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Retinol-Binding Protein-4 in Women With Untreated Essential Hypertension

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BACKGROUND

Retinol-binding protein-4 (RBP4) is a novel adipokine able to modulate the action of insulin in several tissues. A variable degree of insulin resistance characterizes the vast majority of hypertensive (HYP) patients. The aim of this study was to evaluate the relationship between RBP4 and essential hypertension, exploring potential links between RBP4 and other adipokines with some proxies of early vascular damage in female naive HYP patients.

METHODS

Serum RBP4, leptin, adiponectin, and resistin levels were determined in 35 HYP and 35 normotensive lean women with normal glucose tolerance paired by age and body mass index (BMI) served as controls (CTL); carotid intima-media thickness (IMT) was also measured.

RESULTS

A striking difference was observed in RBP4 levels between HYP and CTL with significantly higher levels in the former than in the latter. No relationship was observed between glomerular filtration rate (GFR) and RBP4. Adiponectin levels were slightly but significantly lower in HYP than in CTL, whereas no differences were observed in resistin and leptin concentrations between the two groups of women. In the whole study group, a strong linear relationship was observed between IMT value and both RBP4 ($\rho = 0.321$, $P = 0.0076$) and resistin ($\rho = 0.340$, $P = 0.0048$); these two adipocytokines, together with cholesterol, were the only variables independently related to IMT ($r^2 = 0.24$; $P = 0.004$) by a stepwise analysis.

CONCLUSIONS

RBP4 levels are increased in naive HYP women and correlated with the degree of IMT suggesting a participation of this adipocytokine in the modulation of the atherosclerotic process exerted by the adipose tissue as endocrine organ.

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Adipose tissue is the main site of inflammatory and proatherogenic mechanisms that play a fundamental role in the pathogenesis of diffuse vascular damage;[1,2] moreover, it secretes many types of adipokines that contribute to influence the cardiovascular risk coupled with hypertension, dyslipidemia, and diabetes,[3] even by modulating the action of insulin in other tissues.[4]

The role of adipocytokines in essential hypertension has not been fully clarified: for example, chronic hyperleptinemia increases blood pressure via sympathetic nervous system–independent effects, such as oxidative stress, nitric oxide deficiency, enhanced renal Na^+ reabsorption, and overproduction of endothelin,[5] whereas resistin, another protein secreted by adipocytes and able to mediate insulin resistance in rodents,[6,7] does not differ between hypertensive (HYP) and normotensive (controls CTL) individuals and does not seem to correlate with the degree of insulin sensitivity in hypertension.[8] Regarding adiponectin, an adipocyte-derived plasma protein acting as an endogenous antiatherogenic factor and showing cardioprotective effects,[9,10] it was found decreased in the HYP state;[11] nevertheless, some epidemiological studies evaluating the role of adiponectin in cardiovascular disease have been contradictory.[12,13]

Retinol-binding protein-4 (RBP4) is a novel adipokine able to modulate the action of insulin in several tissues.[14,15] Experimental studies have shown that increased RBP4 expression impairs insulin signaling in muscle and increases gluconeogenesis in animals,[16] suggesting its involvement in the pathogenesis of insulin resistance. However, data in humans are less conclusive: serum RBP4 levels have been described elevated in obesity, type 2 diabetes, and polycystic ovary syndrome,[17,18] but other authors have failed to confirm these observations;[19,20] a relationship of RBP4 with features of the metabolic syndrome has also been inconsistently demonstrated.[21,22]

Essential hypertension is a common clinical condition linked with a high cardiovascular risk and characterized by a variable degree of insulin resistance; however, no information are so far available on the relationship between RBP4 and essential hypertension. Therefore, the present study was designed to address this specific issue, and to explore potential links between RBP4 and other adipokines with some proxies of early vascular damage in naive HYP patients.

METHODS

Thirty-five healthy women (CTL) and 35 neodiagnosed, untreated HYP women fulfilling the inclusion criteria were consecutively enrolled among patients attending the outpatient

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clinic for metabolic disease of our unit. Inclusion criteria were age <65 years, normal glucose tolerance after an oral glucose tolerance test and body mass index (BMI) <27 kg/m^2, and no ongoing acute or chronic illness requiring any chronic drug treatment. Diagnosis of essential hypertension was made on the basis of family history, absence of clinical signs suggestive for secondary hypertension, and the presence of a seated systolic blood pressure (SBP) ≥140 mm Hg and/or a diastolic blood pressure (DBP) ≥90 mm Hg in at least three separate clinic visits. Clinic blood pressure was measured in the sitting position with a mercury sphygmomanometer after a 10-min rest; the mean of two measurements was used for statistical analysis.

All participants were examined the morning after a 14-h overnight fast. Data on lifestyle were collected by a standard questionnaire, and all subjects underwent a complete clinical examination. Height and weight were recorded, and body composition was determined by bioelectrical impedance using a BIA TBF-300 instrument (Tanita, Tokyo, Japan). Venous fasting blood samples were drawn to measure fasting plasma glucose and insulin, lipid profile, serum creatinine, uric acid, and high sensitive C-reactive protein; serum samples were stocked for RBP4, IL-6, leptin, adiponectin, and resistin determination. Glucose 75 g in 200 ml water was administered immediately after baseline sampling and blood samples for plasma glucose and insulin estimation were drawn at 0, 30, 90, and 120 min for calculation of indexes of insulin sensitivity. The homeostasis model assessment (HOMA) index (=fasting plasma glucose (mmol/l) × fasting plasma insulin (mU/l)/22.5) was used as a proxy for insulin resistance. The oral glucose insulin sensitivity (OGIS) index was calculated as previously described.[23] An estimation of glomerular filtration rate (GFR) was obtained by the simplified Modification of Diet in Renal Disease Study prediction equation, which incorporates serum creatinine, body weight, age, and race. An informed consent for the study was obtained from all participants.

Laboratory methods. Plasma glucose and serum lipids were assayed by standard enzymatic methods. Plasma insulin was measured using radioimmunoassay (Linco Research, St Charles, MO). High sensitive C-reactive protein was determined by nephelometric analysis. Plasma concentrations of RBP4 were determined by a commercially available enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden-Nordenstadt, Germany) with an intra-assay coefficient of variation (CV) of 4.6% and an interassay CV of 5.1%. We validated the results by western blot analysis in a subset of 10 controls and 10 HYP women. Briefly, sera were diluted 1:20 in sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer and heated at 100 °C for 5 min. Samples (5 µl) and molecular weight markers were electrophoresed on 15% Trys–Glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA). After a blocking step using bovine serum albumin 3% in Tris–Tween buffered saline (Tris-buffered saline and Tween-20 0.05%) for 1 h at room temperature, blots were washed three times in Tris–Tween buffered saline and incubated overnight at 4 °C with primary antibody anti-human RBP4 (cat. no. ab57620; Abcam, Cambridge, MA) diluted 1:400. The bands' detection was performed incubating the blot with horseradish peroxidase–conjugated secondary antibody (cat. no. AP308P; Chemicon, Temecula, CA) diluted 1:4,000 for 1 h at room temperature, followed by enzymatic chemiluminescence kit (cat. no. 34075; Pierce Biotechnology, Rockford, IL). We obtained a good agreement between plasma RBP4 quantities measured by enzyme-linked immunosorbent assay and western blotting ($r = 0.71$, $P < 0.005$).

Adiponectin concentration was measured using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Linco Research; intra-assay CV, 3.4%, and interassay CV, 4.2%). Resistin concentrations were measured using a radioimmune assay kit (Linco Research; intra-assay CV, 2.8% and interassay CV, 6%). Circulating leptin levels were measured by enzyme-linked immunosorbent assay (R&D Systems; intra-assay CV, 3.0%, and interassay CV, 4.9%).

Within 1 week from the day of the blood drawn, all the subjects underwent an ultrasound scan of the carotid arteries to obtain a measurement of the intima-media thickness (IMT). Examinations were performed by a high-resolution B-mode ultrasound images (ACUSON 128 XP; Acuson, Mountain View, CA) with a 10 MHz linear probe by a sonographer blinded to the subject's identity. All ultrasound images were obtained with the subject in the supine position with the neck mildly extended. B-mode evaluations came from echographic images of the far wall in the first centimeter of common carotid arteries, proximal to the bulb dilation, in lateral projection. Four standardized points 5, 10, 20, and 30 mm from the carotid bulb were measured in both arteries and averaged to calculate the mean IMT for each subject.

Statistical analysis. Values are expressed as mean ± s.d. or median and interquartile range. Group comparisons were performed using the nonparametric Mann–Whitney *U*-test or the unpaired *t*-test, for variables with non-normal or normal distribution, respectively, and χ^2 for categorical variables. Relationships between variables were assessed using Spearman's correlation analysis and multiple linear regression analysis. The statistical significance was determined on a probability level of <0.05. Data were analyzed using the program StatView for Windows (SAS Institute, San Francisco, CA).

RESULTS

The two groups of women were well matched for age and clinical characteristics, as shown in **Table 1**, differing only by mean SBP and DBP values as by selection criteria. The frequency of family history for type 2 diabetes did not differ between the groups (CTL = 7 vs. HYP = 12; $\chi^2 = 1.822$; $P = 0.179$). Similarly, the metabolic parameters defining the degree of insulin sensitivity in the two groups of women, reported in **Table 2**, resulted superimposable in terms of either HOMA or OGIS (both P = ns).

Table 1 | Anthropometric and biochemical parameters of blood pressure values in CTL and HYP women

	CTL ($n = 35$)	HYP ($n = 35$)
Age (years)	46.9 ± 6.3	47.4 ± 5.0
BMI (kg/m^2)	25.7 ± 1.4	25.0 ± 1.6
Waist circumference (cm)	79 ± 6	78 ± 6
Fat mass (%)	28.1 ± 5.6	26.3 ± 3.2
Smoking (%)	25.7	22.9
Systolic pressure (mm Hg)	131.4 ± 6.8	148.0 ± 7.9*
Diastolic pressure (mm Hg)	79.9 ± 3.7	91.0 ± 4.5*
Total cholesterol (mg/dl)	216 ± 18	220 ± 13
HDL-cholesterol (mg/dl)	54 ± 8	57 ± 9
LDL-cholesterol (mg/dl)	136 ± 20	137 ± 16
Triglycerides (mg/dl)	130 ± 23	132 ± 18
Creatinine (mg/dl)	0.91 ± 0.12	0.94 ± 0.13
Uric acid (mg/dl)	4.4 ± 1.1	4.6 ± 1.0
hs-CRP (mg/l)	0.38 ± 0.13	0.41 ± 0.10

Data are expressed as mean ± s.d.
HDL, high-density lipoprotein; hs-CRP, high sensitive C-reactive protein; LDL, low-density lipoprotein; BMI, body mass index; CTL, control; HYP, hypertensive.
*$P < 0.001$ vs. CTL.

Table 2 | Metabolic parameters of blood pressure values in CTL and HYP women

	CTL ($n = 35$)	HYP ($n = 35$)
Fasting glucose (mg/dl)	84 ± 8	84 ± 10
2-h Glucose (mg/dl)	108 ± 14	111 ± 13
Fasting insulin (μU/ml)	16.4 ± 6.7	18.0 ± 6.4
HOMA index	2.05 ± 0.81	2.24 ± 0.75
OGIS index (ml/min/m^2 bsa)	492 ± 45	492 ± 49

Data are expressed as mean ± s.d.
HOMA, homeostasis model assessment; OGIS, oral glucose insulin sensitivity; CTL, control; HYP, hypertensive.

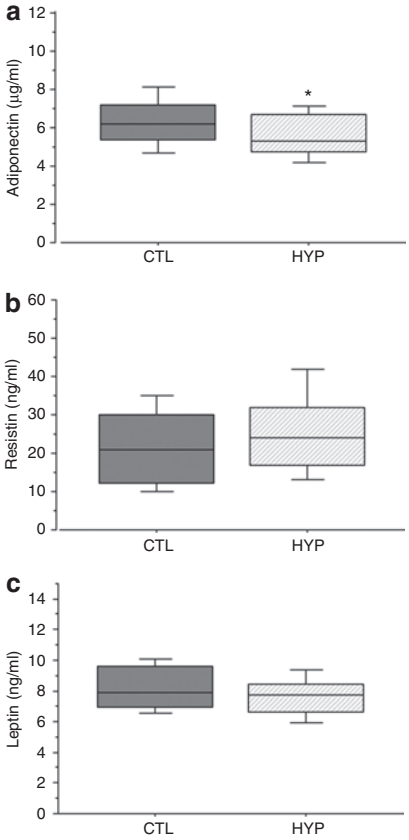

Figure 1 | Adiponectin, resistin, and leptin circulating concentrations in control (CTL) women (dark gray box plot) and hypertensive (HYP) women (light gray box plot). Data are expressed as median and interquartile range. *$P < 0.05$ vs. CTL.

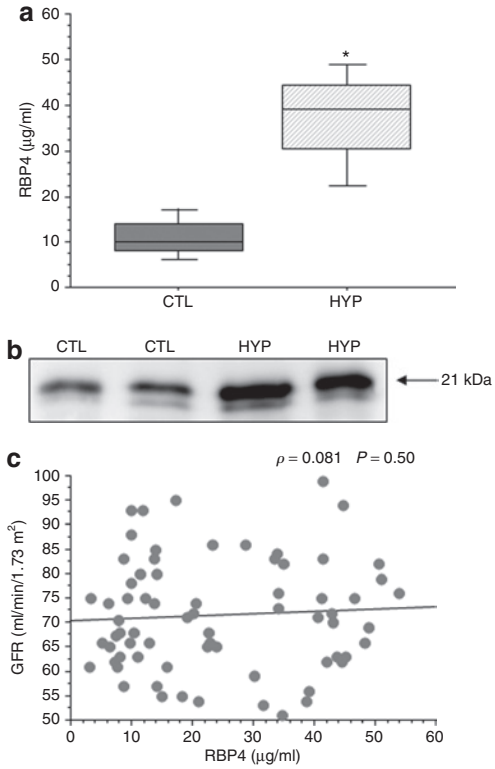

Figure 2 | Retinol-binding protein-4 (RBP4) levels in the two groups and its relationship with kidney functions. (**a**) Retinol-binding protein-4 (RBP4) circulating concentrations in control (CTL) women (dark gray box plot) and hypertensive (HYP) women (light gray box plot). Data are expressed as median and interquartile range. *$P < 0.0001$ vs. CTL. (**b**) Representative western blot for RBP4 protein performed on plasma of two CTL and two HYP women. (**c**) Linear relationship between RBP4 levels and glomerular filtration rate (GFR) in the whole study group.

Figure 1 shows adiponectin, resistin, and leptin concentrations in the two study groups. Adiponectin levels were slightly but significantly lower in HYP than in CTL, whereas no differences were observed in resistin and leptin concentrations between the two groups of women.

A striking difference was observed in RBP4 levels between HYP and CTL with significantly higher levels in the former than in the latter (Figure 2a). In Figure 2b, a representative western blot showing the difference between HYP and CTL is reported. In the presence of a normal kidney function in the whole study population, as shown by the estimated GFR

Table 3 | Matrix of univariate correlations between adipocytokines, IMT, and anthropometric and metabolic parameters

	BMI	Waist	SBP	DBP	IMT	OGIS	RBP4	RST	ADP
BMI	—								
Waist	0.60*	—							
SBP	−0.17	0.06	—						
DBP	−0.21	−0.01	0.66*	—					
IMT	−0.09	0.04	0.09	0.19	—				
OGIS	−0.33*	−0.23*	0.03	0.03	0.01	—			
RBP4	−0.19	−0.07	0.67*	0.74*	0.32*	0.22	—		
RST	−0.13	0.05	0.22	0.15	0.34*	0.22	0.11	—	
ADP	−0.31*	−0.39*	−0.33*	−0.27*	−0.01	0.06	−0.17	−0.19	—
LPT	0.29*	0.36*	−0.08	−0.07	−0.08	−0.19	−0.22	0.13	−0.10

ADP, adiponectin; BMI, body mass index; DBP, diastolic blood pressure; IMT, intima-media thickness; LPT, leptin; OGIS, oral glucose insulin sensitivity; RBP4, retinol-binding protein-4; RST, resistin; SBP, systolic blood pressure.
*$P < 0.05$.

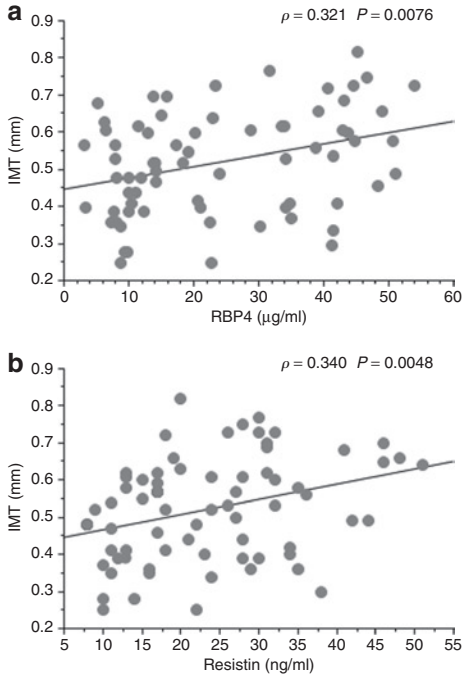

Figure 3 | Linear relationships between plasma concentration of (**a**) retinol-binding protein-4 (RBP4) or (**b**) resistin and intima-media thickness (IMT) in the whole study group.

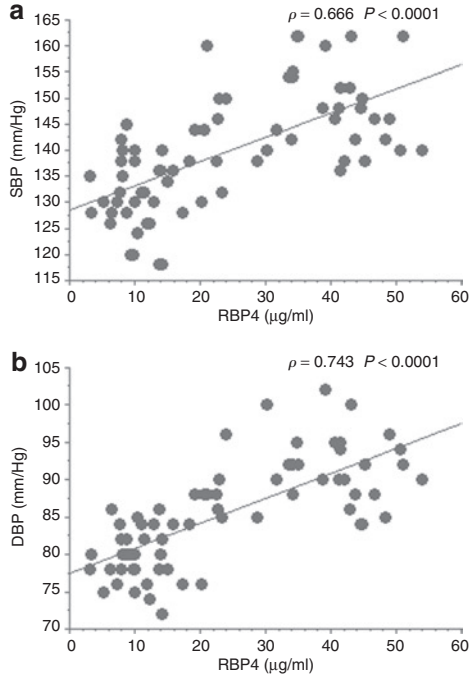

Figure 4 | Linear relationships between plasma concentration of retinol-binding protein-4 (RBP4) and (**a**) systolic blood pressure (SBP) or (**b**) diastolic blood pressure (DBP) in the whole study group.

calculated by the Modification of Diet in Renal Disease formula (median (interquartile range) was 70 (17) ml/min/1.73 m^2 in CTL and 71 (15.25) ml/min/1.73 m^2 in HYP, P = ns); no relationship was observed between GFR and RBP4 (**Figure 2c**).

We then considered the relationship between IMT value, as by the Methods section, and the measured adipocytokines. Mean IMT was 0.499 ± 0.127 mm in CTL and 0.543 ± 0.148 mm in HYP (P = ns); in the whole study group, a strong direct correlation was observed between IMT value and RBP4 and resistin (**Figure 3a,b**), but not with age, BMI, waist, total or low-density lipoprotein cholesterol, fasting plasma insulin and glucose or HOMA and OGIS indexes (**Table 3**). In addition, using a stepwise analysis, RBP4, resistin, and total cholesterol were the only variables independently related to IMT (r^2 = 0.24; P = 0.004) in a model also including blood pressure (as systolic, diastolic, or mean), BMI, and age.

Concerning the link between adipocytokines, anthropometric parameters, and blood pressure, in the whole group, RBP4 was strongly directly related with SBP and DBP values (**Figure 4a,b**), whereas no significant relation was observed with BMI and waist.

Adiponectin was inversely related with BMI, waist, SBP, and DBP; leptin was strongly related to the degree of adiposity,

but not with blood pressure values. Resistin did not show any correlation with the above reported parameters (**Table 3**).

DISCUSSION

This study is a quite comprehensive evaluation of the adipocytokine pathway in female nonobese, naive HYP patients. What is shown here for the first time is that these untreated HYP patients have increased RBP4 levels, and that this adipocytokine correlates with IMT, thus suggesting its possible use as a proxy of early vascular impairment in this kind of patients.

Many reports confirm a direct role for RBP4 as a mediator of insulin resistance in humans,[24–26] supported by positive correlations between RBP4 levels and the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, or type 2 diabetes, and in nonobese nondiabetic subjects with a strong family history of type 2 diabetes.[27] However, our two study groups, even not large, were strictly matched for degree of glucose tolerance, age, and BMI, thus likely excluding the influence of any metabolic alteration on RBP4 levels. It is interesting to underline that in these subjects with the same degree of insulin sensitivity (as suggested by HOMA and OGIS indexes, similar in the two groups), a clear relationship between these parameters and RBP4 was not observed, most likely due to the small range of insulin sensitivity. This suggests that in HYP women the link between blood pressure and RBP4 is not mediated by the degree of insulin sensitivity. Alternatively, we have to consider that we did not perform a direct measurement of insulin sensitivity in our study subjects, for example, by doing a euglycemic hyperinsulinemic clamp study; therefore, it is possible that HOMA and OGIS indexes might not be able to catch a fine difference in the degree of insulin sensitivity among these women. Another important point is to show that, in the presence of a normal glomerular filtration as shown by estimated GFR, this parameter does not affect RBP4 concentrations, which seem to be influenced by kidney function only when filtration function is reduced, at least in type 2 diabetic patients.[28]

The correlation between RBP4 and IMT is quite novel, and deserves attention. It has been recently shown that the early increase in IMT seen in healthy subjects without evidence of plaques or focal thickening in the carotid artery is likely to be a response to elevated pressure rather than to low shear stress.[29] In our HYP patients with presumably short duration of disease, even though the only clinical parameters directly related with RBP4 were systolic and diastolic values, the relationship between RBP4 and IMT was independent of such values, therefore suggesting a direct effect of RBP4 increment on the IMT, even within the normal range and in absence of any significant difference between CTL and HYP individuals. These results also agree with a recent study[30] performed in a large cohort of elderly subjects with subclinical cardiovascular disease, where RBP4 was related to intima-media and plaque fat content, determined by an index of echogenicity of the vessel wall. It is also important to point out that the increased levels of RBP4 cannot be attributed to an subclinical inflammatory status, as our HYP patients had C-reactive protein levels (a marker of inflammation) in the normal range, and similar to controls.

In our HYP subjects apparently not carrying other risk factors, elevation of adipocytokines such as RBP4 may play a role in reinforcing the risk of atherogenesis; we might suggest, as a speculation, an enhanced lipid peroxidation, or an increased presence of small dense low-density lipoprotein particles, conditions potentially related with increased RBP4 levels[31] and already described in HYP patients.[32,33] However, the results from this study cannot be extended to the general population; therefore, despite the significant association between RBP4 and atherogenesis demonstrated in this study, a direct causal relationship between this adipocytokine and the progression of this degenerative process cannot be concluded from the results. Moreover, it remains to clarify whether or not retinol is involved, given that low retinol levels have been described in arteriosclerotic patients,[34] whereas the Los Angeles Atherosclerosis Study did not reveal any role in retinol levels in determining the progression of IMT in a large cohort of subjects free of symptomatic cardiovascular disease at baseline.[35]

Several studies have tried to clarify the relationship between adipose tissue amount and distribution and adipocytokines production. Similar to other adipocytokines, RBP4 is expected to be associated with body fat distribution rather than weight *per se*. Graham *et al.*[27] reported that RBP4 was more highly correlated with waist-to-hip ratio than with BMI. In our subjects, these correlations have not been observed, suggesting a minor role of fat distribution in determining RBP4 in normal-weight subjects.

Beside RBP4, looking for the relationship between IMT and the other adipocytokines, resistin was the only one to be related with IMT, thus confirming a recent observation obtained in a larger cohort of treated HYP patients.[36]

Despite higher serum leptin levels in women, leptin and blood pressure associations have been reported more frequently in men than in women, regardless of hypertension and adiposity;[37] this might explain the lack of such observation in our study. Conversely, our observation of lower adiponectin levels in HYP women confirms previous reports[11,38] and reinforces the hypothesis that hypoadiponectinemia can relate to hypertension independently of classical factors.

The cross-sectional nature of our study limits determinations of temporality or causality. Therefore, a longitudinal study is needed to better explain the relationship of serum RBP4 with blood pressure values and carotid impairment in hypertension, possibly evaluating the role of coexisting conditions like, for example, endothelial dysfunction and the putative effect of anti-HYP therapy on the levels of this potentially new marker.

In conclusion, this newly identified link between RBP4, blood pressure values, and carotid IMT in naive HYP women reinforces the direct role of adipose tissue as a metabolic organ directly involved in the pathogenesis of early vascular damage in the course of hypertension.

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