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WHAT IS FATE OF HEMOGLOBIN SUBFRACTIONS IN PATIENTS OF SICKLE CELL ANEMIA? HEMOGLOBIN ANALYSIS, HBA1C AND JUDGING CONTROL OF DIABETES

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ABSTRACT

Glycated haemoglobin (HbA1c) testing has been widely used by clinicians to analyse the glycaemic control. Its accurate interpretation is highly important as treatment initiation and titration is dependent on its value as it corresponds to an average estimated blood glucose level calculated from its percentage. Variations in red blood cells (RBC) life span can alter the HbA1c values. Conditions such as anaemia and haemoglobinopathies can increase the turnover of erythrocytes, lowering the measured HbA1c values. On the other hand, conditions that decrease the RBC turn over, lead to higher values of HbA1c. Thus, the method for screening and evaluating glycaemic control in subgroups of population with haemoglobinopathies needs refinement. This study aims at emphasizing the interference of common haemoglobin variants and anaemia with HBA1c levels and further diabetes monitoring.

ABBREVIATIONS	DEFINITION
RBC	Red Blood Cell
HBA1C	Glycated Hemoglobin
HPLC	High Performance Liquid Chromatography
SCD	Sickle Cell Disease
SCT	Sickle Cell Trait
IDA	Iron Deficiency Anemia

1. INTRODUCTION

Diabetes is one of the most researched disease entities with recent updates, and still, it outnumbers other lifestyle diseases in terms of its impact on human health and as a public health pandemic of importance. Glycated hemoglobin (HbA1c) testing has been widely used by clinicians to analyze glycemic control (1). Its accurate interpretation is highly important as treatment initiation and titration are dependent on its value, as it corresponds to an average estimated blood glucose level calculated from its percentage. This current interpretation of HbA1c values and calculated glucose levels assumes that all patients have the same red blood cell (RBC) lifespan (2). Variations in red blood cell (RBC) life span can alter the HbA1c values. The HbA1c measured in blood is the mean for RBCs, which includes reticulocytes, to the oldest RBCs (3).

HbA1c is a subfraction of HbA1 and predominant glycated hemoglobin maintained throughout the life span of red blood cells, i.e., 120 days (4). Therefore, the value of HbA1c is determined by the plasma glucose level and RBC lifespan. The production of HbA1c is dependent on RBC survival time and its exposure to blood glucose (5). Hence, many conditions other than hyperglycemia can affect its production and final measured values. Irrespective of the method used to measure HbA1c, its lower levels can be seen in those conditions where the life span of RBCs is shortened (5) (6). Anemia, highly prevalent in countries like India, can increase the turnover of RBCs, lowering the measured HbA1c values. On the other hand, conditions that decrease the RBC turnover will lead to higher values of HbA1c (5) (7). In Iron deficiency anemia, the most common nutritional anemia and highly prevalent in India, especially in females, the RBC (hypochromic) life span is decreased to approximately 46-85 days (8). If such patients present with signs and symptoms of hyperglycemia with raised random blood glucose levels, the next most practical method to evaluate hyperglycemia is the measurement of HbA1c levels, which can yield false results affecting the diagnosis of pre-diabetes and diabetes (9). Similarly, in patients with sickle cell hemoglobinopathy, as there is the presence of abnormally shaped hemoglobin with altered survivability and fetal hemoglobin fraction, the HbA1c values can be highly variable (10). As our hospital caters to patients from tribal populations with a high prevalence of sickle cell disease and trait, their HPLC analysis is routinely done, and variable HbA1c levels are observed. This study aimed at the interpretation of HbA1c values in such patients and learning about the interference of Hb variants in HbA1c measurement (11).

2. METHODOLOGY

A prospective observational study was conducted at Dhiraj General Hospital, a tertiary care center, in a semi-rural area of Vadodara city in Gujarat, India, during the period of three years, i.e., 2016-2019, after approval from the Sumandeep Vidyapeeth Institutional Ethics Committee (SVIEC). The study included a total of 823 adult (>18 years) patients with anemia (sickle cell anemia and/or iron deficiency anemia) who were willing to participate voluntarily after written and informed consent. This hospital caters to patients from rural areas of the district with a high demographic prevalence of sickle hemoglobinopathy, especially from the tribal population (Bhil, Bariya, Vasava, Varli, Chaudhuri, Koli, etc.), and is a multi-specialty hospital with facilities of a sickle clinic running separately twice a week. The study didn't include patients with chronic liver or kidney disease, pregnant patients, or those who received blood transfusions in the last 3 months. Out of 823 patients, 537 had sickle hemoglobinopathy and 286 had iron deficiency anemia. All patients who had sickling solubility tests positive underwent Hb electrophoresis by high-performance liquid chromatography (HPLC). The patients with iron deficiency anemia underwent Iron profile studies, zinc protoporphyrin levels, and HPLC was performed for Hb analysis. The subfractions of hemoglobin were analyzed. The average values of hemoglobin and fractions of hemoglobin with subfractions of HbA1 were calculated and compared among the two groups of patients. The comparison was also made among patients with sickle hemoglobinopathy, as out of 537, 120 patients had sickle cell disease (HbSS) and 417 had sickle cell trait (HbAS). The data analyzed via the statistical package for scientific studies (SPSS version 20) was presented in the form of mean with standard deviation. The chi-square test was applied, and a p-value < 0.05 was considered statistically significant.

2.1 Objectives of the study

This study aims to investigate the interference of common hemoglobin variants and iron deficiency anemia with HbA1c levels and further diabetes monitoring.

2.2 Inclusion Criteria

The inclusion criteria for the patients were as below:

1. Adults > 18 years.
2. Patients are willing to participate in the study and give consent.
3. Patients with abnormal hemoglobin, as shown on HPLC, or patients with iron deficiency anemia, as shown on lab investigations (hemogram and iron studies).

2.3 Exclusion Criteria

The exclusion criteria for the patients were as below:

1. Patients with age <18 years.
2. Patients do not consent to participation in the study.

2.4 Statistical analysis

All the patients were carefully examined, and necessary investigations were sent for parameters used in the study. The collected data was compiled in MS Excel for further statistical analysis. All the necessary statistical analyses were performed.

The data analyzed via the statistical package for scientific studies (SPSS version 20) was presented in the form of mean with standard deviation. The chi-square test was applied, and a p-value < 0.05 was considered statistically significant.

3. RESULTS:

A total of 823 patients with anemia were taken for study, out of which 65.3% had sickle cell hemoglobinopathy (22.3% of patients had sickle cell disease and 77.6% had sickle cell trait), and 34.7% of patients had iron deficiency anemia out of the total patients. The average hemoglobin levels (gm/dl) measured in patients with sickle cell disease, sickle cell trait, and iron deficiency anemia were 8.36±2.16, 10.31±1.91, and 9.28±2.8, respectively. Overall average hemoglobin level in patients with sickle cell anemia (537) was 9.87±2.13.

As described in Tables 1 and 2 below, it was found that the average HbA1c levels in patients with sickle cell disease were higher (7.16±2.82) compared to sickle cell trait (5.16±0.68) and iron deficiency anemia (5.2±0.7), though the average hemoglobin levels were lower in patients with sickle cell disease and iron deficiency anemia when compared to sickle cell trait, and this comparison was statistically significant. Though sickle cell disease patients have shorter life spans of RBCs and should have lower HbA1c levels, an inverse relationship was observed instead (Table 1). The lower the hemoglobin in sickle cell disease patients, the higher the HbA1c. The possible reason that can be postulated is the possibility of another fraction interfering with the measurement, or there may be another Hb variant that is silent, with the same eluting time as HbA1c, probably a thalassemia trait, and probably not detected on HPLC.

As shown in Tables 1 and 2 below, the fetal hemoglobin levels were higher in patients with sickle cell hemoglobinopathy and much higher in sickle cell disease patients. HbF has been found to interfere with HbA1c, which is discussed below. Another important observation made from these results was the value of labile HbA1c, which was found to be higher in patients with sickle cell disease (5.17±13.02), suggesting the possibility of falsely higher levels of HbA1c in these patients. The HbA0, which is the initial pure form of HbA, is highest (81.8±9.59) in patients with iron deficiency anemia when compared to patients with sickle cell trait and disease, implying that these patients should have increased glycation of Hb compared to the non-anemic population. However, in our study, the levels of HbA1c in IDA patients were higher when compared to patients with sickle cell disorder (SCD plus SCT), as shown in Table 2 below, though when compared to patients with sickle cell disease, it was found to be lower.

HbA2, a normal variant of adult hemoglobin, is normally found between 1.5% and 3.1%. In our study, the value of this fraction was found to be higher in sickle cell disorder patients compared to IDA (Table 2). It was also found that patients with sickle cell disease had a higher value (4.15±8.73) compared to the rest of the groups (Table 1), implying that there may be a presence of beta thalassemia trait along with sickle cell disease in these patients.

Table 1—Hb fractions in SCD, SCT, and IDA.

HPLC VARIABLE S	SICKLE CELL DISESAE (n=120)	SICKLE CELL TRAIT (n=417)	IRON DEFICIENC Y ANAEMIA (n=286)
Hb	8.36±2.16	10.31±1.91	9.28±2.8
HbA1a	4.12±1.73	0.98±0.73	1.28±0.8
HbA1b	6.28±8.60	1.20±0.80	1.7±1.3
HbA1c	**7.16±2.82,**	**5.16±0.68**	**5.2±0.7**
Labile A1c	5.17±13.02	0.61±0.39	1.2±4.5
HbA0	6.15±10.06	58.73±25.37	81.8±9.59
HbA2	4.15±8.73	3.38±1.11	2.6±0.7
HbF	15.90±6.58	1.22±1.30	1.5±2.2
HbS	67.31±12.92	28.67±6.30	4.75±7.45

Table 2: Hb analysis in patients with sickle hemoglobinopathy and IDA.

HPLC VARIABLES	SICKLE CELL DISESAE (n=120)	IRON DEFICIENC Y ANAEMIA (n=286)
Hb	9.87 ±2.13	9.28±2.8
HbA1a	1.60 ±1.7	1.28±0.8
HbA1b	0.83 ± 1.46	1.7±1.3
HbA1c	**4.79 ± 2.15**	**5.2±0.7**
Labile A1c	0.55±1.65	1.2±4.5
HbA0	46.97±31.67	81.8±9.59
HbA2	3.54±4.52	2.6±0.7
HbF	4.28±6.92	1.5±2.2
HbS	37.07 ±18.244	4.75±7.45

4. DISCUSSION

HBA1C SYNTHESIS AND RED BLOOD CELL LIFE SPAN

Glycated hemoglobin (HbA1c) has been considered the most convenient and practical way for the diagnosis of pre-diabetes and diabetes, and also for the analysis of glycemic control in diabetics (1). It has taken the role of a "gold standard" investigation, easy to perform, inexpensive, and corresponding to average blood glucose levels (2). But there are limitations, a few aspects probably less discussed, which we here aim to highlight and provide insights for further studies. As there are differences in population groups in terms of ethnicity, race, and associated disorders, the interpretation of HbA1c can be variable (12). The HbA1c production is the result of the irreversible, slow, non-enzymatic reaction between glucose present in RBC and N-terminal valines of the beta-globin chain of mature hemoglobin (13). The normal range of HbA1c ranges between 4 and 5.5%, implying that out of 100 erythrocytes, 4-5 cells have glucose attached to their beta-globin chain (13). Therefore, the value of HbA1c is dependent on three factors: the hemoglobin glycation rate, the hemoglobin content (quantitative and qualitative) of reticulocytes when released from bone marrow, and the average life span of erythrocytes (14). A normal RBC has several stages of progression in circulation—reticulocytes (short-lived), mature RBCs (long-lived), and terminal senescent RBCs (short-lived)—in a hematologically normal individual. We can use this information and imply that the exposure time of RBCs to glucose also determines the glycation rate of hemoglobin. This suggests that anemia is a very important associated factor to be considered and corrected before interpreting the results of HbA1c in diabetics and non-diabetics as well (5).

SICKLE CELL HEMOGLOBINOPATHY AND HBA1C

Sickle cell disorders are hemolytic anemias, with decreased RBC survival and increased RBC turnover, providing less time for RBC exposure to glycation. Sickle cell disease patients have very little or absent HbA (the adult Hb fraction) and higher fetal hemoglobin with an S-window on HPLC analysis of Hb fraction (15). Whereas sickle cell trait patients have a heterozygous condition with the presence of adult hemoglobin but less than normal. Under acute crisis, these cells elongate and transform into sickle shapes owing to low oxygen tension, leading to polymerization, altering the structure of RBCs, and resulting in more fragile RBCs with a shorter life span of about 20 days (16). Hence, altering the values of HbA1c and interfering with its interpretation falsely lowers the values. In our study, the average HbA1c was found to be lower in patients with sickle cell disorder in comparison to iron deficiency anemia patients. But when we studied the Hb analysis of sickle cell disease (HbSS) patients, we found quite the opposite results, with higher values of HbA1c instead, also HbA2, labile HbA1c, and fetal Hb were found higher (17). These patients had lower HbA1 levels but still were found to have increased glycation of the HbA1 fraction, raising various questions regarding this unexpected finding (1). The exact reason for this finding is not well understood, but we have postulated a few theories: the presence of another Hb variant interfering with the measurement of HbA1c, as the HbA2 fraction, was also found to be higher, suggesting the probability of the presence of beta thalassemia trait as well, and increased oxidative stress due to low oxygen tension leading to increased glycation of Hb, as most patients with sickle cell disease presented with an acute crisis (18). These proposed postulations just provide insight and need validation with further studies. The higher values of HbA1c found in sickle cell disease patients can lead to overdiagnosis of diabetes and need to be correlated with fasting and post-prandial blood glucose levels. Also, we would like to recommend the measurement of glycated albumin or fructosamine instead of or in addition to other methods of glycaemic control investigation that are not altered by the presence of hemoglobinopathies and RBCs' life span (17). There are very few studies that are by our findings **(Table 3)**.

Table 3- Three Studies Citing the Effect of Sickle Cell Disorders on HbA1C.

STUDY	PARTICIPANTS	STUDY TYPE	RESULTS	CONCLUSION
Lacy et al (2017) (19)	4620	Retrospective cohort	In this retrospective cohort study of 4620 African Americans, for any given fasting or 2-hour glucose concentration, individuals with sickle cell trait had significantly lower hemoglobin A1c values, 5.72% vs 6.01%, than those without sickle cell trait.	Among African Americans with sickle cell trait, hemoglobin A1c concentration may systematically underestimate past glycemia.
Kweka et al (2019) (20)	480	Cross-sectional	Participants with SCT were 88% less likely to be diagnosed with diabetes by HbA1c compared to those without SCT (OR=0.12, 95% CI (0.1,0.2), P <0.001).	HbA1c systematically underestimate the prevalence of diabetes among people with SCT.
Bleyer et al (2010) (21)	885	Retrospective observational	The study included data from 385 African American (AA) participants—109 with SCT, 22 with hemoglobin C trait, and 254 without any hemoglobinopathy—and 500 European American patients. The researchers employed a multivariate repeated-effects regression model to analyze the relationship between HbA_1c and simultaneous serum glucose measurements. The findings revealed that the relationship between HbA_1c and serum	Sickle cell trait does not impact the relationship between HbA1c and serum glucose concentration. In addition, it does not appear to account for ethnic difference in this relationship between African Americans and whites.

			glucose did not differ between African American subjects with and without SCT. However, the relationship differed significantly between African American subjects without SCT and European Americans, with a P-value of 0.0002.	

Our population in the study belonged mostly to rural areas, mostly from tribal groups with an increased prevalence of sickle cell hemoglobinopathy, though the prevalence of sickle cell trait is higher than sickle cell disease, as observed in our study. These patients are clinically more silent than sickle cell disease patients. And now, due to migration for employment, there is a constant mixing of population, and such patients with sickle cell trait may be underdiagnosed or misdiagnosed as diabetics or pre-diabetics by false interpretation of HbA1c values if not evaluated for associated hemoglobinopathy.

Zhu et al. published in 2009 about the false elevation of HbA1c due to S-beta+-thalassemia interference; they concluded that if a significant increase in HbA1c is detected and HbS is >50%, S-beta (+)-thalassemia should be suspected (22). This finding seems very relevant and indicates a need for careful HbA1c analysis.

FETAL HEMOGLOBIN AND HBA1C

HbF is the hemoglobin of intrauterine life, and levels progressively decrease after birth from 60-95% to less than 1% in adults who are hematologically normal (23). The elevated levels of HbF are seen in pathological conditions like hemoglobinopathies, leukemia, and hereditary persistence of HbF (23). HbF doesn't have beta chains and has gamma chains instead. 60% of glycation of HbA occurs at amino-terminal valine residues of the beta chain, whereas the terminal residue for HbF is glycine, and it has been found that the N-terminal residue of the gamma chain is glycated at a slower rate, leading to lower glycated hemoglobin (24). This interference has been observed in methods using boronated affinity, as it measures the ratio of glycated to non-glycated hemoglobin regardless of fraction (25). In our study, elevated HbF is found in sickle cell disease and trait patients. A positive relation is observed between HbA1c and HbF levels in patients with sickle cell disease; the exact cause and mechanism for this are still a matter of evaluation.

VARIABILITY AMONG METHODS OF HPLC

The total glycated hemoglobin consists of HbA1c, which is glycation at the beta chain, and Hb glycation at other sites, like at the alpha chain and epsilon amino groups of lysine residues as well. There are various methods of HPLC analysis, broadly divided into four categories: immunoassay-based, ion exchange-based, boronated affinity, and enzymatic assays (26). Immunoassays measure specifically HbA1c, with antibodies recognizing the structure of the N-terminal glycated amino acid of the Hb beta chain, whereas ion exchange separates based on charge differences between glycated HbA1 and other fractions (27). The affinity chromatographic methods measure total glycated hemoglobin and tend to pose the least interference from other Hb variants (28). In our study, the available method in our laboratory was ion exchange HPLC. The study by Zhu et al as discussed above used both ion exchange and immunoassay methods and concluded that the sickle–beta thalassemia trait interferes with the Bio-Rad turbo assay (ion exchange), causing falsely elevated HbA1c, as it measures the HbA1c fraction only of glycated Hb, whereas the immunoassay method used measures all Hb variants that are glycated at the beta chain and epitopes similar to that of HbA1c (22).

IRON DEFICIENCY ANEMIA AND HBA1C

In our country, it is very important to evaluate diabetic patients based on HbA1c, as there is a high prevalence of diabetes and iron deficiency anemia. In our study, we found the value of HbA1c in IDA patients slightly higher than in sickle cell hemoglobinopathy, though we didn't compare it with the non-anemic population, as our focus was to study the Hb analysis among these two groups of patients. However, we recommend a separate study of comparison between non-anemic and IDA patients. The study conducted by Nitin et al. in 2012 regarding the effect of iron deficiency anemia on glycated hemoglobin in 50 Indian patients with IDA found a lower mean baseline HbA1c in patients with IDA (4.6%) than in the control group (5.5%), which was in contrast to previous studies, where HbA1c was higher in patients with IDA (29). They attributed this finding to certain unknown variables (29). They also observed an increase in HbA1c after treatment of IDA for 2 months (29). In our study, we found HbA1c of 5.2% in IDA patients, slightly higher when compared to patients with sickle cell trait and lower than those in patients with sickle cell disease. There is a clear effect of IDA on HbA1c levels; theoretically, it should increase, owing to decreased turnover and increased glycation due to oxidative stress and production of malondialdehyde, also a marker of oxidative stress (30).

Another study by Kalairajan et al. in 2019, conducted in a South Indian province among 120 patients, showed that IDA was associated with decreased HbA1c (4.6%) when compared to the control group (5.4%) (31). The author claimed the cause to be nutritional variability due to racial differences and low socioeconomic conditions (31). Whereas the studies in the Western world established either no association or a reverse association of HbA1c and IDA. A cross-sectional study by Ford et al. conducted on the American population in 2011 found higher HbA1c levels in IDA patients. The authors also cautioned to be careful while making a diagnosis of diabetes in such patients, especially those who have values near the threshold (32). These discrepancies suggest the effect of racial differences, and probably countries like ours need to

develop another method for the evaluation of their diabetic population. Needless to say, another method for evaluation of hyperglycemia and screening for diabetes in these patients is recommended.

5. CONCLUSION

The method for screening and evaluating glycemic control in such subgroups of the population needs refinement. The interference of Hb variants and IDA needs further studies with comparison to control groups in sub-populations based on associated factors and racial differences.

CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS DISCLAIMER:

None

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