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Research Article

Retinol Binding Protein-4: a Novel Predictor of Insulin Resistance in Lean Subjects

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Abstract

Background : Retinol-binding protein 4 (RBP4),which is an adipokine related to impaired glucose tolerance, has been associated with insulin resistance (IR) and β-cell function in subjects with obesity or diabetes. RBP4 has been shown to induce insulin resistance, and plasma RBP(4) values are increased in type 2 diabetes mellitus, obesity, metabolic syndrome, and cardiovascular disease .Some researchers claimed that Elevated plasma RBP4 levels have been predominantly found in men and women with abdominal adiposity even in the non-diabetic state. **Objective:** In our study we assessed RBP4 levels in healthy lean subjects in comparison to obese and diabetic patients ,and also determined whether any correlations exist between RBP4 levels and the metabolic profile **Subjects and Methods:** RBP4, Fasting glucose, Fasting insulin, homeostasis model assessment (HOMA)-IR,Hemoglobin A1c, cholesterols (Total, HDL and LDL), triglycerides and BMI were measured in in a group of 30 healthy lean subjects and other same number and sex-matched two groups of obese and patients with type 2 DM. **Results:** The three groups included in the study highly significantly differed from each other and between groups regarding fasting insulin and HOMA-IR ($p<0.001$). All groups highly significantly differed in their BMI, fasting blood glucose (FBG), HbA1c, total and LDL-cholesterol ($p<0.001$), and significantly in triglycerides levels (TG) ($p<0.05$) but not in HDL-cholesterol ($p>0.05$). As regards RBP4, a highly significant difference was found between Group I when compared to either group II or Group III, being higher in the former group ($p<0.001$).The mean levels were (49 ± 8.14), (33.4±6.8), and (27.1± 7) respectively. **Conclusion:** We conclude that RBP4 is elevated in the serum of lean and obese subjects who are being at high risk of the development of frank diabetes and appears to identify possible development of insulin resistance and associated cardiovascular risk factors in lean subjects.

Keywords: Obesity; insulin resistance; RBP4.

Introduction

Retinol binding protein 4 (RBP4) is a member of the lipocalin family of proteins that transport retinol from the liver to the peripheral tissues and plasma RBP4 levels positively correlate with retinol levels **[1].**Recent studies aimed to quantify RBP4 serum standards in population with a wide range of body mass index (BMI) and glucose tolerance level **[2].**

The complex pathogenesis linking obesity, type 2 diabetes mellitus (T2DM), hypertension and dyslipidemia involve mechanisms ranging from increased insulin resistance (IR) through adipocytes production of pro-inflammatory and pro-oxidants factors. Adipose tissue is the largest endocrine organ of human body which regulates glucose homeostasis, steroid production, immune system action, hematopoietic and reproductive function. Despite a strong epidemiological association, overweight does not explain itself only type 2DM development **[3,4 ,5].**

Adipokines have an important role in the development of obesity-related co-morbidities not only in adults but also in children and adolescents. Retinol binding protein 4 (RBP4) is suggested to link obesity with its co-morbidities, especially insulin resistance, type 2

diabetes (T2D), and certain components of the metabolic syndrome. However, data, especially resulting from the clinical studies, are conflicting **[6,7].**

In addition to lower insulin sensitivity, a negative effect on secretion of β-cells caused by RBP4 is suggested. On the other hand, there is a variety of recent studies that did not find in RBP4 an accurate biomarker of IR and metabolic syndrome (MS) in diabetic or normal subjects **[8].**

Aim of the Work:

This study aimed to assess the level of retinol binding protein 4 in a group of healthy lean subjects in comparison to obese population and diabetic patients.

Subjects and Methods

Study design:

This cross sectional study, was conducted on 90 individuals selected from outpatients clinic of Internal Medicine and Endocrinology Unit of Ain Shams University Hospitals from October 2014 to March 2015 .They were divided into the following groups: Group I: which included 30 healthy non-diabetic lean subjects, Group II: including 30 obese non diabetic subjects and Group III: including 30 obese patients with type II DM.

Overweight and obesity were defined according to the World Health Organization criteria on the basis of the body-mass index (the weight in kilograms divided by the square of the height in meters**) [9].**

Definitions of normal glucose tolerance, impaired glucose tolerance, and type 2 diabetes were based on the American Diabetes Association criteria for glucose values obtained after an overnight fast and a two-hour oral glucose-tolerance test (OGTT), conducted with a standard loading dose of 75 g**[10].**

Healthy subjects were recruited. The inclusion criteria were an age of 25 to 55 years, a body-mass index of 22 to 30, normal glucose tolerance, fasting triglyceride level less than 150 mg/dl and the absence of known endocrine or metabolic disease.

Exclusion criteria:

Patients with type 1 DM, patients using incretins ,insulin or steroids, and those with significant liver or kidney function impairment.

All subjects were subjected to the following:

1-Clinical Assessment by full history taking and thorough clinical examination including measuring of blood pressure and waist circumference, calculation of body-mass index (BMI) using the Quetelet formula (weight in kilograms divided by the square of height in meters.

2-Laboratory Investigations including: fasting glucose, fasting insulin, glycated hemoglobin, Kidney function tests including serum creatinine and blood urea nitrogen (BUN), liver function tests including AST and ALT, lipid profile including Total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides, Retinol Binding Protein-4 (RBP4) and assessment of insulin resistance (IR) by calculating homeostasis model assessment Score (HOMA) which is calculated using the formula = {Fasting glucose (mg/dl) x fasting insulin (mU/L)}/ 405. IR was considered to be present in cases with HOMA ≥ 3.0 **[11,12].**

Six milliliters of venous blood were collected under complete aseptic precautions from each subject. The collected blood was divided among an EDTA tube for glycated hemoglobin and a plain test tube for serum separation. After clotting, samples were centrifuged at 1000 xg for 15 minutes and sera were separated. Hemolysed samples were discarded, repeated freezing and thawing was avoided. All individuals were enrolled in this study after an informed oral and written consent.

Analytical Methods:

1. Fasting glucose, Total cholesterol (TC), HDL cholesterol (HDLc), and triglycerides (TG) were measured by enzymatic assays on Synchron CX-9autoanalyzer (Beckman Instruments Inc.; Scientific Insturments Division, Fullerton, CA 92634, 3100, USA).LDL-cholesterol levels were calculated using Friedewald's equation: LDLc = TC – HDLc - TG/5 (mg/dl) **[13].**

2. Hemoglobin A1c percent was determined using the Bio-Rad D-10™ Hemoglobin A1cProgram on D-10™ instrument (Bio-Rad Clinical Diagnostics Group, 4000 Alfred Nobel Drive Hercules, California94547) which depends on ion-exchange high-performance liquid chromatography (HPLC) technique. The D-10 Hemoglobin A1cProgram utilizes principles of ion-exchange high-performance liquid chromatography (HPLC). The samples are automatically diluted on the D-10 and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured

3. Fasting serum insulin was measured with a two-site chemiluminescent enzyme immunometric assay for the IMMULITE® 1000 automated analyzer (Siemens Healthcare Diagnostics Inc., Los Angeles, CA, USA).

4. RBP4 was measured with a competitive enzyme-linked immunoassay kit supplied by (Phoenix Pharmaceuticals, Inc., Belmont, CA). Results are expressed in ng/mL (normal range 2-28ng/ml) .The immunoplate in this kit is pre-coated with a secondary antibody, whose nonspecific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody. This primary antibody's Fab fragment will then be competitively bound by both the biotinylated peptide and the targeted peptide in either the standard peptide solution or the unknown sample. The biotinylated peptide interacts with streptavidin-horseradish peroxidase (SA-HRP) which catalyzes the substrate solution. Addition of the stop solution should change the color in each well from blue to yellow. The intensity of the resulting yellow color is directly proportional to the amount of biotinylated peptide-SA-HRP complex, but inversely proportional to the amount of the targeted peptide (in either the standard peptide solution or the unknown sample). This is due to competition between the biotinylated peptide and the target peptide for binding with the primary antibody. A standard curve was established by plotting the measured absorbance as a function of the various known standard peptide concentrations. Unknown RBP4 concentration in samples was determined via extrapolation based on this standard curve.

Statistical Methods

All data were analyzed using software (version 11, SPSS Inc., Chicago, Illinois). Parametric data were expressed as mean and standard deviation ($\bar{X} \pm SD$). Comparative statistics was done between two groups by Chi squared test and across all cohorts (Lean, Obese and Diabetics) by one-way ANOVA and post-hoc Tuckey analysis. Correlation analysis was performed by Pearson's correlation (r). P values<0.05 were considered significant, whereas values<0.01 or <0.001 were considered highly significant.

Results

Results are presented in tables (1-3) and figure (1).

The three groups included in the study highly significantly differed from each other and between groups regarding fasting insulin and HOMA-IR ($p<0.001$). All groups highly significantly differed in their BMI, fasting blood glucose (FBG), HbA1c, total and LDL-cholesterol ($p<0.001$), and significantly in triglycerides levels (TG) ($p<0.05$) but not in HDL-cholesterol ($p>0.05$). As regards RBP4, a highly significant difference was found between Group I when compared to either group II or Group III, being higher in the former group ($p<0.001$).The mean levels were (49 ± 8.14), (33.4±6.8), and (27.1± 7) respectively.

Correlation analysis between different studied parameters in all studied three groups revealed that RBP4 significantly positively correlated with BMI, fasting blood glucose, fasting insulin, HOMA-IR, HbA1c and total and LDL-cholesterol (r=0.4, 0.5, 0.6, 0.6, 0.5, 0.5 and 0.6 respectively; ($p<0.001$).

Table (1): Descriptive and Comparative Statistics of the Various Studied Parameters in the Three Groups using Chi-Square test for age and ANOVA followed by post-hoc Tukey's test forsex and BMI.

		Groups						ANOVA		TUKEY'S Test		
		Group I (Lean) n=30		Group II (obese) n=30		Group III (Type II DM) n=30		F or X^2	p-value	I&II	I&III	II&III
Sex	Female	15	50%	15	50%	15	50%	0.000	1.000			
	Male	15	50%	15	50%	15	50%					
Age (years)	Range	30 - 46		32 - 52		32 - 54		6.612	0.003	0.394	0.002	0.067
	Mean ±SD	39.1 ± 5.7		41.8 ± 6.8		46.4 ± 6.7						
BMI	Range	18 - 25		30 - 38		18 - 40		60.611	<0.001	<0.001	0.001	<0.001
	Mean ±SD	21.4 ± 2		34 ± 2.7		26 ± 5.4						

p> 0.05: Non-significant difference; p < 0.05: Significant difference;
p < 0.01, <0.001: Highly significant difference

Table (2): Descriptive and Comparative Statistics of the Various Laboratory Data in the Three Studied Groups using ANOVA Test followed by post-hoc Tukey's Test.

		Groups			ANOVA		TUKEY'S Test		
		Group I (Lean) n=30	Group II (Obese) n=30	Group III (Type II DM) n=30	F	p-value	I&II	I&III	II&III
HbA1c (%)	Range	5 - 6.4	5.1 - 6.2	6 - 9.5	44.526	<0.001	0.992	<0.001	<0.001
	Mean ±SD	5.7 ± 0.5	5.7 ± 0.5	7.4 ± 0.9					
FBG (mg/dL)	Range	71 - 100	70 - 100	110 - 181	134.284	<0.001	0.241	<0.001	<0.001
	Mean ±SD	83 ± 9	89 ± 10	145 ± 18					
F.insulin (µU/mL)	Range	5 - 12	8 - 22	14 - 25	89.850	<0.001	<0.001	<0.001	<0.001
	Mean ±SD	7.6 ± 2.2	15.7 ± 3.3	19.9 ± 3.2					
RBP4 (ng/mL)	Range	16 - 30	8 - 20	7 - 19	45.063	<0.001	<0.001	<0.001	0.050
	Mean ±SD	49 ± 8.2	33.4 ± 6.8	27.1 ± 7					
LDL-C (mg/dL)	Range	70 - 132	75 - 187	75 - 189	14.577	<0.001	<0.001	<0.001	0.943
	Mean ±SD	100 ± 19	139 ± 31	142 ± 29					
HDL-C (mg/dL)	Range	39 - 58	28 - 50	28 - 161	0.702	0.500			
	Mean ±SD	46 ± 6	40 ± 7	48 ± 37.5					
TC (mg/dL)	Range	101 - 195	156 - 233	160 - 275	17.563	<0.001	<0.001	<0.001	0.399
	Mean ±SD	165 ± 22	197 ± 20	207 ± 27					
TG (mg/dL)	Range	79 - 159	75 - 118	48 - 166	3.324	0.043	0.244	0.628	0.036
	Mean ±SD	110 ± 24	98 ± 11	117 ± 32					
HOMA-IR	Range	0.9 - 2.4	1.9 - 5.4	2.2 - 9.7	101.449	<0.001	<0.001	<0.001	<0.001
	Mean ±SD	1.5 ± 0.5	3.4 ± 0.9	6.9 ± 1.9					

p> 0.05: Non-significant difference; p < 0.05: Significant difference;
p < 0.01, <0.001: Highly significant difference

Figure (1): Comparison between RBP4 Levels(ng/mL) in the Three Studied Groups

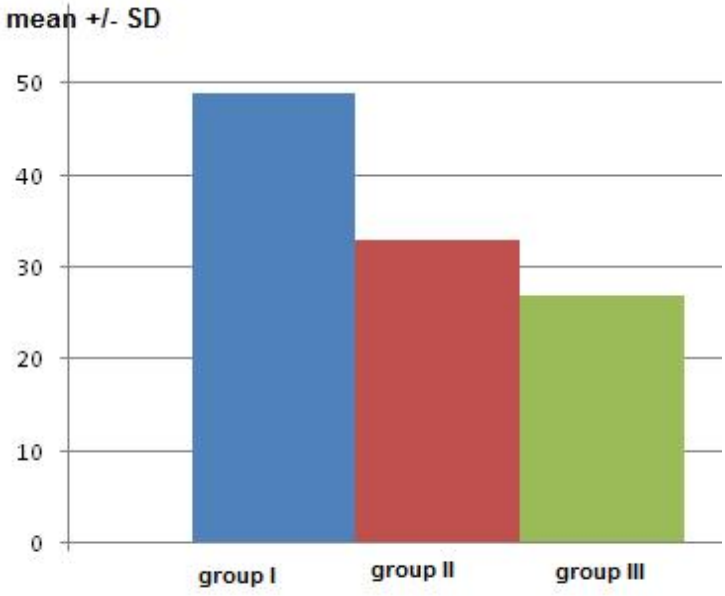

Table (3): Correlation Study between RBP4 (ng/mL) and the Other Assessed Parameters in All Subjects Included Collectively and in Each Group:

Compared Parameter	RBP4							
	All n=90		Group I (Lean) n=30		Group II (Obese) n=30		Group III (Type II DM) n=30	
	r	p	r	p	r	P	r	P
Age (years)	0.324	0.012*	0.095	0.689	0.064	0.789	0.108	0.651
BMI	0.446	<0.001*	0.192	0.418	-0.272	0.247	0.183	0.440
FBG (mg/dL)	0.524	<0.001*	0.296	0.206	0.500	0.025*	0.103	0.664
F.insulin (µU/mL)	0.620	<0.001*	0.042	0.862	0.211	0.372	0.319	0.171
HOMA-IR	0.587	<0.001*	0.081	0.734	0.392	0.087	0.133	0.575
HbA1c (%)	0.457	<0.001*	0.395	0.085	0.016	0.946	0.039	0.871
LDL-C (mg/dL)	0.524	<0.001*	0.186	0.433	0.289	0.216	0.002	0.993
HDL-C (mg/dL)	0.072	0.584	0.138	0.560	0.306	0.189	0.251	0.286
TC (mg/dL)	0.574	<0.001*	0.124	0.603	0.557	0.011*	0.015	0.950
TG (mg/dL)	0.018	0.893	0.020	0.934	0.216	0.360	0.028	0.906

p> 0.05: Non-significant difference; p < 0.05: Significant difference;
p < 0.01, <0.001: Highly significant difference.

Discussion

With the epidemics of obesity, much effort has been put into unraveling the mechanisms by which obesity causes its co-morbidities. Biologically active factors secreted from the adipose tissue have both local and systemic effects on the metabolism, immune system, and endocrinology **[14].**

There is a growing evidence, that there is adipocyte-derived cytokines, known as adipokines, seem to interfere with the crosstalk between adipose tissue, insulin resistance and CAD (cardiovascular disease). **[15].**

Retinol-Binding Protein-4 (RBP4), a adipokine/hepatokine, initially identified by Yang et al. **[16],** is the main transport protein for retinol (vitamin A) in the circulation. Of importance, RBP4 does not interact only with retinol. Formation of a complex with transthyretin – a carrier of thyroid hormone and retinol – prevents glomerular filtration of RBP4 and its subsequent excretion through the kidney **[17].**

Most, but not all studies, have demonstrated its link with insulin resistance and obesity **[16,18].** Recent studies in human found that plasma RBP4 levels were elevated in subjects with IGT or type 2 diabetes mellitus and that RBP4 was related to various clinical parameters known to be associated with insulin resistance **[19, 1].**

RBP4 has been shown to induce insulin resistance, and plasma RBP4 values are increased in type 2 diabetes mellitus, obesity, metabolic syndrome, and cardiovascular disease**[20,21].**

Some researchers claimed that Elevated plasma RBP4 levels have been predominantly found in men and women with abdominal adiposity even in the non-diabetic state **[14, 22].**Similarly, high RBP4 levels in obese children seem to be related to adipose tissue mass, to the differentiation of adipocytes, and to multiple risk factors for adiposity-related co-morbidities [**23, 24].**

As Retinol binding protein 4 (RBP4) is regarded as a novel cardio-metabolic risk factor, **[25],**we believe that the importance of RBP4 should be assessed in more diverse groups. Therefore, our study aimed to assess the levels of RBP4 in a group of healthy non obese non diabetic lean subjects to be compared with other two groups of obese and diabetics and to search for possible association of serum RBP4 levels in such individuals with insulin resistance and lipid profile as components of metabolic syndrome and cardiovascular risk factors.

Unexpectedly, we found that RBP4 is significantly higher in healthy lean subjects than obese and diabetics. However, we found that the magnitude of increase in serum RBP4 correlates with insulin resistance among all studied individuals. Moreover, the serum RBP4 level is correlated with a cluster of cardiovascular risk factors accompanying insulin resistance as part of the metabolic syndrome (BMI, fasting blood glucose (FBG), HbA1c, total and LDL-cholesterol, and significantly in triglycerides levels but not in HDL-cholesterol.

Our findings are consistent with those of Graham et al., 2006 **[22]** who showed that the relationship between the serum RBP4 level and insulin resistance was independent of obesity, and that non-obese subjects also exhibited increased serum RBP4 levels. They explained that this group of lean subjects had decreased expression of GLUT4 (glucose transporter 4) in adipocytes which predicts increased serum RBP4 levels and insulin resistance. Insulin resistance does not develop in all obese persons, and genetic background contributes strongly to insulin resistance, even in non obese persons **[22, 26].**

Several other studies found no correlation between circulating RBP4 levels, the level of obesity, and the amount and distribution of adipose tissue **[27, 28].**

A previous study has shown that circulating RBP4 and TTR (transthyretin) were not affected by human obesity or T2DM, compared to lean controls **[22].**

RBP4 induces expression of pro-inflammatory cytokines in mouse and human macrophages and thereby indirectly inhibits insulin signaling in co-cultured adipocytes**[25,29].**

Our possible explanation of such high RBP4 in lean persons, with the low adipose tissue mass is possibly of having low expression of pro-inflammatory cytokines and good insulin sensitivity. However, a study by Norseen et al., was performed on three groups of non-diabetics; insulin- sensitive, insulin resistant lean humans and in insulin-resistant obese subjects; 8 persons each. They measured RBP4 by mass spectrometry immunoassay and their assayed mean values were very close to ours. Their data also showed that total RBP4, levels were increased by 2.5- to 3-fold in both lean and obese insulin-resistant subjects and that the RBP4/retinol ratio is elevated in insulin-resistant humans independent of obesity **[29].**

On the other hand, the mean serum RBP4 levels reported by Takashima et al, **[30]**appeared high (47.43 ± 24.33, range 6.29-208.73), reflecting the fact that

most RBP4 assays were developed to detect low RBP4 levels in patients with vitamin A deficiency and have not been validated for measuring elevated RBP4 levels. We would note that different commercial RBP4 assays reported widely different values for RBP4 in identical serum samples from subjects with insulin resistance. Quantitative Western blotting of the same samples of the same researchers yielded RBP4 values that correlated most strongly with insulin resistance. Therefore, we recommend thorough cross-validation of assays for measurements of elevated RBP4 levels. Since serum-collection tubes containing clot-activating agents or tubes with plasma anticoagulants may cause spurious effects, we recommend the use of additive-free collection tubes.

Correlation analysis between different studied parameters in our three studied groups revealed that RBP4 significantly positively correlated with BMI, fasting blood glucose, fasting insulin, HOMA-IR, HbA1c and total and LDL-cholesterol. On the other hand, RBP4 was not associated with IR or metabolic indices in each group. Such findings agreed with those of Shim et al., 2010, who revealed no significant correlation of RBP4 with waist circumference, HDL cholesterol, ApoB/ApoAI ratio, and the HOMA index **[12].**

Similarly, Ulgen et al., 2010, also performed age and sex-adjusted multiple linear regression models in obese group and showed no significant association of serum RBP4 levels with BMI, waist-to-hip-ratio, blood pressure, cholesterol, triglycerides, fasting glucose, 2-hour glucose, insulin resistance (as assessed by HOMA-IR), or insulin secretion . They said that obesity might already be associated with elevated RBP4 levels which then show no additional correlation with metabolic markers **[31].**

However, a recent prospective study by Sun et al., 2014, was performed on Chinese adults aged 50–70 years indicated that plasma RBP4 is independently associated with the 6-year risk of developing type 2 diabetes**[32].**

Therefore, the ability to assess a person's risk of impaired glucose tolerance and type 2 diabetes in lean persons by measuring RBP4 before the onset of the disease would provide a rational means for implementing preventive lifestyle interventions or pharmacologic treatment. Our conclusion agrees with Norseen et al who assumed that the lowering of RBP4 levels improves glucose homeostasis **[29].**

Since circulating transthyretin (TTR) is a critical determinant of plasma retinol-binding protein 4 (RBP4) levels, recently, Zemany et al., 2015, also suggested that decreasing circulating TTR levels or altering TTR-RBP4 binding improves glucose metabolism and insulin sensitivity and could be a potential therapeutic approach for the treatment of type 2 diabetes **[33].**

The concentration of serum RBP4 varies by gender, generally lower in women than in men, both among adults and children **[34, 35, 36].** Therefore we included both genders equally in each group.

Conclusion

We conclude that RBP4 is an adipocytes-secreted molecule that is elevated in the serum of lean and obese subjects who are being at high risk of the development of frank diabetes and appears to identify possible development of insulin resistance and associated cardiovascular risk factors in lean subjects.These finding provide a rational for anti-diabetic therapies aimed at lowering serum RBP-4.

Limitations of study

Other interfering factors, such as levels of retinol, iron and even renal function appear to be important and should be investigated in future studies. Differences in results of this study regarding others can also be caused by different methods of RBP4 quantification. Moreover, since the cross-sectional design of the study represents a limitation, we recommend further prospective studies to be done on lean persons with elevated RBP4 levels.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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