Introduction Glycated hemoglobin (HbA1c) is a parameter broadly employed in the assessment of glycemic control in diabetes. The 2010 “Standards of medical care in diabetes”, published by the American Diabetes Association (ADA), recommended performing the HbA1c test at least every 6 months in patients in whom disease is clinically stable, while subjects after modifications of therapy or in whom glycemic goals have not been met should be tested every 3 months. Moreover, the ADA suggested the HbA1c assay be implemented in the diagnosis of diabetes and in the detection of an increased risk of developing this disease. Among various approaches employed to measure the concentration of HbA1c, high-pressure liquid chromatography is considered to be a reference method. HbA1c tests might not be clinically reliable in some circumstances. In cases when HbA1c levels do not correlate with glycemia and clinical symptoms, the results should be interpreted with caution, several conditions known to influence the measurement should be taken into account, and use of another diagnostic method, or even testing another marker of glycemic control, e.g., fructosamine or 1,5-anhydroglucitol, should be considered.

AG (mg/dl) = 28.7 × HbA1c – 46.7
AG (mmol/l) = 1.5944 × HbA1c – 2.5944

Some clinical studies suggested that the effect of plasma glucose levels on HbA1c measurement may vary over a 120-day period. For example, the degree of glycemia immediately before the test will have a stronger impact on the final result than glycemia levels from earlier time points. Average glycemia measured at 1, 2, and 3 months prior to the test will affect 50%, 40%, and 10% of the final value, respectively.

Values of HbA1c can be lower than the actual ones in patients experiencing large daily fluctuations in their glucose levels, and therefore, can be falsely closer to the target values. Similarly, average glycemia in these patients can also be close to the target value. In such cases, measurement of HbA1c does not seem to be a proper method of analyzing glycemic control in these patients. A recent report has indicated that fasting glucose levels and 2-hour glucose levels after ingesting 75 g of glucose were characterized by much higher variability in each patient than HbA1c levels in the same patients.

It is widely accepted to express HbA1c as a percentage of the total level of hemoglobin. For individuals with normal glucose tolerance HbA1c...
Glycation of hemoglobin with the formation of HbA1c (modified from Niederau CM, et al.)

![glycation diagram]

 usually falls within 4.1%–6.5%, although some reports show lower values, namely <4%.5,7

The International Federation of Clinical Chemistry and Laboratory Medicine proposed mmol HbA1c/molHbA as an alternative unit8-11 to express the levels of HbA1c. When using such units, however, it has to be taken into account that normal values for healthy individuals will be lower by 1.3–1.9 as compared to the percentage values (%).10,11

Glycated hemoglobin is not only a retrospective parameter of diabetes control, but also an independent indicator of an increased risk for long-term complications in this disease. Diabetes Control and Complications Trial (DCCT) as well as United Kingdom Prospective Diabetes Study (UKPDS) showed that development and progression of long-term complications in both type 1 and type 2 diabetes strongly correlated with the levels of HbA1c.12,13

FinnDiane, a study conducted in Finland and involving patients diagnosed with type 1 diabetes, revealed that variability in HbA1c measurement is a strong predictor of complications such as microalbuminuria and diabetic nephropathy as well as cardiovascular manifestations.14 A larger variability of HbA1c measurements was related to the younger age at the time of the study and initial diagnosis, shorter duration of the disease, lower sensitivity to insulin, dyslipidemia, higher initial values of HbA1c, smoking (both current and former), lower socioeconomic status, and lower physical activity.14 In the case of diabetic nephropathy and retinopathy, fluctuations of glycemia did not seem to increase the risk of these serious complications;15 nevertheless, in a study published by the same group variability in HbA1c measurements was a distinct predictor of both nephropathy and retinopathy, even after HbA1c values decreased.16

Several studies assessing the status of diabetes control indicated that HbA1c values in the studied populations are usually higher than target values.17-23 A multicenter Polish study, ARETAUS1, which involved patients diagnosed with diabetes within the previous 2 years, revealed the average levels of HbA1c to be indeed higher than target values, i.e., higher than 6.5% value recommended by the 2009 guidelines of the Polish Diabetes Association. Interestingly, the patients receiving diabetologist care had slightly lower HbA1c values than the patients that received general practitioner care only without specialized support (7% vs. 7.1%).24

The 2010 American Diabetes Association (ADA) “Standards of medical care in diabetes” recommended including HbA1c assay in the analytical panel routinely used to establish the diagnosis of diabetes,25 and set the diagnostic cutoff point at 6.5% detected twice. This modification was introduced by the ADA as a result of the International Expert Committee report on the role of HbA1c assay in the diagnosis of diabetes.26 According to the above-mentioned standards, HbA1c value between 5.7% and 6.4% is an important risk factor for developing diabetes, as well as for developing cardiovascular disease.25 Advantages of HbA1c assay over fasting and 2-hour glucose measurements include higher sample stability prior to the test, lower biological variability, no influence of short-term fluctuations of glycemia, and feasibility (can be measured at any time of the day and without fasting).25

The 2010 ADA standards recommend performing the HbA1c test at the time when the diagnosis is made and at least every 6 months in clinically stable patients with good metabolic control. Those patients whose therapy has changed or who are not reaching their glycemia target values should be evaluated every 3 months.

According to the Polish Diabetes Association, the criteria for good glycemic control include HbA1c ≤7%, fasting and preprandial glycemia = 3.9–6.1 mmol/l (70–110 mg/dl), glycemia measured 2 h after a meal <8.9 mmol/l (<160 mg/dl). These criteria are slightly modified for type 1 diabetes and recently diagnosed type 2 diabetes: HbA1c ≤6.5%, fasting and preprandial glycemia = 3.9–6.1 mmol/l (70–110 mg/dl), glycemia measured 2 h after a meal <7.8 mmol/l (<140 mg/dl).27

The ADA suggests that target HbA1c values should be below 7%.25 Maintaining such goals correlates with a decreased risk of diabetic complications, such as microangiopathy and neuropathy. It should constitute a realistic target for patients who have recently been diagnosed with diabetes, who have a long-life expectancy and no significant cardiovascular disease, providing that achieving such levels is not compromised by

**TABLE** Relationship between the percentage of HbA1c and average plasma glucose concentration (confidence interval: 95%) (modified from Nathan DM, et al.)

<table>
<thead>
<tr>
<th>HbA1c (%)</th>
<th>Average plasma glucose concentration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mg/dl</td>
</tr>
<tr>
<td>5</td>
<td>97 (76–120)</td>
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<tr>
<td>6</td>
<td>126 (100–152)</td>
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<tr>
<td>7</td>
<td>154 (123–185)</td>
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<tr>
<td>8</td>
<td>183 (147–217)</td>
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<tr>
<td>9</td>
<td>212 (170–249)</td>
</tr>
<tr>
<td>10</td>
<td>240 (193–282)</td>
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<tr>
<td>11</td>
<td>269 (217–314)</td>
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<tr>
<td>12</td>
<td>298 (240–347)</td>
</tr>
</tbody>
</table>
in the United States.\textsuperscript{12,13,33} HPLC was used in the DCCT and UKPDS studies to establish a relationship between HbA\textsubscript{1c} levels and prevalence of long-term diabetic complications, and therefore became a reference point for the standardization of other HbA\textsubscript{1c} assays. In this method, which uses cationic ion-exchange resins, separation of hemoglobin fractions is based on their charge, and uses cationic ion-exchange resins. HbA\textsubscript{1c} has a lower positive charge as compared with other fractions and elutes faster from the ion-exchange column. Pre-glycohemoglobin has a similar mobility; therefore, it should be removed prior to the measurement. Results obtained using this method can be influenced by temperature and pH. Previously referenced normal values of HbA\textsubscript{1c} in healthy individuals as well as target values for diabetic patients were all established using HPLC.

Affinity chromatography relies on interaction of glycohemoglobin with boric acid derivatives bound covalently to the column matrix. Glycated hemoglobin contains more cis-diol groups and displays higher affinity to boric acid; therefore, it elutes faster than other hemoglobin fractions. Variations of temperature and pH do not affect the results of this method as much as ion-exchange chromatography. Moreover, it is not influenced by the presence of labile form of glycated hemoglobin, aldimine (Schiff base) as well as other forms of hemoglobin, including HbF, HbC, and HbS. This method measures total HbA\textsubscript{1c} but calibration and proper formulas make it possible to use the results to calculate HbA\textsubscript{1c}.

HbA\textsubscript{1c} can also be measured by agarose gel electrophoresis, a method that utilizes differences in charge between molecules. Electric field induces separation of glycohemoglobin into fractions characterized by different eluotropic mobility. This mobility is directly proportional to their electric charge and inversely proportional to their size. Agarose gel acts as a carrier and a molecular sieve and different fractions of glycohemoglobin move through it according to their size and electric charge.\textsuperscript{34}

Immunological methods are based on antigenic specificity of glycated hemoglobin and the potential to bind monoclonal antibodies to its specific
epitopes. Such methods correlate well with HPLC; nevertheless, the detected levels are usually lower, possibly because of high specificity of the binding to HbA<sub>1c</sub>. Additionally, immunological methods are unaffected by various hemoglobinopathies and the presence of aldime. 34

As mentioned earlier, the NGSP, founded in the United States in 1996, recommended HPLC as a reference method for the measurement of HbA<sub>1c</sub>. The program evolved and incorporated several other countries, including Canada, United Kingdom, New Zealand, Australia, Sweden, and Japan. As a result, HPLC became a widely used HbA<sub>1c</sub> detection method, and results obtained with other methods are being standardized based on HPLC results. 33

A proper method for measuring glycated hemoglobin should allow for measurement and presentation of the result as an “HbA<sub>1c</sub> equivalent”, be certified by the NGSP, and constantly undergo intra- and interlaboratory quality control process (coefficients of variation <5%). 27

**Factors influencing the measurement of HbA<sub>1c</sub>:**

**Coexistent diseases** Numerous coexistent diseases may influence the result of HbA<sub>1c</sub> measurement long before the sample arrives at the laboratory. Several types of anemia are known to exert such an effect. In the case of hemolytic anemia, characterized by abnormally shorter erythrocyte life span, exposure time for protein glycation will be shorter, and therefore measurement can underestimate the actual values. Similar situation occurs in sickle cell anemia (caused by an abnormal type of hemoglobin, HbS). On the other hand, in deficiency-related anemias, hemoglobin metabolism slows down significantly, leading to longer glycation exposure and, therefore, overestimation of actual values. 35

Transfusion of erythrocyte concentrate increases the erythrocyte turnover rate, resulting in lower than actual HbA<sub>1c</sub> results, while in polycythemia, the results can be overestimated due to a longer than normal life span of erythrocytes. Interestingly, similar situation can occur in patients who underwent splenectomy. 3 Hemolysis of erythrocytes, occurring in patients on hemodialysis, had an opposite effect, leading to underestimated HbA<sub>1c</sub> values. 36

A recent case report described a diabetic patient with coexistent Evans syndrome (autoimmune hemolytic anemia and autoimmune thrombocytopenia). 37 HbA<sub>1c</sub> values in this case were significantly lower than self-controlled glycaemia values. Autoimmune hemolysis of erythrocytes was the underlying cause for their shorter life span and, consequently, shorter exposure to glycation.

Hypertriglyceridemia is a known factor leading to overestimation of HbA<sub>1c</sub> results. As mentioned above, lipemic blood samples can have higher than the actual measurement of HbA<sub>1c</sub>. Hyperbilirubinemia, providing it does not exceed 20 mg/dl, should not affect HbA<sub>1c</sub> results. 37 Aldimine, a labile precursor of glycated hemoglobin (pre-HbA<sub>1c</sub>), constitutes 5% to 8% of total HbA<sub>1c</sub> in healthy individuals, while in diabetic patients, it can increase up to 30%, and significantly overestimate HbA<sub>1c</sub> results. 1 One report observed racial differences in diabetic patients, namely the percentage of HbA<sub>1c</sub> was lower in white subjects than in the black population. 35,36

**Hemoglobinopathies, variants and derivatives of hemoglobin** Physiologically, total hemoglobin consists of 98% of hemoglobin A formed by 2 α and 2 β chains, and approximately 2% of hemoglobin A2, formed by 2 α and 2 δ chains. Half of the newborn’s hemoglobin consists of fetal hemoglobin, containing 2 α and 2 δ chains.

Different methods of HbA<sub>1c</sub> measurement may be affected differently by various hemoglobinopathies. While HPLC and immunochromatography can either overestimate or underestimate the values, chromatographic separation may reveal some additional peaks (e.g., in the case of the presence of HbO Padova). The presence of hemoglobin Graz led to underestimation of the results obtained using all analytical methods. In other hemoglobinopathies (Sherwood Forest, O Padova, D, S) the results were either over- or underestimated, depending on the employed method. 35,36

An interesting case report was published in 1995. Both a diabetic patient and her healthy niece were positive for a clinically silent variant of hemoglobin Sherwood Forest. 39 When measured by HPLC, their glycated hemoglobin was very high (52%). When latex agglutination assay was used, HbA<sub>1c</sub> was found to be within normal values.

Hemoglobin J-Meerut was described in a Japanese patient with type 2 diabetes. Her glycated hemoglobin was 3.7% and did not correlate with the average values of glycemia. 40 Moreover, the value of HbA<sub>1c</sub> measured a month earlier was 5% higher, suggesting high variability of measurements in this hemoglobinopathy.

A variant of hemoglobin J-Baltimore was described for the first time in the United Kingdom and was responsible for underestimated HbA<sub>1c</sub> results in a patient with type 2 diabetes. 41 Irish authors described an Etobicoke hemoglobin which contains a variant of α-globin chain, and emphasized the necessity of using other than HPLC measurement methods in patients with rare hemoglobin variants. 42

Over 700 variants of hemoglobin have been described, and similarly to hemoglobinopathies, they can influence the measurement of HbA<sub>1c</sub>. Some of the variants are mainly observed in certain populations, e.g., 9% of African American population is positive for HbS. The most common hemoglobin variants include HbE, HbD, Hbc, and HbS. 43,44 HbE and HbD are considered the second and the fourth most common variants. Hemoglobinopathies and hemoglobin variants significantly influencing HbA<sub>1c</sub> measurements are Hb
In case of hemoglobin S, a mutation in β-globin chain leads to substitution of glutamic acid with valine at position 6. Hemoglobin C arises when the same position is lysine instead of glutamic acid, while hemoglobin E has a similar glutamic acid to lysine substitution at position 26. In all these cases, HPLC is not reliable and affinity chromatography is a method of choice for the measurement of HbA1c.

Remnant fetal hemoglobin can be detected in about 1% of white population, and can also influence the results of HbA1c. Methods that are based on the differences in electrical charge between HBA and HbA1c, such as HPLC and agarose gel electrophoresis, will yield falsely high results. The separation of fetal hemoglobin and glycated hemoglobin will be difficult, because they are characterized by a similar charge. Therefore, a method of choice in such cases will be affinity chromatography. Indeed, as it was reported earlier, 2 diabetic patients with remnant fetal hemoglobin were tested for glycated hemoglobin using agarose gel electrophoresis; however, the percentage was high and did not correlate with the values of glycemia, which were close to normal. Nevertheless, when affinity chromatography was employed, HbA1c values were also close to normal.

Several hemoglobin derivatives, such as pre-HbA1c, carbamoyl-Hb, and acetylo-Hb, are known to influence the measurement of HbA1c. Carbamoyl-Hb can be found in uremic patients as a product of hemoglobin reaction with urea, while acetylo-Hb arises as a consequence of reaction with salicylic acid in individuals with high intake of salicylates. In both cases, HPLC and electrophoresis will overestimate the HbA1c results, while affinity chromatography will be a more accurate method. In order to adjust HPLC results obtained in uremic patients, 0.06% for each 1 mmol/l of urea should be deducted from the obtained percentage of HbA1c. In case of uremia, interference with HbA1c measurement is probably due to the changes in hemoglobin structure. In such cases, the results should be interpreted with caution, and using the measurement of fructosamine as an alternative assay may become a viable option.

In conclusion, discrepancies between self-monitored blood glycemia and HbA1c results should always indicate a possibility of hemoglobinopathy. In such cases, an alternative method to HPLC should be considered, preferably affinity chromatography.

HbA1c measurement and drug interactions
Vitamin E administered to diabetic patients can potentially reduce glycation of proteins, irrespectively of changes in glycemia values. As previously reported, such reduction in percentage of HbA1c was dose-dependent, because it was more pronounced in a group of patients receiving 1200 mg daily of vitamin E as compared with the group receiving 600 mg per day. This effect can be explained by antioxidative properties of vitamin E, which can block the nonenzymatic glycation of proteins by interfering with the oxidation of glucose.

There is no consensus as to how vitamin C affects HbA1c testing. One report indicated that vitamin C is able to block protein glycation by competing with glucose in binding to proteins. Other authors claimed that this supplement is not able to influence any of the measurement methods of HbA1c. A recent report described falsely low percentage value of HbA1c in a hepatitis C patient treated with ribavirin. A long-term alcohol abuse, as well as the previously mentioned excessive intake of salicylates, can interfere with HbA1c measurement. Finally, loop diuretics administered in a population of the elderly (>81 years), nondiabetic individuals had a hyperglycemic effect reflected by increased HbA1c values.

Conclusions
Discrepancies between self-monitored glycemic values and percentages of HbA1c should warrant a critical assessment of the usefulness of the employed method, taking into account potential factors confounding the measurement, validation of the measurement with a different HbA1c method, and finally, testing for alternative markers of diabetes control, such as fructosamine or 1,5-anhydroglucitol. Fructosamine is a retrospective glycemic marker, which reflects the blood glucose concentration within 1 to 2 weeks prior to the test. Glycation occurs in extracellular domain and involves albumin; therefore, this parameter is not going to be affected by various hemoglobinopathies. 1,5-anhydroglucitol (1,5-AG) is a monosaccharide known to undergo glomerular filtration in the kidneys and subsequent reabsorption in renal tubules; at the final stage, it is excreted with glucose back to plasma. It has a lower degree of affinity to membrane glucose transporter than glucose itself; therefore, hyperglycemia almost entirely blocks its reabsorption in tubules. As a result, 1,5-AG is excreted with urine, and its concentration in plasma sharply drops. Therefore, there is an inverse correlation between 1,5-AG plasma concentration and average level of glycemia. Recently, a test measuring this component in plasma was introduced and is able to determine average glycemia values within 10 to 14 days prior to the test.

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Difficulties in interpreting HbA1c results

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ARTYKUŁ POGŁĄDOWY

Trudności w interpretacji wyników HbA$_{1c}$

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SŁOWA KLUCZOWE
cukrzycy, hemo­globina glikowana, wysokosprawna chromatografia cieczowa

STRESZCZENIE
Hemoglobina glikowana (HbA$_{1c}$) jest parametrem, który znajduje szerokie zastosowanie w ocenie wyrównania cukrzycy. W zaleceniach klinicznych Amerykańskiego Towarzystwa Diabetologicznego (American Diabetes Association – ADA) z 2010 roku wskazuje się na celowość wykonywania oznaczeń HbA$_{1c}$ przynajmniej raz na 6 miesięcy u osób ze stabilnym przebiegiem choroby, a raz na 3 miesiące u pacjentów, u których nastąpiły zmiany w leczeniu cukrzycy i u których nie osiągnięto docelowych wartości glikemii. Ponadto, w zaleceniach ADA z 2010 roku oznaczanie HbA$_{1c}$ dołączone do panelu badań służących rozpoznaniu cukrzycy oraz podwyższonego ryzyka wystąpienia cukrzycy. Spośród kilku metod używanych laboratoryjnych oznaczania HbA$_{1c}$, za referencyjną uważa się metodę wysokosprawnej chromatografii cieczowej. W pewnych stanach klinicznych, oznaczenia HbA$_{1c}$ mogą być niemieszalne. W przypadkach, gdy wartość HbA$_{1c}$ nie koreluje z pomiarami glikemii i stanem klinicznym pacjenta, należy zachować ostrożność w interpretacji wyniku, wziąć pod uwagę obecność stanów zakłócających oznaczenia HbA$_{1c}$, rozważyć zastosowanie innej metody laboratoryjnej lub oznaczenie alternatywnego parametru wyrównania glikemii, np. fruktozaminy lub 1,5-anhydroglucitolu.