

Interaction of iNOS Gene (C150T) Polymorphism and Endothelial Dysfunction in Pathophysiology of Metabolic Syndrome

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Objective: To study the endothelial dysfunction by measuring Nitric Oxide and Endothelin-1, and inter-genotypic variation of inducible Nitric Oxide Synthase gene (C150T) polymorphism among the study subjects.

Methods: 50 diagnosed cases of metabolic syndrome as per International Diabetes Federation (IDF) criteria and 50 healthy volunteers as control were enrolled. Nitric Oxide, Endothelin were measured and PCR-RFLP was done to identify the iNOS gene C150T polymorphism and its effect on serum nitric oxide levels.

Results: Subjects with metabolic syndrome had lower NO levels (16.3 ± 10.3 vs 20.9 ± 11 μ M, $p = 0.032$) and higher endothelin (2.68 ± 1.73 vs 1.98 ± 0.82 fmol/ml, $p = 0.011$). The frequency of mutant T allele (10% vs 9%) was higher in cases. Serum nitric oxide levels were lower in cases expressing the Mutant T allele as compared to wild type C allele. However, the differences were not statistically significant.

Conclusions: The present study demonstrated that iNOS C150T polymorphism did not show significant association with metabolic syndrome. Serum nitric oxide levels could be influenced by factors other than genetic polymorphism of iNOS gene (C150T) which cause endothelial dysfunction in metabolic syndrome and associated co-morbidities.

Key words: Metabolic Syndrome, Endothelial dysfunction, iNOS gene (C150T) polymorphism, Nitric oxide, Endothelin-1.

INTRODUCTION

Metabolic Syndrome (Met S) consists of groups of metabolic abnormalities such as central obesity, dyslipidemia, hypertension, insulin resistance, proinflammatory and prothrombotic state, which increases the risk of diabetes mellitus (DM) and cardiovascular disease (CAD) [1].

Prevalence of Met S increases rapidly worldwide due to changing life style and unidentified environmental factor and genetic factor. It has been estimated that approximately 1 out of 4 adults has Met S. Its prevalence varies from 8 to 24.2% in males and from 7 to 46.5% in females. In India, its prevalence varies from 24.9% in Northern India to 41% in Southern India [2].

In various studies, it has been reported that endothelial dysfunction is one of the key components in development of Met S. Endothelial dysfunction is defined as decrease in vasodilatory substances like nitric oxide (NO) and increase in vasoconstrictive substances such as Endothelin-1 (ET-1) [3]. Other causes of endothelial dysfunction in Met S are increase in pro-inflammatory and pro thrombotic mediators like various cytokines, interleukins, Plasminogen Activator Inhibitor-1 (PAI-

1) and decrease in protective adipokines such as adiponectin [2].

NO is the most important vasodilator substance produced by endothelial cells. NO is produced by conversion of L-arginine to L-citrulline by Nitric Oxide Synthase (NOS). There are three isoenzymes of NOS: NOS I or neuronal NOS, NOS II or inducible NOS, which is induced by proinflammatory cytokines and NOS III or endothelial NOS. In case of inflammatory-conditions, activation of a Ca²⁺- independent inducible NOS (iNOS) enzyme occurs, resulting in NO production over longer time periods and in larger quantities, which may have both cytotoxic and cytoprotective effects [4].

Various genetic variations such as Single Nucleotide Polymorphism (SNP) may affect the iNOS gene expression and thus may play an important role in pathogenesis of endothelial dysfunction in Met S. The iNOS gene has SNP in both regulatory and coding region. SNP in promoter region might affect the level of gene product, while SNP in coding region might affect the enzymatic activity [5].

Several types of polymorphisms have been identified in the promoter region of the iNOS gene: G to C

at -954, C to T at -1173, and tandem repeat number polymorphism of (TAAA)_n and (CCTTT)_n [4, 6]. A report by Johannesen [7] showed that C/T polymorphism in exon 16 was one of the most frequent SNP among 10 polymorphisms of human iNOS gene identified in a Danish population and was linked to Type 1 Diabetes Mellitus. In terms of functional importance, the deletion mutants retained maximal NO activity at lower concentrations of free Ca²⁺ compared with the wild-type. Identified C/T polymorphism in E16 of iNOS was located at the N-terminal of six amino acids from the deletion reported by Daff *et al.*, and the amino acid change in exon 16 might be of functional interest [7]. Although iNOS could be involved in Met S, its exact role in the pathogenesis of the disease is still unknown. So an attempt has been made to find the association of C150T SNP in iNOS gene, leading to an amino acid substitution ser608leu, in Met S [9, 10].

The present study was conducted to evaluate the endothelial dysfunction through determination of serum levels of NO and ET-1 in subjects with Met S. The genetic influence of SNP of iNOS gene on endothelial dysfunction was further evaluated.

MATERIAL AND METHODS

Study Design:

The study was conducted in the Department of Biochemistry in collaboration with the Department of Medicine, Lady Hardinge Medical College & Smt. Sucheta Kriplani Hospital, New Delhi from period of 2012 to 2015. The study was approved by the ethical committee of LHMC, New Delhi. We enrolled 50 cases of Met S and 50 age and sex matched healthy controls.

Inclusion Criteria:

The diagnosed case of Met S as per IDF (International Diabetic Federation) criteria [11]. The IDF Guidelines are as follows: Central Obesity (Waist circumference: ≥ 90 cm for Males and ≥ 80 cm for Females). Plus any two or more of the following: 1. Hypertriglyceridemia-Fasting Triglycerides ≥ 150 mg/dl or specific medication, 2. Low HDL cholesterol: ≤ 40 mg/dl (Males), and ≤ 50 mg/dl (Females), or specific medication, 3. Hypertension: Blood pressure ≥ 130 mmHg Systolic or ≥ 85 mmHg Diastolic or previous diagnosis or specific medication and 4. Fasting plasma glucose ≥ 100 mg/dl or specific medication or previously diagnosed type 2 diabetes. If BMI is > 30 kg/m², central obesity can be assumed and waist circumference does not need to be measured.

Exclusion Criteria:

Subjects with hepatic disease, renal disease, other endocrine diseases, alcoholism, infectious diseases or receiving any medication were excluded.

Physical and Clinical Examination:

Bilingual informed written consent was taken from the patients. Detailed clinical history with special reference to metabolic syndrome and thorough clinical examination of patient was conducted. Necessary anthropometric measurements were taken which include height, weight, waist circumference, hip circumference, body mass index and waist hip ratio.

Sample Collection:

Six millilitres of venous blood sample was collected from the subjects under sterile conditions after overnight fasting. The blood samples were processed immediately for separation of plasma and serum. Serum samples were stored in aliquots at -40°C for NO and Endothelin-1 estimations. Whole blood for DNA analysis was stored at -40 degree Celsius until DNA was isolated.

Lab Investigations:

For routine haematological and biochemical investigations, samples were immediately analysed by using automated clinical chemistry analyzer (Beckman Coulter systems; CX series) by standard kits and reagents. Electrolytes were measured by ISE method using Roche AVL 1980. Repeated freeze-thaw cycles were avoided. NO in plasma was determined indirectly by the measurement of its stable decomposition product nitrite (NO₂-), employing the modified Griess method by Mathew *et al.* [12].

Serum ET-1 was determined using the commercially available, human ET-1 Enzyme Immuno Assay kit by BIOMEDICA GRUPPE ELISA kit (Vienna, Divischgasse, Austria).

The study of iNOS gene (C150T) polymorphism was done by extracting DNA from whole blood by spin column based QIAGEN DNA extraction kit from Germany. A polymerase chain reaction (PCR) was carried out to identify the genotypes of iNOS SNP using the following primer pairs;

(Sense): 5'-TGTAACCAACTTCCGTGGTG-3' and
(Anti-Sense): 5'GTCTCTGCGGGTCTGAGAG-3'

(Primers were supplied by Biochrom International, GeNei™).

Amplification consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C , 30 seconds at 60°C and 30 seconds at 72°C , then a final extension at 72°C for 5 minute. Amplified products (288bp) were run on 2% agarose check gel. The Cytosine (C) to Thymine (T) transversion produces a recognition site for *Tsp509I* restriction enzyme, so the resulting 288-bp amplification product was digested with 1U of fastdigest *Tsp509I* restriction enzyme/10 μ l of PCR product for 10 minutes at 65°C .

The products were then run on a 3% agarose gel. Restriction Fragment Length Polymorphism (RFLP) was identified with ethidium bromide staining. Homozygous wild-type individuals (CC) showed 113 bp and 175 bp fragments, heterozygous mutant individuals (CT) showed three bands: 113 bp, 142 bp and 175 bp.

STATISTICAL ANALYSIS

All statistical analyses were performed with the SPSS software programme version 20. For comparing the mean of various variables, we used unpaired 2-tailed student's t-test. Pearson's correlation was used to find the association among various study variables. Frequencies of genotypes were compared with chi-square tests. A $p \leq 0.05$ was considered statistically significant.

Table 1 Demographic and anthropometric parameters of study population

Parameters	Cases (n = 50) (mean ± SD)	Control (n = 50) (mean ± SD)	<i>p</i> value
Height (meters)	1.56 ± 0.08	1.64 ± 0.11	0.000*
Weight (Kg)	68.9 ± 13.5	68.1 ± 13.3	0.755
BMI (Kg/m ²)	28 ± 5.4	25 ± 4.4	0.002*
Waist Circumference (cm)	99 ± 8.9	92 ± 8.9	0.001*
Hip Circumference (cm)	103.8 ± 12.4	99 ± 8.5	0.041*
Waist Hip Ratio	0.96 ± 0.09	0.93 ± 0.04	0.025*
Systolic BP	132 ± 12	122 ± 9	0.000*
Diastolic BP	86 ± 8	81 ± 5	0.001*
Pulse Rate	82 ± 8.9	77 ± 6.2	0.003*

**p* value ≤ 0.05 is considered statistically significant.

Table 2 Biochemical profile of study population

Parameters	Cases (n = 50) (mean ± SD)	Controls (n = 50) (mean ± SD)	<i>p</i> value
Total Cholesterol (mg/dl)	240.7 ± 91.5	246.5 ± 87	0.781
Triglyceride (mg/dl)	182.7 ± 112	141.5 ± 64.8	0.026*
HDL (mg/dl)	38.8 ± 10	49.2 ± 11	0.00*
LDL (mg/dl)	36.5 ± 22.4	28.3 ± 13	0.026*
VLDL	38.6 ± 10	49.1 ± 11.2	0.023*
FPG (mg/dl)	181.3 ± 70.9	96.3 ± 7.5	0.000*
PPBG (mg/dl)	232.4 ± 94	140.74 ± 142	0.000*

**p* value ≤ 0.05 is considered statistically significant.

Table 3 Special parameters of study population

Parameters	Cases (n = 50) (mean ± SD)	Controls (n = 50) (mean ± SD)	<i>p</i> value
Nitric Oxide (µM)	16.3 ± 10.3	20.9 ± 11	0.032*
Endothelin (fmol/ml)	2.68 ± 1.73	1.98 ± 0.82	0.011*

**p* value ≤ 0.05 is considered statistically significant.

RESULTS

The demographic, anthropometric profile, lipid and Glycemic profile are shown in Tables 1 and 2.

The mean plasma NO levels were significantly lower in Met S group (16.3 ± 10.3 µM) as compared to controls (20.9 ± 11 µM) which may suggest endothelial dysfunction in Met S. The mean plasma ET-1 levels were significantly higher in study group (2.68 ± 1.73 fmol/ml) than controls (1.98 ± 0.82 fmol/ml) (Table 3).

Homozygous wild type CC genotype was found in 40 subjects (80%) of the study group and 41 subjects (82%) of the control group. Heterozygous mutant type CT genotype was found in 10 subjects (20%) of the study group and 9 subjects (18%) of the control group.

No homozygous mutant TT was found in any group probably due to smaller sample size. The frequency of C allele (91%) was higher in control group and T allele (10%) was higher in study group. The difference was not statistically significant as shown in Table 4. Intergenotypic variations of plasma NO levels were analysed in cases and controls as shown in Table. 5, 6 and 7.

The mean plasma NO levels of wild type CC genotype in the Met S group was 16.2 ± 10.1 µM while in the control group was 20.3 ± 10.8 µM. However the difference between the two was not statistically significant as shown in Table 5. The mean plasma NO levels of mutant type CT genotype in the Met S group was 12 ± 11.2 µM while in the control group was 23.8

Table 4 Distribution of genotype of ser608leu polymorphism in study and control group

Genotype	Case (N = 50)		Control (N = 50)		Level of Significance
	N	Frequency %	N	Frequency %	
CC	40	80	41	82	P value = 0.799
CT	10	20	9	18	
TT	0	-	0	-	

Table 5 Intergenotypic variation of plasma NO level in study and control group

Genotype	Levels of NO (μM)						
	Cases (n = 50)			Controls (n = 50)			P value
	N	Mean	SD	N	Mean	SD	
CC + CT	50	16.3	10.3	50	20.9	11	0.032*
CC	40	16	10.1	41	20.3	10.8	0.068
CT	10	12.4	11.2	9	23.8	12.2	0.321

*p value \leq 0.05 is considered statistically significant.

\pm 12.2 μM as shown in table 5. The difference between the two was also not statistically significant. This finding showed that NO levels were lower in Mutant CT genotype in cases as compared to control group. As shown in Table 6, the levels of NO were lower in mutant CT genotype of cases ($12 \pm 11.2 \mu\text{M}$) as compared to wild type CC genotype in cases which suggested that T allele in cases might be associated with decrease production of NO, however the difference was not statistically significant. We found that serum ET-1 was significantly correlated with Diastolic Blood pressure ($r = 0.286$, $p = 0.044$).

DISCUSSION

The present study was designed to elucidate the role of NO, ET-1 and their relation along with the intergenotypic variations of mean serum NO levels in iNOS (C150T) gene polymorphism pertaining to endothelial dysfunction in Met S. In the present study, significant low levels of mean serum NO and higher levels of ET-1 were observed in Met S cases as compare to healthy control. However the intergenotypic variations of NO levels in iNOS (C150T) gene polymorphism did not show significant difference, which could be due to various known and unknown factors other than iNOS (C150T) gene polymorphism, in causing endothelial dysfunction in Met S cases.

Met S is responsible for the global epidemics of type 2 diabetes [13] and cardiovascular disease. The higher prevalence of Met S in India suggests the possible role of non-conventional risk factors in its etiopathogenesis as the conventional risk factors cannot account for all the cases in the Indian population. There is also the possibility of genetic predisposition to Met S among Indians.

The present study was undertaken to evaluate the role of novel biomarkers along with the frequency of iNOS ser608leu polymorphism as predisposing factors for endothelial dysfunction in Metabolic Syndrome. The endothelial dysfunction is a key component of

Table 6 Intergenotypic variation of plasma NO level in cases of Metabolic Syndrome

Genotype	N	Mean (μM)	SD	p Value
CC	40	16.2	10	0.301
CT	10	12.4	11.2	

Table 7 Intergenotypic variation of plasma NO level in control group

Genotype	N	Mean (μM)	SD	p Value
CC	41	20.4	10.7	0.392
CT	9	23.9	12	

metabolic syndrome as observed by Deedwania *et al.* [14]. The important mediators of endothelial function are NO and ET-1 among others. In our study we observed significant difference between the mean serum NO and ET-1 levels in cases and controls. This decrease in nitric oxide and increase in ET-1 may cause endothelial dysfunction and contribute to development of metabolic syndrome. This decrease in NO levels could be due to a number of factors like decrease in eNOS production, the inhibition of the co-factors necessary for eNOS synthesis, inactivation of NO by reactive oxygen species (ROS) or genetic influence [15]. Previously we have studied serum NO and Endothelin-1 levels in Met S [3].

iNOS is one of the three isoenzymes of NOS, which produces NO under inflammatory conditions [16, 17]. iNOS gene first described in macrophages, has been detected in virtually every cell type, including endothelial cells, vascular smooth muscle cells, fibroblasts, and cardiac myocytes and the NO that it produces can perform both beneficial and detrimental actions. On the one hand, it can eliminate infiltrating microorganisms, reduce thrombosis, and improve blood supply to injured tissues. In contrast, excess production of NO can cause tissue damage and contribute to the development of a wide spectrum of diseases [18].

The iNOS gene is estimated to be 37 kb long and consists of 26 exons which vary in length from 51 to 586 bp and 25 introns which range in size from approximately 100 bp to 6 kb [18, 19]. The iNOS gene is under the transcriptional control of a number of inflammatory mediators, including cytokines and lipopolysaccharide. In the 5'-flanking region of the iNOS gene multiple consensus sequences for the binding of transcription factors that mediate responsiveness to cytokines have been identified, including those for nuclear factor (NF)- κ B, IFN- γ , NF-1, IL-6 [18].

iNOS expression occurs during a variety of infectious diseases [20], as well as in many autoimmune and chronically inflammatory diseases like rheumatoid

arthritis [21], asthma [22], diabetes mellitus and a number of other diseases. iNOS gene expression has been reported to be affected by various gene polymorphisms [5]. Several types of polymorphisms have been identified in iNOS gene. G Kumaramanickavel *et al.* [23] studied pentanucleotide allelic polymorphism of the inducible nitric oxide synthase (iNOS) gene in association with retinopathy in Asian Indians. iNOS (C150T) gene polymorphism was found to be associated with cigarette- and alcohol- induced gastric cancer in Chinese population [24], and with *H. pylori* associated gastric cancer in Japanese population [25]. The mutation has been studied for pre-eclampsia although it did not show any linkage [26].

In a recent Indian study done by Hazam *et al.* showed that iNOS (C150T) gene polymorphism was associated with increased risk of increased risk of Hepatitis E Virus related acute hepatitis and liver failure [27]. Bhardwaj *et al.* also studied same polymorphism in Ischemic Heart disease (IHD) patients and found that T allele (mutant allele) might be the susceptibility allele for IHD in Indian population [10].

iNOS gene expression studies has been done in Type 1 Diabetes Mellitus by Johannasen *et al.* [7] in Danish Population suggesting that iNOS polymorphism may influence insulin resistance. The role of Ser608Leu polymorphism in diabetes mellitus and its complications has been reported. As discussed, we have proven the role of NO in Met S associated endothelial dysfunction. We evaluated the role of C150T polymorphism leading to amino acid change ser608leu in iNOS, to further explain the molecular mechanism behind the endothelial dysfunction in Met S. This C to T substitution in the iNOS gene, might leads to decrease in iNOS expression and hence decreased NO levels. Hence, it is reasonable to assume that human iNOS gene may be an important candidate gene for the development of metabolic syndrome. To our best of knowledge, this study was the first one in India to examine the significance of C/T iNOS gene polymorphism in Met S.

Distribution of genotype of iNOS (C150T) gene polymorphism in our study population shows that wild type CC and mutant CT genotype does not differ significantly in Met S cases and healthy controls. Our control iNOS genotype frequency is coincident with the results presented by Shen *et al.* [21] which represents the genotype frequency in Asia. The frequency of C allele was 90% in Met S group and 91% in control group.

Our study population was in Hardy Weinberg equilibrium. Our study results coincide with the study done by others [25]. The non significant difference in the intergenotypic variation of serum NO levels with iNOS (C150T) gene polymorphism shows that development of endothelial dysfunction in Met S may be a multifactorial phenomena and not just related to iNOS (C150T) gene polymorphism and other known risk factors.

We also observed that mutant CT genotype had lower NO levels than wild type CC genotype in cases, though the difference was not statistically significant. This difference was less evident in controls suggesting the influence of yet unidentified factors on gene expression.

CONCLUSION

Our study highlights the role of endothelial dysfunction in the pathogenesis of metabolic syndrome. The causative influence of iNOS (C150T) gene polymorphism could not be established in our study. The smaller sample size, genetic variability of South-Asian population and the influence of yet unidentified but nonetheless confounding factors on the molecular mechanisms involved in metabolic syndrome could be the possible explanation behind this negative association. It may be interpreted that there are certain factors other than genetic predisposition in the form of iNOS (C150T) gene polymorphism, which are responsible for endothelial dysfunction in Met S. In addition, this result should be interpreted cautiously.

It is imperative to validate the findings through well designed large scale studies that can interpret our findings with considerable statistical significance.

CONFLICT OF INTEREST

No conflict of interest to declare.

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