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ANALYSIS OF KEY SUGAR PHOSPHATE INTERMEDIATES IN GLYCOLYSIS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY TO ASSES AND EVALUATE POSSIBLE ALTERATIONS IN METABOLITE POOL OF ERYTHROCYTES IN TYPE 2 DIABETES

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Keywords:

Type 2 diabetes, Sugar phosphate intermediates, Glycolysis, LC-MS/MS, Erythrocytes

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ABSTRACT: In previous studies, it has been shown that the activities of the glycolysis cycle enzyme are significantly altered with changes in kinetic attributes of hexokinase, phosphorfructokinase and lactate dehydrogenase. Moreover, the kinetic parameters of the terminal enzyme lactate dehydrogenase also changed significantly. In view of these observed changes, it was of interest to know if and how the flux of intermediates of the glycolytic pathway was also affected. With this objective, a simple, rapid and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed for quantification of five important glycolysis intermediates of rate-limiting steps [*viz.* glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-bis-phosphate (FBP), 3-phosphoglyceric acid (3PGA), and pyruvate in human erythrocytes. Liquid chromatography was performed using Supelco Ascentis Express RP amide (250 mm × 4.6 mm, 5µm) column with mobile phase, 10mM ammonium acetate in water and acetonitrile. The detection of metabolites was carried out by tandem mass spectrometry. In erythrocytes from Type 2 diabetic subjects, there was a significant increase in intracellular concentrations of G-6-P and 3-PGA, with a marked decrease in FBP and pyruvate. The results suggest that the metabolic flux of glycolysis is altered significantly in the erythrocytes from Type 2 diabetic patients. The significance of these findings in relation to adenosine triphosphate (ATP) synthesis and loss of membrane deformability, which lead to diabetic complications, is discussed.

INTRODUCTION: Previous studies have shown structural and functional alterations in erythrocytes from type 2 diabetic individuals [1]. This included significant alterations in enzyme activities as well as substrate saturation kinetics of hexokinase (HK) and phosphofructokinase (PFK) [2, 4]. Hence it was of interest to find out whether the flux of intermediary metabolites was also influenced by diabetic state.

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With this view in mind, an LC-ESI/MS/MS method was developed for simultaneous quantification of key metabolites of glycolysis. The details of this method and the results of quantification of the metabolites are described here.

MATERIALS AND METHODS: Glucose-6-phosphate (G-6-P) (purity ≥ 98%), fructose-6-phosphate (F-6-P) (purity ≥ 98%), fructose-1,6-bisphosphate (FBP) (purity ≥ 98%), 3-phosphoglyceric acid (3-PGA) (purity ≥ 98%) and pyruvic acid (purity ≥ 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetone, acetonitrile, methanol, ammonium acetate, and other chemicals were of HPLC grade and were

purchased from Millipore Sigma (Burlington, Massachusetts, USA).

Sample Collection: For the present study, EDTA-blood was collected after overnight fasting from 12 age and sex-matched healthy non-diabetic controls and 40 clinically diagnosed Type 2 diabetic subjects after informed written consent.

The main inclusion criteria for diabetic subjects were glycated hemoglobin (HbA1c) > 7.0%, fasting blood glucose (FBG) > 126 mg/dL and age 40-60 years.

The mean age was 49.13 ± 1.70 and 50.41 ± 1.81 years, respectively, for the control and diabetic groups. The values of FBG levels were 96.8 ± 1.76 and 252.2 ± 12.28 mg/dL, respectively, for the two groups.

The corresponding values of HbA1c were 4.89 ± 0.13 (30 mmol/mol) and 7.7 ± 0.21% (61 mmol/mol). The Type 2 diabetic subjects were advised to continue their treatment with prescribed anti-diabetic agents. The study was conducted following Helsinki Convention Guidelines.

Sample Preparation: The RBC count was taken for individual samples in control as well as diabetic group, and it was found that for both the group the RBC count was comparable.

Erythrocytes from 1 mL whole blood were sedimented by centrifugation at 400 × g for 10 min, and the plasma was carefully decanted. The buffy coat was discarded following gentle swirling with 0.9 % NaCl solution.

The packed cells were repeatedly washed and re-suspended to the original volume using 0.9% NaCl solution. 100 µL aliquots of erythrocyte suspension were diluted 1:10 with 0.9% NaCl solution and used for further analysis.

The metabolites were released from erythrocytes by two to three freeze and thaw cycles, following which the samples were vortex and sonicated for 30 sec on ice and subsequently centrifuged for 5 min at 11,000 × g at 10 ºC.

After this, the supernatant was transferred to a new tube. Complete deproteinization was achieved by adding ice-cold acetone to RBC lysate (4:1 vol/vol), then centrifuged at 11,000 × g at 4 ºC for 10 min.

The supernatant containing glycolytic intermediates was transferred to glass vials and evaporated to dryness under the gentle stream of nitrogen. Finally, the extracted intermediates were diluted with 100 µL acetonitrile: water (50:50 vol/vol) solution before subjecting to LC-MS/MS analysis.

Analysis of Sugar Phosphate Intermediates by LC-ESI-MS/MS: Chromatographic separation of major glycolysis intermediates was achieved on Shimadzu LC 20-AD LC System using Supelco Ascentis Express RP amide (250 mm x 4.6 mm, 5 µm) column heated to 45 ºC.

The mobile phase, A, was: 10mM ammonium acetate in water: acetonitrile (95:5 vol/vol) and B, was: 10 mM ammonium acetate in water: acetonitrile (5:95 vol/vol). The buffer pH was adjusted to 9.0 using 30% aqueous ammonia solution. The initial gradient was 1% B for 0-0.2 min, 0% - 70% B for 0.2-1 min, 70% B, 1 - 6 min, 70% - 1% B, 6 - 7 min, 1% B for 7-12 min.

The flow rate was set to 0.8 mL/min and was split after the analytical column in a ratio of 1:2, resulting in an inlet flow into the tandem mass spectrometer of 200 µL/min; 10 µl of the sample was injected onto the column, and the total run time was 12 min [5, 6]. The total equilibration time was 3 min. Electrospray ionization tandem mass spectroscopic method (ESI-MS/MS) was developed with Sciex 4000 QTrap mass spectrometer. Mass spectrometric conditions for five glycolysis metabolites were optimized by direct infusion of their standards independently. The ion source parameters were set as follows: ion spray voltage, 5500 V, source temperature was set at 500 ºC, nebulizer gas and collision gas at setting approximately at 40 and 30 psi, respectively. Detection and quantification of the sugar phosphates were performed by multiple reaction monitoring (MRM-mode) in positive mode, and pyruvate was quantified by negative MRM-mode. The MRM transitions (Q1/Q3) settings for the different sugar phosphates were G-6-P: m/z – 259.7/-97; F-6-P: m/z - 259.2/-97; FBP: m/z – 339.4-97; 3-PGA: m/z - 185.4/-79: and Na-pyruvate; m/z – 87.2/-43.45 **Table 1.**

TABLE 1: SPECIFIC COMPOUND- DEPENDENT MS PARAMETERS USED IN MULTIPLE REACTION MONITORING (MRM) FOR SUGAR PHOSPHATE AND MRM FOR PYRUVATE

S. no.	Metabolite	MRM Transition		Ionization Mode	Collision energy (v)	Declustering Potential (v)
		Q1	Q3			
1	G-6-P	259.7	97	+ ve	7	60
2	F-6-P	259.2	97	+ ve	7	60
3	FBP	339.4	97	+ ve	7	80
4	3-PGA	185.4	79	+ ve	7	80
5	Pyruvate	88	43	- ve	-23	-25

Abbreviations: G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; FBP: fructose-1,6-bisphosphate; 3-PGA: 3-phosphoglyceric acid.

Statistical Analysis: Results were integrated and calculated using linear regression analysis by the Analyst 1.4.2 software. The results are expressed as mean ± standard error (SEM) and were analyzed for significance by Student's t-test using Graph Pad Prism version 5.0, and p values < 0.05 were considered to be statistically significant.

Quantification and Validation Procedure: The method was partially validated according to the Guidelines of the International Conference of Harmonization (ICH) M10 for partial validation for bio-analytical methods. Briefly, the method was validated for matrix effects, linearity, the limit of detection (LOD) and limit of quantification (LOQ), accuracy, and inter-and intra- day precision.

Preparation of Calibration Curve: Standard solutions of G-6-P, F-6-P, FBP, 3-PGA, and Na-pyruvate, were prepared by dissolving the individual standards in methanol at a final concentration of 1 mg/mL, and aliquot were stored refrigerated. The different concentrations of the four sugar phosphates and pyruvate were spiked in a matrix to obtain a seven-point calibration curve and extracted with the same procedure as that of samples (details given in 'Sample Preparation). Final dilutions were carried out using the diluents, acetonitrile: water (50:50 v/v). Calibration curves were obtained by plotting the analyte peak area (y) versus concentrations of the standard (x). The weighting factor used was $1/X^2$. The acceptance criterion for accuracy of the calibration standards was ≤ 15%. The correlation coefficient (R2) was calculated as the value of the joint variation between x and y. The correlation coefficients for all runs were>0.99 +/- 0.01.

Preparation of Quality Control Sample: The Quality control samples: LQC (Lower Quality Control Sample), MQC (Middle-Quality Control Sample), and HQC (Highest Quality Control Sample) were prepared in 3 different sets with 6 replicates. While HQC concentration was taken as 80% of the highest concentration of the calibration curve standard, MQC was taken at 50%, and the LQC concentration was taken as 1.5 times the LOQ level of the calibration curve.

Recovery Study: The recovery of all four sugar phosphates and pyruvate were determined by comparing peak areas of extracted low-quality control (LQC) and high-quality control (HQC) samples with post-extracted spiked LQC and HQC samples. The extracted LQC and HQC were prepared as a set of six samples for each sugar-phosphate and pyruvate standard in a matrix initially. Then ice-cold acetone was added in 1:4 volume and extracted with the same procedure as that of samples. For post-extracted LQC (n=6) and HQC (n=6) sets, the non-spiked matrix was extracted by the same procedure as that of samples, and then the respective concentrations of four sugar phosphate and pyruvate standard were spiked to extracted matrix independently. The percent recovery of all five analytes was calculated by dividing analyte peak areas of the extracted QC samples with those of post-extracted spiked samples.

Limit of Detection (LOD) and Limit of Quantification (LOQ): The LOD is the lowest concentration of the analyte that can be detected. The LOD is calculated with a peak-to-peak signal-to-noise ratio. The LOQ determined for the experiment is equivalent to the 10 times higher of LOD.

Accuracy and Precision: The accuracy and precision set was prepared in a matrix with six replicates of LQC, MQC, and HQC samples for four each of sugar phosphates and pyruvate.

The % RSD of six replicates should be <15% of the mean. The intra-assay and inter-assay precision were carried out with different sets of injections (n=6).

Matrix Effect: Matrix effect is defined as the suppression or enhancement of ionization of analytes by the presence of matrix components in the biological sample. The matrix effect was determined by preparing two different sets for the neat standards and matrix spike standards.

For each set six LQC and six HQC were prepared. In the first set (A), standards of G-6-P, F-6-P, FBP, 3-PGA and Na-pyruvate were prepared in acetonitrile: water (50:50 vol/vol). In the second set (B), the matrix samples were prepared the same as the extracted QC samples (details given in 'Recovery study'). Six different lots of human (EDTA) RBCs were used for the second sets. Matrix effects were determined by dividing the results of set B by those for A (B/A × 100%).

RESULTS:

LC Method Development and Mass Spectrometry Optimization: earlier, determination and quantification of metabolites of glycolysis by enzymatic methods with photometric detection was a preferred choice for analyzing metabolites because of the high sensitivity of the specific techniques. However, these methods do not permit simultaneous estimation of different compounds in the same sample [7]. Recently, different methods like isotachophoresis, capillary electrophoresis with UV indirect spectrophotometric detection, High-performance liquid chromatography (HPLC) and LC-MS/MS methodology have been developed for simultaneous estimation of glycolysis metabolites [5, 6, 8, 9]. However, these reported LC-MS methods were highly sophisticated and needed specialized chemicals and derivatizing agents for the determination of glycolysis metabolites, which increases the cost of analysis. Keeping this in mind, for simultaneously determining the glycolysis metabolites with high polarity index, low in vivo concentration, and complex matrix structure, a simple and reliable Mass Spectrometry (MS) with Electrospray Ionization (ESI) method was developed. The selected m/z ratios for the five different compounds are shown in **Table 1.** Total ion chromatogram of a sugar phosphate metabolites spiked in matrix of control RBCs is shown in **Fig.1.**

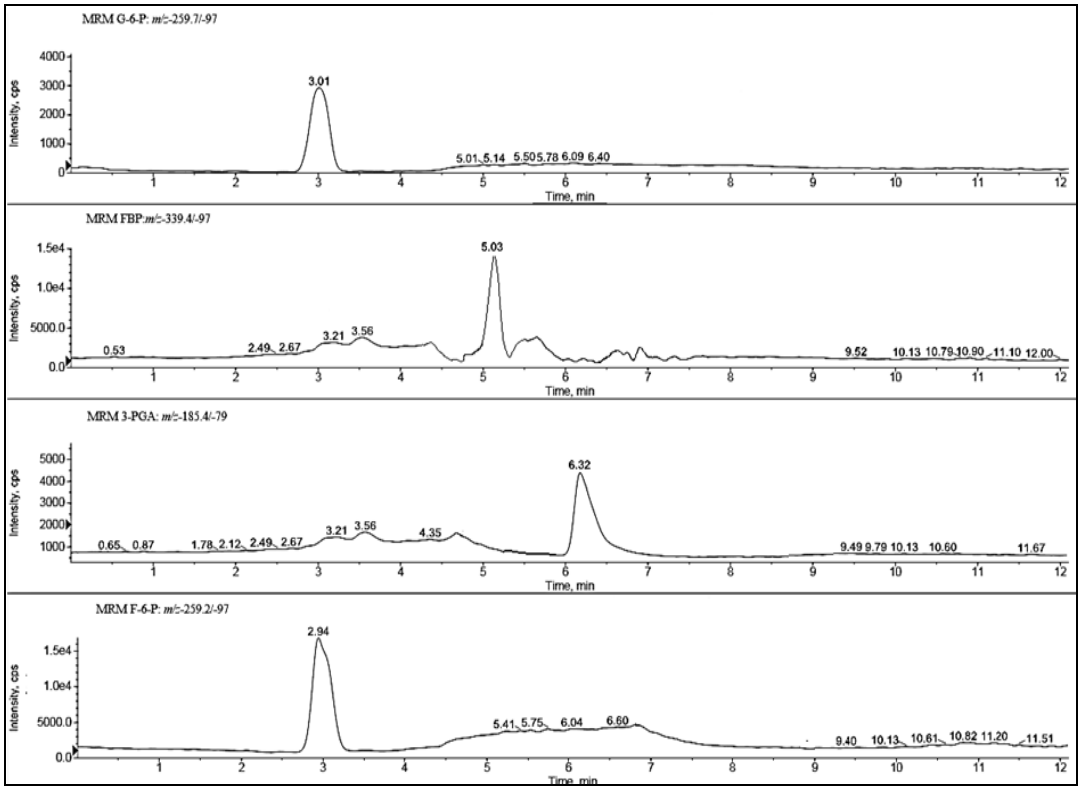

FIG. 1: ION CHROMATOGRAM OF SUGAR PHOSPHATE METABOLITES SPIKED IN MATRIXOF CONTROL RBCS. MRM TRANSITIONS ARE GIVEN FOR EACH INDIVIDUAL SUGAR PHOSPHATE AT LOQ CONCENTRATIONS

Hyphenation of LC with MS detection provides influential analytical techniques for biological samples. LC offers a wide range of columns and allows fast analysis with efficient resolution while MS detects analyte with high selectivity and sensitivity [10].

The LC-MS/MS methods reported so far are mostly for bacteria and other biological samples, whereas very few methods are reported for erythrocytes; these methods use the ion pair modifier and expensive LC columns which increases the cost of analysis [5, 11].

Method Validation:

Linearity: The seven-point calibration curves were obtained for four sugar phosphates and pyruvate. The linearity ranges and coefficients for the same are given in **Table 2.** Linear plots were obtained for the given concentration ranges for all five sugar-phosphate metabolites data not shown in **Table 2.**

TABLE 2: VALIDATION PARAMETERS I.E., LINEARITY RANGE, CORRELATION COEFFICIENT, LOD AND LOQ, PERCENT RECOVERY AND INTRA-DAY AND INTER-DAY PRECISION ASSAY FOR ANALYZED GLYCOLYTIC SUGAR-PHOSPHATE METABOLITES

Metabolite	Linearity range µmol/L	Correlation coefficient (R)	LOD µmol/L	LOQ µm*ol/L*	% Recovery		Intra-day precision % RSD			Inter-day precision % RSD		
					LQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
G-6-P	3.5-70	0.993	0.35	3.5	99.67	99.70	6.20	5.81	5.3	6.4	4.12	5.0
F-6-P	3.3-65.6	0.995	0.33	3.3	98.28	99.42	6.41	4.70	2.2	6.19	3.98	1.8
FBP	2.5-49.3	0.995	0.25	2.5	99.47	99.53	1.53	1.64	2.1	1.6	2.26	2.0
3-PGA	4.3-86.9	0.996	0.43	4.3	99.42	99.51	7.97	6.22	5.1	8.3	5.41	4.3
Pyruvate	0.91-18.2	0.995	0.1	0.9	99.78	101.89	1.96	3.01	2.6	1.85	1.39	2.9

Number of replicates performed at each concentration, n = 6.

Recovery: The recovery studies were performed by injecting six replicate injections of LQC and HQC samples. The developed method shows good recoveries for four sugar phosphates and pyruvate. The respective percent recoveries are given in **Table 2.**

LOD and LOQ: The LOQ determined for the experiment is equivalent to 10 times higher than LOD. The LOD and LOQ for sugar phosphates and pyruvate in the RBC matrix are given in **Table 2.**

Accuracy and Precision: The Accuracy and Precision were assessed by analysis of six replicates of QC samples at three different concentrations (LQC, MQC, HQC). All results were within the acceptance criteria. The % RSD for LQC, MQC and HQC for four sugar phosphates and pyruvate are also shown in **Table 2.**

Matrix Effect: The matrix interference was observed in standard spiked in the control matrix. And because of this, although all targeted sugar phosphates were successfully eluted in 7 min., the total run time was extended to 12 min. to eliminate the matrix interference **Fig. 1.** No interference signals were detected around the retention times of the analytes.

DISCUSSION: Earlier studies have shown that the enzyme make-up as well as the kinetic attributes of the key irreversible enzymes *viz.* hexokinase and phosphofructokinase altered significantly in Type 2 diabetic RBCs [2-4]. In view of this, the present studies were designed to determine whether the intra-RBC concentrations of sugar phosphate metabolites of glycolysis are also altered in the diabetic state and its implications for ATP synthesis.

With this purpose in mind, a new method was developed for simultaneous rapid quantification of targeted four sugar phosphates metabolites *i.e.* G-6-P, F-6-P, FBP, 3-PGA, and Pyruvate in the RBCs from Type 2 diabetic subjects, and normal controls by using LC-MS/MS technique. The results are given as µg/RBCs in 1ml blood and µM/RBCs in 1 ml blood in **Table 3.**

It is clear that in the diabetic RBCs the content of G-6-P increased two folds. The content of 3PGA also almost doubled. But, the contents of F-6-P were unchanged. However, the content of FBP and pyruvate decreased by more than half. Other researchers have also reported the concentrations of glycolysis intermediates as absolute quantities [9, 12].

However as is well recognized the metabolic / enzyme activities and enzyme kinetics are dependent on the intracellular concentration and not on the content. In view of this, it was important to find out the intracellular concentrations of the said metabolites. This was achieved by computations based on the reported intracellular volume of RBC in normal controls. Thus, it has been reported that in normal individuals, the intracellular volume of an RBC is 90×10^{-15} L[13]. In earlier studies, it has been reported that the mean diameter of RBCs the RBCs in normal non-diabetic individuals is 7.73 ± 0.15 µm and height of 2.32 ± 0.12 µm. As against this, these values for diabetic RBCs are diameter 6.71 ± 0.05 µm and height 1.35 ± 0.028 µm [1]. By taking into account the dimensions of diabetic RBCs, by comparison with the reported value for control, the intracellular volume of the diabetic RBCs was computed to be 47.8×10^{-15} L. Based on these values, we then computed the intracellular molar concentrations of the metabolites. These values are presented in **Fig. 2.** Thus, the molar concentration of G-6-P was almost 5 fold higher in the diabetic RBCs with a 3.5 fold increase in 3-PGA concentration and around 52% increase in the concentration of pyruvate; concentration of FBP was unchanged **Fig. 2.**

TABLE 3: THE CONCENTRATIONS OF MAJOR GLYCOLYSIS INTERMEDIATE S IN RBCS FROM CONTROL AND TYPE 2 DIABETIC SUBJECTS

Metabolite	Content /RBCs in 1ml blood			
	µg		µmoles	
	Controls (12)	Diabetics (40)	Controls (12)	Diabetics (40)
G-6-P	31.03 ± 0.0084	65.45 ± 0.006 **	0.11 ± 0.00002	0.232 ± 0.00002*
F-6-P	18.31 ± 0.003	18.85 ± 0.0003	0.060 ± 0.00001	0.062 ± 0.00002^{NS}
FBP	38.58± 0.046	17.62 ± 0.031*	0.095 ± 0.0001	0.043 ± 0.0001*
3-PGA	19.09 ± 0.0087	36.34 ± 0.002*	0.083 ± 0.0004	0.158 ± 0.0001*
Pyruvate	9.79 ± 0.76	4.15 ± 0.44*	0.089 ± 0.0069	0.038 ± 0.0040*

The results are given as mean ± SEM of number of observations indicated in parentheses.*, p< 0.0001, **, p <0.005, ***, p <0.01.

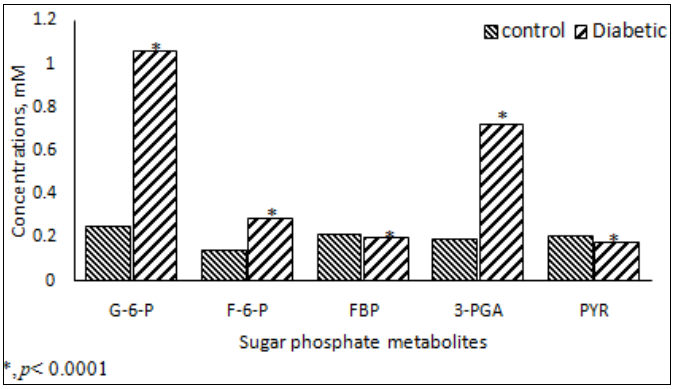

FIG. 2: HISTOGRAM REPRESENTING INTRA-CELLULAR CONCENTRATION OF SPECIFIC SUGAR-PHOSPHATE METABOLITES IN INDIVIDUAL RED BLOOD CELL

Taken together, the results would suggest that significant changes occurred in the metabolic pool/metabolomics of diabetic RBCs. At this point, it may be pointed out that Kono *et al.* reported that the ATP content of RBCs in control and diabetic groups were almost comparable, with the respective values being 1630 and 1473 µmol/ml RBCs. If one takes the liberty of converting these values to molar concentration, these values turn out to be around 3 mM and 6 mM, respectively, for the control and diabetic groups.

The changes in the metabolite concentrations that we observe here represent the steady-state dynamic equilibrium. This would suggest that in spite of their reduced cytoplasmic volume, the diabetic RBCs have a greater capacity to retain G-6-P, which is rapidly converted to F-6-P and FBP. Ultimately this high turnover rate is reflected in increased 3-PGA concentration **Fig. 2.** The concentrations of the end product pyruvate are comparable in the two groups, suggesting that the diabetic RBCs rapidly convert pyruvate to lactate, which is consistent with an earlier report on increased lactate dehydrogenase activity in the diabetic RBCs [4]. Taken together, the results point out to an increased turnover of metabolites and enhanced glycolysis rate in the diabetic RBCs, which may ultimately lead to lactic acidosis, a well-established feature of Type 2 diabetes [14].

Clinical Application: We have reported earlier that the structure of the RBC membrane is abnormally different in the diabetic group as compared with the normal non-diabetic group *i.e.*, quantitatively, the membrane proteins are almost double without any significant changes in the lipid content 1.

As is well recognized, there is loss of RBC deformability in diabetes. The RBCs lose their elastic properties and are either not able to squeeze through capillaries or are unable to reform/regain their shape after passing through the capillaries. Subsequently, the RBCs break and undergo fragmentation [15]. It is very well known that ATP has several important functions in the RBCs, including binding of oxygen to hemoglobin, restoration of glutathione level, maintenance of Na+-K+ pump, and restoration of RBC reform ability [16]. It has been reported that the binding of ATP to spectrin plays an important role in reform ability [17].

This would suggest that possibly the restoration of spectrin conformation is an energy-dependent process. Therefore, one wonders whether, in the diabetic RBCs, the binding of ATP to spectrin results in futile cycles. The altered flux of glycolysis is the indication of the non-linear flow of glycolytic cycle in Type 2 diabetic RBCs, which affects the ATP synthesis; additionally, there may be a significant loss in ATP content due to the futile spectrin-ATP cycle referred to above. This possibility, however, needs to be carefully evaluated. From the literature survey, it is apparent that RBCs are the basic underlying cause that primarily contributes to developing diabetic complications [18, 19]. However, the clinical relevance of RBCs has been only limited to the measurement of the extent of glycosylated hemoglobin in diabetes. The glycosylated hemoglobin can only reflect and/or relate to the mean blood sugar level for a long time but does not consider glycemic variability or the incidence of other diabetes-induced metabolites with deleterious effects. Therefore, glycosylated hemoglobin is the least important parameter to predict diabetic complications [20, 23].

The present work provides strong evidence showing that the metabolomic profile of RBCs in Type 2 diabetic patients is significantly altered in terms of the intra-erythrocyte molar concentrations of glycolysis intermediates. These metabolomic changes could be potential indicators of diabetes-induced changes in RBCs, which can be helpful in the early prognosis of diabetic complications. Hence, it is anticipated that the observed altered metabolic patterns may offer new biomarkers for research in Type 2 diabetes based on RBCs and related diabetic complications. Thus, finally, it may be concluded that in diabetic RBCs, there are not only structural and functional abnormalities but the flux of glycolysis is also altered, leading to loss of deformability which further leads to diabetic complications.

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Contribution of Authors: All authors contributed equally to this work.

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