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Endothelial Nitric Oxide Synthase (T786C) Gene Polymorphism TT Genotype is Associated with High Nitric Oxide Levels and Low HOMA-IR Levels in Coronary Artery Disease Patients

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ABSTRACT

BACKGROUND: Data suggest eNOS -786T/C gene polymorphism is distinct in specific population group, ethnicity and geographic region and perhaps this genetic variability might produce different results on exposure to various environmental factors 9-14. Besides there is hardly any data available in Indian population. Sofurther research is needed to explore the complex interaction between environmental factors and eNOS -786T/C gene polymorphism in susceptibility to insulin resistance in patients with CAD in Indian population.

AIM: This study aimed to study the association of Insulin Resistance, Nitric Oxide and intergenotypic variation of endothelial Nitric Oxide Synthase (T786C) gene polymorphism among coronary artery disease subjects.

METHODOLOGY: This study consisted of 60 adult patients of with documented CAD and 60 age and sex matched healthy subjects as controls.Fasting Serum Insulin was measured by ELISA,fasting Serum nitric oxide by modified Griess reaction and fasting plasma glucose on fully automated chemistry analyzer (Hitachi 902) HOMA-IR was calculated mathematically by using formula given by Matthew et al. The eNOS gene loci was amplified by using PCR and by RFLP. A p-value <0.05 was considered significant. Statistical analysis was performed with the help of SPSS version.

RESULTS: Intergenotypic levels of nitric oxide were significantly lower in TC genotype than TT both in study as well as control groups. Also, TC genotype showed significantly higher HOMA-IR levels as compared to TT genotype in both study as well as control group. Moreover, CC genotype could not be found in both cases and control groups..Thus, TT genotype may have a protective role in development of insulin resistance and coronary artery disease as seen by high Nitric Oxide levels and low HOMA-IR levels in TT genotype than TC genotypes in both case as well as control groups.

CONCLUSIONS: Endothelial nitric oxide synthase (t786c) gene polymorphism tt genotype is associated with high nitric oxide levels and low homa-ir levels in coronary artery disease patients.

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INTRODUCTION

Coronary artery disease (CAD) is described as our modern "epidemic". By 2025, this is expected to account for 34% of all male deaths and 32% of all female deaths in India[1]. Studies suggest that most CAD event rates are noted in individuals with one or more CAD risk factors[2]. However, at least 25 percent of coronary patients have sudden death or myocardial infarction without prior symptoms[3]. Hence, there is a need to focus attention on additional markers to predict coronary risk. It is well accepted that endothelial dysfunction occurs in response to cardiovascular risk factors and precedes the development of atherosclerosis. Increase in prevalence of insulin resistance, Type 2 Diabetes Mellitus (T2D) and CAD has been associated with an increase in western lifestyles and urbanization[4-7].

The complex interaction between various genetic, environmental factors and life style modification factors are responsible for triggering insulin resistance and further development of T2D and CAD.[8] The precise cause and mechanism of Insulin resistance still remains to be elucidated and there is need to identify other causative risk factors and modify treatment.[9]

Nitric oxide (NO) is synthesisized from L-arginine by a family of enzymes, Nitric oxide synthases (NOS), and is responsible for maintaining basal vascular tone. In addition to relaxing vascular muscle cells, NO inhibits platelet and leukocute adhesion to vascular endothelium, inhibits vascular smooth muscle cell migration and growth, and limits the oxidation of atherogenic low density lipoprotein[12]. Experiments with Endothelial Nitric Oxide Synthase (eNOS) knockout mice

have shown decreased NO synthesis and insulin resistance. Insulin resistance has also been suggested to contribute to the pathogenesis of coronary artery disease (CAD)[6]. Recent studies worldwide suggest an association between eNOS -786T/C gene polymorphism and genetic susceptibility to insulin resistance.[10]Data suggest eNOS -786T/C gene polymorphism is distinct in specific population group, ethnicity and geographic region and perhaps this genetic variability might produce different results on exposure to various environmental factors[11]. Besides there is hardly any data available in Indian population. Sofurther research is needed to explore the complex interaction between environmental factors and eNOS -786T/C gene polymorphism in susceptibility to insulin reistance in patients with CAD in Indian population.[15]

AIMS

This study aimed to study the association of Insulin Resistance, Nitric Oxide and intergenotypic variation of endothelial Nitric Oxide Synthase (T786C) gene polymorphism among coronary artery disease subjects.

METHODOLOGY:

The study was conducted in Department of Biochemistry and Department of Cardiology, VardhmanMahavir Medical College and Safdarjung hospital, New Delhi.

This was a hospital based case – control (observational) study conducted on patients attending Cardiac OPD in Safdarjung Hospital, New Delhi.

The study population consisted of 60 adult patients of either sex with documented CAD. Consisted of 60 age and sex matched healthy subjects.

INCLUSION CRITERIA:. Angiographically proven cases of coronary artery disease were included in the study.

EXCLUSION CRITERIA:

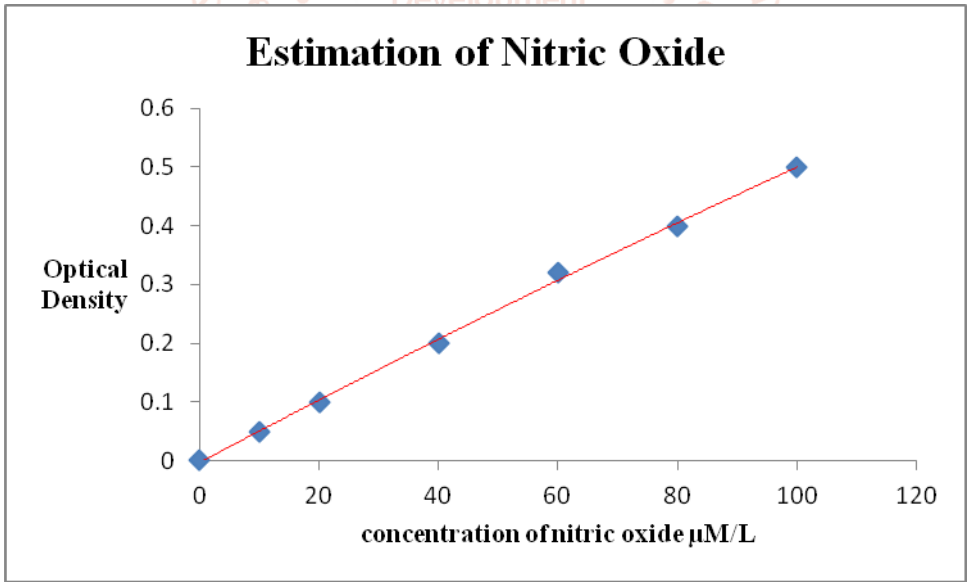
1. Diagnosed cases of Type 1 & 2 Diabetes mellitus**.**
2. Patients with congenital heart diseases.
3. Chronic kidney and liver disease**.**
4. Any history of debilitating illness**.** Any history of drugs affecting NO levels.

The study was conducted after institutional Ethical Committee approval and informed consent was taken from all patients and controls.

Bilingual informed written consent was taken from the patients. Detailed clinical history with special reference to Coronary Artery Disease and thorough clinical examination of patient was conducted. Necessary anthropometric measurements like height, weight and body mass index were taken.

Venous blood was collected from subjects under sterile conditions after overnight fasting. The whole blood collected in EDTA vacutainer was transferred to eppendorf and stored at -20 degree Celsius till DNA was extracted for PCR and RFLP. Nitric oxide in serum was determined indirectly by the measurement of its stable decomposition product nitrite (NO_2), by employing the Griess reaction according to the modified method of Mathew et al[13].

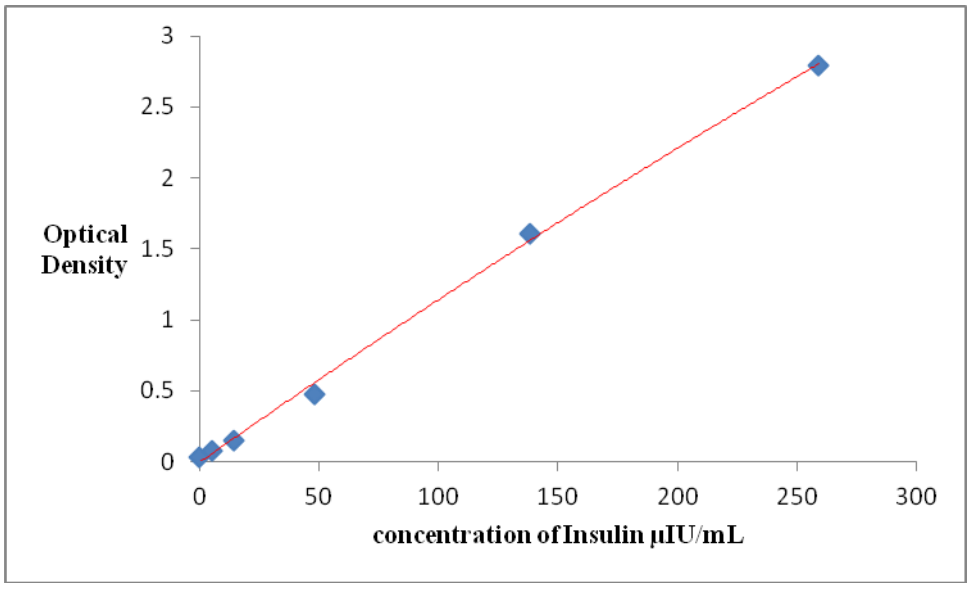
FIGURE 1: Standard curve of Nitric oxide obtained at 543nm



Total nitrite was determined from the standard slope constructed from known standard concentration and their corresponding absorbance values.

Fasting Serum Insulin Estimation: The estimation of serum Insulin was performed by sandwich ELISA technique. The kit was procured from Calbiotech Inc., CA.

FIGURE 2:Standard curve of serum insulin obtained at 450 nm



Estimation of Blood Glucose

Blood Glucose estimation was done by Glucose oxidase peroxidase method using commercially available kit Randox GL 7952 on automated chemistry analyser Hitachi 902.

HOMA-IR (Homeostasis Model of Assessment- Insulin Resistance):

Insulin resistance was calculated mathematically by using formula given by Matthew et al[14].
fasting Glucose(mg/dl) x fasting Insulin(µU/mL) / 405
Manual DNA extraction by the method of Daly's et al [15].

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION:

The required region of NOS3 gene from genomic DNA was amplified by Polymerase Chain Reaction in MJ Research PTC-100™(Peltier Thermal Cycler).

PRIMER SEQUENCE FOR NOS3

FORWARD PRIMER:- 5'- GTCTCTCAGCTTCCGTTTCTT-3'

REVERSE PRIMER:- 5'- CCTTGAGTCTGACATTAGGGTATC-3'

These primers were used to amplify a 458bp product for T-786C (rs2070744)

REAGENTS REQUIRED

1. Template DNA (e.g. genomic DNA) : 200-300ng
2. Forward and reverse PCR primers (Active Oligos) : 0.3pM
3. $MgCl_2$ (present in PCR Buffer) : 20 mM
4. dNTPs (a mixture ofdATP, dCTP, dGTP, and dTTP) : (Thermo Fischer Scientific) 200µM
5. 10× PCR buffer (Thermo Fischer Scientific) : 1X
6. TaqDNA polymerase (Dream Taq, Thermo Fischer Scientific) : 0 .3pM
7. Water to make total volume up to 20 µl.

The thermal cycling conditions were carried out in a PTC-100™ (Peltier Thermal Cycler) machine as follows-
Denaturation at 95^{0}C for 2 min,30 cycles of denaturation at 95^{0}C for 30 sec,annealing at 58^{0}C for 30 sec,elongation at 72^{0}C for 90 sec,followed by a final elongation step at 72^{0}C for 10 min.

The PCR products were analysed in a 2% agarose gel in a 1X TAE buffer system.This product was digested with restriction enzyme separately to reveal the genotype for the SNP.

The condition for digestion were as follows:
The PCR product (10 µl) was digested individually with 1µl (10unit/µl) of Msp1 (Krishgen) with 2µl of 10x restriction buffer and 18µl of nuclease free water incubated at 37^{o}C for 16 hours. Digested product was analysed in a 2% agarosegel.The TT genotype produced two fragments of 303bp and 155bp, while TC genotype produced three fragments of 257bp, 155bp and 46bp.

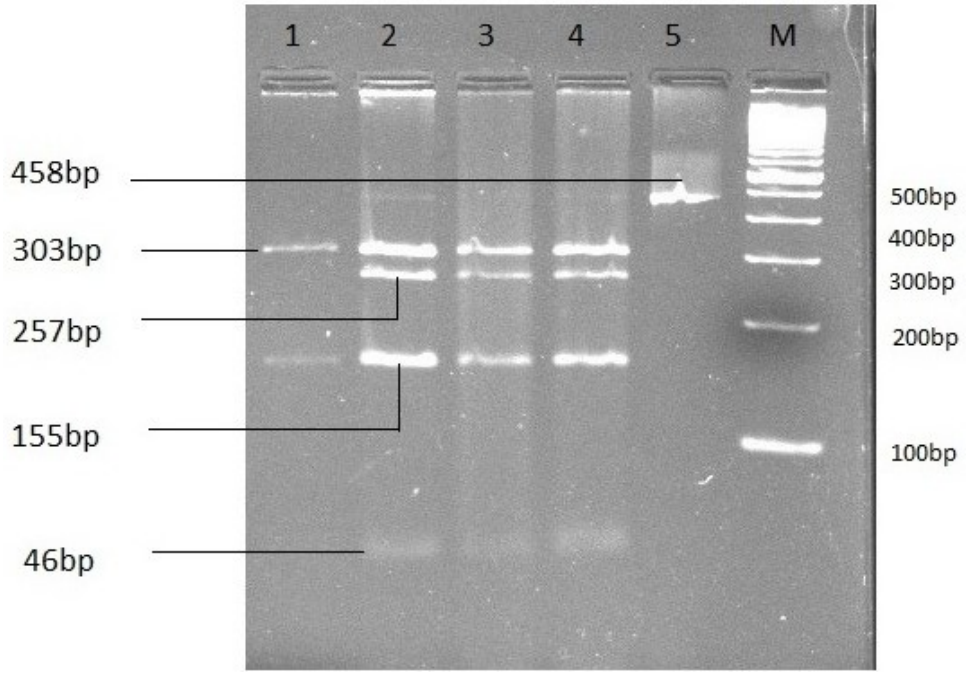


FIGURE 3: Ethidium bromide-stained agarose gel used for genotyping PCR product digested with Msp1.Lane1: Homozygote (TT) showing two bands at 303bp and155bp.

Lane 2,3& 4: Heterozygotes (TC) showing four bands at 303bp, 257bp, 155bp and 46bp.Lane M: molecular wt marker

STATISTICAL ANALYSIS:

A p-value <0.05 was considered significant. Statistical analysis was performed with the help of SPSS version 20. The data was subjected to t-test & dichotomous variables and allele frequencies were analyzed by Chi-Square test. For correlation of two continuous variables, correlation coefficient was used.

RESULTS:

Total 120 subjects were included in this study, out of which 60 subjects were selected in the study group with the Mean age of 63.28 years as well as equal number of subjects were selected in the control group, which were age and sex matched. Selection criteria have been described in material and methods. The study population consisted of 56.66% males and 43.33% females whereas the control group consisted of 60% males and 40% females. The mean systolic blood pressure was higher in the cases 131.93±0.91 mm of Hg as compared to the controls 126.16±1.03 mm of Hg, with statistically significant difference (p=0.000) .

The mean fasting plasma glucose was higher in the cases (95.33±2.92 mg/dl) as compared to the controls (93.06±3.95 mg/dl), with statistically significant difference (p=0.001).

The mean fasting serum insulin was higher in the cases (10.41±4.72mIU/L) as compared to the controls (7.10±2.94mIU/L) , with statistically significant difference (p=0.000).

The mean HOMA-IR was higher in the cases (2.46±1.16) as compared to the controls(1.63±0.69) , with statistically significant difference (p=0.000).

Endothelial Nitric Oxide (eNOS) gene variants (T786-C) was determined in both groups. TT genotype was found in 32 subjects in the study group (53.33%) and in 38 subjects in the control group (63.33%), whereas the TC genotype was found in 28 subjects in the study group (46.66%) and in 22 subjects in the control group (36.66%) respectively. No CC genotype was found in any group. The genotype distribution was in Hardy Weinberg equilibrium. The frequency of T allele was 76.67% in the study group and 81.67% in the control group while the frequency of C allele was 23.33% in the study group 18.33% in the control group respectively.

TABLE 1: INTERGENOTYPIC VARIATION OF SERUM NITRIC OXIDE LEVEL IN STUDY AND CONTROL GROUP

Genotype	Levels of NO(µM)						
	Cases (n=60)			Controls (n=60)			
	N	Mean	SEM	N	Mean	SEM	p value
TT+TC	60	17.80	0.95	60	22.48	0.83	0.0003
TT	32	22.96	0.66	38	25.83	0.74	0.006
TC	28	11.90	1.11	32	16.69	1.08	0.004

*p≤0.000 is statistically very significant

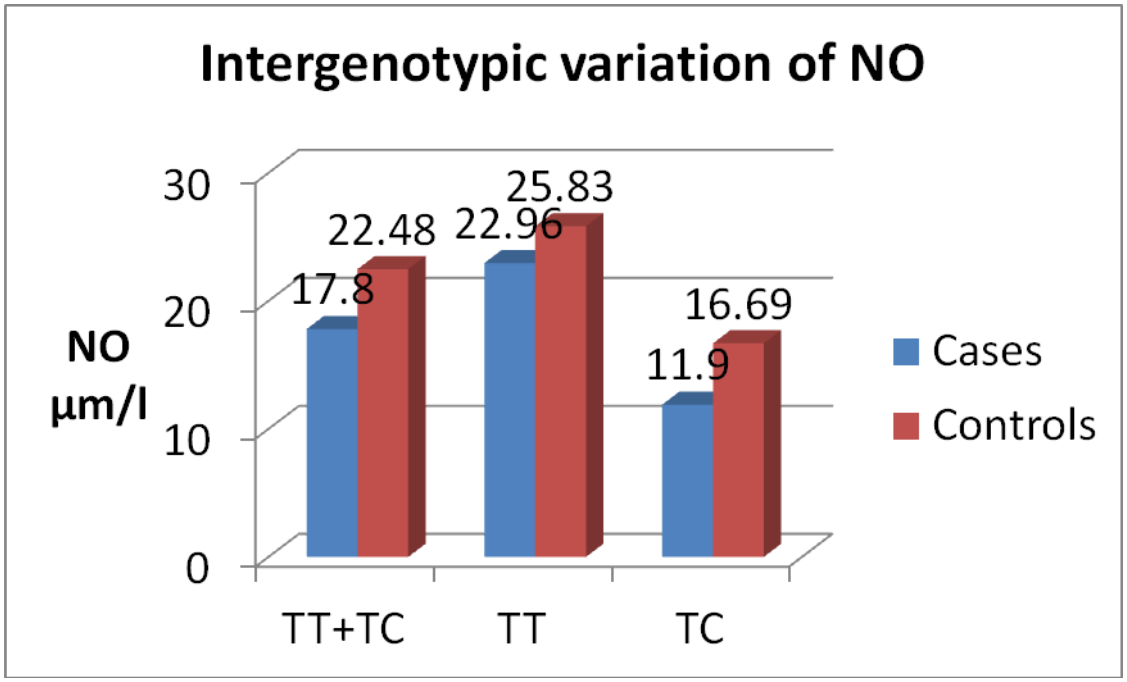
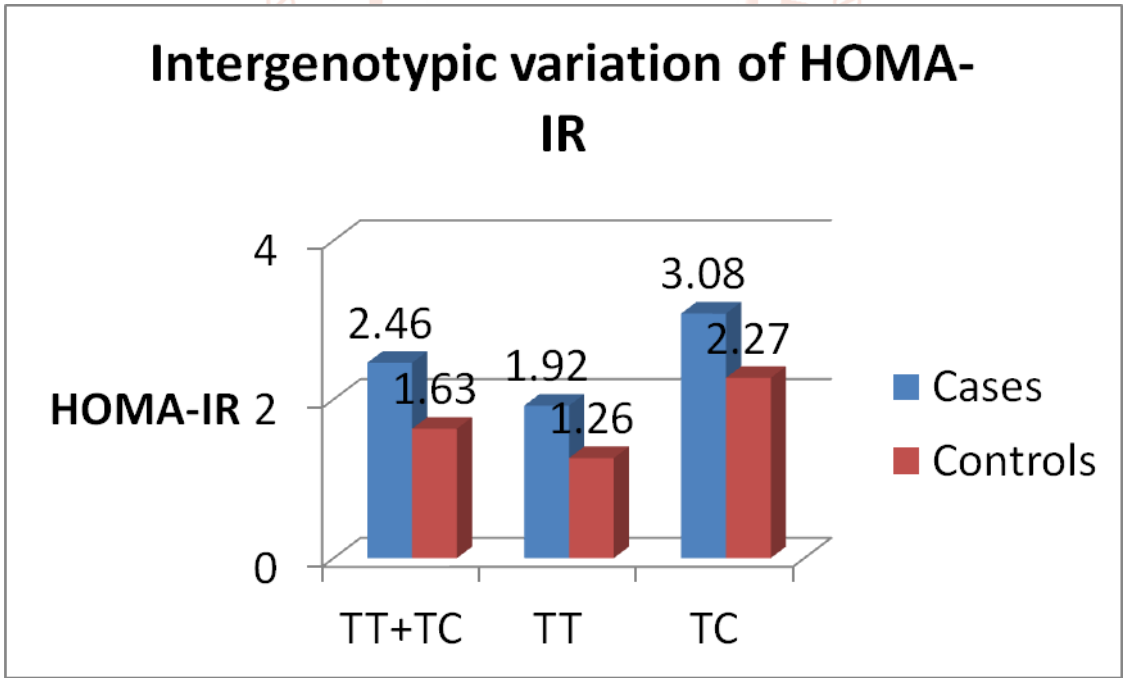


FIGURE 4: INTERGENOTYPIC VARIATION OF NO IN CASES AND CONTROLS

TABLE 2: INTERGENOTYPIC VARIATION OF HOMA-IR IN STUDY AND CONTROL GROUP

Genotype	HOMA-IR						
	Cases (n=60)			Controls (n=60)			
	N	Mean	SEM	N	Mean	SEM	p value
TT+TC	60	2.46	0.15	60	1.63	0.09	0.0001
TT	32	1.92	0.09	38	1.26	0.07	0.0001
TC	28	3.08	0.26	22	2.27	0.12	0.0137

*p≤0.000 is statistically very significant



*p≤0.000 is statistically very significant

FIGURE 5: INTERGENOTYPIC VARIATION OF NO IN CASES AND CONTROLS

FIGURE 6: CORRELATION BETWEEN HOMA-IR WITH NO

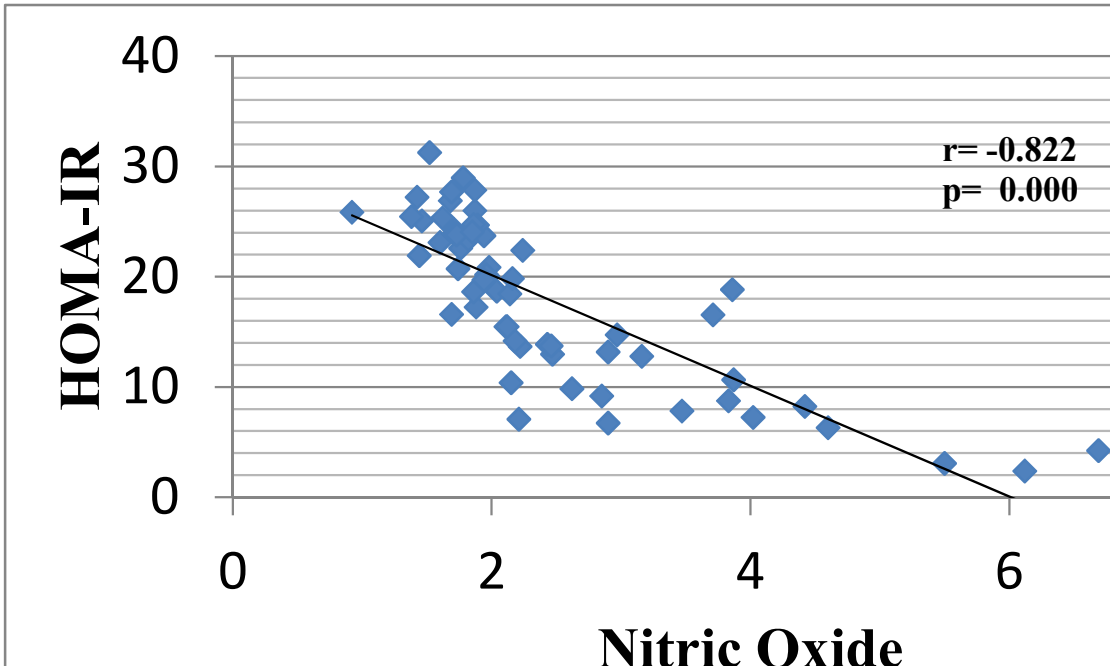


TABLE 3:Chi-square for CAD*HOMA-IR

	HOMA-IR		Total
	≤ 2.000	≥ 2.001	
Contrls	43	17	60
Cases	30	30	60
Total	73	47	120

X^2=5.911 p=0.015 Odds ratio =2.52

TABLE 4: Chi square test for CAD * Nitric Oxide Crosstabulation

	Nitric Oxide		Total
	TT	TC	
Control	52	8	60
Cases	39	21	60
Total	91	29	120

X^2=7.685 p=0.006 Odds Ratio=3.5

DISCUSSION

We found a negative correlation between Nitric oxide and HOMA-IR with Pearson's coefficient r= -0.791 and R^2=0.626 which was statistically very significant p=0.000.

A link between insulin homeostasis and endothelial function has been long documented. Endothelial function is modulated by insulin through the stimulatory effects of the hormone on NO production. Vecoli et al [16] showed a link between a genetic endothelial dysfunction and abnormalities in glycometabolic profile in patients with both ischemic and non-ischemic cardiomyopathy, thus confirming the impact of a defective eNOS(T−786→C) gene in the development of insulin resistance and heart failure. Shah et al[17] demonstrated that 27VNTR (a/b) eNOS polymorphism carrying 'a' allele alone and in association with T-786C and G894T eNOS polymorphism is associated with increased risk of Diabetic nephropathy in Asian Indians Type 2 diabetic patients.

We determined eNOS (T-786C) gene variants in both the groups. TT genotype was found in 32 subjects of the study group (53.33%) and in 38 subjects of the control group (63.33%), whereas the TC genotype was found in 28 subjects of the study group (46.66%) and in 22 subjects of the control group (36.66%) respectively. No CC genotype was found in any group. The genotype distribution was in accordance with Hardy Weinberg equilibrium.[18-19]

The frequency of T allele was 76.67% in the study group and 81.67% in the control group while the frequency of C allele was 23.33% in the study group and 18.33% in the control group.

Yoshimura et al[20-22] demonstrated the relationship between insulin resistance and polymorphisms of the endothelial nitric oxide synthase gene in patients with coronary artery disease. They showed that insulin sensitivity is not affected by eNOS polymorphisms directly, but through changes in endothelial function and eNOS production in skeletal muscle.

Nakayama et al[23-24] reported that T-786C mutation is associated with coronary spasm and acute myocardial infarction in the absence of coronary artery stenosis. These findings were also supported by Alirezaet al[27] in a study on Iranian population which showed that low levels of NO and increased frequency of T-786C polymorphism might be a risk factor in progression of coronary artery disease in the studied subjects. A metaanalysis performed over 26 studies involving 23028 subjects reported lack of influence of T-786C variant on ischaemic heart disease (IHD) risk, but a very small effect of the variant cannot be excluded, since they found only a 73% power to detect an OR of 1.2 at a significance level of 5%. Gommaet al have reported T-786C polymorphism to be associated with coronary in-stent restenosis in patients with CAD. In North Indian population, Arunet al[25] demonstrated that T-786C CC genotype is associated with risk of hypertension. Han and his colleagues showed that T-786C is associated with increased risk of CAD in Chinese population[26]. Colombo et al[27] provided evidence that the Glu(298)-->Asp and T-786C polymorphisms of the eNOS gene are associated with the presence and severity of angiographically defined CAD in the Italian population and that those individuals carrying both eNOS variants simultaneously might have a higher risk of developing CAD.

CONCLUSIONS

It was observed that intergenotypic levels of nitric oxide were significantly lower in TC genotype than TT both in study as well as control groups. Also, TC genotype showed significantly higher HOMA-IR levels as compared to TT genotype in both study as well as control group.Moreover, CC genotype could not be found in both cases and control groups. This may be due to smaller sample size or low prevalence of CC genotype in North Indian population.Our findings suggest that TT genotype may have a protective role in development of insulin resistance and coronary artery disease as seen by high Nitric Oxide levels and low HOMA-IR levels in TT genotype than TC genotypes in both case as well as control groups.

SUGGESTIONS:

Worldwide prevalence of CAD is increasing and INDIA is set to become the country with highest cases of CAD, We suggest earlier identification of even one C allele in the genotype and measurement of Nitric oxide and HOMA-IR levels may be helpful in finding the people at risk of CAD. Further workup for CAD may be suggested to these patients to bring down the burden of morbidity and mortality due to CAD.

These are preliminary findings, requiring further analysis with a larger sample size. Further research can be done to measure activity of endothelial nitric oxide synthase activity. This study could be extended to other genes related to insulin resistance and/or coronary artery disease to conclusively determine exact genetic factors leading to CAD.

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