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ORIGINAL ARTICLE

Oxidative Stress Markers and Divergent SOD1/SOD2 mRNA Expression in Type 2 Diabetes: A Case–Control Study

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is accompanied by oxidative stress, but few studies have examined lipid peroxidation, erythrocyte superoxide dismutase (SOD) activity, and SOD1/SOD2 mRNA expression together. In this cross-sectional case–control study, serum malondialdehyde (MDA) was measured using a TBARS assay, erythrocyte SOD activity by pyrogallol autoxidation inhibition, and peripheral blood mononuclear cell SOD1 and SOD2 mRNA expression by RT-qPCR. The primary analysis included 174 participants after eligibility review (114 with T2DM and 60 controls). Analyses of glycaemic tiers (n = 240), disease duration and treatment regimen (n = 120), and metabolic-syndrome strata (n = 235) were exploratory. All four markers differed between the T2DM and control groups (all $p < 0.001$). After adjustment for BMI and smoking, the T2DM group had higher MDA (adjusted ratio 3.02, 95% CI 2.55–3.57), lower erythrocyte SOD activity (0.64, 0.57–0.72), lower SOD1 mRNA expression (0.39, 0.30–0.49), and higher SOD2 mRNA expression (1.91, 1.55–2.37); all adjusted $p < 0.001$. Significant ordered trends were observed across glycaemic tiers and disease-duration tertiles. No treatment-regimen comparison remained significant after correction for multiple testing. Controls with and without metabolic syndrome did not differ on any marker. T2DM was associated with greater lipid peroxidation, reduced erythrocyte SOD activity, lower SOD1 mRNA expression, and higher SOD2 mRNA expression in peripheral blood mononuclear cells. The exploratory findings and case–control discrimination model require validation in independent cohorts.

Keywords: Type 2 diabetes mellitus, Malondialdehyde, Superoxide dismutase, Oxidative stress, Glycaemic spectrum, Metabolic syndrome

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Introduction

Type 2 diabetes mellitus (T2DM) accounts for more than 90% of diabetes cases. An estimated 537 million adults were living with diabetes in 2021, and this number is projected to reach 784 million by 2045(1). T2DM is characterized by insulin resistance, impaired pancreatic β-cell function, and chronic hyperglycaemia (2,3). Its major complications include nephropathy, retinopathy, neuropathy, and cardiovascular disease, which contribute to mortality, organ failure, and healthcare costs (3,4). Vascular injury may progress despite glucose-lowering treatment, suggesting that glycaemic control does not fully address all processes associated with chronic hyperglycaemia (5–7). The pathways linking chronic hyperglycaemia to tissue damage remain incompletely understood.

Oxidative stress results from an imbalance between reactive oxygen species (ROS) production and antioxidant defence and is associated with hyperglycaemia-related tissue injury (7,8). Hyperglycaemia can increase ROS production through the polyol, advanced glycation end-product, protein kinase C, and hexosamine pathways (4). Malondialdehyde (MDA), a product of lipid peroxidation, is widely used as a circulating marker of oxidative damage (9). Circulating MDA has been associated with HbA1c, fasting plasma glucose, and diabetic complications (9).

Superoxide dismutase (SOD) is a major enzymatic defence against superoxide radicals (10). Three isoforms are expressed in mammals: Three SOD isoforms are expressed in mammals: cytosolic and nuclear SOD1 (CuZnSOD), mitochondrial SOD2, and extracellular SOD3 (10). Erythrocyte SOD activity, which mainly reflects SOD1 activity, provides an accessible measure of antioxidant capacity. Previous studies have reported lower erythrocyte SOD activity in T2DM and associations with HbA1c, disease duration, and microvascular complications (11–13). SOD2 genetic variation, including the Val16Ala substitution, may alter mitochondrial import of SOD2 and has been associated with T2DM and its complications (14,15). These associations do not establish a causal role for SOD.

Less is known about SOD1 and SOD2 transcription in circulating cells. Peripheral blood mononuclear cells (PBMCs) are exposed to the circulating metabolic environment and provide an accessible source for measuring gene expression (16). However, evidence combining PBMC SOD1 and SOD2 mRNA expression with circulating lipid peroxidation and erythrocyte SOD activity in the same participants remains limited. It remains unclear whether T2DM affects antioxidant defence uniformly or produces different changes in SOD isoforms and biological compartments.

This study measured TBARS-derived serum MDA, erythrocyte SOD activity, and PBMC SOD1 and SOD2 mRNA expression. The primary objective was to compare these markers between participants with T2DM and controls, including adjustment for BMI and smoking. Exploratory secondary analyses assessed marker patterns across glycaemic and disease-duration groups, treatment regimens, and metabolic-syndrome categories. The internally cross-validated discrimination of individual and combined oxidative markers was also evaluated.

Methods

Sex matching. Unequal exclusions during the eligibility review described in section 2.2 disrupted the matching structure. The primary dataset therefore included 174 participants (114 T2DM and 60 controls) and was analyzed using unmatched methods. Exclusion details are presented in Table 1.

No a priori sample-size calculation was performed; all eligible participants were included. For the primary two-group comparison (114 T2DM and 60 controls), a retrospective minimum-detectable-effect calculation indicated 80% power at two-sided $\alpha = 0.05$ to detect $|\text{Cliff's } \delta| \geq 0.26$ using the normal approximation to the Mann–Whitney U statistics.

Participant eligibility criteria

Participants were aged 30–65 years. T2DM cases were recruited from the outpatient diabetes clinic. Eligibility required an existing clinical diagnosis of T2DM, verified during the eligibility review by FPG ≥7.0 mmol/L or HbA1c ≥6.5%, according to the American Diabetes Association criteria (2). Controls were recruited from individuals attending general health assessments at the same institution and had no history of diabetes.

They were excluded if FPG was ≥5.6 mmol/L or, among those with FPG <5.6 mmol/L, if HbA1c was

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≥6.5%. Controls with FPG <5.6 mmol/L and HbA1c of 5.7–6.4% were retained because the predefined exclusion criteria did not exclude prediabetes defined solely by HbA1c.

Table 1. Eligibility exclusions applied post-enrolment (n = 240 → 174). FPG = fasting plasma glucose; ADA = American Diabetes Association criteria.

Criterion	Definition	n meeting criterion	Groups affected
Age outside 30–65 years	Measured age at enrolment	10	Both (7 ctrl, 3 T2DM)
FPG ≥ 5.6 mmol/L	ADA impaired fasting glucose threshold	51	Controls only
HbA1c ≥ 6.5% (FPG < 5.6)	ADA diabetes threshold; FPG below IFG cut-off	7	Controls only
T2DM diagnostic criteria not met	FPG < 7.0 mmol/L AND HbA1c < 6.5%	3	T2DM only
Analytic dataset retained	114 T2DM + 60 controls	174	—

Exclusion criteria were not mutually exclusive. Five control participants outside the eligible age range also met a glycaemic exclusion criterion. Therefore, the 71 criterion occurrences corresponded to 66 unique excluded participants: 60 controls and six participants with clinically diagnosed T2DM.

A prespecified sensitivity analysis was conducted using the 44 controls with both FPG <5.6 mmol/L and HbA1c <5.7%. Exclusion criteria for all participants were type 1, secondary, or gestational diabetes; chronic kidney disease; hepatic disease; active malignancy; haematological disorders; and regular antioxidant-supplement use during the preceding three months. These conditions were excluded because they could affect oxidative status. The study workflow, including participant selection, laboratory assessments, statistical analyses, and the principal findings, is summarized in Figure 1.

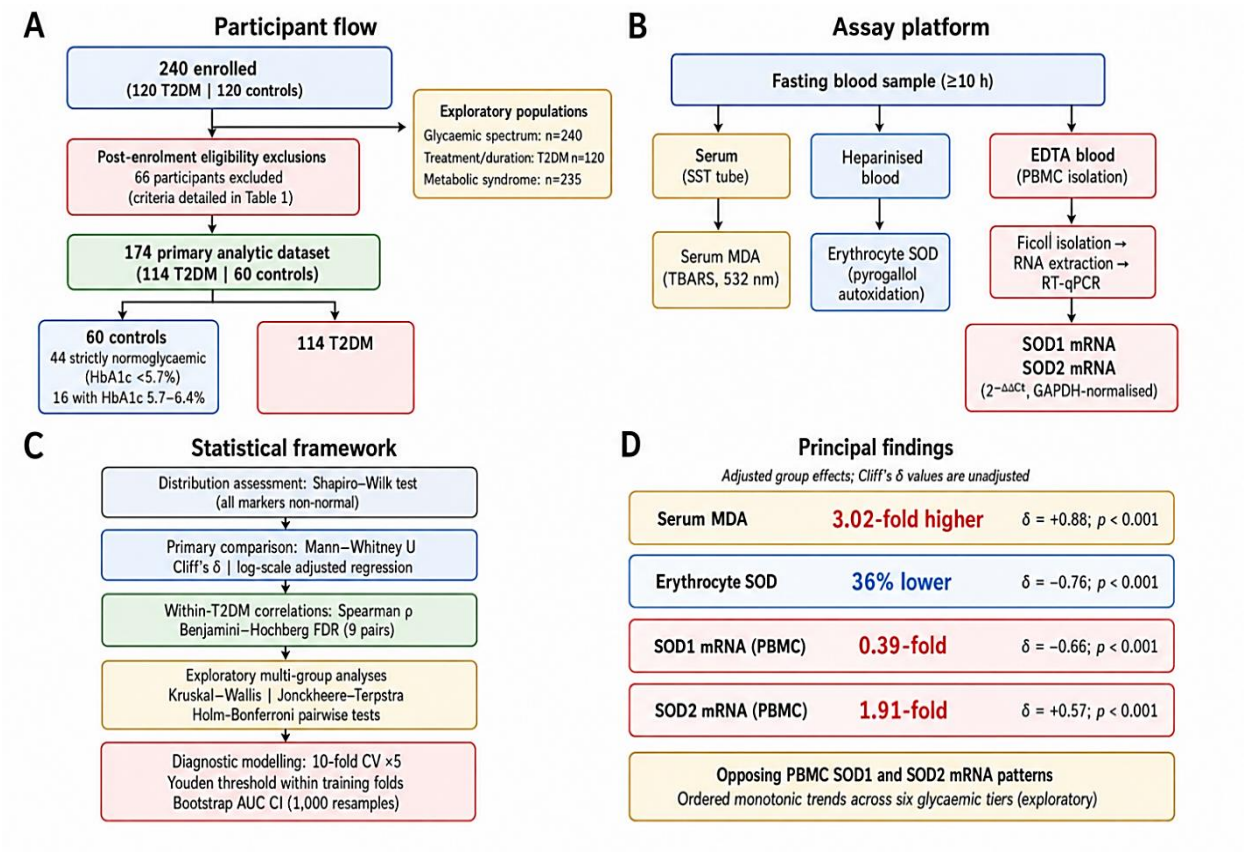

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Figure 1. Study overview. Four-panel schematic showing (A) participant flow and analytic populations; (B) laboratory assessment of serum MDA, erythrocyte SOD activity, and PBMC SOD1 and SOD2 mRNA expression; (C) primary and secondary statistical analyses; and (D) summary of the principal study findings. AUC, area under the curve; BMI, body mass index; FDR, false discovery rate; MDA, malondialdehyde; PBMC, peripheral blood mononuclear cell; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; TBARS, thiobarbituric acid-reactive substances.

Clinical and biochemical characterization

A questionnaire recorded disease duration, treatment, antihypertensive use, and smoking. Current smoking was defined as ≥1 cigarette/day for ≥6 months. Weight and height were measured in light clothing without footwear; BMI was calculated as kg/m². Blood pressure was the mean of two seated readings taken five minutes apart with a calibrated aneroid sphygmomanometer. After a ≥10-hour fast, 10 mL of venous blood was collected; insulin-treated participants were sampled before the morning dose. Samples were processed within two hours and allocated to EDTA tubes (4 mL; haematology, HbA1c, and PBMC isolation), sodium heparin tubes (3 mL; erythrocyte preparation and SOD assay), and serum-separator tubes (3 mL; FPG, lipids, and MDA assay).

Fasting plasma glucose was measured by automated glucose oxidase assay and HbA1c by high-performance liquid chromatography. Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were measured enzymatically. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation when triglycerides were <4.5 mmol/L. Haemoglobin was measured by the cyanomethaemoglobin method. Metabolic syndrome was defined by the 2009 Joint Scientific Statement criteria, with BMI ≥30 kg/m² as a prespecified surrogate because waist circumference was not measured. Other components were triglycerides ≥1.7 mmol/L; HDL <1.0 mmol/L in men or <1.3 mmol/L in women; blood pressure ≥130/85 mmHg or antihypertensive treatment; and FPG ≥5.6 mmol/L or diabetes treatment.

Serum malondialdehyde assay

Serum MDA was estimated by the modified TBARS assay of Ohkawa et al. (17). Serum was separated by centrifugation (1,500 × g, 10 minutes, 4°C) and stored at −20°C for ≤4 weeks. Serum (0.2 mL) was mixed with 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of 0.8% thiobarbituric acid. The volume was adjusted to 4.0 mL with water and heated at 95–100°C for 60 minutes. After cooling, 1.0 mL of water and 5.0 mL of n-butanol:pyridine (15:1, v/v) were added. The mixture was vortexed and centrifuged at 1,000 × g for 10 minutes. Upper-layer absorbance at 532 nm against a blank was converted to TBARS-derived MDA using a 1,1,3,3-tetraethoxypropane curve (µmol/L). Duplicate measurements were averaged; a coefficient of variation >5% prompted repetition.

Erythrocyte superoxide dismutase activity assay

Erythrocyte SOD was assayed by pyrogallol autoxidation inhibition (9). Erythrocytes were isolated from heparinized blood (600 × g, 10 minutes, 4°C), washed three times with phosphate-buffered saline (0.9% NaCl, pH 7.4), and lysed with four volumes of ice-cold water. After centrifugation (10,000 × g, 15 minutes, 4°C), haemolysate was stored at −80°C. The assay contained 2.95 mL of 0.05 M Tris-HCl (pH 8.2), 20 µL of 1:100 haemolysate, and 30 µL of pyrogallol (final concentration, 0.3 mM). Absorbance at 420 nm was monitored for five minutes at 25°C against a haemolysate-free control. One unit equaled 50% inhibition/min/mL. SOD was expressed as U/gHb; haemoglobin was measured by the cyanomethaemoglobin method at 540 nm. Samples were assayed in duplicate.

PBMC isolation, RNA extraction, and RT-qPCR for SOD1 and SOD2

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood over Ficoll-Paque (1.077 g/mL; 400 × g, 30 minutes, room temperature; no brake). Cells were washed twice with phosphate-buffered saline (300 × g, 10 minutes), snap-frozen in liquid nitrogen, and stored at −80°C. Processing was completed within three hours of venepuncture. RNA was extracted using a column kit with DNase I digestion. Inclusion required A260/A280 ≥1.8, A260/A230 ≥1.9, and visible 28S and 18S bands at an approximate 2:1 ratio; other samples were excluded. cDNA was synthesized from 500 ng RNA using

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random hexamers at 25°C for 10 minutes, 42°C for 60 minutes, and 70°C for 10 minutes. Duplicate SYBR Green qPCR used 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

A 65–95°C melt curve in 0.5°C increments assessed specificity. Exon-junction primers targeted SOD1 (gene ID 6647), SOD2 (gene ID 6648), and GAPDH. Serial dilution of pooled cDNA confirmed 95–105% amplification efficiency. Fold change was calculated by the 2^−ΔΔCt method, normalized to GAPDH and the control-group median. Replicates differing by >0.5 cycles were repeated; target Ct values >35 were excluded. Primer sequences, and amplicon sizes are presented in the supplementary Table S1.

Analytic populations and exploratory categorisation framework

The primary population included 174 eligible participants. This population was used for group comparisons, adjusted models, within-T2DM correlations, and discrimination analyses. Exploratory populations comprised all enrollees for the glycaemic spectrum (n = 240), clinically diagnosed T2DM participants for treatment and duration analyses (n = 120), and the metabolic-syndrome analysis (n = 235). These populations were non-confirmatory. Excluded participants did not re-enter the primary dataset.

Scheme 1: Glycaemic spectrum. Participants were assigned to six mutually exclusive tiers using ADA FPG and HbA1c thresholds (Table 2). Screen-detected T2DM (G2b) required FPG ≥7.0 mmol/L or HbA1c ≥6.5%, without previous diabetes diagnosis or glucose-lowering treatment.

Table 2. Glycaemic spectrum tier definitions (Scheme 1). All 240 enrolled participants allocated to six tiers on the basis of ADA fasting plasma glucose and HbA1c thresholds.

Tier	Label	ADA glycaemic threshold	Diagnosis status	n
G1	Normoglycaemic	HbA1c < 5.7% AND FPG < 5.6 mmol/L	No diabetes	45
G2	Prediabetes	HbA1c 5.7–6.4% OR FPG 5.6–6.9 mmol/L	No diabetes	44
G2b	Screen-detected T2DM	FPG ≥ 7.0 mmol/L OR HbA1c ≥ 6.5%	Undiagnosed, untreated	31
G3	Well-controlled T2DM	HbA1c < 7.0% (on treatment)	Known, treated	22
G4	Suboptimal T2DM	HbA1c 7.0–9.0% (on treatment)	Known, treated	51
G5	Poorly controlled T2DM	HbA1c > 9.0% (on treatment)	Known, treated	47

Scheme 2: Treatment regimen. T2DM participants were grouped as metformin monotherapy, metformin plus sulphonylurea, or insulin-based treatment, including insulin alone or with oral agents.

Scheme 3: Disease duration. Duration from diagnosis to enrolment was classified as short (1.4–5.9 years), medium (6.0–9.7 years), or long (9.8–30.0 years), with 40 participants per group. Duration and HbA1c showed limited correlation (Spearman ρ = 0.19).

Scheme 4: Metabolic syndrome. Groups comprised controls without metabolic syndrome (n = 97), controls with metabolic syndrome (n = 23), and T2DM with metabolic syndrome (n = 115), using the criteria in section 2.3. Five T2DM participants without metabolic syndrome were omitted because reliable inference was not possible.

K-means clustering (k = 2–6) of BMI, HbA1c, FPG, triglycerides, HDL, and age at diagnosis was rejected because all silhouette scores were <0.22.

Statistical methods

Analyses were conducted in Python 3.12 using pandas, scipy, statsmodels, and scikit-learn according to a plan finalized before eligibility exclusions. All marker distributions were non-normal by Shapiro–Wilk testing (all $p < 0.05$); primary analyses therefore used non-parametric methods. Primary comparisons used two-sided Mann–Whitney U tests. Effect size was reported as Cliff's delta (δ): negligible, |δ| <0.147;

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small, 0.147–0.329; medium, 0.330–0.473; and large, ≥0.474. The four prespecified primary comparisons were not adjusted for multiplicity. Log-scale ordinary least-squares models included case–control status, BMI, and current smoking. MDA was natural-log transformed; SOD activity and SOD1 and SOD2 fold changes were $\log_2$ transformed. HbA1c was excluded from the primary model because it partially defined case status and could be an intermediate variable; a sensitivity model included HbA1c. Coefficients were exponentiated to fold changes with 95% confidence intervals. Variance inflation factors <3 indicated limited collinearity.

Statin, ACE inhibitor/ARB, and aspirin exposure, physical activity, diet, and vitamin intake were unavailable for consistent adjustment. Regular antioxidant-supplement use was an exclusion criterion. Data on other medications, physical activity, diet, and vitamin intake were insufficient for covariate adjustment. Nine prespecified Spearman correlations were assessed within T2DM: MDA with HbA1c, duration, SOD, and SOD2; SOD with HbA1c; SOD1 with HbA1c and SOD; and SOD2 with duration and HbA1c. Benjamini–Hochberg correction was applied across nine tests, with $q < 0.05$ indicating significance. Ordered groups in Schemes 1 and 3 were assessed using Kruskal–Wallis and two-sided Jonckheere–Terpstra trend tests. Schemes 2 and 4 were assessed using Kruskal–Wallis tests only. Pairwise Mann–Whitney U tests used Holm–Bonferroni correction within each scheme and marker. Cliff's delta was calculated for each comparison. Logistic regression evaluated individual and combined oxidative markers in the primary dataset (n = 174) using stratified 10-fold cross-validation repeated five times. The Youden-optimal threshold was selected in each training fold and applied to its test fold. Cross-validated AUC was the primary metric; 95% confidence intervals used 1,000 stratified bootstrap resamples. HbA1c and FPG were included only as reference anchors because they partially defined case status. Sensitivity analyses comprised restriction to the 44 strictly normoglycaemic controls (HbA1c < 5.7%); exclusion of participants with either MDA or SOD values below the pooled cohort-wide 5th percentile or above the pooled 95th percentile (n = 144); $\log_2$ -scale SOD regression using HC3 heteroscedasticity-consistent standard errors; and MDA regression additionally adjusted for total cholesterol, LDL, HDL, and triglycerides. Two-sided $p < 0.05$ indicated significance unless FDR or Holm–Bonferroni correction was applied. Non-parametric outcomes are reported as medians and interquartile ranges.

Results

Study participants and data cleaning

Post-enrolment eligibility review excluded 66 participants, leaving 174 in the primary analytic dataset (114 with T2DM and 60 controls). Exclusion reasons are presented in Table 1. Because unequal exclusions disrupted the intended matching, unmatched analyses were used. Among the retained controls, 44 were strictly normoglycaemic and 16 had HbA1c values within the prediabetic range. Age, sex distribution, and current smoking did not differ significantly between groups. Participants with T2DM had higher BMI, FPG, HbA1c, systolic and diastolic blood pressure, total cholesterol, triglycerides, and LDL cholesterol; lower HDL cholesterol and haemoglobin; and more frequent antihypertensive use (all $p < 0.001$; Table 3). Among controls, median FPG was 4.06 mmol/L (IQR 2.86–4.79) and median HbA1c was 5.10% (IQR 4.28–5.70).

Oxidative stress markers: T2DM versus controls

All four markers differed between groups (Table 4; Figures 2 and 3; all $p < 0.001$). Participants with T2DM had higher serum MDA and SOD2 mRNA expression and lower erythrocyte SOD activity and SOD1 mRNA expression. The SOD1 and SOD2 mRNA changes were in opposite directions.

Covariate-adjusted regression

All four group differences remained significant after adjustment for BMI and smoking (Table 4). Additional adjustment for HbA1c attenuated the estimates without changing their direction (Supplementary Table S6). HbA1c was not included in the primary model because it partially defines case status and may lie on the pathway between T2DM and oxidative stress. All variance inflation factors were below 3.0, indicating limited collinearity.

Within-group correlations in T2DM patients

Seven of nine pre-specified correlations remained significant after false discovery rate correction (Table 5; Supplementary Figure S1). The strongest associations linked MDA positively with disease

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duration and inversely with SOD activity; SOD1 mRNA also correlated positively with SOD activity. MDA–SOD2 and SOD2–HbA1c were not significant after correction.

Table 3. Baseline characteristics of the primary analytic cohort

Characteristic	T2DM (n = 114)	Control (n = 60)	Test	p value
Age, years	47.24 ± 7.51	45.84 ± 7.52	Welch t	0.244
Male sex, n (%)	66 (57.9)	34 (56.7)	χ^2	1.000
BMI, kg/m²	30.09 ± 4.34	27.10 ± 3.45	Welch t	<0.001
Disease duration, years	7.4 (5.4–10.8)	—	—	—
Current smoking, n (%)	38 (33.3)	13 (21.7)	χ^2	0.152
Antihypertensive use, n (%)	45 (39.5)	0 (0.0)	χ^2	<0.001
FPG, mmol/L	9.80 (8.40–11.71)	4.06 (2.86–4.79)	Mann–Whitney U	<0.001
HbA1c, %	8.65 (7.60–9.80)	5.10 (4.28–5.70)	Mann–Whitney U	<0.001
Systolic blood pressure, mmHg	137.28 ± 15.19	121.95 ± 10.08	Welch t	<0.001
Diastolic blood pressure, mmHg	86.03 ± 9.09	79.80 ± 8.55	Welch t	<0.001
Total cholesterol, mmol/L	5.19 ± 1.11	4.55 ± 0.64	Welch t	<0.001
Triglycerides, mmol/L	2.13 ± 0.97	1.31 ± 0.53	Welch t	<0.001
HDL cholesterol, mmol/L	0.99 ± 0.21	1.28 ± 0.33	Welch t	<0.001
LDL cholesterol, mmol/L	3.47 ± 0.98	2.75 ± 0.57	Welch t	<0.001
Haemoglobin, g/dL	13.16 ± 1.55	13.97 ± 1.47	Welch t	<0.001

Values are mean ± SD, median (IQR), or n (%). Continuous variables were compared using Welch's t-test when normally distributed in both groups and the Mann–Whitney U test otherwise. Categorical variables were compared using continuity-corrected χ^2 tests. BMI, body mass index; FPG, fasting plasma glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; T2DM, type 2 diabetes mellitus.

Table 4. Primary biomarker comparisons and covariate-adjusted estimates

Marker	T2DM, median (IQR)	Control, median (IQR)	Cliff's δ	Adjusted ratio (95% CI)
Serum MDA, µmol/L	6.41 (4.85–9.36)	2.24 (1.59–3.19)	+0.88	3.02 (2.55–3.57)
Erythrocyte SOD, U/gHb	859 (705–1016)	1349 (1108–1559)	−0.76	0.64 (0.57–0.72)
SOD1 mRNA, fold change	0.34 (0.23–0.55)	1.00 (0.61–1.96)	−0.66	0.39 (0.30–0.49)
SOD2 mRNA, fold change	2.01 (1.24–2.98)	1.00 (0.66–1.48)	+0.57	1.91 (1.55–2.37)

Values are median (IQR). Cliff's δ and unadjusted p values are based on Mann–Whitney U comparisons. Adjusted ratios compare T2DM with controls in models including BMI and current smoking. All unadjusted and adjusted p values were <0.001. MDA was natural-log transformed; erythrocyte SOD activity and SOD1 and SOD2 mRNA expression were $\log_2$ transformed. BMI, body mass index; CI, confidence interval; IQR, interquartile range; MDA, malondialdehyde; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; U/gHb, units per gram haemoglobin.

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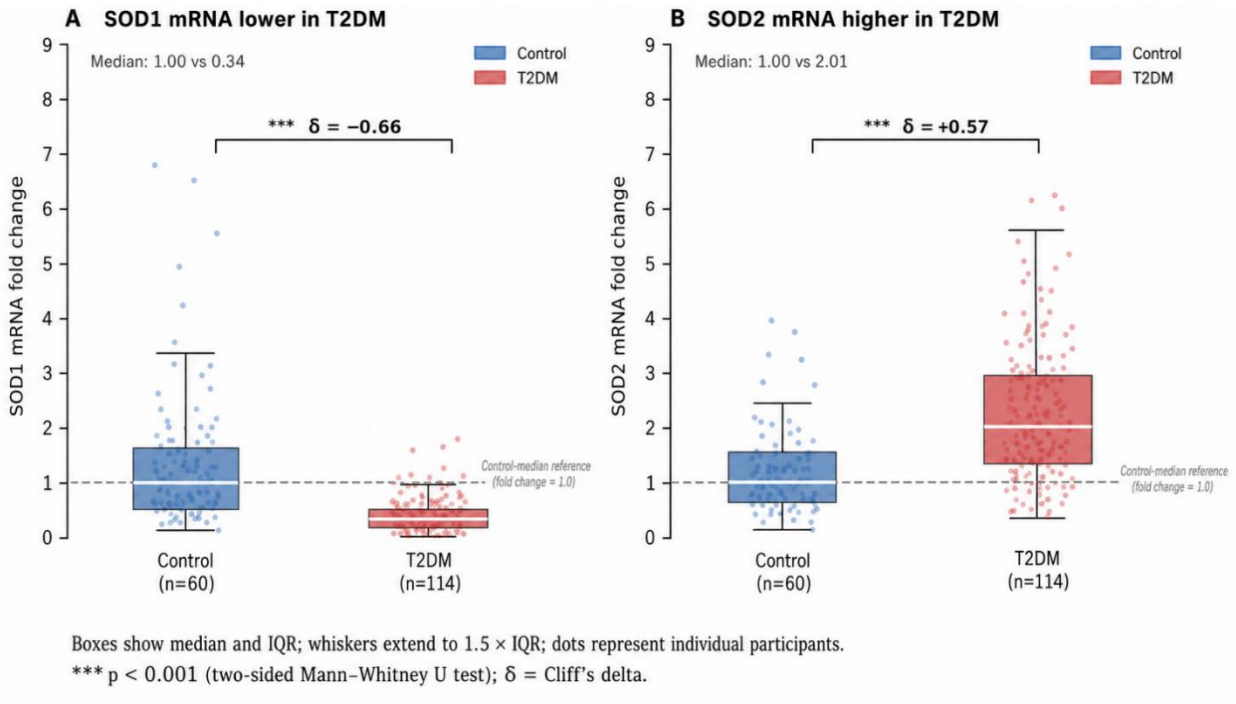

Figure 2. Serum malondialdehyde and erythrocyte superoxide dismutase activity in participants with T2DM and controls. The primary analytic dataset included 114 participants with T2DM and 60 controls. (A) Serum MDA and (B) erythrocyte SOD activities are presented. Boxes represent medians and IQRs; whiskers extend to 1.5 × IQR, and points represent individual participants. Numerical results are reported in Table 4. ***IQR, interquartile range; MDA, malondialdehyde; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus.***

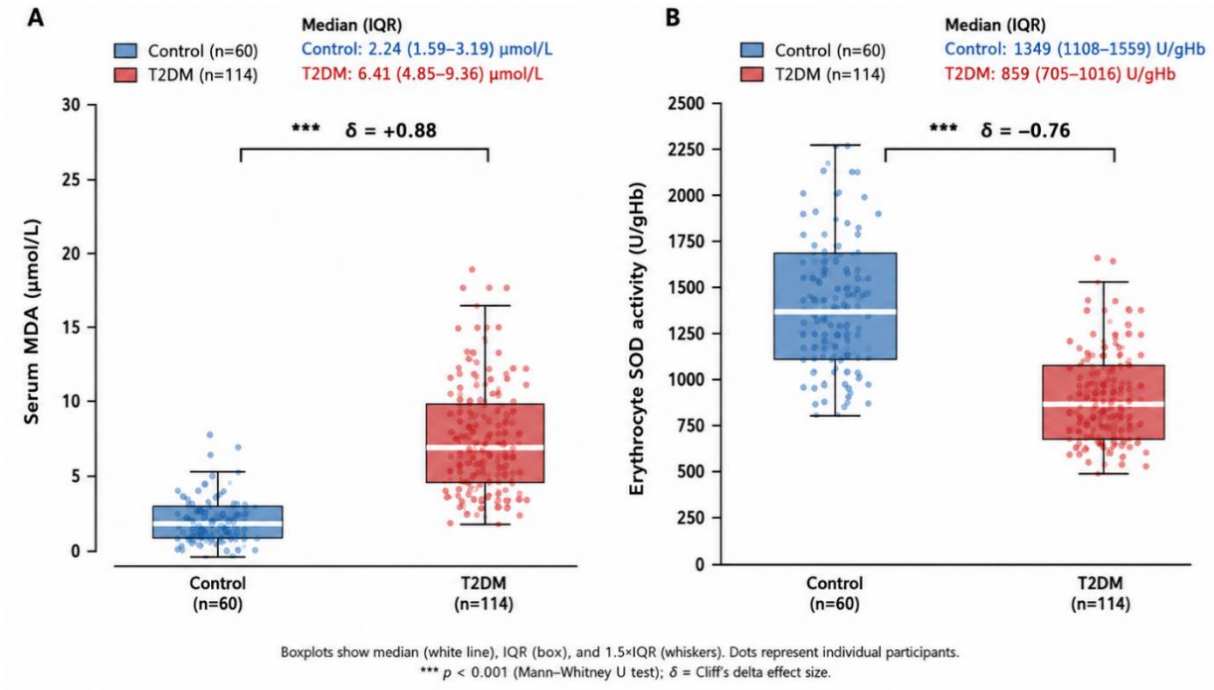

Figure 3. SOD1 and SOD2 mRNA expression in participants with T2DM and controls. The primary analytic dataset included 114 participants with T2DM and 60 controls. (A) SOD1 and (B) SOD2 mRNA fold-change values were calculated using the 2^−ΔΔCt method, normalized to GAPDH, and calibrated to the control-group median. Boxes represent medians and IQRs; whiskers extend to 1.5 × IQR, and points represent individual

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participants. The dashed line indicates the control-median reference at fold change = 1.0. Numerical results are reported in Table 4. ***GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IQR, interquartile range; PBMC, peripheral blood mononuclear cell; RT-qPCR, reverse-transcription quantitative PCR; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; T2DM, type 2 diabetes mellitus.***

Table 5. Spearman rank correlations within the T2DM group (n = 114). FDR correction applied across all nine pairs using the Benjamini-Hochberg procedure.

Pair	Spearman ρ	Raw p	BH-FDR q	Significant?
MDA – HbA1c	+0.55	<0.001	< 0.05	Yes
MDA – Disease duration	+0.69	<0.001	< 0.05	Yes
MDA – SOD activity	−0.64	<0.001	< 0.05	Yes
MDA – SOD2 mRNA	+0.17	0.076	0.076	No
SOD activity – HbA1c	−0.47	<0.001	< 0.05	Yes
SOD1 mRNA – HbA1c	−0.29	0.002	< 0.05	Yes
SOD1 mRNA – SOD activity	+0.58	<0.001	< 0.05	Yes
SOD2 mRNA – Disease duration	+0.39	<0.001	< 0.05	Yes
SOD2 mRNA – HbA1c	+0.18	0.052	0.059	No

Sensitivity analyses

Sensitivity analyses supported the direction and statistical significance of the primary findings (Supplementary Table S6). Restriction to the 44 strictly normoglycaemic controls yielded adjusted T2DM-to-control estimates of 3.05-fold for MDA (95% CI 2.54–3.67), 0.65-fold for erythrocyte SOD activity (0.58–0.72), 0.42-fold for SOD1 (0.32–0.55), and 1.79-fold for SOD2 (1.41–2.28); all $p < 0.001$. Additional adjustment for HbA1c attenuated the estimates, but all remained significant: MDA, 1.74-fold (1.37–2.22); SOD, 0.76-fold (0.66–0.88); SOD1, 0.49-fold (0.34–0.72); and SOD2, 1.78-fold (1.27–2.49); all $p < 0.001$.

After excluding participants with either MDA or SOD values outside the pooled cohort-wide 5th–95th percentiles (n = 144), MDA remained 2.33-fold higher (95% CI 1.98–2.74) and SOD activity remained lower by 320 U/gHb (95% CI −408 to −232) in T2DM; both $p < 0.001$. Log_2 -scale SOD regression using HC3 standard errors produced a T2DM-to-control estimate of 0.64-fold (95% CI 0.58–0.70; $p < 0.001$). Additional adjustment of the MDA model for total cholesterol, LDL, HDL, and triglycerides yielded an estimate of 3.26-fold (95% CI 2.62–4.06; $p < 0.001$), and none of the lipid covariates was statistically significant.

Exploratory glycaemic-spectrum analysis (enrolled cohort; n = 240)

All 240 enrolled participants were allocated to the six glycaemic tiers defined in Table 2. Marker distributions are shown in Figure 4, with numerical summaries and omnibus and trend-test results provided in Supplementary Table S2. All four markers differed across tiers and showed monotonic trends: MDA and SOD2 increased, whereas SOD activity and SOD1 decreased (all Kruskal–Wallis and Jonckheere–Terpstra $p < 0.001$). The three untreated tiers did not differ significantly. After Holm correction, all four markers differed between the screen-detected T2DM and treated, well-controlled T2DM groups.

Exploratory disease-duration analysis (enrolled T2DM subset; n = 120*)*

Disease-duration tertiles were balanced at 40 participants each. Marker distributions are shown in Figure 5, with numerical summaries and trend-test results provided in Supplementary Table S3. Disease duration and current HbA1c were weakly correlated (Spearman ρ = 0.19). All four markers showed significant ordered trends with disease duration: MDA and SOD2 increased, whereas SOD activity and SOD1

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decreased. After Holm correction, MDA differed across all three duration tertiles and SOD activity differed between the long-duration group and both shorter groups. SOD1 and SOD2 differed only between the short- and long-duration groups.

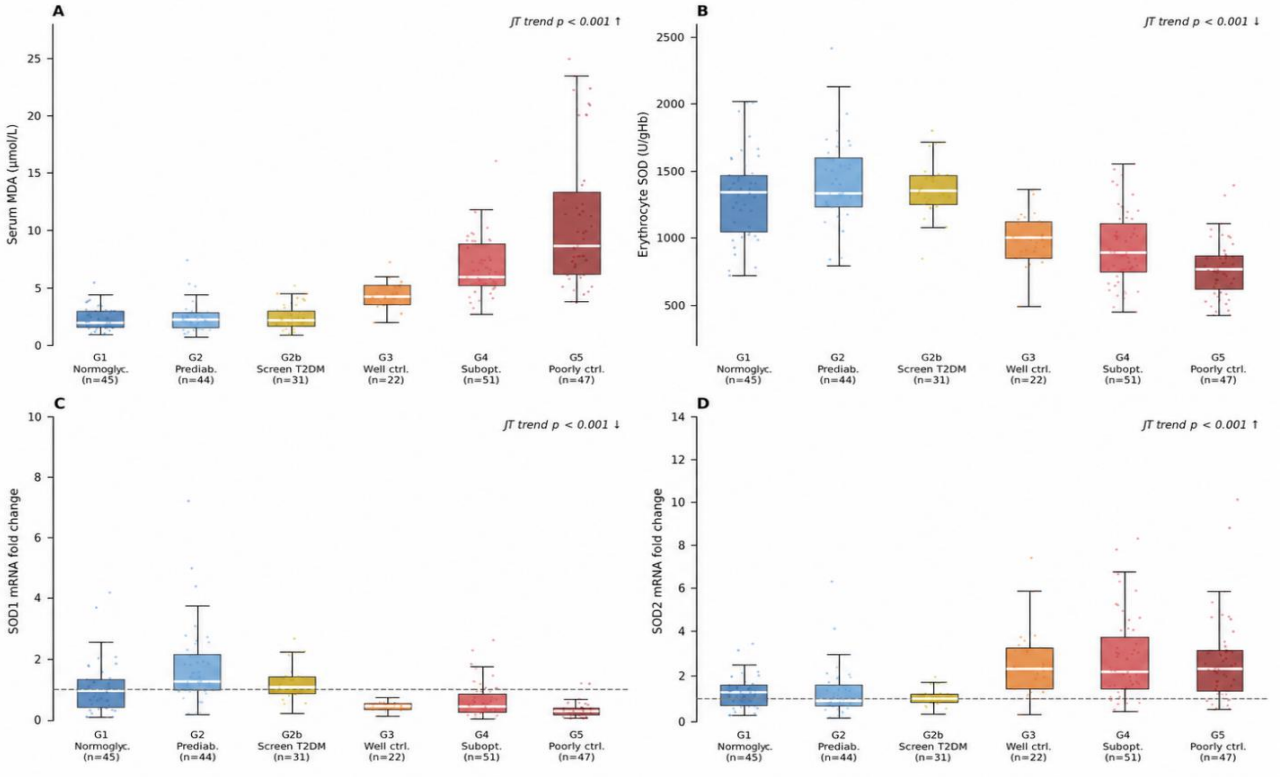

Figure 4. Oxidative-stress markers across six glycaemic categories. Box-and-strip plots show serum MDA, erythrocyte SOD activity, and PBMC SOD1 and SOD2 mRNA expression across the six categories in the enrolled cohort (n = 240). Centre lines indicate medians, boxes represent IQRs, whiskers extend to the most extreme values within 1.5 × IQR, and points represent individual participants. Glycaemic-category definitions are provided in Table 2. Numerical summaries and statistical tests are reported in Supplementary Table S2. ***IQR, interquartile range; MDA, malondialdehyde; PBMC, peripheral blood mononuclear cell; SOD, superoxide dismutase*****.**

Exploratory treatment-regimen analysis (enrolled T2DM subset; n = 120)

Numerical summaries by treatment regimen are provided in Supplementary Table S4. No pairwise comparison survived Holm correction; the nominal SOD2 omnibus signal (p = 0.027) therefore did not establish a regimen-specific difference. HbA1c values were similar across regimen groups.

Exploratory metabolic-syndrome analysis (n = 235)

Marker distributions are shown in Figure 6, with numerical summaries in Supplementary Table S5. All four markers differed across the three strata (Kruskal–Wallis p < 0.001). Holm-adjusted comparisons showed no significant differences between controls with and without metabolic syndrome, whereas both control subgroups differed from the T2DM-plus-metabolic-syndrome group for all four markers. Precision was limited for the smaller control subgroup with metabolic syndrome (n = 23).

Five participants with T2DM but without metabolic syndrome were excluded from this analysis because the subgroup was too small for reliable comparison.

Cross-validated case–control discrimination of oxidative stress markers

Cross-validated performance is summarized in Table 6. MDA was the strongest individual oxidative marker (CV-AUC 0.933, 95% CI 0.90–0.96), while the combined MDA–SOD model achieved the highest discrimination among the evaluated oxidative-marker

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models (CV-AUC 0.953, 95% CI 0.93–0.98). HbA1c and FPG showed higher discrimination because they partially defined case status; therefore, they are presented only as reference anchors and not as direct comparators.

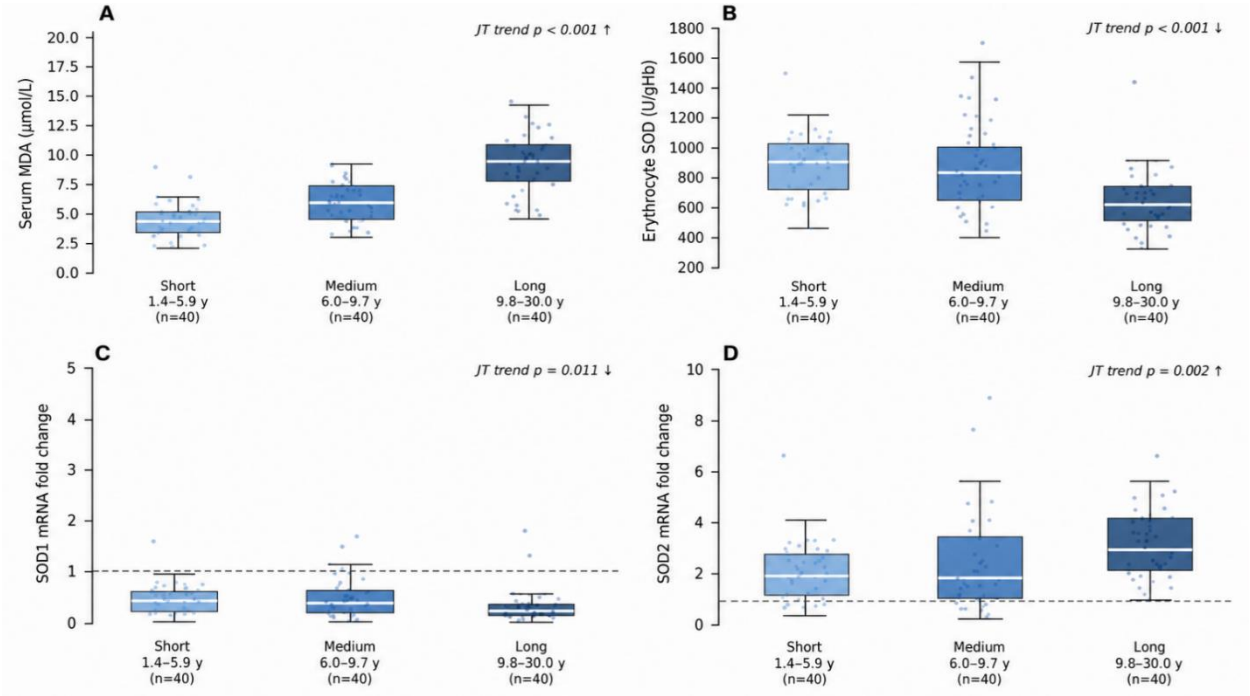

Figure 5. Oxidative-stress markers by disease-duration tertile in participants with T2DM. Box-and-strip plots show serum MDA, erythrocyte SOD activity, and PBMC SOD1 and SOD2 mRNA expression across short (1.4–5.9 years), medium (6.0–9.7 years), and long (9.8–30.0 years) disease-duration groups (n = 40 each). Boxes represent medians and IQRs; whiskers extend to 1.5 × IQR, and points represent individual participants. Dashed lines indicate the control-median reference at fold change = 1.0. Numerical summaries and statistical tests are reported in Supplementary Table S3. IQR, interquartile range; MDA, malondialdehyde; PBMC, peripheral blood mononuclear cell; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus.

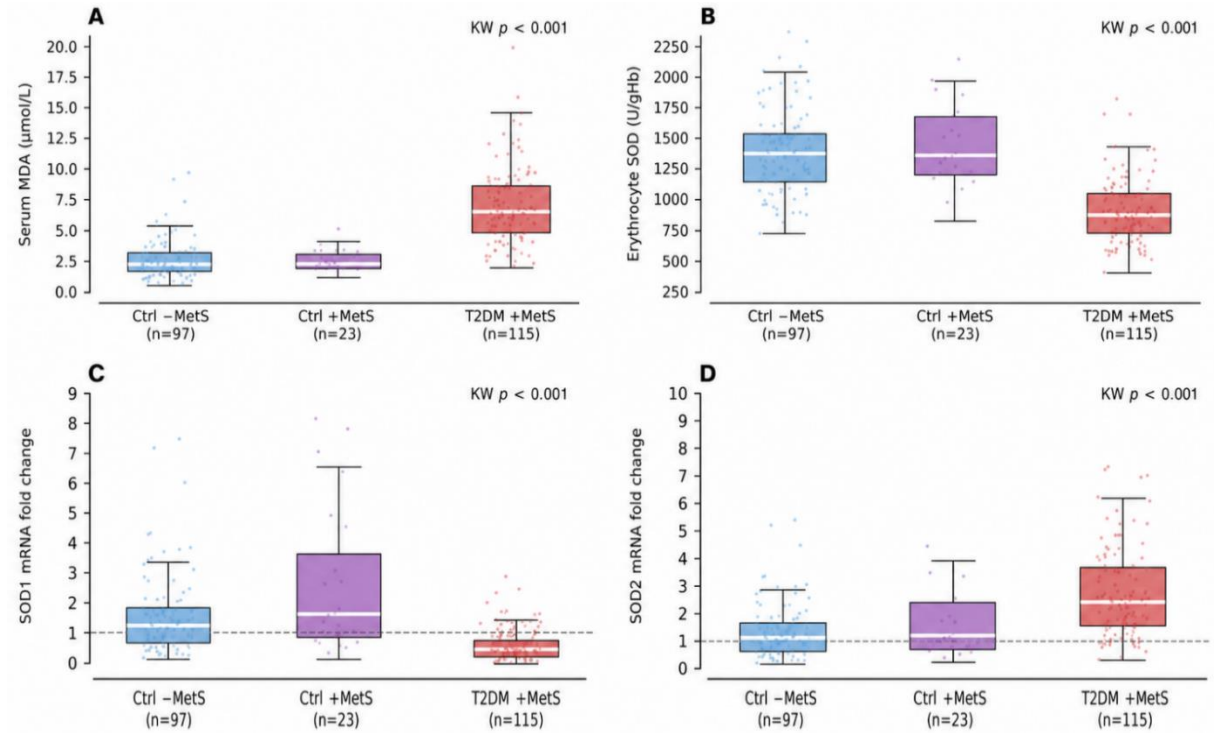

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Figure 6. Oxidative-stress markers across metabolic-syndrome strata. Box-and-strip plots compare controls without metabolic syndrome (n = 97), controls with metabolic syndrome (n = 23), and participants with T2DM and metabolic syndrome (n = 115). The five participants with T2DM but without metabolic syndrome were not included in this analysis. Boxes represent medians and IQRs; whiskers extend to 1.5 × IQR, and points represent individual participants. Dashed lines indicate the control-median reference at fold change = 1.0. Numerical summaries and statistical tests are reported in Supplementary Table S5. IQR, interquartile range; MDA, malondialdehyde; MetS, metabolic syndrome; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus.

Discussion

In this case–control study, T2DM was associated with higher serum MDA, lower erythrocyte SOD activity, lower PBMC SOD1 mRNA expression, and higher SOD2 mRNA expression. T2DM was associated with higher MDA, lower SOD activity, lower SOD1 expression, and higher SOD2 expression after adjustment for BMI and smoking. These findings were maintained in the sensitivity analyses. Exploratory analyses showed ordered marker variation across glycaemic categories and disease-duration tertiles, but no clear differences between treatment regimens or between controls with and without metabolic syndrome.

The higher MDA concentration in T2DM and its positive associations with HbA1c and disease duration agree with previous studies linking chronic hyperglycaemia to lipid peroxidation (17–21). Lower erythrocyte SOD activity and its inverse associations with HbA1c and MDA are also consistent with earlier T2DM studies (22). Possible explanations include oxidative inactivation, non-enzymatic glycation, and loss of copper or zinc cofactors (23). Low SOD activity has been associated with diabetic complications in other cohorts (24). However, the present study did not assess prognosis or complication risk.

The findings were maintained in the sensitivity analyses. Restriction to strictly normoglycaemic controls did not materially change the four group differences. The MDA and SOD findings also persisted after excluding values outside the pooled 5th–95th percentiles. Primary models adjusted for BMI and smoking; sensitivity models additionally considered HbA1c or lipid covariates. The SOD result was supported by HC3 modelling, and the MDA association remained significant after adjustment for total cholesterol, LDL, HDL, and triglycerides.

SOD1 and SOD2 mRNA expression changed in opposite directions. Higher SOD2 mRNA expression may reflect signaling through pathways involving FOXO3a–PGC-1α or NF-κB (25,26). SOD2 genetic variation may also contribute (13,14,27). These pathways and variants were not measured in this study. They should therefore be considered as hypotheses for further investigation.

Superoxide dismutase-1 (SOD1) mRNA expression was positively associated with erythrocyte SOD activity. This finding indicates parallel variation in two different biological compartments but does not show a regulatory relationship. Altered miR-146a/NF-κB signalling or glucose-dependent O-GlcNAcylation may contribute to changes in SOD1 expression (14,28). Neither mechanism was assessed in the present study.

The exploratory glycaemic analysis showed ordered marker variation across glycaemic categories. The three untreated categories did not differ significantly, whereas all four markers differed between screen-detected T2DM and treated, well-controlled T2DM. Differences among treated categories were clearer for MDA and erythrocyte SOD activity. Disease duration, treatment exposure, and participant selection may have contributed to these patterns. These exploratory analyses included enrolled participants who did not meet the final eligibility criteria for the primary analysis and should be interpreted separately from the primary findings.

In the treatment-regimen analysis, SOD2 showed a nominal omnibus difference (p = 0.027). However, no Holm-adjusted pairwise comparison was significant for SOD2 or any other marker. Treatment was not randomized, and glycaemic control was similarly suboptimal across the regimen groups. Because treatment was not randomized, treatment effects cannot be inferred from this analysis. Previous intervention studies have shown that antioxidant-related biochemical measures may change without a consistent improvement in HbA1c (29).

Controls with and without metabolic syndrome did not differ significantly for any marker. In contrast, both control groups differed from the T2DM-plus-metabolic-syndrome group for all four markers after Holm correction. These findings do not support an independent association between metabolic syndrome and the measured markers. The small number of controls with metabolic syndrome and the use of BMI instead of waist circumference should also be considered.

Among the oxidative-marker models, the combined MDA–SOD model provided the highest internally cross-validated case–control discrimination, with a CV-AUC of 0.953 (95% CI 0.93–0.98). Adding SOD1 or SOD2 did not materially improve

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performance. HbA1c and FPG had higher discrimination because they partly defined case status and were included only as reference measures.

This study has several limitations. Its cross-sectional design does not permit causal or temporal conclusions. Recruitment was conducted at a single centre, and no prospective sample-size calculation was performed. Unequal post-enrolment exclusions disrupted the intended matching and may have introduced selection bias. The unequal group sizes also reduced precision. The exploratory analyses used enrolled populations of 240, 120, and 235 participants that did not retain all eligibility restrictions of the primary analysis. These analyses were exploratory and should be interpreted separately from the primary analysis.

The TBARS assay is not specific for MDA and may detect other reactive compounds. The results should therefore be interpreted as TBARS-derived estimates of lipid peroxidation. BMI was used instead of waist circumference, which may have caused metabolic-syndrome misclassification. Bulk PBMC measurements may have been influenced by differences in cell composition. Moreover, mRNA expression does not establish SOD1 or SOD2 protein abundance or isoform-specific activity. PBMC transcript levels also cannot be directly equated with erythrocyte SOD activity.

The primary regression models adjusted for BMI and smoking. Sensitivity models additionally considered HbA1c or lipid covariates, but residual confounding remains possible. Relevant factors such as statin use, ACE inhibitor or angiotensin-receptor blocker use, aspirin, diet, physical activity, and vitamin intake were not fully assessed. Finally, the case–control discrimination models received internal cross-validation only. Their performance and clinical value require assessment in independent cohorts with prospective outcomes.

In this case–control study, participants with T2DM had higher serum MDA, lower erythrocyte SOD activity, lower PBMC SOD1 mRNA expression, and higher SOD2 mRNA expression than controls. Exploratory analyses showed ordered marker variation across glycaemic categories and disease-duration groups. Future multi-centre longitudinal studies should examine whether these transcriptional changes are also present at the protein level and whether they are related to clinical outcomes.

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