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Serum Retinol Binding Protein 4 (Rbp4) Level in Patients with Gestational Diabetes Mellitus

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ABSTRACT: INTRODUCTION: Gestational Diabetes Mellitus by definition is any degree of glucose intolerance with onset or first recognition during pregnancy[1]. It is characterized by insufficient insulin levels to meet the demands in later pregnancy.Retinol binding protein-4 is a novel marker in the pathogenesis of GDM. RBP4, a retinol transporter, plays an important role in dysregulation of insulin sensitivity in GDM.**MATERIALS AND METHODS:** It is a case control study done in 90 pregnant women attending the diabetology OP. They were divided in to 60 cases and 30 controls based on the OGTT report. 6ml of fasting venous blood was drawn and Serum was separated and analysed for Fasting blood Glucose by spectrophotometric method, HbA1c by Immunoturbidimetry and Serum RBP4 by ELISA method. Data was analysed using SPSS software version 16.0 .The results were statistically analysed by using t-test and pearson correlation.**RESULTS:** Serum retinol binding protein 4 (RBP4) levels are higher in patients with gestational diabetes mellitus compared to the control group with statistically highly significant P-value of <0.001. Rise in RBP4 level correlates with rise in HbA_1c levels, showing a highly significant correlation. Family history is significant among the cases when compared to the control group.Previous history of GDM is significant in patients with GDM in present pregnancy.
CONCLUSION: Elevated Sr.RBP4 levels in GDM indicates it as a marker of insulin resistance. These findings provide a rationale for choosing anti-diabetic therapies aimed to lower serum RBP4 levels.

KEY WORDS: Gestational diabetes mellitus, Retinol binding protein-4, insulin resistance

I. INTRODUCTION :

Gestational Diabetes Mellitus by definition is any degree of glucose intolerance with onset or first recognition during pregnancy[1]. It is characterized by insufficient insulin levels to meet the demands in later pregnancy. The significance of GDM is because of its maternal and fetal complications like polyhydramnios, preeclampsia and overt diabetes in future, fetal complications like birth trauma, macrosomia, childhood obesity and diabetes[29]. Apart from the Asian race to be a risk factor for GDM, the other possible risk factors are advanced maternal age, obesity, high parity, polycystic ovarian syndrome (PCOS), family history of diabetes, obstretic history of stillbirth, congenital malformation and macrosomia[30].

Retinol binding protein-4 is a novel marker in the pathogenesis of GDM. RBP4, a retinol transporter, plays an important role in dysregulation of insulin sensitivity in GDM.

This study has been undertaken to find the level of serum retinol binding protein- 4 in GDM and its association with HbA_1C has been evaluated. The raised levels also impair insulin signaling and induce gluconeogenic enzymes in the liver[31]. Impairment of lipid metabolism is a risk factor for cardiovascular diseases.RBP4 role in lipid metabolism and metabolic syndrome has been demonstrated in various studies. There is a positive correlation between RBP4 and LDL cholesterol, TGL and hepatic lipase activity in patients with Type 2 diabetes mellitus and cardiovascular disease[32].

The prevalence of GDM in India is highly variable because of differences in living conditions, socio-economic status and dietary habits. A random survey done among the cities of India in 2002-2003, showed a prevalence of 16.55 per cent[33]. In a study done in Tamilnadu, GDM was present in 17.8 per cent women in urban, 13.8 per cent in semi-urban and 9.9 per cent in rural areas[2]. Indian

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women have a 11 fold increase in risk of developing GDM, compared to the cacausians[3].

Diabetes mellitus is a metabolic disorder. It is characterized by chronic hyperglycemia. There is a defect in either insulin secretion or its action or both. It causes derangement of carbohydrate, lipid and protein metabolism[34]. The chronic hyperglycemia is associated with long-term damage and dysfunction of various organs.

In Gestational diabetes mellitus, many changes in the metabolism of mother occur in such a way as to provide sufficient energy and nutrition to the fetus. Fetus mainly depends on the maternal glucose which reaches it through the placenta, because of which the mother develops a state of "Insulin resistance" during mid-pregnancy[35]. This state of insulin resistance progresses through the third trimester. At one stage there is reduced state of consumption of glucose by maternal tissue and increased gluconeogenesis, ensuring sufficient supply of glucose to the fetus.[4]The resulting positive maternal-fetal glucose gradient, facilitates the transfer of glucose through the placenta. In a proportion of pregnancies, this state of "Insulin resistance" is greatly increased , and Gestational diabetes mellitus develops[36].

"When the biological effects of insulin are less than expected for glucose disposal in skeletal muscle and adipose tissue and suppression of endogenous glucose production primarily in the liver, it is said to be insulin resistance." Insulin resistance may be due to a decrease in the number of insulin receptors or impairment in post-receptor signaling of insulin receptors[37].

A good **screening test** has a positive likelihood ratio of atleast 6. Screening on the basis of risk factors seems to be inefficient[58]. In other words, a diagnostic test only in women with risk factors will miss many women with GDM.

Serum Retinol binding protein-4 is a specific carrier protein which belongs to lipocalin family, of kernel type, calycin superfamily[38].It is coded by chromosome no: 10. Its location is 10q23.33 in cytosol.The protein weighs 23 kDa and has 201 amino acids.It consists of 4 chains which are linked by 3 disulphide bonds. It is a monomer.It undergoes methylation as a post translational modification. It is secreted by hepatocytes and adipocytes[39].It is the transporter of Retinol (Active form of vitamin A) from the liver to the peripheral tissues.It is bound to transthyretin (prealbumin), which is a carrier of thyroid hormone.RBP4 has two receptors namely

1) Stimulated by Retinoic Acid 6(STRA6)
2) RBP4 Receptor 2(RBPR2)

Its physiological functions are[1]

1) It facilitates transport of insoluble retinol from storage site to the peripheral tissues
2) RBP protects bound retinol from oxidation[40].
3) The synthesis of RBP regulates release of retinol from the liver and mediates specific uptake by target tissue.

In present the levels of Serum Retinol Binding Protein 4 (RBP4) was estimated in patients with Gestational diabetes mellitus and its level was correlated with HbA1c.

AIM:

To estimate the level of serum retinol binding protein 4 (RBP4) in women with gestational diabetes mellitus.

OBJECTIVE:

1) To correlate the level of serum retinol binding protein 4 (RBP4) and HbA_1c in patients with gestational diabetes mellitus.
2) To correlate the level of serum retinol binding protein 4(RBP4) with Fasting blood glucose and family history.

II. MATERIALS AND METHODS:

This is a case control study and the study protocol was approved by the Institutional Ethics Committee of Madras Medical College, Chennai .This study was conducted during the period from October 2106 to June 2017 in the Institute of Diabetology,Institute of Biochemistry at Madras Medical College,Chennai.

STUDY POPULATION

90 pregnant women attending the diabetology OP were selected for the study. They were divided in to 60 cases and 30 controls based on the OGTT report. Of the cases,it was further subgrouped as 30 primiparous women and 30 multiparous women. Informed written consent was obtained both from the control group and cases group.

INCLUSION CRITERIA:

- GROUP I : Primi with gestational diabetes mellitus of age 21 to 40 years
- GROUP II : Multi with gestational diabetes mellitus of age 21 to 40 years.
- GROUP III : Age and parity matched healthy pregnant women.

EXCLUSION CRITERIA:

- Women with pre-existing diabetes or hypertension.
- Patients with liver disease.
- Patients with acute illness; infection.

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- Known case of cardiovascular disease.
- Smoking, alcoholism.
- Patients with history of fetal anomalies.
- Other endocrinological disorders.

SAMPLE COLLECTION:

6 mL of blood sample was collected from all subjects after overnight fasting. 3mL of was transferred to serum tube and 3 mL was transferred to K_2-EDTA tube. After adequate clotting, the serum sample was centrifuged and serum was aliquoted.

Serum Retinol Binding Protein 4 was measured by Enzyme linked immunosorbent assay (Bioassay Technology Laboratory, Shangai, China) – non-competitive sandwich.(ELISA method).

Plasma Glucose was estimated by Glucose oxidase- peroxidase method. (GOD/POD) END POINT METHOD.

The kit was calibrated using XSYS0034 XL MULTICAL CALIBRATOR.

HbA1c was measured by Immunoturbidimetry.

III. RESULTS AND STATISTICAL ANALYSIS :

90 pregnant women attending the diabetology OP were selected for the study. They were divided in to 60 cases and 30 controls based on the OGTT report. Of the cases, it was further subgrouped as 30 primiparous women and 30 multiparous women.

STATISTICAL ANALYSIS :

- Data was analysed using SPSS software version 16.0 and P value less than 0.05 was considered statistically significant. Continuous variables were presented as mean ± SD (standard deviation) and categorical variables were represented as frequencies and percentages.
- Height, weight, BMI, FBS, HbA_1c and RBP4 were compared between the study groups by Student t-test.
- Family history and previous history were analysed by Chi square test.
- Correlation of parameters namely RBP4 with HbA1c were found out by Pearson correlation analysis.
- Stepwise linear regression analysis was performed to evaluate the mathematical relationship between RBP4 and other variables in patients with GDM.

TABLE-1 : Age distribution of study Subjects

Age Group (in Years)	PRIMI		MULTI		CONTROL	
	N	%	N	%	N	%
≤ 20 Years	1	3.33	0	0	3	10.00
21 - 30 Years	27	90.00	20	66.67	18	60.00
31 - 40 Years	2	6.67	9	30.00	9	30.00
41 - 50 Years	0	0	1	3.33	0	0
TOTAL	**30**	**100**	**30**	**100**	**30**	**100**
Mean	25.67		30.13		27.63	
Standard Deviation (sd)	3.38		4.90		4.99	

The mean age of distribution of subjects taken up in the study is about 27 years. The mean age of the primi was 25 years and that of multi was 30 years. Age group of 20 to 30 years is fertile period and it's a high time to intervene.

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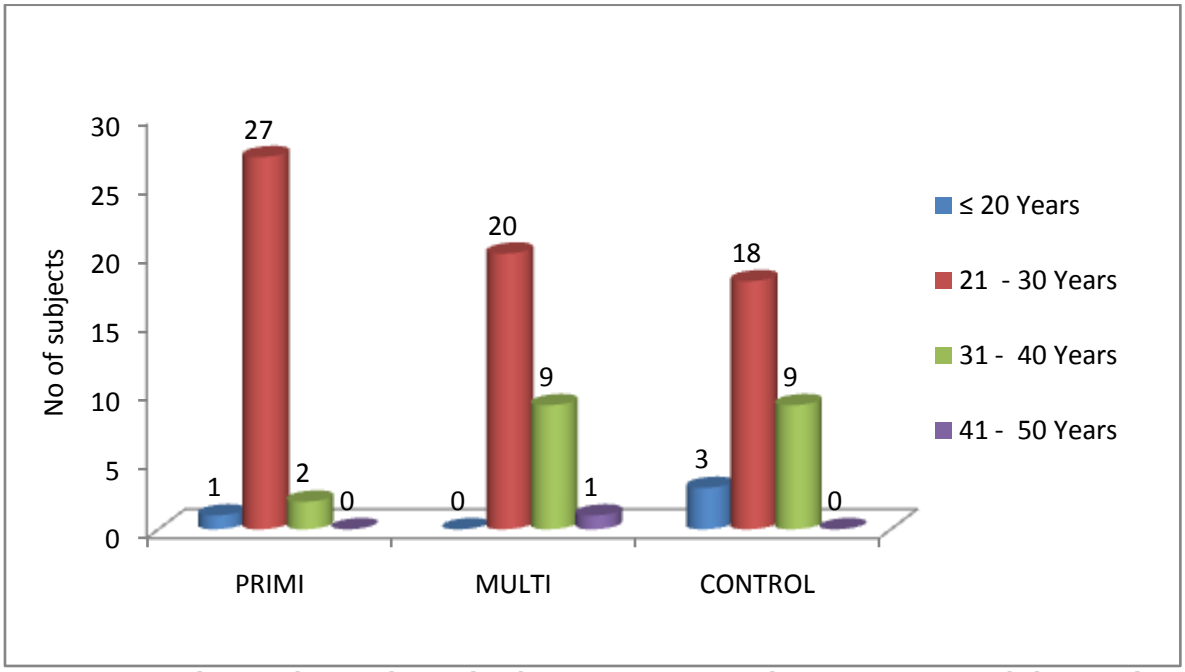

Figure 1 Bar diagram showing the age distribution among primi and mutiparous cases with the control.

TABLE 2

BMI distribution of study Subjects

BMI (kg/m^2)	**CASE**		**CONTROL**	
	Number	**Percentage**	**Number**	**Percentage**
Under Weight	1		1	
Normal Weight	11		11	
Over Weight	20		10	
Obese	28		8	
TOTAL	**60**	**100**	**30**	**100**
Mean	29.92		26.53	
Standard Deviation (sd)	5.37		4.68	
t-value	2.93			
P-value	0.05			
Significant	Significant			

The mean BMI of the cases was 29.92 and that of the controls was 26.53. The P value was 0.05, which is statistically significant.

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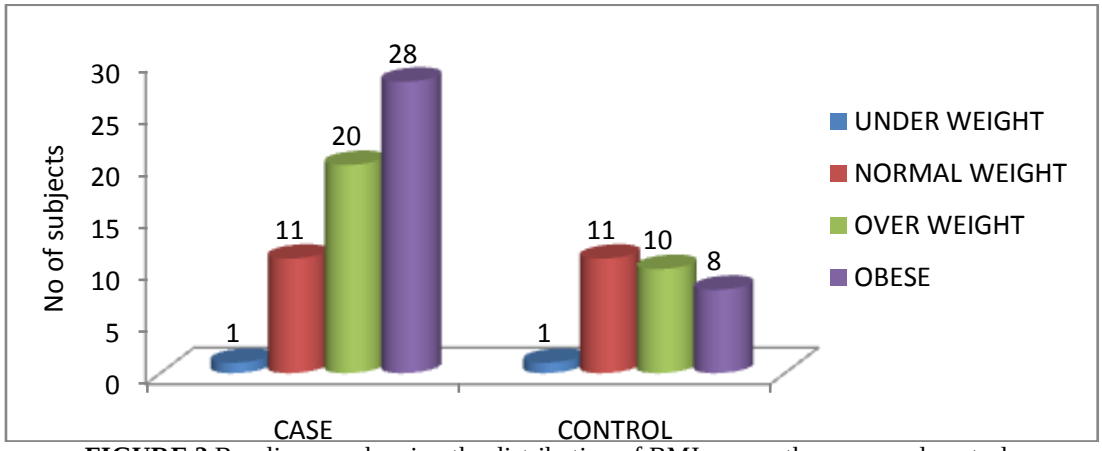

FIGURE 2 Bar diagram showing the distribution of BMI among the cases and controls

TABLE-3: Family History of Diabetes

	CASE		CONTROL	
	Number	**Percentage**	**Number**	**Percentage**
NO	35	58.33	25	83.33
YES	25	41.67	5	16.67
TOTAL	**60**	**100**	**30**	**100**
Chi-square	5.63			
p-value	0.02			
Significant	Significant			

Family history of diabetes was significant among the cases when compared to the controls.

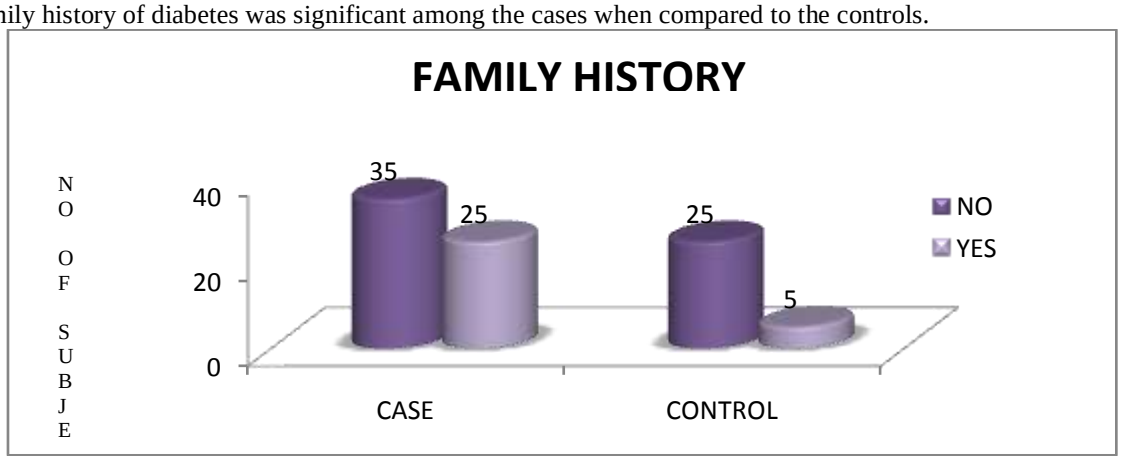

FIGURE 3 Bar diagram showing the association of family history with GDM

TABLE-4 : FBS

FBS (mg/dL)	**CASE**		**CONTROL**	
	Mean	**Sd**	**Mean**	**sd**
FBS	107.78	19.02	75.83	13.27
t-value	8.24			
P-value	0.000			
Significant	Highly Significant			

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The normal fasting blood glucose level is 70 to 100 mg/dL. The mean fasting blood sugar among the GDM patients was 107.78 mg/dL and that of the control group was only 75.83 mg/dL. In normal pregnant women, it indicates absolute control of blood glucose. The variation of blood glucose was statistically highly significant with a P value of <0.001.

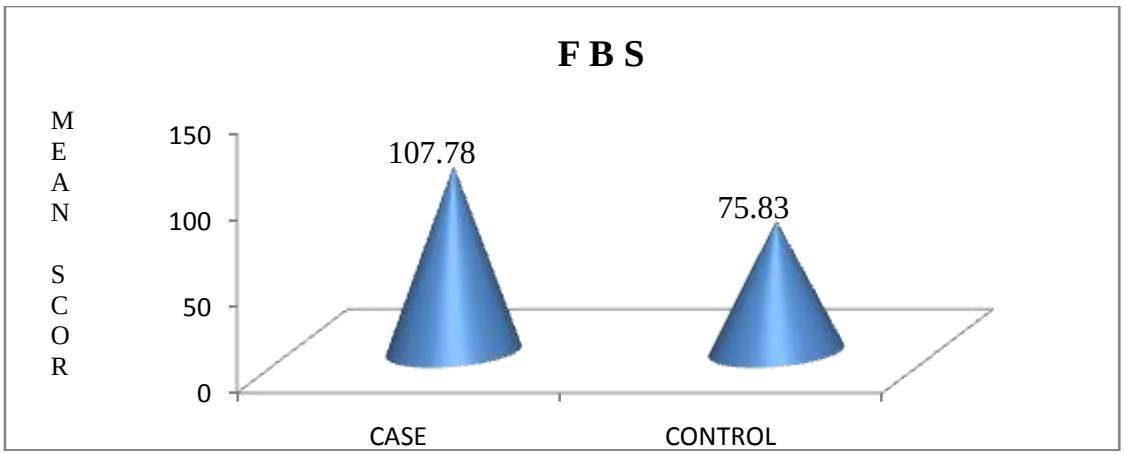

FIGURE 4 Bar diagram showing the distribution of FBS among the two groups

TABLE-5 : HbA_1c

HbA_1c (%)	**CASE**		**CONTROL**	
	Mean	**Sd**	**Mean**	**sd**
	6.52	0.90	5.11	0.53
t-value	7.88			
P-value	0.000			
Significant	Highly Significant			

A value of <5.7% is considered normal.The mean HbA_1c value in the GDM cases was 6.52% which varies from that of normal pregnant women with highly significant P value of <0.001.

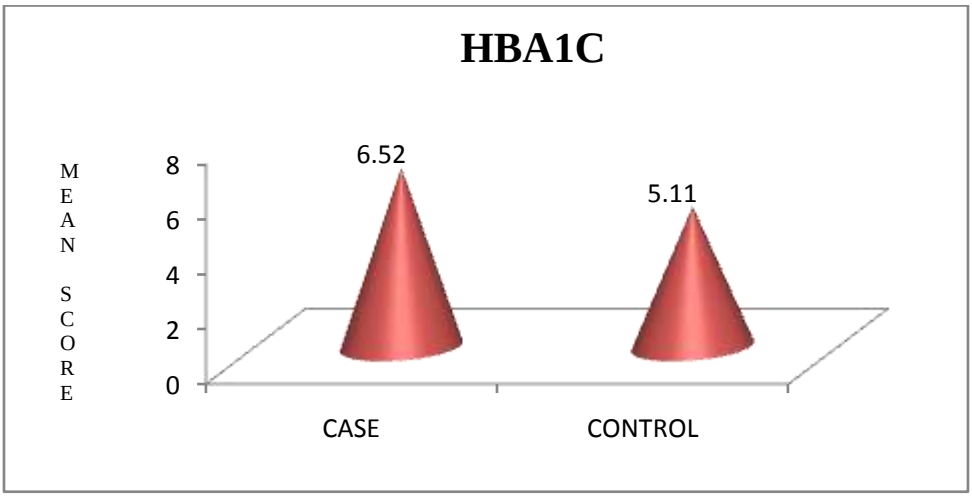

FIGURE 5 Bar diagram showing the distribution of HbA_1c levels between the cases and control groups

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TABLE-6: RBP4

RBP4 (mg/L)	CASE		CONTROL	
	Mean	**Sd**	**Mean**	**sd**
	50.56	12.62	22.71	5.87
t-value	11.46			
P-value	0.000			
Significant	Highly Significant			

According to previous studies, RBP4 levels of <40 mg/L during pregnancy was considered normal. In this study the mean RBP4 level in the pregnant women with GDM was 50.56 mg/L which was very high when compared to the mean in the control group, which was only 22.71 mg/L. It was shown to be statistically highly significant with a P value <0.001.

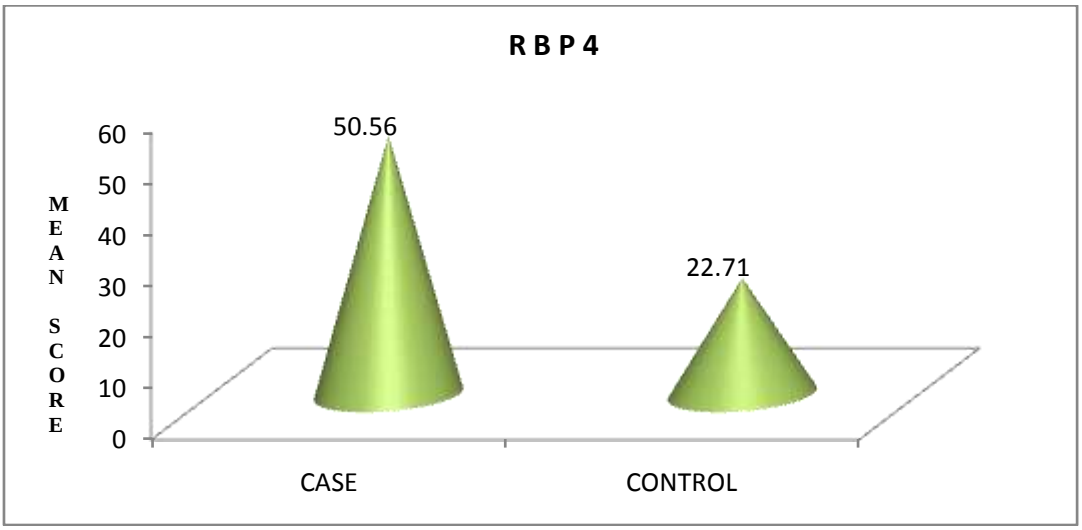

FIGURE 6 Bar diagram showing the distribution of RBP4 among the two groups

TABLE-7 : Correlation with RBP4

	CASE		CONTROL	
	r	**P-value**	**r**	**P-value**
HbA1c	0.44	0.000**	0.38	0.04*

Note : ** Correlation is Significant at 0.01 * Correlation is Significant at 0.05
Correlation analysis showed that Sr.RBP4 levels positively correlated with HbA1c levels (r=0.44) with a P value of < 0.001, which is statistically highly significant.

TABLE - 8 : STEPWISE LINEAR REGRESSION ANALYSIS OF INDEPENDENT PREDICTORS OF RBP4

Unstandardized Coefficients			
Variables	**B**	**T**	**p-value**
BMI	0.039	0.606	0.545

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FBS	0.148	1.866	0.064
HbA1c	7.675	7.348	**0.000**

Stepwise linear regression analyses revealed that HbA_1c and triglycerides (r^2 =0.279) were independent predictors of RBP4.

IV. DISCUSSION :

This study on Serum RBP4 levels in pregnant women with gestational diabetes mellitus was taken up and done as 2 groups with cases(including primiparous and multiparous) and normal pregnant women as control group in the age group of 21 to 40 years.

The prevalence of gestational diabetes mellitus (GDM) has increased by 10-100% in the past 20 years[6]. Apart from its adverse effects on infants during the newborn period ,it also contributes to the current trend of increasing obesity and diabetes[7].In the study done by Coustan DR et al, it was shown that the prevalence of GDM usually reflects the frequency of type 2 diabetes in that population[8,9]. The risk factors for Gestational diabetes mellitus are obesity, advanced maternal age, family history of diabetes and ethnicity.

In India, Gestational diabetes is more common in urban women than those living in rural areas[10].

Relatively only a few published studies are available regarding the genetic susceptibility of gestation diabetes. The available data suggest that GDM has a familial tendency. There is a tendency for GDM to recur in atleast 30% of women with previous history[41]. These suggest that GDM develops in those who are genetically predisposed.

This case control study has been undertaken to estimate serum Retinol binding protein-4 (RBP4) levels in patients with gestational diabetes mellitus and to correlate its level with lipid profile and HbA1c levels.

RBP4, secreted by liver and the adipocytes, is a new adipokine which has been shown to contribute to insulin resistance .Serum RBP4 levels have already shown to be elevated in obesity and Type 2 diabetes[42]. These suggest that the correlation between levels of Sr.RBP4 and insulin resistance has been consistent. But the data about Sr.RBP4 levels in pregnant women are limited. So the present study was taken up to show the association of RBP4 with GDM.

We included pregnant women in the age group of 21 to 40 years. The cases and controls were selected based on their OGTT results which they undergo routinely during prenatal examination.

Adipose tissue is recognized as an endocrine organ[43]. The expansion of adipose tissue due to obesity triggers chronic low grade inflammation. In study done by Clement,k; Viguerie and Cancello, R; Henegar has shown that weight loss decreases macrophage infiltration and expression of pro- inflammatory cytokines in adipose tissue[11].

Table -2 shows the distribution of weight and BMI among the cases and control. There was statistically significant difference in the distribution of weight and BMI between the cases and control group. The mean weight of the gestational age matched pregnant women with GDM was 68.43kg and those without GDM was 60.63kg. Also the BMI of the cases and control women showed significant difference. The mean BMI of cases was 29.92 and that of controls was 26.53 with a P-value of 0.05.

These values signifies the association of obesity as one of the underlying pathology in the development of GDM. In the Study done by Bhartha JL et al,it was found that, measurement of visceral adiposity correlates better than subcutaneous fat or BMI with insulin resistance. In our study, BMI was shown to be a significant indicator.

Gestational diabetes mellitus itself is characterized by amplification of low-grade inflammation. This is supported by the increased levels of pro-inflammatory cytokines like TNFα and IL-6 in GDM[44]. It is also known that obesity is strongly associated with inflammation, which contributes to insulin resistance of GDM.

Table 3 shows the comparison of family history between the cases and control. This study shows that, family history of T2DM was significantly more prevalent in the GDM group with a P value of 0.02. This clearly indicates the genetic predisposition in the etiopathogenesis of GDM. This is supported by a previous study in Danish twins by Poulsen et al in 2005. It was shown that both the twins showed major genetic components in both traits. In study done by Lambrinoudaki I et al showed that more than 75% variation in insulin secretion trait and 53% of peripheral insulin sensitivity are explained by genetic components[12].

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GDM develops when the genetic predisposition of pancreatic islet β-cell impairment is unmasked by the increased insulin resistance during pregnancy[13,45].

Table 4 and 5 shows the comparison of fasting blood glucose levels and HbA_1c between the cases and controls. The serum fasting blood glucose and HbA_1c levels were significantly higher in the GDM group. Their P value in comparison to the control group was highly significant.

Normally, during pregnancy, there is decrease in fasting blood glucose level compared to the non- pregnant women due to

1) The anabolic effect of insulin[14]
2) Increase in plasma volume
3) Increase in feto-placental glucose utilization[15]
4) Decrease in substrates for gluconeogenesis, mainly alanine.

The target HbA_1c value to be achieved in GDM is 6%.[16] But study done by Carr S et al and Coustan DR et al has shown that measurement of HbA_1c is less sensitive in GDM. But in this study HbA1c was shown to be significantly correlated with GDM patients.

Table 6 shows the comparison of serum RBP4 levels between the patients with GDM and control pregnant women. Serum RBP4 levels were significantly elevated in GDM with a statistically highly significant P value of <0.001. This is consistent with the results obtained in various studies including the one by Arash Hossein-Nezhad et al[17]. But in our study the association between RBP4 and HOMA-IR was not done.

In study done by Chan et al and colleagues, found higher RBP4 levels in GDM but the levels of RBP4 did not correlate with insulin resistance[17]. But in a study by Choi et al colleagus, it was stated that a significant high RBP4 levels in women with previous history of GDM were related with insulin resistance.

This study does not agree with the results of the study done by Ueland et al and colleagues which reported low level of RBP4 in women with GDM, which could be due to different levels of BMI[18].

RBP4 (Retinol Binding Protein-4) is secreted by adipocyte which is elevated even before the development of frank diabetes[19]. Serum RBP4 is elevated in obesity as well as in type 2 diabetes mellitus, and is one of the main contributor of insulin resistance.

It has been shown in previous studies that RBP4 in pregnancy strongly correlates with insulin resistance (fasting insulin and HOMA-IR)[20]. Our present finding of markedly elevated RBP4 levels in GDM suggest that, it may be an early marker in the natural history of T2DM with potential implications for the screening and prevention of the disease. But BMI matched strategy is more credible.

In the study done by Choi et al and colleagues, it was found that RBP4 levels after pregnancy in women with previous history of GDM correlated positively with systolic blood pressure, abdominal fat as well as fasting insulin concentration. It was also reported that, RBP4 levels independently correlates with fasting plasma glucose levels in women with previous history of GDM.

Table 7 shows the correlation between RBP4 with HbA1c.It shows that RBP4 levels correlates positively with HbA1c levels, with statistically highly significant P value of <0.001.

Table 8 shows the stepwise linear regression analysis of independent predictors of serum retinol binding protein 4 (RBP4) in GDM. Stepwise linear regression analysis has revealed that HbA_1c were independent predictor of serum RBP4 concentrations in pregnant women.

Studies have shown that elevated level of RBP4 indicates the chance for developing type 2 diabetes mellitus after pregnancy[21,22]. It is proven that GDM women have 17% to 63% of increased risk of developing T2DM in the next 5 to 16 years. 23% of Iranian women with history of GDM were shown to develop frank diabetes or impaired glucose tolerance by 6 to 12 weeks after delivery[23].

It should also be noted that GDM increases the risk of hypertension, susceptibility to atherosclerosis and coronary artery disease (CAD)[24]. Persistent hyperinsulinemia causes raised levels of Sr.triglycerides, free fatty acids and LDL. It also decreases the HDL levels. Elevated free fatty acid levels in the blood causes activation of the innate immune system to release pro-inflammatory cytokines like TNF- α, IL-6 and IL-1β[25]. These cytokines leads to alteration in insulin sensitivity resulting in disruption of glucose homeostasis.

Initially there is insulin signaling by these cytokines in liver, muscle and adipose tissue. There is decrease in the functioning liver X receptors (LXRs) as well as increased cholesterol accumulation[26]. These act as a stimulant for the increased hepatic release of inflammatory markers like CRP, Plasminogen inhibitor -1, α1- acid glycoprotein and haptoglobin. They also stimulate fibrinogen, adding up to cause coronary artery disease[27].Cytokines also increases VLDL and FFA

production, resulting in diabetic dyslipidemia and increased plaque accumulation.

Retinol binding protein -4 is shown to be elevated in the serum before the development of frank diabetes. It serves as an important tool to identify insulin resistance as well as the cardiovascular risk in these subjects[28]. These findings provide a rationale for choosing anti-diabetic therapies aimed to lower serum RBP4 levels.

Our present study suggests that higher levels of RBP4 in GDM may be considered as an early marker in the natural history of type 2 diabetes mellitus, which may have potential implications for screening and also in the prevention of the disease.

V. CONCLUSION :

From this study we conclude that Serum retinol binding protein 4 (RBP4) levels are higher in patients with gestational diabetes mellitus compared to the control group with statistically highly significant P-value of <0.001.Rise in RBP4 level correlates with rise in HbA_1c levels, showing a highly significant correlation.Mean fasting blood glucose levels are significantly higher among the GDM patients compared to the control group.Family history is significant among the cases when compared to the control group.Previous history of GDM is significant in patients with GDM in present pregnancy.

VI. LIMITATIONS OF THE STUDY

In our study serum RBP4 was measured by ELISA method. Measurement by Western blot technique is the gold standard for determination of RBP4. RBP4 is bound to both retinol and transthyretin in plasma. So measuring these would have been more useful. But it is an elongated procedure. The present study did not measure fasting insulin level to directly determine the HOMA-IR levels. A measure of HOMA-IR directly indicates insulin resistance. If this was also taken up and done in our present study and correlated with RBP4 levels, then it will still show that RBP4 is a marker for insulin resistance directly. But in our present study RBP4 as marker of insulin resistance has been shown indirectly.

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