

Neuregulin 4 (NRG4) – the batokine with clinical relevance in Gestational Diabetes Mellitus

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ABSTRACT

Background: Neuregulin4 (NGR4) is a brown fat adipokine that modulates carbohydrate and lipid metabolism, regulate insulin sensitivity. The relationship between NGR4 and gestational diabetes mellitus (GDM) remains unclear due to discrepancy in studies. The objective of this study was to find out association between NRG4 and insulin level in women with GDM and their relationship with other metabolic parameters.

Methods: In this comparative cross sectional study, pregnant women (n=80), coming for regular antenatal check-ups between 24 and 28 weeks of gestation were recruited. After informed signed consent, interview based proforma was filled with demographic data and antenatal history. Anthropometric measurements were taken; weight recorded on first visit was considered pre-pregnancy weight. Blood (5ml) was drawn after 12 hours fast for estimation of fasting blood sugar (FBS), insulin, NRG4 and lipid profile and secondly after one hour post-consuming 50gm glucose, for glucose challenge test. Statistical analysis was done using SPSS version20, Mann Whitney and Spearman correlation for finding association between variables, whereas, Kruskal Wallis Test for differences between groups.

Results: Median NRG4 level (0.98) in healthy group was significantly high ($p<0.038$) compared to GDM (0.94). The NRG4 had inverse weak association with GDM. The mean insulin and homeostasis assessment model of insulin resistance (HOMA IR) values had weak direct association with NRG4. Cholesterol, LDL had inverse relationship with NRG4. All parameters of lipid profile were high in GDM compared to non GDM, but only cholesterol was statistically significant ($p<0.03$). The FBS in healthy group was significantly low ($p<0.02$) compared to GDM. The association between insulin and HOMA IR was statistically significant ($p<0.05$). The difference between BMI was statistically significant with age ($p<0.015$), parity ($p<0.035$). The difference between FBS and education was found significant ($p<0.049$).

Conclusion: Serum NRG4 level were found low in GDM compared to non-GDM ($p<0.03$), with a weak and direct association with insulin. Future studies are required with bigger sample size to establish NRG4 as a potential biomarker for GDM.

Keywords: Adipose Tissue, Brown; Diabetes, Gestational; Pregnancy

Background:

Gestational diabetes mellitus (GDM), the most common metabolic disease during pregnancy, has a prevalence of 8% in Pakistan [1], whereas, universally it is between 16-27% [2]. Recently, a brown fat adipokine, neuregulin4 (NRG4), has been identified, related indirectly with patients having GDM, type1diabetes mellitus (T1DM), type2diabetes mellitus (T2DM),non-alcoholic fatty liver disease (NAFLD), and coronary artery diseases [3, 4].

NRG4, belongs to the epidermal growth factor (EGF) family of extracellular ligands [5]. Harari et al were the first to report NRG4, a fourth neuregulin, having high mRNA expression in the pancreas. They found out that NRG4 acts through ErbB-4 and its pattern of expression together with its strict specificity to growth factor, suggest a physiological role distinct from that of the known ErbB ligands [6]. NRG4 is secreted by all types of adipose tissues upon cold stimuli but is highly expressed in brown adipose tissue (BAT) [7]. NRG4 after release from the brown fat binds to EGF receptor ErbB4 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4) [4] present on the liver and inhibits de novo synthesis of lipids to get rid of high amount of lipids circulating in the blood and promotes fatty acid oxidation [8]. At the same time it increases glucose utilization through GLUT (glucose transporters) 1 and 4 for rapid uptake of glucose by the cells [9]. It is responsible for regulating the homeostasis of bodily metabolic responses either in a paracrine or endocrine manner [10]. NRG4 stimulates cell proliferation, inhibits apoptosis and improves cell energy metabolism4) It helps to mitigate hepatic lipogenic signaling, maintain lipid and glucose homeostasis, prevent obesity, and improve insulin resistance (IR) in mice [10]. Moreover, it has a role in angiogenesis and nerve innervation [10], and protects the intestinal epithelial cell from inflammation [11].

Limited research is available regarding NRG4 association with GDM, and that too with contradictory results. Women with GDM have sevenfold chances of developing T2DM in postpartum. Estimation of NRG4, if found low in GDM, it can be used as a possible biomarker for predicting the chance of developing diabetes postpartum [12]. Hence, through strict diet and lifestyle modification, during this time, NRG4 secretion can be enhanced to maintain glucose homeostasis. The objective of this study was to find out association between levels of NRG4 and insulin in women with GDM and to find out their relationship with other metabolic parameters.

Methods:

This comparative cross sectional study was conducted from June 2018 to February 2019, after the approval from ethics review committee of Ziauddin University. Through a non- probability consecutive technique 84 pregnant women who came for their regular antenatal check-ups between 24 to 28 weeks of gestation were recruited from the gynaecology and obstetrics OPD and Family Medicine department at Dr. Ziauddin Hospital Karachi Pakistan, after they signed an informed consent. Sample size was calculated using open-epi calculator. Women with generalized or chronic inflammation, pre-existing diabetes, under treatment with anti-diabetic or insulin-sensitizing agents were excluded.

An interview based proforma was filled with demographic data, anthropometric measurements, detailed antenatal and previous obstetric history. Weight recorded at the first visit was considered as pre pregnancy weight. Venous blood (5ml) was drawn after 12 hours fast for fasting blood glucose (FBS), insulin levels, NRG4 and lipid profile. All women were given 50gm glucose and 5ml blood after one hour was drawn for glucose challenge test (GCT). The flow chart of data collection procedure is shown in Figure1.

Body fat percentage and body mass index (BMI) was measured using bioelectrical impedance through Omron analyser (HBF 306C). BMI values which are recommended by WHO were considered normal. Body fat percentage values created by Gallagher et al were considered normal [13]. NRG4 and fasting insulin level was estimated using enzyme-linked immunosorbent assays (ELISAs) as per the manufacturer's instructions (Phoenix Pharmaceuticals, Burlingame, CA,USA) and chemiluminescence (Siemens IMMULITE®1000Chemiluminescent) respectively. Lipid profile was done using Merck's company semi-automated spectrophotometric analyzer at 546nm wavelength. Lipid profile values were considered normal that are recommended by National Cholesterol Education Program's (NCEP's) ATPIII (Adult Treatment Panel III) guidelines 2018 for managing cholesterol in adults. FBS normal value were taken normal according to American diabetic association (ADA) 2019 guidelines, FBS between 100-126mg/dl as pre-diabetic and greater than 126 mg/dl as diabetic. GCT values were taken normal according to American diabetic association (ADA) 2019 guidelines.

Statistical program for social science (SPSS) version 20 was used for data analysis. The quantitative variables were expressed as median (interquartile ranges). 84 pregnant women were divided into two groups using Mann Whitney's Test to find the association of quantitative parameters between the GDM and healthy group. Spearman Correlation was used to find an association of NRG4 and insulin with GDM and metabolic parameters and Kruskal Wallis Test to find the difference between age, parity, education and ethnicity with glucose and non-glucose parameters. P value less than 0.05 was considered statistically significant.

Results:

Out of 84 subjects, 37 had GDM with a significant low (p 0.038) median (interquartile range) NRG4 level of 0.94(0.08)ng/ml compared to 47 healthy pregnant women [NRG4 0.98(0.10)ng/ml]. The FBS in healthy group was significantly low (p 0.02) compared to GDM. The mean insulin and HOMA IR values between two groups were non-significant. (Table1)

Median cholesterol was statistically high ($p < 0.03$) in GDM [220(66.50)mg/dl] compared to healthy pregnant women [210(64)mg/dl]. Mean triglycerides (TGs), low density lipoproteins (LDL), high density lipoproteins (HDL) and very low density lipoproteins (VLDL) were also high in GDM but the results were not significant. (Table1).

Insulin had significant strong direct association with body fat% whereas with cholesterol, HDL and LDL had a significant inverse association. BMI had significantly associated with body fat and FBS. GCT was directly associated with HOMAIR, FBS, cholesterol, TGs, and LDL with p value < 0.05 (Table2)

NRG4 was found to have indirect association with GDM. (Figure2)

NRG4 was found to have weak direct association with insulin, homeostasis assessment model of insulin resistance (HOMA IR). Cholesterol and LDL had an inverse weak association. NRG4 had no association with FBS, BMI, TGs and HDL. (Table2 and 3)

Relationship of BMI with age and parity was found significant with $p < 0.015$ and $p < 0.035$ respectively. (Table4)

Association between insulin and HOMA IR is shown in Figure3.

Discussion:

Novel BAT-secreted adipokine NRG4 was found indirectly associated with GDM in this study. This is the first study in the Pakistani population evaluating NRG4 in GDM. Consistent with the preceding research of Karlish et al [3] we observed significantly lower levels of plasma NRG4 in women with GDM compared to the healthy pregnant non GDM females. Many experimental studies on animals and human have revealed the association between NRG4 and glucose levels, but the majority of the researchers are uncertain about the exact mechanism of underlying association. However, research on exploring its relationship with glucose metabolism revealed the underlying metabolic pathways that are involved in glucose and fat homeostasis [10].

Microarray analysis used on mouse model revealed that an over expression of NRG4 leads to decreased expression of enzymes required for gluconeogenesis in the liver [14]. These researches on animals studied the expressions of NRG4 levels in the tissue than its concentration in the plasma. Glucose tolerance on NRG4 knockout mice and observation on high glucose levels after feeding high fat diet demonstrated that transgenic mice with high expressions of NRG4 had low glucose concentration compared to control wild type mice showing involvement of NRG4 in glucose and fat homeostasis [10]. In contrast one study conducted by Kurek et al in 2017, regarding association of NRG4 with GDM published contradictory findings, showing significant elevation in NRG4 levels in GDM pregnant females. The divergence in their findings might be due to difference in methodology, genetic, racial or lifestyle differences in the study population.

The role of NRG4 at maintaining lipid and glucose homeostasis suggest that NRG4 may work as a defending metabolic adipokine. In obese mice low expressions of mRNA of NRG4 were observed which caused fatty liver, dyslipidemia and increased IR [10, 15]. This establishes an inverse relation of NRG4 with FBS and body fat mass [5]. Studying the lipid profile reveals that NRG4 has an inverse relation with TGs but is directly related to HDL [12]. This explains why obese human beings in general have low NRG4 levels along with negative correlation with dyslipidemia and IR [10]. We found an inverse association of NRG4 with Cholesterol and LDL in our study. However, association of NRG4 with insulin and HOMA IR was found direct but it was not significant.

The relationship of NRG4 with diabetes has been ascertained by studying its correlation with FBS, metabolic and other parameters. Majority of the researchers observed low expressions of NRG4 mRNA in visceral and subcutaneous adipose tissue of individuals with impaired glucose tolerance and T2DM, even patients with newly diagnosed T2DM were found to have low NRG4 levels [16]. Whereas, fasting glucose, fasting insulin and HOMA IR were indirectly associated with NRG4. Researchers concluded that serum NRG4 level is elevated in pre-diabetics and diabetics. In Chinese population comparison of quartiles of serum NRG4 concentrations showed an odds ratio (OR) of 3.005 in diabetes prevalence, after adjusting age and gender. However, other parameters were also found significantly correlating with serum NRG4 ($P < 0.05$), including BMI, alanine aminotransferase, HDL, TGs, uric acid, GFR etc. [17] In our study we did not observe any association of NRG4 with FBS, TGs, and HDL. All the results were statistically not significant.

A number of studies investigated association of circulating NRG4 levels on metabolic diseases such as NAFLD, GDM, obesity and T2DM [3,12,18,19]. Regarding Metabolic syndrome (MS), NRG4 in various studies was shown strongly associated. Researchers assert that the OR remained significant for the association of plasma NRG4 concentration with the presence of MS even after adjusting the potential confounders, including all measures of IR. This shows that low levels of NRG4 in plasma is adding to the risk of MS independently of IR, suggesting that plasma NRG4 may protect against MS via IR-independent mechanisms. Experimental evidence suggests that compounding factor for MS oxidative stress which is increased by reduction in antioxidant defenses established by correlation of high inflammation status (IR, T2DM, hypertension, obesity and dyslipidemia) and decrease in levels of antioxidant enzymes [20]. This occurs through down regulation of NRG4 gene expression in patients with high oxidative stress [21]. Yan et al's studies [12] on NRG4 levels in T2DM with and without MS revealed that T2DM with MS had low levels of NRG4. This shows that decrease in NRG4 levels is playing a crucial part in the pathophysiology of MS. Presence of NRG4 in blood helps in maintaining metabolic balance of glucose and lipids in both animal and humans hence preventing or delaying the detrimental effects of metabolic diseases.

In our study HOMA IR and fasting insulin levels were directly related to circulating NRG4 in blood as documented by Kurek et al. [22] NRG4, maintains lipid and glucose homeostasis through ERB3 and ERB4 receptors present in the liver. It also up

regulates GLUT1 and GLUT3 transporters in skeletal muscle to increase glucose uptake hence maintaining glucose homeostasis [5, 10]. The insight in target receptors of NRG4, can therefore, lead to hypothesis that NRG4 is involved in glucose and fat homeostasis. However, the exact mechanism of NRG4 is still not fully understood. Overall, studies conducted on NRG4, so far had diverse variables such as varying sample size, ELISA techniques, and patient characteristics; and ethnic disparity and hence results are also discrepant. Secondly, the pathophysiological processes of metabolic diseases are possibly affecting the signaling pathways activating NRG4 release or maybe they are creating its resistance at the level of receptor are a cause of low or high levels of NRG4 in metabolic diseases. There are some limitations in this study. The study sample size was small, NRG4 cut-off value could not be calculated and used GCT for diagnosis of GDM rather than OGTT.

Conclusion:

The study concludes that since NRG4 was found high in healthy women compared to GDM, therefore, rise in NRG4 can improve glucose status in patients with GDM. This suggests that awareness sessions could be conducted amongst pregnant women and who plan to conceive to increase brown fat activity through strictly modifying diet and lifestyle. Enhanced NRG4 secretion maintain glucose homeostasis and chances of complications during pregnancy may be avoided. Future studies, with bigger sample size are required to establish NRG4 as a potential biomarker for GDM.

Abbreviations:

NRG4: Neureglin4

GDM: Gestational diabetes mellitus

T1DM: Type1 diabetes mellitus

T2DM: Type2 diabetes mellitus

NAFLD: Non-alcoholic fatty liver disease

EGF: Epidermal growth factor

BAT: Brown adipose tissue

IR: Insulin resistance

FBS: Fasting blood sugar

GCT: Glucose challenge test

BMI: Body mass index

HOMA IR: Homeostatic model assessment of insulin resistance

TGs: Triglycerides

LDL: Low density lipoproteins

HDL: High density lipoproteins

VLDL: Very low density lipoproteins

OD: Odds ratio

MS: Metabolic syndrome

DECLARATIONS:

1. Ethics approval and consent to participate:

This study was approved by ethics review committee of Ziauddin University Pakistan. Verbal and written signed consent was taken from all the patients.

2. Consent for publication:

Not applicable

3. Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

4. Competing interests:

The authors declare that they have no competing interests.

5. Funding:

Ziauddin University gave Rs. 1, 50,000 as a seed money for the project. Rest was self-financed.

6. Author's contribution:

HA conceived the idea and did bench work and statistics. SI contributed in sample collection/patient counseling. SB contributed in sampling and manuscript writing. STA and RR contributed in writing and overall formatting. MAS contributed in sample collection and bench work.

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Table1: Comparison of Diabetic Parameters in pregnant females with and without GDM.

Variables	Median(Interquartile Range)		P-Value
	Non-GDM(47)	GDM(37)	
NRG4 (ng/ml)	0.98(0.10)	0.94(0.08)	0.038*
FBS(mg/dl)	79(18)	92(36)	0.020*
BMI (kg/m2)	24.20(6)	25.7(7.90)	0.238
Insulin(μU/ml)	3.92(3.58)	4.74(6.19)	0.428
HOMA IR	0.75(0.80)	1.02(1.84)	0.093
Body Fat (%)	26(11.90)	28.10(11.95)	0.296
Cholesterol (mg/dl)	210(64)	220(66.50)	0.030*
TGs (mg/dl)	170(41)	174(49.50)	0.151
HDL (mg/dl)	51(11)	52(10.50)	0.407
LDL(mg/dl)	114.20(44)	133.20(64.50)	0.135
VLDL(mg/dl)	34(8.20)	34.80(9.90)	0.151

Mann- Whitney's Test. *P<0.05 statistically significant

Table 2: Association of Different Variables with NRG4

Variables	NRG4		Insulin		BMI		GCT	
	R	P value	r	P value	r	P value	r	P value
HOMA IR	0.13	0.115	0.94	0.04	0.215*	0.025	0.235*	0.16
Body fat%	0.07	0.264	0.19*	0.042	0.665**	0.000	0.86	0.218
FBS (mg/dl)	0.069	0.268	0.16	0.071	0.240*	0.014	0.311**	0.002
Cho(mg/dl)	-0.14	0.102	-0.24*	0.013	-0.051	0.324	0.269**	0.007
TGs(mg/dl)	-0.02	0.419	0.07	0.254	-0.061	0.289	0.243*	0.13
HDL(mg/dl)	-0.09	0.185	-0.15	0.084	-0.021	0.425	-0.009	0.467
LDL(mg/dl)	-0.12	0.138	-0.26**	0.008	-0.013	0.453	0.213*	0.026

Spearman correlation

** correlation is significant at 0.01 level. * correlation is significant at 0.05 level *P value < 0.05 statistically significant

Table3: Association of Different Variables with NRG4

Parameters	r	P-Value
Insulin (µU/ml)	0.170	0.062
BMI	0.021	0.423

Spearman correlation

Table4: Quantitative Variable of the Study population

Variable [N (%)]	NRG4 (ng/ml)	Body fat (%)	BMI	FBS (mg/dl)	Insulin (µIU/ml)	HOMAIR	GCT (mg/dl)
AGE							
≤20 [22(20.8)]	0.99[0.10]	26.25 [10.77]	22.45[9.40]	90[24]	2.75[2.88]	0.65[0.65]	133[32.25]
21-30 [49(46.2)]	0.97[0.10]	28.2 [12.75]	25.70[8.20]	81[22]	4.61[5.26]	1.02[1.11]	132[29.50]
≥ 31 [13(12.3)]	0.92[0.18]	28.1 [15.65]	26.7[6.45]	77[26]	3.97[19.01]	0.68[0.69]	150[35]
P-Value	0.153	0.097	0.015*	0.309	0.070	0.136	0.828
PARITY							
Nullipara 20(18.9)	0.98[0.14]	24.25[11.23]	22.1[4.85]	83[31.5]	3.47[3.21]	0.68[0.79]	127.5[29]
Primipara 16 (15.1)	1.01[0.14]	27.05[11.03]	24.35[3.38]	83.5[17]	4.69[5.13]	0.96[0.75]	129.5[24.25]
Multipara 37 (34.9)	0.95[0.09]	28[11.75]	26.7[6.55]	79[22]	4.25[5.43]	.86[1.26]	139[35.5]
Grand multipara 11(10.4)	0.96[0.06]	32.5[19]	25[16.5]	84[63]	3.5[3.53]	0.72[0.44]	153[58]
P-Value	0.362	0.116	0.035*	0.816	0.416	0.57	0.41
ETHNICITY							
Urdu speaking 2(1.9)	1.08[-]	26.95	21.05	60	5.81	0.90	123.5

Sindhi 7 (6.6)	0.98[0.11]	25.5[9.90]	21.4[5.40]	75[24]	2.71[7.39]	0.46[2.07]	125[38]
Punjab 14 (13.2)	0.93[0.14]	29[12.9]	25.95[6.02]	79[21.25]	4.31[4.27]	0.83[0.87]	1148[39.75]
Pushtu 61 (57.5)	0.97[0.1]	27.6[13.10]	24.59[6.50]	85[28]	4.09[4.98]	0.81[0.88]	137[28.50]
P- Value	0.44	0.458	0.105	0.099	0.938	0.861	0.093
EDUCATION							
Un- educat ed 35(33.0)	0.96[0.06]	28.1[9.60]	25.3[7.80]	83[31]	3.5[3.52]	0.74[0.74]	127[40]
Relig ion study 24(n)	1.02[0.19]	28.15[13.93]	24.3[6.32]	86[18.75]	4.85[5.67]	1.03[1.23]	139.5[34.50]
≤Clas s10 21(n)	0.94[0.08]	22[10.80]	24.59[6.55]	81(5.86)	4.25[5.86]	0.89[0.98]	146[27.50]
≥ Class 12 th 4(n)	1.03[0.28]	23.55[17.63]	24[10]	68[0.75]	3.23[4.52]	0.55[0.76]	126[20]
P- Value	0.072	0.271	0.627	0.049*	0.668	0.495	0.068

Kruskal Wallis Test; P- Value < 0.05 is significant

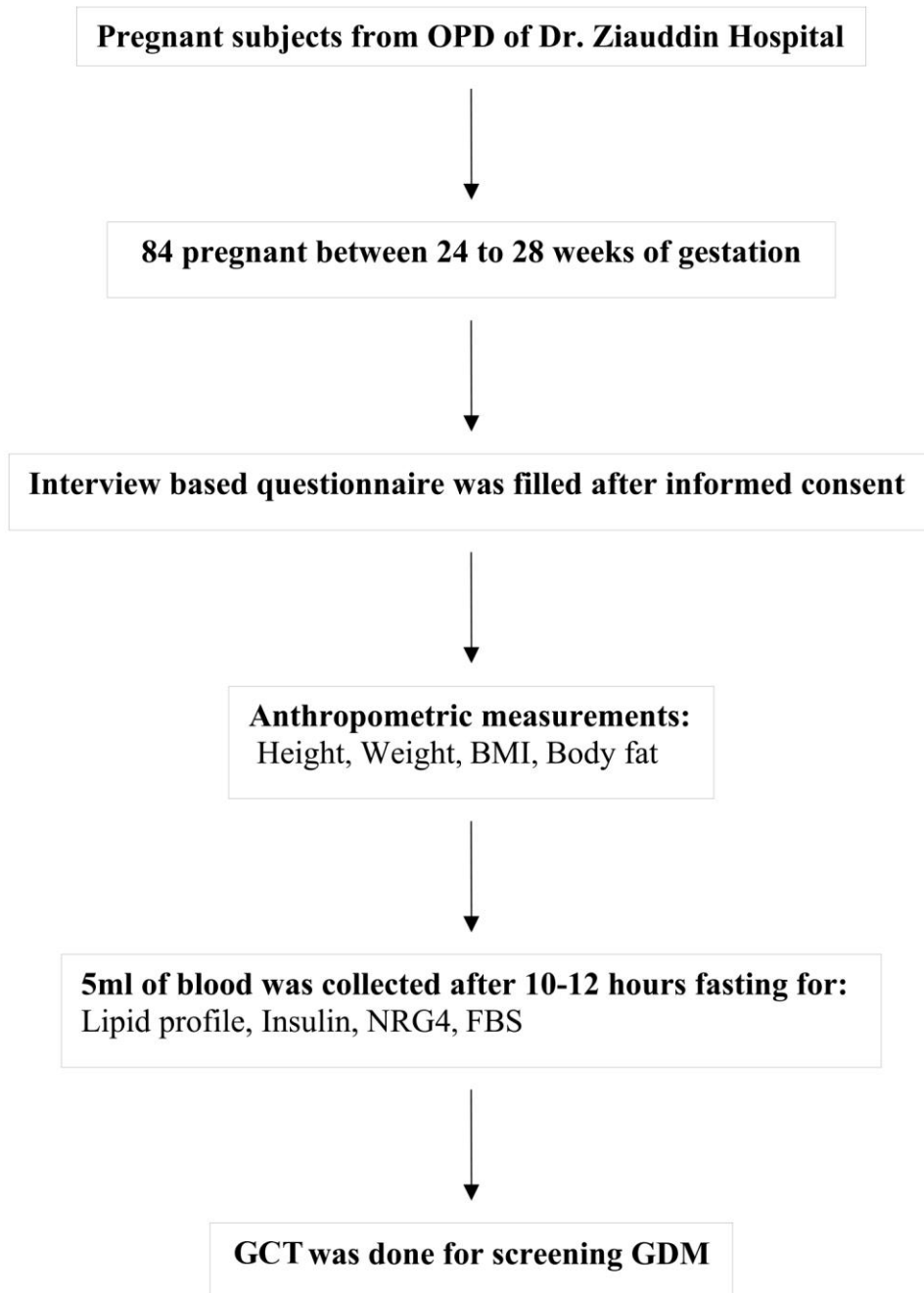


Figure1: Flow chart of study population

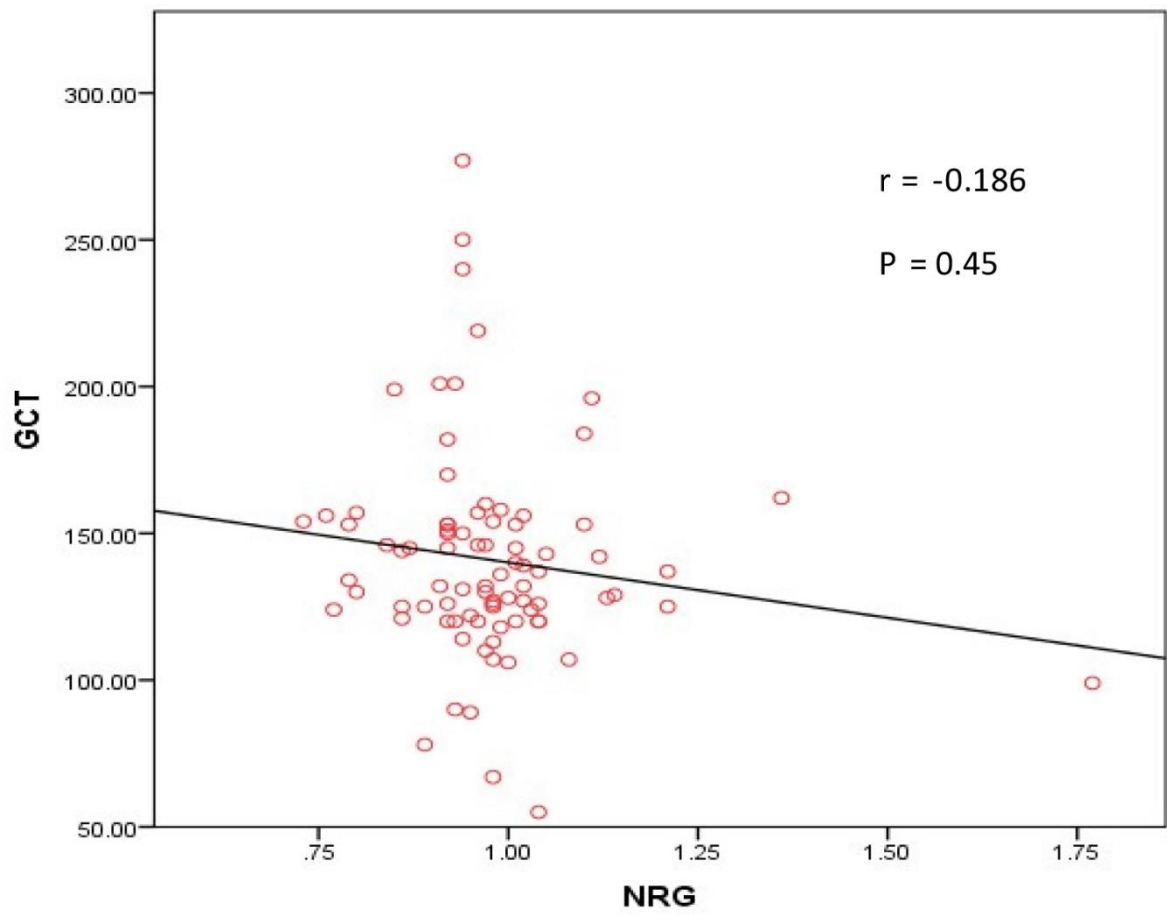


Figure2: Scatter Plot between NRG4 and GDM

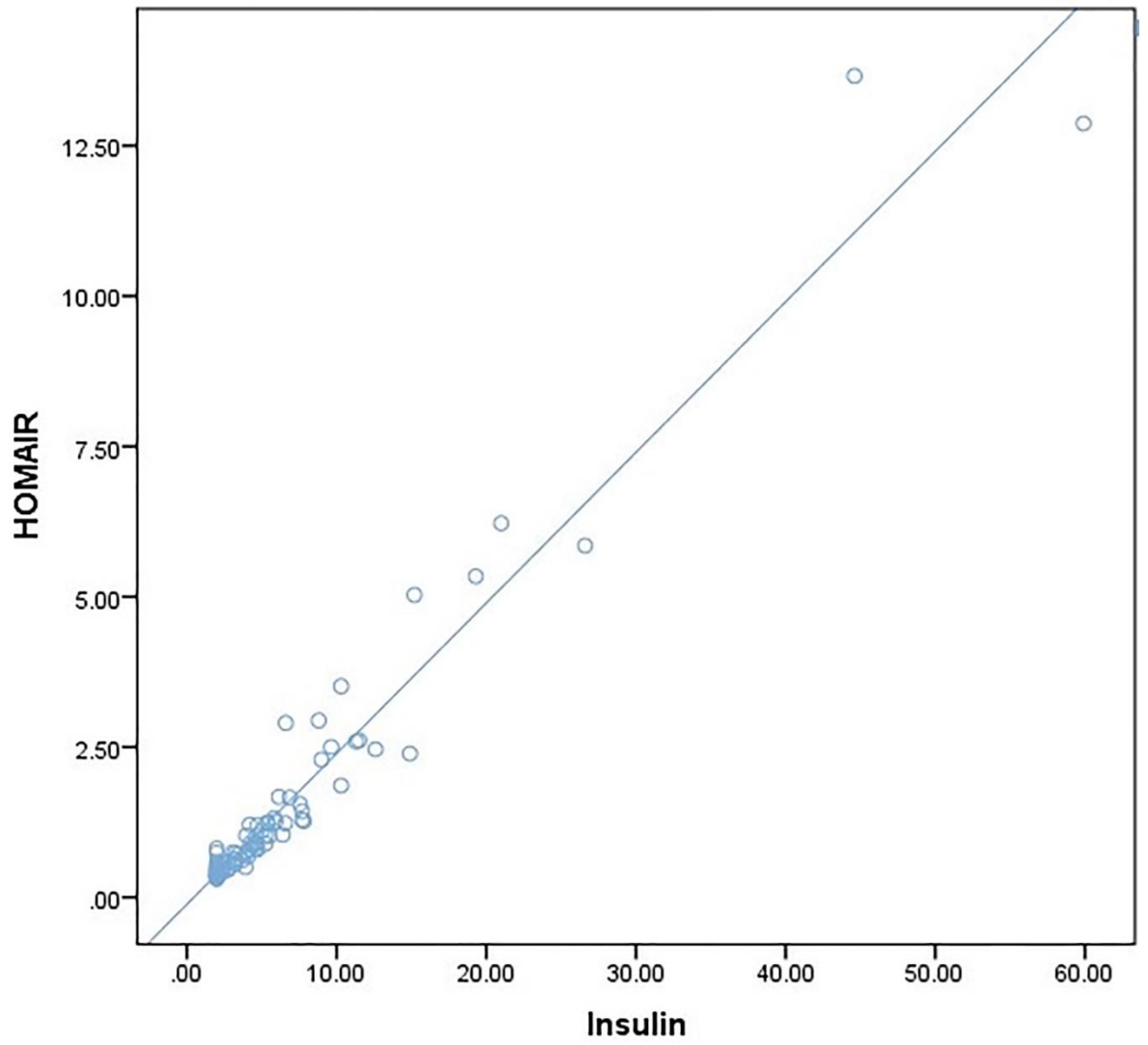


Figure3: Correlation of Insulin with HOMA IR