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ÍNDICES DE VOLUME PLAQUETÁRIO E RISCO DE DOENÇA CARDIOVASCULAR EM PARTICIPANTES DO ESTUDO LONGITUDINAL DE SAÚDE DO ADULTO – ELSA-BRASIL

Universidade Federal de Minas Gerais
Programa de Pós-Graduação em Patologia
Belo Horizonte - MG
2015

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Tese apresentada ao Programa de Pós-Graduação em Patologia da Faculdade de Medicina da Universidade Federal de Minas Gerais como requisito parcial para obtenção do título de Doutor

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Belo Horizonte
2015

Maluf, Chams Bicalho.
M261i Índices de volume plaquetário e risco de doença cardiovascular em participantes do Estudo Longitudinal de Saúde do Adulto – ELSA- Brasil [manuscrito]. / Chams Bicalho Maluf. - - Belo Horizonte: 2015.
48f.: il.
Orientador: Pedro Guatimosim Vidigal.
Coorientador: Sandhi Maria Barreto.
Área de concentração: Patologia.
Tese (doutorado): Universidade Federal de Minas Gerais, Faculdade de Medicina.

1. Plaquetas. 2. Volume Plaquetário Médio. 3. Valores de Referência. 4. Doenças Cardiovasculares/prevenção & controle. 5. Dissertações Acadêmicas. I. Vidigal, Pedro Guatimosim. II. Barreto, Sandhi Maria. III. Universidade Federal de Minas Gerais, Faculdade de Medicina. IV. Título.

NLM: WH 300

Ficha catalográfica elaborada pela Biblioteca J. Baeta Vianna – Campus Saúde UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA

UFMG

FOLHA DE APROVAÇÃO

ÍNDICES DE VOLUME PLAQUETÁRIO E RISCO DE DOENÇA CARDIOVASCULAR EM PARTICIPANTES DO ESTUDO LONGITUDINAL DE SAÚDE DO ADULTO (ELSA – BRASIL)

CHAMS BICALHO MALUF

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em PATOLOGIA, como requisito para obtenção do grau de Doutor em PATOLOGIA, área de concentração PATOLOGIA INVESTIGATIVA.

Aprovada em 14 de dezembro de 2015, pela banca constituída pelos membros:

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AGRADECIMENTOS

Ao Professor Pedro Guatimosim Vidigal orientador deste trabalho por ter proporcionado minha iniciação na vida acadêmica, pela paciência, dedicação e competência. Guardo um eterno reconhecimento pela confiança e amizade.

À Professora Sandhi Maria Barreto, pela confiança depositada em mim nesses anos de trabalho, pela dedicação e principalmente pela enorme contribuição à minha formação.

Ao ELSA e sua equipe, especialmente a Vanessa e ao Rodrigo por terem compartilhado comigo as dificuldades e as vitórias do dia a dia.

Aos participantes voluntários do ELSA cuja contribuição possibilitou este trabalho.

Às alunas de iniciação científica, que trabalharam neste estudo, contribuição fundamental para a conclusão deste projeto.

Ao Setor de Hematologia do Serviço de Medicina Laboratorial do HC-UFMG, pela realização dos exames e contribuição ao desenvolvimento deste trabalho.

Agradeço, de forma especial, o Departamento de Propedêutica Complementar da Faculdade de Medicina- UFMG- pelo acolhimento, aprendizado constante e amizade.

Ao Leones, meu grande amor, pela compreensão, incentivo e companheirismo.

Aos meus amados filhos, Raul e Raquel, que cresceram junto comigo nestes anos de estudo.

Aos meus pais e irmãos, pelo apoio incondicional.

E a todos aqueles que, direta ou indiretamente, colaboraram para que este trabalho fosse realizado.

RESUMO

Uma maior compreensão do desenvolvimento da doença aterosclerótica, relacionada às funções pró-inflamatórias e pró-trombóticas das plaquetas, tem levado a novas estratégias para controle da doença. Nesse sentido, os índices que avaliam a atividade plaquetária são marcadores potenciais para as doenças cardiovasculares (DCV). As plaquetas maiores são mais reativas, sendo os índices que medem o volume plaquetário marcadores indiretos da reatividade plaquetária. O objetivo deste estudo foi determinar a relação dos índices de volume plaquetário (IVP) com fatores de risco para a aterosclerose e com o escore de risco cardiovascular de *Framingham* (FRS). Todas as variáveis utilizadas neste estudo fazem parte dos dados coletados de 3115 participantes na linha de base do Estudo Longitudinal de Saúde do Adulto (ELSA-Brasil), realizada entre 2008-2010, no Centro de Investigação de Minas Gerais. O volume plaquetário médio (VPM), o coeficiente de variação do volume plaquetário (PDW do inglês, *platelet distribution width*) e a porcentagem de macroplaquetas (P-LCR do inglês, *platelet large cell ratio*) foram mensurados de forma padronizada segundo critérios previamente definidos. Foi determinado o intervalo de referência para esses três parâmetros na população do estudo, e os resultados foram apresentados no primerio artigo. No segundo artigo os participantes que não possuíam diagnóstico prévio de doença cardiovascular foram distribuídos de acordo com sua exposição aos diferentes fatores de risco e calculados o risco cardiovascular em 10 anos com base na equação derivada pelo FRS (2008). As seguintes variáveis foram incluídas no escore de risco: idade, sexo, pressão sanguínea sistólica, colesterol total, colesterol HDL, tabagismo, diabetes e utilização de medicamentos anti-hipertensivos. A regressão linear múltipla foi utilizada para aferir a associação entre os índices plaquetários e o FRS após considerar também as variáveis que não fazem parte do escore, mas que se associam ao risco cardiovascular (escolaridade, uso de álcool e atividade física). A análise de regressão linear múltipla mostrou que o VPM, PDW e o P-LCR correlacionam de forma independente ($p \leq 0,01$) com o escore do FRS após o ajuste para variáveis de confusão. Um aumento de uma unidade no VPM, PDW, ou P-LCR aumentou a média do FRS em 0,59%, 0,40% e 0,08% respectivamente. Embora o aumento no escore de risco seja discreto ele pode ser importante considerando que a estimativa de risco cardiovascular pelo escore resulta de uma complexa interação de múltiplos fatores e as plaquetas desempenham papel importante na patogênese da aterosclerose. Os diabéticos apresentaram maior VPM, PDW e P-LCR ($p \leq 0,004$), e hipertensos apresentaram maior PDW e P-LCR ($p \leq 0,045$). A relação dos IVP com o FRS parece ser devido particularmente à associação desses índices com diabetes e hipertensão, dois importantes fatores de risco para DCV contidos no FRS. O seguimento prospectivo desta população pode ajudar a esclarecer se os IVP têm, na verdade, uma relação causal com DCV em grupos com e sem diabetes e hipertensão arterial.

Palavras-chave: plaquetas, volume plaquetário médio, intervalo de referência, doença cardiovascular, escore de risco de *Framingham*

ABSTRACT

The greater understanding of the development of atherosclerotic disease, related to pro-inflammatory and pro-thrombotic functions of platelets, has led to new strategies for control disease. In this sense, the indices that evaluate platelet activity are potential markers for cardiovascular disease (CVD). Platelet function correlates with its volume therefore indices which measure the platelet volume are indirect markers of platelet reactivity.
The objective of this study was to determine the relationship between platelet volume indices (PVI) with atherosclerosis risk factors and the Framingham risk score (FRS). Baseline data (2008-2010) of 3115 participants enrolled in the Brazilian Longitudinal Study of Adult Health were used. Mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR) measurements were strictly controlled. Reference intervals for these three parameters in the study population were determined and pre-analytical interfering factors were analyzed as the impact of time on samples collected in EDTA. The cohort was distributed according to risk factors and the general FRS was estimated. Using the following variables: age, sex, systolic blood pressure, total cholesterol, HDL cholesterol, smoking, diabetes, and use of antihypertensive medications. Multiple linear regression analysis was used to estimate the association between PVI and FRS after adjusting for potential confounders, i.e. variables that are not part of the FRS, but can increase CVD risk and may be related to PVI measures. The multiple linear regression analysis showed that MPV, PDW and P-LCR independently correlated ($p \leq 0.01$) with FRS after adjustment for confounding variables. One unit increase in MPV, PDW, or P-LCR increased the FRS by 0.59%, 0.40%, and 0.08%, respectively. Diabetics had higher ($p \leq 0.004$) MPV, PDW, and P-LCR, and hypertensive individuals had higher ($p \leq 0.045$) PDW and P-LCR. Although the adjusted coefficients of determination of the model were small, risk for CVD results from a complex multi interaction of various risk factors, and platelets play a key role in the pathogenesis of atherosclerosis. The results show that increased PVI is independently associated with higher CVD risk based on the FRS, and is statistically associated with diabetes and systolic hypertension. It appears that the relationship of PVI measures with the FRS is mostly due to their association with diabetes and hypertension, which are part of the FRS. Prospective follow up of this population may help to clarify whether PVI is causally related to CVD in patients with and without diabetes and arterial hypertension.

Keywords: platelet, mean platelet volume, reference range, cardiovascular disease, Framingham risk score

SUMÁRIO

1 INTRODUÇÃO

1.1 PLAQUETAS

As plaquetas são fragmentos citoplasmáticos do megacariócito, com diâmetro de 2 a 4μm. Circulam, em média, por 7 a 10 dias, sem interação com o endotélio vascular normal. Quando ocorre lesão endotelial, as plaquetas são ativadas e rapidamente aderem ao sítio de injúria, ativando e atraindo outras plaquetas, formando um tampão hemostático. Esse mesmo processo fisiológico, quando em contato com lesões ateroscleróticas, pode levar à oclusão vascular pelo trombo plaquetário e complicações cardiovasculares[1,2,3,4,5].

As plaquetas podem interagir com um grande número de diferentes tipos celulares como as células endoteliais, neutrófilos, monócitos, células dendríticas, linfócitos T citotóxicos e várias células tumorais e estão envolvidas em muitos processos fisiopatológicos além da hemostasia e trombose, como inflamação, promoção da aterosclerose, defesa do hospedeiro e até mesmo o crescimento de tumor e metástases[6,7,8].

1.2 PLAQUETAS NA ATEROSCLEROSE

A aterosclerose é uma doença inflamatória crônica, de origem multifatorial. Alterações na função do endotélio em resposta a ações mecânicas, imunológicas e químicas refletem o primeiro passo fisiopatológico da aterosclerose. Assim, a formação da placa aterosclerótica inicia-se com a agressão ao endotélio vascular por fatores como elevação de lipoproteínas aterogênicas, hipertensão arterial e tabagismo. A disfunção endotelial rompe o equilíbrio,

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[3] Kaplan ZS, Jackson SP. The Role of Platelets in Atherothrombosis. Hematology (ASH Education Program 2011:51-61
[4] Ruggeri Z M. Platelets in atherothrombosis. Nat Med 2002, 8:1227-1234.
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levando a uma predisposição da parede vascular a vasoconstrição, aderência leucocitária, ativação plaquetária, pró-oxidação, trombose, inflamação vascular e aterotrombose[9,10,11].

Na fase inicial da aterosclerose, a ativação da plaqueta pode ser atribuída à redução das propriedades antitrombóticas do endotélio e ao aumento dos mediadores pró-trombóticos e pró-inflamatórios, incluindo fator tissular e citocinas circulantes. Adicionalmente, após lesão da parede vascular, a ativação plaquetária pode ser iniciada pela ligação da glicoproteína (GP) Ib com o receptor endotelial P-selectina e o fator de *von Willebrand* (VWF) (Figura 1) .

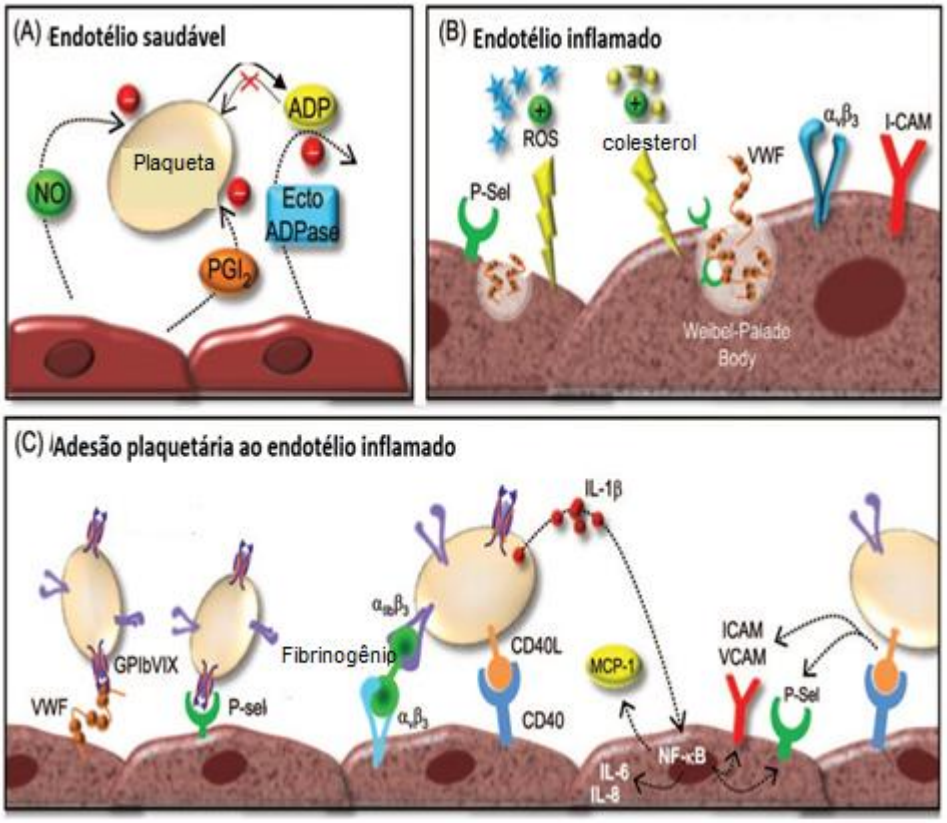

Figura 1 - Representação do envolvimento das plaquetas na fase inicial da aterosclerose

ADP, adenosina difosfato; PGI_2, prostaciclina; NO, óxido nítrico; VWF, fator de *von Willebrand*; ICAM, molécula de adesão intercelular; ROS, espécies reativas ao oxigênio*, P*-Sel*, P*-selectina*; GP,* glicoproteína*; EctoADPase,* ecto-adenosinadifosfatase*; CD,* do inglês *cluster of differentiation; MCP,* proteína quimiotáxica de monócitos*;* ICAM-1, molécula de adesão intercelular*; VCAM,* molécula de adesão vascular*;* IL, interleucina.

Fonte: modificado de Kaplan & Jackson, 2011.[12]

[9] Huo YQ, Ley KF. Role of platelets in the development of atherosclerosis. Trends Cardiovas Med 2004, 14:18-22.
[10] Jennings LK. Role of Platelets in Atherothrombosis. Am J Cardiol 2009, 103:4A-10A.
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A plaqueta tem sido apontada como uma importante mediadora do processo inflamatório. A ativação plaquetária resulta em uma liberação local de mais de 300 proteínas, uma grande proporção delas são conhecidas mediadores inflamatórios. Essas substâncias alteram as propriedades quimiotáticas e adesivas das células endoteliais. Modelos *in vivo* de aterogênese têm sugerido que essas interações entre células endoteliais e plaquetas ativadas são fatores críticos na iniciação da aterosclerose[13,14]. Há evidências de que as plaquetas ativadas aderidas ao endotélio inflamado podem aumentar o recrutamento de leucócitos, sua ativação e transmigração, aumentando, assim, a resposta inflamatória do processo aterosclerótico [13].
O endotélio lesado aumenta a permeabilidade da íntima às lipoproteínas plasmáticas favorecendo sua retenção no espaço subendotelial. Retidas, as partículas de LDL (LDL, do inglês *low-density-lipoproteins*) sofrem oxidação, causando a exposição de diversos neo-epítopos, tornando-as imunogênicas. Os monócitos, induzidos por proteínas quimiotáticas, migram para o espaço subendotelial onde se diferenciam em macrófagos, que por sua vez captam as LDL oxidadas. Os macrófagos repletos de lípides, denominados células espumosas, são o principal componente das estrias gordurosas; lesões iniciais da aterosclerose.

A placa aterosclerótica plenamente desenvolvida é constituída por um núcleo lipídico, rico em colesterol e uma capa fibrosa, rica em colágeno. A placa aterosclerótica estável caracteriza-se por predomínio de colágeno, organizado em capa fibrosa espessa, escassas células inflamatórias e núcleo lipídico de proporções menores com pouco risco de ruptura. As instáveis apresentam atividade inflamatória intensa, especialmente nas suas bordas laterais, com grande atividade proteolítica, núcleo lipídico proeminente e capa fibrótica tênue com maior risco de ruptura. A ruptura de placas instáveis expõe material lipídico altamente trombogênico formando um trombo plaquetário que pode levar à oclusão vascular e complicações cardiovasculares[15,16].

Está bem estabelecido o papel da plaqueta na trombose arterial, uma complicação aguda de lesões ateroscleróticas crônicas. Já na iniciação e desenvolvimento da placa aterosclerótica, o

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[16] Diretrizes. V Diretriz Brasileira sobre dislipidemias e prevenção da aterosclerose. Departamento de Aterosclerose da Sociedade Brasileira de Cardiologia. Disponível em: http://www.cardiol.br/ Acesso em 10 fev 2014.

seu papel parece estar relacionado a um maior recrutamento de leucócitos para locais de inflamação das células endoteliais[17].

Uma maior compreensão e reconhecimento das vias de desenvolvimento da doença aterosclerótica, relacionadas às atividades pró-inflamatórias e pró-trombóticas das plaquetas, têm levado ao desenvolvimento de novas estratégias para controle da doença. Nesse sentido, os índices que avaliam a atividade plaquetária são marcadores potenciais para estratificação de risco das doenças cardiovasculares e podem ser úteis no monitoramento da eficácia do tratamento com drogas antiplaquetárias[18,19].

1.3 ÍNDICES DE VOLUME PLAQUETÁRIO

Alguns estudos correlacionam a função plaquetária com o seu volume, pela medida da velocidade de agregação plaquetária, após estímulo com diferentes agonistas, demonstrando que plaquetas maiores são mais reativas[20,21]. Plaquetas com volume aumentado são metabólica e enzimaticamente mais ativas. Essas plaquetas liberam mais fatores pró-trombóticos como P-selectina, serotonina, adenosina difosfato (ADP) e beta tromboglobulina e apresentam uma maior produção de tromboxano A2 (TxA2) além de expressarem mais receptores de adesão como a GPIb e GPIIb-IIIa[22,23,24,25].

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Vários índices de volume plaquetário (IVP) são facilmente medidos nos aparelhos automatizados que realizam o hemograma[26]. Esses instrumentos são capazes de determinar, além da contagem plaquetária, índices de volume, como volume plaquetário médio (VPM), coeficiente de variação da distribuição do volume plaquetário (PDW, do inglês, *platelet distribution width*) e porcentagem de macroplaquetas (P-LCR, do inglês, *platelet large cell ratio*).

O tamanho plaquetário é determinado durante a megacariocitopoiese e a trombopoiese por múltiplos fatores. A trombopoietina, regulador primário da trombopoiese, e citocinas, como as interleucinas (IL) 3 e 6, influenciam a ploidia do megacariócito e podem levar à produção de plaquetas maiores e mais reativas, explicando como estados pró-inflamatórios podem aumentar o VPM e criar condições pró-trombóticas[27]. O tamanho das plaquetas parece não ser influenciado por sua maturação na circulação[28]. Em condições fisiológicas estáveis ocorre uma relação inversa entre o VPM e o número de plaquetas, a trombopoiese é regulada para manter uma massa plaquetária constante. Entretanto, quando o equilíbrio entre produção e destruição plaquetária é rompido, uma resposta primária parece ser a produção de plaquetas mais reativas, mais densas e maiores, nesses casos o VPM aumentado seria marcador de um maior *turnover* plaquetário[29,30,31]. Uma nova compreensão da regulação da megacariocitopoiese e da trombopoiese surge com a identificação das variantes genéticas associadas com as plaquetas e o volume plaquetário. Em um estudo de Johnson (2011), 84% da variação na contagem plaquetária e 75% na variabilidade do VPM, foi atribuída a fatores genéticos[32]. Estudos realizados em populações européias evidenciaram a presença de pelo menos 12 loci em diferentes cromossomas associados com a função, contagem e volume plaquetário[33,34]. Recentemente, Shameer *et al*. (2014) revelaram que algumas variantes que

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[33] Kunicki TJ, Nugent DJ. The genetics of normal platelet reactivity. Blood 2010, 116:2627-2634

influenciam as plaquetas e o VPM tem efeitos pleiotróficos e são associadas com diversos fenótipos incluindo infarto do miocárdio[35].

1.4 IMPORTÂNCIA CLÍNICA DOS ÍNDICES DE VOLUME PLAQUETÁRIO

Diversos estudos relacionam doenças hematológicas e não hematológicas com os IVP, sendo o VPM o índice que é mais amplamente avaliado nos diferentes estudos.

1.4.1 ÍNDICES DE VOLUME PLAQUETÁRIO E DOENÇAS CARDIOVASCULARES

Chu *et al*. (2010) realizaram uma revisão sistemática com meta-análise de 16 estudos que sugerem que o VPM elevado é associado com infarto agudo do miocárdio (IAM) (média de aumento: 0,92 fL, 95% IC: 0,67-1,16, p<0,001), mortalidade após IAM e reestenose após coronarioangioplastia, sendo o VPM um potencial biomarcador de prognóstico em pacientes com doenças cardiovasculares (DCV)[36]. Em outra revisão, Lippi *et al*. (2013) analisaram dados que relacionam o VPM ao diagnóstico da doença coronariana isquêmica. A partir de 123 citações, somente três estudos foram selecionados, pois, segundo os revisores, apenas esses continham informações suficientes para a avaliação. Esses autores sugerem que o VPM possa ser útil na abordagem de doenças isquêmicas nos serviços de emergência em combinação com outros marcadores, merecendo futuras investigações[37]. Sansanayudh *et al*. (2015) em revisão recente incluiu 40 estudos numa meta-análise que considera a relação entre o VPM e a doença arterial coronariana (DAC). O VPM foi significativamente maior nos pacientes com DAC quando comparados com o grupo controle (média de aumento: 0,70 fL,

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[34] Soranzo N, Spector TD, Mangino M, Kühnel B, Rendon A, Teumer A, Willenborg C, Wright B, Chen L, Li M, et al: A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. Nat Genet 2009, 41:1182-1190.

[35] Shameer K, Denny JC, Ding K, Jouni H, Crosslin DR, de Andrade M, Chute CG, Peissig P, Pacheco JA, Li R, et al: A genome- and phenome-wide association study to identify genetic variants influencing platelet count and volume and their pleiotropic effects. Hum Genet 2014, 133:95-109.

[36] Chu SG, Becker RC, Berger PB, Bhatt DL, Eikelboom JW, Konkle B, Mohler ER, Reilly MP, Berger JS. Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis. J Thromb Haemost 2010, 8:148-156.

[37] Lippi G, Mattiuzzi C, Comelli I, Cervellin G. Mean platelet volume in patients with ischemic heart disease: meta-analysis of diagnostic studies. Blood Coagul Fibrinolysis 2013, 24:216-219

95% IC: 0,11-0,81), sugerindo que o VPM possa ser útil na estratificação do risco cardiovascular, por meio de escores, combinado a outros fatores de risco[38].

1.4.2 ÍNDICES DE VOLUME PLAQUETÁRIO E FATORES DE RISCO CARDIOVASCULAR

Estudos sobre os IVP e sua associação com fatores de risco, como hipertensão arterial sistêmica, dislipidemia, uso de álcool, síndrome metabólica, obesidade e tabagismo são descritos na literatura com resultados controversos[39,40,41,42,43,44,45,46,47,48]. As diferenças metodológicas bem como a padronização das medidas e os diferentes índices estudados dificultam a análise comparativa.

Em relação ao *diabetes mellitus* (DM), Zaccardi *et al*. (2015), em revisão da literatura com meta-análise, sugerem que indivíduos diabéticos tendem a ter valores elevados de VPM e PDW sem diferença na contagem plaquetária. No processo de revisão foram selecionados estudos com IFG (IFG, do inglês *impaired fasting glucose*), IGT (IGT, do inglês *impaired glucose tolerance*), síndrome metabólica e DM tipo 2; desses 40 estudos com o VPM e 30 estudos com contagem plaquetária, além de 4 estudos com PDW e nenhum estudo com P-

[38] Sansanayudh N, Numthavaj P, Muntham D, Yamwong S, McEvoy M, Attia J, Sritara P, Thakkinstian A. Prognostic effect of mean platelet volume in patients with coronary artery disease. A systematic review and meta-analysis. Thromb Haemost 2015, 114.
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[47] Markovic D, Carevic V, Bonacin D, Sekulic BP, Sapunar A, Fabijanic D. Correlation between mean platelet volume and total risk of cardiovascular disease. Signa Vitae 2013, 8:49-55.
[48] De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, Coppo L, Iorio S, Venegoni L, Rondano E, et al. Mean platelet volume and the extent of coronary artery disease: Results from a large prospective study. Atherosclerosis 2009, 206:292-297.

LCR[49]. Os estudos também sugerem que o grupo de diabéticos com pior controle glicêmico está associado a um maior aumento do VPM quando comparado ao diabético controlado[50,51].

1.4.3 ÍNDICES DE VOLUME PLAQUETÁRIO E OUTRAS CONDIÇÕES CLÍNICAS

Kaito *et al*. (2004) investigaram o significado dos três IVP: VPM, PDW e P-LCR, no diagnóstico de trombocitopenia, comparando pacientes com anemia aplástica e trombocitopenia imune[52]. Todos os índices foram significativamente mais elevados nos pacientes com trombocitopenia imune. Muitas síndromes hereditárias são associadas com macrotrombocitopenia como a Síndrome de Bernard-Soulier, anomalia de May-Hegglin, Síndrome de Epstein, Síndrome de Fechtner, Síndrome de DiGeorge, macrotrombocitopenia Mediterrânea benigna e Doença de von Willebrand tipo-plaqueta[53,54].

Alguns estudos têm demonstrado a importância do VPM em outras condições clinicas, como na doença de Crohn podendo ser utilizado como marcador de atividade da doença[55], doenças reumáticas[56], doenças renais[57], doenças hepáticas[58], demência[59].

Estes índices plaquetários não são utilizados de forma rotineira pelos médicos assistentes, muitas vezes sendo omitidos no laudo dos resultados dos exames. Isto pode ser atribuído, pelo

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[49] Zaccardi F, Rocca B, Pitocco D, Tanese L, Rizzi A, Ghirlanda G. Platelet mean volume, distribution width, and count in type 2 diabetes, impaired fasting glucose, and metabolic syndrome: a meta-analysis. Diabetes Metab Res Rev 2015, 31:402-410.
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[52] Kaito K, Otsubo H, Usui N, Yoshida M, Tanno J, Kurihara E, Matsumoto K, Hirata R, Domitsu K, Kobayashi M. Platelet size deviation width, platelet large cell ratio, and mean platelet volume have sufficient sensitivity and specificity in the diagnosis of immune thrombocytopenia. Br J Haematol 2005, 128:698-702.
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[55] Shen J, Ran ZH, Zhang Y, Cai Q, Yin HM, Zhou XT, Xiao SD. Biomarkers of altered coagulation and fibrinolysis as measures of disease activity in active inflammatory bowel disease: A gender-stratified, cohort analysis. Thromb Res 2009, 123:604-611.
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[58] Cho SY, Lee A, Lee HJ, Suh J-T, Park TS. Mean platelet volume in Korean patients with hepatic diseases. Platelets 2012, 23:648-649.
[59] Wang R-T, Jin D, Li Y, Liang Q-C. Decreased mean platelet volume and platelet distribution width are associated with mild cognitive impairment and Alzheimer's disease. J Psychiat Res 2013, 47:644-649.

menos em parte, à falta de padronização do procedimento, o que leva a grande variabilidade dos resultados limitando seu uso na prática clínica.

1.5 DESAFIOS METODOLÓGICOS NA MEDIDA DOS ÍNDICES DE VOLUME PLAQUETÁRIO

Lancé *et al*. (2012), fizeram uma revisão do VPM como marcador de DCV e realizaram análise em relação aos procedimentos pré-analíticos e os métodos de análise descritos nos artigos[60]. Entre 2006 e 2011, estes autores identificaram 126 publicações. Destas, 63 foram excluídas porque não descreviam o método de análise e duas por serem artigos de revisão. Nos artigos selecionados, foram avaliados três tópicos do projeto metodológico: a) anticoagulante utilizado, b) tempo de armazenamento da amostra e c) método analítico utilizado, que são provavelmente as variáveis de maior impacto nos resultados encontrados. Nos 61 artigos analisados, 39 descreveram os três tópicos, porém mais de 10 diferentes intervalos de armazenamento foram descritos e somente dois artigos apresentavam uma padronização metodológica adequada, segundo os autores. Esta grande variabilidade gera confusão na interpretação dos diferentes estudos e pode tornar a comparação dos resultados impossível.

Estudos têm mostrado que fatores pré-analíticos, como anticoagulante, tempo e temperatura de armazenamento bem como o método de análise interferem significativamente no VPM[61,62].

Existem duas tecnologias principais usadas para avaliação do tamanho plaquetário: 1) impedância elétrica, onde as células em uma suspensão são contadas ao passar por um orifício, gerando um pulso elétrico cuja intensidade é proporcional ao volume da célula; 2) método óptico de dispersão ou absorção da luz, que utiliza um sistema com sinal laser, que mede a intensidade e o ângulo da luz dispersa quando incide numa suspensão celular, quantificando e determinando o tamanho das células. Estudo avaliando as medidas do volume plaquetário, em dois instrumentos com diferentes tecnologias (impedância elétrica versus

[60] Lancé MD, Sloep M, Henskens YMC, Marcus MAE. Mean Platelet Volume as a Diagnostic Marker for Cardiovascular Disease Drawbacks of Preanalytical Conditions and Measuring Techniques. Clin Appl Thromb Hemost 2012, 18:561-568.
[61] Dastjerd MS, Emami T, Najafian A, Amini M. Mean platelet volume measurement, EDTA or citrate? Hematology 2006, 11:317-319.
[62] Diaz-Ricart M, Brunso L, Pino M, Navalon F, Jou J, Heras M, White J, Escolar G. Preanalytical treatment of EDTA-anticoagulated blood to ensure stabilization of the mean platelet volume and component measured with the ADVIA counters. Thromb Res 2010, 126:e30-35.

método óptico) mostrou diferença de até 40% entre os dois sistemas[63]. O método óptico tem melhor precisão e exatidão para trombocitopenias severas, já a impedância reconhece interferentes celulares e plasmáticos[64].

Mais recentemente, a citometria de fluxo com uso de anticorpos monoclonais anti-proteínas específicas expressas na superfície das plaquetas, também passou a ser aplicada na determinação do número e avaliação das características específicas das células trombocíticas[65]. Alguns analisadores já utilizam os três princípios metodológicos.

O anticoagulante padrão para o hemograma, ácido etilenodiaminotetracético (EDTA), mantém condições ótimas para contagem celular e diferencial de leucócitos, mas não preserva a ultra-estrutura e a capacidade funcional das plaquetas. Na presença de EDTA, o VPM aumenta em um padrão tempo-dependente[66,67]. A exposição da plaqueta ao EDTA por períodos prolongados resulta em distorção de sua morfologia[68], incluindo dilatação do sistema canalicular, progressiva tendência a mudança de forma e aglutinação plaquetária. Todas essas transformações externas são compatíveis com ativação plaquetária[69].

Para a adequada padronização das fases pré-analítica e analítica, na coorte do Estudo Longitudinal de Saúde do Adulto (ELSA) - Minas Gerais (MG) foi avaliado, em um estudo suplementar, o impacto do tempo entre a coleta da amostra e a medida dos IVP. A medida desses índices, foi realizada em 2 tempos: com até 1 h após a colheita e repetido após 6 h. Comparamos o valor inicial do VPM com o valor após seis horas da colheita. A média do VPM na distribuição com valores após seis horas da colheita de sangue foi 0,35 fL maior que na distribuição com os valores iniciais (teste t para amostras pareadas, foi estatisticamente significante com $p<0,001$). Adicionalmente foi realizada uma análise do impacto do armazenamento das amostras até 2h, nos valores dos IVP, comparando 6 grupos de amostras

[63] Jackson SR, Carter JM. Platelet Volume - Laboratory Measurement and Clinical-Application. Blood Rev 1993, 7:104-113.
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[68] Frojmovic MM, Milton JG: HUMAN-Platelet Size, Shape, and Related Functions In Health And Disease. Physiol Rev 1982, 62:185-261.
[69] Diaz-Ricart M, Brunso L, Pino M, Navalon F, Jou J, Heras M, White J, Escolar G. Preanalytical treatment of EDTA-anticoagulated blood to ensure stabilization of the mean platelet volume and component measured with the ADVIA counters. Thromb Res 2010, 126:e30-35

analisadas com diferentes tempos de armazenamento: até 20 min, 21- 40 min, 41-60 min, 61-80 min, 81-100 min e 101-120 min, não sendo observada nenhuma tendência de elevação nos valores dos IVP com o armazenamento máximo de até 2h (dados não apresentados).

Outro estudo preliminar avaliou se a análise realizada por mais de um instrumento, que utiliza a mesma metodologia, apresentava resultados equivalentes. Esse comparativo demonstrou que os resultados de exames hematológicos realizados em diferentes equipamentos, que utilizam a impedância elétrica, são equivalentes, portanto comparáveis[70].

Assim, na padronização do exame de hemograma para o estudo ELSA – MG definiu-se o uso do K3-EDTA como anticoagulante e as amostras foram mantidas à temperatura ambiente até o momento da análise, que foi realizada em tempo inferior a 2 horas após a coleta. O método analítico utilizado foi a impedância elétrica. Essa padronização consiste em etapa essencial para a realização deste e de futuros estudos que envolvam os IVP, assim como para sua aplicação na tomada de decisão da prática clínica.

1.6 RISCO CARDIOVASCULAR

A identificação dos fatores de risco que aumentam a incidência de DCV é uma das contribuições mais importantes da epidemiologia do século XX. A estimativa do risco de doença aterosclerótica resulta da somatória de diferentes fatores de risco mais a potenciação causada por sinergismos entre eles. Diante da complexidade dessas interações, diversos escores de risco foram criados na tentativa de melhorar a predição do risco cardiovascular. Vários modelos têm sido desenvolvidos, como o escore de risco de *Framingham* (FRS, do inglês: *Framingham Risk Score*), o escore de risco de Reynolds e o SCORE (*Systematic Coronary Risk Evaluation*). O primeiro foi proposto, em 1998, pelo *The Framingham Heart Study*, para a predição da doença coronariana em pacientes assintomáticos. Em 2002 e 2008 esse escore foi revisado e incluídos na avaliação de desfecho, outras manifestações de doença aterosclerótica, além das cardíacas, como doença cerebrovascular isquêmica (CVI), insuficiência cardíaca e claudicação intermitente. Esse escore atualizado permite avaliar o risco de DCV em geral e o risco de eventos cardiovasculares individuais (CVI, doença arterial periférica, doença coronariana e insuficiência cardíaca) em 10 anos. As variáveis atualmente

[70] Maluf CB, Silva IO, Vidigal PG. Understanding commutability: important quality requirement for clinical laboratories. J Bras Patol Med Lab 2011 -47:595-601.

analisadas pelo FRS são idade, sexo, pressão sanguínea sistólica, colesterol total, HDL (HDL, do inglês: *high-density lipoprotein)* colesterol, tabagismo, diabetes e uso ou não de medicação anti-hipertensiva[71,72,73].

Além dos fatores de risco tradicionalmente conhecidos e utilizados pelo FRS, outros biomarcadores são descritos na literatura como úteis na predição do risco cardiovascular, como a proteína C reativa ultra-sensível, homocisteína, magnésio sérico, microalbuminúria e o volume plaquetário médio[74].

[71] Pencina MJ, D'agostino RB, SR., Larson MG, Massaro JM, Vasan RS. Predicting the 30-Year Risk of Cardiovascular Disease The Framingham Heart Study. Circulation 2009, 119:3078-U3061

[72] Wilson PWF, D'agostino RB, Levy D, Belanger AM, Silbershatz H, KanneL WB. Prediction of coronary heart disease using risk factor categories. Circulation 1998, 97:1837-1847

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2 JUSTIFICATIVA

As plaquetas desempenham um papel importante no mecanismo fisiopatológico da doença aterosclerótica e aquelas de maior volume são potencialmente mais reativas e produzem mais fatores pró-trombóticos. Assim, podemos considerar o VPM e os outros IVP como o PDW e o P-LCR marcadores indiretos da reatividade plaquetária e dessa forma, avaliar se esses índices são úteis na estratificação do risco cardiovascular.

Os resultados encontrados nos diferentes estudos, que avaliam o volume plaquetário, não são consistentes, possivelmente pelas diferentes populações e métodos utilizados, além da ausência ou grande variabilidade na padronização dos fatores pré-analíticos. O ELSA-Brasil é um estudo prospectivo multicêntrico, desenvolvido em instituições de ensino superior e pesquisa, em seis estados brasileiros com objetivo primário de investigar o desenvolvimento de doenças crônicas, principalmente DCV e o DM[75,76]. O ELSA- MG oferece a oportunidade de avaliar a relação entre os índices VPM, PDW e P-LCR%, mensurados em sua linha de base, de forma padronizada segundo critérios bem definidos, em indivíduos adultos brasileiros, com descrição de suas características demográficas, comportamentais, clínicas e laboratoriais relacionadas ao risco cardiovascular.

[75] Aquino EML, Barreto SM, Bensenor IM, Carvalho MS, Chor D, Duncan BB, Lotufo PA, Mill JG, Molina MDC, Mota ELA, et al. Brazilian Longitudinal Study of Adult Health (ELSA-Brasil): Objectives and Design. Am J Epidemiol 2012; 175:315-324.
[76] Schmidt MI, Duncan BB, Mill JG, Lotufo PA, Chor D, Barreto S M, Aquino EML, et al. Cohort Profile: Longitudinal Study of Adult Health (ELSA-Brasil). Int J Epidemiol 2014; 43:1-8

3 APRESENTAÇÃO DO CONTEÚDO

O presente trabalho reúne estudos que foram realizados visando investigar os índices de volume plaquetários, VPM, PDW e P-LCR%, incluindo os fatores pré-analíticos e analíticos envolvidos na sua mensuração, a definição dos intervalos de referência, bem como sua relação com fatores de risco cardiovasculares. Este trabalho envolveu os participantes do Centro de Investigação de Minas Gerais do ELSA-Brasil.

O primeiro artigo publicado na revista *Platelets* (Fator de Impacto: 2.98; WebQualis: Área de Medicina II: extrato B1), relata a padronização e a determinação dos intervalos de referência dos índices de volume plaquetários[77]. O Segundo artigo, publicado na revista *Clinical Chemistry and Laboratory Medicine* (Fator de Impacto: 2.707; WebQualis: Área de Medicina II: extrato B1), aborda a associação do volume plaquetário com o Escore de Risco de *Framingham*[78].

[77] Maluf CB, Barreto SM, Vidigal PG. Standardization and reference intervals of platelet volume indices: Insight from the Brazilian longitudinal study of adult health (ELSA-BRASIL). Platelets 2014; 0: 1-8.
[78] Maluf CB, Barreto SM, dos Reis RC, Vidigal PG. Platelet volume is associated with the Framingham risk score for cardiovascular disease in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil). Clin Chem Lab Med 2015; doi: 10.1515/cclm-2015-0686. [Epub ahead of print]

4 ARTIGO 1 - *STANDARDIZATION AND REFERENCE INTERVALS OF PLATELET VOLUME INDICES: INSIGHT FROM THE BRAZILIAN LONGITUDINAL STUDY OF ADULT HEALTH* (ELSA-BRASIL)

http://informahealthcare.com/plt
ISSN: 0953-7104 (print), 1369-1635 (electronic)

platelets

Platelets, Early Online: 1–8
 DOI: 10.3109/09537104.2014.942620

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METHODS PAPER

Standardization and reference intervals of platelet volume indices: Insight from the Brazilian longitudinal study of adult health (ELSA-BRASIL)

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Abstract

Platelet volume indices (PVI) are associated with hematological and non-hematological diseases, notably cardiovascular and cerebrovascular diseases. The establishment of PVI reference intervals (RIs) are essential to evaluate whether these indices are useful in clinical practice. Healthy-associated RIs have not yet been established for the Brazilian population. Here, we determined RIs of PVI for a health adult population, participants of the Brazilian Longitudinal Study of Adult Health ELSA-Brasil. A total of 580 individuals out of an initial sample of 3115 subjects constituted the healthy reference sample. To be part of the study, individuals had to fulfill the following criteria: blood count within 2 hours of collection, no use of continuous medication, self-rated health as good or very good, no reported diagnosis of diabetes and/or arterial hypertension, not smoking, lack of metabolic syndrome, body mass index (BMI) <30 kg/m^2, and platelet, hemoglobin, and creatinine beyond reference values. The RIs are mean platelet volume (MPV): 8.9–11.8 fL, platelet distribution width (PDW): 9.6–15.3 fL, platelet large cell ratio (P-LCR): 15.6–39.5%. These parameters were not significantly affected by age, gender, smoking, obesity, and alcohol abuse. However, significant differences were found among self-rated race/color groups. Standardization of measurement procedures and the establishment of healthy-associated PVI RIs are essential to be able to support clinical decision-making from laboratorial test results. This study at the baseline of the ELSA Brasil reported herein may contribute to future efforts aiming to evaluate whether PVI values are associated with clinical conditions in the Brazilian population.

Abbreviations: GP: glycoprotein; MPV: mean platelet volume; PDW: platelet distribution width; P-LCR: platelet large cell ratio; PVI: platelet volume indices; RI: reference intervals; ELSA-Brasil: Brazilian Longitudinal Study of Adult Health; CLSI: Clinical and Laboratory Standards Institute; EDTA: ethylenediaminetetraacetic acid; BMI: body mass index.

Keywords

Brazil, clinical laboratory techniques, mean platelet volume, platelets, platelet distribution width, reference range

History

Received 30 May 2014
Revised 26 June 2014
Accepted 3 July 2014
Published online 6 August 2014

Introduction

Platelets play a key role in atherothrombosis, the main cause of most unstable coronary syndromes. Prothrombotic and proinflammatory function of platelets are important factors in the prevention of atherothrombosis [1]. Platelet volume has been investigated in connection with both thrombosis and inflammation [2]. Platelet volume is determined by multiple factors during megakaryocytopoiesis and thrombopoiesis [3]. Thrombopoietin, the primary regulator of thrombopoiesis, and cytokines such as interleukins 3 and 6, affect the ploidy of megakaryocytes and can lead to the production of larger and more reactive platelets [4]. Genetic studies have identified loci involved in the platelet function, count, and volume and results from these studies may lead to new insights on megakaryocytopoiesis and thrombopoiesis regulation [5–9].

Larger platelets are metabolically and enzymatically more active, because they contain more α-granules that release prothrombotic factors such as P-selectin, adenosine diphosphate, and β-thromboglobulin. Additionally, they produce more thromboxane A2 and exhibit increased expression of adhesion glycoprotein (GP) Ib receptor and GP IIb and IIIa [10–12]. All these characteristics contribute to the high thrombotic potential of these cells.

Automated hematology analyzers have made the measurement of several indices of platelet volume easy, inexpensive, and available in most clinical laboratories. These analyzers provide platelet count and several indices, such as mean platelet volume (MPV), platelet distribution width (PDW), and platelet large cell ratio (P-LCR) [3, 13].

Several studies have related hematological and non-hematological diseases with platelet volume indices (PVI) [14–21]. Moreover, there is an increasing number of publications correlating platelet indices with cardiovascular and cerebrovascular diseases [2, 3, 22–27]. The Chu et al. meta-analysis of 16

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Table I. Participant exclusion criteria and number of excluded individuals.

	Number of patients ($n = 3115$)	
Exclusion criteria	Excluded (n)	Kept (n)
Time between blood collection and examination >2 hours	241	2874
Continuous use of any medication	1779	1095
Self-rated health as regular, bad, or very bad	93	1002
Reported diagnosis of diabetes mellitus	5	997
Reported diagnosis of arterial hypertension	69	928
Presence of metabolic syndrome[a]	45	883
Smoker[b]	103	780
Body mass index (BMI) $\geq 30\,kg/m^2$	100	680
Platelet count level beyond reference values[c]	51	629
Hemoglobin level beyond reference values[d]	44	585
Elevated serum creatinine[e]	5	580

[a]Three of the five criteria of the National Cholesterol Education Program (ATPIII criteria) [40].
[b]Currently smoking.
[c]Platelet count ≤ 150 or $\geq 450 \times 10^9$/l.
[d]Hemoglobin: ≤ 13.0 or ≥ 17.0 g/dl in men; ≤ 12.0 or ≥ 15.0 g/dl in women.
[e]Serum creatinine: ≥ 1.3 mg/dl in men; ≥ 1.1 mg/dl in women.

studies revealed that high MPV was associated with mortality from acute myocardial infarction and re-stenosis after coronary angioplasty. These authors suggested that MPV might be a potential biomarker for prognosis in patients with manifest cardiovascular disease [24].

Despite the great interest of the scientific community and the relative ease with which platelet indices can be obtained, PVI parameters are not routinely used in clinical practice, and are often omitted in test results. However, standardization of PVI determination procedures is required, as is the establishment of reference intervals (RIs) in the healthy population, a time-consuming task that should ideally be conducted in every clinical laboratorial service. Indeed, studies have shown that pre-analytical factors such as storage time, anti-coagulant use, and the analysis method itself can significantly interfere with these indices [3, 25, 28, 29]. Furthermore, RIs often differ among populations of different nations [30–36].

The aim of this study was to determine the RIs of PVI for Brazilian adults included in the Longitudinal Study of Adult Health (ELSA-Brasil) in accordance with the recommendations of the consensus document: "How to define and determine reference intervals in the clinical laboratory," proposed by the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute (CLSI) [37].

Methods

Population

ELSA-Brasil is the first large multi-center cohort in the country. Participants are civil servants from universities and research institutions located in six different states. The main study objectives are to investigate the incidence and progression of cardiovascular disease and diabetes and their risk factors. All active or retired employees of the institutions involved aged between 35 and 74 years were eligible for the study. Exclusion criteria were current or recent (<4 months prior to the first interview) pregnancy, intention to quit working at the institution in the near future, severe cognitive or communication impairment, and, if retired, residence outside of a study center's corresponding metropolitan area.

All the 15 105 participants of the cohort answered a comprehensive questionnaire about their general health conditions, family health issues, medication use, smoking, alcohol consumption, physical activity, mental health, and many other factors. In addition, they were submitted to a series of clinical and laboratorial tests. Baseline examination was performed between 2008 and 2010. Follow-up and ascertainment of incidence of diseases are made by annual phone interviews (commencing by the second year of participation) and repeated interviews and examinations every 3–4 years. The second wave of the cohort will be completed by mid-2014. The research protocol was approved by the ethics committee of each participating institution and by the National Research Ethics Committee. All participants signed an informed consent which included the storage of biological samples. Details on study design and cohort profile can be found elsewhere [38, 39].

The present study was restricted to cohort participants in the state of Minas Gerais for whom PVI data were collected. Individuals who presented any potential or currently known factors that can affect PVI were excluded in a stepwise manner [37] (Table I).

Serum samples

Venous blood sampling was performed in the morning after 12- to 14- hour-fast, following the CLSI *Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard* [41]. The venipuncture was performed with a scalpel for multiple vacuum collection, with the application of a tourniquet for a maximum of 1 minute. The sampling tubes were identified with bar codes, assuring secrecy, safety for the participant, and traceability [42]. The time between sampling and exam procedure was strictly controlled to be within 2 hours, and the tubes containing K3 salt of ethylenediaminetetraacetic acid (EDTA) were kept at room temperature until the analysis.

In the presence of EDTA, MPV grows in a time-dependent pattern. In this study, blood samples were collected with EDTA, and all the samples were analyzed within 2 hours of collection, thus minimizing the undesirable effects of EDTA on PVI estimates.

Platelets volume indices (PVI) analysis

The PVI measurements were performed with XE 2100 D hematologic analyzers (Sysmex, Kobe, Japan), which use impedance technology to estimate particle count and volume. The quality of the results was validated by internal quality procedures and participation in proficiency programs of the Brazilian Society of Pathology and Laboratory Medicine, and of the College of American Pathologists. An equivalence test between the analyzers

DOI: 10.3109/09537104.2014.942620

employed in the measurements was previously performed to ensure the commutability of results [43].

All particles detected with sizes between 2 and 30 fL are regarded as platelets. Based on these data, the PVI are calculated using the following formulae: MPV(fL) = plateletcrit/platelet count, with plateletcrit being the ratio between the overall volume of platelets and the overall volume of blood in one individual. PDW and P-LCR indices were obtained from the histogram of platelet size distribution. PDW was defined as the distribution width at the level of 20% considering that the peak of the histogram is 100%. The percentage of platelets bigger than 12fL was defined as P-LCR [14].

Statistical analysis

The Skewness and Kurtosis tests were applied to evaluate distribution of PVI values. The non-parametric method was used to determinate the RIs, calculated as the values at the 2.5 and the 97.5 percentiles of the PVI distribution. Because there is no consensus about the effects of pre-collection factors such as smoking, body mass index (BMI), and alcohol abuse on PVI, a sensitivity analysis was carried out comparing the RIs obtained without and with the inclusion of individuals presenting these conditions, while maintaining the other exclusion criteria listed in Table I. Excessive alcohol intake was defined as reported consumption of more than 210 g alcohol/week for men or 140 g alcohol/week for women. Statistical differences between subgroups defined by socio-demographic characteristics and pre-collection factors were verified by the Kruskal–Wallis non-parametric test and by the *t*-test for mean. We assessed the need to recommend a specific race/skin color reference range for PVI using the Harris/Boyd statistical approach [37]. Following this approach, we calculated the z score from PVI means and SD and compared to a critical value (z^*). If the calculated z exceeds z^*, separate RI should be recommended. In addition, statistical differences between PVI SD of each subgroup were verified since separate RIs are recommended if the larger SD exceeds by 1.5 times the smaller SD, or whether larger SD/(larger SD – smaller SD) is less than 3. A p value lower than 0.05 was considered statistically significant, and analysis was performed using the STATA-9.0 statistical package (College Station, TX).

Results

The reference healthy population comprised 580 of the 3115 individuals presenting PVI data. The socio-demographic characteristics of this population are presented in Table II.

More than half of the studied population was men; about 80% of the individuals were aged between 35 and 54 years and most self-defined as being white. There was no statistical difference between men and women with regard to age distribution (mean age of men = 47.2 years SD = 6.9 years, and mean age of women = 47.9 years SD = 8.2 years).

The histograms with the distribution of the MPV, PDW, and P-LCR values are presented in Figure 1. As exhibited, the parameters values dispersion is fairly close to a normal distribution.

There were no statistical differences in the mean PVI values according to gender or age. The analysis based on self-declared race/skin color showed that white individuals presented mean MPV, PDW, and P-LCR values lower than those of individuals self-declared black or pardo (mixed skin color/brown; Table III).

Next, we performed a sensitivity analysis comparing the RIs of PVI obtained with and without the inclusion of individuals reporting tobacco and alcohol use and high BMI values (>30 kg/m^2), while maintaining the other exclusion criteria listed in Table I. The aim was to identify whether or not these variables could have an effect on the PVI of the population studied herein. Thus, we included 76 smoking and 86 obese individuals who also fulfilled all the inclusion criteria shown in Table I and compared with the data obtained for the reference individuals. It should be mentioned that CLSI recommends that each subgroup of pre-analytical variables should be composed of at least 120 individuals [37] and our sample was somewhat short of this recommendation. Table IV shows there are no statistically significant differences between these groups for all of the PVI parameters evaluated (MPV, PDW, and PLCR).

Table II. Demographical characteristics of the 580 reference individuals.

	Frequency	
Characteristics	*n*	%
Gender		
Female	247	42.59
Male	333	57.41
Age group		
35–54 years	465	80.17
≥55 years	115	19.83
Self-rated race/skin color		
White	267	46.03
Pardos[a]	211	36.38
Black	71	12.24
Others[b]	31	5.34
Schooling		
<11 years	40	7.06
11–14 years	163	28.10
≥15 years	377	64.84

[a]"Brown" or of mixed color.

[b]Includes native indigenous ($n = 2$), Asian descendent ($n = 18$), and missing information ($n = 11$).

Among the 580 reference individuals, we identified 33 with excessive alcohol intake. The remaining participants either did not use or made occasional use of alcohol. Again, level of alcohol intake does not seem to affect the PVI parameters evaluated (Table IV).

Table V shows the RIs for the PVI determined by non-parametrical analysis for the reference individuals ($n = 580$, as described in Table I). Following the Harris/Boyd statistical approach [37], we calculated z for PDW (=2.38) and MPV (=2.71) and they did not exceed the critical value z^* (=4.54), while the P-LCR calculated z (=4.94) was higher than z^*. However, the analysis of P-LCR SD values indicated that separate RI is not necessary.

Discussion

Laboratorial test values can vary depending on the ethnic background of a population, geographic location, age, gender, diet, smoking, and alcohol use among other factors [37]. Pre-analytical variables, including the preparation of the individual, sampling procedures, and manipulation of samples, besides the method of analysis, can also have an impact in the laboratorial test values, especially in PVI. For this reason, standardization and control for these factors are fundamental in studies designed to determine RIs for PVI. This study establishes the healthy-associated RIs for PVI (MPV, PDW, P-LCR) in Brazilian adults (35+ years) from the ELSA-Brasil study data using standard pre-analytical and analytical factors according to the CLSI recommendations [37].

The ELSA-Brasil study data allowed an adequate selection of a healthy reference sample because the cohort collected information that included various sources of biological variation. Because individuals with anemia, low platelet count, and high serum

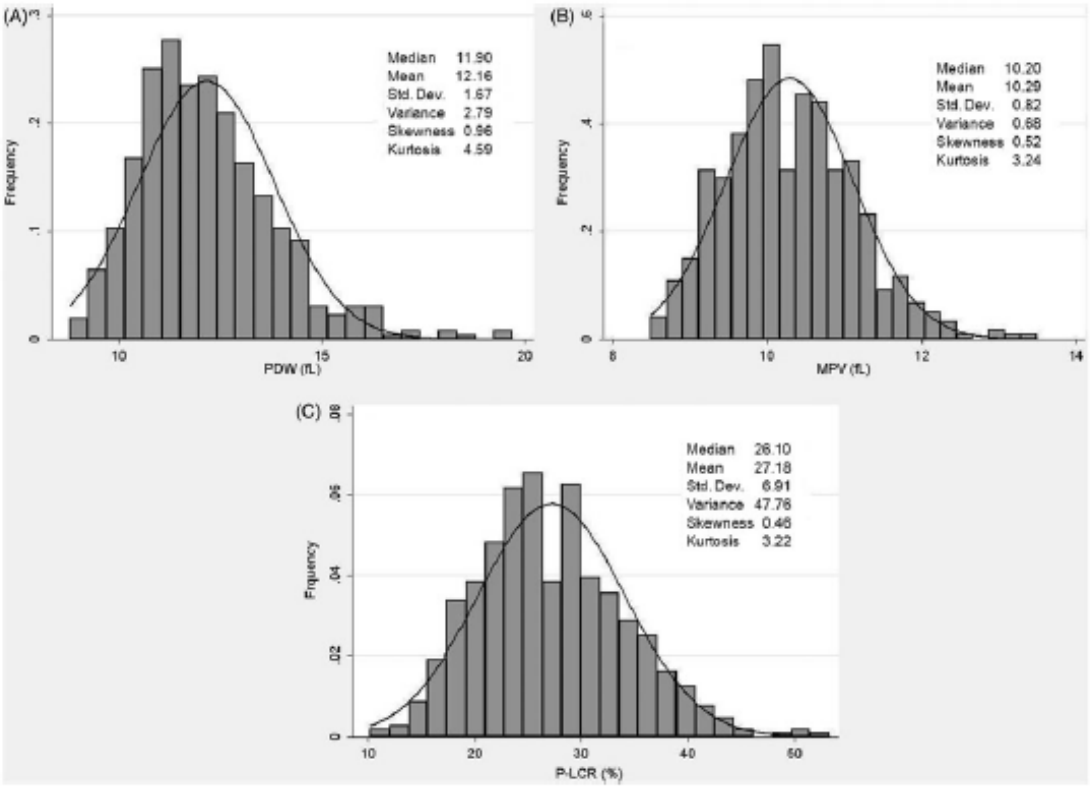

Figure 1. Distribution curves for (A) PDW (platelet distribution width) values; (B) MPV (mean platelet volume) values; (C) P-LCR (platelet large cell ratio) values.

Table III. Mean and 95% confidence intervals of platelet volume indices according gender, age group, and self-rated race/color.

Demographic variable		MPV(fL)	PDW (fL)	PLCR (%)
Gender				
Male ($n=333$)	Mean (SD) 95% CI	10.27 (0.80)	12.19 (1.65)	27.12 (6.60)
		10.19–10.36	12.02–12.37	26.41–27.83
Female ($n=247$)	Mean (SD) 95% CI	10.31 (0.86)	12.12 (1.70)	27.26 (7.33)
		10.21–10.42	11.91–12.33	26.34–28.18
	p Value[c]	0.579	0.597	0.812
	p Value[d]	0.790	0.401	0.983
Age group (years)				
35–54 ($n=465$)	Mean (SD) 95% CI	10.29 (0.84)	12.17 (1.70)	27.13 (7.06)
		10.21–10.36	12.01–12.32	26.49–27.77
≥ 55 ($n=115$)	Mean (SD) 95% CI	10.31 (0.76)	12.14 (1.56)	27.39 (6.29)
		10.17–10.45	11.86–12.43	26.22–28.56
	p Value[c]	0.796	0.887	0.720
	p Value[d]	0.517	0.977	0.510
Race/skin color[a]				
White ($n=267$)	Mean (SD) 95% CI	10.20 (0.82)	12.00 (1.61)	26.49 (6.76)
		10.10–10.30	11.81–12.19	25.68–27.31
Other[b] ($n=282$)	Mean (SD) 95% CI	10.39 (0.82)	12.34 (1.74)	27.94 (7.08)
		10.30–10.49	12.14–12.55	27.11–28.77
	p Value[c]	0.007	0.018	0.015
	p Value[d]	0.006	0.019	0.012

MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; SD, standard deviation; CI: confidence interval.
[a]Self-declared race/skin color (information missing for 31 individuals).
[b]Black and Pardos (“Brown” or of mixed color).
[c]p Value obtained by t-test for parametrical distribution.
[d]p Value obtained by Kruskal–Wallis test for non-parametrical distribution.

DOI: 10.3109/09537104.2014.942620

Table IV. Mean and 95% confidence intervals of platelet volume indices according to pre-collection factors.

Pre-collection factor		MPV (fL)	PDW (fL)	P-LCR (%)
Current smoker ($n=656$)				
No ($n=580$)	Mean (SD) 95% CI	10.29 (0.82)	12.16 (1.67)	27.18 (6.91)
		10.22–10.36	12.03–12.30	26.62–27.75
Yes ($n=76$)	Mean (SD) 95% CI	10.32 (0.83)	12.08 (1.64)	27.13 (6.81)
		10.13–10.51	11.70–12.45	25.58–28.69
	p Value[b]	0.741	0.669	0.955
	p Value[c]	0.701	0.740	1.000
BMI ($n=666$)				
<30 kg/m^2 ($n=580$)	Mean (SD) 95% CI	10.29 (0.82)	12.16 (1.67)	27.18 (6.91)
		10.22–10.36	12.03–12.30	26.62–27.75
≥30 kg/m^2 ($n=86$)	Mean (SD) 95% CI	10.32 (0.81)	12.15 (1.60)	27.76 (6.91)
		10.15–10.50	11.81–12.50	26.27–29.24
	p Value[b]	0.731	0.959	0.472
	p Value[c]	0.735	0.990	0.529
Excessive alcohol consumption[a] ($n=461$)				
No ($n=428$)	Mean (SD) 95% CI	10.31 (0.80)	12.21 (1.64)	27.37 (6.73)
		10.23–10.39	12.05–12.36	26.73–28.01
Yes ($n=33$)	Mean (SD) 95% CI	10.14 (0.86)	12.05 (1.75)	25.99 (7.07)
		9.84–10.45	11.43–12.67	23.46–28.47
	p Value[b]	0.251	0.589	0.249
	p Value[c]	0.227	0.465	0.245

PVI, platelet volume indices; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; SD, standard deviation; CI, confidence interval; BMI, body mass index.
[a]Defined as more than 210 g alcohol/week for men, and more than 140 g alcohol/week for women.
[b]p Value obtained by t-test for parametrical distribution.
[c]p Value obtained by the Kruskal–Wallis test for non-parametrical distribution.

Table V. Reference intervals for the platelet volume indices.

		Reference interval	
PVI	Median	2.5 percentile	97.5 percentile
MPV (fL)	10.2	8.9	11.8
PDW (fL)	11.9	9.6	15.3
P-LCR (%)	26.1	15.6	39.5

PVI, platelet volume indices; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell ratio.

creatinine were not included in the sample studied, we managed to exclude possible interference of analytical factors such as red blood cell fragments, thrombocytopenia, and microcytosis, which are associated with higher MPV values. The exclusion of individuals who made use of continuous medication guarantees, to a great extent, the exclusion of most chronic diseases, including thyroid and chronic obstructive pulmonary diseases.

The selection of a reference sample of healthy individuals is probably the biggest challenge in studies aimed at determining RIs for biological analytes. Indeed, the use of convenience populations such as medical students, blood donors, or a hospitalized population has been criticized and rejected because of uncontrolled selection factors that distinguish these subgroups. Also, the use of an unequivocal ''golden standard'' of young and healthy adults has been criticized because probably it is not representative of the general population in which the parameters will be clinically employed [32, 37, 44].

Table VI lists the published studies attempting to determine RIs for PVI. Most of the studies concentrated on MPV intervals [30, 32, 34, 36], a couple included PDW RIs [31], one concentrated on PDW [45], and none investigated P-LCR RIs. Ours is the first to include all three PVI parameters to determine healthy-associated RIs. PVI RIs varied among the studies. This may be explained by differences in the population selection criteria, in the pre-analytical factors, and in the analyzer equipment used. All studies used the standard anti-coagulant EDTA. Platelet exposure to EDTA for long periods of time results in distortions of its morphology, including progressive expansion of the canaliculus system. This process can lead to changes in shape and agglutination pattern of the platelets [10, 22, 46]. Thus, differences in the time interval between the collection of samples and analysis may be explained in some of the observed variation in PVI RIs among published studies.

The methodology employed by the analyzer may also help to explain some of the RI variations. It has been suggested that MPV obtained by the impedance method is time-dependent, with a maximal variation within 2 hours of collection with EDTA [3]. Using the optical system, MPV results are approximately 10% lower than those obtained with impedance. It is plausible that, in the presence of EDTA, the shape and the permeability of platelets' plasmatic membrane change, causing a dilution of their cytoplasmic material with consequent decrease in optical density [3, 13].

Genetic factors seem to have a considerable influence on the heterogeneity of platelet indices. Studies in Europe found at least 12 loci in different chromosomes associated with MPV, indicating an association between MPV values and the number of these alleles [8]. We found a statistical difference in MPV, PDW, and P-LCR according to self-declared race/skin color. Self-declared white individuals presented mean MPV, PDW, and P-LCR values lower than those of individuals self-declared black or pardo (mixed skin color/brown). Nevertheless, specific race/skin color reference ranges for PVI does not appear to be necessary for the study population. Differences related to ethnicity/skin color are hard to interpret in Brazil, probably because the Brazilian population is highly heterogenic, a result of five centuries of great miscegenation of Europeans, Africans, and Native Americans [47]. Most population genomes are mosaics of different filo-geographic origins. Nevertheless, Brazilian European ancestors predominate independently of the color phenotype, ranging from 60.1% in the Northeast to 79.5% in the South of the country [48]. As a result, classifications based on phenotype such as skin tone, eyes, hair, lips, and nose formats are not adequate to determine the geographic origin of most Brazilian ancestors [49]. Undoubtedly, it is very important to determine

Table VI. Reference intervals for platelet indices in samples with EDTA from different studies.

Study	Population		Time[a]	Equipment	Method	Reference intervals		
	N	Country of origin				MPV (fL)	PDW (fL)	P-LCR %
ELSA-Brasil	580	Brazil	<2 hours	Sysmex XE-2100[b]	Im	8.9–11.8	9.6–15.3	15.6–39.5
Zhang & Huang [36]	11 395	China	<2 hours	Sysmex XE-2100[b]	Im	11.8[h]	NA	NA
Lippi et al. [34]	1822	Italy	<2 hours	ADVIA 2.120[c]	Op	7.7[h]	NA	NA
Subhashree et al. [33]	500	India	<4 hours	Sysmex KX-21[b]	Im	8.0–13.15	8.9–16.40	NA
Cho et al. [35]	7044	Korea	<2 hours	ADVIA 2.120[c]	Op	7.1–9.8	NA	NA
Demirin et al. (2011)	326	Turkey	<6 hours	CD 3700 SL[d]	Im	7.2–11.7	NA	NA
Farias et al. [45]	231	Brazil	<2 hours	AbxPentra 120[e]	Im	NA	10.1–17.9	NA
Wakeman et al. [50]	250	United Kingdom	<1 hour	Sysmex XE-2100[b]	Im	9.4–12.2	NA	NA
Van den Bossche [30]	308	Belgium	<4 hours	AbxPentra 120[e]	Im	6.8–10.0	NA	NA
				Gen-S[f]	Im	7.6–10.7	NA	NA
				SE-9500[g]	Im	9.4–12.9	NA	NA
				CD-4000[d]	Im & Op	6.9–10.6	NA	NA
				ADVIA 120[c]	Op	6.4–9.7	NA	NA
Brummitt & Barker [31]	122	United Kingdom	<1 hour	ADVIA 120[c]	Op	6.6–10.4	40.1–65.8	NA

MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet large cell ratio; Im, impedance method; Op, optical method; NA, data not available.
[a]Time of interval between collection of sample and analysis.
[b]Sysmex XE-2100 and KX21 (Sysmex, Kobe, Japan).
[c]ADVIA 120 and 2.120 (Bayer, Tarrytown, NY).
[d]Cell Dyn 3700 SL and 4000 (Abbott Diagnostics, Santa Clara, CA).
[e]Abx Pentra 120 (Abx, Montpellier, France).
[f]Gen-S (Coulter Electronics, Miami, FL).
[g]SE-9500 (Toa Medical Electronics Co., Kobe, Japan).
[h]Only data regarding MPV mean were available.

reference values for every admixed populations such as that of Brazil, but future genetic studies will be necessary to fully explain the differences observed in the present study regarding PVI and ethnicity/skin color.

No significant differences were found in the mean PVI according to sex. This finding agrees with some studies [30, 34, 35, 50] but contrasts with others [31, 33, 51, 52]. The explanation for these discrepancies is not clear and will need further investigation.

An increase of MPV values with age has been reported elsewhere [34, 35, 53]. However, we did not find significant difference in MPV in relation to age. This divergence may be due to lack of power, as 80.2% of our study participants were aged 35–54 years.

We found that PVI means are not influenced by pre-collection factors such as smoking habit, obesity (BMI $\geq$ 30), and alcohol. The CLSI recommends that each subgroup of pre-analytical variables should be composed of at least 120 individuals [37]. However, our sample was somewhat short of this recommendation with smoking ($n = 76$), obesity ($n = 86$), and excessive alcohol use ($n = 33$), which may explain the absence of effect of the pre-collection factors analyzed on the PVI parameters.

In any case, studies that correlate PVI with these factors are difficult to compare due to diversity of the populations analyzed. Markovic et al. did not find a relation between MPV values and obesity or smoking habit in a population with multiple risk factors for cardiovascular disease [54]. De Luca et al. also did not report any relation between smoking and increased mean MPV in patients with coronary disease [53]. On the other hand, Coban et al. observed higher MPV among obese individuals and lower MPV after weight loss [55, 56]. Kario et al. studied 142 elderly individuals and found that MPV and platelet count were higher in smokers than non-smokers [57]. Ciancarelli et al. showed an increase in MPV with moderate consumption of wine [58]. To our knowledge, only the study by Demirin et al. used alcohol consumption as an exclusion criterion for determining reference values for MPV parameters [32].

Standardization of measurement procedures and the establishment of healthy-associated PVI RIs are essential to be able to support clinical decision-making from laboratorial test results. These intervals must be analyzer-specific and variables such as the anti-coagulant used and the time between collection and analysis must be standardized and controlled. Pre-collection factors such as smoking, alcohol abuse, and obesity may not interfere with PVI values, although further studies will be needed to establish this beyond doubt. The measurement of these indices at the baseline of the ELSA-Brasil reported herein may contribute to future efforts aiming to evaluate whether PVI values are associated with clinical conditions in the Brazilian population.

Acknowledgements

The authors thank the staff and participants of the ELSA study for their important contributions. This article was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability.

Declaration of interest

The ELSA-Brasil baseline study was supported by the Brazilian Ministry of Health (DECIT – Science and Technology Department) and the Brazilian Ministry of Science and Technology (FINEP, Financiadora de Estudos e Projetos and CNPq, National Research Council), grants 01 06 0010.00 RS, 01 06 0212.00 BA, 01 06 0300.00 ES, 01 06 0278.00 MG, 01 06 0115.00SP, 01 06 0071.00 RJ. S. M. B. is a research fellow of the National Research Council (CNPq, grant no 300159/99-4). The authors report no conflict of interest.

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5 ARTIGO 2 - *PLATELET VOLUME IS ASSOCIATED WITH THE FRAMINGHAM RISK SCORE FOR CARDIOVASCULAR DISEASE IN THE BRAZILIAN LONGITUDINAL STUDY OF ADULT HEALTH* (ELSA-BRASIL)

DE GRUYTER Clin Chem Lab Med 2015; aop

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Platelet volume is associated with the Framingham risk score for cardiovascular disease in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil)

DOI 10.1515/cclm-2015-0686
Received July 17, 2015; accepted October 6, 2015

Abstract

Background: Platelet volume indices (PVI), an easy and inexpensive surrogate measure of platelet function, have been associated with cardiovascular diseases (CVD) and their risk factors. However, results are conflicting because of the lack of standardized procedures. The purpose of this study is to investigate the relationship of PVI with the Framingham risk score (FRS).

Methods: Baseline data (2008–2010) of 3115 participants enrolled in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil) were used. PVI measurements were strictly controlled. The cohort was distributed according to risk factors and the general FRS was estimated. Multiple linear regression analysis was used to estimate the association between PVI and FRS.

Results: Mean platelet volume (MPV), platelet distribution width (PDW) and platelet large cell ratio (P-LCR) independently correlated ($p \leq 0.01$) with FRS after adjustment for confounding variables. One unit increase in MPV, PDW, or P-LCR increased the FRS by 0.59%, 0.40%, and 0.08%, respectively. Diabetics had higher ($p \leq 0.004$) MPV, PDW, and P-LCR, and hypertensive individuals had higher ($p \leq 0.045$) PDW and P-LCR.

Conclusions: Increased PVI was independently correlated with higher CVD risk based on the FRS, diabetes, and systolic hypertension. Prospective follow up of this cohort is warranted to confirm that PVI is associated with the development of CVD.

Keywords: cardiovascular disease; Framingham risk score; mean platelet volume; platelet distribution width; platelets.

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Introduction

Platelet pro-inflammatory and pro-thrombotic functions play a key role in the development of atherosclerotic plaques and in the formation of intra-arterial thrombi. Seeking to understand such mechanisms, many researchers have focused their interest on measuring platelet functions [1]. Platelet function can be indirectly estimated from platelet volume indices (PVI), which are easily obtained with automated hematology analyzers [2]. These analyzers provide platelet counts and other measurements, such as mean platelet volume (MPV), platelet distribution width (PDW) and platelet-large cell ratio (P-LCR). Larger platelets, when compared to smaller ones, are metabolically and enzymatically more active, because they contain more α-granules that release prothrombotic factors such as P-selectin, adenosine diphosphate and β-thromboglobulin. Additionally, they produce more thromboxane A2, and exhibit elevated expression of adhesion glycoprotein (GP) Ib receptor as well as GP IIb and IIIa [3].

Multiple factors determine platelet volume during megakaryocytopoiesis and thrombopoiesis [4]. Thrombopoietin, the primary regulator of thrombopoiesis, and cytokines such as interleukins 3 and 6 affect the ploidy of megakaryocytes and can lead to the production of larger, more reactive platelets [5]. Platelet age does not seem to correlate with volume [6]. Under physiological conditions, the number of platelets is inversely proportional to MPV. Interestingly, thrombopoiesis is regulated to keep a constant level of platelet mass. Previous studies show, however, that when the balance between platelet production and depletion is lost, the primary response seems to be the production of bigger, denser, and more reactive

platelets [3, 7, 8]. In this case, higher MPV values act as a marker of increased platelet turnover.

Genetic studies have identified the loci involved in function, count and mean platelet volume; results from these studies may help bring new insights to megakaryocytopoiesis and thrombopoiesis regulation, [9–12]. Studies performed in European populations identified at least 12 loci in different chromosomes associated with function, volume and platelet count [9, 10]. Furthermore, genetic variants, which affect platelets and MPV, have a pleiotrophic effect and are associated with many phenotypes including ischemic heart disease [13].

Several studies have related cardiovascular diseases (CVD) and their risk factors with PVI [8, 14–22]. Yet, these studies have conflicting results, probably because of methodological differences and a lack of measurement standards. Despite the relative ease with which platelet indices can be obtained, clinical decision-making requires standardization of PVI measurement procedures. Indeed, studies have shown that pre-analytical factors such as storage time, anticoagulant use, and the analysis method itself can significantly interfere with these indices and reference intervals [23–26].

Effective CVD prevention depends on the identification of asymptomatic individuals at risk. CVD risk is estimated based on the presence of different risk factors and the potential interaction between these factors. Due to the complexity of these interactions, researchers have developed a variety of clinical risk scores with the intention of improving cardiovascular risk prediction. The general Framingham risk score (FRS) represents the most used score and includes the variables age, total and high-density lipoprotein (HDL) cholesterol, systolic blood pressure, treatment for hypertension, smoking, and diabetes status [27].

Considering the important role of platelets in atherosclerotic disease, and the fact that larger platelets are more reactive and produce more prothrombotic factors, it is biologically plausible that PVIs may provide useful markers of cardiovascular risk. Our purpose in this study was to investigate whether PVI measures are associated with cardiovascular risk as defined by FRS, and to identify which risk factors may influence this association.

Materials and methods

Population

The Brazilian Longitudinal Study of Adult Health (ELSA-Brasil) is the first large multicenter cohort of adults in the country. Participants are government employees from universities and research institutions located in six different states. The main ongoing ELSA-Brasil objectives are to investigate the incidence, progression and risk factors of CVD and diabetes. All active or retired employees of the institutions involved aged between 35 and 74 years were eligible for the study. Exclusion criteria included current or recent (<4 months prior to the first interview) pregnancy, intention to quit working at the institution in the near future, severe cognitive or communication impairment, and, if retired, residence outside the metropolitan area of a study center. All the participants of the cohort answered a comprehensive questionnaire about their general health conditions, family health issues, medication use, smoking, alcohol consumption, physical activity, and mental health, among other factors. In addition, they were submitted to a series of clinical and laboratory tests. Baseline examination was performed between 2008 and 2010. Follow-up and ascertainment of disease occurrence are currently conducted by annual phone interviews commencing in the second year of participation. Repeated interviews and examinations are conducted every 3–4 years thereafter. The second part of the cohort was completed in 2014. The research protocol was approved by the Ethics Committee of each participating institution and by the National Research Ethics Committee. All participants signed an informed consent that included a permission for the storage of biological samples. Details on study design and cohort profile can be found elsewhere [28, 29].

We used the baseline data (2008–2010) of 3115 government employees aged 35–74 from a university and a research institution located in the state of Minas Gerais and enrolled in ELSA-Brasil, for whom PVI data were collected. Presence of CVD was defined based on the report of coronary revascularization and/or medical diagnosis of myocardial infarct and/or stroke and/or heart failure. Presence of diabetes mellitus (DM) was defined as per the American Diabetes Association or by the reported use of insulin or hypoglycemic medication identified in the baseline survey of the ELSA study. Presence of hypertension was defined by the use of antihypertensive medication in the last 2 weeks or average systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg, obtained in three different measures after 5 min of rest.

Of the 3115 participants, 241 were excluded because the interval between blood collection and analysis was >2 h. Of the remaining 2874 individuals, 109 had a previous CVD diagnosis and thus were not eligible for the FRS estimation [27].

Serum samples

Venous blood sampling was performed in the morning after 12–14 h of fasting, following the Clinical and Laboratory Standards Institute's (CLSI) Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture: Approved Standard [30]. Venipuncture was performed with a scalpel for multiple vacuum collections, with the application of a tourniquet for a maximum of 1 min.

Sample tubes containing the tripotassium salt of ethylenediaminetetraacetic acid (EDTA) were identified with bar codes, and blood samples were kept at room temperature until the PVI measurements were performed. The time between sampling and the test procedure was strictly controlled to be within 2 h. In the presence of EDTA, MPV increases in a time-dependent pattern, and the 2-h limit between collection and analyses observed in this study minimizes the undesirable effects of EDTA on PVI estimates.

The plasma/serum for biochemical analysis was stored at −80 °C and sent to the central laboratory of ELSA-Brasil located at the University Hospital of the University of São Paulo [31].

Platelet volume indices analysis

PVI measurements were performed in XE2100 D hematologic analyzers (Sysmex, Kobe, Japan) that use impedance technology to estimate particle count and volume. The quality of the results was validated by internal quality procedures and by participation in proficiency programs of the Brazilian Society of Pathology and Laboratory Medicine and of the College of American Pathologists. An equivalence test between the analyzers used was previously performed to ensure the commutability of results [32]. Intra- and inter assay coefficients of variation were <1.6% e <2.2% for PVI measurements, respectively.

All particles detected with sizes between 2 and 30 fL were regarded as platelets. Based on this data, the platelet volume indices were calculated using the following formulae: MPV (fL)= plateletcrit/platelet count, with plateletcrit being the ratio between the overall volume of platelets and the overall volume of blood in one individual. PDW and P-LCR indices were obtained from the histogram of platelet size distribution. PDW was defined as the distribution width at the level of 20%, considering that the peak of the histogram is 100%. The percentage of platelets larger than 12 fL was defined as P-LCR.

Biochemical analysis

An automated chemical analyzer (ADVIA Chemistry; Siemens, Deerfield, IL, USA) was used to determine serum total cholesterol and HDL cholesterol by the enzymatic colorimetric method as well as fasting glucose and glucose post-load by the hexokinase method. Glycated hemoglobin (HbA_{1c}) was measured by high pressure liquid chromatography (Bio-Rad Laboratories, Hercules, CA, USA).

Cardiovascular risk

The study population, the members of which had no previous diagnosis of CVD, was distributed according to their exposure to different risk factors (age, sex, systolic blood pressure, total and HDL cholesterol, smoking, diabetes status, and use of antihypertensive medications). Their probability of having cardiovascular events in 10 years was estimated using the sex-specific equations of the general FRS proposed by D'Agostino et al. [27].

Statistical analysis

Distributions of the study variables are presented as frequencies and mean and standard deviation (SD) of platelet count and PVI for all participants were calculated. The Shapiro-Wilk test was applied to evaluate the normality of the PVI distributions. In comparisons between subgroups with and without different risk factors of CVD, the Kruskal-Wallis and Wilcoxon tests were performed because PVI indices are not normally distributed. Additionally, multiple linear regression analysis was used to estimate the independent association of PVI measures with the FRS, after adjusting for potential confounders, i.e. variables that are not part of the FRS, but can increase CVD risk and may be related to PVI measures. The variables entered in the multiple regression analysis using the forward approach. Statistical assumptions to perform multiple linear regressions were checked by residual analysis. p-Values lower than 0.05 were considered statistically significant and the analysis was performed using the R software version 3.1.0 (Vienna, Austria).

Results

Table 1 shows the socio-demographic, behavioral, clinical and laboratory characteristics of the study population.

The median PVI measures, 1st interquartile and 2nd interquartile values for the entire study population were, respectively, 10.25 fL, 9.7 fL, 10.8 fL for MPV; 12.0 fL, 11.0 fL, 13.2 fL for PDW and 26.9%, 22.4%, 31.8% for P-LCR. Comparisons between subgroups with and without different CVD risk factors included in the FRS showed that PVI was associated only with diabetes and systolic hypertension. Diabetics had higher MPV ($p=0.004$), PDW ($p<0.001$), and P-LCR ($p=0.002$) medians. A high median MPV was marginally associated with systolic hypertension ($p=0.068$), whereas an association was observed for higher median PDW ($p=0.009$) and P-LCR ($p=0.045$). No significant difference was observed between the groups regarding other risk factors: age, smoking, total cholesterol, and HDL-cholesterol. When comparing men and women, median PDW was significantly higher among men, although no sex differences were observed for MPV and P-LCR medians (Table 2).

In the multiple linear regression analysis, MPV (adjusted $R^2=0.084$; $p=0.013$), PDW (adjusted $R^2=0.086$; $p<0.001$), and P-LCR (adjusted $R^2=0.085$; $p=0.004$) were independently associated with the FRS after adjustment for education, skin color, alcohol use, and physical activity (Table 3). We observed that one unit increase in MPV, PDW, and P-LCR increased the FRS by 0.59%, 0.40%, and 0.08%, respectively. The set of variables included in the multivariate model explained about 8% (adjusted $R^2=0.08$) of the variability of cardiovascular risk stratified by FRS, regardless of the PVI evaluated.

Discussion

Our results show that increased PVI measures are independently associated with higher CVD risk based on the FRS. Regarding the risk factors included in the FRS, the results

Table 1: Baseline characteristics of the studied individuals.

Characteristics	Participants n=2874
Sex (female), n (%)	1482 (54)
Age group, n (%)	
35–44 years	577 (21)
45–54 years	1119 (41)
55–64 years	812 (29)
65–74 years	254 (9)
Self-rated race/skin color, n (%)	
White	1325 (48)
Pardos[a]	965 (35)
Black	359 (13)
Others[b]	79 (3)
Schooling, n (%)	
Incomplete elementary school	119 (4)
Complete elementary school	139 (5)
Complete high-school	865 (31)
University degree	1639 (59)
Smoking status, n (%)	
Never smoked	1628 (59)
Former smoker	800 (29)
Current smoker	334 (12)
Arterial hypertension, n (%)	960 (35)
Diabetes mellitus, n (%)	440 (16)
HDL-cholesterol, n (%)	
Men <40 mg/dL (1.04 mmol/L); women <50 mg/dL (1.30 mmol/L)	514 (19)
Total cholesterol, n (%)	
≥240 mg/dL (6.24 mmol/L)	586 (21)
Alcohol consumption, n (%)	
None	709 (26)
Moderate	1804 (65)
Excessive[c]	247 (9)
Physical activity[d], n (%)	
Low	2054 (74)
Moderate	424 (15)
High	271 (10)
Platelet, $\times 10^3/\mu L$, mean (SD)	228.5 (53.2)
MPV, fL, mean (SD)	10.33 (0.84)
PDW, fL, mean (SD)	12.24 (1.76)
P-LCR, %, mean (SD)	27.53 (7.06)

[a]Mixed color. [b]Includes native Brazilians and people of Asian heritage. [c]Defined as more than 210 g alcohol/week for men and more than 140 g alcohol/week for women. [d]Defined as per the International Physical Activity Questionnaire (IPAQ)- Short Form, according to the IPAC Guidelines for Data Processing and Analysis. MPV, Mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell; HDL, high-density lipoprotein. Some percentages do not totalize 100% due to loss of information.

indicate that median PVI measures are higher among participants with diabetes and systolic hypertension.

Systematic reviews and meta-analyses suggest that MPV is a potential biomarker for prognosis in patients with manifest CVD [18]. The diagnostic accuracy of MPV in patients admitted to the emergency room with suspected ischemic heart disease was analyzed, and the outcome suggests that MPV does not have enough accuracy for efficient triage of patients when used as stand-alone test. However, in combination with other markers, it might be useful in screening for ischemic disease in emergency services [20]. In addition, some studies indicated that increased MPV at admission is an independent predictor of mortality and poor outcome following ST-segment elevation myocardial infarction treated with primary percutaneous coronary intervention or thrombolytic agents [7, 33, 34]. Sansanayudh et al. evaluated its use in cardiovascular risk stratification and risk prediction, and their results showed that MPV may be helpful in risk stratification. However, between-study variation was high and broad heterogeneity was observed within studies, so comparisons have limited value and reaching a conclusion is difficult [8].

On the other hand, a large prospective study conducted with a population in Italy used procedures of pre-analytical standardization of PVI determination, storage time and anticoagulant that were very similar to ours. However, this previous work did not find that MPV was a marker of platelet reactivity or a risk factor for coronary artery disease (CAD) [17]. PDW was also evaluated in the study but similarly showed no correlation with the incidence of CAD and carotid lesions [35]. More recent work evaluated whether the combination of MPV and PDW could improve prognostic information in the prediction of prevalence and extent of CAD. However, the authors concluded that the combination of MPV and PDW could not be considered a risk factor for CAD [36].

The association of high PVI values with systolic hypertension and the presence of diabetes is consistent with results reported by numerous authors [17, 22, 37–40]. In diabetes, multiple factors are responsible for platelet activation and release of pro-inflammatory substances and pro-thrombotic agents, such as systemic inflammation, oxidative stress, altered calcium metabolism, decrease in nitric oxide bioavailability and increased phosphorylation of cellular proteins. Platelets from diabetic patients are hyperactive, which can be explained by their greater volume. In hypertension, increased platelet volume can be explained by increased platelet activation, the secondary effects of renin-angiotensin systems, shear forces, endothelial dysfunction and decreased bioavailability of nitric oxide present in hypertensive patients [41].

We found no significant age or sex-related difference in MPV and P-LCR values. Nonetheless, higher levels of PDW were observed among men. These findings agree with some studies, but differ from others [2, 7, 17, 21, 42].

Table 2: Median and interquartile ranges of PVI for each CVD risk factor group.

Risk factors	MPV, fL				PDW, fL				P-LCR, %			
	1º quartile	Median	3º quartile	p-Value	1º quartile	Median	3º quartile	p-Value	1º quartile	Median	3º quartile	p-Value
Sex				0.392				0.002				0.135
Male (n=1280)	9.7	10.3	10.8		11.1	12.1	13.3		22.7	27.2	32.0	
Female (n=1482)	9.7	10.2	10.8		10.9	11.9	13.1		22.2	26.8	31.7	
Age group				0.695				0.588				0.718
35–44 years (n=577)	9.7	10.2	10.8		11.0	11.9	13.1		22.1	26.7	31.3	
45–54 years (n=1119)	9.7	10.2	10.8		11.0	12.0	13.1		22.5	26.9	31.6	
55–64 years (n=812)	9.7	10.3	10.9		11.0	12.0	13.2		22.3	27.1	32.2	
65–74 years (n=254)	9.8	10.3	10.8		11.1	12.1	13.3		23.2	27.5	32.0	
Current smoker				0.139				0.681				0.222
No (n=2428)	9.7	10.2	10.8		11.0	12.0	13.2		22.3	26.9	31.8	
Yes (n=334)	9.8	10.3	10.9		11.1	12.0	13.1		23.2	27.4	31.9	
Diabetes mellitus				0.004				<0.001				0.002
No (n=2322)	9.7	10.2	10.8		11.0	11.9	13.1		22.3	26.7	31.7	
Yes (n=440)	9.8	10.4	10.9		11.2	12.3	13.4		23.2	28.0	32.6	
Arterial hypertension				0.068				0.009				0.045
No (n=1801)	9.7	10.2	10.8		11.1	11.9	13.1		22.1	26.7	31.6	
Yes (n=960)	9.8	10.3	10.9		11.1	12.1	13.3		22.8	27.4	32.1	
Total cholesterol				0.639				0.471				0.656
<240 mg/dL (6.24 mmol/L) (n=2175)	9.7	10.2	10.8		11.0	12.0	13.2		22.3	26.9	32.0	
≥240 mg/dL (6.24 mmol/L) (n=586)	9.7	10.3	10.8		11.0	12.0	13.0		22.4	27.2	31.2	
HDL cholesterol				0.986				0.311				0.658
Men <40 mg/dL (1.04 mmol/L) and women <50 mg/dL (1.30 mmol/L) (n=514)	9.7	10.2	10.8		11.1	12.0	13.2		22.6	27.2	31.5	
Men ≥40 mg/dL (1.04 mmol/L) and women ≥50 mg/dL (1.30 mmol/L) (n=2247)	9.7	10.2	10.8		11.0	12.0	13.2		22.3	26.9	31.9	

PVI, Platelet volume indices; CVD, cardiovascular diseases; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell; HDL, high-density lipoprotein.

Table 3: Multiple regression analysis of PVI with Framingham risk score.

Variables	PVI									
			MPV		PDW		P-LCR			
	β (95% CI)	p-Value	AjR^2	β (95% CI)	p-Value	AjR^2	β (95% CI)	p-Value	AjR^2
			0.084			0.086			0.085
Intercept	3.665 (−1.175; 8.505)	0.138		4.809 (2.011; 7.606)	<0.001		7.556 (5.890; 9.222)	<0.001	
PVI	0.591 (0.126; 1.056)	0.013		0.406 (0.184; 0.628)	<0.001		0.081 (0.025; 0.136)	0.004	
Schooling									
Incomplete elementary school	11.229 (9.210; 13.247)	<0.001		11.180 (9.164; 13.197)	<0.001		11.234 (9.217; 13.252)	<0.001	
Complete elementary school	5.830 (3.983; 7.676)	<0.001		5.826 (3.981; 7.670)	<0.001		5.834 (3.988; 7.680)	<0.001	
Complete secondary school	0.789 (−0.127; 1.705)	0.091		0.805 (−0.110; 1.721)	0.084		0.810 (−0.119; 1.713)	0.088	
Self-rated race/skin color									
Black	−0.532 (−1.810; 0.744)	0.414		−0.517 (−1.792; 0.757)	0.426		−0.533 (−1.809; 0.743)	0.413	
Pardo[a]	−1.079 (−1.976; 0.182)	0.018		−1.094 (−1.991; −0.198)	0.016		−1.115 (−2.012; −0.218)	0.015	
Other[b]	−0.737 (−3.134; 1.660)	0.547		−0.793 (−3.189; 1.602)	0.516		−0.755 (−3.150; 1.641)	0.537	
Alcohol consumption									
No	−0.985 (−1.916; 0.053)	0.038		−0.964 (−1.895; 0.033)	0.042		−0.972 (−1.904; −0.041)	0.041	
Excessive[c]	6.145 (4.740; 7.550)	<0.001		6.123 (4.719; 7.527)	<0.001		6.132 (4.717; 7.536)	<0.001	
Physical activity[d]									
Moderate	0.304 (−0.792; 1.400)	0.586		0.304 (−0.791; 1.399)	0.587		0.300 (−0.796; 1.395)	0.592	
High	−0.838 (−2.171; 0.495)	0.218		−0.822 (−2.153; 0.510)	0.226		−0.924 (−2.258; 0.410)	0.175	

[a]Mixed color. [b]Includes native indigenous and Asian descendent. [c]Defined as more than 210 g alcohol/week for men and more than 140 g alcohol/week for women. [d]Defined using the International Physical Activity Questionnaire (IPAQ)-Short Form, according to the IPAC Guidelines for Data Processing and Analysis. PVI, Platelet volume indices; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet-large cell; AjR^2, adjusted coefficient of determination; CI, confidence interval.

The explanation for these discrepancies is not clear. Perhaps these differences represent more of an analytical variability than a biological one and require further investigation. In relation to age, the divergence may be due to the relatively small age variability lack of power among our study participants, aged between 35 and 74 years.

Our results showed no correlation of PVI with dyslipidemia and smoking in descriptive analysis. Several studies investigating the relationship between MPV and these same risk factors have reported conflicting results [14–17, 21]. The findings in these studies are difficult to relate to ours due to methodological differences such as the diversity of the population, standardization of measures and analyses of indices.

The identification of PVI as a potential biomarker for cardiovascular risk deserves special attention because of its easy and inexpensive measurement. However, standardization of measurement procedures is crucial to study design, because this may be the source of heterogeneity and high PVI variability in the literature.

Although the adjusted coefficients of determination of the model were small, the model explained about 8% of the variability of cardiovascular risk by FRS. Thus, we cannot discount the possibility that PVI contributes to CVD risk prediction. CVD results from a complex multi interaction of various risk factors, and platelets play a key role in the pathogenesis of atherosclerosis. Larger platelets produce more thromboxane and several studies reported significant reduction of cardiovascular events related to the use of agents, such as aspirin, which have an inhibitory effect on platelet thromboxane production [43]. Recently, Pignatelli et al., by measuring the urinary excretion of 11-dehydro-thromboxane B2 in a large population affected by non-valvular atrial fibrillation, reported the evidence that Mediterranean diet are associated with reduced risk of cardiovascular events by inhibitory effect in vivo platelet activation of Mediterranean diet components [44].

Prospective follow up of this population will enable the identification of new CVD cases and will provide an opportunity to estimate the predictive value of PVI measures in a large number of individuals. Therefore, two issues are critical for the investigation of PVIs as biological markers of platelet reactivity: standardization of the pre-analytical phase and the definition of an analytical method of reference with greater precision and accuracy of measurement.

In summary, our results show that increased PVI is independently associated with higher CVD risk based on the FRS, and is statistically associated with diabetes and systolic hypertension. It appears that the relationship of PVI measures with the FRS is mostly due to their association with diabetes and hypertension, which are part of the FRS. Prospective follow up of this population may help to clarify whether PVI is independently associated with the development of CVD and can add accuracy to CVD risk stratification.

Acknowledgments: The authors thank the staff and participants of the ELSA study for their important contributions. This manuscript was reviewed by a professional science editor and by a native English-speaking copy editor to improve readability.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Financial support: The study was supported by the Brazilian Ministries of Health (DECIT) and of Science and Technology (FINEP/CNPq). SMB is a research fellow of the National Research Council (CNPq, grant no. 300159/99-4).

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Funding source: The ELSA-Brasil baseline study was supported by the Brazilian Ministry of Health (Science and Technology Department DECIT) and the Brazilian Ministry of Science and Technology (FINEP, Financiadora de Estudos e Projetos and CNPq, National Research Council), grants 01 06 0010.00 RS, 01 06 0212.00 BA, 01 06 0300.00 ES, 01 06 0278.00 MG, 01 06 0115.00 SP, 01 06 0071.00 RJ. S.M.B. is a research fellow of the National Research Council (CNPq, grant number 300159/99-4).

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6 CONSIDERAÇÕES FINAIS

Este é o primeiro estudo a propor um intervalo de referência, para três IVP (VPM, PDW, P-LCR), em uma população adulta brasileira sadia com adequada seleção dos indivíduos referência e padronização metodológica.

A avaliação prévia dos interferentes pré-analíticos com padronização dos procedimentos de coleta, manuseio de amostras e do método analítico foi condição fundamental para se estabelecer os estudos com os IVP. A utilização da população do ELSA- Brasil permitiu a exclusão das principais situações clínicas e seus fatores de risco associados com VPM aumentado, bem como potenciais interferentes analíticos como fragmentos de hemácias ou microcitose, fontes de variação biológica conhecidas da literatura.

Estudos referentes aos IVP em diversas condições clínicas são descritos há pelo menos duas décadas. Apesar disso, a aplicação clínica destes índices pode ser limitada se os fatores relacionados à metodologia e aos interferentes pré-analíticos não forem padronizados. A inclusão desses índices na linha de base do projeto ELSA possibilitou avaliar transversalmente a relação entre os parâmetros de volume plaquetário e o perfil de risco cardiovascular. Além disso, permitirá a avaliação prospectiva desses índices com desfechos relacionados às doenças cardiovasculares em participantes dessa coorte.

As plaquetas de maior volume são mais reativas e produzem mais fatores pró-trombóticos. Considerando o importante papel das plaquetas na doença aterosclerótica é biologicamente possível que os IVP sejam úteis como marcadores de risco cardiovascular. Porém, foi observado que o aumento nos valores dos IVP associa-se estatisticamente com aumento da pressão arterial e com a presença de DM. Hipertensão arterial e DM são dois importantes fatores de risco cardiovascular que fazem parte do FRS. Assim, há a possibilidade de que a associação encontrada entre os IVP e o FRS se deva à sua associação com esses dois fatores de risco, que poderiam atuar como fatores de confusão.

Recente revisão sistemática com meta-análise sugere que indivíduos com DM tendem a ter valores VPM e PDW superiores sem diferença na contagem de plaquetas[79]. A associação de altos valores de VPM e PDW com diabetes é consistente com os resultados do presente estudo. Em diabéticos, vários fatores são responsáveis pela ativação plaquetária e liberação de substâncias pró-inflamatórias e agentes pró-trombóticos, como: inflamação sistêmica, estresse oxidativo, metabolismo do cálcio alterado, diminuição da biodisponibilidade do óxido nítrico e aumento da fosforilação de proteínas celulares[80]. Além disso, as plaquetas de pacientes diabéticos parecem ter uma vida média menor, em estudo de Watala *et al*., as plaquetas de indivíduos normais apresentaram vida média de 13,4± 4,8 dias e de pacientes diabéticos 8,2 ± 3,8 dias, podendo o VPM aumentado ser marcador de um maior *turnover* plaquetário nesses pacientes[81,82,83]. Muitos estudos mostram o aumento do VPM correlacionado ao aumento da hemoglobina glicada (HbA1c)[84,85,86] , o que poderia ser explicado pela expansão osmótica da plaqueta induzida pela hiperglicemia[87]. Todos esses fatores podem explicar o maior volume das plaquetas no DM tipo 2.

O volume plaquetário parece ser mais elevado nos pacientes com hipertensão arterial sistêmica, conforme descrito por diferentes autores e observado no presente

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estudo[88,89,90]. Nesse grupo o aumento do volume plaquetário pode ser explicado pela maior ativação plaquetária, secundária aos efeitos do sistema renina-angiotensina, força de cisalhamento, disfunção endotelial e diminuição da biodisponibilidade do óxido nítrico, presente nos pacientes hipertensos[91].

O seguimento prospectivo desta população pode ajudar a esclarecer se os IVP apresentam uma relação causal com DCV em grupos com e sem diabetes e hipertensão arterial.

A identificação do volume plaquetário como potencial biomarcador de risco cardiovascular recebe atenção especial pela sua fácil execução e baixo custo. Porém, além dos problemas relacionados com os fatores de confusão que podem estar presentes na análise estatística e a padronização das medidas, outro ponto que merece ser debatido é a variabilidade analítica dos métodos utilizados que podem impactar na significância clínica dos resultados. Baseada na variação biológica, segundo a *Sociedad Española de Bioquímica Clínica Y Patologia Molecular*[92], para atender a especificação desejável da qualidade analítica, a medição do VPM deve apresentar o coeficiente de variação (precisão) menor ou igual a 2,1%; o erro sistemático (bias) menor ou igual a 2,3%; e erro total menor ou igual a 5,8%. Para o PDW e o P-LCR não estão descritos nas diretrizes de controle da qualidade analítica as especificações da qualidade como são descritas para o VPM. Considerando os resultados do presente estudo, é possível observar que a diferença dos valores do VPM entre os participantes sem e com diabetes foi de 0,2 fL, valor este que é menor que o erro total permitido para o método analítico. Assim, para que estes índices possam ser utilizados na prática clínica, será necessária a melhoria dos métodos de medição.

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Especificamente, na avaliação do P-LCR, há que se destacar que esse índice corresponde ao percentual de plaquetas com volume superior a 12 fL, padronizado pelo analisador hematológico automático utilizado. O valor de 12 fL corresponde ao maior valor de referência do VPM proposto pelo fabricante do instrumento. É possível supor que, se o analisador permitisse a utilização do maior valor de referência do P-LCR definido para essa população, igual a 11,8 fL[93], os resultados da avaliação da sua associação com os fatores de risco estudados poderiam ser diferentes.

Concluindo, duas questões se destacam como críticas para a utilização dos IVP como marcadores biológicos de reatividade plaquetária. A primeira seria a etapa pré-analítica, relacionada com o anticoagulante EDTA utilizado em exames hematológicos e o tempo entre a coleta e a análise. Estes fatores certamente limitam a medição desses índices na rotina de realização de exames laboratoriais. A segunda questão diz respeito à imprecisão das medidas realizadas, pois, os métodos atualmente disponíveis não são suficientemente sensíveis para mensurar as pequenas elevações do volume plaquetário nas diferentes condições clínicas em que estes parâmetros têm sido estudados.

[93] Maluf CB, Barreto SM, Vidigal PG. Standardization and reference intervals of platelet volume indices: Insight from the Brazilian longitudinal study of adult health (ELSA-BRASIL). Platelets 2014; 0: 1-8.

ANEXO A - ATA DO EXAME DE QUALIFICAÇÃO

UNIVERSIDADE FEDERAL DE MINAS GERAIS

UFMG

ATA DO EXAME DE QUALIFICAÇÃO DA ALUNA

CHAMS BICALHO MALUF

Realizou-se, no dia 16 de setembro de 2014, às 14:00 horas, a definir, da Universidade Federal de Minas Gerais, a apresentação do exame de qualificação da aluna **CHAMS BICALHO MALUF**, número de registro 2012652250, intitulado *Correlação dos parâmetros de volume plaquetário com perfil de risco cardiovascular e marcadores de doença aterosclerótica em participantes do Estudo Longitudinal de Saúde do Adulto (ELSA) no Estado de Minas Gerais*, perante a Comissão Examinadora composta pelos professores: Prof(a). Antonio Luiz Pinho Ribeiro (UFMG), Prof(a). Daniel Dias Ribeiro (UFMG).E com a participação dos professores Prof(a). Pedro Guatimosim Vidigal - Orientador (UFMG) e Profª Sandhi Maria Barreto – Coorientadora (UFMG) como membros suplentes,Terminada a apresentação, foi considerada:

(X) aprovada ( ) reprovada

e, para constar, foi lavrada a presente ata que, lida e aprovada, vai assinada pelos membros da Comissão.

Prof(a). Antonio Luiz Pinho Ribeiro (Doutor)

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CONFERE COM ORIGINAL
Centro de Pós-Graduação
Faculdade de Medicina - UFMG

ANEXO B - APROVAÇÃO DO PROJETO ELSA-BRASIL PELO COMITÊ DE ÉTICA EM PESQUISA DA UFMG

Universidade Federal de Minas Gerais
Comitê de Ética em Pesquisa da UFMG - COEP

Parecer nº. ETIC 186/06

Interesse: Prof. (a) Sandhi Maria Barreto
Depto. De Medicina Preventiva e Social
Faculdade de Medicina -UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP, aprovou no dia 28 de junho de 2006 o projeto de pesquisa intitulado **"ELSA - Estudo longitudinal da saúde do adulto."** bem como o Termo de Consentimento Livre e Esclarecido do referido projeto.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Dra. Maria Elena de Lima Perez Garcia
Presidente do COEP/UFMG

ANEXO C – ATA DA DEFESA

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA

UFMG

ATA DA DEFESA DE TESE DA ALUNA
CHAMS BICALHO MALUF

Realizou-se, no dia 14 de dezembro de 2015, às 14:00 horas, Sala 715 - Faculdade de Medicina da UFMG, da Universidade Federal de Minas Gerais, a defesa de tese, intitulada *ÍNDICES DE VOLUME PLAQUETÁRIO E RISCO DE DOENÇA CARDIOVASCULAR EM PARTICIPANTES DO ESTUDO LONGITUDINAL DE SAÚDE DO ADULTO (ELSA – BRASIL)*, apresentada por CHAMS BICALHO MALUF, número de registro 2012652250, graduada no curso de MEDICINA, como requisito parcial para a obtenção do grau de Doutor em PATOLOGIA, à seguinte Comissão Examinadora: Prof(a). Pedro Guatimosim Vidigal - Orientador (UFMG), Prof(a). Sandhi Maria Barreto - Coorientadora (UFMG), Prof(a). Roberta Carvalho de Figueiredo (UFSJ), Prof(a). Daniel Dias Ribeiro (UFMG), Prof(a). Sandra Guerra Xavier (UFMG), Prof(a). Cristina Dickie de Castilhos (UFRGS).

A Comissão considerou a tese:

(X) Aprovada

( ) Reprovada

Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.

Belo Horizonte, 14 de dezembro de 2015.

Prof. Pedro Guatimosim Vidigal (Doutor)

Prof(a). Sandhi Maria Barreto (Doutora)

Prof(a). Roberta Carvalho de Figueiredo (Doutora)

Prof. Daniel Dias Ribeiro (Doutor)

Prof(a). Sandra Guerra Xavier (Doutor)

Prof(a). Cristina Dickie de Castilhos (Doutora)

ANEXO D – FOLHA DE APROVAÇÃO

UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA

UFMG

FOLHA DE APROVAÇÃO

ÍNDICES DE VOLUME PLAQUETÁRIO E RISCO DE DOENÇA CARDIOVASCULAR EM PARTICIPANTES DO ESTUDO LONGITUDINAL DE SAÚDE DO ADULTO (ELSA – BRASIL)

CHAMS BICALHO MALUF

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em PATOLOGIA, como requisito para obtenção do grau de Doutor em PATOLOGIA, área de concentração PATOLOGIA INVESTIGATIVA.

Aprovada em 14 de dezembro de 2015, pela banca constituída pelos membros:

Prof(a). Pedro Guatimosim Vidigal - Orientador
UFMG

Prof(a). Sandhi Maria Barreto - Coorientadora
UFMG

Prof(a). Roberta Carvalho de Figueiredo
UFSJ

Prof(a). Daniel Dias Ribeiro
UFMG

Prof(a). Sandra Guerra Xavier
UFMG

Prof(a). Cristina Dickie de Castilhos
UFRGS

Belo Horizonte, 14 de dezembro de 2015.