Effect of *in vitro* Glycation on Hemoglobin Variants and Influence of Hemoglobin Traits on Glycated Hemoglobin Dosage by Chromatographic Method

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The accuracy of glycated hemoglobin (HbA1c) dosage is essential for the proper monitoring of treatment in diabetic patients. Cromatographic methods are widely used for HbA1c dosage. Hemoglobin variants can influence the HbA1c test results. Purpose of the research was in vitro glycation of blood samples, especially those containing hemoglobin traits, to reveal peaks that could correspond to glycated hemoglobin variants. Another purpose was evaluation of persistent HbF incidence and hemoglobinopathies in the population of Transylvania. The practical approach is increase the accurary of HbA1c measurement by chromatographic methods identifying these interferences.

Keywords: diabetes mellitus, glycated hemoglobin, hemoglobinopathies, in vitro glycosylation, HPLC

Hemoglobinopathies represent the commonest single-gene genetic disorders in human pathology wordwide. Over 1000 human hemoglobin variants with single amino acid substitutions have been discovered, in the majority of cases through their clinical and/or laboratory manifestations. These modifications alter the structure and also the biochemical properties of the hemoglobin, their physiological effects ranging from insignificant to severe, having important practical implications in hematology [1, 2].

The incidence of hemoglobinopathies and thalassemia is quite different depending on the studied population. The most frequent are HbS (sickle cell disease), HbC, HbD, and HbE. HbS is most prevalent in Africa, HbD among Indian population, and HbE in Southeast Asia. HbF (fetal hemoglobin) and HbA_2 fractions can be elevated in thalassemia (a disease affecting the synthesis of hemoglobin alpha or beta subunits). Beta thalassemias can also occur in the presence of HbS and HbE, and the combined sickle/beta thalassemia trait occurs most frequently in the geographical area of the Mediterranean sea [3].

Some hemoglobin variants can be diagnosed using high pressure liquid chromatography (HPLC) equipments, such as the different generations of the Variant system, but scientific data are very limited in current medical literature regarding the glycated forms of different hemoglobin traits [4].

In diabetic patients the accuracy of HbA1c measurement is a very important goal, the presence of hemoglobin variants and thalassemias can cause inaccuracies in HbA_{1c} measurements by affecting factors as red blood cell survival and glycosylation rates [3].

Experimental part

The aim of this study was to develop a method to study the in vitro glycation of hemoglobin in blood samples of diabetic individuals and those having hemoglobinopathies in the Canadian population of Alberta, to identify peaks that could correspond to glycated hemoglobin variants. Another purpose was the evaluation of the incidence of persistent HbF and hemoglobinopathies in the population of Transylvania. The most important aim was to increase the accurary of the HbA1c measurement by chromatographic procedures by identifying these interferences.

Proper conditions for in vitro glycation were achieved by incubating the samples (using blood collected on EDTA-K_2 anticoagulant diluted with saline) with a glucose solution of 25 mmol/L at 37°C in a water bath and performing repeated chromatograms to follow the kinetics of the process on the Variant II analyzer using the Hemoglobin A1c program. All the recommendations included in the Variant II Hemoglobin Testing System Instruction Manual were respected [5].

The study was performed on 150 blood samples from healthy patients and diabetics, and we used 27 blood samples taken from patients with hemoglobinopathies living in Canada. We followed in particular the percentage change in glycosylated hemoglobin and the intermediate form known as the labile Schiff base, and also the P3 fraction, the initial values being used as reference (the area of HbA was considered 100%). A normal chromatogram of the Variant II analyzer contains the following fractions: A1a, A1b, LA1c, A1c, P3 and Ao (from left to right).

Regarding the research performed in Romania, 1540 samples were analyzed (mainly from diabetic individuals) using the Variant I equipment, for evaluation of persistent HbF and the presence of hemoglobinopathies in the population of Transylvania. The chromatogram of the Variant I analyzer contains peaks corresponding to A1a, A1b, F, A1c, Ao fractions. Operation on the equipment was corresponding to the recommendations of the production company [6]. Bilevel Lyphochek Immunology Plus Control was used for quality control purposes on both HPLC analyzers.

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Results and discussions

Research results on the Variant II equipment

Characteristics of the normal chromatograms

Retention times and average normal percentages of hemoglobin fractions on the Variant II analyzer are presented in table 1 and a chromatogram of a diabetic patient can be observed on figure 1.

Study of in vitro glycosylation on blood samples from patients with hemoglobinopathies

We conducted the study of in vitro glycation in 27 cases of pathological hemoglobin variants: homozygous and heterozygous Hb E and Hb S, and heterozygous Hb C, Hb D, Hb G Coushatta, Hb Camden.

- Patients with Hb E

We followed the process of in vitro glycosylation in case of one homozygous HbE sample and seven heterozygous HbE. Depending on the levels of HbA1c, the age of the sample and storage conditions, we obtained different graphics for these samples.

In case of an older sample preserved frozen having HbA1c of 7.3%, we obtained during the in vitro glycosylation an unknown peak with retention time of 1.35 (between A1c and P3 peaks), which kinetically resembles the evolution of the A1c faction, and LA1c evolution is similar to P3 kinetics (the last two might be increased do to hemolysis). Table 2 and figure 2 show the kinetics of the described fractions.

At a homozygous HbE blood sample after incubation with glucose solution, we have achieved an unknown peak with increasing trend with retention time 1.38.

A fraction with increasing trend has been observed also in case of other blood samples from heterozygous HbE patients, but the downward slope was not quite as broad for the analyzer to recognize it as a separate peak.

It should be mentioned that the P3 fraction showed a retention time slightly different from normal (1.63-1.66) in the blood sample from homozygous HbE (1.61) and heterozygous HbE patients (1.62), which might be explained by the overlapping of glycosylated pathological hemoglobin variants on this peak.

- Patients with HbS

We followed the process of in vitro glycosylation in blood samples collected from two homozygous and nine heterozygous HbS patients.

In case of homozygous HbS patients we obtained similar variation of fractions LA1c and P3, data on the changes in percentage compared to their baseline values are given in table 3. Because these samples did not show HbA, only HbS and a small amount of HbF, LA1c and HbA1c fractions couldn't be reported to the amount of HbA, so they were reported to HbS. P3 fraction was also reported to HbS in these samples.

The very high percentage of LA1c values compared to those of the P3 fraction is due to the fact that in the initial chromatogram and that obtained after 2 h of incubation, the fraction LA1c has not yet appeared. LA1c fraction forms a distinct peak just after 4.5 h of incubation with the glucose solution.

In heterozygous HbS samples we obtained a similar pattern for fractions P3 and LA1c, and increasing evolution of the HbA1c fraction. The percentage changes of these fractions are included in table 4 and the kinetics is represented in figure 3.

- Patients with Hb C

We followed the in vitro glycosylation in two blood samples from heterozygous HbC patients. One of those was a frozen sample which presented massive hemolysis and accumulation of degradation products exhibited a negative effect on the results obtained.

Table 1

FRACTIONS APPEARED ON A NORMAL CHROMATOGRAM, THEIR PERCENTAGE AND TYPICAL RETENTION TIMES

Name of the fraction	Corresponding area (%)	Retetion time (min)
A1a	0.7	0.17
A1b	1.6	0.26
LA1c/CHb	1.3	0.76
A1c	5.4	0.95
P3	4.7	1.64
Ao	88.0	1.76

Table 2

EVOLUTION OF LA1c, HbA1c, P3 FRACTION AND OF AN UNKNOWN PEAK (%) HAVING THE RETENTION TIME 1.35 (BETWEN A1c AND P3 PEAKS) COMPARED TO THE INITIAL VALUES DURING IN VITRO GLYCOSILATION IN A HETEROZYGOUS Hb E SAMPLE

Hours	LA1c%	A1c%	P3%	Unknown%
5.5	2.190	-0.078	1.295	1.100
9	3.070	0.160	2.360	1.870
21	4.940	0.062	4.470	2.180
25	5.220	0.030	4.450	2.100
38	5.700	0.110	4.720	2.280

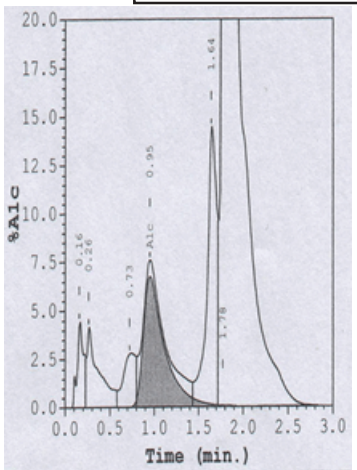

Fig. 1. Normal chromatogram obtained on the Variant II equipment

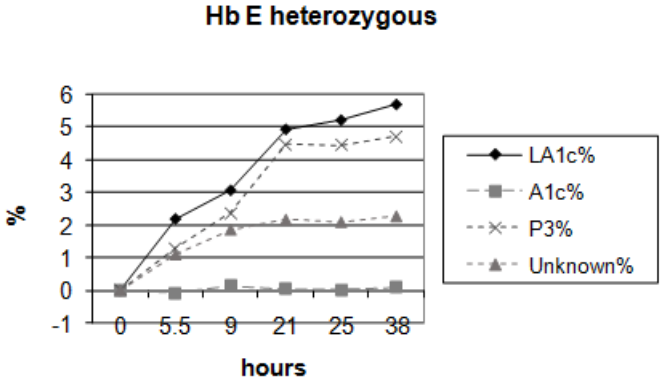

Fig. 2. Graphical representation of the kinetics obtained in case of in vitro glycosilation in a heterozygous Hb E sample

Table 3

EVOLUTION OF FRACTIONS LA1c, HbA1c AND P3 (%) COMPARED TO THE INITIAL VALUES DURING IN VITRO GLYCOSYLATION IN HOMOZYGOUS HbS SAMPLES

Hours	LA1c%	A1c%	P3%
2	-	0.022	0.150
4.5	2.820	0.439	0.300
10	3.160	0.480	0.650
16.5	2.980	0.175	0.480
21	3.140	0.603	0.670
24.5	3.120	0.560	0.550
43	2.880	0.687	0.390

Table 4

EVOLUTION OF LA1c, HbA1c AND P3 (%) FRACTIONS COMPARED TO THE INITIAL VALUES DURING IN VITRO GLYCOSYLATION IN HETEROZYGOUS HbS SAMPLES

Hours	LA1c%	A1c%	P3%
3	1.78	0.17	0.80
5.5	2.28	0.39	1.08
9	2.93	0.40	1.83
24	2.05	0.30	1.73
31	2.03	0.45	1.75
51	1.88	0.90	1.67

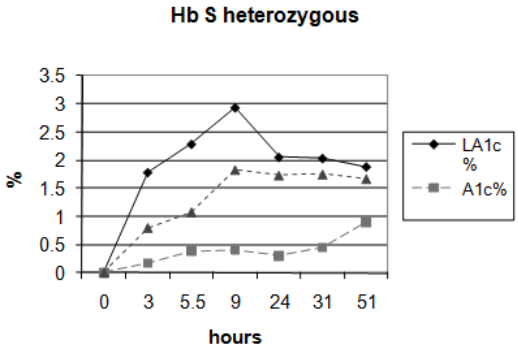

Fig. 3. Graphical representation of the kinetics obtained in case of in vitro glycosilation in heterozygous Hb S samples

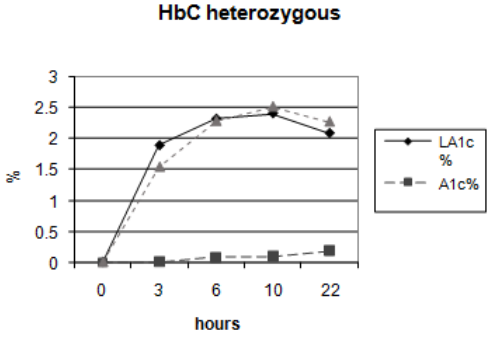

Fig. 4. Graphical representation of the kinetics obtained in case of in vitro glycosilation in heterozygous Hb C samples

The evolution of LA1c, A1c and P3 factions in the other, fresh sample are presented in table 5, their percentage change compared to the corresponding baseline values can be observed. Figure 4 shows that the LA1c fraction has an almost identical evolution compared to the P3 fraction during the experiment.

- Patients with HbD

We studied in vitro glycosylation on four blood samples taken from heterozygous HbD patients. Table 6. shows the percentage differences of fractions LA1c, A1c and P3 in heterozygous HbD patients during incubation with the glucose solution. A similar kinetics of the LA1c and P3 peaks can be observed in figure 5. Following these samples we found that the retention time of P3 in heterozygous HbD patients is 1.67-1.68, higher than in patients without hemoglobinopathies (1.63- 1.66).

- Patients with HbG Coushatta and Hb Camden

In case of the blood sample taken from a heterozygous HbG Coushatta patient after incubation with glucose solution appeared an unknown peak with obvious tendency to increase with retention time 1.17-1.19-1.2. LA1c

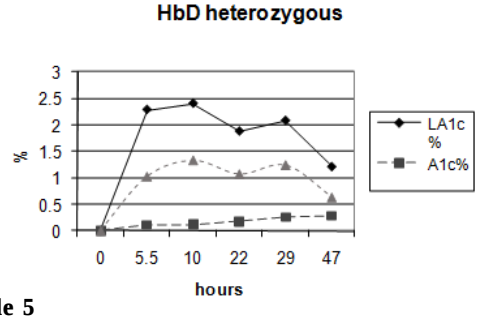

Fig. 5. Graphical representation of the kinetics obtained in case of in vitro glycosilation in heterozygous Hb D samples

Table 5

EVOLUTION OF LA1c, HbA1c AND P3 (%) FRACTIONS COMPARED TO THE INITIAL VALUES DURING IN VITRO GLYCOSYLATION IN BLOOD SAMPLES FROM HETEROZYGOUS HbC PATIENTS

Ore	LA1c%	A1c%	P3%
3	1.880	0.005	1.530
6	2.306	0.083	2.270
10	2.393	0.097	2.501
22	2.076	0.185	2.260

Table 6

EVOLUTION OF FRACTIONS LA1c, HbA1c AND P3 (%) COMPARED TO THE INITIAL VALUES DURING IN VITRO GLYCOSYLATION IN BLOOD SAMPLES FROM HETEROZYGOUS HbD PATIENTS

Hours	LA1c%	A1c%	P3%
5,5	2.286	0.110	1.021
10	2.403	0.120	1.330
22	1.880	0.180	1.070
29	2.080	0.260	1.240
47	1.210	0.280	0.630

Table 7

EVOLUTION OF FRACTIONS LA1c, A1c, P3 AND OF AN UNKNOWN PEAK (%) HAVING RETENTION TIME 1.17-1.19-1.2 COMPARED TO THE INITIAL VALUES DURING *IN VITRO* GLYCOSYLATION IN A SAMPLE CONTAINING HbG COUSHATTA TRAIT

Hours	LA1c%	A1c%	P3%	Necunoscut%
5.5	3.359	0.556	0.024	1.793
9	2.442	0.324	0.402	2.265
21	2.547	0.401	0.409	1.828
25	2.417	0.344	0.777	1.961
29	2.523	0.375	1.013	2.093
47	3.043	0.730	0.794	-

Table 8

EVOLUTION OF LA1c, P3 FRACTIONS AND OF TWO PEAKS NAMED A1a AND A1b (%) WITH RETENTION TIMES 0.23 AND 0.37, RESPECTIVELY, COMPARED TO THE INITIAL VALUES DURING *IN VITRO* GLYCOSYLATION IN A HETEROZYGOUS Hb CAMDEN SAMPLE

Hours	LA1c%	P3%	A1a%	A1b%
5.5	1.990	1.210	0.950	2.290
9	2.870	1.970	1.660	4.070
21	3.550	6.320	5.200	12.002
25	3.910	7.997	5.940	16.030
29	4.090	9.530	7.390	19.520

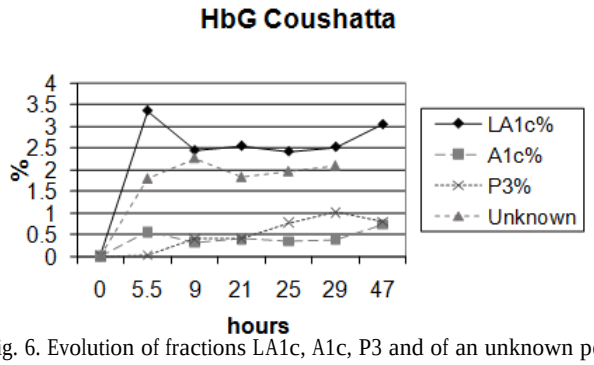

Fig. 6. Evolution of fractions LA1c, A1c, P3 and of an unknown peak (%) during in vitro glycosylation in a sample containing HbG Coushatta trait

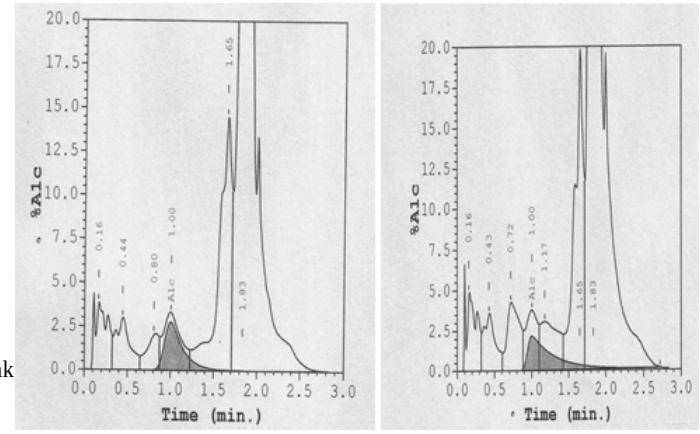

Fig. 7. Heterozygous Hb G Coushatta blood sample: chromatograms obtained before (left) and after (right) in vitro glycosylation

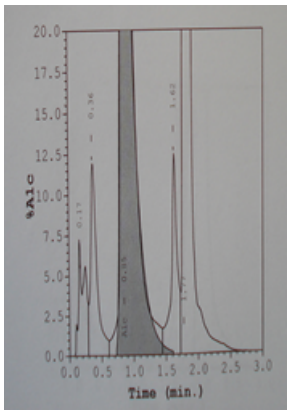

Fig. 8. Falsely elevated A1c peak in a heterozygous Hb Camden patient

fractions, A1c and P3 had a similar kinetics compared to those found in other samples containing pathological hemoglobins. The kinetics of in vitro glycosylation in the blood sample containing HbG Coushatta can be followed in figure 6, the percentage changes of different fractions compared to the baseline values are shown in table 7. Figure 7. shows two chromatograms containing the increasing unknown peak in the blood sample of the heterozygous HbG Coushatta patient.

The blood sample of a heterozygous Hb Camden patient initially presented an unknown peak with retention time of 0.23, which was then called A1a by the computer attached to the analyzer, but this time retention is much higher than that found in case of the A1a fractions of the blood samples from patients without hemoglobinopathies or in case of other hemoglobinopathies (0.15-0.17).

Besides this there was another peak called A1b with retention time 0.37, much higher than the retention time of the A1b faction on chromatograms of blood samples from patients without hemoglobinopathies or with other hemoglobinopathies (0.25 to 0.27). These two fractions presents a fast kinetics, similar to the P3 fraction. Table 8 contains the percentage changes of the fractions compared to the baseline values.

The percentages in table 8 were obtained by fractions A1a and A1b reported to A1c (the A1c fraction has been considered 100% for this calculations) and P3 fraction has been reported to Hb A (which was considered 100% in this case).

For the blood sample from the patient with Camden Hb the A1c fraction could not be determined, because this pathological hemoglobin variant is co-eluted with HbA1c giving a rate of 46-48% of the total hemoglobin (fig. 8).

We evaluated the retention times corresponding to the LA1c, A1c and P3 fractions during experiments on blood samples from patients with hemoglobinopathies, and those without pathological hemoglobin variants.

The data obtained showed that in the samples from patients without hemoglobinopathies, the retention times of LA1c, and P3 and A1c fractions are between 0.72-0.79, 0.92-1.03 and 1.63-1.66, respectively, while in the samples of heterozygous HbE and HbC patients the retention time found for the P3 fraction was in the range 1.61- 1.62, which is lower than normal, and for patients with heterozygous HbD the P3 fraction has often a retention time included in the range of 1.67-1.68, these values being higher than normal.

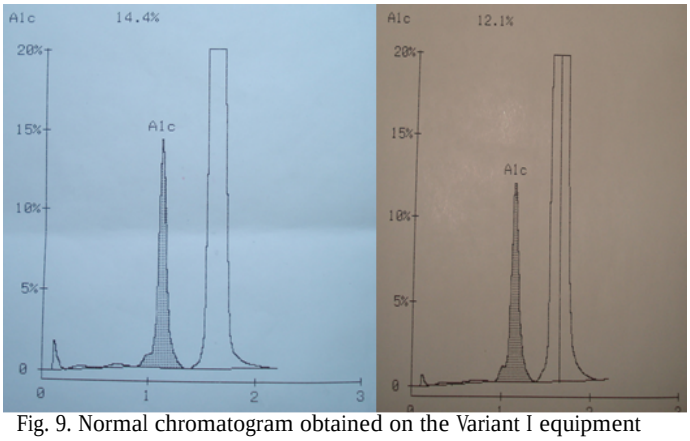

Fig. 9. Normal chromatogram obtained on the Variant I equipment in a diabetic patient presenting metabolic imbalance (left) and heterozygous HbTyne (right)

Research results on the Variant I equipment

Based on our study made on 1540 subject we can conclude that the prevalence of persistent HbF is around 1.8% in the population of middle Transylvania, all these patients presenting an important increase of the HbA1b fraction, so we presume that this peak contains the glycated fraction of HbF. These two fractions can co-elute with HbA1c using certain chromatographic methods, leading to falsely elevated results. We found one case of HbTyne – the chromatogram is visible in figure 9 (Hb electrophoresis was also performed for proper diagnosis) - and two cases of heterozygous HbS, with low percentage representation of the pathological hemoglobin variant.

Based on data presented we can conclude that the P3 fraction is directly related to the glycosylation process, its evolution over time is very similar to the kinetics of the LA1c fraction in blood samples from patients with normal and pathological hemoglobins.

HbA1c fraction shows on most of the graphics values around the baseline in the first hours of incubation with glucose solution, after that presenting a clear, but slow increase of its concentration.

One of the problems we faced during this study was that sometimes the analyzer separated two distinct peaks, sometimes it merged them into a single one, so we encountered sometimes difficulties to follow the evolution of the different fractions. This disadvantage would probably be solved by some enhancements made on the software attached to this analyzer.

In case of populations having a high prevalence of hemoglobinopathies, HPLC is the recommended method for HbA1c measurement, because it is easy to perform, economical and reliable. Hemoglobin variants detected by HPLC should be reported to the physician to improve the management of diabetic patients [7].

By analyzing the in vitro glycosylation process in samples with pathological hemoglobins, an interesting topic is the analysis of unknown peaks occurring in heterozygotes of HbE and HBG Coushatta, which based on their kinetics, could correspond to glycosylated variants of these pathological hemoglobins. Their research might open new perspectives in the research on hemoglobin glycation and their final identification should be carried out with alternative methods.

HPLC is widely used in chemical, pharmaceutical analysis and in clinical laboratories, but sometimes does not give the best result because of some interferences [8], in spite of being the reference method for HbA1c measurement. Some hemoglobin variants (such as HbWayne or HbCamden) can coelute with the HbA1c fraction on HPLC, causing falsely elevated HbA1c values [9]. To evaluate glycated hemoglobin in populations with a high prevalence of hemoglobinopathies, we should use the HPLC method, which is easy to perform, economical, and reliable. Based on an algorithm, hemoglobin variants visualized on HPLC should be reported to the physician to improve the management of patients. If a satisfactory method for hemoglobin glycation rate appreciation could be developed, it could provide a test for diabetic patients who are suspected of being *fast glycators* to optimize their treatment.

Specialists recommend that initial HbA_{1c} requests within the hospital information system should be tested by a method capable of detecting the most common Hb variants (Hb S, Hb C, Hb E, and Hb D). HbA_{1c} orders that represent subsequent requests would be identified and an automatic notification given to the physician for those samples previously identified as having an interfering variant that affects interpretation of the Hb A_{1c} test. In such cases, HbA_{1c} results should not be reported and an alternative method to monitor average blood glucose levels should be used. Although recommendations have been provided regarding the potential reporting of misleading HbA_{1c} values in the presence of Hb variants that alter red blood cell life span, studies demonstrated the importance of fully understanding the limitations of the method chosen to determine Hb A_{1c} results. Improvements to immunoassays have largely eliminated analytic interferences from the most common Hb variants, which include HbS and HbC. An unintended consequence, however, is the ability of these Hb variant interference-free methods to measure and report HbA_{1c} values for patients lacking HbA [10].

Conclussions

Based on the kinetics of unknown fractions during in vitro glycation observed in case of heterozygous HbE and HbG Coushatta patients, these could correspond to the glycated fraction of these pathological hemoglobin variants. Modified retention times of known fractions (P3, A1b peaks) could also be due to these pathological hemoglobins or their glycated form. Future studies on these peaks could open new perspectives in hemoglobin research, and could contribute to software updates capable of recognizing these fractions, enhancing the accuracy of HbA1c test results. Pathological hemoglobin variants and persistent HbF can disturb the result of HbA1c dosage leading to falsely elevated or decreased values, so their possible presence should be taken into consideration in diabetic and non-diabetic individuals.

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