

## Study of inflammatory markers and TNF- $\alpha$ g308a and gene polymorphism in gestational diabetes mellitus

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### Abstract

**Introduction:** The prevalence of Gestational diabetes mellitus (GDM) is increasing world wide. It has been said that plasma levels of TNF- $\alpha$  is regulated by single nucleotide polymorphism in promoter region of TNF- $\alpha$  gene in development of GDM. The study was done to determine Tumor Necrosis Factor- $\alpha$  (TNF) levels and its gene Polymorphism in GDM cases and controls and to correlate them with each other.

**Materials and Methods:** A total of 60 patients were selected comprising 30 diagnosed GDM cases and 30 apparently healthy pregnant women from ANC matched for age and gestation. OGTT was conducted and glucose estimated by GOD-POD method (Beckman AU-480 auto-analyzer) and serum TNF- $\alpha$  levels using sandwich ELISA. TNF- $\alpha$  308(G/A) gene polymorphism was analyzed by extracting DNA from whole blood (using QIAGEN DNA mini kit) followed by DNA amplification by PCR and RFLP using restriction enzyme NcoI.

**Results:** TNF- $\alpha$  levels were significantly increased in GDM cases (28.93 $\pm$ 20.96 pg/ml) as compared to controls (19.4 $\pm$ 11.6 pg/ml). TNF- $\alpha$  polymorphism revealed GG genotype in 87% cases and 93% controls; AA 3% of cases and 7% of controls and GA 10% of cases and absent in controls. However, the difference was statistically insignificant. TNF- $\alpha$  levels did not significantly correlate with the polymorphism. ROC analysis showed area under the curve of 0.613 for prediction of GDM.

**Conclusion:** In GDM significant increase of TNF- $\alpha$  levels signifies the role of inflammation in the etio-pathogenesis. The TNF- $\alpha$  G308A gene Polymorphism in particular is however not significantly associated with GDM.

**Keywords:** Gestational diabetes mellitus, TNF- $\alpha$  G308A gene polymorphism.

### Introduction

Gestational diabetes mellitus (GDM) is characterized by impaired glucose tolerance of different severity. It is recognised suddenly during late second or early third trimester gestation. It can lead to increased risk of maternal and fetal complications.<sup>1</sup> Glucose intolerance may be because of metabolic changes in pregnancy and increased insulin requirement during pregnancy.<sup>2</sup> Worldwide the prevalence of gestational diabetes mellitus is increasing rapidly and it is more in developing countries like India. Women with unmanaged gestational diabetes are at increased risk of developing type 2 DM (or, very rarely, latent autoimmune or type 1) after pregnancy. They may have higher incidence of preeclampsia and cesarean section. Their offspring are prone to developing childhood obesity, with type 2 diabetes later in life. The exact etiopathogenesis of underlying gestational diabetes remain unknown. Increased insulin resistance is seen in GDM. Action of insulin on insulin receptor is interfered by hormone produced during pregnancy at the level of the cell signalling pathway.<sup>3</sup>

As inflammation plays a crucial event in the etiopathogenesis of GDM. Various studies confirmed the role of inflammatory cytokines like TNF- $\alpha$  and NF- $\kappa$ B in mediating inflammation-induced insulin resistance.<sup>4</sup> Scientific studies in the last few years has revealed that NF- $\kappa$ B is activated by inflammatory cytokines such as TNF- $\alpha$ .<sup>5</sup> Literature has shown that plasma levels of TNF- $\alpha$  is

regulated by single nucleotide polymorphisms in promoter region. This polymorphism leads to guanine (G) being replaced by adenine (A) in 308 position leading to a higher rate of TNF gene transcription.<sup>6</sup> Our study is hospital based observational cross sectional study. Aim of our study was to evaluate the pro inflammatory milieu in GDM and also attempts to analyze the effect of TNF- $\alpha$  (308) G/A gene polymorphism on the severity of insulin resistance in GDM.

### Material and Methods

The study was done in the Department of Biochemistry and Department of Obstetrics and Gynaecology, Lady Hardinge Medical College and Smt. Sucheta Kriplani Hospital, New Delhi. 60 subjects were enrolled after prior informed consent which comprised of 30 cases of GDM and 30 healthy pregnant female as controls. Institutional ethical clearance was sought. Samples were taken after overnight fasting and processed accordingly for routine biochemical investigations, special parameters such as TNF- $\alpha$  and NF- $\kappa$ -B while genomic DNA was extracted from whole blood for the said gene polymorphism.

O'Sullivan's and Mohan diagnostic criteria (1986) for Oral Glucose Tolerance Test (in which 100gm of glucose was given) was used for diagnosis of GDM in our study: Fasting  $\geq$  95mg/dl, 1hr  $\geq$  180mg/dl, 2hr  $\geq$  155mg/dl, 3hr  $\geq$  140mg/dl,  $\geq$  2 abnormal values are met or exceeded; GDM is diagnosed.

Known case of DM, Pre-eclampsia, Hypothyroidism, PROM (premature rupture of membrane), history of ongoing chronic inflammatory pathology patients were excluded from study. Routine blood investigations-complete blood count, erythrocyte sedimentation rate, blood sugar, liver and kidney function tests, lipid profile tests were measured by AU-480 clinical chemistry auto analyser using Beckman and Randox standard reagents and kits.

TNF- $\alpha$  level and NF-KB levels were measured by using DIACLONE (France) and QAYEE-BIO commercial Sandwich ELISA kit respectively. DNA extraction was done by using DNA extraction kit (QIAGEN) and DNA was amplified for TNF- $\alpha$  gene by using PCR, which was later screened for RFLP. Primer pairs were as follows: Forward Primer 5'-AGGCAATAGGTTTTGAGGGCCAT-3' and Reverse Primer 5'-TCCTCCCTGCTCCGATTCCG-3'. 107bp pcr product was digested with restriction enzyme NcoI. 10 $\mu$ l pcr product was mixed with 1 $\mu$ l of NcoI enzyme and incubated for 1hr at 37°C. Digested product was run on 2% agarose gel. In homozygous (GG) genotype two bands of 80bp and 27bp are obtained. In homozygous (AA) genotype, single band of 107bp is obtained and in heterozygous (GA) state two bands of 107bp and 80bp are obtained.

The studied data was analyzed by appropriate statistical methods using 20 version of Statistical Package for Social Sciences (SPSS). Data were compared by using student t-test. An Allelic frequency of the genotype was compared with biochemical parameters using appropriate statistical tools. Quantitative data was expressed as mean  $\pm$ SD/ $\pm$ SE of mean and qualitative data as proportions. The p value of < 0.05 was considered as statistically significant.

## Results

In our study BMI was significantly higher in cases (2.53 $\pm$ 0.42) as compared to the controls (2.32 $\pm$ 0.366) with statistical *p*-value of 0.043. In study cases mean serum uric acid value was significantly higher (5.39 $\pm$ 1.51) as compared to the controls (4.60 $\pm$ 1.43) with *p*-value = 0.044.

Other routine biochemical investigations was comparable in cases and controls. In cases the mean value of haemoglobin was significantly higher (11.6 $\pm$ 1.19 gm/dl) in

comparison to the control group (10.8 $\pm$ 1.70 gm/dl) with *p*-value of 0.030. The mean value of OGCT as significantly higher in studied cases (163.37 $\pm$ 33.607) as compared to the controls (93.50 $\pm$ 20.48) with *p*-value of <0.001. Similarly HbA1C levels were also increased in cases (5.63 $\pm$ 0.883) as compare to controls (4.49 $\pm$ 0.610) with *p*-value <0.001. The graph (Fig. 1) illustrates values of sugar profile among the groups. The difference between cases and controls was found to be statistically significant for all three parameters. Lipid profile assay showed significantly higher triglyceride levels in cases (313.97 $\pm$ 119.76) as compare to controls (229.53 $\pm$ 105.69) with *p*-value = 0.005. There was no significant difference between cases and controls in the levels of cholesterol and HDL.

In the studied cases, the serum TNF- $\alpha$  value was significantly higher (28.93 $\pm$ 20.96 pg/ml) as compared to the control group (19.40 $\pm$ 11.66 pg/ml). The difference between the studied cases and controls was statistically significant with *p*-value=0.034. The NF- $\kappa$ B level in the cases was higher 4.67 $\pm$ 2.85ng/ml as compare to control group 4.00 $\pm$ 0.743ng/ml but the difference between the two was statistically insignificant with *p* of 0.220. There was no significant correlation between NF  $\kappa$ -B and TNF- $\alpha$  (Table 2). In our study on 308 (G/A) gene polymorphism of TNF- $\alpha$ , we found GG was more (93.33%) in control as compare to case (86.6%) though difference was (*p*> 0.05). AA genotype frequency was 3.33% in cases and 6.66% in the control group. GA genotype frequency was 10% in cases and 0% in control group as there was no GA because of small sample size. There was no statistically significant (*p*= 0.182) difference in the frequency of distribution of genotype in case and controls. The mean TNF- $\alpha$  level in the study group was 28.93 $\pm$ 20.96pg/ml and in the control group was 19.40 $\pm$ 11.66 pg/ml. The difference between these two was statistically significant (*p* value=0.034). GG genotype was found in 26 subjects (86.6%) of case and 28 subjects (93.3%) of control group. AA genotype was found in 1 subject (3.3%) of study group and 2 subjects (6.6%) of control group. GA was found in only 3 subjects. No significant correlation was found (Table 3).

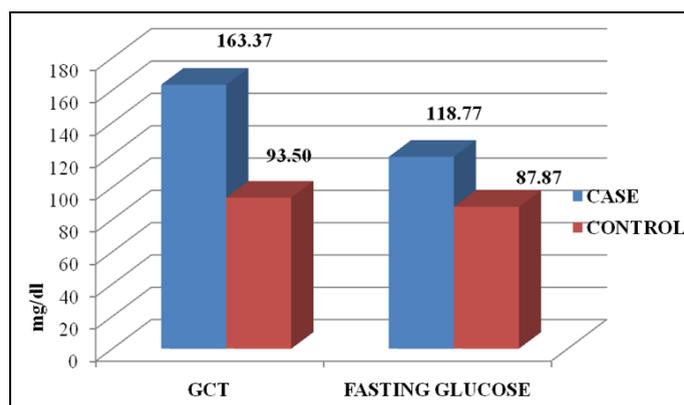


Fig. 1: Glucose profile in case and control group

**Table 2: Serum TNF- $\alpha$  level in case and control group**

Parameter	Case (n=30) MEAN $\pm$ SD (SEM)	Control n=30 MEAN $\pm$ SD (SEM)	p-VALUE
TNF- $\alpha$ (pg/ml)	28.93 $\pm$ 20.96 (3.82)	19.40 $\pm$ 11.66 (2.130)	<b>0.034</b>
NF $\kappa$ -B (ng/ml)	4.673 $\pm$ 2.850 (0.520)	4.006 $\pm$ 0.743 (0.135)	0.220

**Table 3: Genotype of TNF- $\alpha$  308 (G/A) in case and control groups**

Genotype	Control (n=30)		Case (n=30)		p-Value
	n	Frequency %	N	Frequency %	
GG	28	93.33	26	86.6	0.182
AA	2	6.66	1	3.33	
GA	0	0	3	10	

## Discussion

Gestational diabetes mellitus (GDM) is defined as hyperglycemia of variable severity that has recent onset or first recognised during pregnancy due to glucose intolerance. It is common and important complication of pregnancy and that may lead to both maternal and foetal morbidity, mortality. The exact etiopathogenesis of GDM is unknown, but accumulating evidence suggests the multifactorial etiology of the disease and the dominant role of chronic insulin resistance. Molecular and genetic work is underway for better understanding of the disease etiology.

It has been observed in various studies that in obesity, increased age, severe infections, muscle injury, and thermal injury patients, increased circulating TNF- $\alpha$  level have been associated with insulin resistance. TNF- $\alpha$  is an important novel marker of inflammation and has a potential in identifying individuals at high GDM risk. Direct role for TNF- $\alpha$  in the pathophysiology of insulin resistance have been described in literature. It is hypothesised that TNF- $\alpha$  down regulates insulin receptor signaling in adipocytes, liver cells and skeletal muscles. Catalano et al describes that TNF- $\alpha$  activates pathway that increases sphingomyelinase, ceramides and appears to interfere with insulin receptor autophosphorylation. TNF- $\alpha$  also promotes serine phosphorylation of insulin receptor substrate (IRS)-1, thus impairing its association with the insulin receptor.<sup>7</sup>

Insulin receptor and IRS-1 tyrosine phosphorylation is impaired in pregnancy and serine phosphorylation is increased in late gestation in skeletal muscles. In late gestation elevated levels of TNF- $\alpha$  could attenuate insulin signalling, thus causing the decreased insulin sensitivity. However studies to support the role of TNF- $\alpha$  polymorphism in GDM are very limited in literatures and it need to be further explored by study on larger sample sizes in various ethnicity and regions to reach at any concrete conclusion.

In our study BMI in cases was significantly higher (2.53 $\pm$ 0.42) as compare to the controls (2.32 $\pm$ 0.366) with p value = 0.043. Our results show that increasing Body mass

index (BMI) is associated with an increased risk of GDM. Study done by Torloni and coworkers (2009) also support our finding, who proposed that risk of gestational diabetes increases by approximately 1 percent for every 1 kg/m<sup>2</sup> increase in BMI.<sup>8</sup> Chu et al. found that GDM risk increases substantially with increasing pre pregnancy BMI. Increased risk of GDM have been consistently associated with an higher maternal BMI.<sup>9</sup>

In our study mean serum uric acid levels in cases was significantly higher (5.39 $\pm$ 1.51) as compared to the controls (4.60 $\pm$ 1.43) with p value = 0.044. Weisz B et al. demonstrated that higher uric acid levels correlated with insulin resistance in women with hypertensive diseases of pregnancy as well as GDM.<sup>10-11</sup> This finding consistent with our study. The serum uric acid levels decrease significantly from 8<sup>th</sup> week of gestation up to 24 weeks normally during pregnancy due to increased glomerular filtration rate and decreased reabsorption of uric acid from the renal tubules. Hyperuricemia in pregnancy can lead to several complications.<sup>11-12</sup> Uric acid is a marker of oxidative stress, tissue injury and renal dysfunction. Moreover, increased uric acid is also an independent risk factor for cardiovascular disease and is proposed to mediate altered vascular function and inflammation.<sup>13-14</sup>

The mean value of haemoglobin in the study group is significantly higher (11.6 $\pm$ 1.19 gm/dl) as compared to the control group (10.8 $\pm$ 1.70 gm/dl) with p value of 0.030. Rest of the haemogram variable were similar between the two groups. Lao et al. proposed that high haemoglobin levels (more than 13 g/dL) in early pregnancy is an independent risk factor for GDM. This may reflect better nutritional status in these women, as suggested by high iron status.<sup>15</sup> Lao TT et al observed that iron stores are higher in GDM women as compared to women without GDM.<sup>16-17</sup> Fernandez et al proposed tentative mechanism to explain the role of iron in GDM. Iron decreases insulin extraction and metabolism in the liver, which leads to peripheral hyperinsulinaemia.<sup>18</sup> Iron overload results in oxidative stress in pancreatic  $\beta$ -cells, which leads to destruction of the

pancreatic islets.<sup>19</sup> A significantly higher glucose level on GCT of cases was (163.37 $\pm$ 33.607) as compared to the control group (93.50 $\pm$ 20.48) with p value <0.000. Similarly HbA1C levels were also higher in case (5.63 $\pm$ 0.883) as compare to controls (4.49 $\pm$ 0.610) with p value <0.001.

Lipid profile assay shows significantly higher triglyceride of cases (313.97 $\pm$ 119.76) as compare to controls (229.53 $\pm$ 105.69) with p value = 0.005. This finding is supported by study done by Amraei and Azemati who reported a significant increase in the concentration of triglycerides levels in pregnancy complicated by glucose intolerance as compared to normal pregnancy.<sup>20-22</sup> Lipid profile in pregnancy are characterized by marked elevations of total plasma cholesterol and triglyceride levels as a result of increased liver synthesis of triglycerides (TG) and Very Low Density Lipoprotein-Cholesterol (VLDL-C) in response to elevated estrogen levels.<sup>23</sup>

The mean TNF- $\alpha$  level in the cases group was higher (28.93 $\pm$ 20.96 pg/ml) as compared to the control group (19.40 $\pm$ 11.66 pg/ml). The difference between the two groups was statistically significant with p value=0.034. Our finding are similar to the Chang Y et al. and Kirwan et al. who reported higher plasma TNF-alpha levels and lower insulin sensitivity which is predictor of insulin resistance.<sup>24</sup> Hotamisligil et al. and Karasik et al also showed that the pro inflammatory cytokine TNF- $\alpha$  was able to induce insulin resistance.<sup>25-26</sup>

The mean NF- $\kappa$ B level in the study group was 4.67 $\pm$ 2.85ng/ml and in the control group was 4.00 $\pm$ 0.743 ng/ml. The difference was insignificant with p =0.220. NF  $\kappa$ -B levels are higher in case as compare to control groups although it did not come to be statistically significant. Tak et al proposed that transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a key regulator of inflammation and plays a key role in various inflammatory diseases.<sup>27</sup> In support of this Gerondakis et al proposed that NF- $\kappa$ B regulates host inflammatory and immune responses and cellular growth properties by increasing the expression of specific cellular genes encoding for cytokines, major histocompatibility complex (MHC), proteins for antigen presentation, and receptors for neutrophil adhesion and migration.<sup>28</sup> NF $\kappa$ -B is activated by inflammatory mediators, such as TNF- $\alpha$  which can phosphorylate IRS-1 on serine residues and inhibit its function which can explain its effect on insulin resistance. Other inhibitory kinases such as mTOR, p70 S6K1, and protein kinase C are also increased in insulin-resistant states by conditions of nutrient excess.

In our study there was no correlation of NF  $\kappa$ -B with TNF- $\alpha$  which may be due to small sample size. Darnay B et al and Ashikawa K et al proposed that NF- $\kappa$ B is responsible for mediating the inflammatory and insulin resistance inducing actions of TNF- $\alpha$ . This confirms that TNF- $\alpha$  plays an key role in causing insulin resistance and its actions are mediated by NF- $\kappa$ B.<sup>29-30</sup> Genetic predisposition to GDM has been confirmed because GDM occurs in families. Identification of various underlying genetic factors of GDM will help in understanding the mechanisms that contribute to the etio-pathophysiology of the disorder. The relationship

between the presence of SNP in TNF  $\alpha$  and their plasma levels are of the clinical interest because of the pivotal role of these cytokines in the stimulation or regulation of inflammatory and immune responses.

In our study wild type G/G homozygous genotype were found in 26 cases and 28 controls. 3 G/A heterozygous were found in cases while there no heterozygous in controls. 1 A/A homozygous was found in cases and 2 in control groups. In our study the frequency of GG genotype in case and controls were 86.6% and 93.33% respectively. AA genotype frequency was 3.33% and 6.66% in the cases and control group respectively. Frequency of GA genotype was 10% and in control group 0% as there was no GA because of small sample size. There was no statistically significant (p= 0.182) difference in the frequency of distribution of genotype in case and controls. Our findings are in accordance with similar studies conducted by Montazeri S et al. In 2010 in which they evaluated the association between TNF- alpha polymorphism and development of GDM and found no significant difference in genotype and allele frequency of polymorphism at position -308 (G/A) in the promoter region of the TNF-alpha gene between the GDM and controls. In addition they also confirmed that the TNF-alpha levels in the plasma of GDM and control mothers were not significantly different, so it is not an independent risk factor or a predictor for GDM.<sup>31</sup>

Guzmán-Flores JM et.al in 2013 also showed similar results and found that genotype and allele frequencies at the TNF- $\alpha$  G308A gene polymorphisms did not differ significantly between the women with gestational diabetes mellitus and controls.<sup>32</sup> Since the sample size of our study was small, a larger sample size would be better to understand role of TNF- $\alpha$  G308A gene polymorphisms and inflammation in pathogenesis of GDM.

## Conclusion

Maternal weight was the most important critically studied risk factor for GDM. We concluded that higher maternal BMI was consistently associated with an increased risk of GDM. The mean value of haemoglobin in the studied cases was significantly higher than control group. The mean value of GCT was higher in studied cases than controls. HbA1C levels were also significantly higher in cases as compare to controls. Lipid profile assay showed significantly higher triglyceride levels in cases as compared to controls. The findings of high triglyceride levels show a positive association and direct correlation between triglyceride and GDM occurrence. The serum TNF- $\alpha$  level was significantly higher than control group. The difference was statistically significant, there is definite increase levels of proinflammatory cytokines. The NF- $\kappa$ B level in the cases was higher than control group, but the difference between the two was statistically insignificant. The hypothesis that NF- $\kappa$ B is responsible for mediating the inflammatory and insulin resistance inducing actions of TNF- $\alpha$  is not proved in our study. In our study on 308 (G/A) gene polymorphism of TNF- $\alpha$ , we found GG was more (93.33%) in control as compare to case (86.6%) though difference was (p > 0.05).

The polymorphism is not associated significantly with occurrence of GDM.

**Conflict of Interest:** None

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