UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Elevation of HbA1c in hyperglycemic women with decreased iron involves increased osmotic stability and volume variability (RDW) of red cells

Aluno: Breno Batista da Silva

Orientador: Nilson Penha Silva
Co-Orientadora: Nadia Carla Cheik

UBERLÂNDIA - MG

2020

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Elevation of HbA1c in hyperglycemic women with decreased iron involves increased osmotic stability and volume variability (RDW) of red cells

Aluno: Breno Batista da Silva

Orientador: Nilson Penha Silva
Co-Orientadora: Nadia Carla Cheik

Dissertação apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Mestre em Genética e Bioquímica (Área Bioquímica).

UBERLÂNDIA – MG
2020

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

S586 Silva, Breno Batista da, 1996-

2020 Elevation of HbA1c in hyperglycemic women with decreased iron involves increased osmotic stability and volume variability (RDW) of red cells [recurso eletrônico] / Breno Batista da Silva. - 2020.

Orientador: Nilson Penha Silva.
Coorientadora: Nadia Carla Cheik.
Dissertação (Mestrado) - Universidade Federal de Uberlândia, Pós-graduação em Genética e Bioquímica.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.di.2020.641
Inclui bibliografia.

1. Genética. I. Silva, Nilson Penha,1957-, (Orient.). II. Cheik, Nadia Carla,1977-, (Coorient.). III. Universidade Federal de Uberlândia. Pós-graduação em Genética e Bioquímica. IV. Título.

CDU: 575

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

Gizele Cristine Nunes do Couto - CRB6/2091

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Genética e Bioquímica
Av. Pará 1720, Bloco 2E, Sala 244 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
Telefone: +55 (34) 3225-8438 - www.ppggb.ibtec.ufu.br - ppggb@ufu.br

ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
Defesa de:	Dissertação de Mestrado Acadêmico/PPGGB				
Data:	Trinta de setembro de 2020	Hora de início:	09:30h	Hora de encerramento:	10:10h
Matrícula do Discente:	11822GBI002				
Nome do Discente:	Breno Batista da Silva				
Título do Trabalho:	Elevation of HbA1c in hyperglycemic women with decreased iron involves increased osmotic stability and volume variability (RDW) of red cells.				
Área de concentração:	Bioquímica				
Linha de pesquisa:	Biologia Molecular				
Projeto de Pesquisa de vinculação:	Relações entre força muscular, estabilidade de membrana de eritrócitos e variáveis bioquímicas, hematológicas e inflamatórias em pacientes com pré-diabetes e diabetes mellitus tipo 2.				

Aos trinta dias do mês de setembro de dois mil e vinte, às 09:30 horas, reuniu-se via web conferência pela plataforma Google Meet, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES e Resolução de nº 06/2020 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Professores Doutores: Nilson Penha Silva (Orientador), Morun Bernardino Neto e Ricardo Rodrigues. A participação dos dois últimos se deu por epístola. Iniciando os trabalhos o presidente Dr. Nilson Penha Silva apresentou a Comissão Examinadora e o candidato, agradeceu a presença dos participantes, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa. A seguir o senhor presidente procedeu a leitura das epístolas enviadas pelos membros da banca. Em seguida os membros presentes, passaram a arguir o candidato. Ultimada a leitura das epístolas e a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o candidato:

(A) PROVADO.

Esta defesa de Dissertação de Mestrado é parte dos requisitos necessários à obtenção do título de Mestre. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Nilson Penha Silva**, **Membro de Comissão**, em 30/09/2020, às 10:19, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de

outubro de 2015.

Documento assinado eletronicamente por **Morun Bernardino Neto**, **Usuário Externo**, em 30/09/2020, às 10:22, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

Documento assinado eletronicamente por **Ricardo Rodrigues**, **Médico(a)**, em 30/09/2020, às 12:24, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

A autenticidade deste documento pode ser conferida no site https://www.sei.ufu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **2256204** e o código CRC **2CDF605E**.

Referência: Processo nº 23117.053841/2020-60 SEI nº 2256204

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Elevation of HbA1c in hyperglycemic women with decreased iron involves increased osmotic stability and volume variability (RDW) of red cells

ALUNO: Breno Batista da Silva

COMISSÃO EXAMINADORA

Presidente: **Nilson Penha Silva (Orientador)**

Examinadores:

Ricardo Rodrigues

Morun Bernardino Neto

Data da Defesa: 30 / 09 / 2020

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Dissertação foram contempladas

Nilson Penha Silva

DEDICATÓRIA

Dedico este trabalho à minha família, que me mostrou e me mostra o amor genuíno e a alegria de viver

AGRADECIMENTOS

Agradeço a Deus, por todos os dias me dar força para seguir em frente mesmo com diversos obstáculos em meu caminho. Agradeço especialmente aos meus pais, que sempre me apoiaram com muito carinho e educação; se não fosse por eles, provavelmente eu não chegaria onde estou. A minha namorada, que também sempre esteve ao meu lado nos vários momentos difíceis da minha vida acadêmica e pessoal.

E aos meus colegas de laboratório, Sarah Yasminy Luz, Manuela Ortega Marques Rodrigues, Maria Aparecida Knychala e Mario da Silva Garrote Filho, que sempre estiverem presentes comigo e não me deixaram desistir. A todos os professores e, em especial, ao meu orientador, que me ajudou incansavelmente durante todo este percurso; sem a sua experiência não seria possível a realização deste trabalho. Agradeço também, especialmente, a minha coorientadora, que me acolheu no início da graduação e nunca duvidou do meu potencial.

Não posso deixar de agradecer também aos técnicos do laboratório, Maria Conceição Francisco Souto dos Santos e Célia Cristina Souza, pelo apoio essencial nas atividades do LaBFiQ.

Também agradeço o apoio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) com a concessão da bolsa de mestrado.

SUMÁRIO

RESUMO

[A elevação de HbA1c em mulheres hiperglicêmicas com níveis diminuídos de ferro envolve aumento da estabilidade osmótica e da variabilidade de volume (RDW) de células vermelhas]

Introdução: As concentrações de hemoglobina glicada (HbA1c) estão associadas não apenas à glicose, mas também aos níveis de ferro, e isso requer atenção clínica cuidadosa, especialmente em indivíduos hiperglicêmicos. **Objetivo:** Este estudo teve como objetivo analisar as associações de variáveis antropométricas, inflamatórias, regulatórias, metabólicas e hematológicas, com indicadores do *status* do ferro e níveis de HbA1c em mulheres hiperglicêmicas. **Métodos:** Participaram deste estudo transversal prospectivo 143 mulheres (68 normoglicêmicas e 75 hiperglicêmicas). As comparações entre os grupos utilizaram o teste de Mann-Whitney e a busca de associações entre pares de variáveis utilizou o método de correlação de Spearman. **Resultados:** Em mulheres hiperglicêmicas, a diminuição de ferro associa-se ao aumento da HbA1c, e essas alterações estão ambas associadas ao estado inflamatório e envolvem diminuição da concentração de hemoglobina intracelular (CHCM), que por sua vez, envolve aumento da estabilidade osmótica (dX) e da variabilidade de volume (RDW) de eritrócitos, bem como diminuição do catabolismo da hemoglobina. Essa diminuição do catabolismo da hemoglobina não parece ser apenas um processo associado à diminuição das concentrações intracelulares dessa proteína, uma vez que está associada ao estado inflamatório e à diminuição de LDL-colesterol. **Conclusão:** Em mulheres hiperglicêmicas, a elevação da HbA1c com a diminuição do ferro está ligada à inflamação associada à obesidade e envolve mudanças associadas ao aumento da estabilidade osmótica e à variabilidade de volume (RDW) de eritrócitos.

Palavras-chave: Obesidade; inflamação; hiperglicemia; Hemoglobina glicada; deficiência de ferro; eritrócitos; estabilidade osmótica.

ABSTRACT

Elevation of HbA1c in hyperglycemic women with decreased iron involves increased osmotic stability and volume variability (RDW) of red cells

Background: Glycated hemoglobin (HbA1c) concentrations are associated not only with glucose, but also with iron levels, and this requires careful clinical attention, especially in hyperglycemic individuals. **Objective:** This study aimed to analyze the associations of anthropometric, inflammatory, regulatory, metabolic, and hematologic variables with iron status and HbA1c levels in hyperglycemic women. **Methods:** A total of 143 (68 normoglycemic and 75 hyperglycemic) women participated in this prospective cross-sectional study. Comparisons between groups used the Mann-Whitney test, and the search for associations between pairs of variables used the Spearman correlation method. **Results:** In hyperglycemic women, decreased iron associates with increased HbA1c, and these changes are both associated with inflammatory status and involve decreased intracellular hemoglobin concentration (MCHC), which in turn, involves enhanced osmotic stability (dX) and volume variability (RDW) of erythrocytes, as well as decreased hemoglobin catabolism. This decreased hemoglobin catabolism does not seem to be solely a process associated with diminished intracellular concentrations of this protein since it is associated with inflammatory status and decreased LDL-cholesterol. **Conclusion:** In hyperglycemic women, the elevation of HbA1c with decreased iron is linked with obesity-associated inflammation and involves changes associated with increased osmotic stability and distribution width (RDW) in red cells.

Key Words: Obesity; inflammation; hyperglycemia; glycated hemoglobin; iron deficiency; erythrocytes; osmotic stability.

LISTA DE ABREVIATURAS

ALT	Alanine Amino Transferase
A_{max}	Absorbance at 540 nm associated with lysis of the whole population of erythrocytes;
A_{min}	Absorbance at 540 nm associated with residual lysis of the erythrocytes population
AST	Aspartate Aminotransferase
DB	Direct Bilirrubin;
dX	Variation in the concentration of NaCl responsible for total hemolysis
H_{50}	Saline concentration capable of promoting 50% hemolysis;
HbA1c	Glycated Hemoglobin A1c;
HDL	High Density Lipoprotein
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HOMA-β	Homeostasis Model Assessment β Cell Function;
IB	Indirect Bilirrubin;
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
MCH	Mean Corpuscular Hemoglobin;
MCHC	Mean Corpuscular Hemoglobin Concentration;
MCV	Mean Corpuscular Volume;
RDW	Red Cell Distribution Width;
TB	Total Bilirrubin;
TIBC	Total Iron Binding Capacity;
TSI	Transferrin Saturation Index
VLDL	Very Low Density Lipoprotein

1. INTRODUCTION

The blood concentration of glycated hemoglobin (HbA1c) is widely used for screening, diagnosis, and monitoring of hyperglycemia. It is considered as a gold standard to assess long-term blood glucose (WHO, 2011), including by the American Diabetics Association, which recommends the maintenance of the blood levels of HbA1c below 7% in all diabetic patients to avoid the development of micro- and macrovascular complications (AMERICAN DIABETES, 2020).

However, HbA1c is influenced not only by the glucose blood concentration, but also by conditions that cause iron deficiency (ID) (BROOKS; METCALFE; DAY; EDWARDS, 1980; EL-AGOUZA; ABU SHAHLA; SIRDAH, 2002; MADHU; RAJ; GUPTA; GIRI *et al.*, 2017) and/or affect red blood cells (RBC) homeostasis (HUANG; LIU; MAO; CHEN *et al.*, 2018; VIRTUE; FURNE; NUTTALL; LEVITT, 2004), such as insufficient intake and absorption of iron (BOURI; MARTIN, 2018), acute and chronic blood loss (GALLAGHER; LE ROITH; BLOOMGARDEN, 2009), chronic kidney disease (CKD) (LEE; HUANG; CHEN; CHIOU *et al.*, 2018), and hemolytic anemias (ENGLISH; IDRIS; SMITH; DHATARIYA *et al.*, 2015).

This is worrying, since ID affects more than 2 billion people worldwide (MCLEAN; COGSWELL; EGLI; WOJDYLA *et al.*, 2009), predominantly in the female population.

ID, which is not synonymous with anemia, characterizes by a decrease in body iron stores (CAMASCHELLA, 2015) and has three sequential stages. The first stage is characterized by iron depletion and has iron levels and hematologic parameter values that are still within their reference limits. The second stage, characterized by a subclinical ID, begins with normal hemoglobin levels and progressively leads to changes in hematologic parameters, due to the restriction of iron supply for erythropoiesis. Finally, the third stage is ID anemia, which is the most severe condition (SKIKNE; LYNCH; BOREK; COOK, 1984; SUOMINEN; PUNNONEN; RAJAMAKI; IRJALA, 1998).

Although lower iron has been associated with increased HbA1c in individuals without and with diabetes (BROOKS; METCALFE; DAY; EDWARDS, 1980; COBAN; OZDOGAN; TIMURAGAOGLU, 2004; EL-AGOUZA; ABU SHAHLA; SIRDAH, 2002; GRAM-HANSEN; ERIKSEN; MOURITS-ANDERSEN;

OLESEN, 1990), ID anemia was also associated with decreased HbA1c and normalization after iron replacement therapy in a population without diabetes (CETINKAYA ALTUNTAS; EVRAN; GURKAN; SERT *et al.*, 2020). This issue deserves to be well understood, especially in the populations with diabetes, because the prevalence of ID in this disease is relatively high (ALDALLAL; JENA, 2018; LOUTRADIS; SKODRA; GEORGIANOS; TOLIKA *et al.*, 2016) and the assessment of HbA1c is an essential routine in its monitoring (AMERICAN DIABETES, 2020; ASSOCIATION, 2018), but also because the mechanisms by which ID influences HbA1c levels have not yet been fully elucidated (AHMAD; RAFAT, 2013).

A proper understanding of this issue involves the study of the RBC properties that are influenced by the iron blood levels (SANGHANI; HALDANKAR, 2006; YIP; MOHANDAS; CLARK; JAIN *et al.*, 1983), such as the osmotic stability, which increases with decreased hemoglobin content in the erythrocytes (MOHANDAS; CLARK; JACOBS; SHOEHET, 1980; MOHANDAS; EVANS, 1994; MOHANDAS; GALLAGHER, 2008).

Indeed, the evaluation of erythrocytes osmotic stability is of pivotal importance for understanding the role of these cells in many diseases (FESSLER; ROSE; ZHANG; JARAMILLO *et al.*, 2013; LOPEZ DE FRUTOS; CEBOLLA; IRUN; KOHLER *et al.*, 2018; STIER; REICHERT; CRISCUOLO; BIZE, 2015), which comprises different types of erythrocytopathies, but also type 1 (RODRIGUES; DE MEDEIROS; CUNHA; GARROTE-FILHO *et al.*, 2018) and type 2 diabetes mellitus (KUNG; TSENG; WANG, 2009), preeclampsia (AIRES RODRIGUES DE FREITAS; VIEIRA DA COSTA; ALVES DE MEDEIROS; DA SILVA GARROTE FILHO *et al.*, 2018; DE FREITAS; DA COSTA; MEDEIROS; CUNHA *et al.*, 2019), and health interventions such as bariatric surgery (DE ARVELOS; ROCHA; FELIX; DA CUNHA *et al.*, 2013) and physical exercise(PARAISO; DE FREITAS; GONCALVES; DE ALMEIDA NETO *et al.*, 2014; PARAISO; GONCALVES; CUNHA; DE ALMEIDA NETO *et al.*, 2017).

Regardless of the relationship between iron status and HbA1c levels, erythrocytes are part of the scenery. Therefore, this study aims to contribute to the elucidation of this issue by investigating the associations anthropometric,

inflammatory, regulatory, metabolic, and hematologic variables with iron status indicators and HbA1c in hyperglycemic women.

2. GENERAL OBJECTIVE

Elucidate the relationship between iron status and HbA1c levels, by investigating the associations of anthropometric, inflammatory, regulatory, metabolic, and hematologic variables with iron status indicators and HbA1c in hyperglycemic women.

3. SPECIFIC OBJECTIVE

- Identify the anthropometric, inflammatory, regulatory, metabolic, and hematologic variables that suffer changes in patients with hyperglycemia compared to normoglycemic ones.
- Identify the associations of anthropometric, inflammatory, regulatory, metabolic, and hematologic variables with iron status indicators and HbA1c in hyperglycemic women.
- Identify the anthropometric, inflammatory, regulatory, metabolic, and hematologic variables that may be involved in the associations between elevated HbA1c levels and decrease iron status indicators in hyperglycemic women.

4. MATERIALS AND METHODS

4.1. Population

In this cross-sectional study, previously approved by the Research Ethics Committee of the Federal University of Uberlândia under registration number 56557516.0.0000.5152, a population consisted of 143 female volunteers – attended at an outpatient clinic of the Clinical Hospital of that institution – was stratified into the Normoglycemic (FPG < 100 mg/dL, n=68) and Hyperglycemic (FPG ≥ 100 mg/dL, n=75) groups.

Among eligible patients, those with type 1 diabetes mellitus, hereditary erythrocytopathies, nephropathies, cognitive impairment, and a history of alcohol

and other drug abuse, were excluded in the study. All procedures were performed after the participants sign the Free and Informed Consent Form. The patients who, after signing the form, decided to abandon the study, regardless of the reason, were excluded.

4.2. Collection of blood samples

After 8-12 hours of overnight fast, blood samples were collected by an intravenous puncture in evacuated tubes (Vacutainer, BD, Juiz de Fora, MG, Brazil), two tubes containing K_3EDTA, to determine the blood count and osmotic stability of erythrocytes, and two of them containing separation gel for biochemical assays.

4.3. Determination of erythrocytes osmotic stability

A duplicate set of microtubes containing 1.0 mL of 0-1 g.dL^{-1} NaCl solutions was pre-incubated in a thermostated water bath at 37 °C (Marconi, model MA 184, Piracicaba, SP, Brazil) for 10 min. After adding freshly collected whole blood aliquots (0-12 hours after collection) to each of the tubes, they were hermetically sealed, gently shaken and incubated for 30 min in a 37 °C. After incubation and centrifugation at 1,600 x *g* for 10 min (Hitachi Koki, model CF15RXII, Hitachinaka, Japan), the supernatant absorbance was read at 540 nm using a UV-VIS spectrophotometer (Hach, model DR 5000, Düsseldorf, Germany) (PENHA-SILVA; FIRMINO; DE FREITAS REIS; DA COSTA HUSS *et al.*, 2007).

The relationship between absorbance at 540 nm (A) and the concentration of NaCl (X) was adjusted to a sigmoidal regression line according to the Boltzmann equation,

$$A = \frac{A_{max} - A_{min}}{1 + e^{(X - H_{50})/dX}} + A_{min} \quad (1),$$

in which A_{min} and A_{max} are the average absorbance values in the minimum and maximum plateaus of the sigmoid and represent, respectively, the initial and maximum hemolysis rate, H_{50} is the concentration of NaCl capable of promoting 50% of hemolysis, and dX is the variation in NaCl concentration associated with ¼

of the total hemolysis (PENHA-SILVA; FIRMINO; DE FREITAS REIS; DA COSTA HUSS *et al.*, 2007).

H_{50} is an osmotic fragility parameter and, therefore, has an inverse relationship with the osmotic stability, but dX is a stability parameter, as well as the dX/H_{50} ratio.

4.4. Red blood cells count and biochemical analytes determination

Routine blood tests were performed at the Clinical Analysis Laboratory of the Clinical Hospital of the Federal University of Uberlândia.

The complete blood count was obtained using an automated system (Sysmex America Inc., model XN 3.000, Kobe, Japan). The reticulocyte (Rtc) count was performed visually and expressed as a percentage of RBC (Rtc index). HbA1c was quantified by high-performance liquid chromatography (HLPC) using a D10 dual HPLC system (Bio-Rad, Hercules, CA, USA).

Plasma concentrations of high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein cholesterol, total cholesterol, triglycerides, glucose, direct and total bilirubin, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and C-reactive protein were determined using specific commercial kits and an automatic analyzer (Cobas 6000, Roche Diagnostics GmBH, Mannheim, Germany).

Insulin was determined using a chemiluminescence enzyme immunoassay kit (Roche Cobas 8000, Roche Diagnostics, Switzerland).

4.5. Anthropometric assessments

All anthropometric measurements were taken according to the standard proposed by the World Health Organization (WHO, 2008). The body weight and height were taken using a balance coupled to a stadiometer with 0.1 kg and 0.1 cm accuracy, respectively (Filizola, São Paulo, SP, Brazil), with the volunteer standing upright, barefoot and wearing light clothing. The body mass index was estimated by the Quetelet Index, which was obtained by dividing the volunteer's body weight, in kg, by her height, in m^2.

4.6. Muscle strength assessment

Muscle strength was estimated by the handgrip strength measurement using an electronic hand dynamometer (Camry Scale Store, model EH 101, City Industry, CA, USA), and given by the value in kg that the volunteer was able to maintain for at least 3 seconds. Two tests with a 1-minute interval were performed, and the greatest handgrip strength was recorded.

4.7. HOMA-IR and HOMA-β assessments

Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) and Homeostasis Model Assessment β Cell Function (HOMA-β) were calculated using the formulas: HOMA-IR = Fasting Insulin × Fasting Plasma Glucose ÷ 22.5, and HOMA-β = 20 × Fasting Insulin ÷ (Fasting Plasma Glucose - 3.5), with fasting insulin and plasma glucose values given in μIU/mL and mmol/L, respectively (DULLAART; ANNEMA; DE BOER; TIETGE, 2012; MATTHEWS; HOSKER; RUDENSKI; NAYLOR *et al.*, 1985).

4.8. Statistical analysis

Erythrocytes stability analyzes used OriginPro 9.0 (Microcal, Northampton, MA, USA). All other analyzes used version 25.0 of the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). Data distribution analysis used the Shapiro Wilk test. The comparison between groups used the Mann-Whitney test. Correlation analysis used the Spearman method. Results with a p-value <0.05 were considered significant in all tests. The obtention of the correlation matrix exhibited in Figure 2 used the CorrPlot package, version 1.2.1335 (RStudio, Boston, MA, USA), based in R version 3.61 (R Foundation for Statistical Computing, Vienna, Austria).

5. RESULTS

Of a total of 150 volunteers who met the eligibility criteria, three withdrew from the study, two had their blood samples lost, and two underwent tests in laboratories without quality certification, leaving a total of 143 volunteers, who were stratified in the Normoglycemic (n=68) and hyperglycemic (n=75) groups.

Table 1 presents the general characteristics of the normoglycemic and hyperglycemic groups in this study. Some of the significant differences deserve mention.

Hyperglycemic women had higher values for body mass index (BMI), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), hemoglobin A1c (HbA1c), triglycerides (TGC), very low-density lipoprotein cholesterol (VLDL-C), C-reactive protein (CRP), direct bilirubin/total bilirubin ratio (DB/TB), total iron-binding capacity (TIBC), red-cell distribution width (RDW), reticulocyte (Rtc) count, erythrocytes osmotic stability (dX and dX/H_{50} ratio), as well as lower values for iron (Fe), transferrin saturation index (TSI), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), low-density lipoprotein cholesterol (LDL-C), and indirect bilirubin/total bilirubin ratio (IB/TB).

The elevated BMI, HOMA-IR, and CRP values express typical characteristics of prediabetes and T2DM, i.e., obesity, insulin resistance, and inflammation.

The lipid profile characterizes by increased TGC and VLDL-C and decreased LDL-C levels.

Decreased iron and TSI values, as well as the increased TIBC values and the reduction in MCH and MCHC, indicate a tendency towards ID in the hyperglycemic population.

The decrease in the unconjugated bilirubin fraction (IB/TB ratio) is compatible with a reduction in the degradation of heme-proteins.

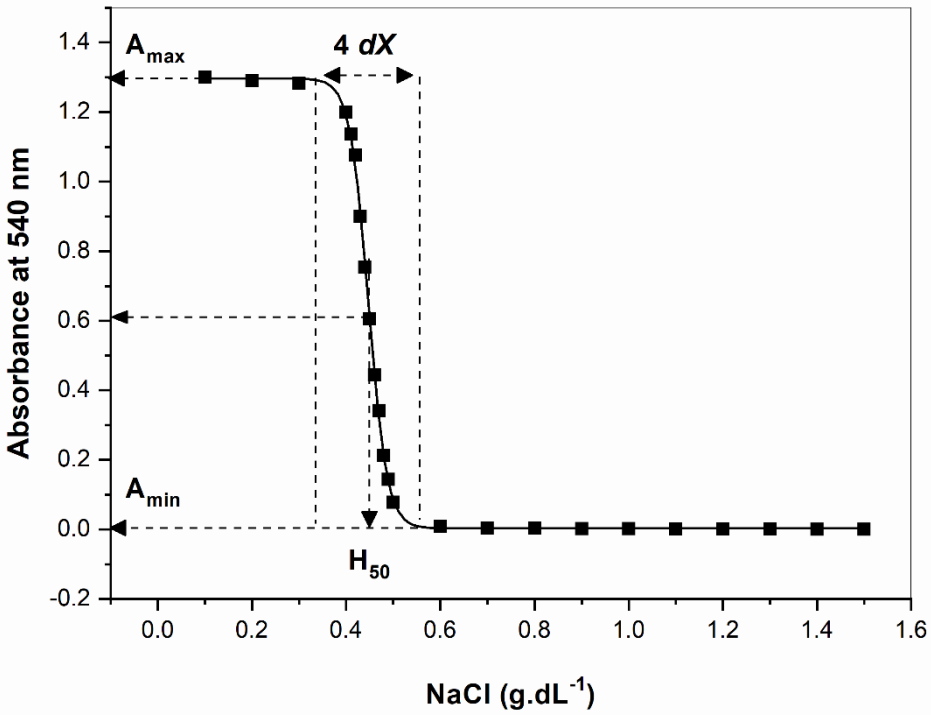
Table 1. Baseline characteristics of the study population

Parameters	Reference Range	Normoglycemics (n = 68)	Hiperglycemics (n = 75)	*p*
Age (years)	-	59 (14)	62 (12)	0.037
Weight (kg)	-	65 (10)	75 (20.1)	<0.001
Height (cm)	-	158 (9)	158 (8)	0.742
Body Mass Index (kg/m^2)	18.5 – 24.9	26.04 (4.58)	30.1 (6.69)	<0.001
Waist Circumference (cm)	< 80	88.25 (12.5)	99 (17)	<0.001
Hip Circumference (cm)	< 0.85	99.5 (8)	105.5 (11.5)	0.001
Waist to Hip Ratio	< 0.7	0.87 (0.09)	0.93 (0.07)	<0.001
Arm Circumference (cm)		29 (3)	31 (5)	0.001
Time After Diagnosis (years)		0 (0)	1.5 (9)	
Force (kg)	> 24	24.25 (6.05)	23.1 (5.8)	0.105
Insulin (μU/mL)	2 - 23	7.33 (5.23)	16.03 (11.62)	<0.001
HOMA-IR	< 2.15	1.6 (1.24)	4.81 (4.56)	<0.001
HOMA-β	167 e 175	98.71 (68.75)	97.76 (95.68)	0.809
Glucose (mg/dL)	≤ 99	92.8 (6.35)	112.1 (35)	<0.001
HbA1c (%)	≤ 5.6	5.55 (0.5)	6.55 (1.65)	<0.001

Triglycerides (mg/dL)	< 150	106.6 (63.45)	145.3 (71.3)	0.003
Total Cholesterol (mg/dL)	< 200	189.3 (49.05)	179.45 (48)	0.122
HDL-Cholesterol (mg/dL)	> 55	55.5 (20.6)	52 (17)	0.310
LDL-Cholesterol (mg/dL)	< 100	108 (43)	91 (51)	0.012
VLDL-Cholesterol (mg/dL)	< 30	21 (12)	29 (13.5)	0.004
AST (U/L)	5 - 40	18.1 (5.8)	18.6 (8.55)	0.738
ALT (U/L)	0 - 50	15.9 (9.5)	17.65 (12.85)	0.047
LDH (UI/L)	120 - 246	182.5 (46)	187 (43)	0.761
C-Reactive Protein (mg/L)	5	2 (2.35)	3.6 (5.9)	0.002
Creatinine (mg/dL)	0.5 - 1.1	0.8 (0.18)	0.78 (0.15)	0.563
Urea (mg/dL)	13 - 43	28.3 (10.65)	29.8 (13.1)	0.617
Uric Acid (mg/dL)	6	4.25 (1.55)	5.25 (2)	<0.001
Direct Bilirubin (mg/dL)	0.1 - 0.4	0.16 (0.09)	0.18 (0.08)	0.172
Indirect Bilirubin (mg/dL)	0.1 - 0.7	0.28 (0.16)	0.28 (0.19)	0.502
Total Bilirubin (mg/dL)	0.2 - 1.1	0.44 (0.23)	0.44 (0.21)	0.805
Direct Bilirubin/Total Bilirubin		0.35 (0.07)	0.39 (0.08)	<0.001
Indirect Bilirubin/Total Bilirubin		0.65 (0.06)	0.61 (0.08)	<0.001
Iron (mmol/L)	11 - 25	17.2 (5.77)	16.15 (7.33)	0.032
Ferritin (ng/mL)	11 - 306	138.5 (158.56)	118.65 (189.21)	0.552
TSI (%)	20 - 55	36.01 (14.04)	28.86 (14)	0.003
TIBC (mcg/dL)	250 – 425	291.04 (66.3)	301.42 (65.07)	0.024
Vitamin B9 (ng/mL)	> 5.4	11.86 (7.15)	11.61 (5.44)	0.817
Vitamin B12 (pg/mL)	211 - 911	387.6 (222.4)	399.55 (241.7)	0.792
Vitamin D (ng/mL)	> 30	27.34 (10.7)	26.25 (10.4)	0.155
Hemoglobin (g/dL)	12 - 16	13.5 (1.35)	13.4 (1.4)	0.559
Hematocrit (%)	36.0 - 47.0	39.4 (4.25)	39.2 (4)	0.819
RBC (million/mm^3)	4.00 - 5.60	4.6 (0.5)	4.6 (0.5)	0.812
MCV (fL)	82.0 - 94.0	86.3 (5.1)	86.8 (5.8)	0.598
MCH (pg)	27.0 - 33.0	30 (1.8)	29.4 (2)	0.010
MCHC (mmol/L)	32.0 - 37.0	34.55 (1.35)	33.9 (1.4)	0.010
RDW (%)	11.0 - 16.5	12.7 (0.9)	13.1 (1.1)	0.015
Reticulocyte Index (%)	0.5 – 1.5	1 (0.9)	1.2 (1)	0.049
Leucocyte Count (10^9 cells/L)	4.5 - 10.0	5.65 (1.75)	6.3 (2.3)	0.003
Lymphocyte Percentage	20 - 40	34 (12.3)	34 (10.4)	0.742
Monocytes Percentage	3 - 10	7.3 (3.4)	7.4 (2.5)	0.956
Platelet Count (10^9 cells/L)	150 - 400	239 (71)	246 (66)	0.632
Mean Platelet Volume (fL)	8.14 - 12.24	10.3 (1.1)	10.3 (1.9)	0.402
A_{min} (ΔOD)	-	0 (0.01)	0 (0.01)	0.791
A_{max} (ΔOD)	-	1.16 (0.12)	1.14 (0.17)	0.808
H_{50} (g/dL NaCl)	-	0.45 (0.03)	0.44 (0.03)	0.238
dX (g/dL NaCl)	-	0.01 (0)	0.02 (0.01)	0.005
dX/H_{50} (g/dL NaCl)	-	0.03 (0.01)	0.04 (0.02)	0.002
dX/Amin (g/dL NaCl)	-	1.14 (5.17)	1.68 (3.3)	0.255

Abbreviations: HOMA-IR, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR); HOMA-β, Homeostasis Model Assessment β Cell Function; HbA1c, glycated hemoglobin A1c; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; ALT, alanine amino transferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TSI, transferrin saturation index; TIBC, total iron binding capacity; DB, Direct Bilirrubin; IB, Indirect Bilirrubin; TB, Total Bilirrubin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; A_{min}, absorbance at 540 nm associated with residual lysis of the erythrocytes population; A_{max}, absorbance at 540 nm associated with lysis of the whole population of erythrocytes; H_{50}, saline concentration capable of promoting 50% hemolysis; dX, variation in the concentration of NaCl responsible for total hemolysis.

* $p<0.05$ indicates statistically significant difference. Values are presented as median (interquartile range) and comparisons between groups were done using the Mann- Whitney test.



The increased dX and dX/H50 mean greater erythrocyte osmotic stability in the hyperglycemic group. A typical osmotic fragility curve of a volunteer study (Fig. 1) shows the meanings of those osmotic stability parameters.

Figure 1. Sigmoidal fitting of a typical curve of hypotonic hemolysis. H_{50} is the NaCl concentration which promotes 50% hemolysis; dX represents 1/4 of the decline in the NaCl concentration responsible for the transition between integer (A_{min}) and lysed cells (A_{max}).

Figure 2 shows the results of the correlation analysis in the hyperglycemic group. Some results deserve mention.

Increased CRP correlated with increased BMI and HOMA-IR, decreased iron and IB/TB, and increased dX and HbA1c. These correlations link inflammatory status to obesity, insulin resistance, a tendency towards ID, and enhanced lifespan, osmotic stability, and HbA1c content in erythrocytes, respectively.

Increased HbA1c, in addition to the association with enhanced CRP, correlated with increased HOMA-IR and decreased iron, TSI, MCH, MCHC, and Amax (iron status markers). These correlations link the high levels of glycated hemoglobin to inflammatory status, insulin resistance, and tendency towards ID, respectively.

The correlation of reduced MCH with reduced IB/TB associates decreased red-cell hemoglobin content with decreased heme-proteins degradation. Since MCH refers specifically to hemoglobin, it is clear that hemoglobin is at least a substantial part of these heme-proteins.

Moreover, the correlations of reduced TSI with reduced values of DB, IB, and TB associate decreased TSI with decreased degradation of heme-proteins.

The correlations of decreased LDL-C with decreased IB/TB and iron status markers (iron, Hb, and Ht) associate the decline in LDL-C with such decreased degradation of heme-proteins and the tendency towards ID.

The correlations of increased RDW with reduced MCHC and increased dX associates increased volume variability with decreased hemoglobin content and increased osmotic stability of erythrocytes, respectively.

The correlations of increased dX with enhanced RDW and decreased iron status markers (Hb, MCH, MCHC, and Ht) associate increased erythrocytes stability with increased volume variability and tendency towards ID, respectively.

Lastly, the correlations of enhanced HOMA-β values with increased iron status markers (iron and TSI), as well as with Rtc count, mean that preservation of insulin secretion is linked to better iron homeostasis and the production of new red blood cells.

Figure 2. Spearman rho coefficients for the correlations of the hyperglycemic group.

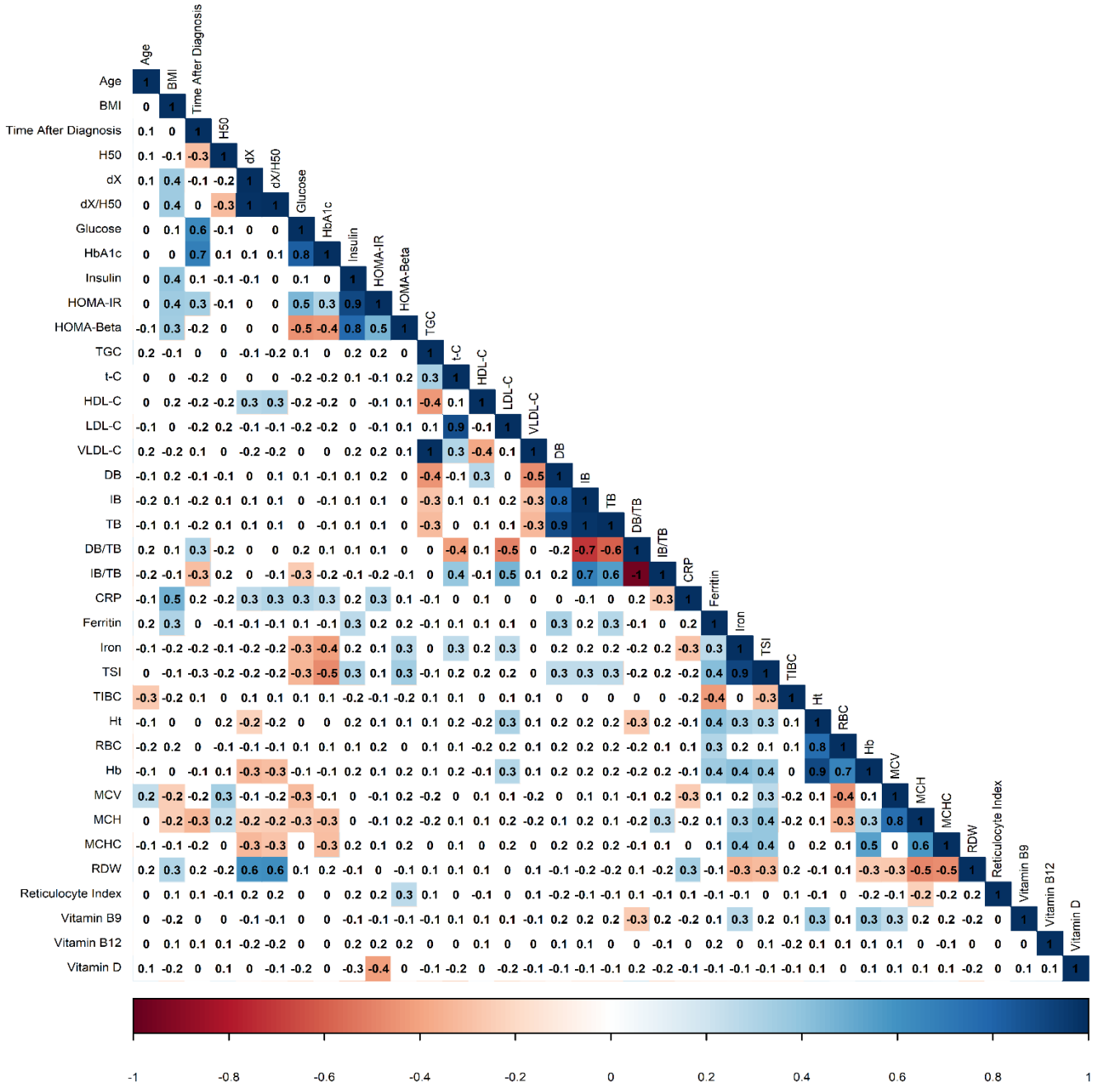


Figure 3 summarizes the main changes and correlations observed in the hyperglycemic group.

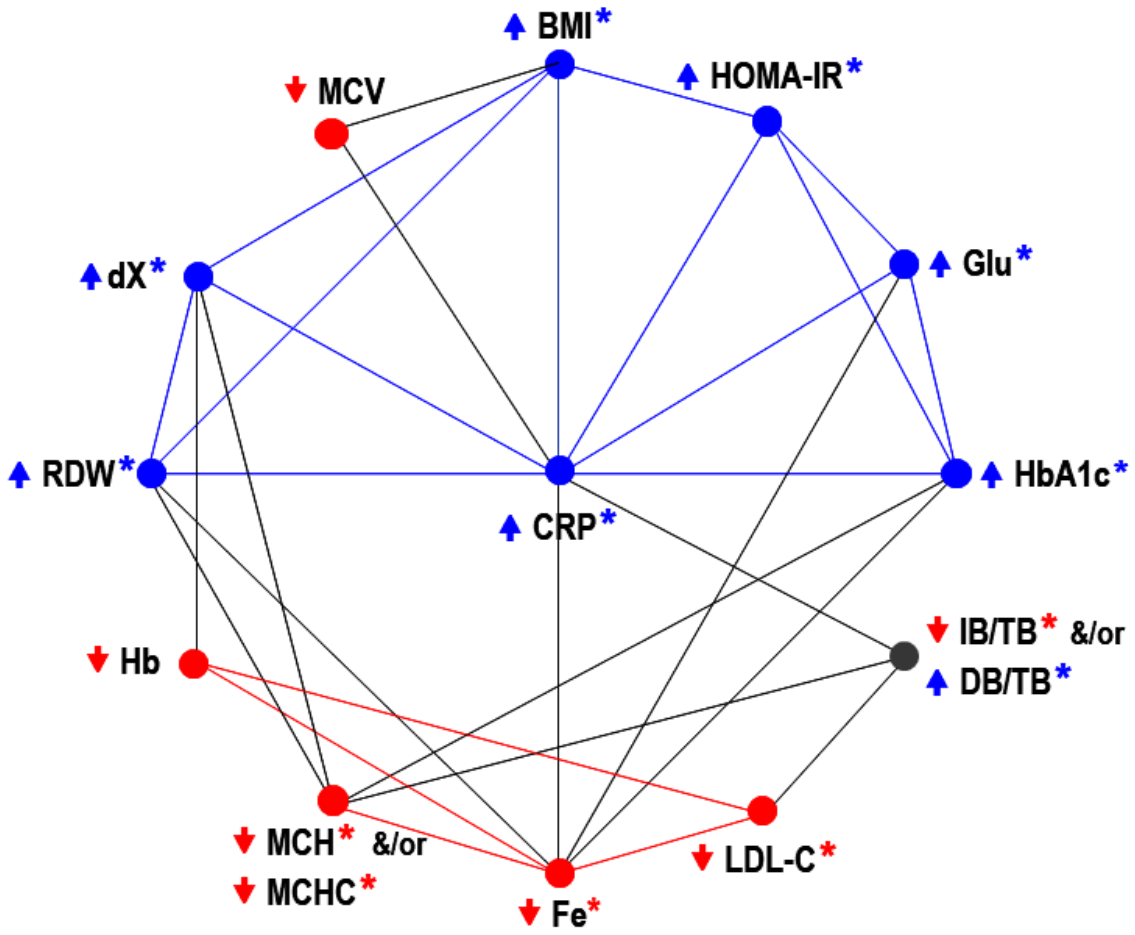


Figure 4. Map of significant ($p<0.05$) Spearman correlations, indicated by lines between variables which values were significantly (*) higher (↑) or lower (↓) in the hyperglycemic group than in the control group. Abbreviations: HOMA-IR, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR); HbA1c, glycated hemoglobin A1c; LDL-C, low-density lipoprotein cholesterol; Fe, iron; DB, Direct Bilirubin; IB, Indirect Bilirubin; TB, Total Bilirubin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; dX, variation in the concentration of NaCl responsible for total hemolysis.

6. DISCUSSION

In this study, increased HbA1c correlated with decreased iron, TSI, MCH, and MCHC, in the hyperglycemic group, which had lower iron levels and TSI concomitantly with higher values of HbA1c and TIBC compared to normoglycemic women. These findings mean that HbA1c increases with a decrease in the levels of iron status indicators, even without ID.

Despite the existence of controversies, with associations of ID anemia with low HbA1c levels and normalization after iron replacement therapy (CETINKAYA ALTUNTAS; EVRAN; GURKAN; SERT *et al.*, 2020), associations of lower serum iron values with higher levels of HbA1c in individuals without and with diabetes have been found in several investigations (BROOKS; METCALFE; DAY; EDWARDS, 1980; COBAN; OZDOGAN; TIMURAGAOGLU, 2004; EL-AGOUZA; ABU SHAHLA; SIRDAH, 2002; HASHIMOTO; OSUGI; NOGUCHI; MORIMOTO *et al.*, 2010; KOGA; MORITA; SAITO; MUKAI *et al.*, 2007; MADHU; RAJ; GUPTA; GIRI *et al.*, 2017; TARIM; KUCUKERDOGAN; GUNAY; ERALP *et al.*, 1999), and even been addressed in a systematic review (ENGLISH; IDRIS; SMITH; DHATARIYA *et al.*, 2015).

In the study by Madhu et al., the HbA1c levels of individuals with ID anemia (n=62) were higher than in the control group (n=60) and correlated negatively with ferritin, Hb, RBC, MCH, and MCHC before supplementation with iron, but declined after the replacement therapy (MADHU; RAJ; GUPTA; GIRI *et al.*, 2017). Likewise, Coban et al. observed that HbA1c levels were higher in individuals without diabetes and with ID anemia compared with the control group and fell after oral administration of iron (COBAN; OZDOGAN; TIMURAGAOGLU, 2004). In the study by Tarim et al., HbA1c was high and associated with ID anemia in pediatric patients with and without T1DM, but declined after iron replacement therapy in both groups, indicating that nutritional iron deficiencies can influence HbA1c values, regardless of whether the patient has diabetes or not (TARIM; KUCUKERDOGAN; GUNAY; ERALP *et al.*, 1999).

Even in premenopausal women, regardless of glucose blood levels, negative correlations between HbA1c and iron, MCV, and MCH have also been

reported, even when ID anemia had not yet been detected (KOGA; MORITA; SAITO; MUKAI *et al.*, 2007).

This evidence indicates that the HbA1c levels overestimate glycemia in populations with a tendency towards ID, and this is worrying, especially in females with diabetes, which are very prone to ID.

Although this issue is known, its mechanism remains unclear (AHMAD; RAFAT, 2013), which deserves consideration, especially in hyperglycemic women populations.

Anyhow, understanding this mechanism involves erythrocyte homeostasis since only HbA1c and not glycated albumin is affected by decreased iron levels (HASHIMOTO; OSUGI; NOGUCHI; MORIMOTO *et al.*, 2010). Nevertheless, this mechanism has to consider other factors such as obesity, which is related to anemia, regardless of diabetes, and with diabetes, regardless of anemia (ADIB RAD; SEFIDGAR; TAMADONI; SEDAGHAT *et al.*, 2019; AIGNER; FELDMAN; DATZ, 2014). Indeed, obesity is a factor associated with the tendency towards ID, since obesity-associated inflammation involves impaired duodenal iron absorption and low expression of duodenal ferroportin and elevated hepcidin levels (AIGNER; FELDMAN; DATZ, 2014).

In the hyperglycemic group of this study, increased BMI correlated with enhanced CRP, which associates obesity with inflammation, and with decreased MCV and MCH, and increased RDW, which associates obesity with a tendency towards ID. Since these correlations were in the hyperglycemic population, such a tendency towards ID could also be linked to diabetes and not specifically to obesity. However, the correlations of enhanced BMI with decreased Hb ($\rho=-0.3$, $p<0.05$) and increased RDW ($\rho=0.4$, $p<0.05$) in the normoglycemic group, reinforce the existence of an association between obesity and tendency towards ID, regardless of diabetes. Nevertheless, indeed, the correlations of enhanced BMI with increased HOMA-IR in both normoglycemic ($\rho=0.4$, $p<0.05$) and hyperglycemic groups show the well-known associations of obesity with the tendency to hyperglycemia and the severity of this condition, respectively.

Although BMI elevation was not associated directly with increased HbA1c, the CRP elevation, in its turn, was correlated directly with enhanced BMI, as previously mentioned, and with increased HOMA-IR, Glu, decreased iron, MCV,

and IB/TB, and increased dX, RDW, and HbA1c. These correlations mean that the inflammation links obesity with insulin resistance, hyperglycemia, a tendency to ID and microcytosis, decreased degradation of heme-proteins, and increased osmotic stability, volume variability, and HbA1c content of erythrocytes, respectively. In other words, inflammation represents a mechanism by which obesity affects metabolic processes and iron homeostasis in hyperglycemia.

It is important to note that those changes and relationships are present in a population that does not have ID anemia, which is characterized by a decrease in iron status indicators, as Hb, below the reference values. Since iron stores are used slowly in a subclinical ID, hematologic indices such as MCV, MCH, and MCHC, begin to decline but may remain within their reference ranges, at least in the initial phase of the process (SUOMINEN; PUNNONEN; RAJAMAKI; IRJALA, 1998). Indeed, as expected for a chronic degenerative condition, the fasting glycemia and HbA1c levels increased with the time after disease diagnosis, which, in turn, correlated with decreased MCH values.

The dX increase in the hyperglycemic group is associated with decreased cell hemoglobin content. Erythrocytes with lower volume and lower hemoglobin content receive less water inflow in a hypoosmolar medium and, therefore, remain intact in this environment (DE FREITAS; DA COSTA; MEDEIROS; CUNHA *et al.*, 2019; MOHANDAS; CLARK; JACOBS; SHOEHET, 1980; MOHANDAS; EVANS, 1994; MOHANDAS; GALLAGHER, 2008). The correlation of enhanced dX with decreased Hb, MCHC, and Ht, and enhanced RDW, expresses the osmotic stability increase with reduced hemoglobin concentration and means that the elevation in the stability of erythrocytes is associated with the tendency towards ID and increased volume variability. Indeed, MCHC was lower in the hyperglycemic group, but MCV was not smaller, and neither showed a negative correlation with dX in this group. Certainly, MCV was not smaller, because it represents the composition of an average population of smaller and larger cells, resulting in increased volume variability. Indeed, the hyperglycemic group had higher Rtc counts and RDW values.

Such a dX increase, although detected *in vitro*, must mean an elevation in the stability of erythrocytes also in the bloodstream, since cells with smaller

volume and lower hemoglobin content are mechanically more stable and better support the shear stress in the blood vessels (MOHANDAS; EVANS, 1994).

A combination of increased stability with decreased content and degradation of hemoglobin, in the face of higher concentrations of glucose, would favor the production of HbA1c (COBAN; OZDOGAN; TIMURAGAOGLU, 2004; EL-AGOUZA; ABU SHAHLA; SIRDAH, 2002; SLUITER; VAN ESSEN; REITSMA; DOORENBOS, 1980). Indeed, in the hyperglycemic group, which presented higher dX, and lower MCHC and IB/TB ratio, decreased MCHC correlated enhanced HbA1c.

Although a decrease in the IB/TB ratio represents a decrease in the general catabolism of heme-proteins, the correlation of decreased IB/TB with decreased MCH shows that hemoglobin is the heme-protein that is determining the change in the fraction of unconjugated bilirubin of the hyperglycemic group, which indeed presented smaller values of IB/TB, MCH, and MCHC. Since 70-90% of blood bilirubin comes from the removal of senescent erythrocytes from the bloodstream and early breakdown of hemoglobin in the bone marrow (NGASHANGVA; BACHU; GOSWAMI, 2019; WESTWOOD, 1991), such a reduction in the unconjugated bilirubin fraction shall indeed reflect decreased catabolism of hemoglobin.

In addition to the association of decreased intracellular hemoglobin concentration with erythrocyte stability, the possible associations of glycemia (LEMOS; MARQUEZ-BERNARDES; ARVELOS; PARAISO *et al.*, 2011) and lipidemia (CAZZOLA; RONDANELLI; RUSSO-VOLPE; FERRARI *et al.*, 2004; CAZZOLA; RONDANELLI; TROTTI; CESTARO, 2011) also deserve consideration.

Hyperglycemia enhances plasma osmolarity, which increases red blood cell stability (LEMOS; MARQUEZ-BERNARDES; ARVELOS; PARAISO *et al.*, 2011). However, it also favors the glycation of hemoglobin and membrane proteins of red cells, which decreases their stability and deformability (KUNG; TSENG; WANG, 2009). Higher glycation levels can change the Hb structure, increasing its aggregation and, consequently, reducing cell deformability (YE; RUAN; YONG; SHEN *et al.*, 2016). Furthermore, even small modifications in the erythrocyte membrane can affect the viscoelastic changes necessary for its passage through

small-diameter capillaries in the body (MOHANDAS; GALLAGHER, 2008). Less deformable erythrocytes are mechanically more fragile (MOHANDAS; CLARK; JACOBS; SHOEHET, 1980), especially in the presence of oxidative stress (MCNAMEE; HOROBIN; TANSLEY; SIMMONDS, 2018) and increased blood pressure (DE FREITAS; DA COSTA; MEDEIROS; CUNHA *et al.*, 2019). Thus, this requires the removal of the damaged cells and acceleration of erythropoiesis.

Although hyperglycemia and diabetes effects in erythrocytes' homeostasis are indeed different, they both are equally controversial. In diabetes, there is mention of a decrease (HUANG; LIU; MAO; CHEN *et al.*, 2018), absence of interference (COHEN; FRANCO; KHERA; SMITH *et al.*, 2008), and even an increase (WU; LIN; GAO; LI *et al.*, 2017) in erythrocytes' lifespan. These differences should not necessarily signify mistakes in one study over another, but rather the complexity of the natural history of diabetes and, indeed, the even greater complexity of the natural history of hyperglycemia in the initiation and progression of diabetes. In this study, the increase in time after diagnosis correlated with enhanced blood glucose and HbA1c levels, as expected, but also with a decline in the IB/TB ratio, which expresses the occurrence of decreased hemoglobin degradation only later in the disease timeline. In addition to the relationship that the decrease in hemoglobin degradation has with the decrease in the intracellular concentration of this protein, an increase in the erythrocyte lifespan could also justify the decrease in the unconjugated bilirubin fraction. Indeed, an extension of erythrocytes' lifespan is considered a cause of increased HbA1c levels (GALLAGHER; LE ROITH; BLOOMGARDEN, 2009), even in healthy individuals (COHEN; FRANCO; KHERA; SMITH *et al.*, 2008). Thus, the higher HbA1c variability in the hyperglycemic group may also reflect in some level the erythrocytes lifespan heterogeneity in this group.

The association of blood lipids with erythrocytes properties is an issue as complex as the evolution of lipidemia in the natural history of T2DM. For this reason, a more objective focus on the present situation of the studied population is necessary. The lower LDL-C levels and higher levels of VLDL-C and TGC in the hyperglycemic group seem to be linked to the development of ID, regardless of T2DM. Indeed, the studies by Verma et al. (VERMA; SHANKAR; MADHU; TANDON *et al.*, 2010) and by Antappanavar et al. (ANTAPPANAVAR; BIRADAR;

PATIL; BIRADAR *et al.*, 2014) also reported decreased LDL-C and increased VLDL-C and TGC in Indian patients without diabetes and with ID anemia when compared to the control group. However, the positive correlations of LDL-C with iron, Hb, and Ht, and between t-C and iron (Fig. 2) observed in the hyperglycemic group of this study reveal an association of cholesterol decline with the tendency towards ID anemia also in the hyperglycemic population. Since Choi et al. (CHOI; KIM; PAI, 2001) demonstrated that t-C was also positively correlated with Hb in patients with ID anemia in general, this means that the decline in cholesterolemia is a condition associated with the development and worsening of anemia itself, regardless of its link with diabetes. Indeed, in the absence of anemia and hyperglycemia (normoglycemic group), the cholesterol levels did not show significant correlations with iron status indicators (data not shown).

Although such a decrease in LDL-C may have a protective effect against atherosclerosis, especially in postmenopausal women, in the hyperglycemic population of this study, it certainly has other implications, since it happened concomitantly with the tendency towards ID.

One of these implications would be worsening liver function associated with increased ALT and AST in this population. As these biomarkers were within their normal reference ranges, and the conjugated bilirubin fraction, given by DB/TB, was also higher in that group, a worsening liver function does not seem to be associated with decreased LDL-C.

Another implication of the decreased LDL-C would be the increased demand for this lipid in the production or maintenance of erythrocytes. Indeed, the hyperglycemic group had an increase in the Rtc index compared to the control group of this study. Furthermore, the positive correlations of LDL-C with iron, Hb, and Ht values may reflect the cholesterol consumption in the maintenance of the erythrocytes in the circulation. Indeed, the positive correlation of LDL-C with the unconjugated bilirubin fraction associates decreased LDL-C with decreased catabolism of hemoglobin. This association suggests that LDL-C consumption is involved in the mechanism by which the organism tries to counteract the tendency towards ID. This mechanism can be an increase in the cholesterol content of the erythrocyte membrane. Indeed, the RDW increase, which correlated with decreased MCHC in this study, was associated with increased cholesterol content

in the RBC membrane by Tziakas et al. (TZIAKAS; CHALIKIAS; GRAPSA; GIOKA *et al.*, 2012).

Although preserving LDL-C levels seems to protect hyperglycemic individuals from developing ID, it is necessary to highlight that exaggeratedly high LDL-C levels can cause hemolytic anemia and morphological atypias (COOPER; ARNER; WILEY; SHATTIL, 1975). However, this is not the case in this study.

Since the levels of vitamins B9 and B12 were not different in the hyperglycemic group, this means that the hematologic changes observed in this group were not due to nutritional deficiencies of these vitamins. However, the positive correlations of vitamin B9 with MCV and Ht shows the influence of that vitamin on these variables.

As increased age correlated with decreased TIBC, both in the hyperglycemic group and the control group (ρ=-0.3, $p<0.05$), this means that age is also associated with changes in iron homeostasis. Indeed, aging has a modest influence on HbA1c levels (NUTTALL, 1999), which is sufficient to compromise the efficiency of HbA1c as a diagnostic criterion for diabetes (WU; LIN; GAO; LI *et al.*, 2017).

As far as we know, this is the first study to collectively assess the relationships among iron status, lipid profile, erythrogram, erythrocyte stability, and HbA1c levels. The dosage of vitamins whose deficiencies can cause anemia, and the sample size, which was even more extensive than those of other studies that analyzed the relationships between iron and HbA1c, are also strengths of this study (COBAN; OZDOGAN; TIMURAGAOGLU, 2004; MADHU; RAJ; GUPTA; GIRI *et al.*, 2017; TARIM; KUCUKERDOGAN; GUNAY; ERALP *et al.*, 1999).

Unfortunately, we did not analyze oxidative stress markers, which should be part of the mechanisms set that associate obesity, inflammation, diabetes, iron status indicators, HbA1c, and erythrocyte properties. Such analyses would be relevant since erythrocytes are essential in the blood antioxidant system, although they are very prone to oxidative stress. Indeed, oxidative stress has been associated with anemia in diabetes without nephropathy (WAGGIALLAH; ALZOHAIRY, 2011), since it is involved in the vascular complications of the disease (RODRIGUES; DE MEDEIROS; CUNHA; GARROTE-FILHO *et al.*, 2018)

and the erythrocytes vulnerability to shear stress damage (MCNAMEE; HOROBIN; TANSLEY; SIMMONDS, 2018).

The exclusively female composition of the study sample is an interesting point because, historically, many experimental and clinical studies exclude females due to their hormonal fluctuations. (MAZURE; JONES, 2015). Furthermore, the correct interpretation of the results requires separation of the population by sex, due to the sex difference in the reference values, especially for the hematologic variables (ROY; SNYDER; STEPHENS-SHIELDS; ARTZ *et al.*, 2017).

In this study, in summary (Fig. 3), hyperglycemic women had decreased iron, MCH, MCHC, LDL-C, and IB/TB, as well as increased BMI, CRP, HOMA-IR, RDW, dX, and HbA1c. Elevated HbA1c was linked to inflammatory status, insulin resistance, and tendency towards ID since increased HbA1c correlated with enhanced CRP, and HOMA-IR, and decreased iron status markers (iron, TSI, MCH, and MCHC), respectively. Inflammatory status was also associated with obesity, insulin resistance, hyperglycemia, a tendency towards ID and microcytosis, decreased hemoglobin catabolism, and increased osmotic stability and volume variability of erythrocytes, since elevated CRP correlated with enhanced BMI, HOMA-IR, and Glu, decreased iron, MCV, and IB/TB, and increased dX, and RDW, respectively. Decreased hemoglobin catabolism was associated with decreased LDL-cholesterol and hemoglobin concentration since reduced IB/TB correlated with decreased LDL-C and MCHC. Such a hemoglobin concentration decrease was associated with increased volume variability, osmotic stability, and glycated hemoglobin in erythrocytes, since reduced MCH or MCHC correlated with increased RDW, dX, and HbA1c. Moreover, preservation of insulin secretion was linked to better iron homeostasis and the production of new red blood cells, since enhanced HOMA-β correlated with increased iron status markers (iron and TSI), as well as with Rtc count.

7. CONCLUSION

In hyperglycemic women, decreased iron associates directly with increased HbA1c. Moreover, these changes are both also associated with decreased intracellular hemoglobin concentration (MCHC), which in turn, involves enhanced osmotic stability (dX) and volume variability (RDW) of erythrocytes, as well as decreased catabolism of heme (decreased IB/TB ratio). Such decreased catabolism of heme does not seem to be solely a process associated with diminished levels of hemoglobin since it is associated with inflammatory status and decreased LDL-cholesterol.

Since decreased iron and increased HbA1c are both linked to obesity-associated inflammation, a balanced diet and exercise could contribute to neutralizing the iron levels' influence on the HbA1c concentrations.

8. REFERENCES

ADIB RAD, H.; SEFIDGAR, S. A. A.; TAMADONI, A.; SEDAGHAT, S. *et al.* Obesity and iron-deficiency anemia in women of reproductive age in northern Iran. **J Educ Health Promot**, 8, p. 115, 2019. DOI: 10.4103/jehp.jehp_371_18.

AHMAD, J.; RAFAT, D. HbA1c and iron deficiency: a review. **Diabetes Metab Syndr**, 7, n. 2, p. 118-122, Apr-Jun 2013. DOI: 10.1016/j.dsx.2013.02.004.

AIGNER, E.; FELDMAN, A.; DATZ, C. Obesity as an emerging risk factor for iron deficiency. **Nutrients**, 6, n. 9, p. 3587-3600, Sep 11 2014. DOI: 10.3390/nu6093587.

AIRES RODRIGUES DE FREITAS, M.; VIEIRA DA COSTA, A.; ALVES DE MEDEIROS, L.; DA SILVA GARROTE FILHO, M. *et al.* Are There Differences in the Anthropometric, Hemodynamic, Hematologic, and Biochemical Profiles between Late- and Early-Onset Preeclampsia? **Obstet Gynecol Int**, 2018, p. 9628726, 2018. DOI: 10.1155/2018/9628726.

ALDALLAL, S. M.; JENA, N. Prevalence of Anemia in Type 2 Diabetic Patients. **J Hematol**, 7, n. 2, p. 57-61, May 2018. DOI: 10.14740/jh411w.

AMERICAN DIABETES, A. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2020. **Diabetes Care**, 43, n. Suppl 1, p. S14-S31, Jan 2020. DOI: 10.2337/dc20-S002.

ANTAPPANAVAR, V. B.; BIRADAR, S. G.; PATIL, V.; BIRADAR, P. M. *et al.* A study of correlation between iron deficiency anaemia and serum lipid profile in Indian adults in BRIMS, Bidar. 1, n. 2, p. 96, 2014. DOI: 10.5455/2349-3933.ijam20140811.

ASSOCIATION, A. D. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. **Diabetes Care**, 41, n. Suppl 1, p. S13-s27, Jan 2018. DOI: 10.2337/dc18-S002.

BOURI, S.; MARTIN, J. Investigation of iron deficiency anaemia. **Clin Med (Lond)**, 18, n. 3, p. 242-244, Jun 2018. DOI: 10.7861/clinmedicine.18-3-242.

BROOKS, A. P.; METCALFE, J.; DAY, J. L.; EDWARDS, M. S. Iron deficiency and glycosylated haemoglobin A. **Lancet**, 2, n. 8186, p. 141, Jul 19 1980. DOI: 10.1016/s0140-6736(80)90019-7.

CAMASCHELLA, C. Iron-deficiency anemia. **N Engl J Med**, 372, n. 19, p. 1832-1843, May 7 2015. DOI: 10.1056/NEJMra1401038.

CAZZOLA, R.; RONDANELLI, M.; RUSSO-VOLPE, S.; FERRARI, E. *et al.* Decreased membrane fluidity and altered susceptibility to peroxidation and lipid

composition in overweight and obese female erythrocytes. **J Lipid Res**, 45, n. 10, p. 1846-1851, Oct 2004. DOI: 10.1194/jlr.M300509-JLR200.

CAZZOLA, R.; RONDANELLI, M.; TROTTI, R.; CESTARO, B. Effects of weight loss on erythrocyte membrane composition and fluidity in overweight and moderately obese women. **J Nutr Biochem**, 22, n. 4, p. 388-392, Apr 2011. DOI: 10.1016/j.jnutbio.2010.03.007.

CETINKAYA ALTUNTAS, S.; EVRAN, M.; GURKAN, E.; SERT, M. *et al.* HbA1c level decreases in iron deficiency anemia. **Wien Klin Wochenschr**, May 6 2020. DOI: 10.1007/s00508-020-01661-6.

CHOI, J. W.; KIM, S. K.; PAI, S. H. Changes in serum lipid concentrations during iron depletion and after iron supplementation. **Ann Clin Lab Sci**, 31, n. 2, p. 151-156, Apr 2001.

COBAN, E.; OZDOGAN, M.; TIMURAGAOGLU, A. Effect of iron deficiency anemia on the levels of hemoglobin A1c in nondiabetic patients. **Acta Haematol**, 112, n. 3, p. 126-128, 2004. DOI: 10.1159/000079722.

COHEN, R. M.; FRANCO, R. S.; KHERA, P. K.; SMITH, E. P. *et al.* Red cell life span heterogeneity in hematologically normal people is sufficient to alter HbA1c. **Blood**, 112, n. 10, p. 4284-4291, Nov 15 2008. DOI: 10.1182/blood-2008-04-154112.

COOPER, R. A.; ARNER, E. C.; WILEY, J. S.; SHATTIL, S. J. Modification of red cell membrane structure by cholesterol-rich lipid dispersions. A model for the primary spur cell defect. **J Clin Invest**, 55, n. 1, p. 115-126, Jan 1975. DOI: 10.1172/JCI107901.

DE ARVELOS, L. R.; ROCHA, V. C.; FELIX, G. P.; DA CUNHA, C. C. *et al.* Bivariate and multivariate analyses of the influence of blood variables of patients submitted to Roux-en-Y gastric bypass on the stability of erythrocyte membrane against the chaotropic action of ethanol. **J Membr Biol**, 246, n. 3, p. 231-242, Mar 2013. DOI: 10.1007/s00232-013-9524-0.

DE FREITAS, M. A. R.; DA COSTA, A. V.; MEDEIROS, L. A.; CUNHA, L. M. *et al.* The role of the erythrocyte in the outcome of pregnancy with preeclampsia. **PLoS One**, 14, n. 3, p. e0212763, 2019. DOI: 10.1371/journal.pone.0212763.

DULLAART, R. P.; ANNEMA, W.; DE BOER, J. F.; TIETGE, U. J. Pancreatic beta-cell function relates positively to HDL functionality in well-controlled type 2 diabetes mellitus. **Atherosclerosis**, 222, n. 2, p. 567-573, Jun 2012. DOI: 10.1016/j.atherosclerosis.2012.03.037.

EL-AGOUZA, I.; ABU SHAHLA, A.; SIRDAH, M. The effect of iron deficiency anaemia on the levels of haemoglobin subtypes: possible consequences for clinical diagnosis. **Clin Lab Haematol**, 24, n. 5, p. 285-289, Oct 2002. DOI: 10.1046/j.1365-2257.2002.00464.x.

ENGLISH, E.; IDRIS, I.; SMITH, G.; DHATARIYA, K. *et al.* The effect of anaemia and abnormalities of erythrocyte indices on HbA1c analysis: a systematic review. **Diabetologia**, 58, n. 7, p. 1409-1421, Jul 2015. DOI: 10.1007/s00125-015-3599-3.

FESSLER, M. B.; ROSE, K.; ZHANG, Y.; JARAMILLO, R. *et al.* Relationship between serum cholesterol and indices of erythrocytes and platelets in the US population. **J Lipid Res**, 54, n. 11, p. 3177-3188, Nov 2013. DOI: 10.1194/jlr.P037614.

GALLAGHER, E. J.; LE ROITH, D.; BLOOMGARDEN, Z. Review of hemoglobin A(1c) in the management of diabetes. **J Diabetes**, 1, n. 1, p. 9-17, Mar 2009. DOI: 10.1111/j.1753-0407.2009.00009.x.

GRAM-HANSEN, P.; ERIKSEN, J.; MOURITS-ANDERSEN, T.; OLESEN, L. Glycosylated haemoglobin (HbA1c) in iron- and vitamin B12 deficiency. **J Intern Med**, 227, n. 2, p. 133-136, Feb 1990. DOI: 10.1111/j.1365-2796.1990.tb00131.x.

HASHIMOTO, K.; OSUGI, T.; NOGUCHI, S.; MORIMOTO, Y. *et al.* A1C but not serum glycated albumin is elevated because of iron deficiency in late pregnancy in diabetic women. **Diabetes Care**, 33, n. 3, p. 509-511, Mar 2010. DOI: 10.2337/dc09-1954.

HUANG, Z.; LIU, Y.; MAO, Y.; CHEN, W. *et al.* Relationship between glycated haemoglobin concentration and erythrocyte survival in type 2 diabetes mellitus determined by a modified carbon monoxide breath test. **J Breath Res**, 12, n. 2, p. 026004, Jan 9 2018. DOI: 10.1088/1752-7163/aa9081.

KOGA, M.; MORITA, S.; SAITO, H.; MUKAI, M. *et al.* Association of erythrocyte indices with glycated haemoglobin in pre-menopausal women. **Diabet Med**, 24, n. 8, p. 843-847, Aug 2007. DOI: 10.1111/j.1464-5491.2007.02161.x.

KUNG, C. M.; TSENG, Z. L.; WANG, H. L. Erythrocyte fragility increases with level of glycosylated hemoglobin in type 2 diabetic patients. **Clin Hemorheol Microcirc**, 43, n. 4, p. 345-351, 2009. DOI: 10.3233/ch-2009-1245.

LEE, M. Y.; HUANG, J. C.; CHEN, S. C.; CHIOU, H. C. *et al.* Association of HbA1C Variability and Renal Progression in Patients with Type 2 Diabetes with Chronic Kidney Disease Stages 3(-)4. **Int J Mol Sci**, 19, n. 12, Dec 18 2018. DOI: 10.3390/ijms19124116.

LEMOS, G. S.; MARQUEZ-BERNARDES, L. F.; ARVELOS, L. R.; PARAISO, L. F. *et al.* Influence of glucose concentration on the membrane stability of human erythrocytes. **Cell Biochem Biophys**, 61, n. 3, p. 531-537, Dec 2011. DOI: 10.1007/s12013-011-9235-z.

LOPEZ DE FRUTOS, L.; CEBOLLA, J. J.; IRUN, P.; KOHLER, R. *et al.* The erythrocyte osmotic resistance test as screening tool for cholesterol-related

lysosomal storage diseases. **Clin Chim Acta**, 480, p. 161-165, May 2018. DOI: 10.1016/j.cca.2018.02.010.

LOUTRADIS, C.; SKODRA, A.; GEORGIANOS, P.; TOLIKA, P. *et al.* Diabetes mellitus increases the prevalence of anemia in patients with chronic kidney disease: A nested case-control study. **World J Nephrol**, 5, n. 4, p. 358-366, Jul 6 2016. DOI: 10.5527/wjn.v5.i4.358.

MADHU, S. V.; RAJ, A.; GUPTA, S.; GIRI, S. *et al.* Effect of iron deficiency anemia and iron supplementation on HbA1c levels - Implications for diagnosis of prediabetes and diabetes mellitus in Asian Indians. **Clin Chim Acta**, 468, p. 225-229, May 2017. DOI: 10.1016/j.cca.2016.10.003.

MATTHEWS, D. R.; HOSKER, J. P.; RUDENSKI, A. S.; NAYLOR, B. A. *et al.* Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. **Diabetologia**, 28, n. 7, p. 412-419, Jul 1985. DOI: 10.1007/BF00280883.

MAZURE, C. M.; JONES, D. P. Twenty years and still counting: including women as participants and studying sex and gender in biomedical research. **BMC women's health**, 15, p. 94-94, 2015. DOI: 10.1186/s12905-015-0251-9.

MCLEAN, E.; COGSWELL, M.; EGLI, I.; WOJDYLA, D. *et al.* Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. **Public health nutrition**, 12, n. 4, p. 444-454, 2009. DOI: 10.1017/S1368980008002401.

MCNAMEE, A. P.; HOROBIN, J. T.; TANSLEY, G. D.; SIMMONDS, M. J. Oxidative Stress Increases Erythrocyte Sensitivity to Shear-Mediated Damage. **Artif Organs**, 42, n. 2, p. 184-192, Feb 2018. DOI: 10.1111/aor.12997.

MOHANDAS, N.; CLARK, M. R.; JACOBS, M. S.; SHOEHET, S. Analysis of Factors Regulating Erythrocytes Deformability. **Journal of Clinical Investigation**, 66, p. 563-573, 1980.

MOHANDAS, N.; EVANS, E. Mechanical Properties of the Red Cell Membrane in Relation to Molecular Structure and Genetic Defects. **Annual Review of Biophysics and Biomolecular Structure**, 23, p. 787-818, 1994.

MOHANDAS, N.; GALLAGHER, P. G. Red cell membrane: past, present, and future. **Blood**, 112, n. 10, p. 3939-3948, Nov 15 2008. DOI: 10.1182/blood-2008-07-161166.

NGASHANGVA, L.; BACHU, V.; GOSWAMI, P. Development of new methods for determination of bilirubin. **J Pharm Biomed Anal**, 162, p. 272-285, Jan 5 2019. DOI: 10.1016/j.jpba.2018.09.034.

NUTTALL, F. Q. Effect of age on the percentage of hemoglobin A1c and the percentage of total glycohemoglobin in non-diabetic persons. **J Lab Clin Med**, 134, n. 5, p. 451-453, Nov 1999. DOI: 10.1016/s0022-2143(99)90165-8.

PARAISO, L. F.; DE FREITAS, M. V.; GONCALVES, E. O. A. F.; DE ALMEIDA NETO, O. P. *et al.* Influence of acute exercise on the osmotic stability of the human erythrocyte membrane. **Int J Sports Med**, 35, n. 13, p. 1072-1077, Dec 2014. DOI: 10.1055/s-0034-1371834.

PARAISO, L. F.; GONCALVES, E. O. A. F.; CUNHA, L. M.; DE ALMEIDA NETO, O. P. *et al.* Effects of acute and chronic exercise on the osmotic stability of erythrocyte membrane of competitive swimmers. **PLoS One**, 12, n. 2, p. e0171318, 2017. DOI: 10.1371/journal.pone.0171318.

PENHA-SILVA, N.; FIRMINO, C. B.; DE FREITAS REIS, F. G.; DA COSTA HUSS, J. C. *et al.* Influence of age on the stability of human erythrocyte membranes. **Mech Ageing Dev**, 128, n. 7-8, p. 444-449, Jul-Aug 2007. DOI: 10.1016/j.mad.2007.06.007.

RODRIGUES, R.; DE MEDEIROS, L. A.; CUNHA, L. M.; GARROTE-FILHO, M. D. S. *et al.* Correlations of the glycemic variability with oxidative stress and erythrocytes membrane stability in patients with type 1 diabetes under intensive treatment. **Diabetes Res Clin Pract**, 144, p. 153-160, Oct 2018. DOI: 10.1016/j.diabres.2018.01.031.

ROY, C. N.; SNYDER, P. J.; STEPHENS-SHIELDS, A. J.; ARTZ, A. S. *et al.* Association of Testosterone Levels With Anemia in Older Men: A Controlled Clinical Trial. **JAMA Internal Medicine**, 177, n. 4, p. 480-490, 2017. DOI: 10.1001/jamainternmed.2016.9540 %J JAMA Internal Medicine.

SANGHANI, S. P.; HALDANKAR, V. A. Comparative analysis of RBC membrane fatty acids, proteins and glycophorin in patients with heterozygous beta thalassemia and iron deficiency anemia. **Indian J Clin Biochem**, 21, n. 2, p. 28-33, Sep 2006. DOI: 10.1007/bf02912907.

SKIKNE, B.; LYNCH, S.; BOREK, D.; COOK, J. Iron and blood donation. **Clin Haematol**, 13, n. 1, p. 271-287, Feb 1984.

SLUITER, W. J.; VAN ESSEN, L. H.; REITSMA, W. D.; DOORENBOS, H. Glycosylated haemoglobin and iron deficiency. **Lancet**, 2, n. 8193, p. 531-532, Sep 6 1980. DOI: 10.1016/s0140-6736(80)91853-x.

STIER, A.; REICHERT, S.; CRISCUOLO, F.; BIZE, P. Red blood cells open promising avenues for longitudinal studies of ageing in laboratory, non-model and wild animals. **Exp Gerontol**, 71, p. 118-134, Nov 2015. DOI: 10.1016/j.exger.2015.09.001.

SUOMINEN, P.; PUNNONEN, K.; RAJAMAKI, A.; IRJALA, K. Serum transferrin receptor and transferrin receptor-ferritin index identify healthy subjects with subclinical iron deficits. **Blood**, 92, n. 8, p. 2934-2939, Oct 15 1998.

TARIM, O.; KUCUKERDOGAN, A.; GUNAY, U.; ERALP, O. *et al.* Effects of iron deficiency anemia on hemoglobin A1c in type 1 diabetes mellitus. **Pediatr Int**, 41, n. 4, p. 357-362, Aug 1999.

TZIAKAS, D.; CHALIKIAS, G.; GRAPSA, A.; GIOKA, T. *et al.* Red blood cell distribution width: a strong prognostic marker in cardiovascular disease: is associated with cholesterol content of erythrocyte membrane. **Clin Hemorheol Microcirc**, 51, n. 4, p. 243-254, 2012. DOI: 10.3233/CH-2012-1530.

VERMA, U.; SHANKAR, N.; MADHU, S. V.; TANDON, O. P. *et al.* Relationship between iron deficiency anaemia and serum lipid levels in Indian adults. **J Indian Med Assoc**, 108, n. 9, p. 555-558, 562, Sep 2010.

VIRTUE, M. A.; FURNE, J. K.; NUTTALL, F. Q.; LEVITT, M. D. Relationship between GHb concentration and erythrocyte survival determined from breath carbon monoxide concentration. **Diabetes Care**, 27, n. 4, p. 931-935, Apr 2004. DOI: 10.2337/diacare.27.4.931.

WAGGIALLAH, H.; ALZOHAIRY, M. The effect of oxidative stress on human red cells glutathione peroxidase, glutathione reductase level, and prevalence of anemia among diabetics. **N Am J Med Sci**, 3, n. 7, p. 344-347, Jul 2011. DOI: 10.4297/najms.2011.3344.

WESTWOOD, A. The analysis of bilirubin in serum. **Ann Clin Biochem**, 28 (Pt 2), p. 119-130, Mar 1991. DOI: 10.1177/000456329102800202.

WHO. **Waist circumference and waist–hip ratio: report of a WHO expert consultation, Geneva, 8–11 December 2008**. 2008.

WHO. WHO Guidelines Approved by the Guidelines Review Committee. *In*: **Use of Glycated Haemoglobin (HbA1c) in the Diagnosis of Diabetes Mellitus: Abbreviated Report of a WHO Consultation**. Geneva: World Health Organization Copyright © World Health Organization 2011.

WU, L.; LIN, H.; GAO, J.; LI, X. *et al.* Effect of age on the diagnostic efficiency of HbA1c for diabetes in a Chinese middle-aged and elderly population: The Shanghai Changfeng Study. **PLoS One**, 12, n. 9, p. e0184607, 2017. DOI: 10.1371/journal.pone.0184607.

YE, S.; RUAN, P.; YONG, J.; SHEN, H. *et al.* The impact of the HbA1c level of type 2 diabetics on the structure of haemoglobin. **Sci Rep**, 6, p. 33352, Sep 14 2016. DOI: 10.1038/srep33352.

YIP, R.; MOHANDAS, N.; CLARK, M. R.; JAIN, S. *et al.* Red cell membrane stiffness in iron deficiency. **Blood**, 62, n. 1, p. 99-106, Jul 1983.