

ORIGINAL ARTICLE

ASSOCIATION OF GLUTATHIONE-S-TRANSFERASE (*GSTM1* and *GSTT1*) AND *FTO* GENE POLYMORPHISMS WITH TYPE 2 DIABETES MELLITUS CASES IN NORTHERN INDIARaza ST^{1,2*}, Abbas S¹, Ahmad A¹, Ahmed F¹, Zaidi ZH³, Mahdi F¹

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is growing in an epidemic manner across the world and India has the world's largest number of diabetic subjects. The present study was carried out to investigate the association of glutathione-S-transferase (*GSTM1*, *GSTT1*) and fat mass and obesity associated (*FTO*) gene polymorphisms with T2DM patients and controls, and its role in increasing the susceptibility to T2DM. A total of 198 subjects (101 T2DM patients and 97 controls) participated in this study. *GSTM1*, *GSTT1* and *FTO* gene polymorphisms in the patients and controls were evaluated by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). We observed significant association of *GSTM1* positive ($p = 0.046$) and *GSTM1* null ($p = 0.046$) genotypes with T2DM, while no significant association was found with the *FTO* gene polymorphism in our study. It seems that the *GSTM1* gene polymorphism can be a predictive marker for early identification of a population at risk of T2DM. The potential role of *GST* and *FTO* gene polymor-

phisms as a marker of susceptibility to T2DM needs further studies in a larger number of patients.

Keywords: Glutathione-S-transferase (*GSTM1*, *GSTT1*); Fat mass and obesity associated (*FTO*) gene; Gene polymorphisms; Type 2 diabetes mellitus (T2DM)

INTRODUCTION

Diabetes has become a common global health problem that affects >170 million people worldwide. It is one of the leading causes of death and disability. It is estimated that by 2030, the number will rise to 366 million (www.who.int). According to Wild *et al.* [1] the 'top' three countries in terms of the number of individuals with type 2 diabetes mellitus (T2DM) are India (31.7 million in 2000; 79.4 million in 2030), China (20.8 million in 2000; 42.3 million in 2030); and the US (17.7 million in 2000; 30.3 million in 2030). Clearly, T2DM has become an epidemic in the 21st century with India leading the world with the largest number of diabetic subjects. Type 2 diabetes mellitus is a non autoimmune, complex, heterogeneous and polygenic metabolic disease in which the body fails to produce enough insulin, characterized by abnormal glucose homeostasis [2]. Its pathogenesis appears to involve complex interactions between genetic and environmental factors [2]. Identification of the genetic components of T2DM is the most important area of diabetes re-

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search because elucidation of the diabetes genes (alleles) will influence all efforts toward a mechanistic understanding of the disease, its complications, and its treatment, cure, and prevention [3].

Researchers have identified a number of genetic factors that may be responsible for selected or more general diabetic patients. It has also been reported that defects in antioxidant defense against oxidative stress play an important role in the etiology of diabetic complications [4,5]. Glutathione (GSH) is the major cellular antioxidant that protects against environmental toxicants as well as reactive oxygen species (ROS) mediated cell injury. Glutathione detoxifies ROS, reduces peroxides and detoxifies multiple compounds through glutathione-S-transferase (GST) conjugation [6]. Three of the *GST* genes, *GSTM1*, *GSTT1* and *GSTP1*, have been found to have functional polymorphisms that are frequently present in the general population. There are several studies that reported significant association to T2DM for both null genotypes of GST [7,8] and others that verified no association between *GSTT1* and *GSTM1* polymorphisms and T2DM [9,10]. Some studies indicated that genetic variations of the *GSTT1* enzyme are associated with the development of end-stage renal disease in diabetes mellitus patients [11]. Recently, part of a genome-wide association study found that several single nucleotide polymorphisms (SNPs) of the fat mass and obesity associated (*FTO*) gene were strongly associated with obesity and T2DM [12,13]. The *FTO* gene, which is located on chromosome 16q12.2, and has nine exons and emerged 450 million years ago [14]. The *FTO* gene is mainly expressed in the hypothalamus and encodes a 2-oxoglutarate-dependent nucleic acid demethylase. It may play an important role in the management of energy homeostasis [14,15], nucleic acid demethylation, and the regulation of body fat masses by lipolysis [16]. A recent meta-analysis [17] in East and South Asians, concluded that the *FTO* minor allele (or a proxy), the risk allele for obesity also increased the risk of T2DM. Similar results were reported in a Scandinavian population [18]. Currently, adequate data is not available dealing with *GSTT1*, *GSTM1* and *FTO* gene polymorphisms and its susceptibility with T2DM patients among north Indian population; thus, we conceived the need for further studies on *GSTT1*, *GSTM1* and *FTO* gene polymorphisms and its association with T2DM in north Indian population.

MATERIALS AND METHODS

Patient Selection. A total of 101 blood samples from T2DM patients and 97 healthy controls were collected at the Diabetic Clinic, Department of Medicine of Era's Lucknow Medical College & Hospital, Lucknow, Uttar Pradesh, India. Written informed consent was obtained from all participants. Data collection was done for each patient on clinical variables including age, alcohol consumption, body mass index (BMI), height, weight, cigarette smoking, family history, *etc.*, patients with overnight fasting plasma glucose of more than 126.0 mg/dL on two consecutive events were included in the T2DM category, while samples having a fasting blood glucose level below 110.0 mg/dL without a family history of diabetes were included in the study as controls. Patients with type 1 presentation, defined as diabetic ketoacidosis, acute presentation with heavy ketonuria (>3+), or uninterrupted requirement of insulin within 1 year of diagnosis, were excluded. Ethical Committee clearances were obtained from the respective departments, prior to the recruitment of subjects in this study.

Biochemical Estimations. The BMI was calculated according to Quetelet equation by using weight in kg, height in m². Fasting plasma glucose and random blood sugar (glucose oxidase-peroxidase method). Serum creatinine concentration was assessed by a kinetic Jaffe's method. Serum cholesterol (cholesterol oxidase-peroxidase), serum triglyceride (glycerol phosphate oxidase-peroxidase-amidopyrine method), high-density lipoprotein (HDL) cholesterol (immunoinhibition) were assessed by XL-300 Transasia Fully Auto Analyzer; Transasia, Mannheim, Germany. Low-density lipoprotein (LDL) cholesterol levels were calculated using the Friedewald formula [19]. Very low-density lipoprotein (VLDL) cholesterol was determined by an enzymatic method. Hb A_{1c} was measured using a semi auto analyzer (Transasia).

DNA Extraction. Peripheral blood (5 mL) was collected from all the subjects in 0.5 M vacutainers with EDTA as anticoagulant. Genomic DNA was isolated from whole blood using the standard phenol-chloroform extraction method [20]. The DNA concentration was determined by spectrophotometer and stored at -20 °C.

ANALYSIS OF POLYMORPHISMS

GSTM1 and *GSTT1* Gene Polymorphisms.

GSTM1 and *GSTT1* genetic polymorphisms were evaluated using a multiplex polymerase chain reaction (PCR) technique (MJ Mini Thermal Cycler; Bio-Rad Laboratories, Hercules, CA, USA). The PCR primers were synthesized according to Arand *et al.* [21]. Primers for the *GSTM1* gene were: 5'-GAA CTC CCT GAA AAG CTAA AGC-3' and 5'-GTT GGG CTC AAA TAT ACG GTG G-3' and for *GSTT1*: 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG GACAT GGC CAG CA-3'. The β -globin locus was used as an internal control to avoid false-negative readings. Primers for the β -globin locus were: 5'-CAA CTT CAT CCA CGT TCA CC-3' and 5'-GAA GAG CCA AGG ACA GGT AC-3'. The PCR reaction was carried out in a total volume of 25 μ L containing 10 pmol of each primer, 2.5 mmol/L of $MgCl_2$, 0.2 mmol/L of each deoxynucleotide triphosphate, 1 unit of Taq polymerase (Bioline Ltd., London, UK) and 100 ng of genomic DNA. Amplification was performed by initial denaturation at 94 °C for 5 min., followed by 30 cycles at 94 °C for 1 min., 64 °C for 1 min. and 72 °C for 1 min., and a final extension of 72 °C for 7 min. The amplified products were identified by electrophoresis in a 1.5% agarose gel and stained with 0.5 mg/mL (concentration) ethidium bromide. The product lengths were 215, 480 and 268 bp for *GSTM1*, *GSTT1* and β -globin locus, respectively.

***FTO* Gene Polymorphism.** The *FTO* SNP (rs993 9609) was genotyped by PCR (MJ Mini Thermal Cycler; Bio-Rad Laboratories) and restriction fragment length polymorphism (RFLP), analyses. Genomic DNA (20 ng) was incubated in a 10 μ L solution containing 1 $\times$ NH_4 buffer, 2.5 mmol/L magnesium, 200 mmol/L each dNTP, 20 pmol forward (5'-AAC TGG CTC TTG AAT GAA ATA GGATTC AGA-3') and reverse (5'-AGA GTAACA GAG ACT ATC CAA GTG CAG TAC-3') oligonucleotide primers [22], and 0.5 U Taq DNA polymerase (Bioline Ltd.). The PCR mix was denatured at 94 °C for 5 min., followed by 20 cycles of 45 seconds at 94 °C, 45 seconds at 61 °C (dropping 0.5 °C per cycle), and 45 seconds at 72 °C. After this, the PCR mix was incubated for 15 cycles at 45 seconds at 94 °C, 45 seconds at 51 °C, and 45 seconds at 72 °C, followed by a final extension at 72 °C for 10

min. This was then incubated at 37 °C for 16 hours with 2 U *ScaI* (New England Biolabs, Hitchin, Hertfordshire, UK). Upon running the final products on a 3.0% agarose gel, the T allele produced a 182 bp band and the A allele produced 154 and 28 bp bands.

Statistical Analyses. All the figures are presented as mean $\pm$ standard deviation (SD). The genotyping data were compared between patients and controls using the χ^2 test. Other variables were compared using the Student's *t*-test for normally-distributed

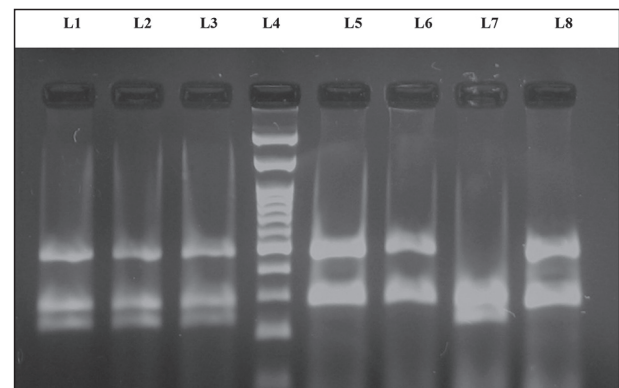


Figure 1. The PCR products analyzed on 2.0% agarose gel. The presence or absence (null) of the *GSTM1* and *GSTT1* genes was detected by the presence or absence of a band at 480 bp (corresponding to *GSTT1*) and a band at 210 bp (corresponding to *GSTM1*). β -Globin is considered as an internal control (260 bp). Lane 4: 100 bp ladder; lanes 1, 2 and 3: *GSTM1* and *GSTT1* wild type genotype; lanes 5, 6 and 8: *GSTM1* null genotype; lane 7: *GSTT1* null genotype.

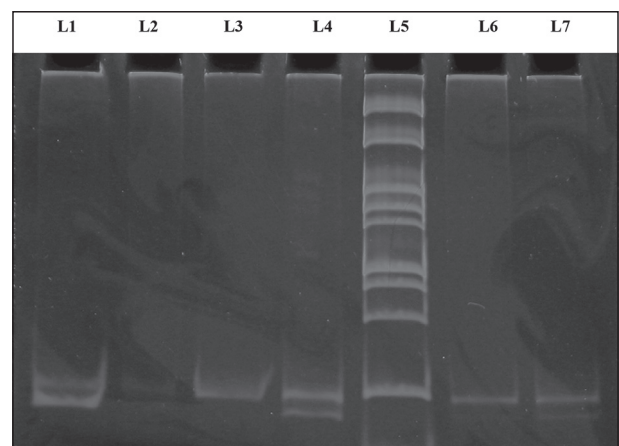


Figure 2. Polyacrylamide gel picture showing digested PCR products for the *FTO* gene polymorphism. Lanes 2, 3, 5 and 7 show the AT genotype (182 and 154 bp); lane 6: shows the TT genotype (182 bp); lane 4 shows the 100 bp ladder; lane 1 shows the undigested PCR product of the *FTO* (182 bp) gene polymorphism.

variables. All statistical tests were performed using the Statistical Package for the Social Sciences, version 12 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Our study included 101 T2DM patients (65 males and 36 females) and 97 controls (53 males and 44 females). The mean age of the T2DM patients and controls were 41.32 ± 11.39 years and 40.13 ± 10.28 years, respectively. The age of onset in T2DM patients was 44.05 ± 11.20 years. Clinical and biochemical profiles of the patients and controls are shown in Table 1. The mean BMI, blood pressure, random blood sugar (RBS), fasting blood sugar (FBS), Hb A_{1c}, total cholesterol, HDL cholesterol, VLDL cholesterol, triglyceride and serum creatinine levels in the diabetic patients were significantly higher than in the control subjects ($p < 0.001$). We have found the significant correlation between the RBS-Hb A_{1c} ($p < 0.001$), RBS-serum cholesterol ($p = 0.007$), RBS-triglyceride ($p = 0.049$), serum creatinine-Hb A_{1c} ($p = 0.002$), serum creatinine-triglyceride ($p < 0.001$), serum creatinine-VLDL ($p < 0.001$), serum cholesterol-triglyceride ($p = 0.001$), serum cholesterol-HDL ($p < 0.001$), HDL-VLDL ($p = 0.019$), VLDL-triglycer-

ide ($p < 0.001$). The frequency of *GSTM1* and *GSTT1* null genotypes was 38.61 and 7.92%, respectively, in T2DM patients, while it was 53.61 and 4.12%, respectively, in the controls. The frequency of positive *GSTM1* and *GSTT1* genotypes was 61.38 and 92.07%, respectively, in T2DM patients as compared to 46.39 and 95.87% in the controls. Odds ratio (OR) for *GSTM1* null genotypes was 0.544 [95% confidence interval (CI) 0.31-0.96, $p = 0.046$, $\chi^2 = 3.97$, power = 0.923, Bonferroni corrected $p = 0.092$], for the *GSTT1* null genotypes 2.0 OR (95% CI 0.58-6.87, $p = 0.405$, $\chi^2 = 0.694$, power = 0.869, Bonferroni corrected $p = 0.810$). The OR for the *GSTM1* positive genotypes was 1.837 (95% CI 1.04-3.23, $p = 0.046$, $\chi^2 = 3.97$, power = 0.923, Bonferroni corrected $p = 0.092$), for the *GSTT1* positive genotypes 0.5 OR (95% CI 0.15-1.72, $p = 0.405$, $\chi^2 = 0.694$, power = 0.869, Bonferroni corrected $p = 0.810$) and the *GSTM1* and *GSTT1* wild genotypes 1.569 (95% CI 0.89-2.75, $p = 0.115$, $\chi^2 = 2.49$, power = 0.918, Bonferroni corrected $p = 0.345$). The frequencies of the *FTO* genotypes AA, AT, TT in T2DM patients were 4.95, 71.28 and 23.76%, respectively, while in the control group these frequencies were 0.00, 75.25 and 24.74%, respectively. The frequency of the A and T alleles in T2DM patients was 40.59 and 59.40% as

Table 1. Clinical and biochemical parameters of type 2 diabetes mellitus patients and controls.

Parameters	T2DM Patients (n = 101)	Controls (n = 97)	p Values
Gender	65 males; 36 females	53 males; 44 females	0.072
Age (years)	41.32 ± 11.39	40.1 ± 10.28	0.12
BMI (kg/m ²)	26.01 ± 4.12	23.99 ± 2.36	<0.001
RBS (mg/dL)	195.44 ± 91.66	118.33 ± 18.55	<0.001
FBS (mg/dL)	190.81 ± 66.24	92.92 ± 14.03	<0.001
Systolic BP (mm Hg)	136.23 ± 14.21	119.96 ± 5.06	<0.001
Diastolic BP (mm Hg)	87.77 ± 6.77	78.22 ± 1.66	<0.001
Serum creatinine (mg/dL)	1.03 ± 0.24	0.88 ± 0.12	<0.001
Hb A _{1c} (%)	6.43 ± 1.54	5.61 ± 0.38	<0.001
Serum cholesterol (mg/dL)	194.92 ± 43.27	166.06 ± 20.52	<0.001
Triglyceride (mg/dL)	237.77 ± 147.44	147.55 ± 40.33	<0.001
HDL (mg/dL)	59.32 ± 12.22	57.33 ± 11.66	<0.001
LDL (mg/dL)	99.22 ± 34.32	92.66 ± 18.88	0.133
VLDL (mg/dL)	47.55 ± 28.22	32.55 ± 10.22	<0.001

For finding p values, the Student's unpaired t -test was applied except in the case of gender comparison where the χ^2 test was applied. BMI: body mass index; RBS: random blood sugar; FBS: fasting blood sugar; BP: blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; VLDL: very low-density lipoprotein.

compared to 37.62 and 62.37% in the controls. The OR for AA was N/A(0) (95% CI NA, $p = 0.077$, $\chi^2 = 3.12$, power = 0.989, Bonferroni corrected $p = 0.231$), for AT 0.816 (95% CI 0.43-1.53, $p = 0.528$, $\chi^2 = 0.40$, power = 0.782, Bonferroni corrected $p = 1.000$), and for TT 0.948 (95% CI 0.50-1.82, $p = 0.872$, $\chi^2 = 0.03$, power = 0.509, Bonferroni corrected $p = 0.509$). The genotype, allele frequencies of the *GSTM1*, *GSTT1* and *FTO* genes and statistical analysis of the patients and controls are also shown in Table 2.

DISCUSSION

GSTM1 and *GSTT1* Gene Polymorphisms.

Type 2 diabetes mellitus is characterized as being a polygenic disorder and generally thought of as a syndrome, rather than a single specific entity. Oxidative stress plays a major role in the pathogenesis of T2DM [23]. Glutathione-S-transferases catalyze the conjugation of glutathione to a wide range of electrophiles and represent a protective mechanism against oxidative stress. The GST family of genes is critical in the protection of cells from ROS because they utilize as substrates a wide variety of products of oxidative stress [24]. The deletion polymorphisms of *GSTT1* and *GSTM1*, which are associated with abolished enzyme activity [25], have been associated with T2DM

when compared to control subjects [9,24]. The majority of case control studies of *GSTM1* and *GSTT1* null genotypes have reported risk associations with disease [26,27]. The frequency of the *GSTM1* null genotype in our study was 38.61%; it is little lower than reported in other populations (Egyptian 58.62%, Dubai 57.5%, North Indian 54.00%) [28-30]. The frequency of the *GSTT1* null genotype was 7.92% in T2DM patients, which is lower when compared to the Dubai (60.0%) and Egyptian (35.0%) populations [7,29]. We have also observed that the genotype frequency of the positive *GSTT1* and *GSTM1* genes was 92.07 and 61.38%, respectively, which is significantly higher in comparison with Dubai where the positive *GSTT1* and *GSTM1* genes was 40.0 and 42.5%, respectively [29].

The *FTO* Gene Polymorphism. The frequency of the *FTO* gene polymorphism is quite variable in different geographic and ethnic groups (Table 3). Previous studies have shown that T2DM risk associated with the A allele of the *FTO* variant (rs 9939609) was strongly mediated by BMI, and has been shown to potentially affect mass rather than height [31]. The *FTO* gene (rs 9939609) was shown to be strongly associated with T2DM risk (OR 1.27; $p = 5 \times 10^{-8}$) in a genome wide association (GWA) scan performed in the UK population [31]. In our

Table 2. Genotype and allele frequencies of the *GSTT1*, *GSTM1* and *FTO* gene polymorphisms in type 2 diabetes mellitus patients and controls.

	<i>n</i>	Frequency (%)	<i>n</i>	Frequency (%)	OR	95% CI	χ^2	<i>p</i> Value	Bonferroni Corrected <i>p</i> Value	Power
<i>GSTM1</i> positive	45	46.39	62	61.38	1.837	1.04-3.23	3.970	0.046	0.092	0.923
<i>GSTM1</i> null	52	53.61	39	38.61	0.544	0.31-0.96	3.970	0.046	0.092	0.923
<i>GSTT1</i> positive	93	95.87	93	92.07	0.500	0.15-0.96	0.694	0.405	0.810	0.869
<i>GSTT1</i> null	4	4.23	8	7.92	2.000	0.58-6.87	0.694	0.405	0.810	0.869
<i>GSTT1</i> positive; <i>GSTM1</i> positive	41	42.27	54	53.47	1.569	0.89-2.75	2.490	0.115	0.345	0.918
<i>GSTT1</i> positive; <i>GSTM1</i> null	52	53.61	39	38.61	0.544	0.31-0.96	4.480	0.034	0.102	0.898
<i>GSTT1</i> null; <i>GSTM1</i> positive	4	4.12	8	7.92	2.000	0.58-6.87	1.250	0.263	0.789	0.937
AA	0	0.00	5	4.95	NA	NA	3.120	0.077	0.231	0.989
AT	73	75.26	72	71.29	0.816	0.43-1.53	0.400	0.528	1.000	0.782
TT	24	24.74	24	23.76	0.948	0.50-1.82	0.030	0.872	1.000	0.509
A	73	37.63	82	40.59	1.133	0.76-1.70	0.360	0.546	1.000	0.825
T	121	62.27	120	59.41	0.833	0.59-1.32	0.360	0.546	1.000	0.825

NA: not available.

Table 3. *GSTM1*, *GSTT1* and *FTO* gene polymorphisms in different ethnic groups.

Ethnicity	Ref.	Alleles (%)				Significant	Genotypes (%)		Alleles (%)			Significant
		<i>GSTM1</i> Positive	<i>GSTM1</i> Null	<i>GSTT1</i> Positive	<i>GSTT1</i> Null	Yes/No	TT	AT	AA	A	T	Yes/No
North Indian	this study	61.38	38.61	92.07	7.92	Yes	23.76	71.29	4.95	40.59	59.41	No
Egyptian	7	38.00	62.00	65.00	35.00	Yes	–	–	–	–	–	–
North Indian	30	46.00	54.00	83.00	17.00	Yes	–	–	–	–	–	–
Egyptian	28	41.38	58.62	82.76	17.24	No	–	–	–	–	–	–
Dubai	29	42.50	57.50	40.00	60.00	Yes	–	–	–	–	–	–
North Indian	32	–	–	–	–	–	39.90	47.60	12.50	64.00	36.00	Yes
South Asian Indian	33	–	–	–	–	–	43.60	42.20	14.20	35.40	64.60	Yes
South African	34	–	–	–	–	–	15.00	55.00	30.00	57.00	43.00	No
Spanish	36	–	–	–	–	–	33.90	48.30	17.80	58.04	41.95	No
Scots	35	–	–	–	–	–	33.20	48.80	18.00	42.4	57.59	Yes

study, we reported that the frequency of the AT genotype (Pro12Ala) was 71.29% in T2DM patients, which is significantly higher when compared with North Indians (47.6%), South Asian Indians (42.2%), South Africans (55.0%), Scots (48.8%) and Spanish (48.3%) populations [32-36]. The frequency of the *FTO* AA genotype described in other populations fluctuates between 14.0 to 18.0% in T2DM patients of North Indians (12.5%), South Asian Indians (14.2%), Scots (18.0%) and Spanish (17.8%) populations [32,33,35,36], but the frequency of the *FTO* AA genotype in our study group was 4.95%, which is considerably lower when compared to other reports. Many factors may account for the different results in similar studies. In our population, the frequency of the TT genotype was 23.76%, which is considerably lower than in South Asian Indians (43.6%) and North Indians (39.9%) [32,33]. There may be differences even in the same population because of genetic, environmental factors and the number of samples analyzed.

Our data suggest that the polymorphism in the *GSTM1* gene might be a risk factor for T2DM, while no significant association was found with the *FTO* gene polymorphism. The potential role of *GST* and *FTO* gene polymorphisms as a marker of susceptibility for T2DM needs further studies in a larger number of patients.

Declaration of Interest. We are grateful for the support of the intramural grant from the Era's Lucknow Medical College and Hospital, Lucknow, Ut-

tar Pradesh, India. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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