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

INFLUÊNCIA DE ÍNDICES HEMATIMÉTRICOS E BIOQUÍMICOS DE PACIENTES SUBMETIDAS À CIRURGIA BARIÁTRICA SOBRE A ESTABILIDADE DE MEMBRANA DE ERITRÓCITOS

Estudante: **Leticia Ramos de Arvelos**

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Orientador: Professor Dr. **Nilson Penha-Silva**

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Professor Dr. **Nilson Penha-Silva**
(Orientador)

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pelo amor e dedicação em todos os momentos de minha vida

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Sumário

Abreviaturas

A_1	Absorvância com valor mínimo de hemólise
A_2	Absorvância com valor máximo de hemólise
A_{540}	Absorvância a 540 nm
BMI	*Body Mass Index* (Índice de Massa Corporal)
D_{50}	Concentração de etanol que causa 50% de hemólise
dD	Variação na concentração de etanol necessária para promover hemólise total
dX	Variação na concentração de NaCl necessária para promover hemólise total
Glu	*Glucose* (glicose)
H_{50}	Concentração de NaCl que causa 50% de hemólise
Hb	*Hemoglobin* (hemoglobina)
HDL-C	*High-Density Lipoprotein Cholesterol* (Colesterol da Lipoproteína de Alta Densidade)
Ht	*Hematocrit* (Hematócrito)
IMC	Indice de Massa Corporal
LDL-C	*Low-Density Lipoprotein Cholesterol* (Colesterol da Lipoproteína de Baixa Densidade)
MCH	*Mean Corpuscular Hemoglobin* (Hemoglobina Corpuscular Média)
MCHC	*Mean Corpuscular Hemoglobin Concentration* (Concentração de Hemoglobina Corpuscular Média)
MCV	*Mean Corpuscular Volume* (Volume Corpuscular Médio)
DGYR	Desvio Gástrico em Y- de-Roux
OMS	Organização Mundial de Saúde
RBC	*Red Blood Cell* (eritrócito)
RDW	*Red Cell Distribution Width* (variação de tamanho dos eritrócitos)
RYGB	Roux-en-Y Gastric Bypass
t-C	*Total Cholesterol* (colesterol total)
TGC	*Triglycerides* (triglicérides)
Vit B12	Vitamina B12
VLDL-C	*Very Low Density Lipoprotein Cholesterol* (Colesterol da Lipoproteína de Muito Baixa Densidade)

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Apresentação

A obesidade é um problema de saúde pública que acomete grande parte da população mundial. Ela é decorrente do acúmulo de gordura corporal, em decorrência do excessivo consumo de energia na dieta. Esse acúmulo de gordura é devido a fatores comportamentais, como vida sedentária, alto consumo de produtos industrializados, além de fatores hereditários, psicológicos e alterações neuroendócrinas.

A Organização Mundial de Saúde (OMS) considera como obesidade um índice de massa corporal (IMC) acima de 30 kg/m^2 e como obesidade mórbida um IMC acima de 40 kg/m^2. O IMC é obtido a partir do cálculo da razão entre o peso (dado em kg) e o quadrado da altura (dada em metros). Os indivíduos com obesidade mórbida estão mais sujeitos a comorbidades, como dislipidemia, doença cerebrocardiovascular aterosclerótica, diabetes, hipertensão, além de problemas articulares e respiratórios. Os métodos para controle da obesidade vão desde o uso de medicamentos, mudança nos hábitos alimentares e no estilo de vida, com a prática de atividade física. Caso todos esses métodos sejam ineficientes, a cirurgia bariátrica torna-se necessária. Até o momento, a cirurgia bariátrica é considerada a forma mais efetiva de perda de peso por pessoas obesas. Porém, a manutenção do peso é um processo que demanda muita disciplina por parte do paciente, além de apoio multiprofissional de nutricionista, psicólogo e médico.

A cirurgia bariátrica mais realizada no Brasil é o desvio gástrico em Y-de-Roux (DGYR). Os pacientes submetidos a essa cirurgia têm maior chance de desenvolver deficiências nutricionais, pois ocorre o isolamento da porção alta do estômago e de parte do intestino, local onde ocorre a absorção de muitos nutrientes advindos da dieta, particularmente de ferro, folato (vitamina B9) e cobalamina (vitamina B12), que são fatores essenciais na eritropoiese. A absorção desses nutrientes é prejudicada pela redução na produção de ácido clorídrico, pepsina e do chamado fator intrínseco. A absorção limitada de ferro pode resultar na produção de hemácias microcíticas e hipocrômicas. Já as deficiências de folato e/ou de cobalamina resultam em macrocitose. Desse modo, a cirurgia bariátrica contribui para que o paciente se torne anêmico.

O Capítulo 1 traz a fundamentação teórica da tese. Nesse capítulo, é feita primeiro uma breve explanação sobre a obesidade e os efeitos da cirurgia bariátrica. Em seguida é feita uma abordagem sobre a composição e o comportamento das membranas celulares, bem como dos fatores que interferem na estabilidade de membrana e descrição da estatística multivariada, os quais certamente sofrem influência da cirurgia bariátrica, particularmente nos eritrócitos.

O Capítulo 2 consiste no uso da análise estatística bivariada e multivariada na investigação das correlações e relações causais entre as variáveis hematológicas e bioquímicas e a estabilidade de membrana de eritrócitos contra a ação caotrópica do etanol em eritrócitos de pacientes que realizaram a cirúrgica bariátrica por desvio gástrico em Y-de-Roux. Este capítulo corresponde ao artigo produzido no processo de qualificação dessa tese e já foi publicado no periódico *Journal of Membrane Biology* no ano de 2013.

O Capítulo 3 analisou a correlação entre a estabilidade osmótica de eritrócitos e vários índices hematológicos e bioquímicos de pacientes submetidos à cirurgia bariátrica por desvio gástrico em Y-de-Roux.

CAPÍTULO 1

FUNDAMENTAÇÃO TEÓRICA

Obesidade

A obesidade tem alcançado proporções epidêmicas mundialmente, sendo um grande problema não apenas para o indivíduo, mas para toda a sociedade. Cerca de 1,6 bilhões de pessoas (com idade igual ou a superior a 15 anos) apresentam excesso de peso, das quais 400 milhões são obesas (Kruger *et al.*, 2014). A OMS estima que até 2015 aproximadamente 2,3 bilhões de adultos estarão com sobrepeso e mais 700 milhões serão obesos. Nos Estados Unidos, 154,7 milhões de pessoas apresentam sobrepeso ou obesidade, e desses 79,9 milhões são homens e 74,8 milhões são mulheres (Go *et al.*, 2013).

No Brasil, os índices também são preocupantes. Aproximadamente 50,8% da população acima de 18 anos apresentam excesso de peso, sendo que 17,5% apresentam obesidade (VIGITEL, 2014). Dentre os obesos, 16,9% são mulheres e 12,4%, homens (IBGE, 2009). Além disso, de 0,5 a 1% dos adultos têm obesidade mórbida (Repetto & Bonatto, 2003). Nós últimos 30 anos houve um crescimento de 255% dos casos de obesidade mórbida no país, o que é um dado bastante preocupante (Oliveira, 2007).

A obesidade é uma doença que envolve o acúmulo de gordura corporal em decorrência de vários fatores, tais como: herança genética, sedentarismo, maus hábitos alimentares e alterações neuroendócrinas (Misra & Khurana, 2008; Marques-Lopes *et al.*, 2004). Há também fatores sociais, comportamentais, culturais e psicológicos (Barreto, 2004). A OMS utiliza como critério para classificação de obesidade o IMC, que é obtido a partir da razão entre o peso e o quadrado da altura. Indivíduos com valores de IMC entre 18,5 e 24,9, 25,0 e 29,9 e acima de 30 kg/m^2 são considerados normais (eutróficos), com sobrepeso e obesos, respectivamente. A OMS também classifica a obesidade da seguinte forma: classe I (IMC de 30,0 a 34,9 kg/m^2); classe II (IMC de 35,0 a 39,9 kg/m^2); e classe III (IMC $\geq$ 40,0 kg/m^2). A classe III é considerada obesidade mórbida (World Health Organization, 2005).

Os valores elevados de IMC estão associados a diversas doenças, tais como: hipertensão arterial sistêmica; diabetes mellitus; doenças coronarianas; artropatia; insuficiências respiratória e cardíaca; varizes e tromboses nos membros inferiores; alguns tipos de câncer, que incluem o de mama, próstata e cólon; além de alterações psicossociais, dentre outras condições (Austin *et al.*, 1991; Kruger *et al.*, 2014; Shikora, 2000).

Cirurgia bariátrica por desvio gástrico em Y-de-Roux

O tratamento da obesidade compreende várias abordagens, tais como: mudança no estilo de vida, intervenção dietética, atividade física, uso de fármacos e intervenção cirúrgica (Buchwald *et al.*, 2004). Destes, a intervenção cirúrgica mostrou-se o método terapêutico mais eficaz e confiável na redução de peso (Shikora, 2000).

As cirurgias bariátricas são classificadas em restritivas e disabsortivas, conforme o mecanismo responsável pela perda de peso. A cirurgia restritiva consiste na diminuição do tamanho do reservatório gástrico, o que diminui a quantidade de alimento ingerido. Já na cirurgia disabsortiva há uma diminuição da absorção de nutrientes devido à redução do comprimento intestinal. As técnicas que combinam as duas modalidades são conhecidas como mistas (Alvarez-Leite, 2004; Vargas-Ruiz *et al.*, 2008).

A intervenção cirúrgica é indicada nas seguintes situações: pessoas com IMC ≥ 35 kg/m^2 com comorbidades ou com IMC ≥ 40 kg/m^2; obesos por cinco anos ou mais; ineficiência dos tratamentos convencionais; avaliação multidisciplinar do risco cirúrgico aceitável e com liberação para cirurgia; orientação e adesão a mudanças de comportamento e aos novos hábitos alimentares após a cirurgia (Buchwald *et al.*, 2004).

Atualmente, a técnica cirúrgica mais utilizada é o desvio gástrico em Y-de-Roux (DGYR), também conhecida por cirurgia de Fobi-Capella. Essa técnica consiste na restrição da capacidade gástrica, com a criação de uma bolsa de aproximadamente 30 a 50 ml por secção da porção mais alta do estômago, separando-a do restante deste órgão. O jejuno é, então, seccionado em uma região de 30 a 60 cm de sua origem e anastomosado àquela bolsa gástrica, de tal forma a ultrapassar o duodeno e jejuno proximal. A alça remanescente do jejuno proximal é, então, ligada ao segmento do qual foi separado a mais ou menos 100 cm da anastomose gastrojejunal gerada na cirurgia (**Figura 1**). Além disso, um pequeno anel de silicone é inserido no pequeno estômago acima da ligação gastrojejunal. Consequentemente, a maior parte do estômago, duodeno e jejuno são excluídas do trânsito intestinal (Silva *et al.*, 2005).

O DGYR proporciona uma considerável redução de peso e com baixa mortalidade, além de uma melhora também considerável nas comorbidades como diabetes mellitus, hipertensão arterial, dislipidemias, distúrbios do sono, dentre outras (Buchwald *et al.*, 2004;

Steffen *et al.*, 2009; Custódio Afonso Rocha *et al.*, 2012) e, consequentemente, uma melhora na qualidade de vida (Dixon *et al.*, 2001; Silva *et al.*, 2005).

O sucesso da cirurgia é mensurado pela perda média de 68,2% de excesso de peso, manutenção de um menor peso e melhora nas comorbidades [Buchwald *et al.*, 2004; Steffen *et al.*, 2009].

Essa cirurgia é considerada padrão ouro pelos especialistas no controle do diabetes para obeso mórbido, pois apresenta uma prevenção, melhora e reversão do diabetes (Geloneze & Pareja, 2006; Colquit *et al.*, 2014).

Fatores nutricionais

O eritrócito (*Red Blood Cell* - RBC) é uma importante célula sanguínea (Mohandas & Gallagher, 2008). A quantidade desta célula em pacientes submetidos à cirurgia bariátrica, geralmente, se encontram abaixo dos níveis considerados normais, o que caracteriza anemia (Ferrer *et al.*, 2014).

A deficiência de ferro, geralmente, é causa primária da anemia neste tipo de paciente (Yale *et al.*, 1993; Bloomberg *et al.*, 2005) e apresenta uma prevalência de cerca de 6 a 50% nos meses ou anos seguintes após esse procedimento cirúrgico (Simon *et al.*, 1989; Halverson, 1986).

As causas da deficiência de ferro são diversas, dentre as quais se destacam a eventual redução da ingestão de carne por intolerância crônica após a cirurgia (Brolin *et al.*, 1994; Avinoah *et al.*, 1992) e a redução na secreção do ácido gástrico no estômago, o que diminui a solubilidade do ferro e a sua consequente absorção pelo duodeno (Conrad & Umbreit, 2000).

A cirurgia bariátrica também reduz a absorção de ferro devido à exclusão do duodeno, principal região do intestino responsável pela absorção de ferro (Sugerman *et al.*, 1989), mas não no jejuno, cuja superfície, entretanto, não é suficientemente grande para compensar na plenitude a exclusão do duodeno (Fleming & Bacon, 2005).

Além disso, a anemia decorrente da cirurgia também pode ser causada pela perda de sangue durante o procedimento cirúrgico.

Deficiências nutricionais

Pacientes submetidos à cirurgia bariátrica apresentam redução na quantidade de reticulócitos, que são células precursoras dos eritrócitos (Ferrer *et al.*, 2014), o que pode contribuir para o desenvolvimento de anemia hipoproliferativa.

A anemia por deficiência de ferro (anemia ferropriva) caracteriza-se por diminuição de: volume corpuscular médio (MCV), hemoglobina corpuscular média (MCH), hemoglobina (Hb), hematócrito (Ht), ferro e transferrina séricos. Essas alterações geralmente resultam em hemácias microcíticas e hipocrômicas. Clinicamente, isso pode estar relacionado aos seguintes sintomas: sensação de fadiga com concomitante diminuição à tolerância ao exercício físico; aspecto pálido da conjuntiva; alterações na aparência da língua (glossite atrófica); alterações na mucosa e submucosa do esôfago, gerando as chamadas teias de esôfago (síndrome de Plummer-Vinson) (Love, 2008).

Há também deficiência das vitaminas A, K, D, E, B1 (tiamina), B9 (ácido fólico) e B12 (cobalamina), bem como do micromineral cobre, em pacientes submetidos a este método cirúrgico, mesmo com a suplementação oral desses fatores nutricionais (Love, 2008; Ferrer *et al.*, 2014). As deficiências de ácido fólico e cobalamina podem levar à anemia macrocítica, o que reflete em aumento do MCV (Batool *et al.*, 2013). No caso da cobalamina, essa vitamina é liberada pela quebra das proteínas presentes nos alimentos pela ação do acido clorídrico e da pepsina presentes no estômago. Então, a cobalamina se liga ao fator intrínseco, que é uma glicoproteína presente no estômago, e em seguida, passa pelo duodeno, e é finalmente absorvida na porção terminal do íleo (Shankar & Sriram, 2010).

Em pacientes submetidos à cirurgia bariátrica, vários fatores levam a deficiência de cobalamina, tais como: menor ingestão de proteínas de origem animal, devido à intolerância alimentar; diminuição na produção gástrica de acido clorídrico e pepsina, importantes na liberação da vitamina do alimento; diminuição da secreção e função do fator intrínseco em decorrência da exclusão do corpo gástrico do processo de digestão (Vargas-Ruiz *et al.*, 2008; Shankar & Sriram, 2010).

A deficiência do acido fólico é resultante da diminuição da quantidade de alimento ingerida. Essa deficiência é menos comum, pelo fato dessa vitamina ser absorvida principalmente no jejuno. Embora a sua absorção seja facilitada pela ação do ácido clorídrico, a cobalamina forma a coenzima (desoxiadenosilcobalamina) que atua na

conversão do acido fólico para a forma absorvível. Assim, a deficiência de cobalamina compromete os níveis de ácido fólico, o que contribui para o quadro de anemia megaloblástica (Shankar & Sriram, 2010).

Membranas celulares

As membranas celulares delimitam o meio interno e externo da célula, o que permite as trocas de substâncias de acordo com as necessidades celulares (Nelson & Cox, 2008; Berg *et al.*, 2008). As membranas celulares são compostas por proteínas, lipídios e outros constituintes, que se organizam em uma estrutura heterogênea formando um "mosaico fluido" (Singer & Nicolson, 1972; Qian *et al.*, 2014). A membrana é formada por diversas classes de lipídios, sendo que os fosfolipídios compõem 95% dos lipídios da membrana (Cooper, 1977).

A composição da membrana é fundamental para a sua funcionalidade. Em mamíferos, o colesterol é importante na regulação da fluidez e funcionalidade da membrana (Roduit *et al.*, 2008). Esse lipídio aumenta a rigidez da membrana e, consequentemente, reduz a sua permeabilidade (Qian *et al.*, 2014).

A forma plana e rígida do colesterol, assim como a natureza apolar do seu anel esteroidal, são fatores que aumentam a quantidade de interações de van der Waals, o que torna a membrana menos fluida (Hubbell & McConnel, 1971; Shinitzky & Inbar, 1976; Raffy & Teissie, 1999).

Os lipídeos da dieta influenciam na composição da membrana celular, em especial do eritrócito, já que essa célula não sintetiza colesterol (Cooper, 1977). Portanto, o nível de colesterol da membrana do eritrócito é resultado da concentração de colesterol nas lipoproteínas plasmáticas, já que essa célula obtém colesterol principalmente a partir da interação com a LDL (*Low Density Lipoprotein*) (Roduit *et al.*, 2008).

O eritrócito com membrana excessivamente rica em colesterol possui uma menor deformabilidade e fluidez. Isto pode alterar suas propriedades reológicas e funcionais (Cazzola *et al.*, 2004; Cazzola *et al.*, 2011).

Estabilidade x funcionalidade

A estabilidade de membrana depende de fatores intrínsecos e extrínsecos. Os fatores intrínsecos, como o próprio nome diz, se referem à composição da membrana. Por sua vez, os fatores extrínsecos se referem ao meio em que as células se encontram e ao estado nutricional do indivíduo a que pertencem (de Arvelos *et al.*, 2013; de Freitas *et al.*, 2014).

A fluidez da membrana é determinada pela proporção entre a quantidade de colesterol e de fosfolipídios, bem como entre a proporção de ácidos graxos saturados (SFA) e insaturados (UFA) presentes nas caudas dos fosfolipídios. Membranas com menores razões de SFA/UFA e colesterol/fosfolipídios são mais fluidas (Allen *et al.*, 2006). Na situação oposta, elas se tornam mais rígidas e osmoticamente mais resistentes, ou seja, apresentam maior estabilidade osmótica.

A melhor funcionalidade de membrana é coincidente com a chamada fluidez crítica, ou seja, a membrana deve ter certo nível de fluidez para que exerça suas funções. Acima desse nível de fluidez, a membrana se torna muito propensa a sofrer fusão e abaixo dele ela se torna propensa a se solidificar (de Freitas *et al.*, 2010).

A funcionalidade da membrana não é necessariamente coincidente com a sua estabilidade, ou seja, dizer que uma membrana é estável não significa que ela esteja exercendo mais adequadamente suas funções. Um aumento na proporção SFA/UFA e/ou colesterol/fosfolipídios até certo ponto torna a membrana osmoticamente mais estável, ou seja, mais resistente à lise em meio hipotônico, mas isso não a torna mais capaz de sofrer as transconformações necessárias ao exercício de suas funções (de Freitas *et al.*, 2010; Lemos *et al.*, 2011; de Arvelos *et al.*, 2013).

Estabilidade osmótica x estabilidade mecânica

A estabilidade osmótica da membrana não é coincidente com a sua estabilidade mecânica *in vivo*. Como exemplo, pode citar-se o que ocorre com o eritrócito. Em meio hipotônico, os eritrócitos mais ricos em colesterol e ácidos graxos saturados são mais resistentes à lise, porque eles apresentam membrana menos fluida, ou seja, mais rígida. Entretanto, no interior dos vasos sanguíneos isso não é tão vantajoso. Pelo contrário,

quanto mais rígida a membrana, menos flexíveis são essas células. Assim, com uma menor deformabilidade, os eritrócitos se tornam mecanicamente menos estáveis, pois se rompem mais facilmente ao passar por vasos de calibres estreitos ou colidirem com as paredes dos vasos ou com outras células sanguíneas (Mohandas *et al.*, 1980).

A idade da célula também contribui para uma menor resistência mecânica. A média de vida dos eritrócitos é de 120 dias. À medida que essas células envelhecem, vão perdendo o formato bicôncavo e com isso se tornam esféricas e menores. Esses fenômenos ocorrem devido ao desgaste das proteínas que compõem seu citoesqueleto e também por perdas de partes da membrana plasmática por vesiculação. Nesse processo, os eritrócitos perdem mais lipídios do que proteínas, tornando-se mais densos (Greenwalt *et al.*, 1984; Waugh *et al.*, 1992).

O envelhecimento da célula, por desgaste, compreende os danos causados pela ação de radicais livres sobre os fosfolipídios de membrana. Como os eritrócitos são células desprovidas de núcleo e organelas, as enzimas antioxidantes responsáveis pela sua proteção não podem ser substituídas por enzimas novas (*turnover*). Com o tempo essas enzimas perdem gradativamente suas atividades (Minetti *et al.*, 2007).

Esse envelhecimento, também contribui para a diminuição da estabilidade osmótica do eritrócito. A mudança do tamanho e do formato de disco bicôncavo para esférico, com a redução da área celular e da espessura da sua membrana devido à diminuição do conteúdo de colesterol, faz com que o eritrócito não suporte o inchamento e estoure mais facilmente em condições hipotônicas (Greenwalt *et al.*, 1984; Waugh *et al.*, 1992).

Os fatores extrínsecos à membrana também interferem bastante na estabilidade osmótica dos eritrócitos. Dentre estes fatores podemos citar a composição do plasma sanguíneo, o pH, a temperatura e a ação de solutos estabilizantes (osmólitos) e desestabilizantes (caotrópicos) (Cunha *et al.*, 2007; Penha-Silva *et al.*, 2008; de Arvelos *et al.*, 2013; Cunha *et al.*, 2014).

Composição e estabilidade de membrana

A composição do plasma sanguíneo tem efeito sobre a composição da membrana do eritrócito, o que afeta sua estabilidade. Indivíduos que têm um plasma rico em LDL-C

colesterol (*Low Dense Lipoprotein Cholesterol*) possuem células cuja membrana é mais rica em colesterol. O LDL atua diretamente na deposição de colesterol nas membranas (Cooper, 1977).

O aumento da idade do indivíduo, que contribui para aumento no teor sanguíneo de LDL-C (Araki & Rifkind, 1980), tende a tornar os eritrócitos mais ricos em colesterol e osmoticamente mais estáveis (Penha-Silva *et al.*, 2007).

A HDL (*High Density Lipoprotein*) também exerce importante papel na estabilidade osmótica da membrana. A HDL atua de modo inverso ao da LDL, ao retirar colesterol da membrana do eritrócito, seja por competir com a LDL pela membrana ou por retirar o excesso de colesterol da membrana para promover seu transporte até o fígado (Ejima *et al.*, 2000; Meurs *et al.*, 2005). Assim, maiores valores de HDL-C devem estar associados a um menor teor de colesterol na membrana do eritrócito, o que a torna menos rígida e osmoticamente menos resistente, porém mais deformável e, em consequência, mais estável mecanicamente e mais adequada ao exercício de suas funções reológicas.

A estabilidade osmótica de membranas tem sido bastante avaliada *in vitro* em nosso laboratório. Isso já foi feito por meio da exposição a variações no pH, temperatura e soluções de agentes caotrópicos, como o etanol (Penha-Silva *et al.*, 2008; Cunha *et al.*, 2007; de Arvelos *et al.*, 2013) e o detergente dodecil sulfato de sódio (SDS) (Fonseca *et al.*, 2010; de Freitas *et al.*, 2014). Também foram feitos estudos com solutos estabilizantes (Penha-Silva *et al.*, 2008; Cunha *et al.*, 2007).

O etanol é um agente caotrópico anfifílico (Fonseca *et al.*, 2006). Em soluções aquosas, ele é capaz de atenuar a força hidrofóbica, acomodando melhor no solvente os grupos apolares dos constituintes das membranas ao formar ligações de van der Waals com os mesmo e ligações de hidrogênio com a água. O etanol também compete com a água pela formação de ligações de hidrogênio com as proteínas e lipídios da superfície da membrana (Sonmez *et al.*, 2013).

Ao atenuar a força hidrofóbica do meio, o etanol contribui para um aumento da fluidez da membrana. As moléculas de etanol também podem se inserir na bicamada lipídica, diminuindo a força hidrofóbica entre os fosfolipídios e aumentando também a fluidez da membrana. Além disso, o etanol desnatura proteínas da membrana, o que contribui para a abertura de poros por onde saem moléculas de hemoglobina do eritrócito (Zavodnik *et al.*, 1994).

O aumento da concentração de etanol no meio faz com que haja aumento da osmolaridade, o que contribui para a estabilização de complexos biológicos, como proteínas e membranas. Isto significa que o etanol apresenta efeitos dualísticos antagônicos, caotropismo e osmoestabilização, sobre essas estruturas. O efeito predominante, caotrópico ou osmoestabilizador, do etanol sobre as membranas de eritrócitos depende da concentração em que esse soluto se encontra em solução salina a 0,9% de NaCl. Um estudo *in vitro* realizado com eritrócitos incubados em soluções salinas fisiológicas com concentrações crescentes de etanol de 0 a 34 $g.dL^{-1}$ mostrou que há predomínio da hemólise na faixa de concentração entre 10 e 20 $g.dL^{-1}$, mas predomínio da estabilização entre 20 e 25 $g.dL^{-1}$, com retorno ao predomínio da hemólise após 25 $g.dL^{-1}$. Desse modo, o etanol interfere na estabilidade das membranas, aumentando ou diminuindo a estabilidade de acordo com sua concentração no meio (Gouveia-e-Silva, 2006). Isso se deve aos efeitos antagônicos dualísticos promovidos pelo etanol (Sonmez *et al.*, 2013).

A exposição crônica ao etanol, enquanto a função hepática é preservada, torna os eritrócitos osmoticamente mais resistentes. Isso acontece não pela ação direta do etanol, mas em decorrência do fato do etanol ser precursor da colesterologênese no fígado, o que eleva os teores sanguíneos de LDL-C e, consequentemente, o teor de colesterol na membrana do eritrócito. Esse é um mecanismo adaptativo (Goldstein *et al.*, 1986).

Estatística

Existem várias ferramentas estatísticas que permitem analisar correlações entre as variáveis. Essas ferramentas são baseadas em regressão linear e compõem o chamado Modelo Linear Generalizado. As principais ferramentas que compõem esse modelo são, na ordem crescente de complexidade, a Regressão Linear Simples, a Regressão Linear Múltipla, a Correlação Canônica e a Modelagem de Equações Estruturais (SEM, do inglês *Structural Equation Modeling*). Cada uma delas apresenta um ou mais avanços em relação à anterior, embora não torne a ferramenta precedente desnecessária. De certa forma, é como se seguissem um processo evolutivo (**Figura 1.3**) (Graham, 2008).

A Regressão Linear Simples investiga a correlação entre apenas duas variáveis, sendo uma delas dependente e a outra independente. A Regressão Linear Múltipla

também investiga uma única variável dependente, denominada *criterion*. Entretanto, existe a possibilidade de correlacioná-la simultaneamente a várias variáveis independentes, denominadas preditores. Na Correlação Canônica a inovação consiste em poder se correlacionar ao mesmo tempo diversas variáveis independentes com diversas outras variáveis dependentes. Entretanto, as variáveis de cada um desses dois tipos encontram-se confinadas em um grupo próprio. Isso limita os possíveis modos com que tais variáveis podem se correlacionar entre si (Graham, 2008).

Na SEM essa limitação não existe, pois as variáveis podem se relacionar entre si de diversas maneiras, não estando, necessariamente, restritas a um grupo. Inclusive, na SEM a classificação de uma variável em dependente ou independente não é fixa, pois uma determinada variável pode ser ao mesmo tempo dependente e independente, conforme a forma com que se relaciona com as demais variáveis do modelo a que pertence (Ulman, 2006).

A SEM é bastante útil na investigação de modelos teóricos construídos a partir de hipóteses. Com essa ferramenta é possível não apenas testar se um determinado modelo teórico é plausível, como também fazer as devidas modificações para que isso seja alcançado. Esses modelos teóricos podem ser esquematizados visualmente por meio dos chamados diagramas de caminho. Nesses diagramas, as variáveis podem ser correlacionadas entre si por meio de correlação causal (seta de sentido único) ou covariância (setas de duplo sentido) (Ulman, 2006).

O sentido das setas no diagrama de caminho devem respeitar as relações de causalidade conhecidas (Ulman, 2006). Por exemplo, sabe-se que o volume corpuscular médio (MCV) dos eritrócitos exerce efeito sobre a distribuição de volumes (RDW) dessas células, e não o contrário. Assim, em um diagrama de caminho, a seta que representa o efeito de uma dessas sobre as outras deve partir de MCV em direção à RDW, e não o contrário. Caso não se soubesse ou não se quisesse definir um sentido de efeito entre as duas variáveis, seta de duplo sentido seria utilizada para representar o efeito entre essas variáveis.

Além disso, em um diagrama de caminho não é adequado incluir correlações que são decorrentes de colinearidade, como é o caso da correlação entre triglicérides (TGC) e VLDL-C. Essa colinearidade ocorre porque o VLDL-C é calculado a partir dos valores de TGC.

Portanto, essa é uma correlação redundante e não acrescentaria praticamente nada ao modelo teórico representado por meio do diagrama de caminho (Ulman, 2006).

As variáveis que estão presentes em um diagrama de caminho podem ser classificadas como observadas e não observadas. As variáveis observadas são aquelas efetivamente medidas e são representadas na forma de um retângulo. Tais variáveis incluem, por exemplo, o índice de massa corporal (BMI) e o MCV (**Figura 1.4**) (Ulman, 2006).

As variáveis observadas, por sua vez, também podem ser classificadas como endógenas e exógenas. Essa classificação depende do fato da variável exercer ou não efeito sobre outra variável, independentemente do fato de receber ou não efeito de alguma outra variável. Assim, têm-se as variáveis endógenas, que são aquelas que recebem efeito de outra variável, enquanto que as exógenas são aquelas não recebem efeito de nenhuma outra variável. No caso do diagrama de caminho exemplificado na **Figura 1.4**, o BMI é a única variável observada exógena. As demais variáveis observadas são endógenas (Ulman, 2006).

Já as variáveis não observadas não são medidas, sendo obtidas por meio de cálculo pelo próprio programa que elabora o diagrama de caminho. Essas variáveis incluem os erros associados às variáveis endógenas. Tais erros são indicados por meio de um círculo (Ulman, 2006).

As variáveis não observadas também incluem as variáveis latentes, que são variáveis obtidas indiretamente a partir de um determinado conjunto de variáveis observadas. Quando um diagrama de caminho não contém variáveis latentes, diz-se que a ferramenta estatística envolvida é a análise de caminho, que é um tipo de SEM (Ulman, 2006).

Na SEM, ao contrário de outras ferramentas estatísticas mais simples, não há um único parâmetro que possa ser utilizado para se determinar se um determinado modelo teórico é satisfatório ou não. Na Regressão Linear Simples, por exemplo, um valor de p abaixo de 0,05 é suficiente para poder considerar os valores encontrados aceitáveis (Ulman, 2006).

Já na SEM há uma vasta quantidade de parâmetros que podem ser utilizados para se indicar se um determinado modelo teórico é plausível ou não. E não basta que apenas um desses parâmetros seja utilizado. É comumente empregado um conjunto deles com

esse propósito. Tais parâmetros fazem parte do que se chama de *model fit*. Maiores informações sobre esses parâmetros estão disponíveis na **Tabela 1.1** (Ulman, 2006).

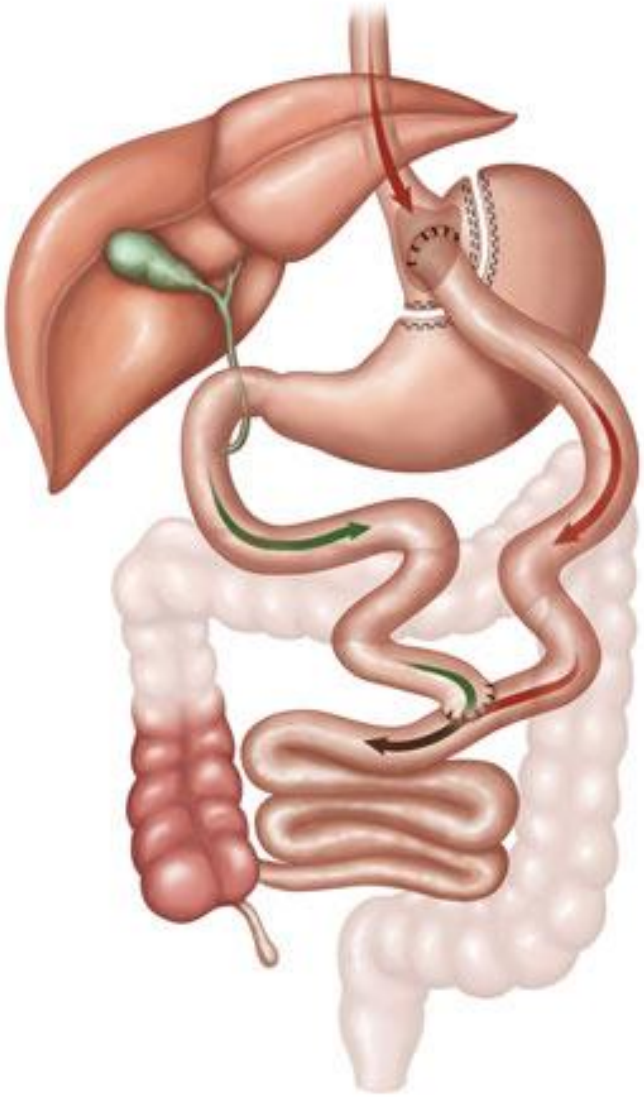

Figura 1. Cirurgia bariátrica por desvio em Y-de-Roux (Fonte: http://www.sbcbm.org.br/wordpress/wp-content/uploads/2014/05/bypass-gastrico.jpg).

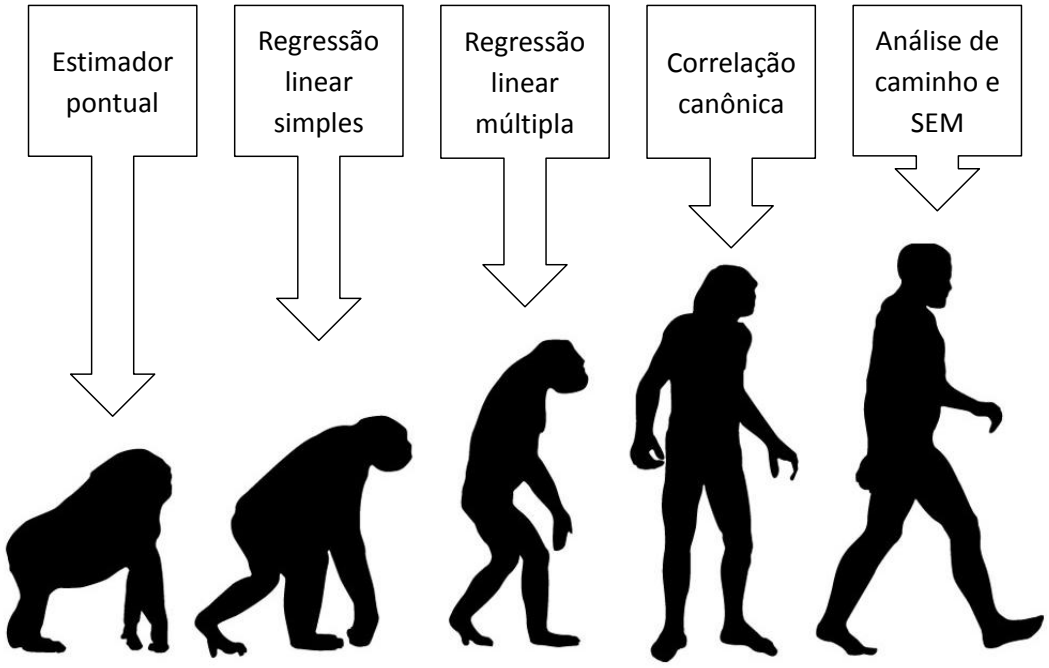

Figura 2. Comparação entre diferentes ferramentas estatísticas [Fonte: Garrote-Filho, 2014].

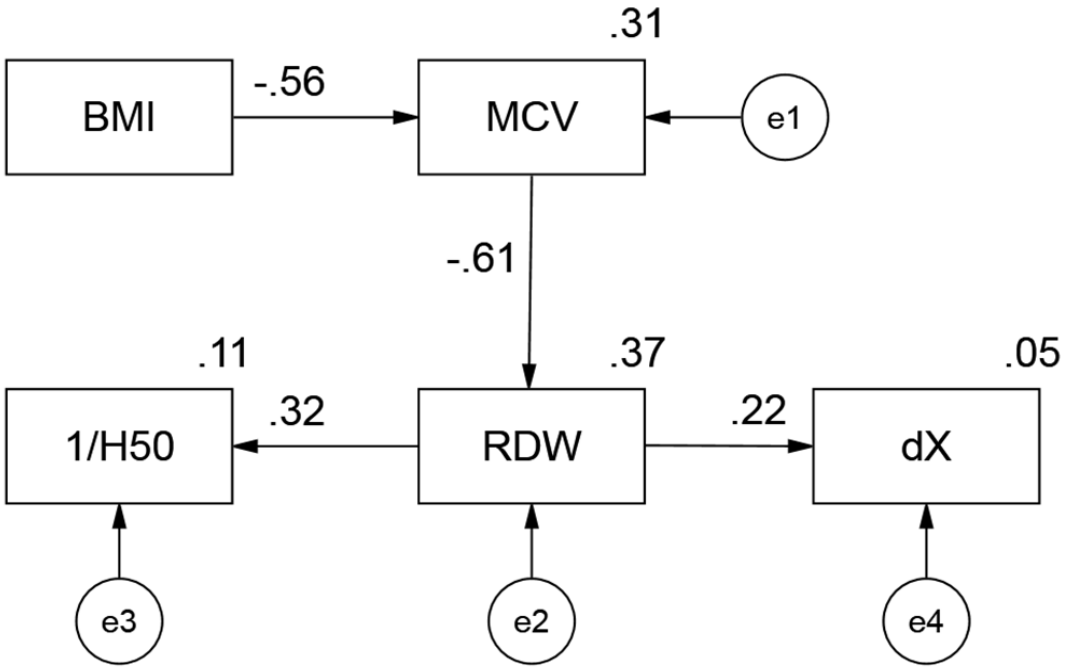

Figura 3: Ilustração de um exemplo de diagrama de caminho, com análise das correlações entre as variáveis de estabilidade de membrana de eritrócitos 1/H50 e dX, as variáveis hematimétricas MCV e RDW e a variável antropométrica BMI [Fonte: Garrote-Filho, 2014].

Tabela 1. Parâmetros de *model fit.*

Tipos de parâmetro	Abreviação	Nome	Valor de referência
Mais citados	CMIN p	Chi-square p	> 0.05
	GFI	Goodness Fit Index	> 0.95
	NNFI	Non Normal Fit Index	> 0.95
	CFI	Comparative Fit Index	> 0.95
	RMSEA	Root Mean Squared Error of Approx.	< 0.05
Importantes	CMIN/DF	-	< 2.00
	NFI	Normal Fit Index	> 0.95
	PCLOSE	-	> 0.05
	SRMR	Standarized Root Mean Residual	< 0.05

(Fonte: Uhman, 2006).

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Capítulo 2

Análises bivariada e multivariada da influência de variáveis sanguíneas de pacientes submetidos a cirurgia de desvio gástrico em Y-de-Roux sobre a estabilidade de membrana de eritrócitos em relação à ação caotrópica do etanol

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

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Keywords: Red blood cell, Membrane stability, Ethanol, Chaotropic action, Bariatric sugery, Feeding restriction

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Resumo

Análises bivariada e multivariada da influência de variáveis sanguíneas de pacientes submetidos a cirurgia de desvio gástrico em Y-de-Roux sobre a estabilidade de membrana de eritrócitos em relação à ação caotrópica do etanol

A estabilidade de membrana de eritrócitos, que é essencial para a manutenção da função dessas células, ocorre em uma região crítica de fluidez, que depende largamente de sua composição e das características do meio. Como a composição da membrana do eritrócito é influenciada por muitas variáveis sanguíneas, a estabilidade de membrana do eritrócito deve ter relações com elas. O presente estudo objetivou avaliar, por análises estatísticas bivariadas e multivariadas, as correlações e relações causais entre variáveis hematológicas e bioquímicas e a estabilidade de membrana de eritrócitos conta a ação caotrópica do etanol. A validade deste tipo de análise depende da homogeneidade da população e da variabilidade dos parâmetros estudados, condições que podem ser satisfeitas por pacientes que sofrem cirurgia bariátrica pela técnica do desvio gástrico em Y-de-Roux, uma vez que eles passam por restrições alimentares que têm grande impacto sobre a composição sanguínea deles. A análise de caminho revelou que um aumento na concentração de hemoglobina leva a uma diminuição da estabilidade da célula, provavelmente através de um processo mediado por um aumento no volume corpuscular médio (MCV). Além disso, um aumento na hemoglobina corpuscular media (MCH) leva a um aumento na estabilidade de membrana do eritrócito, provavelmente porque valores elevados de MCH são associados a menores quantidades de células vermelhas (RBC) e maiores áreas de contato entre a membrana da célula e o etanol presente no meio.

Palavras-chave: Células vermelhas do sangue, estabilidade de membrana, etanol, agentes caotrópicos, cirurgia bariátrica, restrição de alimentação

Abstract

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

The stability of the erythrocyte membrane, which is essential for maintenance of cell functions, occurs in a critical region of fluidity, which depends largely on its composition and the composition and characteristics of the medium. As the composition of the erythrocyte membrane is influenced by several blood variables, the stability of the erythrocyte membrane must have relations with them. The present study aimed to evaluate, by bivariate and multivariate statistical analyses, the correlations and causal relationships between hematological and biochemical variables and the stability of the erythrocyte membrane against the chaotropic action of ethanol. The validity of this type of analysis depends on the homogeneity of the population and on the variability of the studied parameters, conditions that can be filled by patients who undergo bariatric surgery by the technique of Roux-en-Y gastric bypass, since they will suffer feeding restrictions that have great impact on their blood composition. Pathway analysis revealed that an increase in hemoglobin leads to decreased stability of the cell, probably through a process mediated by an increase in MCV. Furthermore, an increase in the MCH leads to an increase in the erythrocyte membrane stability, probably because higher values of MCH are associated to smaller quantities of RBC and larger contact area between the cell membrane and ethanol present in the medium.

Keywords: Red blood cells, membrane stability, ethanol, chaotropic, bariatric surgery, feeding restriction.

Introduction

The membrane stability is essential for maintenance of cell functions, and compromising its integrity can not only affect these functions but also lead to cell death (Mcneil and Steinhardt 1997). In theory, the stability of a membrane can be changed by the same factors that affect protein stability, such as pH, temperature, drugs and stabilizing and destabilizing solutes. Furthermore, other factors, more specifically related to the composition of the membrane, as the contents of phospholipids and cholesterol, may also compromise the structural organization of the amphiphilic system of the membrane, as well as its fluidity (Singer and Nicholson 1972; Cribier et al. 1993; Murray et al. 2012). For the cell to remain physiologically active, it is essential that the membrane fluidity is within ideal limits (Garcia et al. 2005).

The membrane fluidity is essential for determining the shape, deformability and, therefore, the rheological properties of the cell (Aloulou et al. 2006; Maeda et al. 2006; Martínez et al. 1996; Seki et al. 2006; Velcheva et al. 2006; Zilberman-Kravits et al. 2006; Uydu et al. 2012).

Studies both in vitro and in vivo have shown that the cholesterol content in the erythrocyte membrane depends on the plasma lipoproteins (Cooper et al. 1972; Cooper et al. 1975; Cooper 1977) and the fluidity of these cells is markedly reduced with increasing mole fraction of cholesterol/phospholipids (Kroes et al. 1972; Vanderkooi et al. 1974; Shattil and Cooper, 1976; Lemmich et al. 1997; Cazzola et al. 2004). Notably low-density lipoprotein (LDL), which is responsible for the exchange of cholesterol with the erythrocyte membrane (Nikolić et al. 2007), affects the rheology of the blood fluid and therefore predisposes to atherosclerosis more intensely than the levels of total cholesterol (t-C) (Hoefner et al. 2001; Okada et al. 2004).

In spur-cell anemia erythrocytes have higher cholesterol content in their membranes (Cooper 1969), which leads to the development of a phenotype of spurs. Clinical observations show that when healthy red blood cells are transfused into an individual affected with this disease, they take the form of spur-cells due to the transfer of cholesterol from plasma lipoproteins to their membranes (Cooper 1969). Indeed, the relationship between membrane cholesterol and shape of the erythrocyte is evidenced by the correlation existing between content of cholesterol in this cell membrane and the variable

red cell distribution width (RDW). This finding links the RDW not only to the pathogenesis of atherosclerosis, as well as to its pathophysiological complications (Tziakas et al. 2007, 2008; Yu et al., 2010; Tziakas et al. 2011; Nishizaki et al. 2012; Tziakas et al. 2012).

The environment in which the red blood cell is found, therefore, is a decisive factor in determining the composition and fluidity of the cell membrane, and it can be changed by diet, physical activity and many diseases. Thus, the composition and degree of fluidity of the erythrocyte membrane should reflect these changes in blood (Schick and Schick 1985; Martínez et al. 1996; Ozdemirler et al. 2001). Imbalances in the diet, sedentary lifestyle and hereditary factors are conditions that may, alone or in combination, lead to disruption of energy homeostasis and to the development of dyslipidemia, which will affect the molar ratio of cholesterol/phospholipids of the erythrocyte membrane (Martínez et al. 1998; Michalska-Malecka et al. 2008; Spengler et al. 2008). Therefore, the stability of the erythrocyte membrane must have relations with the blood lipid levels.

The red blood cell is a suitable model for evaluation of the stability of the membrane, because it is a biological material which is easily obtainable through a minimally invasive procedure and without harm to the patient. Also, the hemolysis can be followed by spectrophotometric quantification of hemoglobin released in the lysis (Cunha et al. 2007; Penha-Silva et al. 2008; de Freitas et al. 2010; Fonseca et al. 2010; Mansur et al. 2010; Lemos et al. 2011).

This study assessed, through bivariate and multivariate statistical analysis, the correlations and causal relationships between the stability of erythrocytes against the chaotropic action of ethanol and blood variables (hematological and biochemical) in a population of patients undergoing Roux-en-Y gastric bypass (RYGB). After surgery, these patients suffer a drastic food restriction that has a huge impact on the blood variables (Custódio Afonso Rocha et al. 2012). The validity and effectiveness of a correlation analysis depends largely on the existence of a lower inter-individual variability, but with greater variability in the variables, conditions that can best be offered in the population considered in this study in relation to the general population.

Material and Methods

Population

This study was approved by the Ethics Committee in Research of the Federal University of Uberlândia (number 023/08) and all 24 volunteers who participated in the study signed terms of informed consent.

The subjects (8 women with class II obesity and comorbidities and 16 morbidly obese women, with mean age of 36.46±9.8 years) were recruited among the candidates for bariatric surgery of the Obesity Center of Uberlândia (CENTROBESO) who were not diabetic on insulin and not fulfill the general exclusion criteria adopted by the institution (anesthetic risk classified as ASA IV; esophagogastric varices with portal hypertension; significant intellectual limitations in patients without adequate family support; current uncontrolled psychiatric disorder, including abuse of alcohol and illicit drugs).

Collection of blood samples

Blood samples were collected by venipuncture in evacuated tubes (Vacutainer, Becton Dickinson, Juiz de Fora, MG, Brazil) containing EDTA as anticoagulant for determination of erythrogram and evaluation of membrane stability, and without anticoagulant for the biochemical determinations. The procedure of blood collection occurred after a 12 h overnight fast before surgery and on the 14th, 28th, 42nd and 56th days after surgery.

Determination of hematologic and biochemical variables

The erythrogram (automated system Cell-Dyn 3700 Abbott Diagnostics, Abbott Park, IL, USA) and the biochemical assays (automated analyzer Architect C 8000 Abbott Diagnostics, Abbott Park, IL, USA) were done in the Laboratory of Clinical Analyses of the Clinical Hospital of the Federal University of Uberlândia.

Reagents and equipments

The NaCl and ethanol used (Labsynth, Diadema, SP, Brazil) had a purity of 99.5%, which were duly corrected in the preparation of solutions. The mass measurements were made on a digital analytical balance (model 870, AND, Japan). The volume measurements

were made with automatic pipettes (Labsystems, Helsinki, Finland). Incubations were carried out in thermostatic bath model MA 184 (Marconi, Piracicaba, SP, Brazil). The absorbance readings were performed in a digital spectrophotometer model UV-1650 (Shimadzu, Tokyo, Japan). The centrifugations were performed in a centrifuge model CF15RX II (Hitachi Koki, Hitachinaka, Japan).

Determination of stability of human erythrocyte membrane against the chaotropic action of ethanol

Duplicate sets of microtubules (Eppendorf, Hamburg, Germany) containing 1.5 ml of NaCl 0.9 $g.dl^{-1}$ and increasing concentrations of 0-20% ethanol, were prepared and pre-incubated at 37 °C for 10 min. After preincubation, the microtubes received aliquots of 10 µl of whole blood and were carefully homogenized. After incubation for 30 min at 37 °C, the tubes were centrifuged for 10 min at 1600 x *g* and the supernatants were removed for reading of absorbance at 540 nm (A_{540}) (Cunha et al. 2007; Penha-Silva et al. 2007; de Freitas et al. 2008; Penha-Silva et al. 2008; de Freitas et al. 2010; Mansur et al. 2010; Lemos et al. 2011).

Determination of the transition curves of lysis

The dependence of A_{540} with the ethanol concentration was adjusted to a line of sigmoidal regression according to the Boltzmann equation,

$$A_{540} = \frac{A_1 - A_2}{1 + e^{(X - D_{50})/dXe}} + A_2 \qquad (1),$$

in which $\mathbf{A_1}$ and $\mathbf{A_2}$ represent, respectively, the mean values of $\mathbf{A_{540}}$ at the minimum and maximum plateaus, $\mathbf{D_{50}}$ is the concentration of ethanol capable of promoting 50% lysis, and $\mathbf{dX_e}$ is the variation in the chaotropic concentration responsible for converting erythrocytes from a completely integer ($\mathbf{A_1}$) to a completely lysed state ($\mathbf{A_2}$).

Statistical analysis of data

Relations between variables of erythrocyte stability against chaotropic action of ethanol and hematologic and biochemical variables considered in this study were treated statistically by bivariate and multivariate analyses.

In the bivariate correlations, each of the variables for evaluating the stability ($\mathbf{dX_e}$, $\mathbf{D_{50}}$ and $\mathbf{dX_e.D_{50}}$) was fitted by linear regression as a function of each of the different biochemical and hematological variables. From these correlations we obtained the matrices of correlation and significance that were used to build the groups of hematologic and biochemical variables of the multivariate treatment. These analyses were performed using the application Origin 8.0 (Microcal Inc., Northampton, Massachusetts, USA).

Multivariate analyses were made using the application of free distribution GENES (Federal University of Viçosa, Viçosa, MG, Brazil) and divided into two stages: (1) search for correlation and (2) search for cause and effect.

Canonical correlations were used to search for correlations between the mathematical parameters of stability and hematologic and biochemical variables. The group formed by the variables $\mathbf{dX_e}$ and $\mathbf{dX_e.D_{50}}$ constituted the dependent variable and the groups of hematologic or biochemical variables constituted the statistical explanatory variable. These analyses used the Pearson correlation matrices.

The study of relations of cause and effect has been developed through pathway analysis (Wrigth 1923; Li 1975; Khatri et al. 2012) and considered as the dependent variable the product $\mathbf{dX_e.D_{50}}$ and as explanatory variables the same groups of biochemical and hematological parameters used in canonical analysis.

Results and Discussion

In this study, blood samples from volunteers were analyzed before (0) and 14, 28, 42 and 56 days after bariatric surgery. A detailed description of the study population and the evolution of nutritional, hematologic and blood biochemical variables during the eight weeks of the study was previously published (Custódio Afonso Rocha et al. 2012). Table 1 presents the baseline characteristics of the study population. After surgery, there was a decrease in all biochemical parameters (glucose, total cholesterol, LDL-C, VLDL-C and triglycerides), with a tendency to stabilize, although HDL-C have also declined. After surgery, there was also a progressive decrease in the values of most hematologic variables (RBC, Hb, Ht, MCV, MCH and MCHC) and an increase in the RDW (Supplementary Table 1).

The study used patients submitted to bariatric surgery by the technique of "Roux-en-Y Gastric Bypass" (RYGB) due to the large influence of this procedure on the values of hematologic and biochemical variables. Greater variation in these variables is desirable in the search for correlations between them and the behavior of the erythrocyte membrane. The use of lipid and glucose-lowering drugs by some of the volunteers (Table 1) decreases the variability in blood levels of lipids and glucose. Similarly, supplementation of vitamins and minerals recommended as a routine practice for patients after 15 days of surgery (Custodio Alfonso Rocha et al. 2012), interferes with the variability of the erythrogram. Even with these limitations in the study population, the obtainment of the same variance in a group of people in the general population would require a significantly greater number of individuals, which would increase the inter-individual variations of various kinds, making difficult the obtainment of conclusions.

The multivariate statistical analysis was based on the matrix of bivariate correlations (Table 2) between the stability parameters and biochemical and hematologic variables, along with the respective matrix of significance (Table 3). The organization of the groups used in multivariate analyses (Table 4) was based on the nature of the variables (hematological or biochemical) and the nature (direct or reverse) and significance ($P < 0.05$) of the correlations presented in the correlation matrix.

The search for relationships between groups was performed by analysis of canonical correlations. The canonical correlations are a logical extension of multiple linear regressions, from which differs by correlate groups of dependent and independent variables. The base of

such statistical analysis is the construction of a linear combination of variables for each group in order to maximize the correlation between the two groups. The attribution of weights for the independent and the dependent variables allows us to obtain the maximum correlation (canonical correlation) between the two sets of variables (dependent and independent) (Hair et al. 2006). In these analyses, the previously organized groups of variables (Table 4) were the explanatory variable and the stability parameters, $\mathbf{dX_e}$ and $\mathbf{dX_e.D_{50}}$, constituted the dependent variable.

The combined variable of stability, $\mathbf{dX_e.D_{50}}$, was defined as a product of variables $\mathbf{dX_e}$ and $\mathbf{D_{50}}$ to preserve the direct relationships that both individual variables have with stability. These individual variables have different meanings. While $\mathbf{dX_e}$ represents the change in concentration of chaotrope (ethanol) necessary and sufficient to bring the erythrocyte population from an integer to a completely lysed state, $\mathbf{D_{50}}$ is the concentration of chaotropic necessary to promote the lysis of half the population of erythrocytes. A higher value of $\mathbf{dX_e}$ means that is less abrupt the transition of lysis and more stable is the erythrocyte membrane. Moreover, the higher the value of $\mathbf{D_{50}}$, the greater is the stability of the red cell population. The combined variable $\mathbf{dX_e.D_{50}}$ preserves the direct relations of both individual variables with the stability and may represent in an unique manner what the individual variables represent isolatedly.

A canonical correlation analysis does not allow obtaining findings in terms of cause and effect, so that the existence of canonical correlations between the groups of variables only mean the existence of associations which are measurable but not necessarily with a causal relationship between them. Thus, further analyses are necessary to better understand the relationships among the variables that define or influence certain characteristic of interest. It was in this sense that Li (1975) adapted and popularized the analysis of path coefficients, already used by other authors. The importance of the path analysis lies in the fact that it decomposes the effects observed in the correlation analysis in direct and indirect (or mediated) effects, which gives us greater security at the time of choosing the variables that are responsible for a particular effect of interest. It is through path analysis that we can state with certainty that between our findings, there were no false positive or false-negative correlations caused by the interaction of the multiple variables involved in the system. In a biological universe wherein the variables are involved in a

network of multiple influences, this possibility cannot be disregarded (Wrigth 1923; Li 1975; Khatri 2012).

The investigation of the cause and effect has been made using path analysis, in order to determine the existence of direct or indirect relationships of causes between $\mathbf{dX_e.D_{50}}$ and the variables of groups shown in Table 4.

The canonical correlations between the parameters of stability and the groups formed by hematological variables (groups 1 and 3 of Table 4) showed no significant relationship with the first canonical pair (Tables 5 and 7) and therefore will not be considered in this discussion.

The behavior observed for the hemolysis by ethanol seems to dangle from that observed for the hypotonic hemolysis. The osmotic stability of erythrocytes showed a strong correlation with RDW (Bernardino Neto 2011), differently from the stability in ethanol. The difference in the mechanism of action would be the cause of the difference between the osmotic lysis and lysis by ethanol. The lysis induced by ethanol is dependent on the direct contact of this chaotrope with the membrane, where it will promote opening of pores (Chi and Wu 1991). However, the hypotonic lysis is closely related to the osmolarity of the medium and the cell volume, so that the stability of red cells is very sensitive to these two factors and relates inversely to them. In this sense, it is possible that the osmotic stability could reflect better the pathological conditions that have been associated with RDW, including vascular complications in diabetes (Malandrino et al. 2012) and the risk of mortality from cardiovascular diseases (Patel et al. 2010; Nishizaki et al. 2012; Tziakas et al. 2012).

The biochemical parameters arranged in groups 2 and 4 (Table 4) showed significant or borderline correlations with the first canonical pair (Tables 6 and 8).

The variables from Group 2 had a borderline correlation (P = 0.0911) with the group constituted by $\mathbf{dX_e}$ and $\mathbf{dX_e.D_{50}}$, in which the parameters evaluators of the stability against the chaotropic action of ethanol have had canonical loads close together, which means that they contribute in an approximately equal manner to the correlations between these groups, with a slight predominance of the parameter $\mathbf{dX_e.D_{50}}$ in relation to $\mathbf{dX_e}$. Among the biochemical variables, LDL-C was the variable that showed the greatest weight in group 2.

LDL is the lipoprotein involved in exchange of cholesterol with the membrane of the erythrocytes by simple diffusion, directed to the structure with lower molar fraction

cholesterol/phospholipids in order to search for mass balance (Nikolić et al. 2007). This explains why the LDL-C was the most impactful variable in group 2. The direct correlation observed between the variables in group 2 and the stability of erythrocytes can be explained in light of the role of cholesterol in the membrane of the cell. The rigid steroid ring of cholesterol reduces the freedom of lateral movement of lipids and increases the intensity of the intermolecular forces of van-der-Waals, giving greater rigidity to the membrane of these cells (Hubbell and McConnell 1971; Shinitzky 1976; Raffy and Teissié 1999). The higher the plasma levels of LDL-C, the greater the flow of cholesterol to the membrane, which becomes more rigid and stable as long as there are no impairment of fluidity and deformability of the membrane. Stability must not be a property that varies linearly with increasing cholesterol concentration, since an excessive increase in cholesterol would reduce so drastically the membrane fluidity, affecting the deformability of the cell and also increasing its susceptibility to lysis.

The combined parameter $\mathbf{dX_e.D_{50}}$ was more impactful than the single parameter $\mathbf{dX_e}$ in the canonical correlation with the variables of group 2.

The variables of group 4 were those that had most significant correlations with the parameters $\mathbf{dX_e}$ and $\mathbf{dX_e.D_{50}}$ (P = 0.0060). These parameters again showed values of canonical loads close to one another, with $\mathbf{dX_e.D_{50}}$ contributing more to the correlation between the two groups, what corroborates with the assumption of greater coverage of the combined parameter. Blood glucose was the variable that gave greater weight in determining this correlation, followed by blood levels of triglycerides. The HDL-C presented a negative canonical load, contrary to what we saw in the bivariate regression, however without greater significance, given the small size of the absolute value of the load in relation to those of the other variables in its group.

The reverse direction found for the relation between stability and the values of the variable TG is justified, since VLDL-C = TG ÷ 5 (Friedewald et al. 1972).

The correlations between the parameters **dXe** and $\mathbf{dXe.D_{50}}$ and group 4 variables had a stronger influence of glucose, both characterized by decreased stability with increased blood glucose levels. It is likely that this loss in cell stability determined by glucose is related to the glycation membrane proteins, altering the structure and, in consequence, the function of biological molecules that play essential roles in maintaining the structural organization of the membrane amphiphilic system (Shin et al. 2008).

The path analyses to search for cause and effect relationships between stability and blood variables were conducted using the combined parameter of stability (**$dX_e.D_{50}$**), based on the fact that it was associated with larger canonical loads than the single parameter **dX_e**.

The path analyses did not reveal the existence of direct or mediated effects with values above the value of the residual variable for relations in groups 2, 3 and 4 with the parameter **$dX_e.D_{50}$** (Tables 10, 11 and 12). However, several values of direct and mediated effects were greater than the residual variable in the relations of group 1 variables and **$dX_e.D_{50}$**. The variable MCHC presented indirect effects through Hb and MCH greater than the value of the residual variable (Table 9). The variables Ht, Hb, MCV and MCH values had all direct and indirect effects greater than the value of the residual variable, except for the indirect effects through MCHC and the total effect.

The coefficient of determination obtained for group 1 in the pathway analysis was 0.1051 and the magnitude of the residual variable effect was 0.946 (Table 9). The variable Ht showed direct and positive effects on the stability 5.62 times greater than the magnitude of the value of the residual variable. This indicates that there must be a direct causal link between membrane stability of erythrocytes and hematocrit values. This relationship was statistically evident in the calculations of the path analysis. However, the strong and direct correlation between stability and hematocrit observed in the path analysis was not found in bivariate correlation and also did not appear in the value of the total effect in path analysis. This happened because this effect was masked by variables such as MCV and Hb, and other variables that were not involved in this study. This indicates that the use of simple linear correlation to study the relationship between stability of erythrocytes and Ht is not appropriate. There are external factors that are strong enough to prevent the correlation between these variables appear in a bivariate study (Bernardino Neto 2011).

The direct effects of MCV and hemoglobin on erythrocytes stability were negative and respectively 5.72 and 5.69 times greater than the magnitude of the residual variable. This means that the direct effects of Hb and MCV contribute to decrease the values of **$dX_e.D_{50}$** or, in other words, they contribute to reducing the stability of erythrocytes. A higher content of Hb in the erythrocyte induces a higher difference in concentration between internal and external environment of that cell, which must then hold more water and have a higher volume (MCV). The bulkier erythrocytes are less stable, while those less bulky, which have their membrane lipids organized with more approximation and intensification of the

molecular attractive forces of van-der-Waals, are more stable (Cunha et al. 2007; Penha-Silva et al. 2008).

The variable MCH presented a direct effect on the stability 6.67 times greater than the residual variable, with the positive direction, which means that the greater the value of MCH the higher is the stability of the erythrocyte membrane. This finding appears to conflict with the inverse relationship observed between the stability of erythrocyte membrane and hemoglobin concentration. How MCH and Hb could have opposite correlations with the stability being conceptually so close? What is the role of chaotrope (ethanol) in the relationship between MCH and stability, since this inverse relationship does not occur in erythrocyte lysis by hypotonic shock (Bernardino Neto 2011) but only in the lysis by ethanol?

The solution to this apparent paradox can be understood based on a simple analysis. From two different populations of cells with the same content of Hb, the population with higher MCH should have fewer cells (lower RBC count) and thus lower contact surface between cells and medium. If lysis by ethanol occurs by leakage of the membrane phospholipids by direct action of chaotropic on the cell surface, a lower surface of contact between cell and medium should decrease the effectiveness of this mechanism (Fig. 1), requiring a higher concentration of ethanol to promoting lysis, ie higher values of $\mathbf{D_{50}}$ and $\mathbf{dX_e.D_{50}}$. This would explain the direct relationship between MCH and stability of erythrocytes against the chaotropic action of ethanol. Nevertheless, the hypotonic lysis must be solely dependent on salt concentration difference between the internal and external media of the cell but not on the amount of cells. As the concentration of water in the external environment is substantially greater than the concentration of ethanol, the variation in surface contact between the cell membrane and the medium caused by changes in cell count (RBC) does not affect the mechanism of lysis by hypotonic shock with the same intensity as it affects the mechanism of lysis by chaotropic action of ethanol.

LDL-C is correlated closely with the fluidity and therefore the stability of the membrane. Since LDL is the more important lipoprotein in assessing the risk of atherosclerosis, this lipoprotein must be a link between the stability of the erythrocyte and the risk of developing this disease. This should indicate that the stability of the erythrocyte may be a further indirect indicator of the risk of developing atherosclerosis.

As **$dX_e.D_{50}$** was more sensitive to changes in blood variables that affect the stability of the membrane in response to the chaotropic action of ethanol, it should be the elective indicator of the stability of erythrocyte.

The search for causal relations for the stability of the erythrocyte membranes by multivariate analysis revealed that the bivariate regression can produce some questionable results due to the variable being involved in a complex system under the influence of a wide range of factors. This was evident in the analysis of correlation between the stability of the erythrocyte and hemoglobin, in which the direct relationship between these variables could only be verified in pathway analysis after excluding the influence of other variables in the same group. Hemoglobin contributes to reducing the stability of the cell, probably through a process mediated by an increase in MCV. For the specific mechanism of lysis by the action of ethanol, the stability of erythrocyte membrane varies directly with MCH, probably because higher values of MCH are related to lower values of RBC and greater contact surface between the membrane and the chaotropic agent in the solution.

Many other variables of the complex system that is the human blood also exert influence on the stability of the erythrocyte membrane. These variables include plasma concentrations of electrolytes and albumin (Fonseca et al. 2010) and also the proper composition of the cell membrane. Besides these, there are the variables that have influence on the erythrogram, as blood levels of iron, folate and cyanocobalamin (Alves de Rezende et al 2009, Toh et al. 2009). Certainly, the inclusion of all these variables in the analysis should allow obtaining an even more accurate picture of the interrelationship between the stability of red cell membrane against the chaotropic action of ethanol and its natural environment in human blood.

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Table 1 Baseline characteristics of the study population

Characteristic	
Number of subjects (n)	24
Gender (n, %)	
Female	24 (100.0)
Age (y)*	36.5 ± 9.8
BMI (kg/m^2)*	44.3 ± 5.3
Hematologic profile	
Red Blood Cells (million/mm^3)	4.67 ± 0.3
Hemoglobin (g/dl)	13.7 ± 0.8
Hematocrit (%)	40.7 ± 2.5
Mean Corpuscular Volume (fl)	87.2 ± 4.9
Mean Corpuscular Hemoglobin (pg)	29.4 ±2.1
Mean Corpuscular Hemoglobin Concentration (g/dl)	33.7 ± 1.0
Red Cell Distribution Width (%)	15.4 ± 1.0
Lipid profile	
Total cholesterol (mg/dl)	197.54 ± 39.3
High Density Lipoprotein Cholesterol	45.7 ± 9.3
Low Density Lipoprotein Cholesterol	120.6 ±33.7
Very Low Density Lipoprotein Cholesterol	31.3 ± 14.2
Triglycerides (mg/dl)	156.3 ± 70.9
Medical history (n, %)[§]	
Depression	1 (4.2)
Diabetes mellitus	1 (4.2)
Disc herniation	1 (4.2)
Hiatal hernia	1 (4.2)
Hypertension	11 (45.8)
Hypothyroidism	2 (8.3)
Impaired fasting glucose	7 (29.2)
Osteoarthritis	2 (8.3)
Tachycardia	1 (4.2)
Medication (n, %)[§]	
Amlodipine	1 (4.2)
Atenolol	4 (16.7)
Atorvastatin	1 (4.2)
Captopril	1 (4.2)
Ciprofibrate	1 (4.2)
Cyclobenzaprine hydrochloride	1 (4.2)
Enalapril	4 (16.7)
Escitalopram	1 (4.2)
Fluoxetine	2 (8.3)
Hydrochlorothiazide	3 (12.5)
Indapamide	2 (8.3)
Levothyroxine sodium	3 (12.5)
Metformin	4 (16.7)
Omeprazole	2 (8.3)
Potassium losartan	2 (8.3)
Sertraline	2 (8.3)
Simvastatin	2 (8.3)
Without medication	10 (41.7)

*Mean ± sd

[§] Some patients had multiple medical conditions and were under use of different types of drugs.

Table 2 Matrix of correlations (R^2) of biochemical and hematological variables with the parameters of stability against the chaotropic action of ethanol (dX_e, D_{50}, and $dX_e.D_{50}$)

	Glu	TG	t-C	HDL	LDL	RBC	Ht	Hb	MCV	MCH	MCHC	RDW	D_{50}	dXe	$dX_e.D_{50}$
Glu	1.000														
TG	0.004	1.000													
t-C	0.039 §	0.259 §	1.000												
HDL-C	0.065 §	0.000	0.039 §	1.000											
LDL-C	0.089 §	0.088 §	0.908 §	0.000	1.000										
RBC	0.018	0.008	0.007	0.024	0.011	1.000									
Ht	0.002	0.000	0.004	0.001	0.007	0.472 §	1.000								
Hb	0.005	0.003	0.010	0.001	0.018	0.378 §	0.912 §	1.000							
MCV	0.008	0.020	0.001	0.017	0.001	0.089 §	0.239 §	0.267 §	1.000						
MCH	0.004	0.028	0.000	0.012	0.001	0.109 §	0.166 §	0.292 §	0.872 §	1.000					
MCHC	0.006	0.018	0.011	0.001	0.026	0.033	0.006	0.048 §	0.015	0.217 §	1.000				
RDW	0.001	0.000	0.001	0.000	0.002	0.006	0.153 §	0.161 §	0.384 §	0.327 §	0.002	1.000			
D_{50}	0.067 §	0.072 §	0.016	0.004	0.042 §	0.001	0.001	0.001	0.000	0.000	0.001	0.005	1.000		
dX_e	0.062 §	0.011	0.002	0.001	0.007	0.003	0.021	0.021	0.060 §	0.053 §	0.001	0.032	0.052	1.000	
$dX_e.D_{50}$	0.069 §	0.014	0.003	0.001	0.010	0.003	0.020	0.021	0.056 §	0.051 §	0.001	0.032	0.089	0.994	1.000

*Glu: blood glucose; TG: triglycerides; t-C: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; RBC: red blood cell; Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width

§ R^2 values associated to significant correlations (P <0.05)

Table 3 Matrix of significances of biochemical and hematological variables with the parameters of stability against the chaotropic action of ethanol (dX_e, D_{50}, and $dX_e.D_{50}$)

	Glu	TG	t-C	HDL-C	LDL-C	RBC	Ht	Hb	MCV	MCH	MCHM	RDW	D_{50}	dX_e	$dX_e.D_{50}$
Glu															
TG	0.510														
t-C	0.042 §	0.000 §													
HDL-C	0.008 §	0.968	0.041 §												
LDL-C	0.002 §	0.002 §	0.000 §	0.919											
RBC	0.169	0.375	0.379	0.111	0.271										
Ht	0.621	0.853	0.526	0.705	0.391	0.000 §									
Hb	0.458	0.590	0.317	0.704	0.168	0.000 §	0.000 §								
MCV	0.347	0.152	0.750	0.174	0.797	0.002 §	0.000 §	0.000 §							
MCH	0.538	0.087	0.930	0.258	0.740	0.001 §	0.000 §	0.000 §	0.000 §						
MCHC	0.421	0.165	0.287	0.797	0.095	0.061	0.431	0.023 §	0.215	0.000 §					
RDW	0.810	0.893	0.701	0.979	0.633	0.435	0.000 §	0.000 §	0.000 §	0.000 §	0.623				
D_{50}	0.007 §	0.005 §	0.196	0.513	0.033 §	0.696	0.761	0.708	0.976	0.916	0.791	0.458			
dX_e	0.010 §	0.284	0.676	0.742	0.391	0.586	0.135	0.133	0.011 §	0.017 §	0.775	0.063	0.019		
$dX_e.D_{50}$	0.006 §	0.222	0.599	0.811	0.315	0.610	0.143	0.136	0.014 §	0.019 §	0.735	0.064	0.002	0.000	

*Glu: blood glucose; TG: triglycerides; t-C: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; RBC: red blood cell; Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width

§ $P<0.05$ indicating statiscally significant correlations

Table 4 Organized groups for the multivariate statistical analyzes (canonical correlations and path analyzes)

Group	Variables*	Characteristics
Group 1	Hb, Ht, MCV, MCH and MCHC	Hematologic variables whose simple correlations are positive with dX_e and/or $dX_e.D_{50}$
Group 2	t-C, LDL-C	Biochemical variables whose simple correlations are positive with dX_e and/or $dX_e.D_{50}$
Group 3	RBC and RDW	Hematologic variables whose simple correlations are negative with dX_e and/or $dX_e.D_{50}$
Group 4	Glu, TG, HDL-C	Biochemical variables whose simple correlations are negative with dX_e and/or $dX_e.D_{50}$

*Glu: blood glucose; TG: triglycerides; t-C: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; RBC: red blood cell; Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width

Table 5 Canonical correlations and canonical pairs estimated between the stability parameters (dX_e e $dX_e.D_{50}$) and the variables of group 1

	Canonical Pairs	
	1st	2nd
R	0.3284	0.1230
Significance	0.2130	0.8169

Variables*	First Canonical Pair	
dX_e	0.9956	-0.0943
dX_e . D_{50}	0.9850	-0.1723
Ht	0.4494	0.1350
Hb	0.4462	-0.0253
MCV	0.7612	0.4436
MCH	0.7113	0.1823
MCHC	0.0662	-0.5494

*Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC mean corpuscular hemoglobin concentration

Table 6 Canonical correlations and canonical pairs estimated between the stability parameters (dX_e e $dX_e.D_{50}$) and the variables of group 2

	Canonical Pairs	
	1st.	2nd.
r	0.2692	0.0470
Significance	0.0911	0.6322

Variable*	First Canonical Pair	
dX_e	-0.5501	0.8351
$dX_e.D_{50}$	-0.6141	0.7893
t-C	-0.5051	-0.8631
LDL-C	-0.7436	-0.6686

*t-C: total cholesterol; LDL-C: low density lipoprotein cholesterol

Table 7 Canonical correlations and canonical pairs estimated between the stability parameters (dX_e e $dX_e.D_{50}$) and the variables of group 3

	Canonical Pairs	
	1st.	2nd.
R	0.1847	0.0387
Significance	0.4408	0.6936

Variables*	First Canonical Pair	
dX_e	0.9985	-0.0542
$dX_e.D_{50}$	0.9912	-0.1325
RBC	-0.2991	-0.9542
RDW	-0.9742	0.2255

*RBC: red blood cell; RDW: red cell distribution width

Table 8 Canonical correlations and canonical pairs estimated between the stability parameters (dX_e e $dX_e.D_{50}$) and the variables of group 4

	Canonical Pairs	
	1st.	2nd.
r	0.3871	0.1149
Significance	0.0060[§]	0.5043

Variables *	First Canonical Pair	
dX_e	-0.6485	0.7612
$dX_e.D_{50}$	-0.7064	0.7078
TG	0.5446	0.3680
HDL-C	-0.1635	-0.8373
Glu	0.7884	-0.5816

* TG: triglycerides; HDL-C: high density lipoprotein cholesterol; Glu: blood glucose

[§] $P < 0.05$ indicating statistically significant correlations

Table 9 Direct, indirect and total effects of the correlations between the variables of group 1 and the parameter $dX_e.D_{50}$*

Variable§	Effect	Via	$dX_e.D_{50}$	Variable§	Effect	Via	$dX_e.D_{50}$
Ht	Direct		5.3176	MCH	Direct		6.3137
		Hb	-5.1720			Ht	2.1694
		MCV	-2.6294			Hb	-2.9216
	Indirect	MCH	2.5758		Indirect	MCV	-5.0297
		CHCM	0.0505			MCHC	-0.3057
	Total		0.1425		Total		0.2262
Hb	Direct		-5.4152	MCHC	Direct		-0.6565
		Ht	5.0787			Ht	-0.4092
		MCV	-2.7807			Hb	-1.1900
	Indirect	MCH	3.4064		Indirect	MCV	-0.6509
		MCHC	-0.1443			MCH	2.9396
	Total		0.1449		Total		0.0331
MCV	Direct		-5.3831				
		Ht	2.5974				
		Hb	-2.7973				
	Indirect	MCH	5.8992				
		MCHC	-0.0794				
	Total		0.2368				

*Effect of the residual variable: 0.9460; coefficient of determination: 0.1051.

§Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration.

Table 10 Direct, indirect and total effects of the correlations between the variables of group 2 and $\mathbf{dX_e.H_{50}}$*

Variable§	Effect	Via	$dX_e.D_{50}$	Variable§	Effect	Via	$dX_e.D_{50}$
LDL-C	Direct		0.5308	t-C	Direct		-0.4542
	Indirect	t-C	-0.4327		Indirect	LDL-C	0.5056
	Total		0.0981		Total		0.0515

*Effect of the residual variable: 0.9855; coefficient of determination: 0.0287

§LDL-C: low density lipoprotein cholesterol; t-C: total cholesterol

Table 11 Direct, indirect and total effects for the correlations between the variables of group 3 and $dX_e.D_{50}$*

Variable§	Effect	Via	$dX_e.D_{50}$	Variable§	Effect	Via	$dXe.D_{50}$
RBC	Direct		-0.0364	RDW	Direct		-0.1768
	Indirect	RDW	-0.0135		Indirect	RBC	-0.0028
	Total		-0.0499		Total		-0.1796

*Effect of the residual variable: 0.9830; coefficient of determination: 0.0335.

§RBC: red blood cell; RDW: red cell distribution width.

Table 12 Direct, indirect and total effects for the correlations between the variables of group 4 and $dX_e.D_{50}$*

Variable§	Effect	Via	$dX_e.D_{50}$	Variable§	Effect	Via	$dX_e.D_{50}$
Glu	Direct		-0.2675	TG	Direct		-0.1016
	Indirect	TG	-0.0065		Indirect	Glu	-0.0172
		HDL-C	0.0112			HDL-C	-0.0002
	Total		-0.2629		Total		-0.1190
HDL-C	Direct		0.0440				
	Indirect	Glu	-0.0678				
		TG	0.0003				
	Total		-0.0234				

*Effect of the residual variable: 0.9584; coefficient of determination: 0.0814

§Glu: blood glucose; HDL-C: high density lipoprotein cholesterol; TG: triglycerides

Supplementary Table 1 Percentage of change in values of hematologic and biochemical variables at different times after Roux-en-Y gastric bypass (n=24)*

Variables[§]	14th day	28th day	42nd day	56th day
RBC	1.50	-1.71	-3.640	-3.43
Hb	-0.80	-3.94	-5.835	-5.40
Ht	0.59	-2.53	-4.969	-4.50
MCV	-0.91	-0.89	-1.376	-1.16
MCH	-2.21	-2.45	-2.277	-2.04
MCHC	-1.36	-1.48	-0.919	-0.92
RDW	-1.62	2.14	4.481	6.88
Glu	-7.32	-8.60	-6.547	-11.40
t-C	-4.16	-14.83	-14.534	-13.92
HDL-C	-23.01	-18.11	-11.899	-8.05
LDL-C	3.76	-12.52	-14.192	-13.13
VLDL-C	-7.10	-19.10	-19.706	-25.56
TG	-7.09	-19.09	-19.701	-25.57

*Data reported in Custódio Afonso Rocha et al. (2012) with inclusion of two new volunteers

[§]Glu: blood glucose; TG: triglycerides; t-C: total cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; RBC: red blood cell; Ht: hematocrit; Hb: hemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width

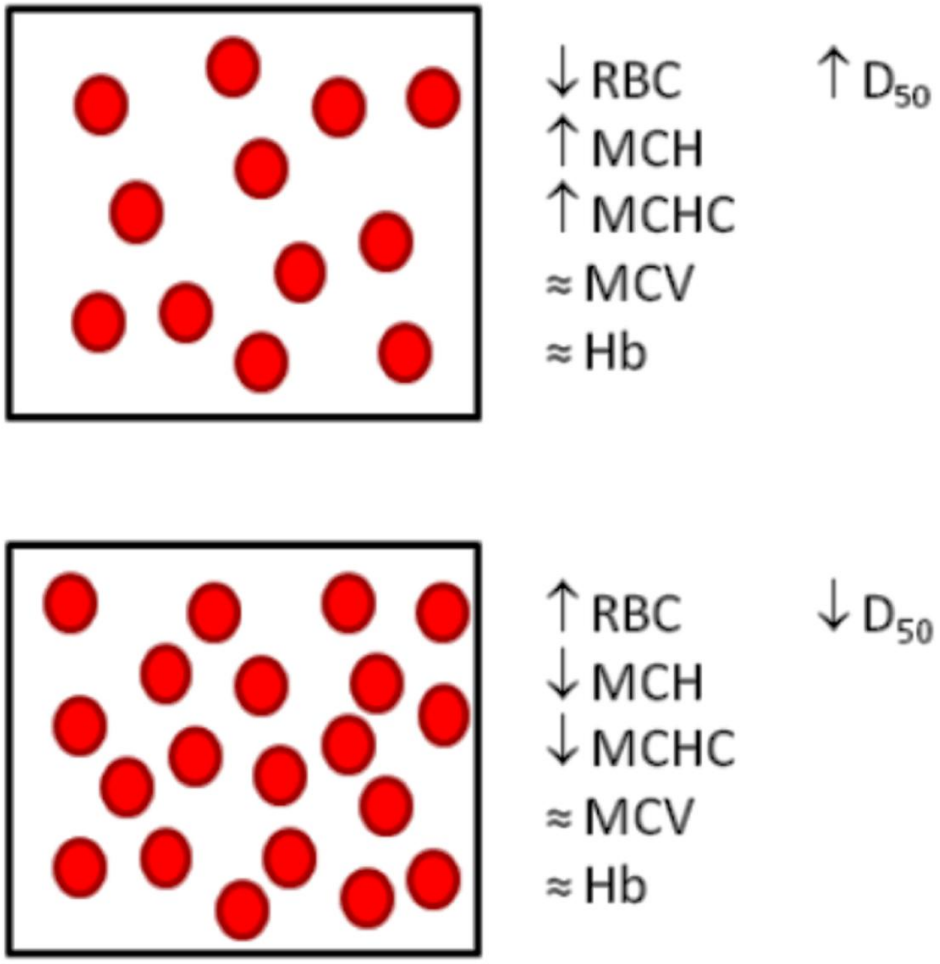

Fig. 1 Analogy used to explain why the MCH has a positive effect on the stability of erythrocytes against the chaotropic action of ethanol. The higher the value of RBC, the greater the contact surface between the cell membrane and medium (ethanol). This makes more efficient the lysis induced by the chaotrope, so that lower concentrations of ethanol are required, ie, the lower the stability (and lower the D_{50}) of the erythrocyte

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Capítulo 3

Efeito da cirurgia bariátrica sobre a distribuição de volumes e a estabilidade osmótica de células vermelhas do sangue

Effect of the bariatric surgery on the distribution width and osmotic stability of red blood cells

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Resumo

Efeito da cirurgia bariátrica sobre a distribuição de volumes e a estabilidade osmótica de células vermelhas do sangue

A necessidade de tratar a obesidade, um crescente problema de saúde pública em todo o mundo, tem levado a um aumento na execução de cirurgia bariátrica, particularmente o desvio gástrico pelo Y-de-Roux. A súbita mudança nos hábitos alimentares, resultante deste tipo de cirurgia, leva a mudanças abruptas no corpo. Este estudo analisou a correlação entre a estabilidade osmótica de eritrócitos e vários índices hematológicos e bioquímicos em uma população constituída de 24 participantes do sexo feminino, antes e em quatro diferentes momentos após a cirurgia, distribuídos ao longo de oito semanas, o que permitiu a geração de 120 pontos amostrais. A estabilidade osmótica de eritrócitos mostrou ser de grande importância para a compreensão do significado da distribuição de volumes das células vermelhas do sangue (RDW), porque as variáveis de estabilidade ($1/H_{50}$ and dX) foram positivamente correlacionadas com este índice hematológico. Entretanto, as variáveis de estabilidade e o RDW parecem sofrer diferentes influências de outras variáveis, como o LDL-colesterol (LDL-C), porque somente o RDW aumentou ao longo do tempo após a cirurgia. Realmente, a variável de estabilidade $1/H_{50}$ apresentou correlação positiva com os níveis sanguíneos de LDL-C, os quais diminuíram ao longo do tempo. A análise de caminho mostrou que o índice de massa corporal (BMI) tem um efeito indireto, mediado pelo RDW, sobre a estabilidade osmótica de eritrócitos. As correlações que as variáveis de estabilidade osmótica apresentaram com RDW podem ajudar na compreensão da origem da habilidade preditiva que este índice hematológico tem em relação a várias condições patológicas.

Palavras-chave: Eritrócitos, estabilidade de membrana, RDW, análise de caminho

Abstract

Effect of the bariatric surgery on the distribution width and osmotic stability of red blood cells

The need to treat obesity, a growing worldwide public health problem, has led to an increase in performing bariatric surgery, particularly the Roux-en-Y gastric bypass. The sudden change in eating habits, resulting from this type of surgery, leads to abrupt changes in the body. This study analyzed the correlation between the osmotic stability of erythrocytes and various biochemical and hematological indices in a population consisting of 24 female volunteers, before and at four different times after surgery, distributed along eight weeks, what allowed the generation of 120 sampling points. The osmotic stability of erythrocytes proved to be of great importance for understanding the meaning of the red-cell distribution width (RDW), because the stability variables ($1/H_{50}$ and dX) were positively correlated with this hematological index. However, the stability variables and RDW seem to suffer different influences from other variables, as the LDL-cholesterol (LDL-C), because only RDW has increased throughout time. Indeed, the stability of variable $1/H_{50}$ showed positive correlation with the blood levels of LDL-C, which declined throughout time. Path analysis showed that the body mass index (BMI) has an indirect effect, mediated by RDW, on the osmotic stability of erythrocytes. The correlations that the osmotic stability variables presented with RDW may help to understand the origin of the predictive ability of this hematological index in relation to various pathological conditions.

Key words: Erythrocytes, membrane stability, RDW, path analysis

Introduction

Bariatric surgery limits the intake and absorption of nutrients that are essential to the human body. And several of these nutrients exert an influence in the erythrocytes. One of those nutrients is iron, whose deficiency results in anemia, which is reflected in a decrease in the RBC (Red Blood Cell) count. Such iron deficiency also results in alteration of other hematologic parameters that include both reduction of MCV (Mean corpuscular volume) as MCH (Mean Corpuscular Hemoglobin), as well as increased RDW (red cell distribution width) (Vaya et al., 2010; Vaya et al., 2005).

The increase in RDW was also observed in various situations that cause health impairment (Hunziker *et al.*, 2012). These situations include cardiovascular disease (Cavusoglu et al., 2010; Ephrem, 2013), diabetes (Malandrino *et al.*, 2012) and aging (Patel et al., 2010), among others. Despite the large number of articles recently published on this subject, the relationship between RDW and these different conditions has not yet been duly clarified.

Red cells have often been used as a study model to investigate the influence of several factors affecting the stability of biological membrane. Such factors include changes in the environment in which the erythrocytes are, as hypotonicity, which results in increased hemolysis (de Freitas et al., 2010; Mascarenhas Netto Rde et al., 2014; Penha-Silva et al., 2007). Other factors that also impair the stability of erythrocytes include the presence of the chaotropes in the environment in which the erythrocytes are. These chaotropes include ethanol (Cunha et al., 2007; de Arvelos et al., 2013; Penha-Silva et al., 2008) and sodium dodecyl sulfate (de Freitas et al., 2014; Fonseca et al., 2010).

The aim of this study is to assess how the osmotic stability of erythrocytes is affected by bariatric surgery using Roux-en-Y Gastric Bypass, which is currently the most frequently performed procedure in patients with morbid obesity (Gasteyger et al., 2008; Shikora, 2000). This has great relevance to public health because the number of obese and surgical interventions have increased over time (Gasteyger et al., 2008).

Materials and Methods

Sample size

This study was approved by the Ethics Committee of the Federal University of Uberlândia (number 023/08). The study population consisted of 24 female participants (36.46 ± 9.8 years), with class II obesity and comorbidities (8) or morbid obesity (16), subjected to bariatric surgery at the Obesity Center of Uberlândia, between August 2009 and September 2010. They all signed terms of informed consent. For inclusion were respected all the exclusion criteria for the conduct of bariatric surgery: anesthetic risk classified by the American Society of Anesthesiology as ASA IV; portal hypertension with esophagogastric varices; significant intellectual limitation in patients without adequate family support; current uncontrolled psychiatric disorders, including abuse of alcohol and illicit drugs. Also diabetic patients using insulin were excluded. The reviews considered in this work were made before surgery and the 14, 28, 42 and 56 days after surgery, totaling 120 sample points. With the removal of discrepant sampling points (outliers), the effective sample was 111.

Collection of blood samples

After overnight fasting for 8 to 12 hours, blood samples were collected by venipuncture in three evacuated tubes (Vacutainer™, Becton Dickinson, Juiz de Fora, MG, Brazil) containing 1.8 $\mathrm{mg/mL}$ K_3EDTA as anti-coagulant, for determination of erythrograms and evaluating the stability of the erythrocyte membrane, and also in tubes without anti-coagulant, for the biochemical assays.

Reagents e equipments

The NaCl used in the experiments (Labsynth, Diadema, SP, Brazil) had a purity of 99.5%, which was duly corrected for preparation of solutions. Mass measurements were made in an analytical balance model 870 (AND, Japan). Volume determinations were made in refractory glass beakers or automatic pipettes (Labsystems, Finnpipette Digital model, Helsinki, Finland). The incubations were done in thermostated water bath model MA 184 (Marconi, Piracicaba, SP, Brazil). Absorbance readings were done with a digital spectrophotometer model UV-1650 (Shimadzu, Tokyo, Japan) under control of the software

UV Probe 2.21. Centrifugations were performed in a centrifuge model CF15RX II (Hitachi Koki, Hitachinaka, Japan). The determination of the erythrogram (erythrocytes count, RBC; hemoglobin, Hb; hematocrit, Ht; mean corpuscular hemoglobin, MCH; mean corpuscular volume, MCV; mean corpuscular hemoglobin concentration, MCHC; and red-cell distribution width, RDW) was done with help of an automated system (Cell-Dyn 3700 Abbott Diagnostics, Abbott Park, IL, USA). The biochemical measurements (total cholesterol, t-C; HDL-cholesterol, HDL-C; LDL-cholesterol, LDL-C; VLDL-cholesterol, VLDL-C; triglycerides, TGC; and glucose, Glu) were done with the help of an automatic analyzer (Architect C 8000 Abbott Diagnostics, Abbott Park, IL, USA) were done in the Laboratory of Clinical Analyses of the Clinical Hospital of the Federal University of Uberlandia.

Measurement of the osmotic stability of erythrocytes

Duplicate sets of 15 mini vials (Eppendorf, Hamburg, Germany) containing 1.5 ml solution with concentrations of 0 to 0.9 $g.dL^{-1}$ NaCl were preincubated for 10 min at 37 °C. After addition of 10 µL aliquots of whole blood and mixing, the tubes were incubated for 30 min at 37 °C and then centrifuged for 10 min at 1600 x *g*. The supernatants were removed for absorbance readings at 540 nm (A_{540}).

The dependence of A540 with the NaCl concentration was adjusted to a sigmoidal regression line according to the Boltzmann equation,

$$A_{540} = \frac{A_{max} - A_{min}}{1 + e^{(X - H_{50})/dX}} + A_{min} \quad (1),$$

where A_{max} and A_{min} represent the mean A_{540} values of the maximum and minimum plateaus, H_{50} is the NaCl concentration capable of promoting 50% hemolysis, and dX is the variation in the concentration of NaCl responsible for lysis of the entire population of erythrocytes (Figure 1).

Blood samples containing erythrocytes that are osmotically more resistant have lower values of H_{50}. Therefore, the variation of the values H_{50} occurs in the opposite direction to the osmotic stability. To avoid confusion and facilitate the interpretation of the results, we used the parameter $1/H_{50}$ instead of H_{50}. The values of $1/H_{50}$ and dX obtained for each patient were used to assess the stability of the erythrocyte membrane.

Statistical analyses

Sample size was estimated to achieve power of 0.80 and significance of 0.05. The data were tested for normality using the Kolmogorov-Smirnov test. All parameters were normally distributed and were correlated using Pearson's correlation analysis. Only correlations with $p \leq 0.05$ were considered significant. The results were also evaluated by path analysis. Path analysis is a type of Structural Equation Modeling, which is currently considered the most advanced multivariate tool that is available. This tool allows representing complex correlations in a visual way through the so-called path diagrams, which are also used to test the validity of the theoretical model represented in this type of diagram. With this tool it is also possible to evaluate the indirect effects among the studied variables.

The osmotic stability parameters were calculated using the Origin Pro 9.0 software (MicroCal, Northampton, MA, USA). This software was also used to analyze the distribution of data, correlations between variables and to compare means. Path analysis was performed using the SPSS software AMOS 21 (IBM, Armonk, NY).

Results and Discussion

Correlations involving time

The effectiveness of bariatric surgery can be assessed by BMI reduction throughout time. This is due to a lower intake and absorption of nutrients related to body weight, which is reflected in the correlation of LDL-C and TGC with time. This and other statistically significant bivariate correlations are shown in Table 1.

Bariatric surgery also limits the absorption of nutrients that exert their effect on hematological parameters. These nutrients include iron, folate and cobalamin. A deficiency of any of these nutrients results in anemia (Macdougall, 1968; Vaya et al., 2005). This explains the decrease in RBC, Hb and Ht, as well as the increase in RDW with the passage of time.

On the other hand, the variables MCV and MCH were not correlated with time, as shown in Table 1. This can be seen in Fig 2, since the straight line that represents the value of MCV is kept substantially parallel to the axis that represents time. Despite not having been shown, the correlation between MCH and time also consists of a line parallel to the time axis and was omitted for not making this figure unnecessarily loaded.

Certainly, the similar behavior observed for MCV and MCH is due to the high collinearity between these two hematological indices, a result of how the variables are determined (Figure 3). Basically, MCH comes from the ratio Hb/RBC, while MCV is the quotient of Ht/RBC. As RBC, Hb and Ht had a negative correlation with time, this means that the decrease in RBC was accompanied by a reduction of Hb and Ht, in such a way that the calculated values of MCV and MCH did not change significantly with time.

The fact that patients tended to anemia and simultaneously presenting a constant MCV suggests the occurrence of deficiency of iron and also folate and/or cobalamin. This is because iron deficiency results in microcytosis, whereas deficiencies of folate and/or cobalamin promote macrocytosis (Prueksaritanond et al., 2013; Song et al., 2010). As the MCV is a calculated parameter, its value would remain the same. On the other hand, the increase in the value of RDW throughout time suggests that the populations of small and large erythrocytes have increased concomitantly. Due to the reduction in RBC, this possibly occurred at the expenses of the normal size erythrocytes. This hypothesis is exemplified in Fig 4.

Osmotic stability of erythrocytes and blood levels of lipids

A positive correlation was found between $1/H_{50}$ and LDL-C. Thus, the decrease in LDL-C due to bariatric surgery results in a decrease of $1/H_{50}$. This reinforces the idea that bariatric surgery causes a reduction in erythrocyte osmotic stability.

Erythrocytes would become osmotically less stable due to bariatric surgery because the LDL-C is a major source of cholesterol for the erythrocyte membrane (Hui & Harmony, 1979; Lange & Slayton, 1982). As there was a decrease in the blood levels of LDL-C, it is also expected that the cholesterol content of the erythrocyte membrane also had decreased. With less cholesterol, the erythrocyte membrane becomes less rigid (Hui & Harmony, 1979; Lange & Slayton, 1982).

Erythrocytes with less rigid membranes, when exposed to a hypotonic environment, are less able to resist against the expansion of volume by the water intake that occurs in those cells. This means that these erythrocytes are osmotically less stable, since they are more easily lysed.

In principle, it seems contradictory that bariatric surgery, which is a procedure considered beneficial to the human health, comes to result in osmotically less stable erythrocytes. In order to allow clarification of this apparent contradiction, it is important to note that the experiments were performed *in vitro* and were done to evaluate the osmotic stability. However, the hypotonic condition that promotes the *in vitro* hemolysis will not happen *in vivo*. In the *in vivo* conditions, the mechanical stability, which is associated to the deformability of these cells, is much more relevant than the osmotic stability (Chasis, Agre & Mohandas, 1988; Vaya et al., 2005).

During its period of existence in the body, red blood cells need to be deformable so that they can pass through capillaries of small diameter. This deformability is greater in erythrocytes with more fluid membranes. This can occur when the membrane becomes less rich in cholesterol (Cazzola et al., 2011; Chabanel et al., 1983; Cooper et al., 1975). Therefore, bariatric surgery would increase the deformability of erythrocytes, which makes these cells more resistant mechanically. And this is beneficial to the body because it allows red blood cells last longer and better perform their functions.

In principle, an increase in the lifetime of the erythrocytes should cause RBC counts increase with time. However, the opposite was observed in this study. This apparent

contradiction occurs because red blood cells are influenced not only by the blood levels of LDL-C, but also by the nutrients necessary for erythropoiesis, such as iron, folate and cobalamin. Deficiencies of these nutrients are known causes of decrease in RBC counts. Apparently, the decrease in RBC counts throughout time suggests that the effect of these nutritional deficiencies on the RBC counts should have been greater than the effect of cholesterol. However, further studies are needed to verify this hypothesis.

Osmotic stability of erythrocytes and RDW

Currently, an increasing number of scientific studies have shown that high values of RDW is related to the worsening of various conditions and diseases that affect the human health status (Cavusoglu et al., 2010; Ephrem, 2013; Hunziker et al., 2012; Malandrino et al., 2012; Patel et al., 2010). Most of these studies emphasize the variation of RDW throughout time.

In this study, $1/H_{50}$ and RDW were positively correlated with each other. This may be a result of a change in the population of red blood cells, with increase in the subpopulation of smaller erythrocytes due to an iron deficiency (Fig 4), probably along with an increase in reticulocytes, which are younger and larger cells, due to deficiency of folate and/or cobalamin. Both microcytic erythrocytes as reticulocytes have in common the fact that they are osmotically more resistant cells (Marks & Johnson, 1958; Vaya et al., 2005).

A positive correlation between $1/H_{50}$ and RDW was also found in other conditions, as in the malaria caused by *Plasmodium vivax* (Mascarenhas Netto Rde et al., 2014). The observation of correlation between these variables in a wide range of conditions should help to understand the origin of the prognostic ability of RDW in these situations.

The positive correlation between $1/H_{50}$ and RDW indicates that this parameter of osmotic stability, in the same way that RDW, is associated with variability between erythrocytes. However, while the variability expressed by RDW is restricted to the volume of red blood cells, the source of variability associated with dX should be much broader, as it also includes all the variables that can affect the osmotic resistance of erythrocytes.

Path analysis

The realization of path analysis allowed elaborate a theoretical model that discover correlations that could not be found through other statistical tools most commonly used, as

multiple linear regression. This theoretical model is shown in the path diagram of Fig 5 and the values of their significance indicators with the respective reference levels are listed in Table 2. This path diagram was constructed from the values of the variables during the study and show how these variables relate to each other and not necessarily with the passage of time after bariatric surgery.

The path diagram shows that BMI has a significant effect on the stability parameters. This effect is indirect, because it is successively mediated by MCV and RDW. This diagram also shows that the correlations between BMI and the stability parameters have the same direction. As bariatric surgery resulted in a decrease of BMI, it means that there was also a reduction of dX and $1/H_{50}$. Therefore, the physiological changes resulting of bariatric surgery are associated with decreased osmotic stability of erythrocytes.

Study limitations

The serum levels of iron, folate (vitamin B9), and cobalamin (vitamin B12) were not measured in this study. A lower intake of these nutrients, deriving from the bariatric surgery, results in an increase in RDW. And indeed there was an increase in RDW after bariatric surgery.

Although patients undergoing bariatric surgery have been advised to eat nutritional supplements on the 15^{th} day surgery, certainly supplementation was not enough to meet all of their demands for iron, folate and cobalamin throughout the study period.

The availability of the amounts of these nutrients would allow the construction of a theoretical model even more complete and that could clarify the apparent contradiction observed in some of the correlations involving the RDW.

Conclusions

The RDW plays a key role in the osmotic stability of erythrocytes in patients undergoing bariatric surgery, because this hematological index is positively related to dX and $1/H_{50}$. Although RDW has increased throughout time, the same did not happen with the osmotic stability, due in part to the reduction in LDL-C levels, especially in the case of $1/H_{50}$, since the correlation between these two parameters was positive. The RDW was also important in mediating the positive indirect effect of the body mass index on the osmotic stability parameters dX and $1/H_{50}$, as was demonstrated by means of path analysis. Correlations between RDW, particularly the osmotic stability parameters of erythrocytes, can help us understand the origin of the predictive ability of this erythrocyte index in various pathological conditions.

Table 1. Significant bivariate correlations

Main parameter	Related parameter	R	p
$1/H_{50}$	RDW	0.32	0.001
	LDL-C	0.24	0.011
dX	RDW	0.22	0.019
Time	BMI	- 0.40	< 0.001
	RBC	- 0.32	0.001
	RDW	0.24	0.012
	Hb	- 0.34	< 0.001
	Ht	- 0.32	0.001
	LDL-C	- 0.24	0.012
	TGC	- 0.29	0.002
BMI	RBC	0.62	< 0.001
	RDW	0.31	0.001
	MCH	- 0.52	< 0.001
	MCV	- 0.56	< 0.001
RBC	Hb	0.62	< 0.001
	Ht	0.69	< 0.001
	MCH	- 0.40	< 0.001
	MCV	- 0.37	< 0.001
RDW	Hb	- 0.34	< 0.001
	Ht	- 0.34	< 0.001
	MCH	- 0.54	< 0.001
	MCV	- 0.61	< 0.001

dX (hemolysis range); $1/H_{50}$ (half hemolysis constant); BMI (Body Mass Index); RBC (erythrocytes count); Hb (total hemoglobin); Ht (hematocrit); MCH (Mean Corpuscular Hemoglobin); MCV (Mean Corpuscular Volume); MCHC (Mean Corpuscular Hemoglobin Concentration); RDW (Red-Cell Distribution Width); TGC (total triglycerides); LDL-C (Low Density Lipoprotein Cholesterol); HDL-C (High Density Lipoprotein Cholesterol).

Table 2. Parameters indicative of a good fit on a path analysis

Tipe of parameter	Abreviation	Name	Reference	Value
Most citated	CMIN p	Chi-square p	> 0.05	0.581
	GFI	Goodness Fit Index	> 0.95	0.980
	NNFI	Non Normal Fit Index	> 0.95	1.024
	CFI	Comparative Fit Index	> 0.95	1.000
	RMSEA	Root Mean Squared Error of Approx.	< 0.05	0.000
Important	CMIN/DF	-	< 2.0	0.825
	NFI	Normal Fit Index	> 0.95	0.947
	PCLOSE	-	> 0.05	0.746
	SRMR	Standarized Root Mean Residual	< 0.05	0.041

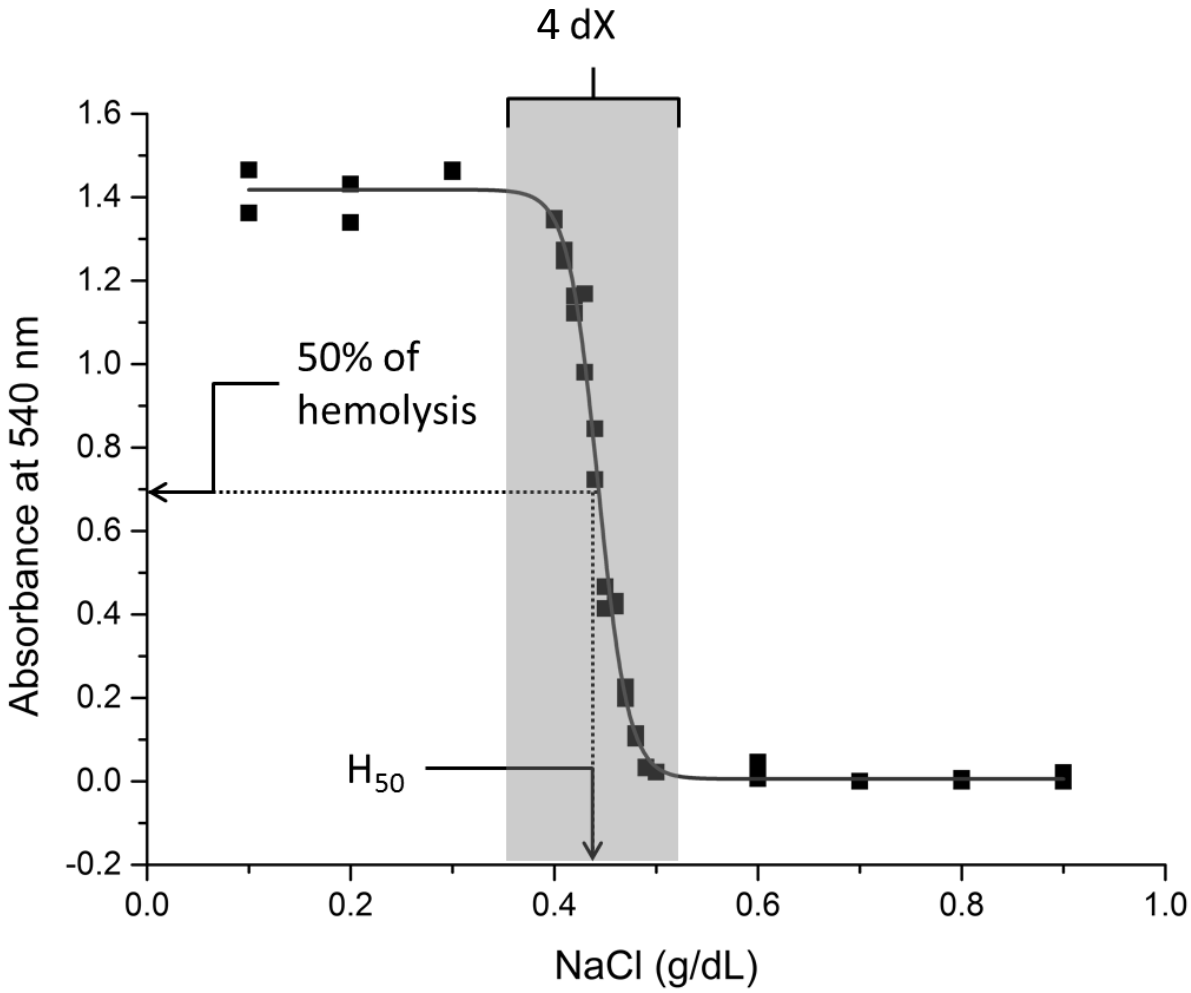

Figure 1. A typical osmotic fragility curve indicating the meaning of the stability variables H_{50} and dX.

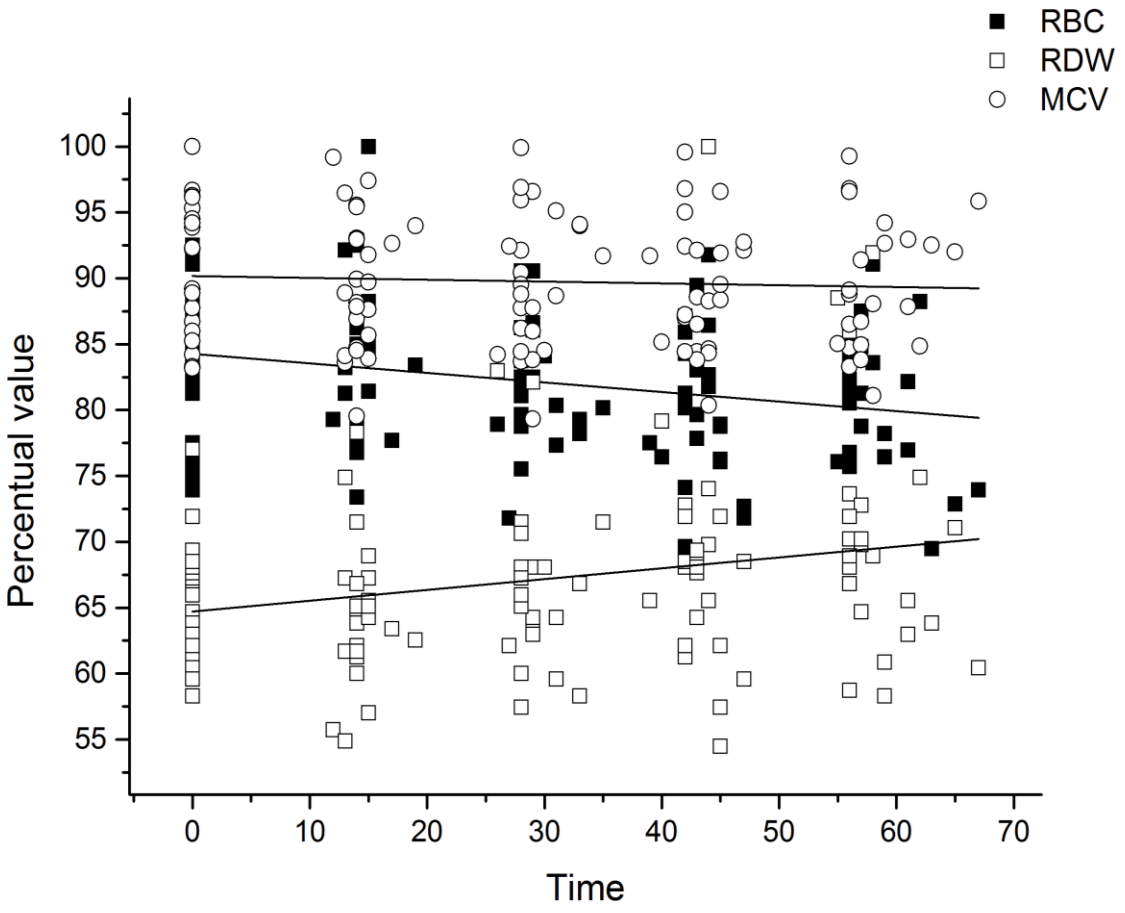

Figure 2. Correlation of the percentage values of RBC, RDW and MCV with the time since the moment of realization of the bariatric surgery.

$$\textbf{MCH}\ (pg) = \frac{\textbf{Hb}\ (g/dL)}{\textbf{RBC}\ (10^6/mm^3)} \times 10$$

$$\textbf{MCV}\ (fL) = \frac{\textbf{Ht}\ (\%)}{\textbf{RBC}\ (10^6/mm^3)} \times 10$$

$$\textbf{MCHC}\ (g/dL) = \frac{\textbf{Hb}\ (g/dL)}{\textbf{Ht}\ (\%)} \times 100$$

$$\textbf{RDW}\ (\%) = \frac{\textbf{1 sd}\ (fL)}{\textbf{MCV}\ (fL)} \times 100$$

Figure 3. Secondary hematimetric variables.

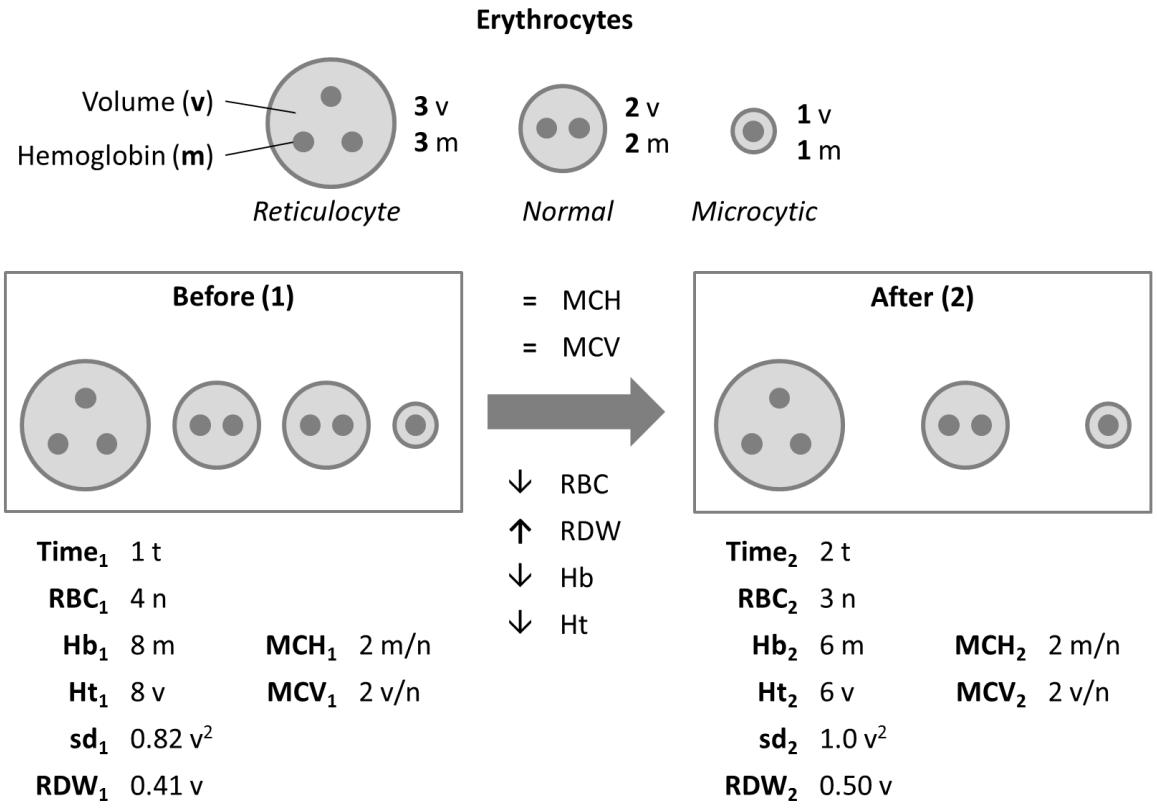

Figure 4. Hypothesis for the variation in the hematological parameters throughout time after bariatric surgery.

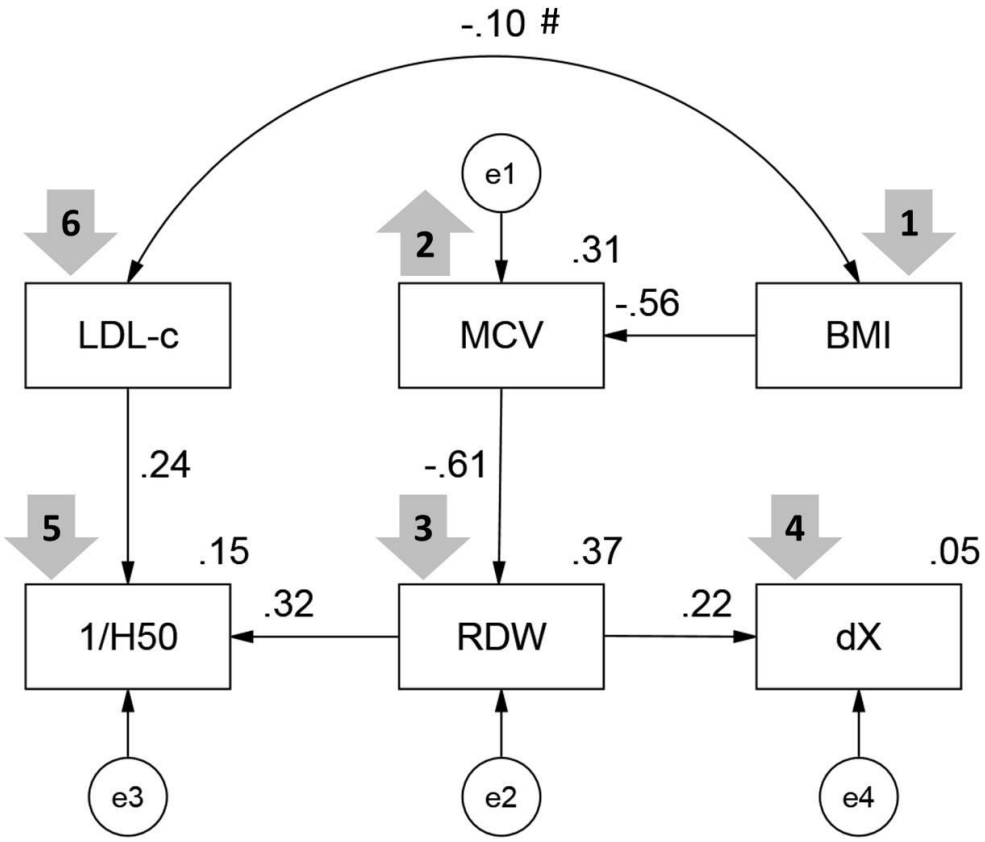

Figure 5: Path diagram presenting interactions of parameters related to erythrocyte osmotic stability. All total, direct and indirect effects are significant, except the path between BMI and LDL-C.

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