, kidney, brain, arterial wall, lung and endothelial cells can synthesize CTRP-1 (Fig. 8B). Interestingly, expression of CTRP-1 was 1.6 and 2.3 higher in the heart as compared to subcutaneous or peri-adrenal fat ($p < 0.01$).

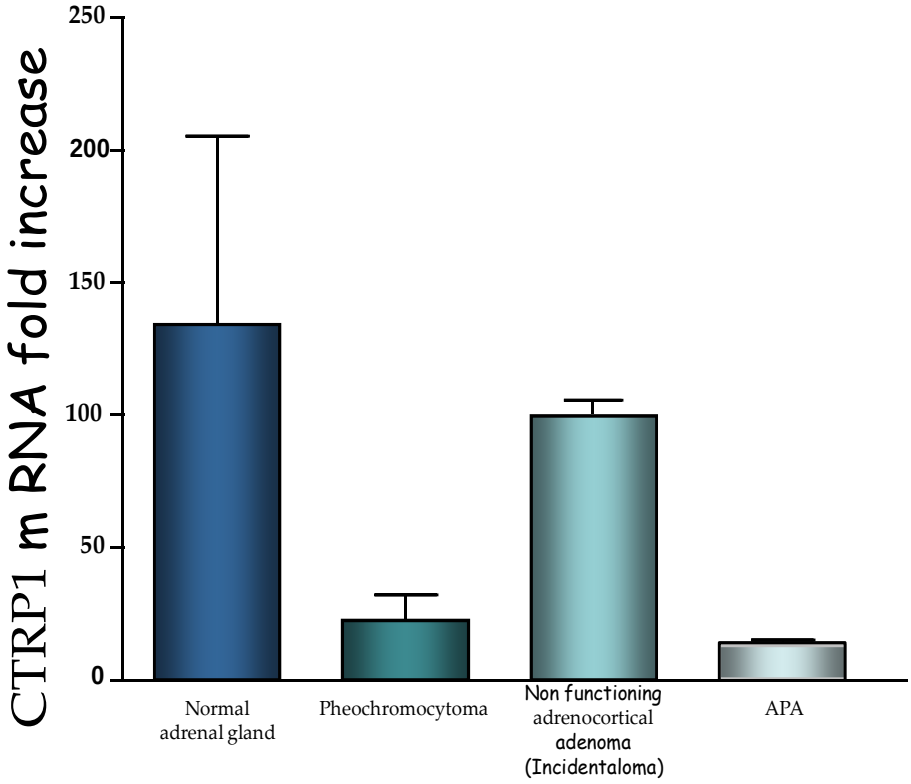


Figure 8A: CTRP-1 gene expression in the normal adrenal gland and adrenal tumours. Levels of CTRP-1 are higher in normal adrenal gland and incidentaloma as compared to pheochromocytoma and APA. CTRP-1 expression was calculated relative to porphobilinogen deaminase (PBGD), used as an internal control, and fibroblasts as tissue reference by comparative Ct ($2^{-\Delta\Delta}Ct$) method. Values were obtained from three independent experiments, each performed in duplicate.

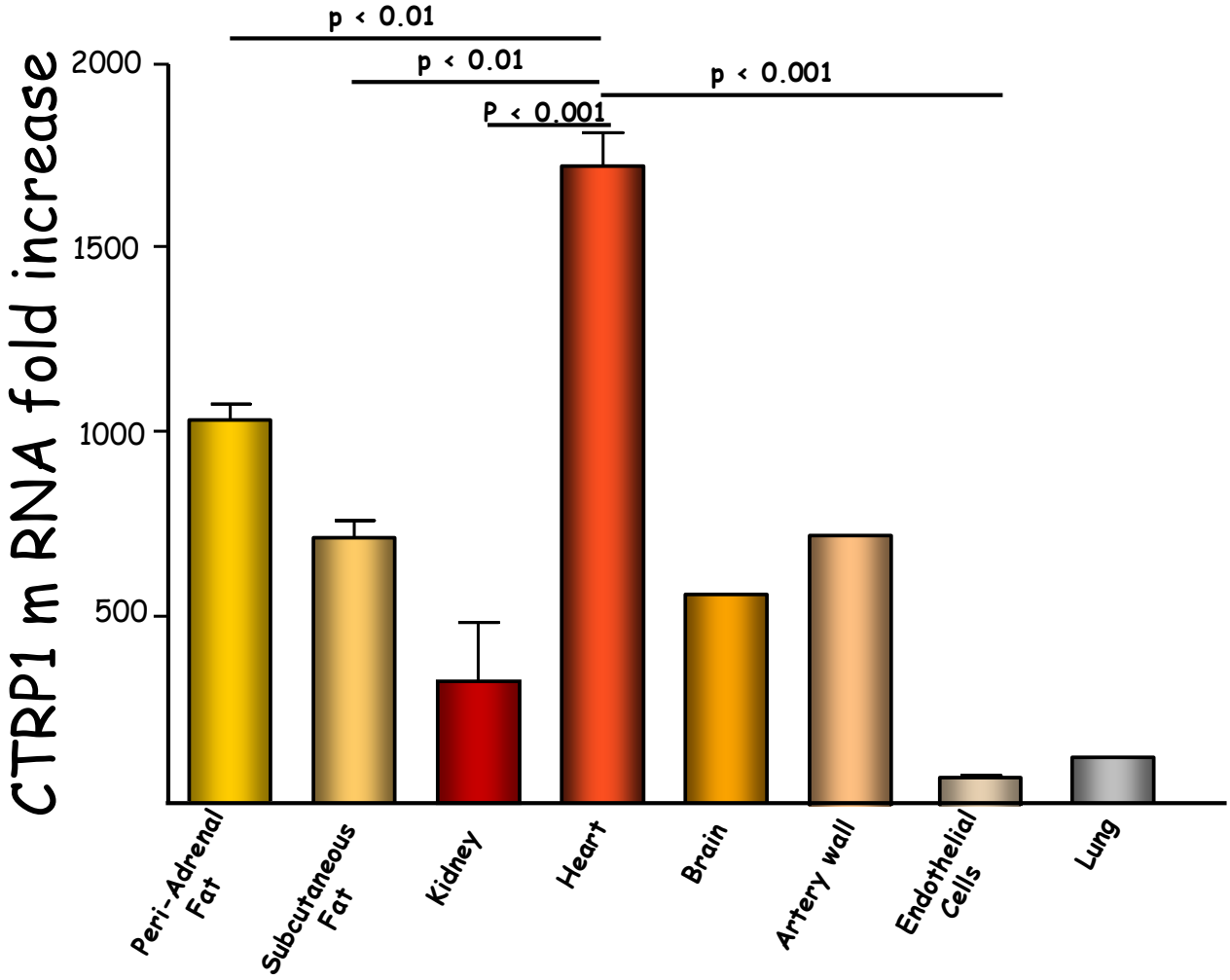


Figure 8B: Expression pattern of CTRP-1 mRNA in different human tissues. CTRP-1 expression was calculated relative to porphobilinogen deaminase (PBGD), used as an internal control and fibroblasts as tissue reference by comparative Ct ($2^{-\Delta\Delta}$Ct) method. All values are represented as the means ± S.E.M. of duplicates.

CTRP-1 Protein Expression

IHC analysis of the sections of the human adrenal gland and peri-adrenal fat showed that CTRP1 protein is produced at both sites (Fig. 9A). A more intense immunostaining was observed at the zona glomerulosa in comparison to the capsule and other inner zones. Moreover, a positive staining was also found in the intima and media layers of the small arteries and in the veins located in the peri-adrenal adipose tissue (Fig. 9B).

Analysis of the large arteries showed intense immunostaining at the internal layer, and in particular at the elastic membrane. Since a positive immunostaining was observed in the media layer, especially in the small arteries, IHC analysis was performed on cultured VSMCs, which showed positive staining in the cytoplasm.

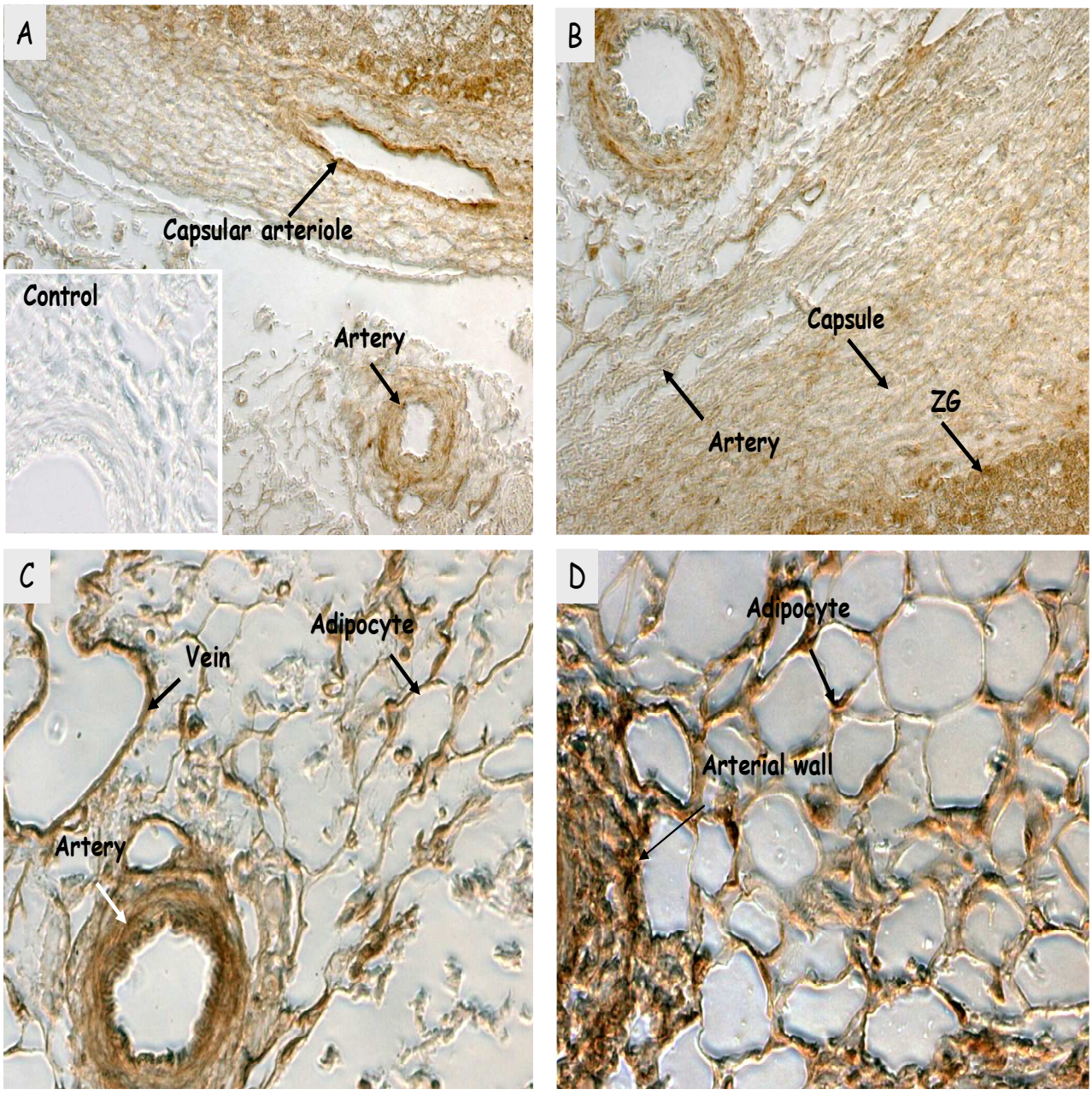


Figure 9A: CTRP-1 is expressed in the zona glomerulosa region of the human adrenal cortex. Expression of CTRP-1 by IHC in adrenal gland (panel A, B) and peri-adrenal fat (panel D). A strong specific CTRP-1 immunostaining is evident in the zona glomerulosa (ZG), artery wall, vein (panel C) and adipocyte (panel D). The control was negative (section of panel A). The image was obtained by contrast of phase microscopy. Magnification x 100.

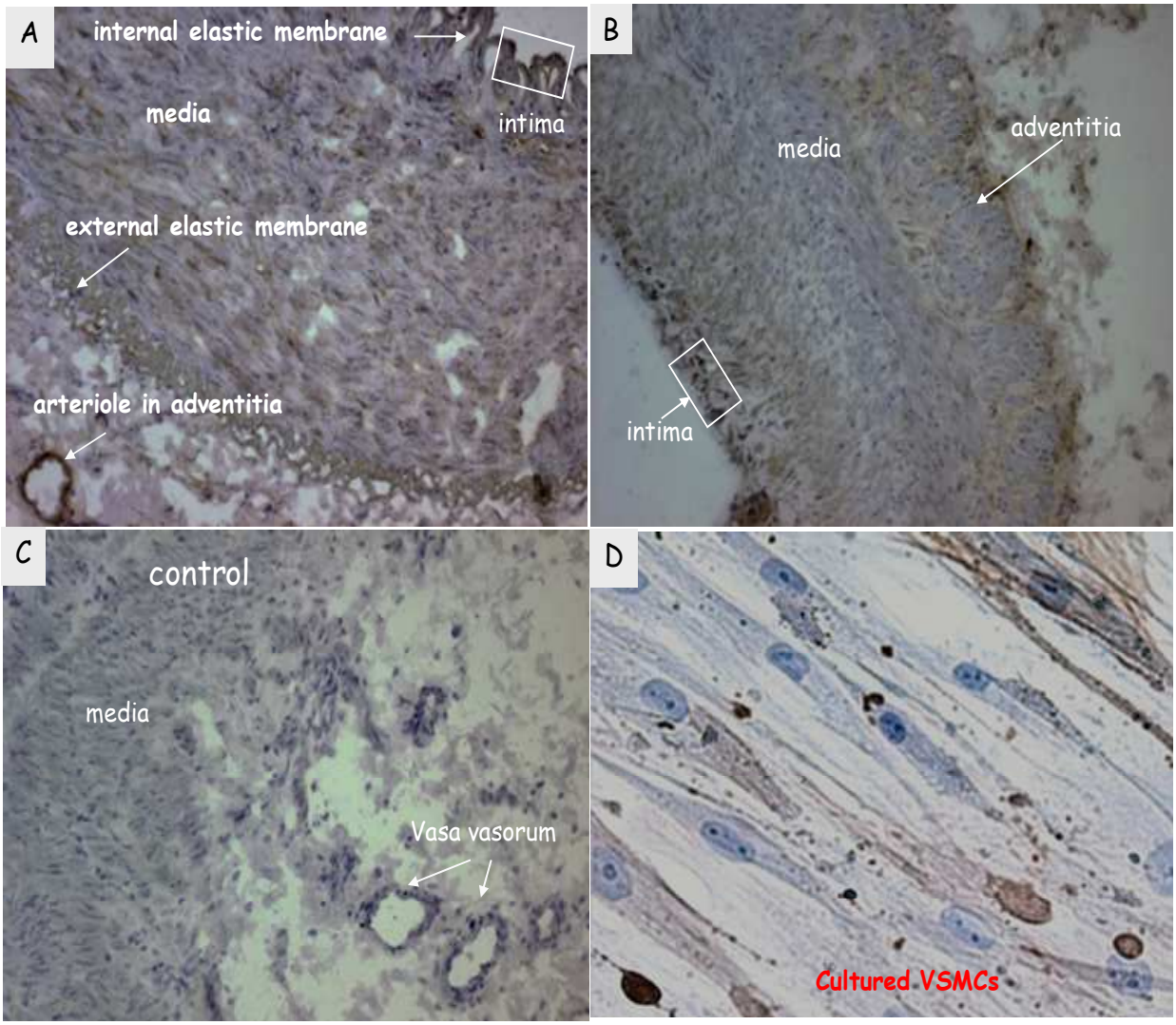


Figure 9B: CTRP-1 expression in the vascular tissue. Panel A shows the highest expression of CTRP-1 (brown colour) in the internal elastic membrane. An intense immunostaining can be also observed in the arteriole located in the adventitia. The panel B confirms the high expression of CTRP-1 in the intima layer. Panel C, which corresponds to the negative control, in which the antibody against CTRP-1 was omitted, shows no specific immunostaining for CTRP-1, in particular in the *vasa vasorum*. Panel D shows smooth muscle cells in cross (*) and longitudinal sections (**). Immunostaining for CTRP-1 is evident in the cytoplasm.

Co-expression of CTRP-1 and CD56 in the cell line H295R, and in the ZG cells

Fixed adrenocarcinoma-derived cell line H295R permeabilized with Triton X-100 (TX-100) as above reported were serially incubated with an anti-rabbit antibody against CTRP-1, a monoclonal antibody against CD56, which is specifically located at the membrane level, and streptavidin-Alexa 488 & 594. We found that CTRP-1 is predominantly, even not

exclusively, expressed at the membrane level (panel A, Fig. 10) similarly to CD56 (panel B, Fig. 10). The overlay confirmed partial co-expression of CTRP-1 and CD56 in H295R cells (panel C, Fig. 10).

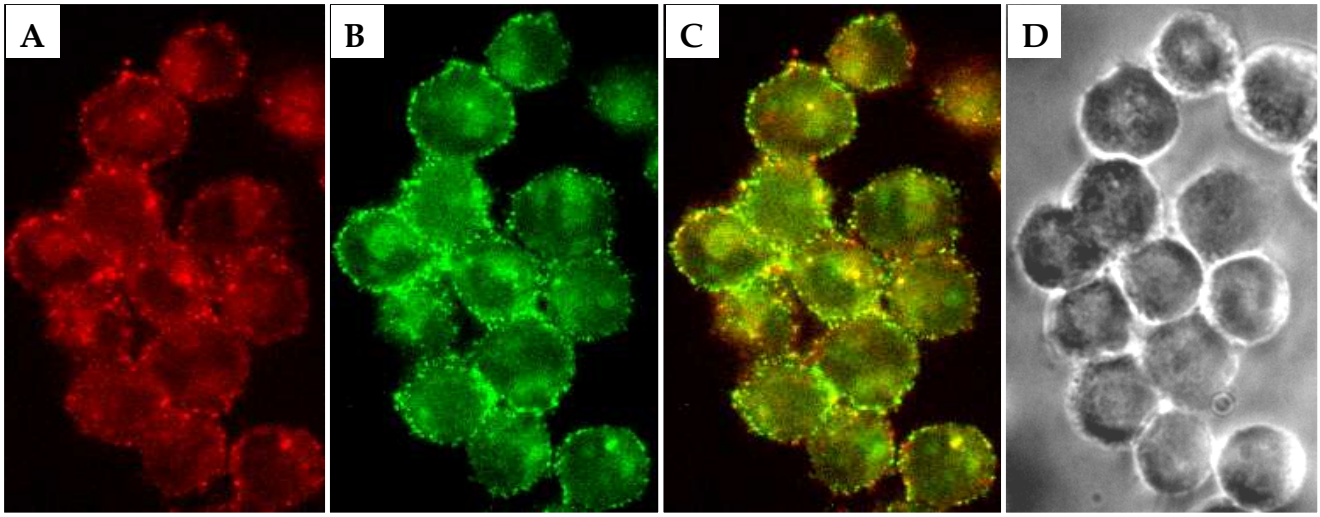


Fig. 10: Representative section of cell line H295R examined at confocal microscopy using an anti-rabbit Alexa 594 antibody against CTRP-1 (panel A), a monoclonal anti-mouse antibody against CD56 marker of membrane (panel B) and the overlay image (panel C) showing the co-expression of CTRP-1 and CD56 at the membrane level. The image D shows more in detail cell morphology.

Since we found co-expression of CTRP-1 and CD56 in H295R cells, we investigated whether CTRP-1 is exclusively expressed in CD56+ cells of the ZG. Analysis at confocal microscopy revealed that CTRP-1 in both CD56+ and CD56- cells, thereby suggesting that CTRP-1 expression is not restricted to the aldosterone-producing cells (Fig. 11). Similarly in the H295R cells, CTRP-1 is expressed mostly, but not exclusively, at the membrane level.

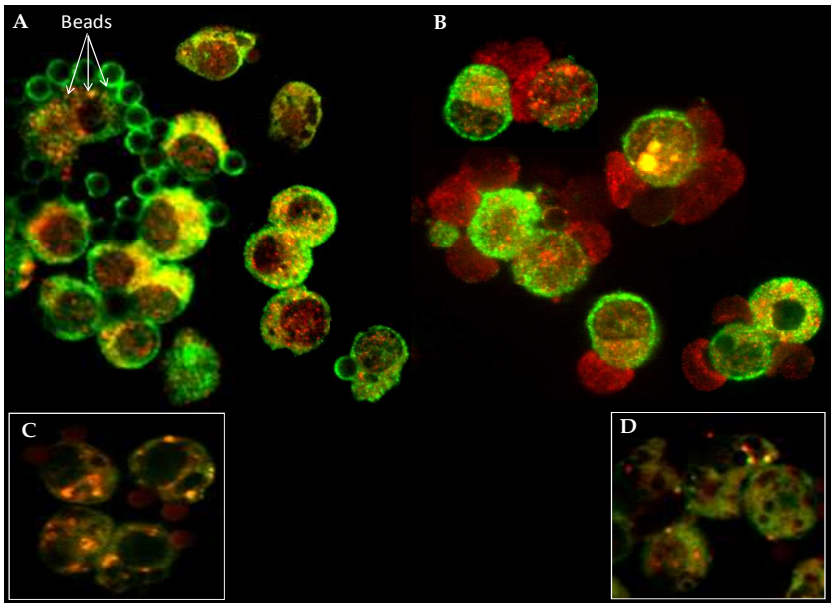


Fig. 11: Representative CD56 Cell obtained by immunoseparation and examined at confocal microscopy using an anti-rabbit Alexa 594 antibody against CTRP-1. Panel (A) shows cortical CD56+. Panel (B) shows cortical $CD56^-$. Panel (C) shows autofluorescence and the panel (D) shows the fluorescence after omission of the primary antibody CTRP-1.

To confirm that CTRP-1 is expressed in both CD56+ and CD56- cells, we measured CTRP-1 expression in an enriched population of CD56+ cells immunoseparated from an APA and normal adrenal gland. We found that CTRP-1 expression was 3.10-fold higher in CD56+ than in CD56- cells ($p= 0.006$) in the normal adrenocortex and, moreover, its expression was 4.46-fold higher in APA cells than in CD56+ cells isolated from the normal adrenal cortex ($p<0.05$). However, CD56- cells showed higher expression of CTRP-1 than CD56+ in the normal adrenal cortex ($p<0.0049$), thereby confirming that CTRP-1 gene expression is not confined to the CD56+ cells (Fig. 12).

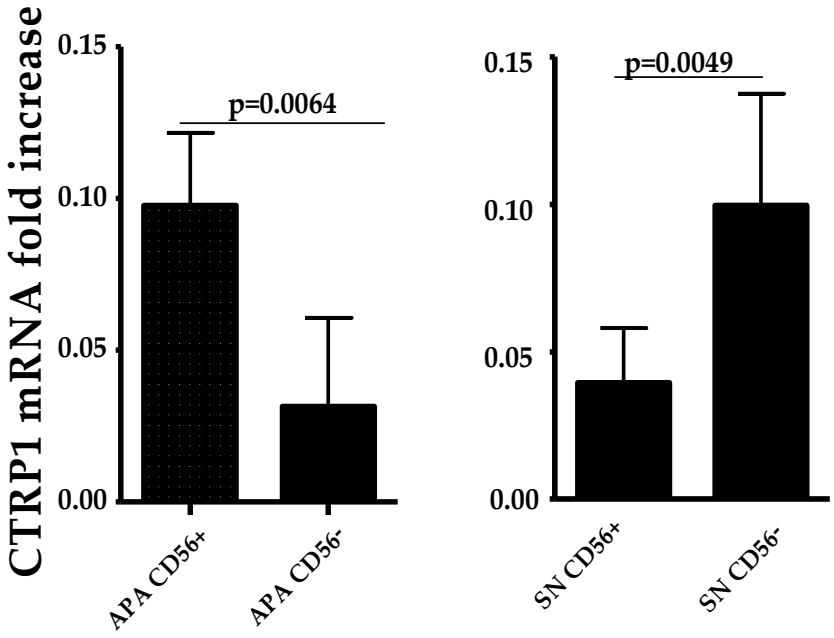


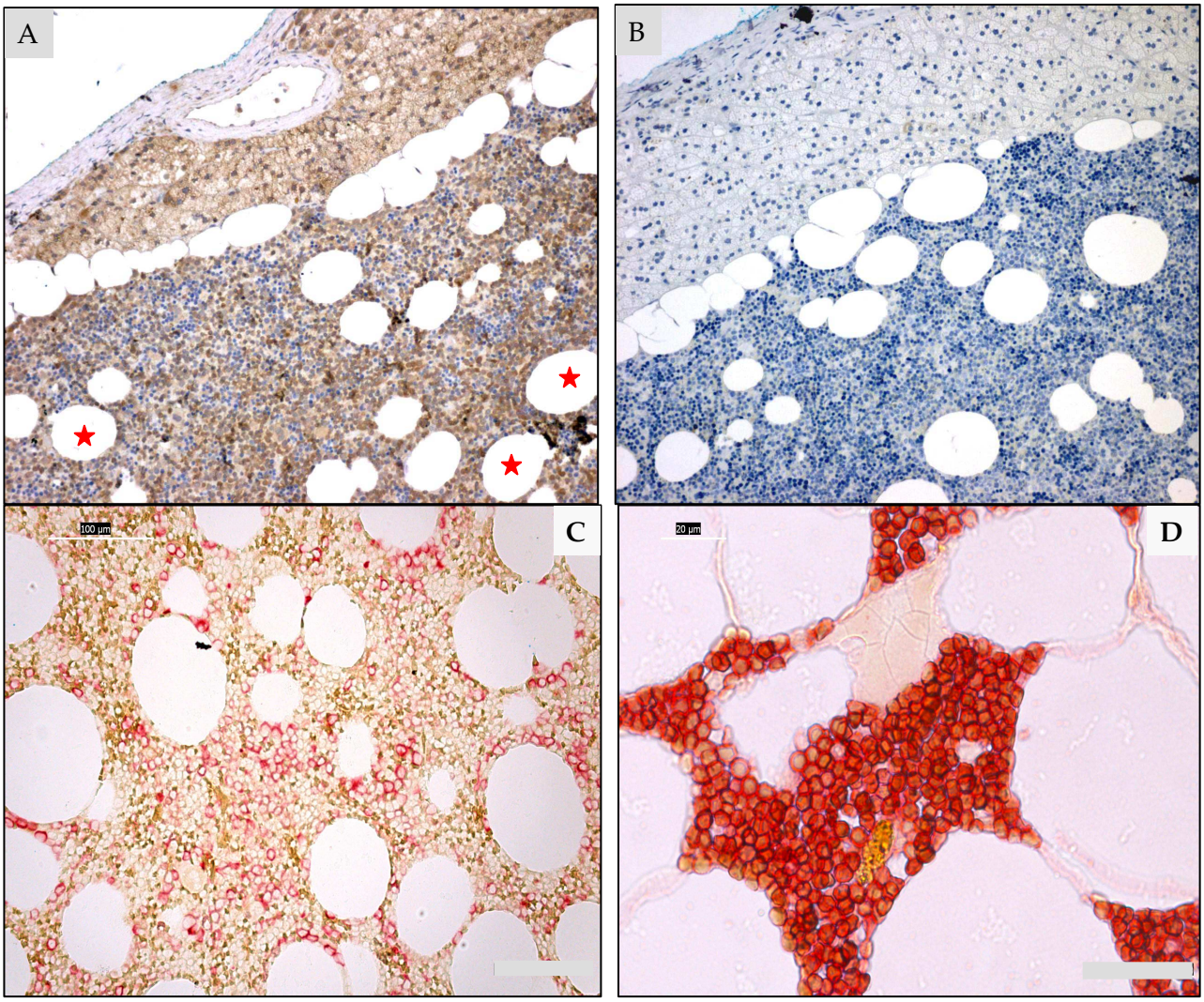
Figure 11: The bar graph shows the relative expression of the CTRP-1 gene in C56+ and CD56- cells isolated from an APA (A) and normal adrenal cortex (B). SN indicate the adrenocortical. Bars indicate CTRP-1 mRNA fold increase.

Expression of CTRP-1 in the Adrenal Myelolipoma

Adrenal myelolipoma (AML) is an uncommon benign tumor that is composed of mature adipose tissue and normal hematopoietic tissue. Since we found marked expression of CTRP-1 in the adipose tissue, we hypothesized that CTRP-1 may be expressed also in the adipocytes of AML where it could trigger the aldosterone secretion. To test this hypothesis we investigated two cases of primary aldosteronism, one due to adrenocortical hypertrophy and the other to an APA, and AML that concurred in the same patient. We therefore, measured the expression of CTRP-1 protein in both AML and APA (or adrenocortical hyperplasia) obtained from these two patients.

IHC studies showed positive immunostaining for CTRP-1 in the AML as well as in the adrenal cortex (Fig. 12, panel A). In the former the staining pertained to both adipocytes (Fig. 12A, panel A asterisks) and some cell lineages of the mature hematopoietic tissue (Fig 12A, panel A). In the latter the staining involved mainly the zona glomerulosa (ZG), and the

medulla (M) but also the zona fasciculata (ZF) and zona reticularis (Fig.12A, panel B). Negative control experiments performed by omitting the primary antibody (Fig.12A, panel C) or after precipitation of the latter with excess antigen confirmed the specificity of the staining. To localize more precisely the CTRP-1 immunostaining in the hematopoietic cell lineages of the AML, double immunostaining experiments were performed using specific markers for the erythropoietic line (glycophorin), the megacariocyte line (CD61) and the white cell line myeloperoxidase (MPO). Even though these experiments provided preliminary evidence for a predominant CTRP-1 expression in the megacariocyte and white cell line, results (not shown) were judged to be inconclusive. Hence, confocal microscopy experiments using the same lineage markers were performed. These studies allowed to conclusively demonstrating the co-expression of CTRP-1 in the white cell line lineage (Fig. 12B).



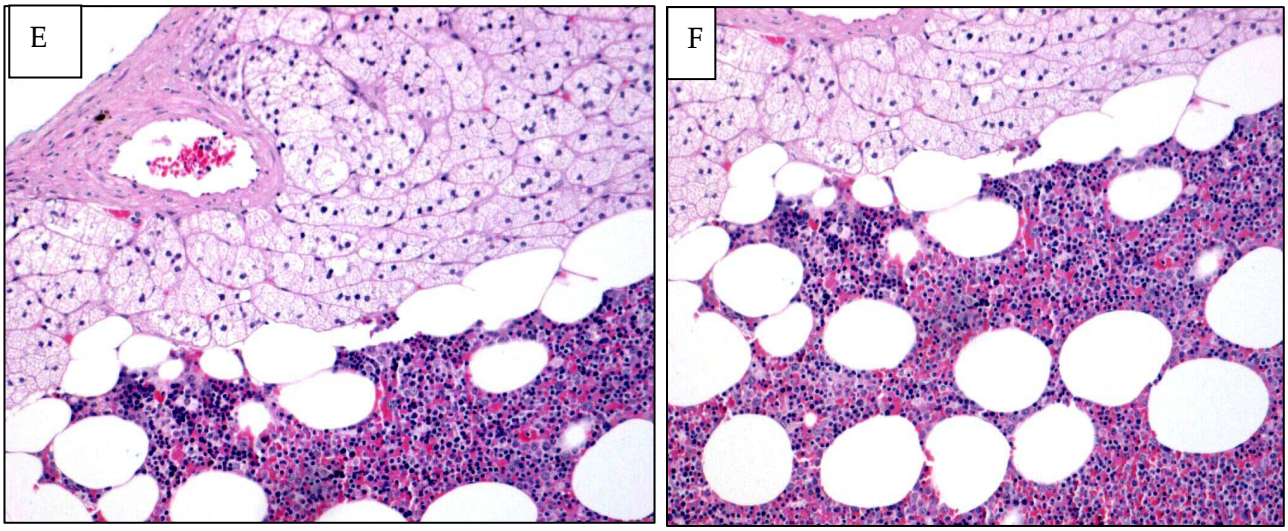


Figure 12: Immunohistochemical staining for CTRP-1 (panel A). The myelolipoma was composed of adipocytes (asterisks) and CTRP-1 peptide (brown). Immunohistochemical double staining for myeloperoxidase (MPO) and CTRP-1 (panel C), glycophorin A and CTRP-1 (panel D). The panels E and F show the hematoxylin and eosin staining. Negative control (panel B) was performed by omitting the primary antibody. Magnification x20 & x40.

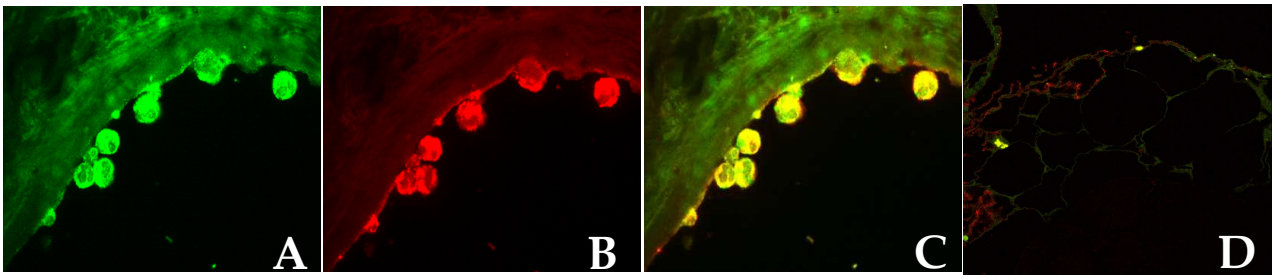


Fig. 12B: Exemplary result of confocal microscopy performed using a monoclonal antibody against CTRP-1 (panel A), an antibody against myeloperoxidase (panel B) and the merged image (panel C) showing the co-expression in monocytes adhering to the endothelium of a small artery within the AML. Panel D shows the negative control.

Task 5: To investigate whether CTRP-1 may affect the aldosterone synthesis.

The addition of the commercially available CTRP-1 protein to cultured H295R cells induced a significant increase in CYP11B2 gene expression after 12 h (+35% as compared to untreated cells; NS) and 24 h (+35% as compared to untreated cells; NS) incubation (fig. 13, panel C). No change was observed in H295R treated with either CTRP-1 pooled fraction A or fraction B, which were obtained from CTRP-1 cloning in *E. coli* BL21, after 12 h (fig. 13, panel A). A

slight but not significant increase in CYP11B2 expression was observed after addition of the pooled fraction B (fig. 13, panel B) and incubation for 24 h.

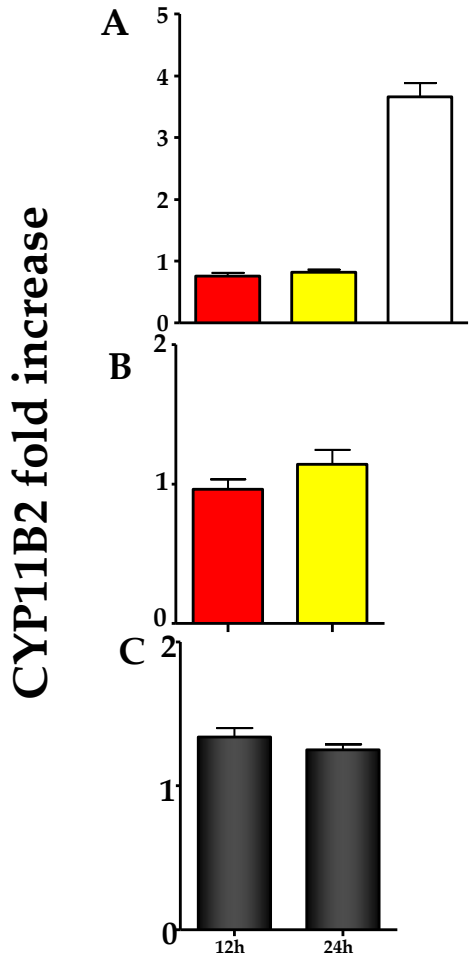


Figure 13: Treatment of H295R with CTRP-1. Panels (A) and (B) show gene expression of CYB11B2 at 12 h and 24 h, respectively, after treatment with 800 ng/ mL pooled fractions A (red bars) and B (yellow bars) obtained in our laboratory. White bar indicates treatment with Angiotensin II (positive control). Panel (C) shows gene expression of CYB11B2 after treatment with 400 ng/ mL commercially availably CTRP-1 protein for 12 h and 24 h.

ELISA assay for measuring levels of CTRP-1 in human serum

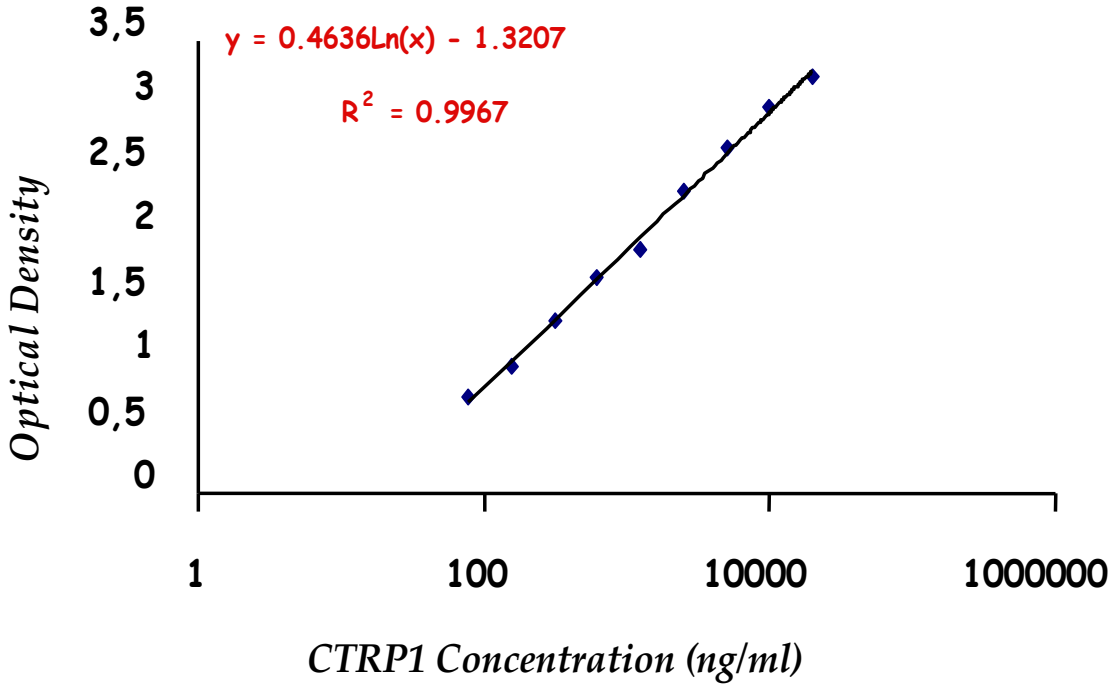
We developed an *in-house* ELISA assay to measure the levels of CTRP-1 in human serum. To this aim, several preliminary experiments were performed to determine the optimal experimental conditions, including the antigen concentration for coating the plate, the

concentration of the first antibody and of the polyclonal conjugated antibody, the temperature and incubation times.

We found that the optimal conditions were: (a) blocking of the plate with 5% milk powder for 2 h at room temperature, (b) incubation with human serum for 1 hour at room temperature, (c) incubation with polyclonal antibody overnight at 4°C and (d) incubation with enzyme substrate for 1 h at 25° C in a constant temperature cabinet. The blank value, estimated in duplicate on each plate, was obtained by identical treatment of wells using 100 μl of PBS in place of serum.

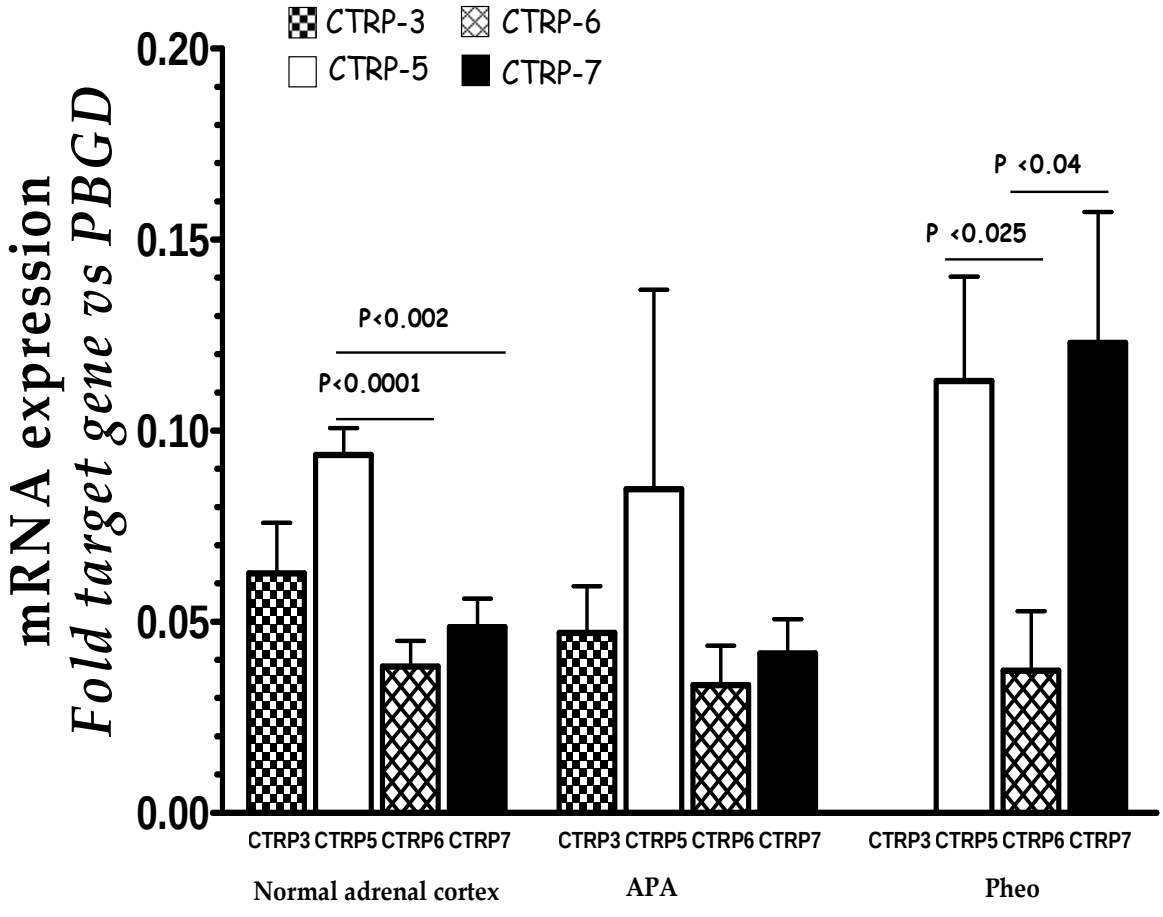
A linear-log relationship was observed between O.D. and CTRP-1 serum concentrations. A similar linear-log relationship was found between O.D. and CTRP-1 plasma concentrations.

By using the ELISA assay developed in our laboratory we measured CTRP-1 serum levels in 6 normotensive lean/normal weight subjects and 4 hypertensive overweight/obese patients, and we found increased levels in the latters (1.68 ± 0.83 vs. 0.56 ± 2.11)



Gene Expression of CTRP-3, CTRP-5, CTRP-6 and CTRP-7

Total RNA was extracted from normal adrenal gland (n=5), APA (n=5), pheochromocytoma (n=5), peri-adrenal (n=5) and subcutaneous (n=4) adipose tissue following the protocol above reported. Gene expression of each CTRP was measured with quantitative real-time RT-PCR. PBGD and β-actin were used as housekeeping genes for the adrenal or adipose tissue, respectively. Although many tissues expressed low levels of CTRP transcripts, quantitative real-time PCR analysis revealed that adipose tissue was the predominant tissue that expressed CTRP-1 (Figure 8B) and CTRP-3 (Figure 14), transcripts among the 12 different human tissues examined (fig 6). Expression levels of CTRP-5, and CTRP-7 transcripts also varied across tree different tissues (normal adrenal cortex, APA and Pheocromocytoma), with high expressing in Pheocromocytoma (fig 14A), whereas others were highly expressed in in subcutaneous and peri-adrenal fat (CTRP-3, fig 14B).



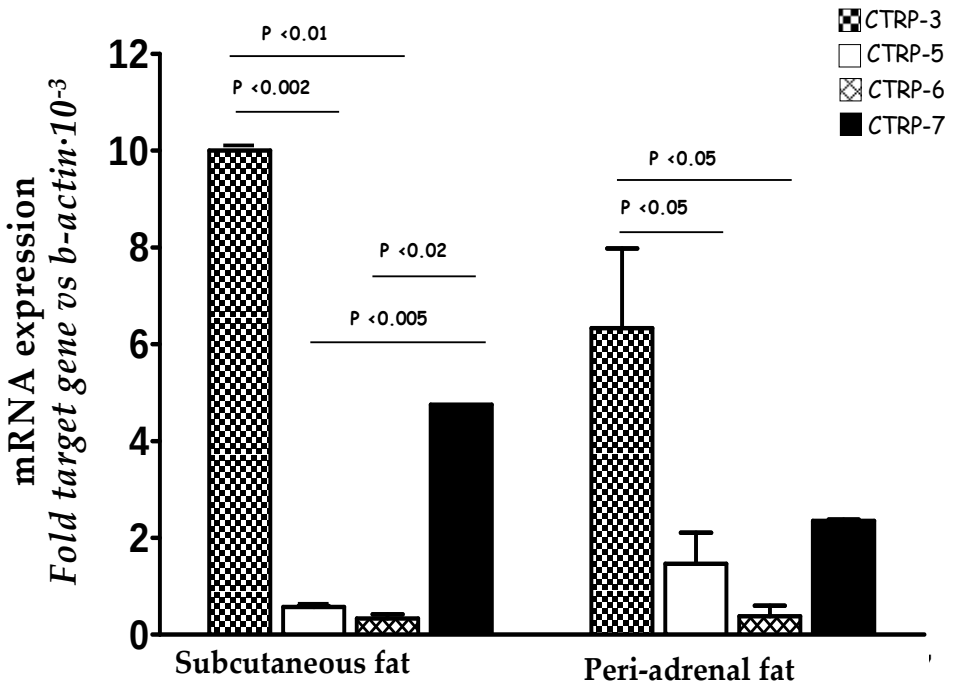


Figure 14: Analysis of CTRP-3, CTRP-5, CTRP-6 and CTRP-7 transcript levels in human tissues, subcutaneous adipocytes and peri-adrenal fat. (**A**) Quantitative real-time PCR analyses of CTRP expression in human tissues. Expression levels of CTRP transcripts in different tissues were normalized to their corresponding PBGD levels. (**B**) Quantitative real-time PCR analyses of CTRP expressions in subcutaneous adipocytes and peri-adrenal fat. The relative expression levels of CTRPs in subcutaneous and peri-adrenal fat normalized to their corresponding β-actin levels.

CTRP proteins expression in the human Adrenal gland

IHC analysis of the sections of the human adrenal gland showed that CTRP-3, CTRP-6 and CTRP-7 protein is produced in the adrenal gland (Fig. 13). A more intense immunostaining was observed at the zona glomerulosa in comparison to the capsule and other inner zones.

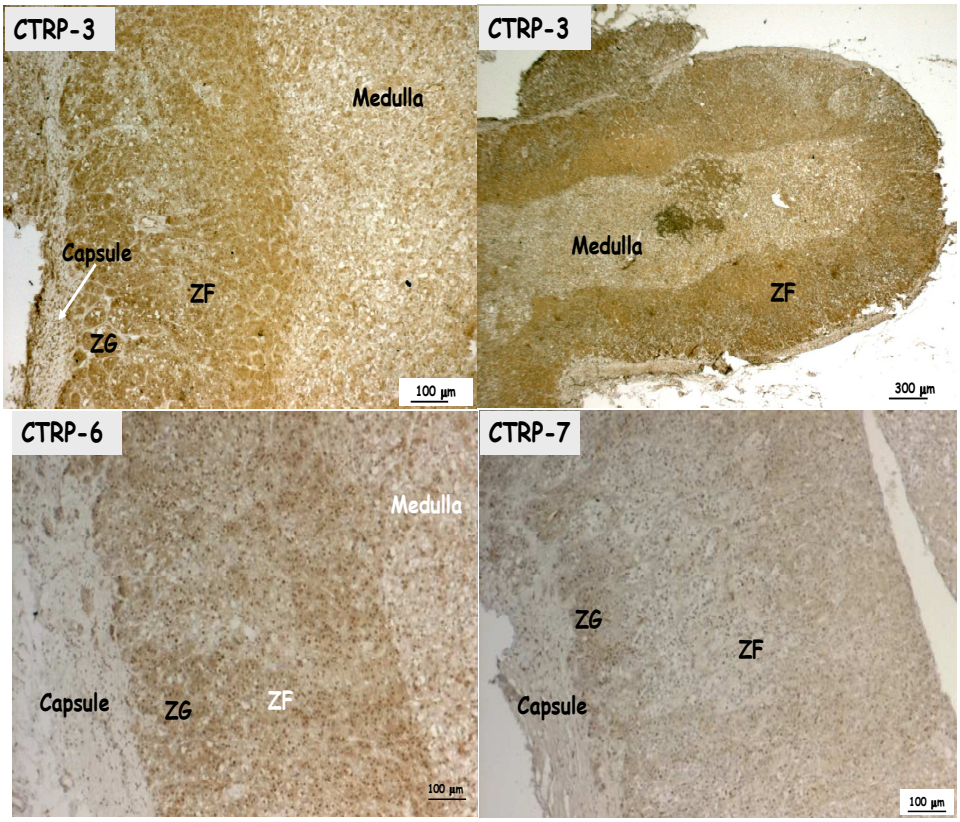


Figure 13: CTRPs were expressed in the zona glomerulosa region of the human adrenal cortex. Expression of CTRP-3 by IHC in the zona glomerulosa (ZG) and zona facciculata (ZF). Magnification, x 100 & 300. Also CTRP-6 and CTRP-7 shown positivity the zona facciculata (ZF). Magnification, x 100. The image was obtained by contrast of phase microscopy.

Discussion

Due to its high prevalence, arterial hypertension is the most important cardiovascular risk factor. However, hypertension alone occurs in less than 20% of the patients, with the majority having at least 2 risk factors, and clustering of the risk factors exponentially increases the probability to develop a cardiovascular event.(Kannel Am J Hypertens., 13: 3S-10S, 2000) Overweight-obesity (OO) and hypertension very often coexist, and both are associated with the metabolic syndrome. Notwithstanding this, the mechanisms underlying hypertension and OO in the metabolic syndrome remain enigmatic.

Some years ago our group showed a relationship between BMI and plasma aldosterone levels in a large population of hypertensive patients, thereby suggesting that aldosterone may be a link between hypertension and OO (33). More recently, the identification of CTRP-1 in the adipose tissue and the evidence that adrenocortical cell line H295 pre-treated with CTRP-1 can increase aldosterone synthesis (67), allowed us to hypothesize that CTRP-1 may be a mediator of the aldosterone production in OO hypertensive patients. Hence, we performed a series of experiments aimed first at investigating the expression of CTRP-1 in a wide array of tissues, particularly peri-adrenal fat and adrenal gland, and then to identify the cells that produce it. We further evaluated if CTRP-1 may affect aldosterone synthesis, and developed an ELISA assay to measure serum levels of CTRP-1 in normotensive lean/normal weight and hypertensive/obese patients. The overall results strongly support our hypothesis.

CTRP-1 is expressed in both the adrenal gland and the adipose tissue

This study first documented the expression of CTRP-1 in the human peri-adrenal gland and ZG. This finding is consistent with our hypothesis that CTRP-1 produced by the adipose tissue, particularly peri-adrenal fat, may stimulate aldosterone synthesis. Because there are many evidences that extra-visceral fat, e.g. epicardial fat, can affect the metabolism of the adjacent organ, it is reasonably to contend that also peri-adrenal gland can modulate hormone production of the adrenal cortex. Hence, excess of both visceral and peri-adrenal fat could enhance aldosterone synthesis, thereby explaining the relationship between BMI and plasma aldosterone levels previously described by our group in the hypertensive patients.

Consistently with Jeon and coll., who found that CTRP-1 is highly expressed in the rat adrenocortex, we observed that CTRP-1 is expressed in the human adrenocortex, mostly ZG. IHC and confocal microscopy clearly showed that both H295R adrenocortical cells and CD56+ cells isolated from the normal gland or the APA markedly express CTRP-1, thereby confirming that aldosterone-producing cells can produce CTRP-1. Use of the bead-based immunoseparation technique with CD56+, a specific marker of the ZG producing

aldosterone cells, allowed us not only to unequivocally identify CTRP-1 in this cell type, but also to localize the adipokine mostly at the membrane level, with a similar pattern to CD56+.

However, RT-PCR analysis showed that also CD56- cells express CTRP-1 gene, even to a smaller extent. Thus, the evidence that CTRP-1 is largely expressed within the adrenal gland, although with zonal gradient and mostly in ZG, suggests that CTRP-1 produced by the ZG cells may regulate aldosterone synthesis in the same cells via an intracrine mechanism. However, complex interactions between CD56+ and CD56- cells, involving paracrine effects, may be also suggested. Interestingly, CTRP-1 expression is higher in CD56+ cells as compared to CD56+ cells in the APA, but lower in normal adrenal gland, thereby suggesting that paracrine mechanisms are predominant under physiologic conditions, whereas intracrine mechanisms regulate the excess aldosterone production in the APA.

CTRP-1 could regulate aldosterone synthesis via paracrine mechanisms

The hypothesis that CTRP-1 could regulate aldosterone synthesis via paracrine mechanisms may be supported by *(a)* the increased aldosterone synthesis in H295R cells after treatment with CTRP-1 and *(b)* the very high expression of CTRP-1 in myelolipomas concurrent to APA.

In contrast to Jeon and coll., (49) we observed only a slight not significant increase in CYP11B2 gene expression after treatment of H295R with CTRP-1. Although either the commercially available CTRP-1 protein or the pooled protein fractions obtained with cloning in our laboratory provided similar results, these findings do not exclude a stimulatory effect of CTRP-1 on the aldosterone synthesis. In fact, at variance with the native protein, both proteins used in our experiments are not glycosylated, and it is well known that post-translational modifications are crucial for protein function. Moreover, the pooled fractions obtained with cloning in our laboratory was only in part purified and, therefore, the actual concentration of CTRP-1 could be very low, not sufficient to induce a significant stimulatory effect. Production of recombinant human CTRP-1 in insect cells and cloning of

CTRP-1 in baculovirus are ongoing in our laboratory at the aim of producing glycosylated protein and clarify this isssue.

The concomitance by chance of AML and primary aldosteronism can be estimated to be extremely low, based on conditional probability calculations. Hence, the observation of the concurrence of AML in two patients with hypertension that was caused by primary aldosteronism raises is the question on why this happened. It might be hypothesized that the AML and APA share a common pathogenic background or, alternatively that one is causally associated with the other. While the first hypothesis might find support in the previous report of concurrent AML and APA in a patient with heterozygosity for the retinoblastoma gene, the present finding would support instead the latter possibility for the following reason.

Of the two patients herein reported, one had primary aldosteronism due to unilateral adrenocortical hyperplasia,(68) while the other had an APA, indicating that AML does not associate just with APA, but also with adrenocortical hyperplasia ipsilateral to the AML. Moreover, our experiments showed a high level of expression of the CTRP-1in cell phenotypes that are comprised in the AML tumor tissue as well as in the adrenocortical tissue both with and without APA. This finding is of relvance since CTRP-1 was recently shown to exert a concentration-dependent secretagogue effect on aldosterone in an adrenocortical carcinoma cell line H215R that is widely used as an in vitro model for assessing the regulation of adrenocortical steroidogenesis. (49)

The finding of CTRP-1 immunostaining and gene expression also in the adrencortical ZG cells and in the APA rises the question if this is AML-derived receptor-bound CTRP-1 or locally produced (in the APA and in adrenocortical ZG) CTRP-1 acting in an autocrine fashion. The confocal microscopy experiments indicate that CTRP-1 is detectable not only on the plasma membrane of ZG and APA cells but also in the cytoplasm (Fig. 10), suggesting that it is synthesized in these cells. This conclusion is supported also by our gene expression experiments that demonstrated the presence of CTRP-1 mRNA in both cell phenotypes (Fig. 11).

Therefore, overall the observation of these two intriguing cases of concomitant AML and primary aldosteronism along with the set of experiments that were undertaken suggest the hypothesis that an enhanced synthesis of CTRP-1 in AML plays a causative role in triggering aldosterone excess production thus leading to primary aldosteronism. Moreover, as we previously described a relationship between excess adipose tissue and plasma aldosterone,(33) which itself is directly related to blood pressure level, the finding that a adipose tissue-rich tumour, as the AML, highly expresses CTRP-1 and is associated with primary aldosteronism due to either unilateral hyperplasia or a to an APA collectively suggests a functional link, which likely involves CTRP-1, between AML, adipose tissue, and hyperaldosteroinim. As the molecular mechanisms of primary aldosteronism, have been elusive thus far, this observation can have important implications for the understanding the pathophysiology of this is disease that is defined "primary" to underline its autonomy from the renin angiotensin system.

Incidentally of a high immunostaining for CTRP-1 also in cell lineages of the mature hematopoietic system suggest an important role of this protein also in bone marrow maturation, a funding that deserves further specific investigation.

CTRP-1 expression is expressed in many cell types and tissues

The finding that CTRP-1 gene expression is not confined to the adipose tissue, but occurs also in the adrenal cortex, heart, kidney, brain, arterial wall, lung and endothelial cells suggests that this adipokine may be syntesized in a wide array of tissues. IHC analysis confirmed that CTRP-1 can be markedly expressed in the arterial wall and the heart, as well as in the vascular smooth muscle cells, thereby strongly suggesting a role of CTRP-1 in the cardiovascular remodelling.

Peri-adrenal and visceral fat and the adrenal gland express more CTRP family members

We first detected expression of CTRP-3, CTRP-5, CTRP-6 and CTRP-7 in the peri-adrenal fat and adrenal gland. However, the expression of these adipokines in the adrenal gland was markedly lower as compared to that of CTRP-1, thereby suggesting a major role for CTRP-1

in the aldosterone production. Of interest, CTRP-3 was markedly expressed in the adipose tissue, thereby suggesting that, in addition to CTRP-1, also CTRP-3 may regulate the aldosterone production in a paracrine manner.

CTRP-1 levels can be measured in the human serum

We developed in our laboratory an ELISA assay to measure the levels of CTRP-1 in the human serum. At variance with the semi-quantitative method previously reported by Jeon and coll., which exploited immunoprecipitation, our method allows quantitative measurements of the CTRP-1 levels. Consistent with our hypothesis that CTRP-1 may be a link between OO, hypertension and aldosteronism, we found that CTRP-1 levels are higher in hypertensive/obese patients than in normotensive lean/normal weight subjects.

Conclusions and prespectives

The findings of this study showing a marked expression of CTRP-1 in the human peri-adrenal fat, in the normal adrenal ZG and the APA are consistent with our hypothesis that CTRP-1 is produced by the adipose tissue, particularly peri-adrenal fat, and that it may stimulate aldosterone synthesis. Moreover, it demonstrates that also ZG cells can produce CTRP-1, thereby suggesting that aldosterone-producing cells may modulate aldosterone synthesis by intracrine/paracrine mechanisms. These contentions are further supported by the demonstration that adrenocortical CD56+ cells strongly expressed CTRP-1 and that the adipose cells of the myelolipomas concurrent to APA are markedly immunostained with an antibody against CTRP-1. Moreover, a slight increase in CYP11B2 gene expression in H295R was observed after treatment with CTRP-1 protein and higher serum levels of CTRP-1 were found in hypertensive/obese patients than in normotensive lean/normal weight subjects.

Limitations: The commercially available CTRP-1 proteins and the pooled protein fractions contained a non- glycosylated form of the protein. We are now producing recombinant human CTRP-1 in insect cells and cloning of CTRP-1 in baculovirus to obtain CTRP-1

glycosylated protein and, therefore, to clarify whether CTRP-1 actually stimulates aldosterone production.

CTRP-1 levels were measured in a few samples of human sera. However, we have the availability of about 1,000 serum samples obtained from the patients participating the PAPY Study. The measurement of CTRP-1 levels in this large population, phenotipically characterized for BMI, blood pressure values and plasma aldosterone levels, could clarify whether CTRP-1 is a link between OO, hypertesnion and aldosteronism.

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