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# Oxidative stress markers as early predictors of diabetes complications in Type 2 diabetic patients

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## ABSTRACT

**Objectives:** Type 2 diabetes mellitus (T2DM) is a complex disease that affects many organs. Oxidative stress plays a key role in the pathogenesis of insulin resistance and β-cell dysfunction. Thus, the present study aimed to use oxidative stress markers as early predictors for the progression of diabetic complications.

**Materials and Methods:** The study sample included 400 individuals (300 T2DM and 100 non-diabetic controls) aged from 35 to 59 years randomly selected from the outpatient clinic of the National Institute for Diabetes and Endocrinology. T2DM patients were divided into subgroups: Subgroup (1) patients without any complications, Subgroup (2) patients with diabetic nephropathy(DN) and Subgroup (3) patients with cardiovascular disorders (CVD). Biochemical markers of fasting blood glucose, glycated haemoglobin (HbA1C), glucose-6-phosphate dehydrogenase (G6PD), lactate, arginase, heme oxygenase-1 (HO-1), haemoglobin (Hb), triglycerides (TG), cholesterol, low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), urea, creatinine, malondialdehyde (MDA), reduced glutathione (GSH), catalase (CAT) and nitric oxide (NO) were performed.

**Results:** DM patients showed significant increases in body mass index, systolic blood pressure, diastolic blood pressure, FBS, HbA1C, cholesterol, TG, LDL-C and glomerular filtration rate, while HDL-C decreased. Significant increases were observed in HO-1, MDA and NO, while G6PD/lactate, GSH and CAT decreased in DM patients. The DN and CVD patients exhibited a significant increase in HO-1, MDA and NO; while G6PD/lactate, GSH and CAT decreased compared with DM patients. Receiver operating characteristic analysis showed that the sensitivity and specificity of oxidative stress markers were 66.67–100%.

**Conclusion:** Hexose monophosphate (HMP)/glycolysis pathways are shifted during DM near glycolysis rather than HMP pathway to produce energy where the amount of glucose enters the cells is low, causing oxidative stress. Oxidative stress markers could be used as early predictors of diabetes complications.

**Keywords:** Diabetes mellitus, Hexose monophosphate, Glycolysis, Oxidative stress, G6PD/lactate ratio

## INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders characterised by elevated glucose levels in the blood (hyperglycaemia) and insufficiency in the production or action of insulin produced by the pancreas.[1] It is considered one of the most dangerous metabolic disorders in the world.[2] In general, 90–95% of diabetes is diagnosed as Type 2 diabetes mellitus (T2DM).[3] The main features of T2DM are insulin resistance, obesity and dysfunction of pancreatic β-cells.[4,5] T2DM is a complex disease which affects many organs besides the pancreas, such as the liver, brain, eye, stomach and kidney.[6] In the early stage of the disease, pancreatic β-cells compensate for insulin

resistance through additional insulin production, leading to a week and dysfunction of β-cells and impaired insulin secretion, causing the hyperglycaemic condition.[6]

Diabetic complications include macrovascular (coronary heart disease, peripheral vascular disease and stroke), microvascular (neuropathy, retinopathy and nephropathy) and both macro- and microvascular (diabetic foot).[7] Hyperglycaemia altered signalling pathways in different tissues creating oxidative stress, advanced glycation products (AGEs), secretion of the pro-inflammatory cytokines and cellular death.[8] It is becoming clear that the main mechanisms for cellular damage of T1DM and T2DM diabetic complications are oxidative stress and inflammation.[9]

Cardiovascular disorders (CVDs) account for more than 80% of the mortality seen in the diabetic population and diabetes equates to an approximately 3-fold increased risk of myocardial infarction compared with the general population.[10] CVD in diabetes includes premature atherosclerosis, manifests as myocardial infarction and stroke as impaired cardiac function is predominantly diastolic dysfunction.[11] A recent study revealed that patients with T2DM compared to T1DM had a significantly higher risk of diabetic complications.[12] Oxidative stress considers the primary cause of the development of diabetes microvascular and cardiovascular complications.[13-15]

Diabetic nephropathy (DN) represents the major cause of end-stage renal failure.[16] Clinically, it is characterised by proteinuria development with a subsequent decline in glomerular filtration rate, which progresses over a long period, often over 10–20 years.[17] The main risk factor for DN is hyperglycaemia, which induces the formation of advanced glycation end products that promote reactive oxygen species (ROS) production.[18] Oxidative stress is associated with metabolic changes and alterations in renal haemodynamics, which have synergistic effects on DN development.[19]

Oxidative stress causes a complex dysregulation of cell metabolism and cell-cell homeostasis; in particular, oxidative stress plays a key role in the pathogenesis of insulin resistance and β-cell dysfunction.[20] In diabetes, oxidative stress changes an enzymatic system, induces lipid peroxidation, impaired glutathione (GSH) metabolism and diminishes Vitamin C concentration.[17]

Because of their propensity to damage lipids, proteins and DNA, oxidative stress plays a key role in initiating and progressing late diabetes complications.[21] It has been observed that oxidative stress induces many diabetic complications, including coronary artery disease, neuropathy, nephropathy, retinopathy and stroke.[17]

Biomarkers of oxidative stress include malondialdehyde (MDA) (polyunsaturated fatty acid peroxidation and hence considered a meaningful sign of lipid peroxide) and[22] catalase (CAT) (a vital component of the antioxidant defence system that supports each other and provides a protective defence against ROS).[23]

One of the primary antioxidant cellular defence systems is the hexose monophosphate (HMP) pathway.[24] The relationship between GSH metabolism and the HMP pathway protects cells against oxidative stress.[25] NADPH, which is produced by glucose-6-phosphate dehydrogenase (G6PD), is also recognised to be a crucial system involved in the maintenance of various important redox and antioxidant defence processes.[26]

We hypothesise that glucose metabolism during diabetes prefers the glycolysis pathway rather than the HMP pathway, leading to depletion in the antioxidants and causing oxidative stress in the tissue.

Thus, the present study aimed to investigate a new mechanism for diabetic complications mediated by oxidative stress by studying glucose metabolism.

## MATERIALS AND METHODS

### Chemicals and reagents

Blood glucose (CAT. NO. GL 13 20), G6PD (CAT. NO. PD 25 26), arginase (CAT. NO. AR 25 34), haemoglobin (Hb) (CAT. No. HG 14 10), triglycerides (TG) (CAT. No. TR 20 30), total cholesterol (CAT. No. CH 12 20), low-density lipoprotein (LDL-C) (CAT. No. CH 12 31), high-density lipoprotein (HDL-C) (CAT. No. CH 12 30), urea (CAT. NO. UR 21 10), creatinine (CAT. No. CR 12 51), MDA (CAT. No. MD 25 29), GSH reduced (CAT. No. GR 25 11), CAT (CAT. No. CA 25 17) and nitric oxide (NO) (CAT. No. NO 25 33) kits were purchased from BIODIAGNOSTIC Company (El Moror St, Dokki, EGY). Heme oxygenase-1 (HO-1) (abx252635), lactate (abx258342) and glycated haemoglobin (HbA1C) (abx253430) kits were purchased from Abbexa (Cambridge Science Park, Cambridge, CB4 0GJ, UK).

### Ethical consideration

The National Institute for Diabetes and Endocrinology (NIDE) approved experimental protocols and procedures in this study and with the Helsinki Declaration of 1975 (Ethical approval No. IDE00242).

All participants in the present study have signed an informed consent form.

### Experimental design

A cross-sectional observational study was performed on 400 individuals (200 males and 200 females) classified as T2DM ($n$ = 300) and non-diabetic controls ($n$ = 100) aged from

35 to 59 years randomly selected from NIDE's outpatient clinic between 2019 and 2020. The study consists of an equal number of males and females. The diagnosis of T2DM was based on clinical records using the criteria recommended by the American Diabetes Association. T2DM patients were divided into subgroups: Subgroup (1) patients without complications, Subgroup (2) patients with nephropathy complications and subgroup (3) patients with cardiovascular disorders (CVD).

DN was defined as the presence of albuminuria (≥30 mg/g of creatinine) and estimated glomerular filtration rate ≤60 mL/min/1.73 $m^2$. Diabetic cardiovascular diseases are diagnosed by electrocardiography, coronary angiography, echocardiography and lipid profile tests in the blood.

### Inclusion criteria

The inclusion criteria include: T2DM, age between 35 and 59 years and HbA1c>9% in the last test performed in the 12 months before the study.

The control individuals were selected from healthy males and females aged between 35 and 59 years.

### Exclusion criteria

The exclusion criteria included not Type 2 diabetes, other immune disorders, other endocrine disorders, smoking and using anti-inflammatory drugs. Furthermore, individuals treated for systemic hypertension, use antioxidants, use not oral hypoglycaemic or insulin and received an investigational drug within 30 days before the study were excluded from the study.

Controls: Individuals with any chronic systemic diseases, smokers, patients under any medication other than oral hypoglycaemic drugs and insulin and individuals with a history of any illness for the past 6 months.

### Demographic characteristics

The demographic characteristics are included determination of age, sex, blood pressure and body mass index (BMI).

### Urine collection and laboratory analysis

Freshly voided urine samples were obtained and stored at 4°C. All urine samples were analysed within 6 h of collection.

### Blood collection and laboratory analysis

Blood was collected by venipuncture after a 6 h fast into a Vacutainer with EDTA-K2. The blood collected was separated by centrifugation (3000 rpm, 15 min) to obtain plasma, stored at −80°C for the biochemical measurements.

### Diabetic markers and Hb

Fasting blood glucose (FBG), arginase and haemoglobin (Hb) were estimated by the colorimetric method,[27,28] while HbA1C was estimated by ELISA method.

### Lipid profile markers

TG, cholesterol, LDL-C and HDL-C were estimated by the colorimetric method.[29-32]

### Kidney function markers

Urine albumin was quantitatively measured according to Cambiaso *et al*.,[33] urea by urease – Berthelot method[34] and creatinine by colorimetric kinetic method.[35] Glomerular filtration rate (GFR) was calculated according to the equation of Levey *et al*.:[36]

$$GFR = 175 \times \left(S_{Cr}\right)^{-1.154} \times \left(age\right)^{-0.203} \times 1.212$$
$$\left[if\ black\right] \text{x } 0.742\ [if\ female]$$

GFR is expressed as mL/min/1.73 $m^2$ of body surface area and serum creatinine ($S_{Cr}$) is expressed in mg/dL.

### Oxidative stress markers

MDA, reduce GSH, CAT and NO were estimated by colorimetric method.[37-40] G6PD was estimated by UV method,[41] while ELISA assays estimated HO-1 and lactate.

### Statistical analysis

Values were expressed as means ± SE. Differences between the four groups were assessed using one-way analysis of variance with the Tukey *post hoc* test. $P < 0.05$ was considered to indicate statistically significant differences. SPSS for Windows (version 20) was used for the statistical analysis.

Receivers operating characteristic (ROC) curves were used to compare the discriminative power of oxidative stress markers to predict the progression of diabetic complications. ROC test was performed between whole diabetic complication groups (DM+DN and DM+CVD) versus diabetes group without complication. Area under the curve (AUC) of the ROC curve was calculated for each test and $P < 0.05$ was accepted as significant.[42]

## RESULTS

### Demographic characteristics

Data represented in [Table 1] showed significant increases ($P < 0.05$) in BMI, systolic blood pressure (SBP) and diastolic blood pressure (DBP) of DM patients as compared to the control group. In comparison, the DN patients exhibited a significant increase in SBP only compared with DM patients.

In CVD patients, BMI, SBP, DBP and disease duration increased significantly compared with DM patients.

### Diabetic markers and Hb

[Table 2] shows significant increases ($P < 0.05$) in FBG and HbA1C, while Hb decreased significantly in DM as compared to the control group. However, DN and CVD patients exhibited a significant increase in FBS, HbA1C and arginase while Hb decreased compared with DM patients.

### Lipid profile markers

A significant increase ($P < 0.05$) was noticed in cholesterol, TG and LDL-C while HDL-C decreased in the DM group compared to the control group [Table 3]. On the other hand, the DN and CVD patients exhibited a significant increase in TG and decrease in HDL compared with DM patients.

### Kidney function markers

Data represented in [Table 4] showed a significant decrease ($P < 0.05$) in the glomerular filtration rate (GRF) of DM patients compared to the control group. The DN and CVD patients significantly increased urea, creatinine and albumin/creatinine (A/C) ratio while GFR decreased compared with DM patients.

### Oxidative stress markers

[Table 5] shows significant increases ($P < 0.05$) in HO-1, MDA, NO while G6PD/lactate, GSH reduced and CAT significantly reduced in DM patients as compared to the control group. The DN and CVD patients exhibited a significant increase in HO-1, MDA and NO; while G6PD/lactate, GSH and CAT significantly decreased compared with DM patients.

### ROCs curve results

ROC analysis for MDA showed that cutoff was >15.83; sensitivity of test 100%, specificity 91.67% and AUC 99% [Figure 1a]. The GSH test showed cutoff ≤440.27; sensitivity 83.33%, specificity 66.67% and AUC 68% [Figure 1b]. Test of CAT showed cutoff ≤1503.21, sensitivity 66.67%, specificity91.67 % and AUC 82% [Figure 1c]. ROC analysis for NO showed cutoff >311.05, sensitivity 100%, specificity 100% and AUC 100% [Figure 1d]. The HO-1 showed cutoff

**Table 1:** Demographic characteristics of the groups.

| Parameter | Control | DM | DM+DN | DM+CVD |
|---|---|---|---|---|
| Age (years) | 47.83±1.19 | 48.9±0.955 | 49.25±1.15 | 50±0.62 |
| Duration (years) | - | 5.91±0.60 | 13.24±0.88** | 21.24±0.78** |
| BMI (kg/m$^2$) | 24.23±0.48 | 28.2±0.86* | 30.37±1.03 | 35.66±1.49** |
| SBP (mmHg) | 115.83±2.1 | 127.5±294* | 138.13±1.35** | 150.83±1.64** |
| DBP (mmHg) | 77.5±2.53 | 82.5±2.40* | 85.83±1.88 | 90.83±1.88** |

*Significant compare to the control group. **Significant compare to the diabetic group. DM: Diabetes mellitus, DN: Diabetic nephropathy, CDV: Cardiovascular diseases, BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure

**Table 2:** Diabetic markers and haemoglobin of the control and diabetic groups.

| Parameter | Control | DM | DM+DN | DM+CVD |
|---|---|---|---|---|
| FBS (mg/dL) | 72.6±2.77 | 154.66±4.06* | 205.24±1.94** | 242.66±2.77** |
| HbA1C (%) | 4.75±0.095 | 8.19±0.19* | 9.55±0.19** | 10.67±0.40** |
| Arginase (U/L) | 90.83±1.36 | 92.78±0.74 | 100.17±3.94** | 109.88±1.35** |
| Hb (g/dL) | 14.69±0.49 | 13.54±0.15* | 12.16±0.41** | 13.26±0.245** |

*Significant compare to the control group. **Significant compare to the diabetic group. DM: Diabetes mellitus, DN: Diabetic nephropathy, CDV: Cardiovascular diseases, HbA1C: Glycated haemoglobin, Hb: Haemoglobin

**Table 3:** Lipid profile markers of the control and diabetic groups.

| Parameter | Control | DM | DM+DN | DM+CVD |
|---|---|---|---|---|
| Cholesterol (mg/dL) | 163.75±12.97 | 209.49±12.65* | 220.16±13.07 | 249.24±13.45** |
| Triglycerides (mg/dL) | 72.99±8.42 | 103.41±10.77* | 131.99±12.78** | 215.08±14.71** |
| HDL (mg/dL) | 67.0±1.48 | 52.08±1.58* | 43.99±0.94** | 36.99±0.99** |
| LDL (mg/dL) | 89.91±6.72 | 137.24±7.03* | 141.24±7.87 | 160.16±8.41** |

*Significant compare to the control group. **Significant compare to the diabetic group. DM: Diabetes mellitus, DN: Diabetic nephropathy, CDV: Cardiovascular diseases, HDL: High-density lipoprotein, LDL: Low-density lipoprotein

**Table 4:** Kidney function markers of the control and diabetic groups.

| Parameter | Control | DM | DM+DN | DM+CVD |
|---|---|---|---|---|
| Urea (mg/dL) | 22.41±1.45 | 26.71±1.22 | 45.08±2.18** | 37.58±1.16** |
| Creatinine (mg/dL) | 0.83±0.025 | 0.85±0.025 | 1.25±0.02** | 0.93±0.0115** |
| A/C ratio (mg/g) | 21.36±0.96 | 27.15±2.4 | 136.6±6.54** | 66.25±1.26** |
| GFR (ml/min/1.73 $m^2$) | 114.28±6.46 | 94.81±1.30* | 66.25±1.26** | 82.50±1.32** |

*Significant compare to the control group. **Significant compare to the diabetic group. DM: Diabetes mellitus, DN: Diabetic nephropathy, CDV: Cardiovascular diseases, A/C: Albumin/creatinine, GFR: Glomerular filtration rate

**Table 5:** Oxidative stress markers of diabetic patients.

| Parameter | Control | DM | DM+DN | DM+CVD |
|---|---|---|---|---|
| MDA (nmoles/ml) | 12.16±0.28 | 14.47±0.086* | 18.43±0.20** | 21.72±0.44** |
| GSH (nmoles/ml) | 449.44±6.96 | 438.65±6.11* | 424.54±6.82** | 416.53±6.14** |
| CAT (U/mL/min) | 1666.11±12.67 | 1572.71±10.51* | 1499.61±10.78** | 1410.43±9.15** |
| NO (µM/mL) | 262.01±1.84 | 300.12±2.80* | 369.73±2.49** | 406.01±8.23** |
| HO-1 (ng bilirubin/mg protein/h) | 51.81±1.23 | 63.04±1.18* | 69.97±0.74** | 77.18±1.11** |
| G6PD/lactate (U/g ) | 140.62±10.0 | 90.93±4.6* | 70.60±2.22** | 50.31±5.54** |

*Significant compare to the control group. **Significant compare to the diabetic group. DM: Diabetes mellitus, DN: Diabetic nephropathy, CDV: Cardiovascular diseases, MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase, NO: Nitric oxide, HO-1: Heme oxygenase-1, |G6PD: Glucose-6-phosphate dehydrogenase

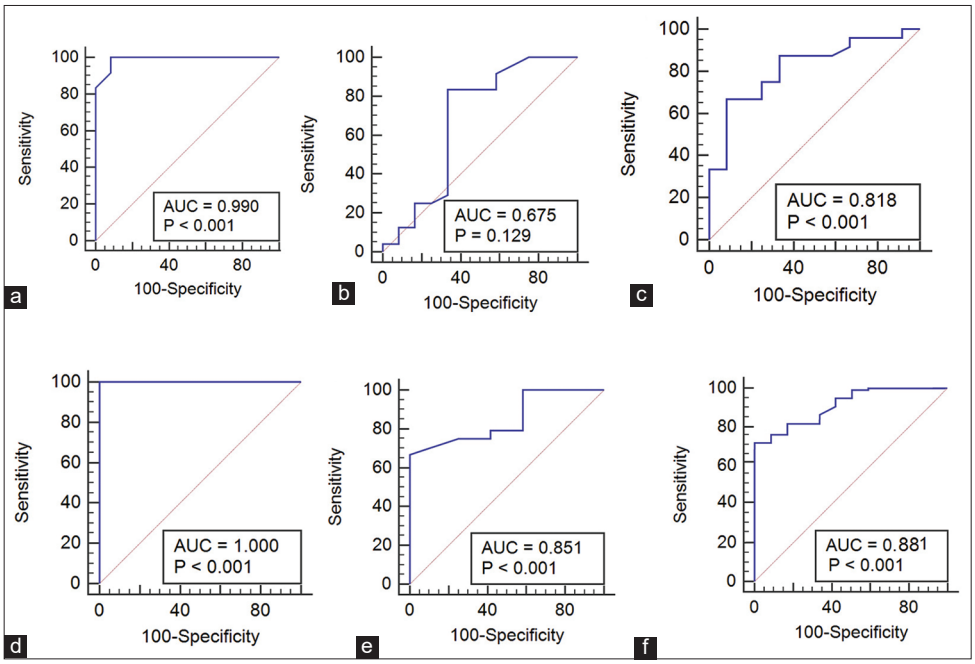


**Figure 1:** Roc analysis of a (MDA), b (GSH) and c (CAT), d (NO), e (HO-1), and f (G6PD/lactate). ROC analysis showed that the sensitivity and specificity of oxidative stress markers were 66.67% to 100%. ROC: Receivers operating characteristic, MDA: Malondialdehyde, GSH: Glutathione, CAT: Catalase, NO: Nitric oxide, HO-1: Heme oxygenase-1, G6PD: Glucose-6-phosphate dehydrogenase.

>68.6, sensitivity 66.67%, specificity 100% and AUC 85% [Figure 1e]. ROC analysis of G6PD/lactate showed cutoff was <90.72, sensitivity 100%, specificity 92.35% and AUC 88% [Figure 1f].

## DISCUSSION

T2DM is a metabolic disease that affects more than 400 million people globally, causing great danger to human

health and the economy.[43] It is associated with insulin resistance and insufficient response of pancreatic β-cells to compensatory insulin needed.[44]

Oxidative stress is considered the primary mechanism of insulin resistance and DM.[45] This oxidative harm is associated with defective glucose metabolism, vascular dysfunction and kidney disease. It results in excess production of ROS and reactive nitrogen species (RNS) more than the buffering capacity of the antioxidant defence systems, leading to cellular and molecular deviations.[46,47] Our study confirmed the presence of oxidative stress within diabetic patients by the increases in HO-1, MDA and NO; while G6PD/lactate, GSH reduced and CAT levels decreased in DM. ROX and RNS produced by glucose oxidation and protein glycation cause enzymes dysfunction, cellular machinery damage and increased insulin resistance.[48] Elevation of MDA content may be due to the accumulative load of reactive oxygen and nitrogen species in hepatic tissue.[49] CAT is one of the regulators of free radical hydrogen peroxide metabolism. Reduced enzyme activity confirms a weakened antioxidant enzyme system in diabetes.[50] According to ROC results, oxidative stress markers (especially NO and MDA) could use as attractive candidates as diabetic complications predictors.

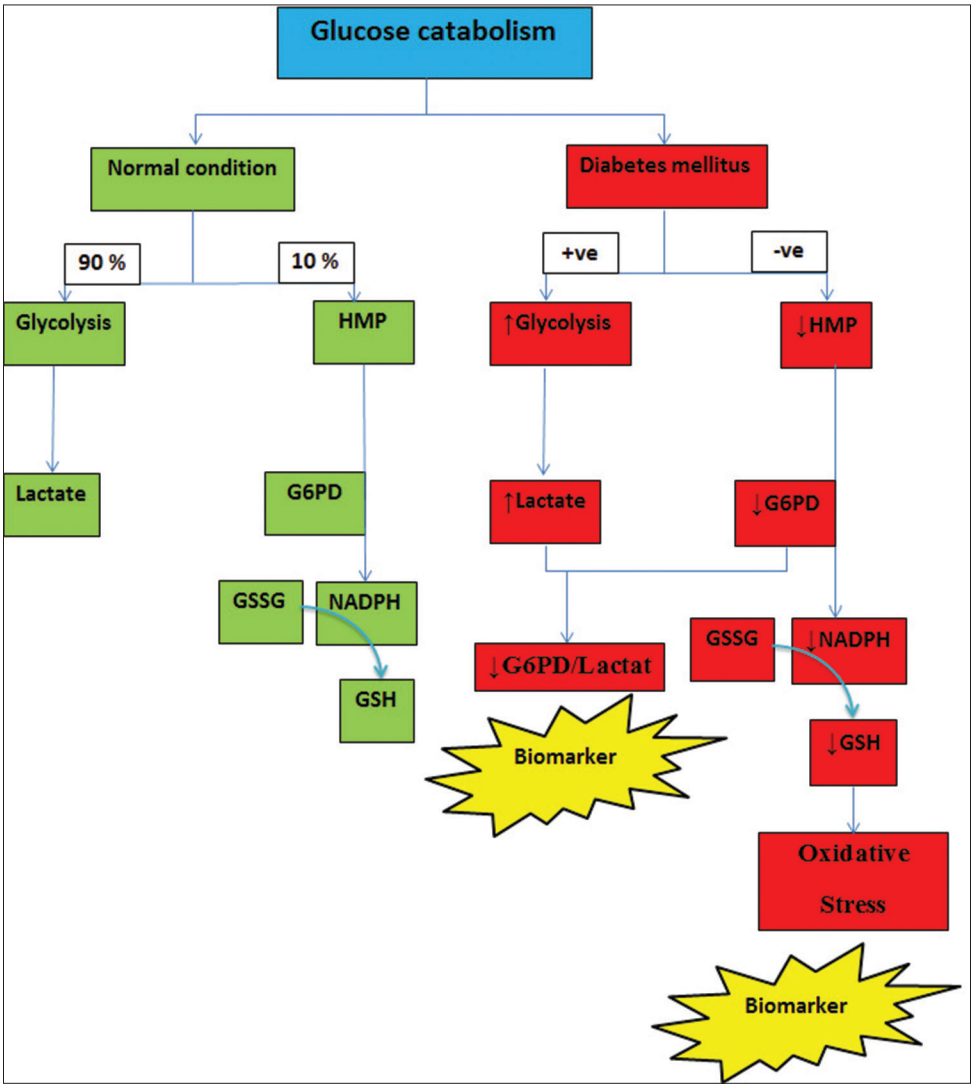


**Figure 2:** Hexose monophosphate/glycolysis pathways shift during Diabetes Mellitus. Under normal physiologic circumstances, 90% of glucose in red blood cells converts to pyruvate or lactate by glycolysis, while the other 10% of glucose is catabolized by hexose monophosphate (HMP). Under diabetes conditions, a low amount of glucose enters the cells; thus, the cells prefer to use most of the glucose to produce energy (glycolysis pathway) rather than antioxidants (HMP pathway), leading to oxidative stress conditions, which could use as a biomarker for diabetic complications. GPD: Glucose-6-phosphate dehydrogenase, GSSG: Oxidised glutathione, GSH: Reduced glutathione.

G6PD/lactate is a novel indicator of both glucose metabolism and oxidative stress. Glucose is the energy source of the red blood cell, and under normal physiologic circumstances, 90% of glucose is catabolised anaerobically to pyruvate or lactate by glycolysis.[51] This pathway produces energy in the form ATP for essential cellular processes. The other 10% of glucose is catabolised by the HMP pathway producing NADPH using the rate-limiting enzyme G6PD. NADPH is required for the regeneration GSH that protects cells against oxidative stress.[51] The novel ratio of G6PD/lactate represents the glucose catabolism by HMP/glycolysis as an expression for using glucose to produce either antioxidants (GSH) or energy. Due to the low amount of glucose that enters the cells in DM, the cells prefer to use most of the glucose to produce energy rather than antioxidants, leading to oxidative stress conditions as a biomarker for diabetic complications [Figure 2]. ROC results confirm our conclusion where the sensitivity and specificity of oxidative stress parameters were 66.67–100%. For G6PD/lactate, the sensitivity was 100% and specificity was 92.35%, which indicated that the parameter is strongly related to any change in glucose metabolism and oxidative stress.

Thus, G6PD/lactate ratio of control group > diabetic group > diabetic complication group. This means that the HMP pathway inhibits in diabetic complication > diabetic group and the glycolysis pathway stimulates in diabetic complication > diabetic group.

Dyslipidaemia is commonly associated with obesity and T2DM. The present study established dyslipidaemia in diabetic patients by increasing cholesterol, TG and LDL-C and decreasing HDL-C. Hypercholesterolaemia and DM cause abnormal ROS production, which consider the common pathogenetic mechanism of endothelial dysfunction and CVD.[52] Furthermore, diabetic oxidative stress in cardiac and vascular tissues leads to chronic inflammation, fibrosis, apoptosis, vascular smooth muscle cell proliferation and arterial stiffness.[53]

Kidney injury was indicated in the present study by increasing urea, creatinine, A/C ratio and the decrease in glomerular filtration rate. Hyperglycaemia increases free radical production, resulting in oxidative stress, which plays an essential role in the pathogenesis of DN.[54-56]

The present study refers for the 1st time to changes in HMP/glycolysis pathways during diabetes, which is an important cause for the formation of oxidative stress in diabetic patients. Clinically, this finding reflects the importance of using antioxidant drugs to prevent diabetic complications.

Limitations of the present study include the small sample size and the effect of gender should be investigated. Prospective follow-up studies with a bigger sample size and other biochemical parameters will confirm the new mechanism of diabetic complications.

## CONCLUSION

HMP/glycolysis pathways are shifted during DM near glycolysis rather than HMP to produce energy where the amount of glucose enters the cells is low, causing oxidative stress. Oxidative stress markers could be used as early predictors of diabetes complications.

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### Declaration of patient consent

Institutional Review Board (IRB) permission obtained for the study.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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