Susceptibility of induced sickle in samples of heterozygous hemoglobin S patients (sickle cell trait) suffering diabetes mellitus type 2

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Abstract

Hemoglobin S is an abnormal protein that induces morphological changes in erythrocyte in low-oxygen conditions. In Mexico, it is reported that up to 13.7% of the population with mutation in one allele are considered asymptomatic (sickle cell trait). The sickle cell trait and diabetes mellitus are conditions that occur together in more than one million patients worldwide. Both diseases possibly produce microvascular changes in retinopathy and acute chest syndrome. The aim of this study was to evaluate the induction of sickle cells in samples of diabetic patients with sickle cell trait to identify altered red cell parameters. We obtained samples of diabetic patients to determine hemoglobin A1c and S; furthermore, red blood cell biometrics data were analyzed. We found that older men with diabetes were susceptible to generate sickle cells and this correlated with reduced red blood cell count and an increase in media cell volume. In samples of women diabetes, there were no differences. We conclude that samples from patients with sickle cell trait and diabetes can cause sickle cells with high frequency in men, with lower red blood cells count and increased mean corpuscular volume as susceptibility parameters. (Gac Med Mex. 2015;151:702-8)

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Introduction

Hemoglobin (Hb) is a protein present in red blood cells (RBC) and its function is to bind and carry oxygen to all tissues of the body[1]. Nearly 400 Hb variants have been described, with differences in one or more amino acids[2]. An Hb variant is known as HbS, which induces changes in the oxygen-binding function, leading to chronic hemolytic anemia when the mutation is found in both alleles. In addition, up to 20% of the African population has been found to produce this type of hemoglobinopathy[3,4]. In Mexico, its variability ranges from 0.6 to up to 13.7% in 5 populations studied across the country; this difference is considered to be caused by miscegenation of Mexican with African populations[5]. HbS partial carrier patients have been reported to be generally asymptomatic, since only from 35 to 45% correspond to this HbS, with the rest corresponding to normal Hb[6].

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The molecular alteration in HbS involves a mutation with a thymine substituting an adenine in the b-chain, which translates into glutamic acid being substituted by valine[7]. This mutation generates a physiological and anatomical modification of RBCs known as sickle cell disease, which occurs in patients with the genic variant in both alleles, which in turn generates a Hb b-subunit able to polymerize in low oxygen conditions in well-defined filamentous structures, ultimately leading to RBC degeneration, by modifying its biconcave shape into a sickle shape known as sickle cell[8,9]. Sickle cell formation induces high adherence between altered cells[10], in addition to coagulation cascades[11] and immune system response[12], with these processes causing partial or total blockage of small-caliber blood vessels that irrigate vital organs, a situation that can end up in acute ischemia or other hemodynamic complications that may lead to death of the patient[13]. Individuals with mutation in a single allele (heterozygous) are considered asymptomatic and are regarded as sickle cell trait patients that don't require treatment; however, thorough investigations suggest susceptibility to lethal effects in acute severe hypoxia conditions, as in the case of sudden death in athletes; usually, death in these individuals occurs under sudden situations during excessive sport activity[14].

Diabetes mellitus (DM) is a serious and growing public health problem in the entire world and is associated with serious acute and chronic complications that negatively impact on both quality of life and survival of affected individuals[15]. In addition, it is characterized by metabolic imbalance[16] and hyperglycemia[17], which involve the generation of oxidative stress[18] and a generalized inflammation phenomenon[19]. It has also been associated with cardiovascular disorders involving endothelial dysfunction[20], coagulation cascades alterations[21] and tissue hypoxia episodes[22]. Sickle cell trait and DM are conditions usually occuring together in over 1 million patients worldwide[23]. This dyad is likely to produce microvascular alterations, thus favoring the generation of sickle cells in clinical case reports on retinopathy and acute chest syndrome in diabetic patients[24]. However, reports are insufficient to understand the pathological events that can produce the DM-sickle cell trait dyad owing to HbS and Hb C. It's because of this that it is important to study the susceptibility of RBCs to degenerate into sickle cells in a group of patients of the Mexican population with DM and sickle cell trait (heterozygous for HbS) to better understand the dyad. The present study is intended to study the susceptibility of RBC to degenerate into sickle cells in patients with type 2 DM.

Methodology

Patient selection and HbA1c and HbS determination

Mexican population patients already diagnosed with type 2 DM were selected, and a blood sample was collected from them with the vacutainer system in tubes containing EDTA as anticoagulant. HbA1c was then determined, in addition to an HbS percentage, corresponding to a single-allele mutation (heterozygosis for HbS or sickle cell trait). HbA1c and HbS values were determined by means of HPLC (Variant II turbo instrument, BioRad) and by electrophoresis (Spife-Helena), respectively.

Clinical data were obtained from Laboratorio Carpemor, S.A. de C.V. According to the General Statute of Health, the study does not represent physical or confidentiality risks and, therefore, no signed consent was required from the patients. The protocol was approved by the Laboratorio Carpemor Ethics and Research Committee.

Blood count

All samples were analyzed by flow cytometry with cytochemistry using the ADVIA® 120 instrument (Hematology System, SIEMENS) in order to obtain the erythrocyte count values, which correspond to the following parameters: RBC count (RBCC), Hb, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

Sickle cell induction test

The sickle cell induction test was carried out with sodium metabisulfite at 2% (J.T. Baker); for this, of the samples selected during the processing of HbA1 in the Variant instrument that also yielded some HbS percentage, a drop of blood was placed on a slide. A drop of 2% sodium metabisulfite reductant solution was then added, the cover glass was placed and, finally, the borders were sealed with wax to observe under the microscope with monitoring at 1, 2 and 24 hours.

Statistical analysis

Data were analyzed with the t-test when the distribution was parametric and with the Mann-Whitney test when the distribution was non-parametric. Statistical analysis was performed using the SigmaPlot 11.0 software,
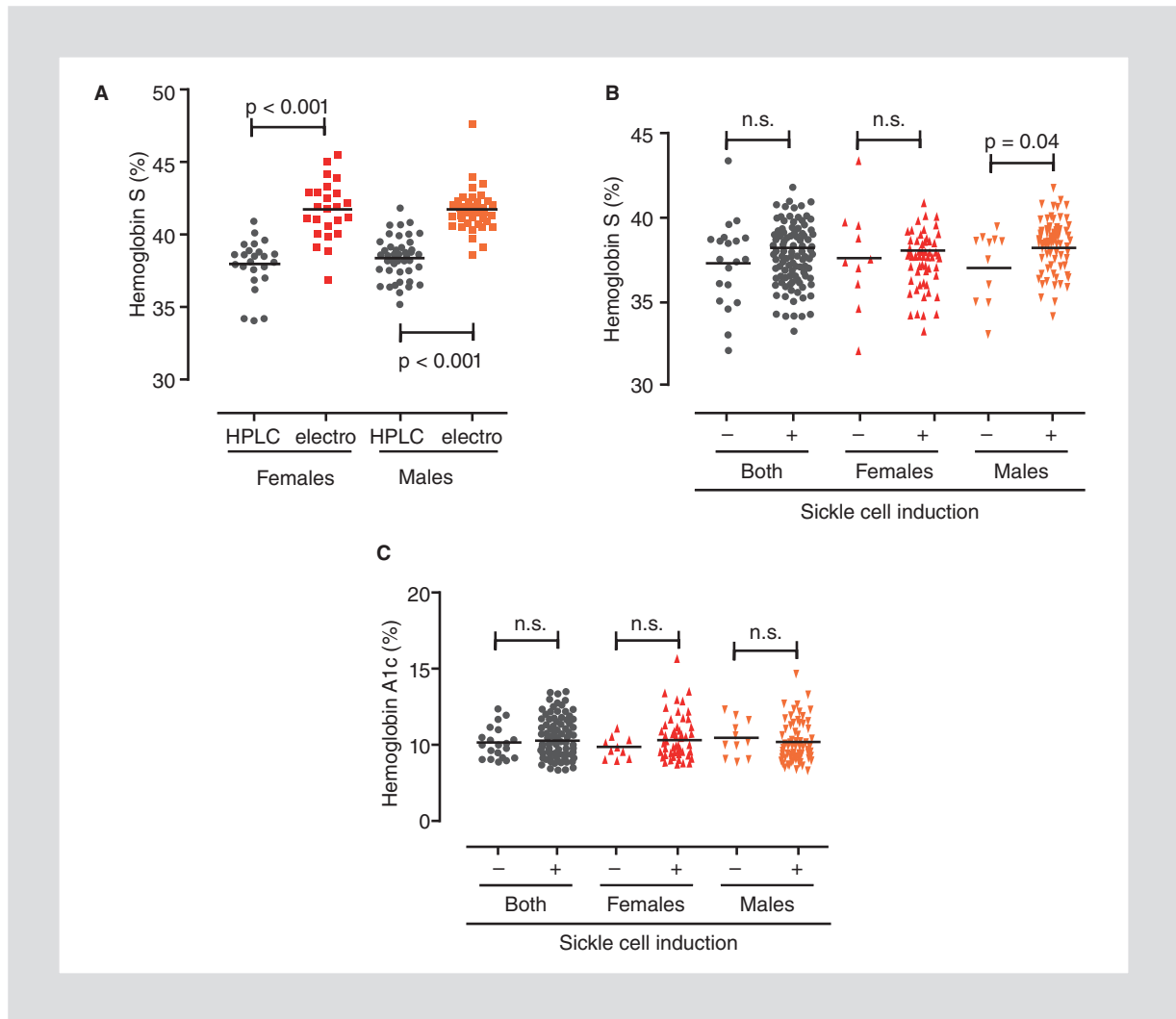
Figure 1. *Hemoglobin S quantification and sickle cell induction in diabetic patients are not associated with glycated hemoglobin variations. A: comparison of hemoglobin S levels as determined by HPLC and electrophoresis; t-test and Mann-Whitney test. B: hemoglobin S levels in sickle cell induction test-positive and negative RBCs; Mann-Whitney test. C: hemoglobin 1Ac levels in sickle cell induction test-positive and negative RBCs; Mann-Whitney test. n.s.: non-significant.*

whereas the graphs were carried out with the Graph-Pad Prism 5.0 software.

Results

One hundred and fifty blood samples from HbS-positive diabetic patients, corresponding to heterozygous individuals with mutation in one allele, were selected and assessed, with 74 samples being from females and 76 from males. Initially, we conducted a comparative analysis in order to assess the HbS-determination variability with the HPLC and electrophoresis techniques in the same samples of diabetic patients. Higher HbS values were found to be observed in both genders with the electrophoresis technique (Fig. 1 A).

In individuals where HbS corresponding to sickle cell trait was identified, the metabisulfite sickle cell-induction test was performed in the diabetic patients' samples. In these analyzed samples, in spite of the presence of HbS, some of them failed to degenerate into sickle cells, but differences were found in some parameters such as age, RBCC and MCV among the population that was positive and negative to the sickle cell induction, as described above.

The HbS content in male samples showed significant differences, with a p-value of 0.04 betweeen those with negative or positive sickle cell induction, i.e., the proportion of HbS was higher in those samples with positive sickle cell induction, which is consistent with RBC degeneration. In contrast, female samples with positive
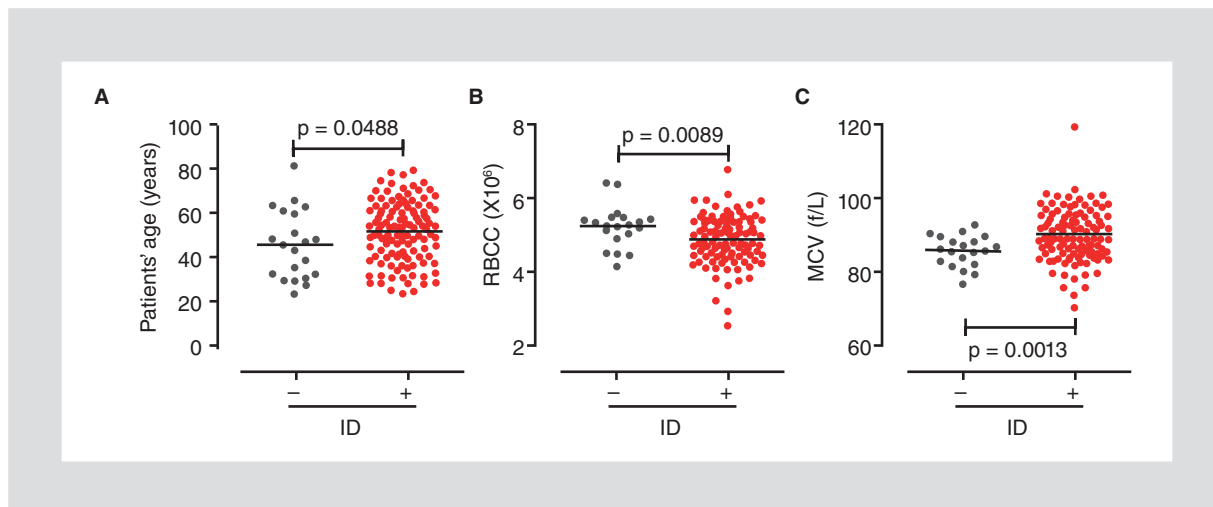
Figure 2. *Diabetic patients with sickle cell trait who are sickle cell induction test-psitive have age-dependent reduced red blood cell count (RBCC) and increased mean corpuscular volume (MCV) . **A:** age of diabetic patients with positive vs. negative sickle cell induction; t-test. **B:** decreased red blood cell count in sickle cell induction-positive diabetic patients; Mann-Whitney test. **C:** increased mean corpuscle volume in sickle cell induction-positive diabetic patients; Mann-Whitney test.*

and negative induction were observed to have no differences in HbS content, which suggests there are additional factors contributing to degeneration into sickle cells in this population (Fig. 1 B).

Based on the two populations defined as positive or negative for the sickle cell induction test and on the fact that they share the diabetes diagnosis and the sickle cell trait, the relationship between HbA1c and HbS in diabetic patients was analyzed; however, the results showed there are no significant differences (Fig. 1 C).

Subsequently, other clinical data associated with the samples undergoing sickle cell induction were analyzed, and individuals with a positive sickle cell-induction test (regardless of gender) were found to be older than those who failed to generate sickle cells during the metabisulfite test (Fig 2 A). Additionally, sickle cell induction-sensitive blood samples were found to correspond to patients with lower RBCC and higher MCV, which suggests that sickle cell induction-sensitive cells generally contain large or macrocytic RBCs (Fig. 2 B and C). The remaining parameters corresponding to the erythrocyte count did not yield significant differences between positive and negative samples to the sickle cell induction test.

When data were stratified based on the individuals' gender, no significant differences were found in sickle cell induction in women with regard to age, with a p-value of 0.9118. This suggests that, in this population, age is not a susceptibility factor favoring sickle cell induction. In contrast, men showed a clear age difference between those who did and did not generate

sickle cells, with a p-value of 0.0023, which suggests that this population is susceptible to generate sickle cells as age of the individuals advances (Fig. 3 A).

Overall, the RBCC and MCV clinical parameters showed significant differences in males, with increased RBCC (p = 0.0149) and higher MCV values (p = 0.0004) in sickle cell induction-positive samples in comparison with induction-negative samples. In turn, no difference was observed between positive and negative samples of women in RBCC (p = 0.985) or in MCV (p = 0.304); therefore, only in males there is clinical correlation between the susceptibility of RBCs to the sickle cell induction test and decreased RBCC, increased MCV and older age of the individuals.

Discussion

Our research group studied sickle cell generation in patients who were heterozygous for HbS and who also suffered from type 2 DM. DM produces cardiovascular and hemodynamic problems that aggravate the patients' health[25], and this is why we studied the sickle cell trait in this population by inducing sickle cells in blood samples.

Initially, we compared the analytical methods used to analyze the percentage of HbS and found significant differences between both techniques; this could be owing to HPLC technique being more sensitive than electrophoresis. These results are consistent with the study carried out by Keren et al., where the high sensitivity of HPLC is demonstrated[26].
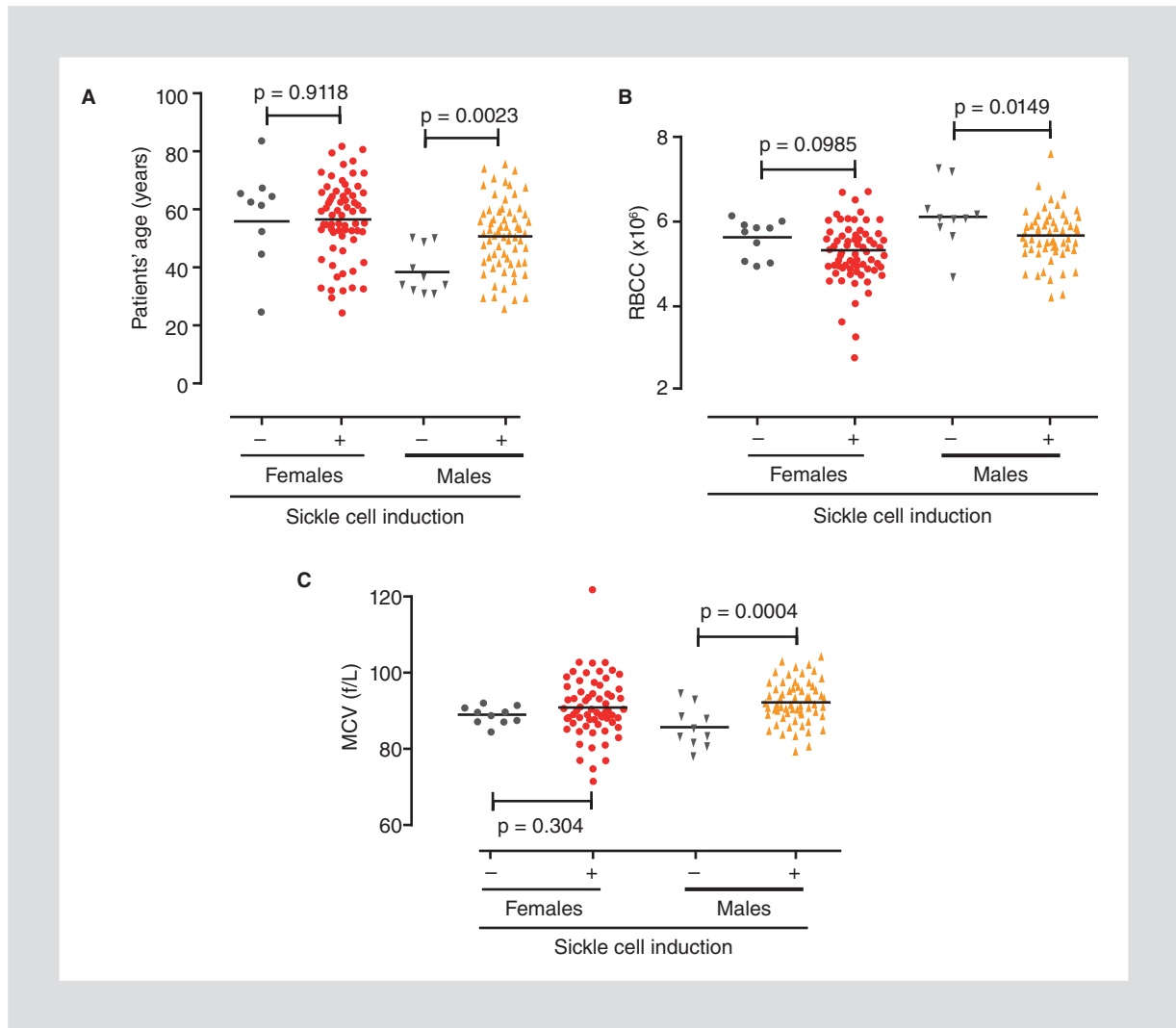
Figure 3. *Male diabetic patients with sickle cell trait who are susceptible to sickle cell induction, but not females, have age-dependent reduced RBC counts (RBCC) and increased mean corpuscular values (MCV).* **A:** *older male patients showed sickle cell induction; t-test.* **B:** *male patients with lower complete blood RBC count showed sickle cell induction; Mann-Whitney test.* **C:** *male patients with higher RBC mean corpuscular volume show sickle cell induction; Mann-Whitney test.*

Our results showed that as the individual's age advances, particularly in males, HbS-containing RBCs are more susceptible to deform into sickle cells. This phenomenon is consistent in previous studies in diabetic patients where cardiovascular and hematological pathological events evolved depending on age[25]. It is important to point out that, in our study, the incidence in the percentage of HbS in diabetic patients is higher in men with RBC-positive versus negative induction, which does not occur in women's samples; this can be explained by the Hb and RBC baseline levels present in men to cover tissue oxygenation requirements[27]. However, studies conducted by Garrote and Santana in 2013 reported that HbS-pesenting female athletes increase the possibilty of sickle cells generation due to

a decrease in iron uptake associated with increased peristaltism, RBC mechanical hemolysis and blood losses by menstrual discharge in childbearing age women; these data have been pointed out as the main causes[28]. On the other hand, sickle cell generation did not show significant differences with regard to HbA1c concentration, with the results being consistent with the study conducted by Ama et al. in 2012[29]. This indicates that the sickle-shaped trait that is characteristic of this condition does not depend on HbA1c increase or decrease; i.e., sickle cell generation is not favored or hindered by Hb non-enzymatic glycation. These data are consistent with previously published reports[24,30]. In this sense, HbS is known to be susceptible to glycation[31-34], but we don't know the proportion of HbS that was

glycated in the analyzed samples and how this non-enzymatic post-translational modification might affect in the HbS polymerization kinetics context, a situation that requires clarification in future studies within the assessed population.

Individuals who were heterozygous for HbS and who had DM showed lower RBCC, which is consistent with typical features of hemoglobinopathies[35]; it is possible for partial presence of HbS to discretely induce RBC destruction throughout the individual's life. MCV was also found to be elevated in samples of sickle cell-generating individuals, and this is due to an increase in hemoglobin synthesis intended to cover oxygenation requirements of the body[36]. In particular, samples corresponding to males were the ones that showed differences in the above mentioned parameters; i.e., this gender appears to be more sensitive to the presence of HbS according to our results. Reports indicate that the sickle cell trait affects essentially the male gender, which increases the frequency of microvascular alterations[37] and accelerates cardiovascular and cerebrovascular disturbances, as well as mortality in Africans with type 2 diabetes[38].

Reports on the DM-sickle cell trait dyad owing to HbS and Hb C are insufficient to clarify the consequences that the coexistence of both conditions can have and under what circumstances can complications be favored in individuals. This is why prospective studies aimed at investigating the consequences of the dyad and the molecular mechanisms involved have to be established. Furthermore, it is necessary for study population to be increased by including non-diabetic patients heterozygous for HbS and only diabetic patients in order to contrast, understand and assess the cardiovascular and hemodynamic consequences shown by this dyad in the Mexican population. Finally, the design of animal models that allow for the sickle cell trait together with diabetes to be simulated will enable deep assessment of the consequences of this dyad from the molecular and pathophysiological point of wiew in order to establish treatment strategies when necessary.

Conclusions

We conclude that samples of patients with sickle cell trait and diabetes can generate sickle cells on smear under the metabisulfite-induction protocol with a frequency higher than 80%. Male patients' induction-positive samples correspond to older individuals with lower RBCC and higher MCV as susceptibility parameters.

Samples obtained from females showed no differences in the erythrocyte count parameters.

Acknowledgements

References

1. Snell FM. Facilitated transport of oxygen through solutions of hemoglobin. J Theor Biol. 1965;8:469-79.
2. Macchiato MF, Tramontano A. VARIANT: a store and retrieval system for human haemoglobin variants. Comput Methods Programs Biomed. 1990;31:113-4.
3. Zeng Y, Huang S. The studies of hemoglobinopathies and thalassemia in China--the experiences in Shanghai Institute of Medical Genetics. Clin Chim Acta. 2001;313:107-11.
4. Lorey FW, Arnopp J, Cunningham GC. Distribution of hemoglobinopathy variants by ethnicity in a multiethnic state. Genet Epidemiol. 1996;13:501-12.
5. Penaloza-Espinosa RI, Buentello-Malo L, Hernandez-Maya MA, et al. Hemoglobin S frequency in five Mexican populations and its importance in public health. Salud Publica Mex. 2008;50:325-9.
6. R HG. Hemoglobin Disorders. En: Nelson textbook of pediatrics; Elsevier; 2000, pp.
7. Wajcman H, Prehu C, Bardakdjian-Michau J, et al. Abnormal hemoglobins: laboratory methods. Hemoglobin. 2001;25:169-81.
8. Dong C, Chadwick RS, Schechter AN. Influence of sickle hemoglobin polymerization and membrane properties on deformability of sickle erythrocytes in the microcirculation. Biophys J. 1992;63:774-83.
9. Rodgers GP, Schechter AN, Noguchi CT, et al. Periodic microcirculatory flow in patients with sickle-cell disease. N Engl J Med. 1984;311: 1534-8.
10. Chien S, Kaperonis AA, King RG, et al. Rheology of sickle cells and its role in microcirculatory dynamics. Prog Clin Biol Res. 1987;240:151-65.
11. Connes P, Tripette J, Chalabi T, et al. Effects of strenuous exercise on blood coagulation activity in sickle cell trait carriers. Clin Hemorheol Microcirc. 2008;38:13-21.
12. Abu-Zeid YA, Theander TG, Abdulhadi NH, et al. Modulation of the cellular immune response during Plasmodium falciparum infections in sickle cell trait individuals. Clin Exp Immunol. 1992;88:112-8.
13. Juncos JP, Grande JP, Croatt AJ, et al**.** Early and prominent alterations in hemodynamics, signaling, and gene expression following renal ischemia in sickle cell disease. Am J Physiol Renal Physiol. 2010;298: F892-9.
14. Ali Z, Troncoso JC, Fowler DR. Recurrent cerebral venous thrombosis associated with heterozygote methylenetetrahydrofolate reductase CT mutation and sickle cell trait without homocysteinemia: An autopsy case report and review of literature. Forensic Sci Int; 2014.
15. Vigneri R. Diabetes: diabetes therapy and cancer risk. Nat Rev Endocrinol. 2009;5:651-2.
16. Pauer J, Fek A, Buday B, et al. Metabolic alteration in healthy men with first degree type 2 diabetic relatives. Orv Hetil. 2013;154:178-86.
17. Klotzbucher E. Glucagon hyperglycemia and disordered insulin utilization in diabetes mellitus in man. Dtsch Gesundheitsw. 1952;7:1487.
18. Matkovics B, Varga SI, Szabo L, et al**.** The effect of diabetes on the activities of the peroxide metabolism enzymes. Horm Metab Res. 1982;14:77-9.
19. Lobner K, Fuchtenbusch M. Inflammation and diabetes. MMW Fortschr Med. 2004;146:32-3, 5-6.
20. Sibireva OF, Kaliuzhin VV, Urazova OI, et al. The biochemical markers of endothelium dysfunction in patients with diabetic nephropathy. Klin Lab Diagn. 2012;8-11.
21. Vazzana N, Ranalli P, Cuccurullo C, et al. Diabetes mellitus and thrombosis. Thromb Res. 2012;129:371-7.
22. Gabbay IE, Gabbay M, Gabbay U. Diabetic foot cellular hypoxia may be due to capillary shunting--a novel hypothesis. Med Hypotheses. 2014;82:57-9.
23. Levitt NS. Diabetes in Africa: epidemiology, management and healthcare challenges. Heart. 2008;94:1376-82.
24. Tsaras G, Owusu-Ansah A, Boateng FO, et al. Complications associated with sickle cell trait: a brief narrative review. Am J Med. 2009;122:507-12.

25. Franch-Nadal J, Roura-Olmeda P, Benito-Badorrey B, et al. Metabolic control and cardiovascular risk factors in type 2 diabetes mellitus patients according to diabetes duration. Fam Pract. 2014.

26. Keren DF, Hedstrom D, Gulbranson R, et al. Comparison of Sebia Capillarys capillary electrophoresis with the Primus high-pressure liquid chromatography in the evaluation of hemoglobinopathies. Am J Clin Pathol. 2008;130:824-31.

27. Maniatis A, Frieman B, Bertles JF. Increased expression in erythrocytes ii antigens in sickle cell disease and sickle cell trait. Vox Sang. 1977; 33:29-36.

28. Bain BJ. Haemoglobinopathy diagnosis: algorithms, lessons and pitfalls. Blood Rev. 2011;25:205-13.

29. Ama V, Kengne AP, Nansseu NJ, et al. Would sickle cell trait influence the metabolic control in sub-Saharan individuals with type 2 diabetes? Diabet Med. 2012;29:e334-7.

30. Bleyer AJ, Vidya S, Sujata L, et al. The impact of sickle cell trait on glycated haemoglobin in diabetes mellitus. Diabet Med. 2010;27:1012-6.

31. Aleyassine H. Glycosylation of hemoglobin S and hemoglobin C. Clin Chem. 1980;26:526-7.

32. Abdella PM, Ritchey JM, et al. Glycosylation of hemoglobin S by reducing sugars and its effect on gelation. Biochim Biophys Acta. 1977; 490:462-70.

33. Abraham EC, Elseweidy MM. Non-enzymatic glycosylation influences Hb S polymerization. Hemoglobin. 1986;10;173-83.

34. Abraham EC, Cameron BF, Abraham A, et al. Glycosylated hemoglobins in heterozygotes and homozygotes for hemoglobin C with or without diabetes. J Lab Clin Med. 1984;104:602-9.

35. Ruiz-Reyes G. Fundamentos de Hematología. Panamericana; 1998. pp 31-44.

36. Kaul DK, Liu XD. Rate of deoxygenation modulates rheologic behavior of sickle red blood cells at a given mean corpuscular hemoglobin concentration. Clin Hemorheol Microcirc. 1999;21;125-35.

37. Ajayi AA, Kolawole BA. Sickle cell trait and gender influence type 2 diabetic complications in African patients. Eur J Intern Med. 2004; 15:312-5.

38. Kolawole BA, Ajayi AA. Prognostic indices for intra-hospital mortality in Nigerian diabetic NIDDM patients. Role of gender and hypertension. J Diabetes Complications. 2000;14:84-9.