RELATIONSHIP OF RETINOL BINDING PROTEIN-4 WITH INSULIN RESISTANCE, LIPID PROFILE AND BODY MASS INDEX

Bushra Noreen[1*], Muhammad Imran Khan[2], Muhammad Shahid Qureshi[3], ZainabIqbal[4], Sadia Sabahat[5], Aroosa Waheed[6],Faheem Hadi[7], Tahir Maqbool[8*]

[1]Department of Physiology, D.G. Khan Medical College, DeraGhazi Khan, Pakistan.
[2]Department of Physiology, D.G. Khan Medical College, DeraGhazi Khan, Pakistan.
[3]Allama Iqbal Teaching Hospital,Dera Ghazi Khan, Pakistan.
[4]Allama Iqbal Teaching Hospital, Dera Ghazi Khan, Pakistan.
[5]Department of Pharmacology, D.G. Khan Medical College, Dera Ghazi Khan, Pakistan.
[6]Avicena Institute of Medical Sciences, Dera Ghazi Khan, Pakistan
[7]University College of Conventional Medicine, Faculty of Medicine and Allied Health Sciences, TheIslamia University of Bahawalpur, Bahawalpur, Pakistan.
[8]Centre of Research in Molecular Medicine, Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan.

***Correspondence**
Dr. Bushra Noreen*

Dr. Tahir Maqbool*

ABSTRACT

Background: Retinol Binding Protein 4 (RBP-4) is an adipokine, RBP-4 was identified as key regulator of obesity related insulin resistance and type 2 diabetes, and certain components of metabolic syndrome. RBP4 is secreted mostly by liver and adipocytes.RBP-4 is a member of lipocalin family transporting vitamin A from liver to peripheral tissues.RBP-4 is linked to obesity and its comorbidities especially insulin resistance, type 2 diabetes, and certain components of metabolic syndrome.The present study is aimed to assess serum level of RBP4 and its correlation with insulin resistance in patients with visceral obesity and type 2 diabetes mellitus.**Methadology:**For the current study forty eight men (n=48) and thirty two women (n=32) age and sex matched controls were substituted. All of the research work was carried out after the approval from the research ethical committee of the Institute of molecular biology and biotechnology (IMBB), The University of Lahore. An informed consent was obtained from the patients before including them into the study. Five ml of blood was taken from cubital vein and stored at -70^{o}C for further analysis. The samples were processed and analyzed for the estimation of LDL/HDL, VLDL and Triglycerides with the help of their respective methods. **Results:**The demographic data of the cohort is shown in table 1. The cohort was composed of men (n=48, 54%) and women (n= 32, 46%) with mean ages of 33.64 ± 12.08 and 31.50 ± 20 years respectively. Means and medians of BMI, waist circumference, fasting blood sugars, serum insulin, HOMA-IR and serum RBP4 levels are also shown. All the above-mentioned variables were skewed as shown by Shapiro Wilk test ($p \leq 0.05$). **Conclusion:**The current study demonstrated the value of RBP4 as an indicator of insulin resistance in type 2 diabetes and viscerally obese subjects as it was found high in viscerally obese group as well as in diabetics. Also, RBP4 had significant positive correlation with FI & HOMA-IR in the viscerally obese group, so it may be a risk factor for type 2 diabetes in this group.

Keywords: Type2DiabetesMellitus Low Density Lipoproteins, High Density Lipoproteins, Very Low Density Lipoproteins, Triglycerides

1.0 INTRODUCTION

Retinol binding protein-4 (RBP-4) is type of adipokine, which is involved in regulation of diabetes and insulin resistance related obesity. It is mostly found in adipocytes and hepatocytes. It is member of lipocalin family that transport vitamin A from hepatocytes to other tissues (Nankam & Blüher, 2021). Visceral obesity is involved in contribution of cardiovascular diseases, diabetes and insulin resistance. RBP-4 impair insulin-stimulated uptake of glucose in muscles and contribute to insulin resistance and diabetes and hence, production of glucose in hepatocytes is increased (Kilicarslan *et al.*, 2020). In adipocytes, its expression in inversely related to glucose transporter-4 and it inhibits insulin induced phosphorylation of insulin receptor substrate-1.

Several endogenous signals (adipokines) are involved in metabolism of glucose, which inturn promote diabetes development and disturb signal pathways related to insulin. Levels of HbA1c and FBG are also raised in patients as compared to healthy individuals. This mechanism is related to higher levels of free fatty acids and impaired glucose due to adipokines effect. In a research work in Turkey, effects of adipokines and fat distribution on insulin resistance were studied (Bilir*et al.*, 2016). In another study, lower levels of HDL and higher levels of LDL and serum cholesterol were observed in patients as compared to control (Bora *et al*., 2015). Similar findings were also found in obese patients (Gomez-Ambrosi*et al.*, 2014). HOMA-IR% was found higher in diabetics as compared to healthy individuals (Awad*et al*., 2013).

Another study displayed higher levels of RBP4 in patients associated with diabetes and obesity. This increase in level is more linked with raised tissue content of visceral adipose as compared to subcutaneous adipose tissue (Budhitresna*et al*., 2013). Degree of obesity is directly related to high levels of RBP4 in serum of diabetics. High waist circumference was also found in diabetics with high levels of RBP4 (Derosa*et al*., 2013). Similar findings were also observed in another study (Chielle*et al.*, 2017). There was some correlation of RBP4 found with other anthropometric parameters, whereas, there was no correlation of RBP4 found with body weight in a study of visceral obese patients. In viscerally obese patients, there was correlation of RBP4 found with body fat percentage, body mass index and body weight (Scribner *et al.*, 2007). In another study, contrary findings were observed in which no correlation of RBP4 was found in viscerally obese patients with waist-to-hip ratio, insulin resistance and BMI (Ulgen*et al.*, 2010). Similarly, correlation of RBP4 with insulin resistance was also observed in some studies (Derosa*et al*., 2013; Choi *et al.*, 2013).

Regarding lipid profile, no correlation of RBP4 was found with low density lipoproteins, high density lipoproteins, cholesterol and triglycerides. In liver fatty acid metabolism, it is postulated that mechanism of RBP4 is somewhat linked with triglycerides via regulation of gene expression involved in lipid metabolism (Scheuer*et al.*, 2015).

In that disorder the excrement was with no taste. T2DM is considered as chronic metabolic disorder. Its prevalence has been expanding in the world. Diabetes mellitus abbreviated as (DM) is one among the oldest diseases which are known by human beings. Approximately 3000 years ago, DM was officially described in an Egyptian manuscript. During the year 1936, the difference between T1DM and T2DM was noticed. In 1988, it was reported that T2DM was a component of metabolic syndrome. The most common form of DM is T2DM (non-insulin dependent DM). It is attributed to insulin resistance, hyperglycemia and relative insulin deficiency. T2DM is the result of reciprocity of some risk factors like genetics, environment and behavior. The patients of T2DM are usually at risk to have short- and long-term complications. This endangerment often leads to death. The increasing ratio of morbidity and

mortality is common in T2DM patients in Pakistan. The main reasons are insidious onset and late recognition of T2DM because of poor resources (Nair *et al*., 2012).

Obesity is one of the major causes of T2DM. Approximately, there is a major contribution of obesity in 55% of cases of T2DM. According to the previous studies, there is an increase in the ratio of obesity in kids from the 1960s to 2000s. The researchers now believe that it has increased the ratio of T2DM in kids and young people. There is another factor which we called Environmental toxins. It also has contributed in the increase of T2DM during the recent decades. Typically, the major destruction of T2DM is insulin resistance. This pathogenesis results in the reduction of the capacity of the insulin receptor sensitivity to the cells. Still, the causes of T2DM are unknown. Usually, it begins with risk factors, co-morbidities status and autoimmune. It increases inflammations too. It ended up with the metabolic syndromes. In past different researches were done to investigate the relationship between inflammation and T2DM incident. They agreed on many findings on the incidence of insulin resistance. It has been done especially in the signal molecules that are involved in the insulin signal mechanism (Jehan*et al.*, 2018). Current study was conducted to assess serum level of RBP-4 and its correlation with diabetes, insulin resistance and obesity.

2.0 MATERIALS AND METHODS

2.1 Study Design

It is cross sectional analytical study.

2.2 Sample Size

Sample size was calculated using the formula for calculating mean

$$n= \frac{\sigma^2(Z_{1-\alpha}+ Z_{1-\beta})^2}{(\mu_o- \mu_a)^2}$$

Where

σ^2 = variance

$Z_{1-\alpha}$=confidence level

$Z_{1-\beta}$=power of test

μ_o= population mean # 1

μ_a= population mean #2

Mean concentrations of RBP4 in obese patients were 33.93 µg/ml and 32.53 µg/ml in non-obese controls (Korek *et al*., 2018). Mean standard deviation (S.D) is 0.7 and variance is 0.49. Confidence interval is taken as 95. Power is taken as 80. The estimated sample size is 4 but for the better power of study we will take sample of 50.

2.3 Inclusion criteria

- Self reported healthy individuals
- Both genders
- Ages 18+

2.4 Exclusion criteria

- Pregnant females
- Patients with BMI less than 18

2.5 Methodology

- Informed consent was taken
- Demographic data was collected

- Blood samples was drawn by venipuncture from any accessible vein and collected in serum vials
- Serum will be separated by centrifugation at 1000 RPM

2.6 Lipid profile tests:

- High density lipoprotein cholesterol (HDL-C)
- Low density lipoprotein cholesterol (LDL-C)
- LDL/HDL Ratio (calculated values)
- Triglycerides.
- Very low density lipoprotein cholesterol (VLDL-C)
- **Body Mass Index** is a simple calculation using a person's height and weight.
- **Formula of BMI:**

$$BMI = kg/m^2$$

- Where
- **Kg=**person's weight in kilograms
- **m^2 =** height in metres squared.
- BMI of 25.0 or more is overweight
- Healthy range is 18.5 to 24.9.

2.7 ELISA:

ELISA was performed according to manufacturer protocol, where 100ul of standard via serial dilution and each sample was added in each well and then incubated for 90minutes at 37 C. After thatsamplewasremoved and 100ul of Biotinylated Detection Ab/Ag was added andincubated for 1 hour at 37 C. Then each well aspirated and washed for three times. Then 100ul of HRP Conjugate was added andincubated for 30 minutes at 37 C. again washed for 5 times and 90ul of Substrate Reagent was added in each well and incubated for 15 minutes at 37 C. Then 50ul of Stop Solution was added in each well. Determined the OD value at 450 nm immediately.

2.8 STATISTICAL ANALYSIS:

The data will be entered and analyzed using P.A.S.W18.0 (formerly SPSS). Mean ±S.E.M will be given for quantitative variables like size, weight etc. Student sample test will be applied. Ap-value of <0.05 will be considered as statistically significant.

3.0 RESULTS

Demographic and Baseline Data (n=80)

The demographic data of the cohort is shown in table 1. The cohort was composed of men (n=48, 54%) and women (n= 32, 46%) with mean ages of 33.64 ± 12.08 and 31.50 ± 20 years respectively. Means and medians of BMI, waist circumference, fasting blood sugars, serum insulin, HOMA-IR and serum RBP4 levels are also shown.

As shown in Table 1 all the above-mentioned variables were skewed as shown by Shapiro Wilk test ($p \leq 0.05$).

Table 4.1: Demographic and baseline data (n=80)

Sr.#	CHARACTERISTIC	FREQUENCY	PERCENTAGE	'P'*
1	Gender	Male: 48 Female :32	54 % 46 %	
		Mean ± SD	Median ± IQR	
2	Age in years*	33.64 ± 12.08	31.50 ± 20.00	0
3	Body Mass Index in kg/m2*	27.35 ±7.48	26.90 ± 15.00	<0.001
4	Waist Circumference inches*	38.61 ±12.06	36.00 ± 19.00	<0.001
5	Fasting blood sugar in mg/dl	29.68 ± 44.13	6.60 ± 70.75	<0.001
6	Serum insulin	44.61± 65.56	21.80± 44.25	<0.001
7	HOMA-IR	34.16± 56.65	13.00± 34.24	<0.001
8	Serum RBP4	55.63±15.65	56.16±26.17	

*p value for Shapiro Wilk test for normality of data

Lipid profile of the participants (n=80)

This demographic data shows the lipid profile of the participants (n=80) in table 2.Mean and Median of Cholesterol HDL,LDL,VLDL and Triglycerides are shown. As shown in Table 1 all the above-mentioned variables were skewed as shown by Shapiro Wilk test ($p \leq 0.05$).

Table 4.2: Lipid profile of the participants (n=80)

SR#	*LIPID PROFILE*	*Mean ± SD*	*Median ± IQR*	*'P'**
1	**CHOLESTEROL in mg/dl**	175.89 ±42.83	175.18± 57.62	0.64
2	**HDL in mg/dl**	40.89± 11.93	40.99± 13.97	<0.001
3	**LDL in mg/dl**	98.05 ± 33.67	92.0 ± 39.11	0.01
4	**VLDL in mg/dl**	37.28 ± 21.66	33.26 ± 28.23	<0.001
5	**TRIGLYCERIDES in mg/dl**	194.15 ± 112.54	168.28 ± 142.77	<0.001

*p value for Shapiro Wilk test for normality of data

Serum Lipid profile levels in male and female subjects

This demographic data shows the serum lipid profile level in male and female subjects in table 3. and also shows the p values of Cholesterol, HDL, LDL, VLDL, TG.

Table 4.3: Serum Lipid profile levels in male and female subjects.

SR#	*LIPID PROFILE*	*Males*	*Females*	*'P'**
1	**CHOLESTEROL in mg/dl**[a]	167.41±39.94	179.60±46.29	0.49
2	**HDL in mg/dl**	35.58±13.82	42.77±10.63	0.01[b]
3	**LDL in mg/dl**	86.62±26.01	96.45±50.08	0.11
4	**VLDL in mg/dl**	30.69±26.01	33.10±38.48	0.82
5	**TRIGLYCERIDES in mg/dl**	164.69±130.84	175.33±182.57	0.82

Pearson correlation showing relationship between RBP4 levels and various metabolic and anthropometric variables

This demographic data shows Pearson correlation showing relationship between RBP4 levels and various metabolic and anthropometric variables in table 4 and correlation coefficient and p values of Age, Gender, Body Mass Index, Waist Circumference, Fasting Blood Sugar, Serum Insulin Level, HOMA-IR, Cholesterol, HDL, LDL, VLDL and TG.

Table 4.4: Pearson correlation showing relationship between RBP4 levels and various metabolic and anthropometric variables.

Sr#	*Variable*	*Correlation Coefficient (r)*	*P value*
1	Age	1	
2	Gender	0.165	0.144
3	Body Mass Index	0.707**	0.000
4	Waist Circumference	0.703**	0.000
5	Fasting Blood Sugar	-0.218	0.052
6	Serum Insulin Level (fasting)	0.295**	0.008
7	HOMA-IR	-0.107	0.347
8	Cholesterol	0.637**	0.000
9	HDL	0.008**	0.944
10	LDL	0.497**	0.000
11	VLDL	0.455**	0.000
12	Triglycerides	0.437**	0.000

Multiple linear regression showing factors affecting serum RBP4 levels in the cohort

This demographic data showsMultiple linear regression showing factors affecting serum RBP4 levels in the cohort, Unstandardized Coefficients, Standardized Coefficients, Significance Probability etc.

Table 4.5: Multiple linear regression showing factors affecting serum RBP4 levels in the cohort.

Variables	*Unstandardized Coefficients*		*Standardized Coefficients*	*Sig.*	*95.0% Confidence Interval for B*	
	B	**Std. Error**	**Beta**		**Lower Bound**	**Upper Bound**
(Constant)	2.69	0.45		<0.001	1.79	3.59
Female Gender	**0.14**	0.07	0.24	**0.03***	0.01	0.28
Age years (Natural Log)	-0.13	0.14	-0.16	0.34	-0.42	0.14
Insulin levels	-0.03	0.04	-0.11	0.42	-0.10	0.04
Body Mass Index kg/m^2 (Natural log)	**0.56**	0.20	0.51	**0.01***	0.16	0.96
Serum LDL level in mg/dl	-0.001	0.001	-0.06	0.64	-0.003	0.002

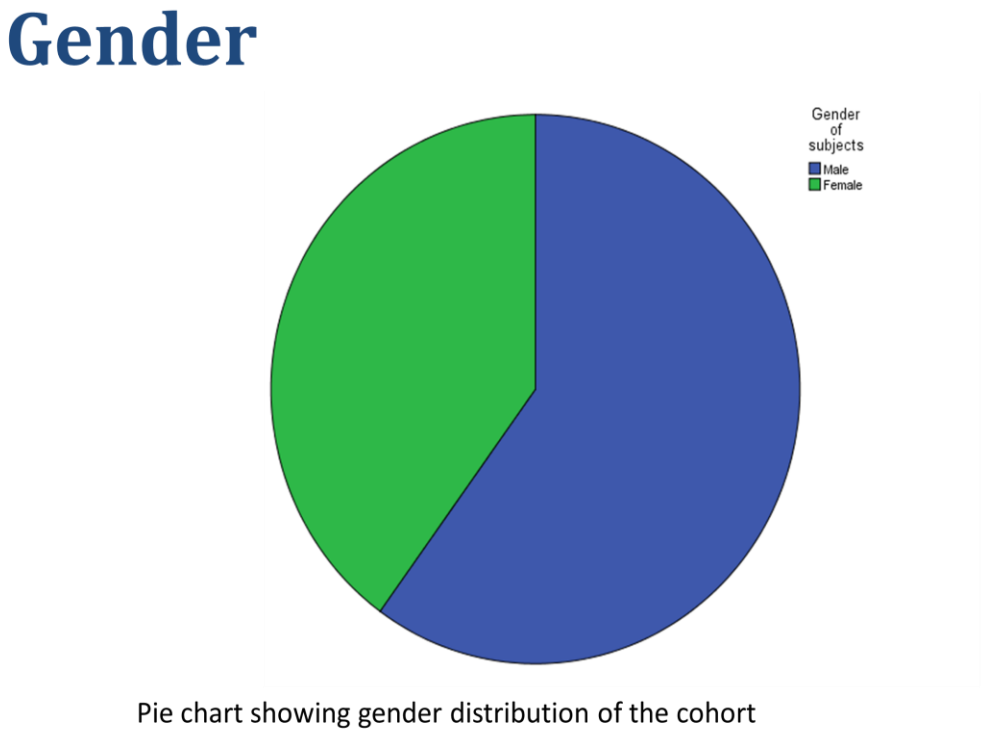

Figure 4.1: Pie chart distribution between male and female.

4.0 DISCUSSION

RBP4 may contribute to diabetes and insulin resistance by elevating glucose production in hepatocytes and by impairing glucose uptake in muscle cells which are stimulated by insulin. It also inhibits phosphorylation of insulin receptor substrate-1 induced by insulin and its expression in inversely linked with glucose transporter-4 in adipose tissue cells. It acts as endogenous molecule, like other adipokines, in disturbing insulin signal pathways and promote progression of diabetes mellitus type 2 (Bilir *et al*., 2016).

Hence, this study was designed to assess RBP4 serum levels correlation between patients (diabetes and obesity) and control. HBA1c and FBG were found higher in patients as compared to control. This may be linked with impairment of metabolism of glucose and high levels of insulin resistance and free fatty acids under effect of adipokines. In comparison with control, other lipid profile markers were also raised in diabetic patients in a study (Bilir et al., 2016). Serum levels of LDL and cholesterol were found higher in a study of obese patients as compared to control, whereas HDL levels were low. High BMI and WC is also related with high dyslipidemia (Derosa*et al*., 2013).

In another study, FI and HOMA-IR were also high in diabetics and obese patients in comparison with control (Chielle*et al*., 2017). Similar findings were observed regarding HOMA-B%. In current study, levels of RBP4 were found higher in diabetics and obese patients as compared to control. There was no difference between viscerally obese patients and diabetics

and there was higher level of RBP4 found in patients in comparison with healthy individuals (Ulgen*et al.*, 2010).

Increased level of RBP4 is closely linked with increase of tissue content in adipose tissue area. In comparison with subcutaneous fat, RBP4 was expressed more in visceral fat in equally both diabetic and obese patients. Patients also expressed increased insulin resistance in which RBP4 level was found higher.

It is found in both studies on DM patients and non DM subjects in the current meta-analysis that a common opposite relation exists among vitamin D deficiency and fatness. Some distinct studies were done on the females from the KSA (Kingdom of Saudi Arabia).They have presented a positive relationship in non DM experimental studies.

Presently free radical reactions have been taken as the unifying link of diabetes and its complications. In this research, in this research a notable high serum MDA levels in T2DM patients was found. Serum MDA level was notably high in T2DM patients with micro albuminuria when they were compared to patients without micro albuminuria. This result is in agreement to preceding studies. This great expansion in MDA tiers may want to be because of accelerated generation of free radicals in diabetes mellitus patients. MDA is a give up product of lipid peroxidation could be accountable for the intermolecular move association of collagen via MDA guides to its counteraction and more required glycation. This starts a vicious cycle as glycated collagen initiates similarly lipid peroxidation releasing more MDA. Superoxide dismutase (SOD), a superoxide radical scavenging enzyme, is viewed above contrary to the destructive reaction of oxygen radicals in the cells (Inouye *et al*., 1999).

The presence of superoxide dismutase (SOD) in a variety of compartments of the body allows it to dismutate O_2 - radicals straight away and protects the cells from oxidative damage. Serum SOD level was extensively lowered in patients of T2DM with micro albuminuria as compared to patients besides micro albuminuria. Literature supports the findings of the research. A widespread inhibition in SOD exercise in T2DM with micro albuminuria may effects in an elevated flux of O_2^- radical and subsequently reflects the tissue damage/injury. The reduce in degree was greater full-size in patients with micro albuminuria. This observation is in accordance with the hypothesis that make bigger MDA stage and reduced GPx level would possibly participate in damage of tissue (Inouye *et al*., 1999).

Formulation of reactive oxygen intermediates (ROI) is one among dominant factors in the onset and the progress of DM and related complications. Previous researches of the last decade signify the importance oxidative stress which has a significant participation in this connection of hyperglycemia and the previously identified pathophysiological characteristics of DM. It is connected to the onset and development of DM linked complications. Currently, a scientific presentation has also showed inactivated signal pathway because of an oxidative stress marker. It is present among the transport system of glucose and insulin receptor. It is noted as abnormal element for the skeletal muscle in the patients of T2DM. So it can be stated that oxidative stress may be a significant undercover characteristic which leads to the onset and development of insulin resistance in persons with T2DM (Lapenna*et al*., 1998).

So the antioxidants may be beneficial in treatment of DM patients and complications. A large number of antioxidants, for example ascorbate, glutathione, acetylcysteine are available. But only two α-lipoic acid and RRR-α-tocopherol are presented in various clinical experimental studies that have advantageous effects on diabetes and its complexities. RRRα-Tocopherol decreases LDL oxidation and autoantibody titres against LDL altered proteins which are famous and disadvantageous risk elements for the demonstration of atherosclerosis (Lapenna*et al*.,

1998). Clinical and epidemiological researches have proposed that there is an indirect relationship of RRR-α-tocopherol and the rate of myocardial infarction. It was also proved that RRR-α-Tocopherol exerted positive effects on flow of blood. α-Lipoic acid is proven in some other researches to interact with the expansion of diabetic polyneuropathy. It has shown that clinically, it lowers the symptoms of neuropathic. It develops autonomic and peripheral functions positively in people with diabetes. It is presented in previous researches that α-Lipoic acid lowers the disproportion of oxidative stress measures and antioxidant capacity in DM patients (Borcea*et al*., 1999). This antioxidant effect can give an explanation for the inoperativeness of the oxidative stress sensitive transcriptional factor, NFkB, in plasma mononuclear cells. It is related to the better working of renal and endothelial factors in diabetic in T2DM patients. Advancementofinsulinstimulated glucose disposal in patients of T2DM strengthens its therapeutic value in the existence of α-lipoic-acid. These experimental and scientific effects advised the significance of research to assess more the hidden importnce of antioxidants for the therapy of DM and related complexities. The clinical trials which are performed at a larger scale like the DCCT Study or the UKPDS Study are in need of considering the long-term effects of such antioxidants in DM patients. There is a clinical and socio-economic burden of DM on patients, society, doctors and even on researches. Diabetes and its problems in the present time therefore demand more innovative treatments. So attainable agents which are helpful in treatment such as antioxidants ought to be examined except lengthen via means of long-term potential studies (Borcea*et al*., 1999; Lapenna*et al.,* 1998).

Lipid peroxidation has importance in-vivo for some reasons particularly. It is because its strong contribution in the advancement of atherosclerosis (Esterbauer*et al.,* 1992). Many research articles are there which can take measurement of lipid peroxidation, like malonicdialdehyde (MDA) through the tests of thiobarbituric acid (TBA) and diene conjugation (Yasuda *et al*., 1997).

Conversely, Baynes (1991) gave proofs about the position of oxidative stress. He did not believe on the early arise of oxidative stress. He argues that it appears in the disease with the growth of diabetes. It is taken as an elementary pathogenic character in the increase and progression of DM. One among the related serious issues in evaluating the queries regarding when, how and why oxidative impairment takes place during the overall tenure of disease and either any chance is there for aggregation of free radical-derived injury of tissue in the specific period of time of the illness is the firmness of the oxidation products (Baynes*et al*., 1991).

But for DNA, it is recognized that alterations of DNA can be remodeled through a process of restoration. This process utilizes enzymes that are related to repair. It works through removal and restoration of the nucleotide (Baynes*et al.,* 1991). In case of RNA, protein and lipids, the proportion of the ratio of the destructed molecules seems the significant element that limited the accretion of O_2 radical destruction. There are damaged protein molecules that lived long, (e.g., collagen). In such case, the outcomes which are given by radical reactions of oxygen can accrue with time. So they can work as an exclusive sensor. They work for uncovering of oxidative stress over time. Different researches are done on glycation of proteins and Maillard reactions of glycated proteins. These studies have created indirect proofs for expanded oxidative alterations of collagen in DM (Suzuki *et al.,* 1999).

5.0 CONCLUSION

The current study demonstrated that RBP4 can be a biomarker or signal of insulin resistance in viscerally obese or type 2 diabetes patients, as it was found high as compared to healthy

individuals. Also, RBP4 had significant positive correlation with HOMA-IR and FI in viscerally obese patients, so it may be a risk factor for type 2 diabetes.

6.0 COMPLIANCE WITH ETHICAL STANDARDS

Current work was conducted at the University of Lahore following all the standards after the approval from Ethical committee of university of Lahore, No.75866.

7.0 FUNDING

There was no funding source, Project was self-funded.

8.0 CONFLICT OF INTEREST

No conflict of interest.

9.0 ETHICAL APPROVAL

Current work was approved from Ethical committee of university of Lahore, No.75866.

10.0 INFORMED CONSENT IN THE MANUSCRIPT.

Informed consent was taken from the patients.

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