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Evaluating the role of cytokinesis-block micronucleus assay as a biomarker for oxidative stress-inducing DNA damage in type 2 diabetes mellitus patients

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Abstract

Background Type 2 Diabetes Mellitus (T2DM) is a common metabolic and lifestyle disorder leading to increased oxidative stress and DNA damage. The present study aims to evaluate the feasibility of utilizing the cytokinesis-block micronucleus assay (CBMN) as a biomarker for assessing the DNA damage induced due to variations in oxidative stress.

Methodology The study group includes diabetic (n = 50) and non-diabetic (n = 50) subjects. The assays for the diabetes-like fasting blood sugar, postprandial glucose and hemoglobin A1c (HbA1c), lipid profiling, and serum ferritin level along with c-reactive protein (CRP) were applied. Further, the CBMN assay was performed to evaluate the micronuclei present in the lymphocytes of control and T2DM groups.

Results Significant imbalance in the glycaemic index, dyslipidemia, increased ferritin levels, and CRP levels, with a significant increase of micronucleus frequency, was found in T2DM patients compared with the control group. Results suggest a trend of positive correlation between HbA1c and the micronuclei, indicating the assay's potential importance as a biomarker for T2DM-induced risk assessment.

Conclusion From the observed results, it can be suggested that the CBMN assay could be used to assess the risk of oxidative stress-induced DNA damage in high glycaemic index diabetic patients.

Keywords Type 2 Diabetes, Cytokinesis-block micronucleus assay, Micronucleus, Oxidative stress, DNA damage

1 Background

Metabolic syndrome (MetS) is one of the world's most important health issues in the twenty-first century [1]. The exact mechanisms driving MetS are not known. The factors contributing to developing MetS may be genetic or environmental and behavioral (e.g., unhealthy lifestyle). MetS is a multifactorial condition that may lead to developing health conditions, including Cardiovascular disease (CVD) [2], Diabetes Mellitus [3], Polycystic ovarian syndrome (PCOS) [4] and fatty liver diseases [5]. Studies suggest that MetS is a serious health condition [6] affecting around 23% of adults, placing them at higher

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risk of the diseases mentioned above. MetS are characterized by central obesity and insulin resistance, leading to elevated glucose, triglyceride levels, and hypertension. As such, Type 2 Diabetes Miletus (T2DM) is one of the critical MetS conditions and a serious public health concern. Diabetes mellitus is a condition characterized by persistent hyperglycaemia caused by insulin secretion, insulin action, or a combination of the two [7]. The number of diabetics is estimated to reach 7079 per 1,00,000 by 2030 [6].

Obesity, coronary artery disease, peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure, and blindness are a few complications resulting in increased disability, reduced life expectancy, and enormous healthcare costs [7]. Atherosclerosis, retinopathy, neuropathy, and nephropathy are all secondary problems associated with T2DM [8–12]. Obesity and MetS patients are also subject to genetic damage [13]. For example, it is reported that there are significant associations between body mass index (BMI), Waist Circumference (WC) and increased levels of oxidative DNA damage. Moreover, genomic instability is associated with high BMI and insulin resistance in PCOS [13]. The non-enzymatic glycation of proteins, lipids, and nucleic acids, which results in Advanced Glycation End products (AGEs), is linked to diabetes mellitus complications [14]. The formation of oxidative stress is one of the acute effects of this harmful action. Oxidative stress causes cellular damage and insulin resistance, and it appears to be one of the main

processes behind linked co-morbidities [15, 16]. Furthermore, compared to healthy people, patients with T2DM have feeble antioxidant defenses [17]. The increased production of reactive oxygen species (ROS) has also been linked to protein glycation [18] and/or glucose auto-oxidation during hyperglycemia [19]. Consequently, oxygen-free radicals created by oxidative stress are causing several types of DNA damage [13]. The imbalance between oxidants and antioxidants could badly affect DNA and other cellular biomolecules, which induce diabetes mellitus and CVD [20–22]. Despite the well-characterized presence of the specific defense systems against radicals, constant ROS production and low antioxidant activity can lead to the loss of balance between ROS formation and the operation of a protective system, resulting in the development of oxidative stress (Fig. 1).

Increased ROS production and oxidative stress may induce endogenous DNA damage transcription interruption and cell-cycle arrest [23, 24]. Lipid peroxidation processes are induced by ROS and lead to DNA-reactive lesions. As oxidative stress is linked to the pathogenesis of T2DM, dyslipidemia, and periodontitis, which causes DNA damage, it appears worthwhile to investigate the prevalence of DNA damage and its relationship with these disorders [25].

The micronucleus (MN) test is one of the most well-known tests for determining DNA damage. Many studies [26–28] have utilized the MN test to assess in vivo chromosome damage. It has been used to evaluate

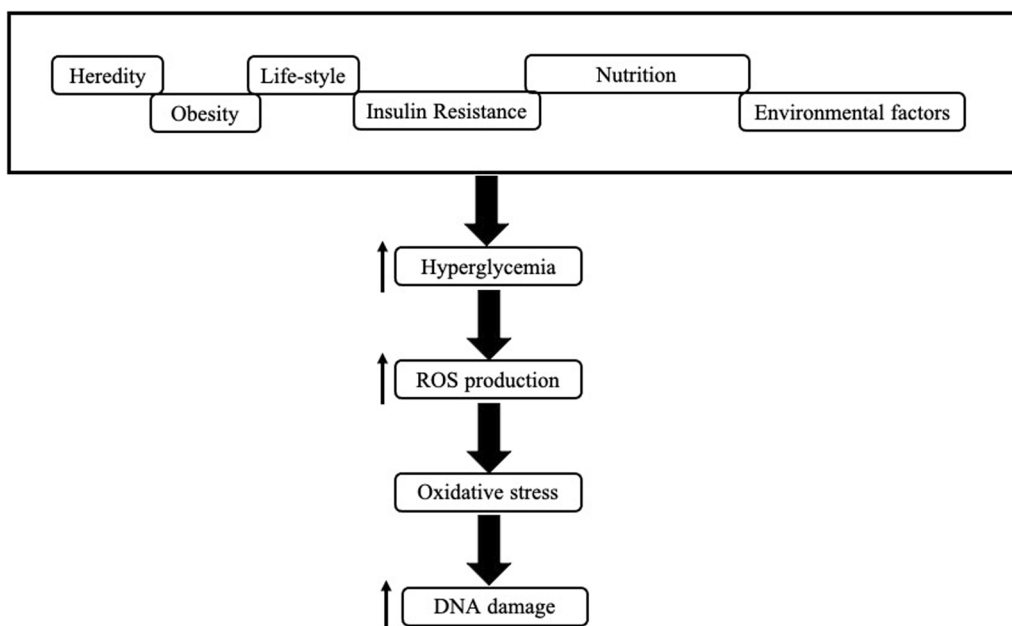


Fig. 1 Schematic representation for DNA damage induction. The figure summarises the overall process of the cause and consequences of T2DM and the relationship between oxidative stress and DNA damage

DNA integrity following cell exposure to radiation, toxic chemicals, and other stressful conditions. The MN test is based on detecting a secondary nucleus, which can result from a trailing acentric chromosome or chromatid fragments caused by the misrepair of DNA breaks or unrepaired DNA breaks during anaphase. The few studies that have looked into MN in T2DM patients have found an increased frequency of MN in diabetic patients [25, 29, 30]. Notably, in all cases, the authors explicitly emphasized the need for more research to back up their findings.

We hypothesized that the presence of T2DM could account for the corresponding increase in DNA damage observed through the MN assay. As a result, this study aimed to see if MN frequency might be used as a biomarker for DNA damage in people with T2DM.

2 Methods

2.1 Sample size

Based on Quintero Ojeda et al. [31], standard deviation (SD) in group I (T2DM) was on the order of 0.21 and in group II (control) was 0.06, with a mean difference of 0.1. For an effect size of 0.7, at the 5% significance level, with 90% power for a two-sided hypothesis, the sample size required is thus 50 in each group, which is a total of 100 patients (Fig. 2). The sample size was calculated using the nMaster 2.0 (India) software.

2.2 Ethical clearance

The study was performed only after obtaining clearance from Central Ethics Committee, Nitte (Deemed to be University), Ref: NU/CEC/2019/0255.

2.3 Sample collection

Patients who have come to Justice K.S Hegde Charitable Hospital for diagnosis and treatment were included. Blood samples were collected from T2DM patients

(n=50) and controls (n=50) using lithium heparinized, Ethylenediaminetetraacetic acid (EDTA), and plain vacutainers (BD, USA).

2.4 Inclusion and exclusion criteria

For type 2 diabetes: Subjects with T2DM according to World Health Organization (WHO) criteria, diagnosed for at least three months before screening, with age group from 30 to 60 years age and sex-matched T2DM and control patients visiting for regular health check-ups and willing to participate in the study, were included in the study. Pregnant women or subjects below 30 or younger were excluded from the study and control groups. At the same time, T2DM patients with immunosuppressants or antibiotics, who underwent X-ray examination or radiation therapy, and T2DM patients with infections (bacterial infections, cancer or hepatitis B or C, HIV) were also excluded.

2.5 Methodology

2.5.1 Estimation of glucose level, lipid profile, ferritin and C-reactive protein (CRP)

Fasting and Postprandial Glucose (PPG), Lipid profile (Total cholesterol, Triglyceride, HDL, and LDL) levels and CRP were estimated according to the instructions from the commercial available kits (Agappe, India) using Multimode microplate and fluorescence intensity bottom reader (Tecan Spark, Bioscreen, Switzerland). The serum ferritin levels were assessed using an ELISA kit obtained from Calbiotech following the instructions mentioned in the datasheet.

2.5.2 Evaluation of antioxidant levels

2.5.2.1 Myeloperoxidase (MPO) assay MPO enzyme activity was determined by the o-dianisidine-H₂O₂ method [32]. Briefly, lysates (20 μL) were added to 0.53 mmol/L o-dianisidine dihydrochloride (Sigma-Aldrich, USA) and

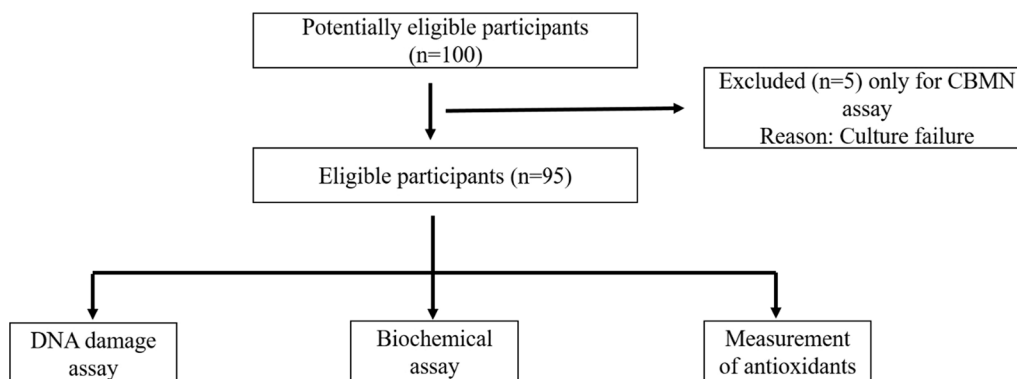


Fig. 2 Flow of participants throughout the study

1.7 mmol/L H₂O₂ in 50 mmol/ sodium phosphate buffer (pH 6.0). After incubation for 10 min at room temperature, the change in absorbance was measured at 510 nm.

2.5.2.2 Glutathione peroxidase (GPx) assay The activity of GPx in erythrocyte lysate was determined as described elsewhere [33]. In brief, the Oxidized glutathione (GSSG) produced during the GPx reaction was immediately reduced by nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase. As a result, the rate of NADPH consumption during the GPx reaction could be equated to the rate of GSSG production. The GPx activity was then measured using a double-beam spectrophotometer (Systonics-2202, India) at 340 nm. After subtracting the blank value, GPx activity was estimated as mmol of NADPH oxidized/minute/mg protein.

2.5.2.3 Superoxide dismutase (SOD) assay The activity of SOD in erythrocyte lysate was determined as described elsewhere [34]. Briefly, this method uses riboflavin to generate superoxide radicals that react with nitro blue tetrazolium chloride in the presence of methionine as an electron donor to form a blue formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction using a double-beam spectrophotometer (Systonics-2202) at 560 nm. The 50% decrease in forming the formazan was taken as one unit of SOD activity, which is expressed as U/mg Hb.

2.5.3 Cytokinesis blocked micronucleus (CBMN) assay

Briefly, the whole blood of about 1 mL was added to the culture medium (RPMI-1640, 20% Fasting Blood Sugar (FBS) and 2% of PHA-M) and incubated at 37°C using a CO₂ incubator. At the 44th hour, cytochalasin-B (6 µg/mL) was added aseptically to arrest at cytokinesis stage and incubated further 28 h. At the 72nd hour, the culture was terminated, treated with pre-chilled KCl (0.075 M), and fixed with Carnoy's fixative [35]. The next day slides were prepared, stained and analyzed. A total of 2000 binucleated cells were scored using 40X magnification (Primo Star, Zeiss, Germany) to calculate MN frequency. The MN were identified using Fenech's [35] criteria: (a) diameter between 1/16 and 1/3 of the mean diameter of the main nucleus; (b) non-refractivity; (c) no linkage or connection to or overlap with the main nucleus; (d) similar staining intensity as the nucleus; and (d) position within the cytoplasm.

2.5.4 Statistical analysis

Data are presented as mean ± SEM. GraphPad Prism 8 statistical tool was used to calculate unpaired t-test to compare the T2DM patients and control subjects. Pearson correlation was performed at the confidence limit of 95%. The significant levels were expressed at $P < 0.0001$ and $P < 0.05$.

3 Results

3.1 Demographic data

Table 1 shows 50 T2DM patients (M=31; F=19) and 50 healthy adults (M=22; F=28) participated in this study.

Table 1 Demographic, glycaemic and lipid profile of the study subjects

	Group	n	Males	P value	Females	P value
Study subjects	D	50	31	ND	19	ND
	C	50	22		28	
Fasting glucose (mg/dL) ± SEM	D	50	165.5 ± 9.07	$P < 0.0001$	146.7 ± 11.11	$P < 0.0001$
	C	50	105.8 ± 1.95		98.2 ± 1.83	
Post prandial glucose (mg/dL) ± SEM	D	50	243.3 ± 16.23	$P < 0.0001$	236.3 ± 19.16	$P < 0.0001$
	C	50	124.4 ± 1.89		126.4 ± 2.17	
HbA1c ± SEM	D	50	8.5 ± 0.4	$P < 0.0001$	8.2 ± 0.38	$P < 0.0001$
	C	50	4.6 ± 0.34		4.1 ± 0.14	
Total cholesterol ± SEM	D	50	176.9 ± 8.53	$P < 0.05$	227 ± 11.001	$P < 0.05$
	C	50	203.4 ± 11.79		195.6 ± 10.98	
Triglyceride ± SEM	D	50	159.5 ± 20.74	$P < 0.05$	220.1 ± 44.21	$P > 0.05$
	C	50	219.6 ± 15.42		192.4 ± 16.03	
LDL ± SEM	D	50	113.2 ± 8.49	$P > 0.05$	153.2 ± 8.83	$P < 0.0001$
	C	50	115.8 ± 13.57		114.5 ± 11.51	
HDL ± SEM	D	50	42.1 ± 1.99	$P > 0.05$	49.7 ± 2.59	$P < 0.05$
	C	50	43.7 ± 2.15		42.7 ± 3.01	

The P-value mentioned in table-1 denotes the significance between the groups and marked in bold

SEM standard error mean, ND not determined, D diabetes, C control; n no. of study subjects, LDL low density lipoprotein, HDL high density lipoprotein

The average ages of T2DM patients and control individuals were 52.2 ± 10.9 and 46.5 ± 7.5 years, respectively. The demographic and laboratory data for T2DM patients and controls are shown in Table 1. Compared to the control group, T2DM patients had significantly higher triglyceride, fasting and postprandial glucose, and HbA1c levels. The traditional risk factors investigated in these people only explain a portion of the greater risk in diabetic subjects.

3.2 Estimation of Ferritin and CRP

According to Fig. 3, the Ferritin level in the control group was 34.69 ± 4.97 ng/mL, which in patients with T2DM was found to be 109.96 ± 12.57 ng/mL. Thus a significant ($P < 0.05$) increase in the ferritin levels was observed

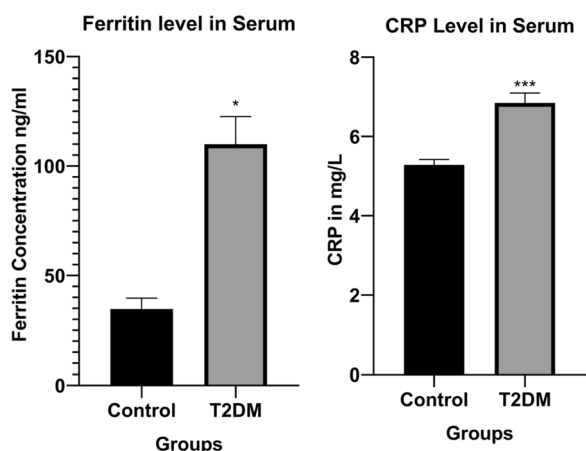


Fig. 3 The serum ferritin and C-reactive protein levels in diabetic patients compared to controls. * P value < 0.05 ; *** P value < 0.0001

in patients with T2DM compared with the sex-matched controls. Similarly, the CRP level in the control group was 5.28 ± 0.221 mg/L, which in patients with T2DM was 6.85 ± 0.38 mg/L. A significant increase in the CRP level was found in the patients with T2DM compared with the sex-matched controls.

3.3 Evaluation of antioxidant activity

The levels of oxidative stress indicators SOD, GPx, and MPO in T2DM subjects were compared to those in the age-matched control group (Fig. 4). The SOD activity was found to be 2.18 ± 0.33 U/mg of Hb in the control group, which significantly decreased ($P < 0.0001$) to 1.43 ± 0.147 U/mg of Hb in patients with T2DM. The GPx activity was found to be 1.40 ± 0.11 μ g of glutathione/min/mg Hb in the control group, which was found to be significantly decreased ($P < 0.0001$) 0.87 ± 0.02 μ g of glutathione/min/mg Hb in patients with T2DM. Further, the MPO levels were 54.14 ± 14.12 pM/L in the control group, which increased significantly ($P < 0.0001$) to 113.93 ± 9.67 pM/L in patients with T2DM. Thus, a significant decrease in SOD and GPx enzyme activity levels and a significant increase in MPO activity in patients with T2DM indicates a significant increase in oxidative stress in patients with T2DM.

3.4 Evaluation of DNA damage using CBMN assay

As illustrated in Fig. 5, a significant increase in the MN frequency ($P < 0.001$) was observed in the diabetic group (0.01 ± 0.0003 MN/Cell) compared with the control group (0.004 ± 0.0005 MN/Cell) which indicates the increase in the DNA damage due to the T2DM condition may be due to oxidative stress.

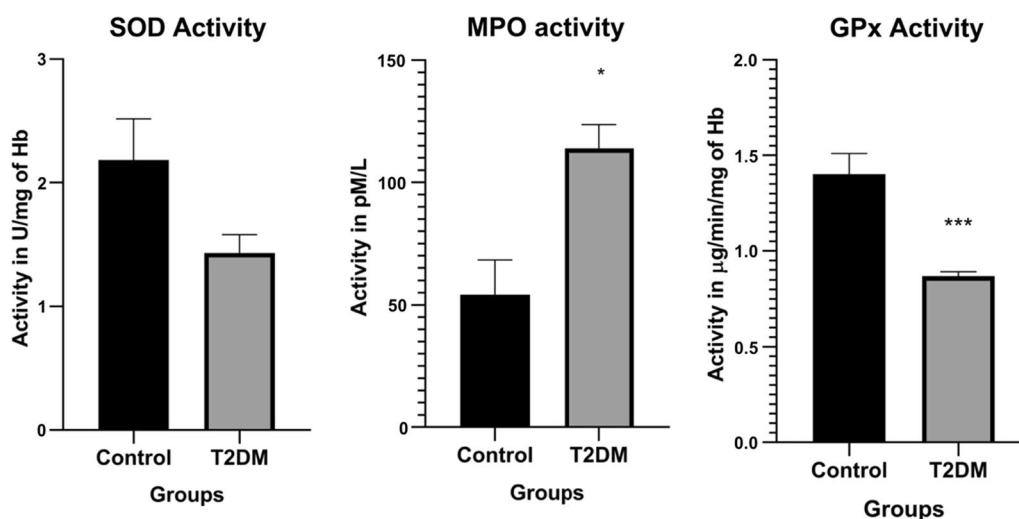


Fig. 4 The obtained SOD, MPO and GPx activity from control and type 2 diabetes mellitus patients. * P value < 0.05 ; *** P -value < 0.0001

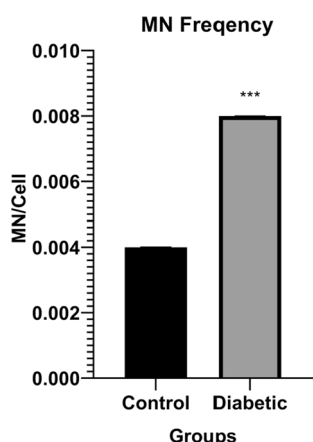


Fig. 5 MN frequency obtained for control and type 2 diabetes mellitus patients. ****P* value < 0.0001

Table 2 Correlation of FBS, PPG and HbA1c with micronuclei

Groups	Correlation coefficient (r)	Significance (<i>P</i> value)
FBS vs. micronuclei	- 0.2310	0.127 (NS)
PPG vs. micronuclei	- 0.2011	0.185 (NS)
HbA1c vs. micronuclei	0.0644	0.682 (NS)

P < 0.05 is considered as statistically significant; NS not significant

3.5 Correlation of MN with controlled and uncontrolled FBS, PPG

Table 2 presents the relationships between FBS, PPG, and HbA1c levels with the number of micronuclei. No significant correlations were observed. Although a positive correlation trend was observed when comparing the MN levels with HbA1c, a trend of negative correlation was observed when comparing the MN with FBS and PPG levels.

4 Discussion

T2DM was an important chronic disease that challenged global health. T2DM (formerly called non-insulin-dependent or adult-onset diabetes) results from the body’s ineffective use of insulin. This type of diabetes is primarily the result of excess body weight and physical inactivity. According to the fact sheet published by the Centres for Disease Control and Prevention, Diabetes is the 7th leading cause of death in the United States (and maybe underreported). T2DM accounts for approximately 90–95% of all diagnosed cases of diabetes. This has increased the importance of identifying the disease’s root cause(s) and potential treatments or preventative measures. In this direction, in this work, we attempted to test the effectiveness of the CBMN assay as a predictive

tool to determine the DNA damage induced in diabetic patients.

The status of the diabetes was evaluated by estimating the glycaemic index using FBS, PPG and HbA1c levels and lipid profiling by estimating total cholesterol, HDL, LDL, and the basic demographic data of age. In the Diabetic group, a significant increase in the FBS, PPG and HbA1c was observed compared with the control group, which is in the lane with the previous studies [36].

Previous studies have proved dyslipidaemias as a common symptom observed in patients with T2DM. According to the literature, 60% of people with diabetes exhibit dyslipidemia, which can be correlated with the severity due to the hyperglycaemic state and level of its control [37–39]. In addition [40], in patients with T2DM, there is a significant increase in triglyceride, total cholesterol, and LDL levels, with a decrease in HDL levels. The current study shows a similar trend in patients with T2DM, indicating an alteration in the lipid profile due to T2DM.

Previous work also showed that when diabetic patients were compared to controls, serum ferritin, a reflector of body iron reserves, was significantly greater in diabetic patients. This increased dramatically as the duration of diabetes increased [36]. This could be due to the development of subclinical hemochromatosis in a diabetic patient with a lengthy history of diabetes. Increased body iron reserves are likely connected with the incidence of glucose intolerance, T2DM, and gestational diabetes [37]. The obtained levels of serum ferritin in the present study for diabetic patients were found to be greater than those in the control group, and this difference was statistically significant (*P* < 0.0001). According to the literature [38], the average ferritin concentration was substantially higher in people with diabetes. Ferritin levels in the blood are linked to T2DM. And this could be a significant and independent predictor of diabetes mellitus development [39]. Another study reported [40] that T2DM people have a higher mean serum ferritin value than control subjects and that plasma ferritin concentrations are related to plasma glucose and glycosylated hemoglobin levels. However, our investigation showed only a minor association between serum ferritin and glycosylated hemoglobin levels. As a result, the cause of elevated serum ferritin levels in diabetic patients and its clinical importance remain unknown. The main question is whether to estimate serum ferritin in all T2DM patients regularly and define a serum ferritin cut-off value for excellent glycaemic management. Though our study is a step in the right direction, we would encourage more research in this area to establish clear criteria.

CRP is used as a biomarker of CVD, and it is usually higher in individuals with T2DM [41]. CRP is an acute-phase reactant mainly synthesized in the liver and

adipocyte and is a multifunctional, non-specific marker for systemic inflammation. It has been reported that elevated plasma CRP is associated with CVD and T2DM [42]; however, the underlying mechanisms between CRP levels and these diseases have not been fully understood. A statistically significant increase in CRP levels was observed in diabetic patients ($p < 0.0001$), which agrees with the previously reported studies [42].

Results previously published [37] suggest that oxidative stress may play an essential role in the pathogenesis of complications associated with the chronic diabetic state. Further, oxidative stress induced by ROS generated due to hyperglycemia is one of the significant targets studied in recent studies on diabetes. Thus, changes in antioxidant enzymes like SOD, GPx, catalase, etc., play an essential role in diabetes management. In a previous study [43] involving T2DM patients with an age-matched control group, antioxidants and antioxidant enzymes such as SOD, GSH-Px, GR, GSH and MDA were analyzed. The results suggested significantly higher lipid peroxidation levels and decreased antioxidant enzyme levels in the serum of diabetic patients compared with the control group. Compared to control people without diabetes, the circulatory and renal tissue of diabetes patients exhibits higher levels of oxidative stress markers [44].

In comparison to Caucasian patients, African-born patients have been reported to have greater levels of circulating lipid peroxide, lower levels of vitamin E, and a faster rate of decrease in renal function [45]. Serum selenium levels in people of Asian ancestry have been found to be somewhat low [46]. Low selenium levels are linked to increased inflammatory chemokines because it is a cofactor for the antioxidant enzyme GPx. Selenium supplementation reduces the inflammatory response and may enhance therapeutic results in conditions of systemic inflammatory diseases [47].

According to another study conducted in T2DM patients [48] ($n = 100$), although a decreased level of SOD was observed in diabetes patients compared to controls, the results were not statistically significant. Glutathione peroxidase and reduced glutathione levels significantly increased ($p < 0.05$) compared with the controls.

In addition, previous study findings [49] suggested that elevated MDA levels in erythrocyte ghost membranes of diabetic patients are due to the high levels of glucose-induced oxidative stress, which will further increase antioxidant enzymes. The authors also suggested that these antioxidant enzymes may be considered markers for vascular injury. There is strong evidence that antioxidants play a significant role in the appearance of secondary symptoms of diabetes, such as nephropathy, neuropathy, retinopathy, etc. The

present study results suggested a significant increase in the MPO activity and decreased level of GPx activity, indicating increased peroxidation levels in diabetic patients compared with the control group and, thus increased levels of oxidative stress due to cellular damage. The levels of SOD activity also decreased, suggesting increased superoxide free radical load in diabetic patients, which further underlines the importance of oxidative stress in diabetic patients, and which is in line with previously published data [50].

Another finding of this study is that MetS patients have more genetic damage overall. Following the above, this is expected, as ROS are genotoxic, and oxidative stress can result in genomic damage [51]. In this study, the CBMN assay was used to assess DNA damage. Comparing T2DM patients to control participants revealed statistically significant ($p < 0.0001$) increased numbers of CBMN in T2DM patients based on their clinical diagnosis. According to our findings, the MN assay revealed dramatically enhanced chromosomal damage in patients with T2DM compared to the control group ($P < 0.0001$). These factors are also linked to T2DM patient's characteristics and components, such as blood sugar, triglycerides, and HDL levels.

Further, most prior studies correlated the HbA1c with the FBS and PPG [52]. The focus of the study is to predict MN formation with the glycaemic index in the present study. Thus, we correlated the FBS, PPG, and HbA1c with MN in the patients and control groups. Although no statistically significant correlations were identified, the results showed a trend of positive correlation of MN with the HbA1c percentage, whereas, with FBS and PPG, a trend of negative correlation was observed. According to the American Diabetes Association (ADA), HbA1c is an essential indicator for long-term glycaemic control, reflecting the preceding two to three months [36]. The ADA also suggests that HbA1c can be correlated to the risk of developing long-term diabetic complications [36]. Thus, we can conclude that both FBS and PPG levels are the one-time indicators that might be the reason for the trend of negative correlation, which, however, does not reflect the cumulative glycaemic index of the patient. The present study included 50 persons with diabetes and 50 controls, and this smaller sample size also limited the potential findings of the research. But in the case of HbA1c, a non-significant positive correlation was observed, which indicated a trend of increase in the MN number with an increase in the HbA1c. Thus we can interpret the findings as indicating that the MN can be used as an additional biomarker to assess the long term risk involved with diabetes.

5 Conclusion

Although the study consists of a limited number of patients, the results show that the CBMN assay can be used as an additional tool to evaluate oxidative stress-induced DNA damage in T2DM patients with a high glycaemic index. Further research with a multi-centric approach must be undertaken with more subjects to confirm and/or reduce inter-individual differences in patients with T2DM.

Abbreviations

ADA	American Diabetes Association
AGEs	Advanced Glycation End products
BMI	Body mass index
CBMN	Cytokinesis-block micronucleus assay
CRP	C-reactive protein
CSSG	Oxidized glutathione
CVD	Cardiovascular disease
EDTA	Ethylenediaminetetraacetic acid
FBS	Fasting blood sugar
GPx	Glutathione peroxidase
HbA1c	Hemoglobin A1c
MetS	Metabolic syndrome
MN	Micronucleus
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
PCOS	Polycystic ovarian syndrome
PPG	Postprandial glucose
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
T2DM	Type 2 Diabetes Mellitus
WC	Waist Circumference
WHO	World Health Organization

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Author contributions

RV and GTS are performed most laboratory experiments and data analysis, AB helped to collect the samples and analyse the samples, biochemical estimations done by SSS and PB. NSK, GTS and RV are involved in the data analysis and GTS, and RV, wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All the data we generated in this paper is available in the body of the manuscript as supporting figures and tables. We do not have any ethical or legal consideration for not to make our data publicly available.

Declarations

Ethics approval and consent to participate

The study was performed only after obtaining clearance from Central Ethics Committee, Nitte (Deemed to be University), Ref: NU/CEC/2019/0255.

Consent to publication

Informed written consent to participate in the study was provided by all participants.

Consent for Publication

Not applicable.

Competing interests

The authors report there are no competing interests to declare.

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