

Glutathione S-Transferase (*GSTT1* rs17856199) and Nitric Oxide Synthase (*NOS2* rs2297518) Genotype Combination as Potential Oxidative Stress-Related Molecular Markers for Type 2 Diabetes Mellitus

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Background: Deregulation of the antioxidant enzymes was implicated in pathogenesis and complications of type 2 diabetes mellitus (T2DM). The data relate the genetic variants of these enzymes to T2DM are inconsistent among various populations.

Purpose: We aimed to explore the association of 13 genetic variants of “superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and nitric oxide synthase (NOS)” with T2DM susceptibility and the available clinical laboratory data.

Subjects and Methods: A total of 384 individuals were enrolled in this work. Different genotypes of the genes mentioned above were characterized using TaqMan OpenArray Genotyping assays on a Real-Time polymerase chain reaction system.

Results: After age- and sex-adjustment, among the studied 13 variants, *GSTT1* rs17856199 was associated with T2DM under homozygote (OR=3.42; 95% CI:1.04–11.2, $p=0.031$), and recessive (OR=3.57; 95% CI: 1.11–11.4, $p=0.029$) comparison models. The *NOS2* rs2297518*A allele was more frequent among the T2DM cohort (58.1% vs 35.4%, $p<0.001$) and showed a dose-response effect; being heterozygote was associated with higher odds for developing DM (OR=4.06, 95% CI=2.13–7.73, $p<0.001$), whereas being AA homozygote had double the risk (OR=9.06, 95% CI=3.41–24.1, $p<0.001$). Combined *NOS2* rs2297518*A and either *GSTT1* rs17856199*A or *C genotype carriers were more likely to develop T2DM. Different associations with sex, BMI, hyperglycemia, and/or hyperlipidemia were evident. The principal component analysis revealed *NOS2* rs2297518*G, old age, dyslipidemia, high systolic blood pressure, and elevated HbA1c were the main classifiers of T2DM patients.

Conclusion: The oxidative stress-related molecular markers, *GSTT1* rs17856199 and *NOS2* rs2297518 variants were significantly associated with T2DM risk and phenotype in the study population.

Keywords: single nucleotide polymorphism, *GSTT1*, *NOS2*, oxidative stress, T2DM

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder that impacts patients' lives worldwide. It is estimated that T2DM affects around 463 million individuals globally, rising to 578 million by 2030.¹ Saudi Arabia is considered one of the top ten countries characterized by a high incidence of diabetes.² Accumulating evidence, including previous studies in this region,^{3–5} indicated

that environmental and genetic risk factors play essential roles in T2DM etiopathology.^{6,7} One of these factors is oxidative stress, which has been implicated in insulin resistance, β -cell dysfunction, and impaired glucose tolerance progressing to T2DM.⁸ Also, oxidative stress has been associated with the various micro-and macrovascular complications of T2DM.⁹ The in vivo antioxidant enzymes: “Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and nitric oxide synthase (NOS)” acted as scavengers for the reactive metabolites which ameliorate the oxidative stress imposed by these reactants on the cells.¹⁰ Several genetic variants associated with T2DM, including those associated with the antioxidant enzymes, have been detected by genome-wide association studies (GWAS), mainly in European descent people.^{11,12} These functional gene variants might be associated with a decline or lack of catalytic activity with subsequent accumulation of reactive oxygen species (ROS).^{8,13} These species play a central role in insulin resistance and β -cell dysfunction, the two major components in T2DM development and increased risk of complications.^{13–16}

The SOD family catalyzes the conversion of superoxide to hydrogen peroxide (H_2O_2), which form H_2O and O_2 by CAT and GPx enzymes. This family comprises the intracellular “CuZn-SOD (SOD1; EC 1.15.1.1)”, mitochondrial “Mn-SOD (SOD2; EC 1.15.1.1)”, and the extracellular “EC-SOD (SOD3; EC 1.15.1.1)”. The gene family coding for these enzymes is located on different chromosomes, “SOD1 on 21q22.11, SOD2 on 6q25.3, and SOD3 on 4p15.3-p15.1”.^{17,18} The *SOD1* rs2234694 (A/C) which mapped near the splice site at the third exon/intron boundary, the *SOD2* rs4880 (A/G) which reported to change the structural conformation of the enzyme “mitochondrial targeting peptide” domain,¹⁹ and the *SOD3* rs2536512 (A/G) that resulted in threonine substitution of alanine, have been associated with the risk of diabetes.^{13,20,21}

The main H_2O_2 -scavenging catalysts include CAT (EC 1.11.1.6) and several thiol-based peroxidases such as GPXs (EC 1.11.1.9). Their deregulated activities were implicated in disease initiation and progression, including DMT2.²² The *CAT* rs7943316 variant was mapped to the promotor region just proximal to the start site (–21A/T).¹⁸ T1DM susceptibility locus has been identified on the chromosome 11p13 near this gene, supporting the idea of *CAT* may play a role in DMT2.²³ *GPX1* is expressed mainly in the red blood cells, whereas *GPX3* and *GPX4* work mainly in high-density lipoprotein cholesterol

fraction and phospholipids, respectively, detoxifying the complex lipid hydroperoxides.^{24,25} The *GPX1* gene (locus 3p21.3) contains the rs1800668G/A variant, which is likely to affect the encoded protein’s activity.²⁶ Although *GPX3* rs3828599 (T/C) and *GPX4* rs713041C/T were not explored yet with T2DM; the latter variant showed risk modulation for diabetic kidney disease and cardiac autonomic neuropathy in patients with Type 1 diabetes.^{27,28}

The GST (EC 2.5.1.18) family of Phase II metabolic isozymes were implicated in detoxifying various electrophilic compounds via catalysis of compounds conjugation with glutathione.²⁹ The *GST* Mu 1 (*GSTM1*) rs1056806 (C/T), *GST* theta 1 (*GSTT1*) rs17856199 (A/C), *GST* Pi 1 (*GSTP1*) rs1695 (A/G), and microsomal *GST3* (*mGST3*) rs2065942 (C/T) gene variants of this family were reported to alter the detoxification process which might increase the risk of T2DM in several populations.^{8,15,30–33}

Lastly, the NOS (EC 1.14. 13.39) family members, which catalyze the NO production from L-arginine, have three isoforms, “neuronal NOS (nNOS/NOS1), inducible NOS (iNOS/NOS2) and endothelial NOS (eNOS/NOS3)”. Impairment of NO production leads to endothelial dysfunction, which eventually contributes to developing several disorders, including insulin resistance and T2DM.⁸ The *NOS2* rs2297518 (A/G) and *NOS3* rs1799983 (G/T) genetic variations were associated with the development of T2DM and/or its microvascular complications in several populations.^{34–37}

As most association studies of the variants mentioned above were mainly investigated in European descent, the authors were inspired to explore for the first time, up to the knowledge of the authors, the genetic profile of a preliminary sample from Middle Eastern (Saudi population) concerning the 13 likely susceptibility loci collectively in the same patient group to unravel their association with T2DM and/or phenotype.

Subjects and Methods

Study Participants

This case-control study enrolled a total of 384 (177 T2DM and 207 controls) individuals with a mean age of 38.7 ± 15.5 (ranged 23 to 83) years. All participants were unrelated Saudi adult population. Informed consent was obtained from all participants before taking part. Uncomplicated cases with a confirmed diagnosis of T2DM who receive antidiabetic medications such as

metformin for their glycemic control were enrolled in the study. The patients attended the Endocrinology and Diabetes center outpatient clinic, King Fahd Armed Forces Hospital (KFAFH), Jeddah, KSA. The patients complicated with other severe clinical disorders were excluded. The participants attending the clinics for a routine check-up and/or blood donation with normal glucose tolerance and without a family history of diabetes have been enrolled in the study as a control group.

All the participants underwent a complete medical history and physical examinations. Anthropometric parameters, including weight and height, and arterial blood pressure (BP), were measured. Body mass index (BMI) was calculated, and accordingly, obesity was defined from a BMI of ≥ 30 kg/m^{2.38} Hypertension was defined as stated previously. Ethical approval was obtained from the institutional review board at the King Fahd Armed Forces Hospital (approval No. REC 201), Jeddah, KSA.

Sample Collection and Biochemical Analysis

Venous blood samples (10 mL) were collected after an overnight fast (10–12 h); 4 mL on EDTA tubes for molecular analysis, 3 mL also on EDTA for glycated hemoglobin A1c (HbA1c) determination (VARIANT II TURBO Hemoglobin Testing System, Bio-Rad, USA), and 3 mL on serum separator tubes for other chemical assays. The later tubes were centrifuged immediately at 3000 rpm \times 12 minutes, and the separated serum was subjected to routine laboratory data analysis, including serum glucose and lipid profile [total cholesterol, HDL-cholesterol (HDL-C), and triglyceride] (Cobas c701, Roche Diagnostics, GmbH, Mannheim). The LDL-cholesterol (LDL-C) levels were calculated according to the Friedewald equation as serum TG levels in all included samples were less than 4.5 mmol/L.³⁹ All abnormal lipid and lipoprotein profiles were justified by the National Cholesterol Education Program and Adult Treatment Panel III (NCEP-ATP III) guidelines.⁴⁰ Low HDL-C level was considered from a value below the 50th percentile (HDL-C < 1.0 mmol/L). Hypertriglyceridemia (HTG) was defined by fasting serum for TG level of ≥ 1.7 mmol/L. Participants who had an abnormal level of one or more lipid profiles, or currently on the lipid-lowering drug, or had a history of lipid disorder was defined as “dyslipidemia”. Serum insulin levels were measured by Electrochemi-luminescence Immunoassay (Cobas e 602 immunoassay analyzer, Roche Diagnostics, GmbH, Mannheim).

Variants Selection and Genotyping

Based on dbSNP (www.ncbi.nlm.nih.gov) search for the SNP minor allele frequency (MAF) > 0.05 and the previous literature that showed evidence of functional significance and association of the specified SNPs with T2DM risk in various populations, the authors selected 13 variants related to *GSTM1*, *GSTT1*, *GSTP1*, *MGST3*, *SOD1*, *SOD2*, *SOD3*, *CAT*, *GPX1*, *GPX3*, *GPX4*, *NOS3* and *NOS2* genes (Table S1). Genomic DNA was extracted from peripheral blood leukocytes using Roche MagNA Pure Compact Nucleic Acid-based Isolation Kit I (Catalog no. 03730964001; Roche Diagnostics GmbH Mannheim, Germany) according to the manufacturer's instructions. Concentration and integrity of the extracted DNA were assessed by Nanodrop 2000/2000c Spectrophotometer V1.0 (Thermo Fisher Scientific Wilmington, DE USA) and gel electrophoresis, respectively. Amplification and allelic discrimination were performed in a GenaTi research center (King Abdulaziz University, King Fahad Medical Research Center) using Quant Studio 12K Flex Real-Time PCR System (Applied Biosystems). Each PCR reaction contained 5 μ L TaqMan GTXpress Master Mix (2x) (Cat no. 4,403,311, Applied Biosystems) mixed with 0.5 μ L TaqMan genotyping assay mix (20x), 2.5 μ L nuclease-free water, and 2.0 μ L gDNA (20 ng/ μ L) in a total reaction volume of 10 μ L/sample. The genotyping assays ID and the ready-to-use probe sequences for the specified study variants are summarized in Table S1. The PCR program was run as follows: enzyme activation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60 °C for 1 minute. All the quality control measurements were followed in each run, including using the appropriate controls.

Statistical Analysis

Data were analyzed using the R software version 4.0.2 (RStudio 3.0.1) and BM Statistical Package for the Social Sciences (SPSS) Statistics for Windows, version 26.0 (Armonk, NY: IBM Corp.).

Quantitative data were expressed as means \pm standard deviation or median and interquartile range, while qualitative data were expressed as numbers and percentages. Shapiro–Wilk test was used to test the normality of the continuous variables. Two-sided Chi-square, Student-t, and ANOVA tests were used for parametric data, while Mann–Whitney U and Kruskal Wallis tests were employed

for non-parametric variables. A p -value of <0.05 was considered statistically significant.

Analysis of allele and genotype frequencies were carried out.⁴¹ Genotype frequencies were assessed in patients and controls for deviation from the Hardy-Weinberg equation (HWE) using an online excel sheet to compare observed versus expected values. A Chi-square test was used to check Goodness-of-fit.⁴² Single and polygenic SNP analyses were performed. Genotype combination analysis was performed using the online SNPStats software (www.snpstats.net).⁴³ The relationship between allele frequencies and T2DM was determined under different inheritance models with logistic regression analysis after adjustment for age, sex, and obesity. Genetic association models included heterozygote comparison, homozygote comparison, dominant model, and recessive models.⁴⁴ Iteration of analysis was performed to test the association with the risk of obesity. Significant results in univariate analysis were plotted as a forest plot using STAT version 16.0 (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC). Next, multivariable regression analysis was performed to include significant molecular markers from univariate analysis with clinical data and laboratory testing. Hosmer-Lemeshow test was used to assess goodness-of-fit. Results were reported as odds ratio (OR) and 95% Confidence interval (CI). Regression models were applied with genetic variants alone then repeated to integrate clinical and laboratory data. Both clinical and laboratory findings were tested for their association with each polymorphism. Multivariate analysis was executed for data exploration in principal component analysis using Psych, Factoextra, FactoMineR, ggplot2, ggpubr, and magrittr packages. Subjects were categorized according to their diabetic status.⁴⁵

Results

Demographic Characteristics of the Study Population

A total of 384 subjects were enrolled in the current study, including 143 women and 241 men accounting for 37.2% and 62.8%, respectively. Comparison between diabetic and non-diabetic cohorts is illustrated in Table 1. Of the diabetic group, males and females were equally reported in the study population; 95 females (53.7%) versus 82 male subjects (46.3%). However, more men were represented in the control group (159 subjects, 76.8%) compared to women volunteers (48 subjects, 23.2%) ($p < 0.001$). The

mean age of the study population was 38.7 ± 15.5 years (ranged 13 to 83 years old). Diabetic cohorts (44.6 ± 17.3 years) were significantly older than non-diabetic controls (33.8 ± 11.7 years) ($p < 0.001$). The mean body mass index (BMI) of the whole population was 28.6 ± 5.64 Kg/m². Diabetic patients had a higher BMI value (29.9 ± 6.01 Kg/m²) than their counterparts (27.6 ± 4.58 Kg/m²) ($p < 0.001$). For the whole study population, the mean systolic/diastolic blood pressure was $124.5 \pm 18.3/72.3 \pm 9.81$ mmHg. Systolic blood pressure was significantly higher in the diabetic group (133.1 ± 18.3 mmHg) than in the control group (117.3 ± 14.8 mmHg) ($p < 0.001$). Similarly, higher measurements of diastolic blood pressure were found in diabetic cases (73.5 ± 10.4 mmHg) than controls (71.4 ± 9.1 mmHg) ($p = 0.047$).

Comorbid Conditions of the Study Population

In the study population, 154 subjects (40.1%) were obese. Of the T2DM patients, 58 cases had hypertension, accounting for 32.8%. None of the controls were hypertensive. Regarding the lipid profile of the study groups, hyperlipidemia was only reported in the diabetic group, representing about 34.5% (N = 61) of diabetic patients (Table 1).

Laboratory Findings of the Study Population

As shown in Table 1, hyperglycemia was observed in the diabetic group. The median random blood sugar level of 6.1 mmol/L (IQR: 5.0–8.0) in individuals with diabetes was significantly higher than non-diabetics (median: 5.7 mmol/L, IQR: 4.79–6.64) ($p < 0.001$). As a long-term marker, the HbA1c level was 6.0% (IQR: 5.3–8.0) in patients with type 2 diabetes higher than controls at 5.3% (IQR: 5.1–5.6) ($p < 0.001$). Also, LDL cholesterol levels in people with diabetes (median: 2.95 mmol/L, IQR: 2.30–3.63) were significantly higher than those in cohorts without diabetes (median: 2.67 mmol/L, IQR: 2.18–3.43) ($p = 0.047$). There was no significant difference between the two groups regarding the serum insulin level or other lipid profile parameters.

Allelic Discrimination Analysis in Diabetics and Non-Diabetics

Genotype distribution was found in agreement with HWE in patients and controls except for *MGST3* rs2065942 (C/T), *SOD1* rs2234694 (A/C), *CAT* rs7943316 (A/T), *GPX1*

Table I Demographic, Clinical, and Biochemical Characteristics of the Study Population

Variables	Controls (n=207)	Patients with T2DM (n=177)	P-value
Demographic data			
Age, years	33.8 ± 11.7	44.6 ± 17.3	<0.001
Female	48 (23.2)	95 (53.7)	<0.001
Male	159 (76.8)	82 (46.3)	
Body mass index, Kg/m ²	27.6 ± 4.58	29.9 ± 6.01	<0.001
Systolic blood pressure, mmHg	117.3 ± 14.8	133.1 ± 18.3	<0.001
Diastolic blood pressure, mmHg	71.4 ± 9.1	73.5 ± 10.4	0.047
Comorbidities			
Obesity	76 (36.7)	78 (44.1)	0.14
Hypertension	–	58 (32.8)	NA
Hyperlipidemia	–	61 (34.5)	NA
Glycemic state			
Fasting serum glucose, mmol/L	5.71 (4.79–6.64)	6.10 (5.00–8.80)	<0.001
Glycosylated hemoglobin (HbA1c), %	5.30 (5.10–5.60)	6.00 (5.30–8.00)	<0.001
Serum insulin, mIU/L	181.8 (84.9–441)	161.0 (68.8–337)	0.10
Lipid profile			
Triglyceride, mmol/L	1.63 (1.07–2.63)	1.45 (0.99–2.09)	0.41
Total cholesterol, mmol/L	4.59 (3.96–5.58)	4.90 (4.24–5.67)	0.30
HDL-cholesterol, mmol/L	1.10 (0.96–1.32)	1.19 (0.99–1.36)	0.15
LDL-cholesterol, mmol/L	2.67 (2.18–3.43)	2.95 (2.30–3.63)	0.047

Notes: Data are presented as median and interquartile range, mean ± standard deviation (SD), or number and percentage. Student's t and Mann–Whitney U-tests were used. Bold values indicate significance at P-value less than 0.05.

rs1800668 (G/A), and *GPX3* rs3828599 (T/C). Allele and genotype frequencies of the 13 polymorphisms were significant for *NOS2* rs2297518 (A/G); rs2297518*A allele was more frequent among diabetic cohorts (58.1% vs 35.4%, $p < 0.001$). While G/G being the most prevalent genotype in healthy controls accounting for 54.4%, A/G was the most prevalent genotype among diabetic patients (59.5% vs 39.6%), followed by A/A (23.4% vs 5.9%, $p < 0.001$) (Table 2). rs2297518*A carriers showed a dose-response effect; being heterozygote with one allele (A/G) rendered higher odds for developing diabetes mellitus (OR = 4.06, 95% CI = 2.13–7.73, $p < 0.001$), whereas being a homozygote carrying two alleles (A/A) had double the risk (OR = 9.06, 95% CI = 3.41–24.1, $p < 0.001$) (Table 3).

Regarding *GSTT1* rs17856199 (A/C) polymorphism, a higher frequency of C/C genotype was observed in diabetic patients, though the p-value did not reach significance ($p = 0.09$) (Table 2). After adjustment by age and sex, homozygote carriers for two copies of the C allele were three times more liable to develop diabetes than non-carriers. On homozygote comparison (C/C vs A/A), odds ratio (OR) was 3.42 (95% CI: 1.04–11.2, $p = 0.031$), and similar risk was found under recessive model (C/C vs A/

A-A/C) with OR at 3.57 (95% CI: 1.11–11.4, $p = 0.029$) (Table 3). Genetic association models analysis did not show a further association of other variants with disease susceptibility.

Polygenic Risk of Diabetes in Association with Gene Variants

Having both *GSTT1* rs17856199*A and *NOS2* rs2297518*A variants were 3.96 times more likely to have diabetes (OR = 3.96, 95% CI = 2.14–7.33, $p < 0.001$), while carrying both *GSTT1* rs17856199*C and *NOS2* rs2297518*A variants had 3 times higher susceptibility of developing diabetes type 2 (OR = 3.08, 95% CI = 1.47–6.48, $p = 0.003$) (Table 4).

Association of Genetic Variants with Clinical and Biochemical Features

In the diabetic group, two polymorphisms were more frequent in males namely *GSTP1* rs1695 G/G (82.4%, $p < 0.001$) and *CAT* rs7943316 A/A (70.0%, $p = 0.034$). Another two variants were associated with higher body mass index: *SOD2* rs4880 G/G genotype (31.70 ± 5.05 Kg/m² in G/G vs 29.06 ± 7.54 Kg/m² in A/A and 30.58 ± 5.53 Kg/m² in A/G, $p = 0.013$), and *SOD3* rs2536512 A/A

Table 2 Genotype and Allele Frequencies of Polymorphisms

Gene/SNP	Parameter	Allele/Genotype	Controls	Cases	P-value
GSTM1 rs1056806 (C/T)	Allele frequency	C	368 (91.5)	318 (91.6)	0.91
		T	34 (8.5)	29 (8.4)	
		Total	402	347	
	Genotype frequency	C/C	168 (83.2)	146 (83.4)	0.80
		C/T	32 (15.8)	26 (14.9)	
		T/T	2 (1)	3 (1.7)	
		Total	202	175	
	HWE	P-value	0.73	0.16	
GSTT1 rs17856199 (A/C)	Allele frequency	A	256 (78.8)	162 (76.4)	0.52
		C	69 (21.2)	50 (23.6)	
		Total	325	212	
	Genotype frequency	A/A	96 (58.2)	61 (55)	0.09
		A/C	64 (38.8)	40 (36)	
		C/C	5 (3)	10 (9)	
		Total	165	111	
	HWE	P-value	0.14	0.36	
GSTP1 rs1695 (A/G)	Allele frequency	A	211 (68.7)	143 (69.8)	0.80
		G	96 (31.3)	62 (30.2)	
		Total	307	205	
	Genotype frequency	A/A	69 (41.8)	49 (44.1)	0.82
		A/G	73 (44.2)	45 (40.5)	
		G/G	23 (13.9)	17 (15.3)	
		Total	165	111	
	HWE	P-value	0.60	0.22	
MGST3 rs2065942 (C/T)	Allele frequency	C	328 (96.5)	225 (98.3)	0.20
		T	12 (3.5)	4 (1.7)	
		Total	340	229	
	Genotype frequency	C/C	159 (93)	111 (96.5)	0.41
		C/T	10 (5.8)	3 (2.6)	
		T/T	2 (1.2)	1 (0.9)	
		Total	171	115	
	HWE	P-value	<0.001	<0.001	

(Continued)

Table 2 (Continued).

Gene/SNP	Parameter	Allele/Genotype	Controls	Cases	P-value
SOD1 rs2234694 (A/C)	Allele frequency	A	324 (97.6)	223 (99.6)	0.07
		C	8 (2.4)	1 (0.4)	
		Total	332	224	
	Genotype frequency	A/A	159 (95.2)	111 (99.1)	0.18
		A/C	6 (3.6)	1 (0.9)	
		C/C	2 (1.2)	0 (0)	
		Total	167	112	
	HWE	P-value	<0.001	<0.001	
SOD2 rs4880 (A/G)	Allele frequency	A	167 (58.2)	121 (61.4)	0.47
		G	120 (41.8)	76 (38.6)	
		Total	287	197	
	Genotype frequency	A/A	44 (26.8)	33 (30.3)	0.52
		A/G	79 (48.2)	55 (50.5)	
		G/G	41 (25)	21 (19.3)	
		Total	164	109	
	HWE	p-value	0.64	0.82	
SOD3 rs2536512 (A/G)	Allele frequency	A	167 (58.2)	121 (61.4)	0.47
		G	120 (41.8)	76 (38.6)	
		Total	287	197	
	Genotype frequency	A/A	44 (26.8)	33 (30.3)	0.52
		A/G	79 (48.2)	55 (50.5)	
		G/G	41 (25)	21 (19.3)	
		Total	164	109	
	HWE	p-value	0.64	0.82	
CAT rs7943316 (A/T)	Allele frequency	A	96 (54.2)	72 (54.5)	0.95
		T	81 (45.8)	60 (45.5)	
		Total	177	132	
	Genotype frequency	A/A	27 (25)	20 (25)	0.98
		A/T	42 (38.9)	32 (40)	
		T/T	39 (36.1)	28 (35)	
		Total	108	80	
	HWE	P-value	0.027	0.08	

(Continued)

Table 2 (Continued).

Gene/SNP	Parameter	Allele/Genotype	Controls	Cases	P-value
GPX1 rs1800668 (G/A)	Allele frequency	A	84 (31.6)	71 (31.7)	0.97
		G	182 (68.4)	153 (68.3)	
		Total	266	224	
	Genotype frequency	A/A	12 (6.2)	12 (7.3)	0.83
		A/G	60 (30.9)	47 (28.5)	
		G/G	122 (62.9)	106 (64.2)	
		Total	194	165	
	HWE	P-value	0.21	0.044	
GPX3 rs3828599 (T/C)	Allele frequency	C	15 (7)	9 (4.9)	0.18
		T	200 (93)	173 (95.1)	
		Total	215	182	
	Genotype frequency	C/C	2 (1)	2 (1.1)	0.45
		C/T	11 (5.4)	5 (2.9)	
		T/T	189 (93.6)	168 (96)	
		Total	202	175	
	HWE	P-value	<0.001	<0.001	
GPX4 rs713041 (C/T)	Allele frequency	C	171 (58.6)	116 (58.6)	0.99
		T	121 (41.4)	82 (41.4)	
		Total	292	198	
	Genotype frequency	C/C	42 (25.8)	27 (24.8)	0.82
		C/T	87 (53.4)	62 (56.9)	
		T/T	34 (20.9)	20 (18.3)	
		Total	163	109	
	HWE	P-value	0.37	0.13	
NOS3 rs1799983 (G/T)	Allele frequency	G	265 (82.3)	165 (77.5)	0.16
		T	57 (17.7)	48 (22.5)	
		Total	322	213	
	Genotype frequency	G/G	107 (65.2)	63 (56.8)	0.17
		G/T	51 (31.1)	39 (35.1)	
		T/T	6 (3.7)	9 (8.1)	
		Total	164	111	
	HWE	P-value	0.97	0.40	

(Continued)

Table 2 (Continued).

Gene/SNP	Parameter	Allele/Genotype	Controls	Cases	P-value
NOS2 rs2297518 (A/G)	Allele frequency	G	159 (64.6)	85 (41.9)	<0.001
		A	87 (35.4)	118 (58.1)	
		Total	246	203	
	Genotype frequency	G/G	92 (54.4)	19 (17.1)	<0.001
		A/G	67 (39.6)	66 (59.5)	
		A/A	10 (5.9)	26 (23.4)	
		Total	169	111	
	HWE	p-value	0.63	0.41	

Notes: Values are shown as number (%). The total number of cases for each investigated variant was not the same according to the genotyping recall success rate. A Chi-square test was used. Bold values indicate significance at P -value < 0.05.

Abbreviation: HWE, Hardy-Weinberg equilibrium.

genotype (32.30 ± 7.08 Kg/m² in A/A vs 30.21 ± 4.82 Kg/m² in G/G and 28.61 ± 5.33 Kg/m² in A/G, $p = 0.013$) (Table 5). In addition, a higher frequency of hyperlipidemia was observed in diabetic patients with *SOD2* rs4880*G/G genotype (56.5% in G/G vs 35.1% in A/A and 24% in A/G, $p = 0.025$) and *NOS2* rs2297518 G/G (63.2% in G/G vs 34.6% in A/A and 31.3% in A/G, $p = 0.039$).

As depicted in Table S2, higher levels of triglyceride were reported in *GPX4* rs713041 C/C cohorts (median = 1.7 mmol/L, IQR = 1.2–2.4), followed by C/T patients (median = 1.5, IQR = 1.2–2.0) and T/T carriers (median = 1.1, IQR = 0.9–1.4) ($p = 0.049$). Higher glycemic state was found in diabetic patients carrying *NOS2* rs2297518 G/G (median = 6.3 mmol/L, IQR = 5.8–7.6) or A/G (median = 5.7 mmol/L, IQR = 5.2–7.4) compared to A/A patients (median = 4.9, IQR = 4.5–5.7) ($p = 0.004$).

Multivariable Analysis

Binary regression analysis was employed and confirmed the dose-effect of the rs2297518*A variant of the *NOS2* gene that was elaborated in univariate analysis. Also, being a female (OR = 2.94, 95% CI = 1.44–6.25, $p = 0.003$) and old age (OR = 1.03, 95% CI = 1.02–1.04, $p = 0.031$) were also predictor risk factors for developing diabetes (Figure 1).

Principal Component Analysis (PCA) was performed to integrate all clinical, laboratory, and molecular features of the individual subject. Patients and controls were distributed in a high dimensional space then plotted across “axis 1” and “axis 2” with the highest variability. Subjects

close to each other had similar features to those dispersed apart. As shown in Figure 2, there was a clear demarcation between diabetic patients and non-diabetic controls with some overlaps. *NOS2* gene variant, older age, dyslipidemia, high systolic blood pressure, and elevated HbA1c were the main characteristics of this group, while the male sex was more represented in control subjects. Other variants were perpendicular to the Subjects’ distribution in the PCA, highlighting their absent role in determining disease outcomes in the present population.

Discussion

The development of diabetes requires the implication of oxidative stress-related signaling in many aspects of disease etiopathology.^{46,47} It has been shown that the antioxidant enzyme-related gene variants are involved in the pathogenesis of T2DM and its complications.⁸ Although several ethnic population-based studies have reported the association between the specified study polymorphisms and T2DM, controversial results have been identified.³² The present study explored the association of 13 antioxidant-related gene variants with T2DM susceptibility and phenotype in a sample of Saudi population. From these polymorphisms being investigated, only the *GSTT1* rs17856199*C and *NOS2* rs2297518*A were significantly associated with T2DM risk.

For rs17856199 (A/C) polymorphism, CC homozygote carriers were three times more liable to develop diabetes than non-carriers, and a similar risk was found under the recessive model. The *GSTT1* is a cytosolic isozyme of GSTs superfamily, which has an imminent role in

Table 3 Risk of Diabetes Mellitus According to Genetic Association Models

Gene/SNP	Genetic Association Model	Comparison Group	Adjusted OR	95% CI	P-value
GSTMI rs1056806 (C/T)	Heterozygote comparison	C/T vs C/C	1.01	0.55–1.86	0.58
	Homozygote comparison	T/T vs C/C	2.65	0.41–16.94	0.45
	Dominant model	C/T-T/T vs C/C	1.09	0.61–1.97	0.76
	Recessive model	T/T vs C/C-C/T	2.64	0.41–16.88	0.30
	Overdominant model	C/T vs C/C-T/T	0.99	0.54–1.82	0.98
GSTT1 rs17856199 (A/C)	Heterozygote comparison	A/C vs A/A	0.89	0.50–1.58	0.08
	Homozygote comparison	C/C vs A/A	3.42	1.04–11.2	0.031
	Dominant model	A/C-C/C vs A/A	1.07	0.63–1.84	0.80
	Recessive model	C/C vs A/A-A/C	3.57	1.11–11.4	0.029
	Overdominant model	A/C vs A/A-C/C	0.80	0.45–1.39	0.42
GSTP1 rs1695 (A/G)	Heterozygote comparison	A/G vs A/A	0.64	0.36–1.15	0.32
	Homozygote comparison	G/G vs A/A	0.87	0.39–1.95	0.45
	Dominant model	A/G-G/G vs A/A	0.69	0.40–1.20	0.19
	Recessive model	G/G vs A/A-A/G	1.08	0.51–2.29	0.84
	Overdominant model	A/G vs A/A-G/G	0.66	0.38–1.15	0.14
MGST3 rs2065942 (C/T)	Heterozygote comparison	C/T vs C/C	0.62	0.16–2.40	0.76
	Homozygote comparison	T/T vs C/C	1.22	0.11–13.71	0.69
	Dominant model	C/T-T/T vs C/C	0.71	0.22–2.34	0.57
	Recessive model	T/T vs C/C-C/T	1.25	0.11–14.05	0.86
	Overdominant model	C/T vs C/C-T/T	0.62	0.16–2.39	0.47
SOD1 rs2234694 (A/C)	Heterozygote comparison	A/C vs A/A	0.31	0.03–2.83	0.24
	Homozygote comparison	C/C vs A/A	NA	NA	NA
	Dominant model	A/C-C/C vs A/A	0.24	0.03–2.03	0.12
	Recessive model	C/C vs A/A-A/C	NA	NA	NA
	Overdominant model	A/C vs A/A-C/C	0.31	0.03–2.86	0.25
SOD2 rs4880 (A/G)	Heterozygote comparison	A/G vs A/A	0.77	0.41–1.44	0.38
	Homozygote comparison	G/G vs A/A	0.59	0.28–1.25	0.54
	Dominant model	A/G-G/G vs A/A	0.71	0.39–1.26	0.24
	Recessive model	G/G vs A/A-A/G	0.69	0.36–1.33	0.26
	Overdominant model	A/G vs A/A-G/G	0.96	0.56–1.66	0.90
SOD3 rs2536512 (A/G)	Heterozygote comparison	A/G vs A/A	0.78	0.42–1.48	0.50
	Homozygote comparison	G/G vs A/A	0.64	0.30–1.36	0.45
	Dominant model	A/G-G/G vs A/A	0.73	0.40–1.33	0.30
	Recessive model	G/G vs A/A-A/G	0.74	0.38–1.42	0.36
	Overdominant model	A/G vs A/A-G/G	0.96	0.55–1.65	0.87

(Continued)

Table 3 (Continued).

Gene/SNP	Genetic Association Model	Comparison Group	Adjusted OR	95% CI	P-value
CAT rs7943316 (A/T)	Heterozygote comparison	A/T vs A/A	1.71	0.79–3.73	0.31
	Homozygote comparison	T/T vs A/A	1.76	0.73–4.24	0.23
	Dominant model	A/T-T/T vs A/A	1.73	0.85–3.55	0.13
	Recessive model	T/T vs A/A-A/T	1.29	0.62–2.70	0.50
	Overdominant model	A/T vs A/A-T/T	1.33	0.69–2.55	0.40
GPX1 rs1800668 (G/A)	Heterozygote comparison	A/G vs A/A	0.80	0.48–1.34	0.70
	Homozygote comparison	G/G vs A/A	0.93	0.35–2.47	0.69
	Dominant model	A/G-G/G vs A/A	0.82	0.51–1.33	0.43
	Recessive model	G/G vs A/A-A/G	1.00	0.38–2.61	1.00
	Overdominant model	A/G vs A/A-G/G	0.81	0.49–1.33	0.41
GPX3 rs3828599 (T/C)	Heterozygote comparison	T/C vs T/T	0.62	0.19–2.00	0.62
	Homozygote comparison	C/C vs T/T	1.73	0.22–13.42	0.54
	Dominant model	T/C-C/C vs T/T	0.78	0.28–2.16	0.63
	Recessive model	C/C vs T/T-T/C	1.77	0.23–13.68	0.59
	Overdominant model	T/C vs T/T-C/C	0.61	0.19–1.98	0.41
GPX4 rs713041 (C/T)	Heterozygote comparison	C/T vs C/C	1.70	0.86–3.37	0.30
	Homozygote comparison	T/T vs C/C	1.43	0.61–3.31	0.52
	Dominant model	C/T-T/T vs C/C	1.63	0.84–3.14	0.14
	Recessive model	T/T vs C/C-C/T	0.98	0.50–1.93	0.95
	Overdominant model	C/T vs C/C-T/T	1.45	0.83–2.51	0.19
NOS3 rs1799983 (G/T)	Heterozygote comparison	G/T vs G/G	1.26	0.71–2.24	0.49
	Homozygote comparison	T/T vs G/G	1.36	0.39–4.78	0.68
	Dominant model	G/T-T/T vs G/G	1.28	0.74–2.21	0.39
	Recessive model	T/T vs G/G-G/T	1.25	0.36–4.32	0.73
	Overdominant model	G/T vs G/G-T/T	1.23	0.70–2.17	0.46
NOS2 rs2297518 (A/G)	Heterozygote comparison	A/G vs G/G	4.06	2.13–7.73	<0.001
	Homozygote comparison	A/A vs G/G	9.06	3.41–24.08	<0.001
	Dominant model	A/G-A/A vs G/G	4.66	2.49–8.73	<0.001
	Recessive model	A/A vs G/G-A/G	3.74	1.56–9.00	0.002
	Overdominant model	A/G vs G/G-A/A	2.17	1.26–3.72	0.004

Notes: OR (95% CI), odds ratio, and confidence interval. Bold values indicate significance at P-value < 0.05. Adjusted covariates: age, sex, and obesity.

detoxifying the phospholipid hydroperoxide, as mentioned earlier. In vitro studies have revealed that activation of GST protein expression can prevent 4-hydroperoxy-nonenal (an end product of phospholipid oxidation)-induced insulin resistance and lipolysis in adipocytes.⁴⁸

Interestingly, on searching some online prediction tools (SIFT, PolyPhen, SNPs3D, and MutPred), Masoodi et al have identified the potential impact of the rs17856199 variant on gene expression and protein structure that affect the detoxification process with subsequent metabolites

Table 4 Genotype Combination Analysis of Risk Alleles for Type 2 Diabetes Mellitus

<i>GSTT1</i> rs17856199	<i>NOS2</i> rs2297518	Total	Controls	T2DM Cases	OR (95% CI)	P-value
A	G	0.5002	0.5983	0.3494	1	—
A	A	0.2574	0.1778	0.3805	3.96 (2.14–7.33)	<0.001
C	G	0.1339	0.1446	0.1191	1.67 (0.78–3.57)	0.18
C	A	0.1085	0.0793	0.151	3.08 (1.47–6.48)	0.003

Notes: Data are presented as the odds ratio and confidence interval. Bold values indicate significance at P -value < 0.05 . Binary logistic regression analysis was adjusted for age, sex, and body mass index.

Table 5 Association of Genetic Variants with Clinical Features of Diabetic Patients

<i>GSTM1</i> rs1056806 (C/T)		C/C (n=61)	C/T (n=40)	T/T (n=10)	P-value
Age, years	Mean \pm SD	45.32 \pm 17.51	44.68 \pm 15.66	41.67 \pm 19.76	0.96
Sex	Female	78 (53.4)	16 (61.5)	0 (0)	0.12
BMI, kg/m ²	Mean \pm SD	30.09 \pm 6.13	30.73 \pm 5.73	29.28 \pm 4.11	0.44
Comorbidities	Obesity	62 (42.5)	15 (57.7)	1 (33.3)	0.32
	Hypertension	50 (34.2)	6 (23.1)	1 (33.3)	0.53
	Hyperlipidemia	48 (32.9)	10 (38.5)	2 (66.7)	0.42
<i>GSTT1</i> rs17856199 (A/C)		A/A (n=49)	A/C (n=45)	C/C (n=17)	P-value
Age, years	Mean \pm SD	40.62 \pm 16.68	42.35 \pm 16.17	32.70 \pm 11.97	0.38
Sex	Female	25 (41)	18 (45)	5 (50)	0.83
BMI, kg/m ²	Mean \pm SD	30.01 \pm 5.41	30.05 \pm 7.66	30.65 \pm 5.64	0.48
Comorbidities	Obesity	27 (44.3)	15 (37.5)	5 (50)	0.70
	Hypertension	14 (23)	16 (40)	2 (20)	0.15
	Hyperlipidemia	20 (32.8)	16 (40)	3 (30)	0.71
<i>GSTP1</i> rs1695 (A/G)		A/A (n=49)	A/G (n=45)	G/G (n=17)	P-value
Age, years	Mean \pm SD	38.87 \pm 15.44	44.76 \pm 17.28	36.60 \pm 17.46	0.17
Sex	Female	16 (32.7)	28 (62.2)	3 (17.6)	0.001
BMI, kg/m ²	Mean \pm SD	30.40 \pm 5.74	29.17 \pm 5.36	32.66 \pm 9.11	0.47
Comorbidities	Obesity	22 (44.9)	19 (42.2)	8 (47.1)	0.93
	Hypertension	13 (26.5)	13 (28.9)	8 (47.1)	0.27
	Hyperlipidemia	15 (30.6)	19 (42.2)	5 (29.4)	0.43
<i>MGST3</i> rs2065942 (C/T)		A/A (n=111)	A/G-G/G (n=4)	P-value	
Age, years	Mean \pm SD	40.92 \pm 16.66	37.00 \pm 3.61	0.53	
Sex	Female	47 (42.3)	1 (25.0)	0.66	
BMI, kg/m ²	Mean \pm SD	30.23 \pm 6.23	29.09 \pm 4.06	0.96	

(Continued)

Table 5 (Continued).

Comorbidities	Obesity	49 (44.1)	1 (33.3)		0.63
	Hypertension	34 (30.6)	0 (0)		0.42
	Hyperlipidemia	39 (35.1)	2 (66.7)		0.40
SOD1 rs2234694 (A/C)		A/A (n=111)	A/C-C/C (n=1)		P-value
Age, years	Mean \pm SD	40.46 \pm 16.57	40		0.90
Sex	Female	46 (41.4)	0 (0)		0.40
BMI, kg/m ²	Mean \pm SD	30.12 \pm 6.13	27		0.64
Comorbidities	Obesity	48 (43.2)	0 (0)		0.38
	Hypertension	34 (30.6)	0 (0)		0.51
	Hyperlipidemia	39 (35.1)	1 (100)		0.18
SOD2 rs4880 (A/G)		A/A (n=37)	A/G (n=50)	G/G (n=23)	P-value
Age, years	Mean \pm SD	37.88 \pm 18.48	42.78 \pm 16.70	40.38 \pm 12.89	0.18
Sex	Female	15 (40.5)	19 (38)	12 (52.2)	0.51
BMI, kg/m ²	Mean \pm SD	29.06 \pm 7.54	30.58 \pm 5.53	31.70 \pm 5.05	0.013
Comorbidities	Obesity	12 (32.4)	23 (46)	14 (60.9)	0.09
	Hypertension	13 (35.1)	16 (32)	5 (21.7)	0.54
	Hyperlipidemia	13 (35.1)	12 (24)	13 (56.5)	0.025
SOD3 rs2536512 (A/G)		A/A (n=33)	A/G (n=55)	G/G (n=21)	P-value
Age, years	Mean \pm SD	44.73 \pm 16.85	37.80 \pm 15.29	40.44 \pm 19.29	0.10
Sex	Female	11 (33.3)	25 (45.5)	9 (42.9)	0.53
BMI, kg/m ²	Mean \pm SD	32.30 \pm 7.08	28.61 \pm 5.33	30.21 \pm 4.82	0.018
Comorbidities	Obesity	19 (57.6)	19 (34.5)	9 (42.9)	0.11
	Hypertension	14 (42.4)	11 (20)	8 (38.1)	0.06
	Hyperlipidemia	10 (30.3)	16 (29.1)	10 (47.6)	0.28
CAT rs7943316 (A/T)		A/A (n=20)	A/T (n=32)	T/T (n=28)	P-value
Age, years	Mean \pm SD	37.78 \pm 15.60	44.60 \pm 15.96	44.29 \pm 17.50	0.33
Sex	Female	6 (30)	12 (37.5)	18 (64.3)	0.034
BMI, kg/m ²	Mean \pm SD	30.19 \pm 4.47	29.66 \pm 4.84	30.12 \pm 6.30	0.70
Comorbidities	Obesity	9 (45)	11 (34.4)	13 (46.4)	0.59
	Hypertension	8 (40)	9 (28.1)	7 (25)	0.51
	Hyperlipidemia	7 (35)	6 (18.8)	9 (32.1)	0.35
GPXI rs1800668 (G/A)		A/A (n=12)	A/G (n=47)	G/G (n=106)	P-value
Age, years	Mean \pm SD	42.23 \pm 17.24	48.93 \pm 16.17	50.42 \pm 20.68	0.11
Sex	Female	53 (50)	29 (61.7)	6 (50)	0.40
BMI, kg/m ²	Mean \pm SD	29.89 \pm 6.42	30.23 \pm 5.42	31.61 \pm 5.34	0.30

(Continued)

Table 5 (Continued).

Comorbidities	Obesity	43 (40.6)	21 (44.7)	9 (75)	0.07
	Hypertension	33 (31.1)	16 (34)	7 (58.3)	0.17
	Hyperlipidemia	33 (31.1)	16 (34)	5 (41.7)	0.74
GPX3 rs3828599(T/C)		T/T (n=2)	T/C (n=5)	C/C (n=168)	P-value
Age, years	Mean \pm SD	45.24 \pm 17.38	39.50 \pm 11.12	43.50 \pm 27.58	0.44
Sex	Female	92 (54.8)	2 (40)	0 (0)	0.25
BMI, kg/m ²	Mean \pm SD	30.19 \pm 6.16	27.29 \pm 0.90	27.03 \pm 1.88	0.48
Comorbidities	Obesity	76 (45.2)	1 (20)	0 (0)	0.24
	Hypertension	55 (32.7)	2 (40)	1 (50)	0.83
	Hyperlipidemia	57 (33.9)	2 (40)	1 (50)	0.86
GPX4 rs713041 (C/T)		C/C (n=27)	C/T (n=62)	T/T (n=20)	P-value
Age, years	Mean \pm SD	41.12 \pm 13.79	39.65 \pm 16.72	43.25 \pm 20.42	0.62
Sex	Female	14 (51.9)	24 (38.7)	8 (40)	0.50
BMI, kg/m ²	Mean \pm SD	31.48 \pm 5.66	29.91 \pm 6.52	29.02 \pm 5.87	0.36
Comorbidities	Obesity	11 (40.7)	28 (45.2)	8 (40)	0.88
	Hypertension	8 (29.6)	20 (32.3)	4 (20)	0.58
	Hyperlipidemia	13 (48.1)	19 (30.6)	6 (30)	0.24
NOS3 rs1799983 (G/T)		G/G (n=63)	G/T (n=39)	T/T (n=9)	P-value
Age, years	Mean \pm SD	41.36 \pm 16.95	39.22 \pm 15.53	47.50 \pm 18.11	0.40
Sex	Female	25 (39.7)	17 (43.6)	5 (55.6)	0.65
BMI, kg/m ²	Mean \pm SD	30.35 \pm 7.07	30.20 \pm 5.19	30.04 \pm 3.09	0.99
Comorbidities	Obesity	30 (47.6)	16 (41)	3 (33.3)	0.64
	Hypertension	20 (31.7)	8 (20.5)	5 (55.6)	0.10
	Hyperlipidemia	23 (36.5)	13 (33.3)	3 (33.3)	0.94
NOS2 rs2297518 (A/G)		A/A (n=26)	A/G (n=64)	G/G (n=19)	P-value
Age, years	Mean \pm SD	38.82 \pm 16.10	42.52 \pm 16.19	40.12 \pm 17.34	0.37
Sex	Female	14 (53.8)	25 (39.1)	7 (36.8)	0.38
BMI, kg/m ²	Mean \pm SD	30.00 \pm 5.22	30.48 \pm 5.12	30.08 \pm 10.13	0.18
Comorbidities	Obesity	10 (38.5)	30 (46.9)	8 (42.1)	0.75
	Hypertension	5 (19.2)	21 (32.8)	7 (36.8)	0.35
	Hyperlipidemia	9 (34.6)	20 (31.3)	12 (63.2)	0.039

Notes: Values are shown as number (%) or mean and standard deviation (SD). A Chi-square or Student's t-tests were used. The total number of cases for each investigated variant was not the same according to the genotyping recall success rate. Bold values indicate significance at P -value < 0.05 .

Abbreviation: BMI, body mass index.

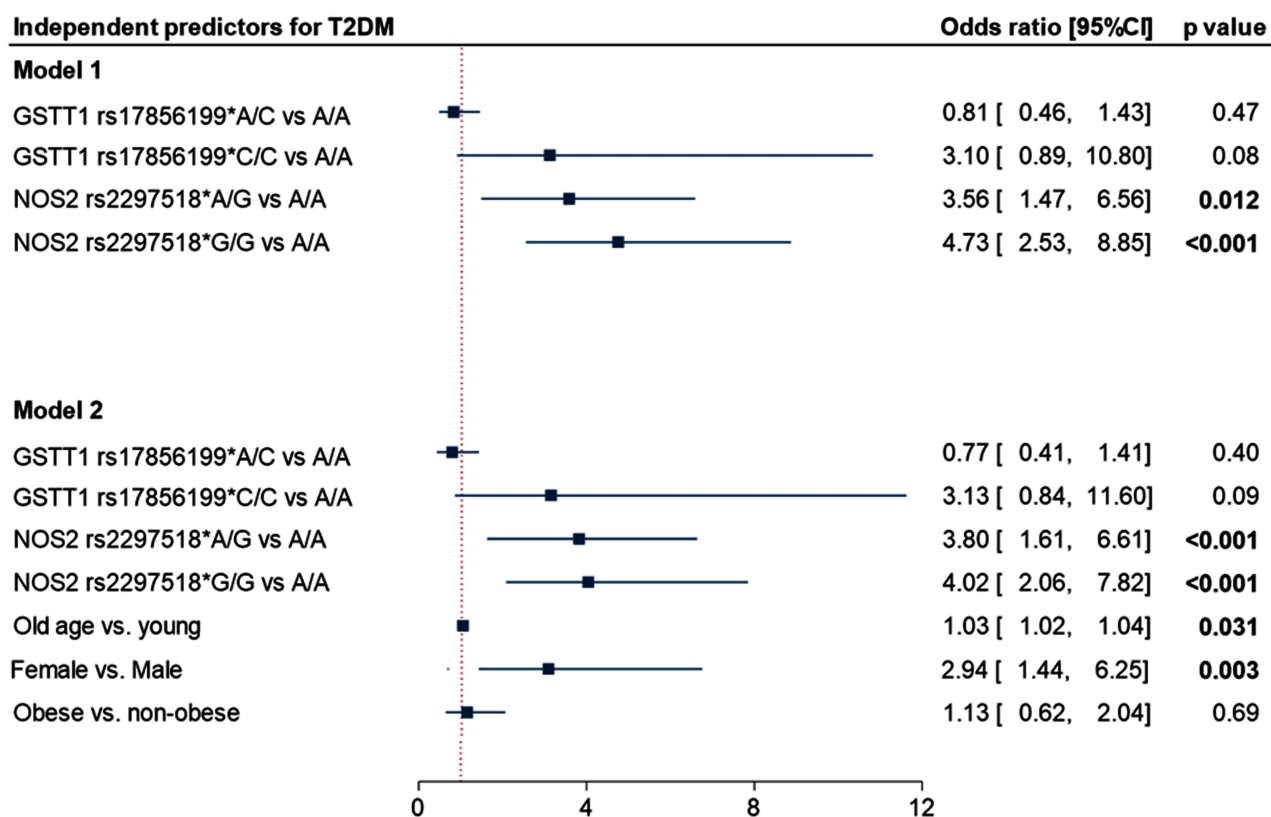


Figure 1 Independent risk factors for type 2 diabetes mellitus. Data are presented as the odds ratio and confidence interval. P-value < 0.05 was considered as statistically significant. Binary logistic regression analysis was applied.

accumulation and risk modification for breast cancer.⁴⁹ We also search these tools to confirm this variant's impact on the structure/function of the related protein. We found that this gene variant results in phenylalanine substitution by cysteine (F45C) associated with a decrease in the encoded protein's hydrophobic interaction (Figure S1).⁵⁰ It is well known that the phenylalanine side chain is fairly non-reactive and is thus rarely directly involved in protein function, but it can play a role in substrate recognition *via* stacking interactions with other non-protein ligands that contain aromatics groups.⁵¹ In this sense, the decreased hydrophobicity of the coded protein at the specified variant position could impact the encoded enzyme's function. Also, running the "Variant Effect Predictor" (https://asia.ensembl.org/Homo_sapiens/Tools/VEP) revealed this variant has potential deleterious effect by SIFT (Sorting Intolerant from Tolerant)⁵² algorithm and probably damaging by PolyPhen (polymorphism phenotyping) annotation with score = 0.999.⁵³ These findings could support the significant association of this SNP with diabetes susceptibility identified for the first time, up to the

authors' knowledge, in the current population. Further animal model or cell line-based testing of this variant can help to confirm our findings.

Regards the *NOS2* variant, the rs2297518*A allele showed a dose-response effect; being heterozygote was associated with higher odds for developing DM, whereas being AA homozygote had double the risk. This dose-effect remained significant in the multivariable analysis. In association with other non-genetic factors such as older age, dyslipidemia, high systolic blood pressure, and elevated HbA1c, this variant showed a significant role in distinguishing the T2DM cohort from controls by PCA. This exon-16 nonsynonymous variant was proved to increase the *NOS2* activity with several implications in many diseases.⁵⁴

The rs2297518 risk allele results in an S608L amino-acid substitution that is located in the catalytic domain of *NOS2* close to the flavin mononucleotide-binding region

which may impact the catalytic activity of *NOS2* and disease susceptibility, as proposed by Dhillon et al.⁵⁵

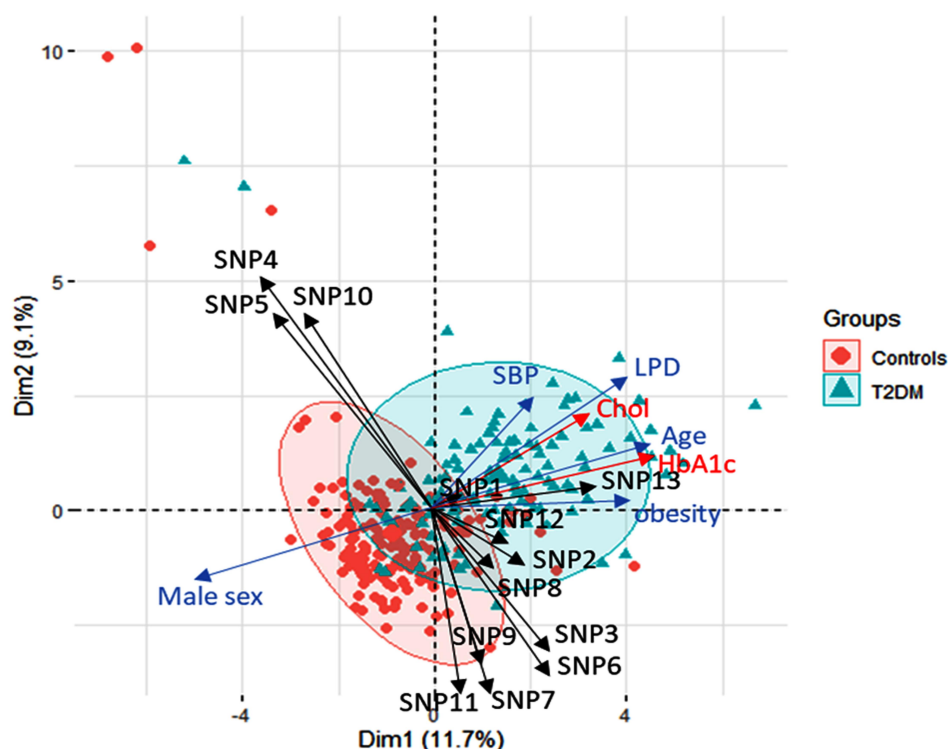


Figure 2 Principal Component analysis for data exploration. Data are plotted across axis 1 and 2, showing the variability of 11.7% and 9.1% of the data. Subjects are represented as circles (controls) or triangles (type 2 diabetic cohorts). Subjects close to each other had similar features to those dispersed apart. An oval ellipse was drawn around each group. Variables are indicated with arrow length, indicating the magnitude of strength, with a longer arrow indicating a greater influence of the factor. Genetic variants are shown in black arrows. Clinical data in blue while the red arrows represent the laboratory testing. All clinical and laboratory data were used in the plot, but fewer arrows with higher effects are only shown to avoid crowdedness. SNP1: *GSTM1* rs1056806, SNP2: *GSTT1* rs17856199, SNP3: *GSTP1* rs1695, SNP4: *MGST3* rs2065942, SNP5: *SOD1* rs2234694, SNP6: *SOD2* rs4880, SNP7: *SOD3* rs2536512, SNP8: *CAT* rs7943316, SNP9: *GPX1* rs1800668, SNP10: *GPX3* rs3828599, SNP11: *GPX4* rs713041, SNP12: *NOS3* rs1799983, SNP13: *NOS2* rs2297518.

Abbreviations: HbA1c, glycosylated hemoglobin; LPD, hyperlipidemia; SBP, systolic blood pressure; Chol, total cholesterol; T2DM, type 2 diabetes mellitus.

Although this variant showed association with T2DM in Caucasians,³⁵ the significant association in the population under the study, to our knowledge, is the first to be identified.

Apart from the SNPs mentioned above, there were no statistically significant differences in the distribution of other studied genetic variants and no impact of these variants on the present cohort's biochemical parameters. These findings are similar to some previous reports,^{13,18,20} but inconsistent with others.^{13,21,56} These susceptibility differences could be contributed by several factors, including the enrolled population's genetic background, the study design and sample size, the genotyping method, and the presence of diabetic complications, among others.

Interestingly, we found that having both *GSTT1* rs17856199*A and *NOS2* rs2297518*A variants were 3.96 times more likely to have DM while carrying both *GSTT1* rs17856199*C and *NOS2* rs2297518*A variants had three times higher susceptibility of developing T2DM. These findings are consistent with the

previous observations, which reported that the “combined or additive effect of multiple gene variants from distinct loci might account for a greater proportion of the disease risk.”^{57,58} Risk alleles interaction in these antioxidant-related gene variants might impact the overall antioxidant activities, thus allowing more susceptibility to oxidative stress and increasing the overall disease risk as proposed by Decharatchakul and colleagues.⁵⁹

The *SOD2* rs4880 GG, and *SOD3* rs2536512 AA genotypes, though they did not show a significant association with T2DM risk in the present cohort, they showed significant association with BMI and/or hyperlipidemia (Table 5), and the former variant also showed an association with blood glucose levels (Table S2). Interestingly, these results are consistent with a recent study that identified the same genotypes' association with increased hypertriglyceridemia risk combined with low high-density lipoprotein cholesterol levels.⁵⁹ *SOD2* rs4880 variant causes valine substitution of alanine in “mitochondrial targeting sequence” as mentioned

previously, affecting the normal importing process of the enzyme into the mitochondrial matrix with a partial halt of the precursor within the inner mitochondrial membrane and decline in active SOD2 enzyme formation with concomitant lower enzyme activity.⁶⁰ Also, other studies suggested that this variant might not be associated with T2DM pathogenesis but could be related to diabetic microangiopathy in terms of microalbuminuria⁶¹ or macular edema.⁶² Dong et al demonstrated that the *SOD3* rs2536512 variant might affect the enzyme plasma activity, increasing susceptibility to oxidative stress associated with obesity.⁶³ The latter finding could, in part, support the association of this SNP with BMI in the present study. Given the essential roles NO plays, including regulation of endothelial cell function and inhibition of the vascular tone and oxidized LDL-C formation, NO production impairment due to *NOS2* rs2297518 variant,³² could result in insulin resistance, T2DM, hypertension, and hypercholesterolemia.⁶⁴ This can also support the significant association of this variant with hyperlipidemia in the present work consistent with the recent report that identified this missense variant as a genetic risk for vulnerable carotid plaque in the Chinese population.⁶⁵

Taken together, although the present study is limited by the relatively small sample size, the cross-sectional design, and lack of inclusion of other known oxidative stress-related variants, the study findings, to the best of our knowledge, are the first to identify the potential association of the oxidative stress-related *GSTT1* rs17856199 and *NOS2* rs2297518 gene variants, individually or in combination, with susceptibility to T2DM in the current population. Also, *SOD2* rs4880, *SOD3* rs2536512, and *NOS2* rs22975 showed significant association with BMI and/or hyperlipidemia. Testing the association of these gene variants with T2DM and considering other genetic and non-genetic factors could help patient stratification by the risk and be scheduled as molecular targets in future individualized therapy. Further large-scale multicenter studies are recommended to confirm the findings.

Data Sharing Statement

All data generated or analyzed during this study are included in this submitted article and [Supplementary Materials](#).

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the IRB at the King Fahd Armed Forces

Hospital (approval No. REC 201), Jeddah, KSA, and informed consent was given by all the patients. Parental informed consent was obtained for participants under the age of 18 (if any).

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article has been submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest for this work.

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