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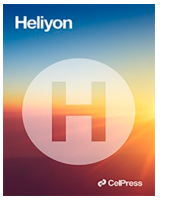

Research article

Thermal and morphological properties of human erythrocytes from patients afflicted with type 1 diabetes mellitus

Péter Gaszler [a,1], Dénes Lőrinczy [a,1], Dávid Szatmári [a], Beáta Bódis [b], Katalin Türmer [a,*]

[a] Department of Biophysics, Medical School, University of Pécs, Pécs, Hungary
[b] Department of Internal Medicine I, Clinical Centre, University of Pécs, Pécs, Hungary

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ABSTRACT

Red blood cells (RBC), are the most unique and abundant cell types. The diameter of RBCs is 7–8 μm. They have an essential role in transporting circulatory oxygen. The RBCs travel through varioussized capillaries throughout the entire body, some significantly smaller than the RBCs due to their incredible deformability. The RBC membrane allows the cell to resist stress as it squeezes the cytoplasm. Permanent stress or altered blood plasma conditions can result in decreased membrane deformability. Type 1 diabetes mellitus (T1DM) is one of the most common chronic diseases. In diabetes, the primary influence is increased glucose levels in the blood plasma that can result in the oxidation of lipids and proteins and the glycation of proteins. The damage changes the conformation and organization of various lipids and proteins, which can result in the loss of function and decreased deformability. Hemoglobin A1c (HbA1c) or glycohemoglobin is a form of hemoglobin found in RBCs. Glucose and fructose can bind to hemoglobin by non-enzymes, and different glycated forms of hemoglobin can be formed. The ratio of glucose-bound (glycated) hemoglobin to total hemoglobin (expressed as a percentage) is a critical laboratory parameter in managing diabetes. It can be used to determine the average blood glucose level of the patient over the past 60–120 days. Here, we investigate the effect of diabetes on RBCs' shape and membrane stability due to microscopy and DSC (Differential Scanning Calorimetry) methods. The comparison of the RBCs from diabetic and non-diabetic patients was classified by the HbA1c, showing that the conditions in diabetes caused atypical cell morphology and then, in a casedependent manner, increased or decreased the thermal stability of cytoplasm or the cell membrane, respectively. It shows the importance of DSC application in routine quality screening of diabetic erythrocytes and that it can be a crucial parameter of T1DM.

1. Introduction

Type 1 diabetes mellitus is major health problem in modern society in childhood and adults too [1–4]. The relationship between type 1 diabetes and chronic microvascular (retinopathy, nephropathy, neuropathy) or macrovascular complications (coronary arteries, changes of lower extremities) needs more investigationSymeonidis and colleagues found that in diabetic patients the rigidity index (RI)

* Corresponding author.Szigeti str. 12, H-7624, Pécs, Hungary.
E-mail address: katalin.turmer@aok.pte.hu (K. Türmer).
[1] equal contribution.

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of RBCs was significantly increased compared to healthy controls. Their results are consistent with other findings, show a direct link between the state of glucose control and membrane rigiditiy of erythrocytes. The same working group studied the viscoelastic behaviour of the red blood cell membrane (RBCM) in cells from patients with beta thalassemia to investigate whether the precipitated or abnormal haemoglobin, which is one of the main features of thalassemic syndromes, influences the membrane viscosity. The altered membrane, is less deformable (the membrane bending modulus is about twice as high as that of normal erythrocytes) and more resistant to flow, so the viscoelasticity of the erythrocytes has also increased. In addition to normal haemoglobin A, pathogenic conditions such as beta thalassemia have higher levels of fetal haemoglobin (HbF) and abnormal haemoglobin [5–7].

Erythrocytes, red blood cells (RBC), are the most unique and abundant cell types Their nucleus and other cellular components (mitochondria, Golgi-apparatus, endoplasmic reticulum) are missing in RBCs, but cytoplasmic processes make up their functions. However, they have cell membranes, soluble hemoglobin, and cytoskeleton with contractile apparatus. The RBC envelope is based on a tight composition of the lipid bilayer and the skeletal system [8,9]. The composition of RBCs' cell envelope plays an essential role in maintaining the integrity of their shape, size, and flexibility. Any lipid bilayer disorder risks the envelope's functionality, thus decreasing its survivability [10]. The skeleton of the RBC envelope is formed primarily by spectrin and actin filaments and myosin bundles arranged triangularly parallel to the membrane. In this case, their binding to the lipid bilayer via tethering sites is formed from the ankyrin-based and the 4.1R-based complexes [11,12]. Ankyrin links the spectrin to band 3 and band 4.1 to glycoforin. Band 3 is an integral membrane protein (25–30 % of total RBC membrane proteins). Band 3 (anion exchange protein 1) and band 4.5 (glucose transporter 1) are two abundant erythrocyte membrane proteins. In diabetic patients, prolonged hyperglycaemia (diabetes not well or not at all controlled), prolonged higher glycated hemoglobin levels result in prolonged oxidative stress through the generation of reactive oxygen species (ROS). If the band 3 protein undergoes structural modifications due to oxidative stress, resulting in inadequate ion transport activity, this will also have detrimental effects on membrane structure, integrity and functionality. This affects the lifespan of RBCs, altering their shape and deformability. Therefore, the importance of the band 3 protein in the physiology and aging of erythrocytes has been emphasized by Remigante, Zheng and Morabito and colleagues as a potential molecular target for therapeutic interventions [13–17].

Red blood cells are normally flexible and disc-shaped, which optimizes their flow properties in vessels and capillaries. They are thicker at the edges and are therefore usually compared to doughnuts. In many inherited disorders, the red blood cells become rounded, oval sickle-shaped [18]. In many hereditary disorders, red blood cells are spherical, oval or sickle-shaped [19–24].

Hemoglobin is an iron-containing metalloprotein in the human RBCs. Due to the iron in its structure, it binds oxygen in the lungs and transports it to the cells and from cells it transports carbon dioxide to the lungs. There are a large number of hemoglobin variants in human blood. About 700 structural hemoglobin variants have been identified, only HbS, HbC and HbE exist in high frequencies in the world. Elevated haemoglobin levels can indicate the presence of various inherited diseases (for example beta thalassemia). The most common type of hemoglobin is a tetramer (contains two α and two β protein subunits, these subunits are made up of 141 and 146 amino acids) called haemoglobin A. Hemoglobin gives the 95–98 % of hemoglobin amount in human blood. Hemoglobin A2 (HbA 2) is a normal variant of hemoglobin A that consists of two alpha and two delta chains. It is found at low levels in normal human blood. Hemoglobin A1c (HbA1c) or glycohemoglobin is a form of hemoglobin found in red blood cells. Glucose is bound to hemoglobin by non-enzymes, and different glycated forms of hemoglobin can be formed. The ratio of glucose-bound (glycated) hemoglobin to total hemoglobin (expressed as a percentage) is a critical laboratory parameter in managing diabetes. It can be used to determine the average blood glucose level of the patient over the past 60–120 days [25,26].

In a higher blood glucose level, more sugar binds to hemoglobin subunits. The binding is initially unstable, but after a few hours, it stabilizes and becomes irreversible (13,14): the sugar can no longer detach from the hemoglobin. As a consequence, the lifetime of the HbA1c form depends on the lifetime of the red blood cells. To allow measurements and, thus, diabetes management to be carried out under standardized conditions worldwide, a working group at the Institute for Collaborative Classification (IFCC) in Münster has chosen HbA1c as the parameter to be measured because it is a stable glycaated form. In HbA1c, besides other valine and lysin residues, glucose possibly binds to the N-terminal of the beta chain of hemoglobin (a valine amino acid) [25,26]. Fructose binds to other sites in the hemoglobin structure [27].

Diabetes management aims in accordance with ADA (American Diabetes Association) and WHO (World Health Organisation) guidelines to keep the HbA1c below 5.7 % (39 mmol/mol) so that long-term complications of diabetes are delayed or do not occur. HbA1c is a good indicator for the diagnosis of diabetes, and values above 6.5 % or 48 mmol/mol (between 5.7 and 6.4 % or 39–46 mmol/mol is prediabetes) support the diagnosis. As the binding is unstable for a while and stabilizes only after a few hours, transient high blood glucose levels are barely detectable by HbA1c. In contrast, the historical average value is visible. In healthy people, the value is between 4 and 5.7 % or 20–39 mmol/mol [28,29].

We collected patients into groups based on their glucose metabolism status, as determined by the HbA1c value.

In this study, we focused on the changes in the morphological and thermal stability of the RBCs.

Although more studies have investigated the changes in the characteristics of the erythrocyte membrane, they have mainly focused on type 2 diabetes mellitus. In this study, we aimed to examine and compare the morphology and thermal stability of red blood cells of patients with type 1 diabetes to those of erythrocytes from patients without diabetes. Many people are already working on finding a cure for type 1 diabetes, even if we cannot play a part in finding a cure for diabetes, but it would be important to be able to make the lives of patients with diabetes easier. Therefore, we were very interested to see how much does long-term high blood sugar levels affect the thermal parameters of RBCs. In addition, we wanted to find a method to check glycated status of patient with diabetes at any time, even within 3 months, with little material requirement and for little money. Many previous papers have used DSC results for other pathological conditions to analyse pathologies. Lőrinczy and his collegues [35,41,42] have been using DSC for years to assess the severity of various diseases using RBCs, blood plasma and serum. This has not yet been done for diabetics, but I believe that the results

of DSC measurements can be used to monitor HbA1c. By utilizing biophysical techniques, such as differential scanning calorimetry (DSC), we investigated the effect of increased glucose conditions on the thermal stability of cytoplasmic and membrane components; the relevant morphological changes of RBCs were studied by light microscopy.

2. Materials and methods

2.1. Patient selection

The blood samples were collected from patients with type 1 diabetes and patients without diabetes at the Clinical Centre, Department of Internal Medicine 1, University of Pécs. All participants signed a written consent form after being informed in detail about the details of the research. 19 patients aged between 25 and 67 years (mean 40 years) were selected, 11 women and 8 men. Participant characteristics are summarized in Table I. We divided the patients into 3 groups based on their HbA1c values: below 5.7 % (39 mmol/mol) is the group of patients without diabetes (n = 4), between 6 and 8 % (42–64 mmol/mol) (patients with mild diabetes) (n = 8), and above 10 % (86 mmol/mol) is severe diabetes (patients with severe diabetes) (n = 7). Patients are using insulin only.

2.2. Blood sample collection and preparation

In this work, we explore the effects of high blood glucose on the thermodynamic properties of RBCs derived from patients with type 1 diabetes to identify calorimetric features associated with diabetes mellitus. These characteristics were compared with the typical thermodynamic parameters of RBCs of controls (patients without diabetes). For this purpose, we utilize freshly isolated RBCs before the measurements. Peripheral blood samples were obtained from patients using routine endocrinological sampling. To avoid blood coagulation, we collected the samples in BD Vacutainer (REF: 368860) blood collection tubes containing K3-EDTA (7.2 mg/mL). RBC sample was washed with 5 mL of erythrocyte storing medium (5 mM KCl, 145 mM NaCl, 1 mM $CaCl_2$, 0.15 mM $MgCl_2$, 10 mM MOPS-HCl, 10 mM glucose, 0.1 % BSA, pH 7.4). Then, they were centrifuged with 1500×*g* at 37 °C for 15 min to separate cells from plasma components. The RBC samples were immediately used for microscopic and DSC measurements [30–33].

2.3. Microscopy of RBC

For better visualization, cells were stained with a Papanicolaou (PAP) Red Stain Kit (Abcam) following the same protocol as we did for epithelial cells [34]. All reagents and materials were equilibrated to room temperature before usage. A dilution containing 50 μL of the blood sample and 950 μL of the erythrocyte medium (1:20) was prepared.

An inverted light microscope (Olympus IX 81, Olympus Europa Gmbh, Hamburg, Germany) was used to visualize and compare the morphological differences of the RBCs with 10 × and 40 × objective lenses.

Table 1
Participant characteristics: Data are mean ± SD or % (*n*). Differences in participant characteristics between patients without diabetes, patients with mild, and severe type 1 diabetes were compared using the One-way Anova Test and Student's T-test to determine whether the difference between the basic characteristics of the 3 groups is statistically significant. Post hoc pairwise comparisons were conducted using the Bonferroni test when significant interactions were found. Bolded characters indicate significance accepted at $p < 0.05$. No significant difference in HbA1c values was found between patients without diabetes and patients with mild diabetes. In contrast, there was a substantial difference between the group of patients without diabetes and the group of patients with severe diabetes (***p=0.0318***) and between the group of patients with mild diabetes and the group of patients with severe diabetes (***p = 0.0394***). The gender of the patients was not considered a factor in the statistical analysis of the data.

	Patients without diabetes (HbA1c <5.7 % or 39 mmol/mol)	Patients with mild type 1 diabetes (HbA1c 6–8% or 42–64 mmol/mol)	Patients with severe type 1 diabetes (HbA1c >10 % or 86 mmol/mol)	*p*
Basic characteristics				
Participants, *n*	4	8	7	
Age (years)	43.00 ± 15.43	37.88 ± 8.59	44.14 ± 15.77	0.630
Female	1(25 %)	5(62.5 %)	5(71.42 %)	
Duration of diabetes (years)	0	20.125 ± 9.83	18.71 ± 9.38	0.781
Weight (kg)	86.5 ± 12.77	76.88 ± 20.47	75.43 ± 22.99	0.666
HbA1c (%)	5.33 ± 0.17	7.25 ± 0,23	14.77 ± 0.74	**0.014**
HbA1c (mmol/mol)	34.75 ± 2.22	55.75 ± 2.38	92.14 ± 13.33	**0.014**
Red Blood Cells (T/L)	4.3 ± 0.18	4.65 ± 0.48	4.73 ± 0.58	0.3634
Hemoglobin	137.25 ± 12.09	143.5 ± 11.51	139.86 ± 15.13	0.7687
Comorbidities				
Lung disease	0	0	0	
Stroke	0	0	1(14 %)	
Heart disease	0	2(25 %)	3(43 %)	
Hypertension	1(25 %)	0	4(57 %)	
Kidney disease	0	0	1(14 %)	
Hypothyroidism	1(25 %)	4(50 %)	2(29 %)	
Hypoglycemic medications				
Insulin	0	8(100 %)	7(100 %)	

2.4. Analysis of microscopic images

ImageJ (developed by NIH, Bethesda, MA, USA) was applied for the quantitative analysis of microscopy images. We determined the average for interpreting the cell diameter and central concavity. First, we measured the average diameter of cells or concavity on the mean in five independent directions per cell, then calculated the average in the population of 20–50 cells from the same sample.

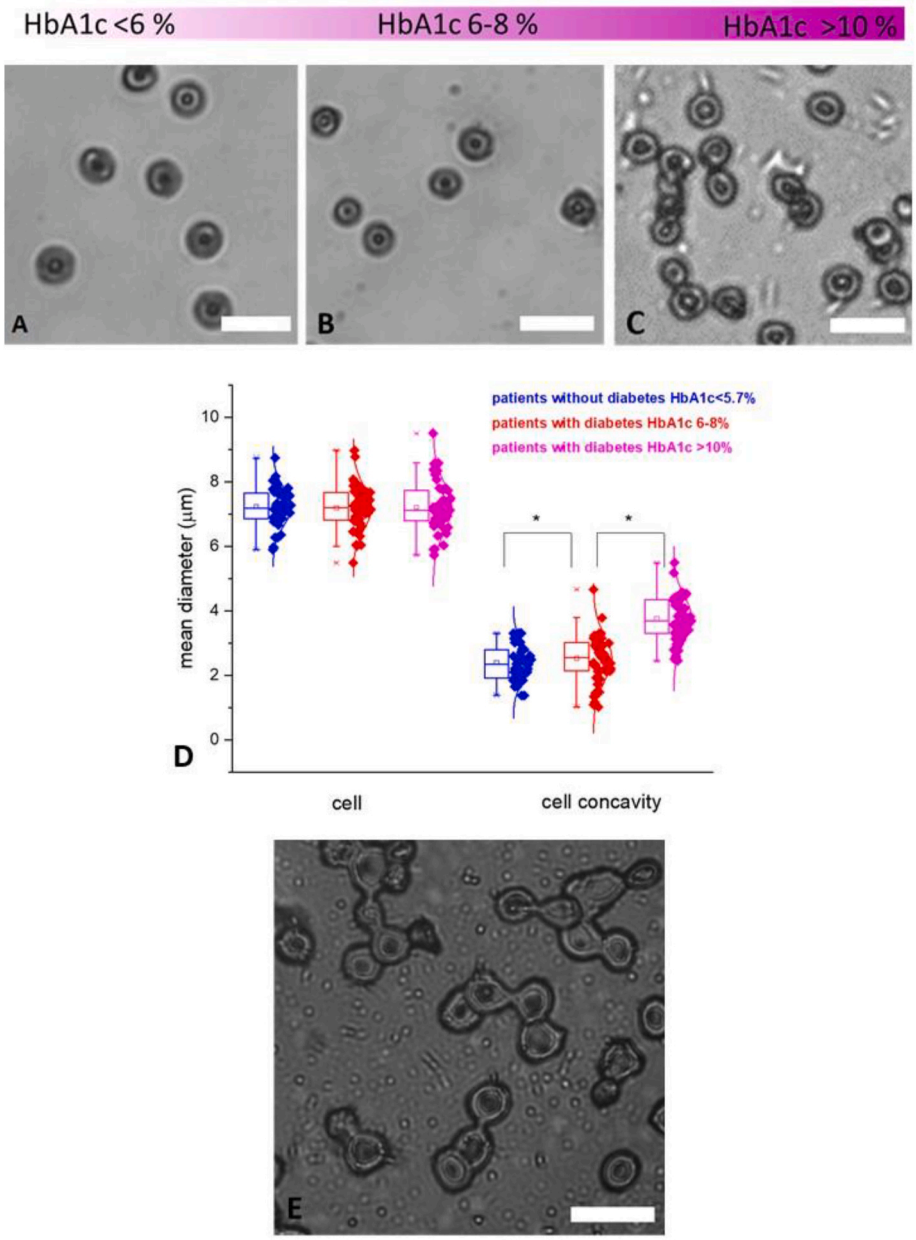

Fig. 1. Microscopic images of blood smears. (A) The erythrocytes from patients without diabetes (HbA1c below 5.7 % or 39 mmol/mol). (B) Erythrocytes of patients with mild diabetes (HbA1c 6–8% or 42–64 mmol/mol). (C) Altered morphological presentation of stomatocytes and discocytes from the sample of patients with severe diabetes (HbA1c above 10 % or 86 mmol/mol). Size bars 14 μm. (D) Box-diagram analysis of cell and central concavity diameters based on images from patients without diabetes, patients with mild and severe diabetes cases. Box normal diagrams represent data with ±SD (error bars), average value (points), and median values (lines). Asterisks mean statistically significant differences between groups. Significance was defined as p values < 0.05. (E) The gender of the patients was not considered a factor in the statistical analysis of the data. A high number of plasma-bridges and cell-cell interactions were observable in the case of patients with severe diabetes. Size bar 14 μm.

2.5. Differential scanning calorimetric (DSC) measurements

Calorimetric methods are ideal for understanding the processes occurring in different materials involving heat effects. These methods are used to characterize the global changes in biological systems. The DSC method can map proteins' conformational changes and spatial stability [35]. The SETARAM Micro DSC-III calorimeter monitored the thermal denaturation of the human RBCs. Conventional Hastelloy batch vessels were used as sample holders. In the first standard zone, the instrument was conditioned by increasing the temperature to 25 °C and holding the temperature for 1200 s (s). A dynamic method was applied to heat, and it cooled the sample. In the heating process, the sample was heated from 25 °C to 100 °C at 0.3 K min^{-1} with a duration of 16000 s. Then, in the cooling process, the sample was cooled from 100 °C to 20 °C at 1 K min^{-1} with a duration of 4800 s. The volume of the samples was 0.5 mL in all cases. The RBC concentration was approximately 5000 cells/mL. The reference was the erythrocyte-storing medium. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. The precision of enthalpy determination in the case of our equipment lies at 1 mJ/g. As the sample and reference are heated, at the beginning of it, the difference in temperature between the sample and the reference is zero because they have a similar heat capacity. Reaching the starting and unfolding range, the supplied heat will evoke a structural change in the sample; this way, a temperature difference will appear between the sample and the reference cell. A control system will supply extra heat into the sample holder (endotherm process) or the reference vessel (exotherm process) to maintain the constant zero temperature difference between the vessels. In the function of temperature, this extra heat flow is presented as a DSC scan, which can provide information on the samples' behavior (unfolding or „crystallization"). A repeated scan of the denatured sample was used as a baseline reference, and this was subtracted from the original DSC curve. After plotting the heat flow values in the function of temperature, the calorimetric enthalpy was calculated from the area under the heat flow curve using a two-point setting SETARAM peak integration.

2.6. Statistical analysis

The statistical analysis was performed in Origin 2018 (OriginLab Corp. Northampton, MA, USA), derived from independent samples. The effects of type 1 diabetes were studied on the population of cells and presented with a box diagram. We aimed to obtain and measure the impact on the population of cells. To compare the characteristics of the different groups (patients without diabetes, patients with mild and severe type 1 diabetes), one-way ANOVA and Student's t-tests were used to determine whether the difference between the baseline characteristics of the 3 groups was statistically significant. Post hoc pairwise comparisons were performed using the Bonferroni test when significant interactions were found. Bold indicates significance accepted at $p < 0.05$. The gender of the patients was not considered a factor in the statistical analysis of the data.

3. Results and discussion

3.1. The varied erythrocyte morphology in the samples from patients with type 1 diabetes

The morphology of RBCs in the peripheral blood smears made from patients without diabetes (HbA1c <5.7 % or 39 mmol/mol) or patients with mild diabetes (HbA1c 6–8% or 42–64 mmol/mol) did not show remarkable differences. However, the cells from patients with severe diabetes (HbA1c above 10 % or 86 mmol/mol) were varied (Fig. 1A, B, C). The central concavity of the RBCs is more

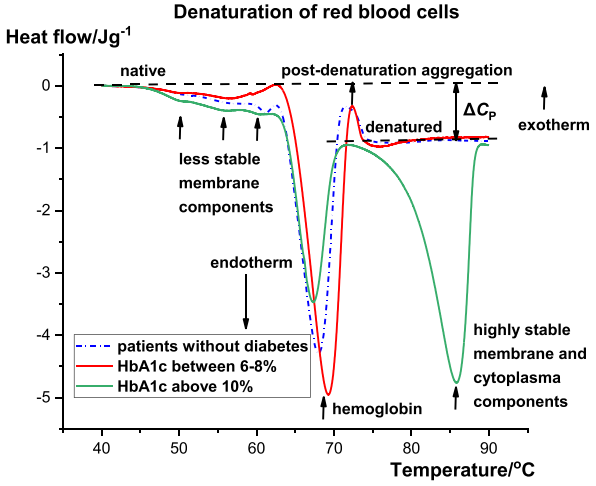

Fig. 2. Hemoglobin, membrane, and cytoplasmic components. DSC scans of RBCs from patients without diabetes (HbA1c below 5.7 % or 39 mmol/mol) (blue dotted line), patients with mild diabetes (HbA1c 6–8% or 42–64 mmol/mol) (red line) and patients with severe diabetes (HbA1c>10 % or 86 mmol/mol) (green line). The curves are the average of five independent samplend normalized on total sample mass. The patients without diabetes are the controls.

extended under conditions caused by diabetes than in regular instances, referring to the typical morphology of discocytes and stomatocytes. In the case of patients without diabetes, the samples contained only 16 ± 2 % discocytes; besides that, other erythrocytes showed normal morphology; in the case of patients with mild diabetes smear contained 46 ± 3 % and, in the severe case, 80 ± 3 % of cells were spherocytes and stomatocytes with plasma bridges and aggregations. The cell diameter and concavity of the RBCs were measured as adequate membrane and cytoplasmic flexibility indicators. The average diameter of the normal cells and discocytes or stomatocytes did not show significant differences in the case of patients without diabetes, and it was 7.23 ± 0.57 μm; in the case of patients with mild diabetes, it was 7.19 ± 0.66 μm; in the case of patients with severe diabetes it was 7.21 ± 0.75 μm (Fig. 1D). The average diameter of the central concavity in the samples from patients without diabetes was 2.39 ± 0.53 μm in the case of patients with mild diabetes and, in the case of patients with severe diabetes, significantly higher, 2.52 ± 0.70 μm and 3.75 ± 0.69. The gender of the patients was not considered a factor in the statistical analysis of the data.

The typical morphology of erythrocytes is considered the most adaptable, while the discocytes with extended central concavity and modified SF are more rigid. Typically, the maximum abundance of spherocytes is 44 %. Healthy control has no significant difference in cell types than in patients with type 1 diabetes without vascular complications. However, in the case of vasculopathy, the spherocytes (60 %) number was increased significantly [22], and some more different shapes like acanthocytes (surface blebbing), distorted forms, and "cup forms" as stomatocytes. After receiving adequate treatment, the erythrocyte morphology was restored to normal [23]. A high number of plasma bridges and cell-cell interactions were observable in case of patients with severe diabetes and within 20 % of cells. A high number of plasma-bridges and cell-cell interactions were observable in the case of patients with severe diabetes.

3.2. Glycation and thermal stability of erythrocytes and hemoglobin

Fig. 2 shows the average of five thermal denaturation curves of RBCs in the case of three different groups of patients. Generally, three small endothermic peaks were observed in the low-temperature range of 55–61 °C, one in the middle range of 67–69 °C, and one in the high range of 75–90 °C. The analysis of the DSC scans results in thermodynamic parameters to explore the structural dynamics of RBCs to the heat denaturation (Table II). The denaturation temperature range ($\boldsymbol{\Delta T}$) is inversely proportional to the cooperativity of thermal domains. The denaturation (or melting $\boldsymbol{T_m}$) temperature and the calorimetric enthalpy ($\boldsymbol{\Delta H}$) are proportional to the components' stability.

As can be seen in the smaller denaturation range, we can find the formerly identified denaturated compounds [36–42]: spectrin, around 50 °C, bands of proteins 2.1, 4.1, and 4.2 around 56 °C, as well as the B3 glycoprotein at ~ 60–62 °C. The running of the scans fluctuates around the control. The remarkable endothermic curves in the middle range are responses of hemoglobin [40]; it exhibited a slightly increased Tm with 1.18 °C in the case of patients with mild diabetes and decreased with 0.73 °C in the case of patients with severe diabetes (Table II). The hemoglobin, the most abundant membrane component of erythrocytes [43], contains four domains and hem-iron complexes (Fig. 3A) and can respond to abnormal conditions.

It shows a high structural sensitivity to the high glucose and fructose concentration due to the several valin and lysin residues that can act as good target points for glycation (Fig. 2B) [26,44]. As previously shown by Saraswathi et al. (PBD ID: 5HY8) [45], one of the most sensitive binding cleft of hemoglobin is the cavity around the Lys138 (α1 domain), and then the fructose binding causes an enormous structural refinement. They identified a particular glucose binding cleft around Thr38 (β2 domain) (Fig. 3B) (PBD ID: 3B75) [46], which did not have any effect on the structure of the fructose-saturated hemoglobin. Usually, the free glucose concentration of blood plasma is 7.5 times higher than the free fructose, which ratio can increase to 10 times in the case of severe diabetes [47] in combination with the absolute increment of both sugar levels. In our interpretation, we can suggest that the fructose binding leads to the high stability of hemoglobin, and the T_m and ΔH of the endothermic peak were increased around 69 °C (Table II.*)* in the case of patients with mild diabetes. However, in the case of patients with severe diabetes, due to the relatively high concentration of glucose, it can compete with fructose on binding clefts by the varied affinities and/or glucose binds to different sites on hemoglobin, thus reducing its structural stability, with decreased T_m and ΔH (Table II).

However, in the high range, there are main effects (Fig. 2). The first exothermic peaks, around 74 °C (Table II), can be observed in control and patients with mild diabetes cases, which could signify the reordering of proteins after endothermic responses. A similar

Table 2
The characteristic thermal parameters of denaturation of thermal domain 1. Symbols: ΔT stands for denaturation range, Tm is the melting temperature, and ΔH is the calorimetric enthalpy normalized on the total sample mass (data are average of five independent samples).

Thermodynamic parameters									
Severity	Low range	Middle range	High range	Low range	Middle range	High range	Low range	Middle range	High range
	ΔT/°C	T_m/°C	ΔH/ Jg^{-1}	ΔT/°C	T_m/°C	ΔH/ Jg^{-1}	ΔT/°C	T_m/°C	ΔH/ Jg^{-1}
Patients without diabetes HbA1c <5.7 % or 39 mmol/mol	16.25	61.14	0.14	9.02	68.07	3.28	13.13	74.51	0.57
Patients with diabetes HbA1c 6–8 % or 42–64 mmol/mol	17.38	56.84	0.36	9.78	69.25	4.05	10.96	74.74	0.62
Patients with diabetes HbA1c >10 % or 86 mmol/mol	18.09	55.53	0.17	9.61	67.31	2.03	17.17	85.84	4.27

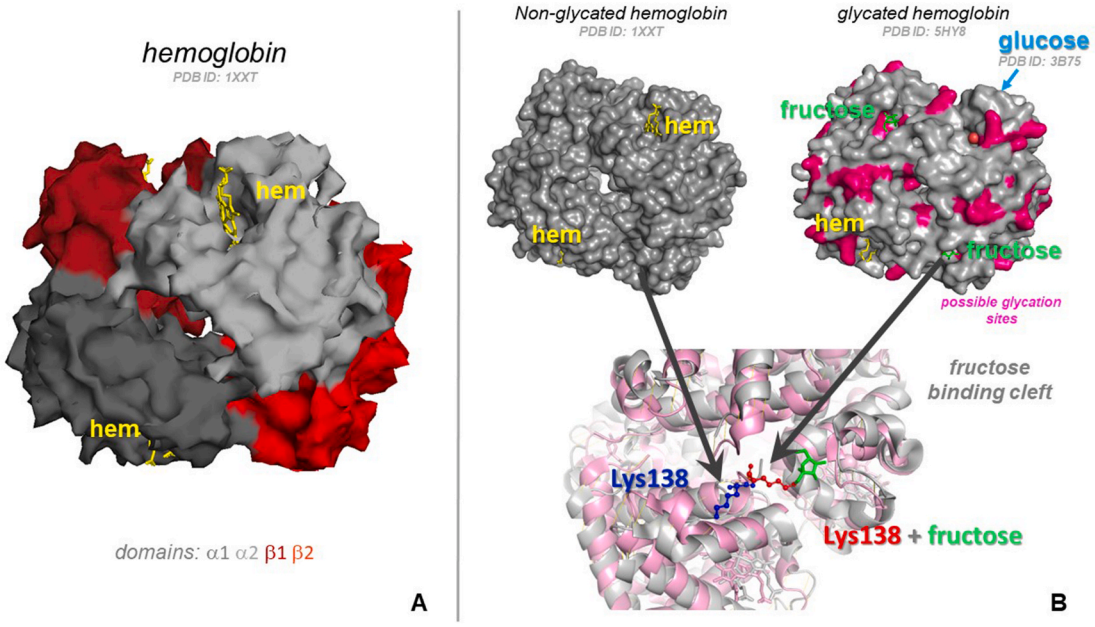

Fig. 3. The modular structure of hemoglobin. (A) The structure of non-glycated hemoglobin (PDB ID: 1XXT) is the composition of four domains α1,2 and β1,2, with the O_2 binding hem complexes localized in all of them. (B) Structural differences between the non-glycated (PDB ID: 1XXT) and glycated (PDB ID: 5HY8) hemoglobin are remarkable. All the valine and lysin residues (magenta) can be target points of glycation. One possible glucose-binding cleft was identified around the residue of Thr38 in β2 chains (PDB ID: 3B75). (lower panel) One structurally sensitive fructose binding cleft was found at Lys138 and neighboring chains in α1, non-glycated (gray), and glycated (pink).

effect can be observed in the case of polymer physics, where the process of recrystallization followed the melting. It was shifted slightly to a higher temperature (by 1 °C) in the patients with mild diabetes case, suggesting that the glycation modified the structure of cytoplasmic polymers. In the study of Todinova et al. [40], there was an exotherm peak at 78 °C; it was interpreted as the aging effect on RBCs. Therefore, exotherms between 71 and 78 °C can be described as rearrangement of cytoplasmic polymers by metabolic and aging effects. Interestingly, after the hemoglobin response, the baseline dropped, representing a significant increase in heat capacity compared to the native state, which can result from molecular aggregations [40–42]. In the case of severe diabetes, there is no exotherm between 71 and 78 °C, but a remarkable endotherm appeared at 86 °C. Compared to the microscopic images, we can suggest that the cells underwent a general rearrangement of membrane and cytoplasmic components, resulting in altered morphology and function. A high number of plasma-bridges and cell-cell interactions were observable in the case of patients with severe diabetes. A similar endotherm effect was observed in our previous study on guinea pigs' RBC [42]. It was observed rarely under normal conditions. Therefore, we can explain the extraordinary peaks at 85–86 °C as the implication of highly stable membrane and cytoplasmic components. They were generated by an adaptation mechanism to the abnormal blood plasma conditions during the maturation and differentiation of erythrocytes. In good agreement with plasma bridges in Fig. 1E, as signs of the modified differentiation and adaptation of cells.

The tendency of the effect on the RBCs caused by the severity of diabetes is more pronounced, characterized by increased ΔT refers to the low cooperativity between the thermal domains, indicating the altered function and structure of cell compartments due to glycation of the cell envelop and cytoplasmic molecules.

4. Conclusion

It is hypothesized that in patients without diabetes (HbA1c under 5.7 % or 39 mmol/mol) and a patients with mild diabetes group (HbA1c 6–8 % or 42–64 mmol/mol), the treatment (insulin injections) significantly affects the state of hemoglobin and membrane components, respectively, together with their morphology and thermal stability. In these cases, there is a short period of high blood glucose, during which some of the sugar is detached from the hemoglobin and does not bind irreversibly. Patients who have severe diabetes do not manage their diabetes with strict regularity, do not obtain insulin, or give it late, so the binding of sugar to hemoglobin becomes irreversible; they essentially have high blood glucose most of the time, which is why there is a remarkable difference in the results of DSC measurements and microscopic images. The binding of glucose and fructose varies the structure of hemoglobin. Alternatively, red blood cells may undergo a rearrangement of compartments during maturation, adapting to persistently high sugar concentrations, resulting in a more rigid membrane composition to more successfully resist the adverse effects of glycation end products in the bloodstream.

The plasma bridges observed in the patients with severe diabetes cases also indicate adaptation to changing conditions. Previously, this nanotube-like membrane or plasma bridge (based on cytoskeletal protrusions) has been observed in many instances among other

cell types (e.g., PC12 cells, dendritic cells, and THP-1 monocytes but has not been observed in RBCs [48,49].

Elevated glucose and high fructose concentrations in the blood can reduce the flexibility of the erythrocytes' membrane and cytoskeletal components, implicating it in harmful conditions and leading to serious long-term complications. Hyperglycemia was associated with increased serum and urinary fructose concentrations in patients with severe diabetes, which decreased with improved glycemia. In diabetic patients, the viscoelasticity of RBCs is altered, their shape is changed and they are more prone to aggregate in the bloodstream due to the altered membrane structure. Several medical studies have wroted that, concave depth, diameter, and rigidity index of RBCs in diabetes mellitus decreased, while the stiffness and viscosity increased, compared to non-diabetes samples. Ciasca, Jin and AlSalhi and collaegues measured the viscoelasticity of RBCs and found increased viscosity and stiffness of diabetic RBC membranes compared to healthy RBCs. Both RBC aggregation and blood viscoelasticity are abnormal in hyperglycemic type 2 diabetics and suggest that altered in vivo blood flow dynamics should be present in these individuals [5,50,51].

Other groups reports that they can't measure significant difference in the rheological behaviour of RBCs from diabetic and healthy subjects, despite significant differences in the non-enzymatic glycosylation of haemoglobin and cell membrane proteins. They therefore think that degree of glycosylation RBCs does not modify the viscoelastic properties of the cells [52]. The increased viscoelastic properties of diabetic RBCs from patients with diabetes are mainly the result of increased erythrocyte membrane rigidity [7].

Therefore, it is thought that the more rigid membrane is caused by the persistence of non-enzymatic glycosylation mainly in the severe diabetes group. Livesey and Sánchez-Lozada and their research teams have studied the effect of patients with diabetes getting more fructose than glucose in their diet. Fructose is often used in regular foods for healthy people too and, in clinical feeds intended for persons with diabetes.

Measuring fructosamine concentrations is advantageous when monitoring the condition with another similar HbA1C test, which is impossible. The fructosamine concentration helps to monitor the effectiveness of diabetes treatment but cannot be used to screen for diabetes. If the patient's fructosamine level is elevated, the patient's average glucose level has been elevated for the past 2–3 weeks. In general, a higher fructosamine level means a higher glucose level. Suppose there is a trend from normal towards higher fructosamine. In that case, this may indicate that the patient's glucose control is inadequate, they have a high sugar level or insufficient insulin, or that insulin treatment has become ineffective [27].

The glycemic response to dietary fructose is low, which may improve concentrations of glycated hemoglobin. A lower HbA1c concentration was found because of the use of fructose. They concluded that fructose may have the added benefit of lowering concentrations of glycated hemoglobin. However, the higher amount of fructose binding to hemoglobin results in a more rigid rbc membrane structure, probably due to the increased amount of triacylglycerol in the membrane [53,54].

Keeping blood sugar levels in the proper range in the medical routine is essential to avoid serious complications. It is not just a matter of whether the patient takes the insulin, but also of taking it at the right time, not later, when blood glucose levels have been high for several hours. Based on our work, DSC measurement could also be used as a routine test to determine the qualitative status of erythrocytes in the blood in the case of patients with diabetes. Evaluating of the DSC curves provides immediate information on the condition of diabetic patients. In severe cases, the results of glycosylation can be clearly seen, and doctors can warn their patients that prolonged high blood glucose levels affect the properties of their RBCs. These measurements can be repeated not only every 120 days, but also every month. This means that changes in patients' bodies can be monitored every 30 days. Lőrinczy and Garbett and their collegues [55–59] have been using DSC for decades. The differences caused by diseases have been researched using calorimetric methods to assess the severity of various diseases using RBCs, blood plasma and serum. I think so that the results of DSC measurements can be used to monitor HbA1c. Mainly time-based follow-up studies were conducted, we are planning to do this in the future. More measurements and a larger number of cases are definitely needed to be able to state this with complete certainty.

CRediT authorship contribution statement

Péter Gaszler: Writing – original draft, Methodology, Data curation. **Dénes Lőrinczy:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation. **Dávid Szatmári:** Writing – original draft, Software, Formal analysis, Conceptualization. **Beáta Bódis:** Writing – review & editing, Validation, Methodology, Conceptualization. **Katalin Türmer:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Informed consent Statement

Not applicable.

Guarantor Statement

Dr. Katalin Türmer is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity and accuracy of the data and analysis.

Data and resource availability statement

The datasets and resource data generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

Institutional review Board Statement

Not applicable.

Data Availability Statement

There is no additional data available to upload.

Ethics Approval

The protocols were approved by the Regional Research Ethics Committee of the Clinical Centre at the University of Pécs (9592-PTE 2023), dated May 15, 2023.

All participants signed a written consent form after being informed in detail about the details of the research.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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