UNIVERSIDADE FEDERAL DO MARANHÃO
CENTRO DE CIIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE
DOUTORADO

CORRELAÇÃO ENTRE OS NÍVEIS SÉRICOS DE 25-HIDROXIVITAMINA D E A ESPESSURA MÉDIO-INTIMAL CAROTÍDEA EM AFRODESCENDENTES HABITANTES DE COMUNIDADES QUILOMBOLAS

NATÁLIA RIBEIRO MANDARINO

SÃO LUÍS

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde como parte dos requisitos para a obtenção do título de Doutor em Ciências da Saúde.

Orientador: Prof. Dr. Natalino Salgado Filho

Co-orientador: Prof. Dr. Francisco das Chagas Monteiro Júnior

SÃO LUÍS

2017

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Mandarino, Natália Ribeiro.
Correlação entre os níveis séricos de 25-hidroxivitamina D e A espessura médio-intimal carotídea em afrodescendentes habitantes de comunidades quilombolas / Natália Ribeiro Mandarino. -2017.
125 f.: il.

Coorientador(a): Francisco das Chagas Monteiro Júnior
Orientador(a): Natalino Salgado Filho
Tese (Doutorado) - Programa de Pós-Graduação em Ciências Da Saúde /CCBS, Universidade Federal do Maranhão, São Luís, 2017.

1. Aterosclerose carotídea. 2. Deficiência de vitamina D. 3. Espessura médio-intimal carotídea. 4. População negra. I. Monteiro Júnior, Francisco das Chagas. II. Salgado Filho, Natalin. III. Título.

Natália Ribeiro Mandarino

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7) Presidente (Orientador) Natalino Salgado Filho

Ao meu querido esposo Francisco Monteiro Júnior, companheiro de todas as horas;

À minha adorada filha Maria Clara, que todos os dias renova minha alegria de viver;

Aos meus amados pais, Edmundo e Maria das Graças, pelo apoio incondicional em todos os momentos importantes da minha vida;

Aos meus irmãos queridos, Renata, Milena e Thiago, pelo incentivo imprescindível nos momentos difíceis.

AGRADECIMENTOS

A todos os habitantes de comunidades quilombolas do município de Alcântara, Maranhão, que aceitaram voluntariamente participar do projeto PREVRENAL;

Ao Prof. Dr. Natalino Salgado Filho, por ter acreditado em meu trabalho e pelo incentivo e apoio constantes em todos os momentos desta jornada;

Ao Prof. Dr. Francisco Monteiro Júnior, pelo incentivo, apoio, dedicação, companheirismo e ensinamentos, essenciais para transformar este sonho em realidade;

À coordenação, na pessoa da Prof[a.] Dra. Flávia Nascimento, e a todos os professores do Programa de Pós-graduação em Ciências da Saúde, pelos ensinamentos compartilhados;

A todos os profissionais e estudantes que compuseram a equipe multidisciplinar do projeto PREVRENAL, pela amizade, dedicação e competência na execução da pesquisa;

Ao bioquímico Johnny Ramos Nascimento, do Laboratório de Imunologia da Universidade Federal do Maranhão, por dispor do seu precioso tempo na realização de dosagens laboratoriais;

Aos bioquímicos Ilma e Renato, do Laboratório de Análises Clínicas do HU da Universidade Federal do Maranhão, pelo trabalho esmerado na realização das dosagens de vitamina D;

Ao Prof. Dr. Rafael, chefe do Departamento de Enfermagem da Universidade Federal do Maranhão, pelo apoio e incentivo ao meu trabalho;

Às Prof[as] Dras. Lúcia Holanda e Flávia, do Departamento de Enfermagem da Universidade Federal do Maranhão, pelo apoio e imprescindível colaboração na disciplina Semiotécnica, em meus momentos de ausência;

À Fundação de Amparo à Pesquisa e ao Desenvolvimento Científico e Tecnológico do Maranhão (FAPEMA), pela viabilização financeira do projeto;

Ao médico angiologista Enedino Fontes, pela competência e inestimável colaboração na realização dos exames de ultrassonografia vascular;

Aos colegas de turma do Doutorado, pelo companheirismo e conhecimentos compartilhados;

Ao médico endocrinologista Wellington Santana da Silva Júnior, pelo incentivo e valiosa colaboração na análise dos dados;

À Prof[a] Dra. Adalgisa de Sousa Paiva Ferreira, pela valiosa colaboração na análise estatística;

À Prof[a.] Ms Joyce Santos Lages, pelo incentivo e decisiva colaboração na viabilização do projeto;

Por fim, a todos aqueles que, direta ou indiretamente, contribuíram para a concretização deste trabalho;

‘Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer’.

Mahatma Gandhi

RESUMO

O papel da vitamina D na regulação do metabolismo ósseo já está bem estabelecido. Entretanto, nos últimos anos, o papel da vitamina D na saúde extraesquelética tem sido amplamente explorado. Na área cardiovascular, a deficiência de vitamina D tem sido associada de forma independente à ocorrência de infarto do miocárdio, acidente vascular cerebral e morte cardiovascular. Os mecanismos para explicar a associação entre hipovitaminose D e doença cardiovascular ainda não estão de todo esclarecidos, sendo postulada sua vinculação com a aterosclerose. No entanto, estudos procurando correlacionar hipovitaminose D com marcadores de aterosclerose têm produzido resultados conflitantes, da mesma forma que pequenos estudos randomizados de suplementação oral para avaliar desfechos intermediários, de modo que há atualmente considerável debate acerca de se a hipovitaminose D representa um novo fator de risco ou seria apenas um marcador inflamatório. No primeiro artigo, já publicado, procurou-se realizar uma revisão abrangente sobre o papel da deficiência de vitamina D na patogenia da doença cardiovascular, incluindo desde aspectos básicos de sua biossíntese até os resultados de estudos de intervenção, por meio de sua suplementação oral. O segundo artigo apresenta os resultados de uma análise transversal de 382 indivíduos habitantes de comunidades quilombolas em Alcântara - MA, participantes da coorte PREVRENAL, apresentando pelo menos um fator de risco cardiovascular, com média de idade de 57.79 (± 15.3) anos e discreto predomínio do sexo feminino, em que se procurou correlacionar os níveis séricos de 25-hidroxivitamina D, a forma circulante estável da vitamina, com um marcador estabelecido de aterosclerose subclinica, a espessura médio-intimal carotídea, e outros fatores de risco cardiovascular. Foram coletados dados sócio-demográficos, sobre estilo de vida, antropométricos e clínicos e realizados exames bioquímicos, incluindo a dosagem de 25-hidroxivitamina D, por meio do ensaio eletroquimioluminiscência. A excreção urinária de albumina foi avaliada por meio da razão albumina / creatinina em amostra isolada de urina. Hipovitaminose D foi definida como níveis séricos de 25-hidroxivitamina D <30 ng / mL. Todos os participantes foram submetidos a exame das artérias carótidas comuns por ultrassonografia de alta resolução para medida da espessura médio-intimal, sendo adotada a média das medidas de ambos os lados. A média dos níveis séricos de 25-

hidroxivitamina D foi de 50.4 (± 13.5) ng / mL, observando-se uma baixa prevalência de hipovitaminose D (<5%). Por correlação linear simples, observou-se uma associação inversa significativa entre os níveis de 25-hidroxivitamina D e a espessura médio-intimal carotídea (r = -0.174, p = 0.001). Entretanto, após análise de regressão múltipla, apenas as variáveis sexo masculino, idade, tabagismo, pressão arterial sistólica, glicemia em jejum e LDL-colesterol permaneceram significativamente associadas com a espessura médio-intimal carotídea. Níveis de 25-hidroxivitamina D se associaram independentemente, de forma positiva com o HDL-colesterol, e inversa com a excreção urinária de albumina. Em conclusão, nesta população afrodescendente, com baixa prevalência de hipovitaminose D, não se observou uma associação independente entre os níveis séricos de 25-hidroxivitamina D e a espessura médio-intimal carotídea, achado que contraria a hipótese do seu papel antiaterosclerótico. Por outro lado, a sua associação positiva com o HDL-colesterol e inversa com a excreção urinária de albumina, também considerada um preditor independente de eventos cardiovasculares, não permite afastar ações de proteção cardiovascular da vitamina neste perfil populacional.

Palavras-chave: Deficiência de vitamina D. Espessura médio-intimal carotídea. Aterosclerose carotídea. População negra.

ABSTRACT

The role of vitamin D in the regulation of bone metabolism is already well established. However, in recent years, the role of vitamin D in extraskeletal health has been widely explored. In the cardiovascular area, vitamin D deficiency has been independently associated with the occurrence of myocardial infarction, stroke and cardiovascular death. The mechanisms to explain the association between hypovitaminosis D and cardiovascular disease are still not fully understood, and their association with atherosclerosis is postulated. However, studies attempting to correlate hypovitaminosis D with atherosclerosis markers have produced conflicting results, in the same way as small randomized trials of oral supplementation to evaluate intermediate outcomes, so there is currently considerable debate about whether hypovitaminosis D represents a new risk or would be just an inflammatory marker. In the first article, a comprehensive review was made of the role of vitamin D deficiency in the pathogenesis of cardiovascular disease, from basic aspects of its biosynthesis to the results of interventional studies, through its oral supplementation. The second article presents the results of a cross - sectional analysis of 382 individuals living in quilombola communities in Alcântara - MA, participants of the PREVRENAL cohort, presenting at least one cardiovascular risk factor, with a mean age of 57.79 (± 15.3) years and a slight predominance, in which the serum levels of 25-hydroxyvitamin D, the stable circulating form of the vitamin, were correlated with an established marker of subclinical atherosclerosis, carotid intima-media thickness, and other cardiovascular risk factors. Socio-demographic, lifestyle, anthropometric and clinical data were collected and biochemical tests were performed, including the dosage of 25-hydroxyvitamin D by means of the electrochemiluminescence assay. The urinary excretion of albumin was evaluated by means of the albumin / creatinine ratio in an isolated sample of urine. Hypovitaminosis D was defined as serum 25-hydroxyvitamin D levels <30 ng / mL. All participants underwent examination of the common carotid arteries by high-resolution ultrasonography to measure the intima-media thickness, and the mean of the measurements on both sides was adopted. Serum levels of 25-hydroxyvitamin D were 50.4 (± 13.5) ng / mL, with a low prevalence of hypovitaminosis D (<5%). By simple linear correlation, there was a significant inverse association between 25-hydroxyvitamin D levels and carotid intima-media thickness ($r = -0.174$, $p = 0.001$). However, after multiple regression

analysis, only the variables male gender, age, smoking, systolic blood pressure, fasting blood glucose and LDL-cholesterol remained significantly associated with carotid intima-media thickness. Levels of 25-hydroxyvitamin D were independently associated positively with HDL-cholesterol and inversely with urinary albumin excretion. In conclusion, in this Afrodescendant population, with a low prevalence of hypovitaminosis D, there was no independent association between serum 25-hydroxyvitamin D levels and carotid intima-media thickness, a finding that contradicts the hypothesis of its antiatherosclerotic role. On the other hand, its positive association with HDL-cholesterol and the inverse association with urinary albumin excretion, also considered as an independent predictor of cardiovascular events, does not allow the exclusion of cardiovascular protection actions of the vitamin in this population profile.

Keywords: Vitamin D deficiency. Intima-media thickness (IMT). Atherosclerosis. African continental ancestry group.

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LISTA DE SIGLAS

AVC	Acidente Vascular Cerebral
CaDDM	*Calcium and Vitamin D for Diabetes Mellitus*
CKD-EPI	*Chronic Kidney Disease Epidemiology Collaboration*
CVD	Cardiovascular Disease
DCCT	Diabetes Control and Complications Trial
DCV	Doenças Cardiovasculares
DM	Diabetes Mellitus
DMF	Dilatação Mediada por Fluxo
DNA	Ácido Desoxirribonucléico
EMIC	Espessura Íntimo-Medial Carotídea
FIND	*Finnish Vitamin D trial*
FMD	*Flow-Mediated Dilatation of the Brachial Artery*
HAS	Hipertensão Arterial Sistêmica
HDL	Lipoproteína de Alta Densidade
HOMA	*Homeostasis Model Assessment of Insulin Resistance*
IC	Insuficiência Cardíaca
IMC	Índice de Massa Corpórea
IMT	Intima-media thickness
LDL	Lipoproteína de Baixa Densidade
LURIC	*Ludwigshafen Risk and Cardiovscular Health*
NHANES	*National Health and Nutrition Examination Survey*
NHANES III	*Third National Health and Nutrition Examination Survey*
PCR-US	Proteína C-Reativa Ultra-Sensível
pro-BNP	Peptídeo Natriurético Cerebral
PTH	Hormônio paratireóideo
RAC	Razão Albumina/Creatinina
RAS	*Renin-Angiotensin System*
RNA	Ácido Ribonucleico
RXR	Ácido Retinóico
SD	Desvio Padrão
SM	Síndrome Metabólica

SRA	Sistema Renina-Angiotensina
TBARS	*Thiobarbituric Acid Reactive Substances*
TIDE	*Thiazolidinedione Intervention with vitamin D Evaluation*
UFMA	Universidade Federal do Maranhão
UNIFESP	Universidade Federal de São Paulo
VDR	Receptor de Vitamina D
VDRE	Elementos de Resposta da Vitamina D
VIDAL	*Vitamin D and Longevity*
VITAL	*Vitamin D and Omega-3 Trial*
VLDL	Lipoproteína de Densidade Muito Baixa
WHI	*Women's Health Initiative*

1 INTRODUÇÃO

A doença cardiovascular representa a principal causa de morte e incapacidade no Brasil e no mundo (YUSUF et al., 2001). Embora o papel dos fatores de risco tradicionais, incluindo hipertensão arterial sistêmica, tabagismo, dislipidemia e diabetes mellitus, já esteja consolidado por vários estudos epidemiológicos, sabe-se que eles podem não explicar completamente o desenvolvimento da doença cardiovascular ou mesmo não estar presentes em muitos indivíduos acometidos pela mesma (KHOT et al., 2003), o que tem suscitado a busca contínua de novos fatores de risco. Evidências crescentes, obtidas nos últimos anos, sugerem que a deficiência de vitamina D possa estar associada a um maior risco de doença cardiovascular, podendo representar um fator de risco emergente ou não-clássico (GINDE et al., 2009).

Apesar da denominação tradicional, a vitamina D é, na verdade, um hormônio, cuja função primordial consiste na regulação da homeostase do cálcio e fósforo, em interação com as paratireoides, rins e intestinos. Em condições normais, apenas aproximadamente 10% da vitamina D necessária são obtidos por ingestão alimentar, sob a forma tanto de vitamina D2 (ergocalciferol) como vitamina D3 (colecalciferol). Assim, a sua principal fonte é representada pela síntese no próprio organismo, que se inicia com a ativação de um precursor cutâneo pela radiação ultravioleta solar e envolve sucessivos processos de hidroxilação a nível hepático e renal (REIS et al., 2009; MARQUES et al., 2010).

O papel da vitamina D na regulação do metabolismo ósseo já está bem estabelecido há décadas. Nesta função, ela promove aumento da absorção intestinal e da reabsorção renal de cálcio, atuando ainda em sua mobilização a partir do osso, na presença do hormônio paratireóideo (PTH). Assim, doenças como o raquitismo e a osteomalácia têm sido classicamente atribuídas à deficiência prolongada de vitamina D (MARQUES et al., 2010).

Entretanto, nos últimos anos vários estudos têm evidenciado que a função da vitamina D no organismo se estende muito além da saúde óssea, incluindo a regulação do sistema imunológico e efeitos anti-proliferativos nas células, podendo ainda desempenhar um papel importante na patogenia da doença cardiovascular (REIS et al., 2009; TEMMERMAN, 2011). Estas ações, ditas não calcêmicas, são

exercidas sobre receptores específicos, que regulam a transcrição do ácido desoxirribonucléico (DNA) em ácido ribonucleico (RNA), semelhantes aos receptores para esteróides, hormônios tireoidianos e retinóides, presentes em inúmeros tipos celulares, incluindo epitélio do intestino delgado, células hematopoiéticas, linfócitos, células epidérmicas, células pancreáticas, miócitos, neurônios, cardiomiócitos, células do músculo liso vascular, endotélio e tecido placentário, além de osteoblastos, osteoclastos e células tubulares renais (MARQUES et al., 2010).

Atualmente se sabe que a forma biologicamente ativa da vitamina D exerce ações modulatórias diretamente sobre mais de 200 genes humanos. Assim, a deficiência de vitamina D tem sido associada a desordens tão variadas quanto doenças auto-imunes, infecções, desfechos materno-fetais desfavoráveis e câncer colo-retal. Especificamente em relação ao risco cardiovascular, a hipovitaminose D tem sido independentemente associada à ocorrência de hipertensão arterial (FORMAN et al., 2007), resistência insulínica e síndrome metabólica (LU et al., 2009; PACIFICO et al., 2011; MIÑAMBRES et al., 2012), diabetes mellitus tipos 1 e 2 (KNEKT et al., 2008), doença arterial periférica (MELAMED et al., 2008), infarto do miocárdio (GIOVANNUCCI et al., 2008), acidente vascular cerebral (PILZ et al., 2008) e mortalidade relacionada (MELAMED et al., 2008).

Estas evidências se tornam particularmente alarmantes quando se considera a alta prevalência da deficiência de vitamina D nas populações já estudadas, atingindo em geral mais da metade dos indivíduos (TARGHER et al., 2006; HOLICK et al., 2006; LU et al., 2009; MITHAL et al., 2009), e o nível endêmico da doença cardiovascular a nível global (YUSUF et al., 2001). Além disso, tem sido descrito que a deficiência de vitamina D é mais prevalente em indivíduos de pela escura, o que pode ser explicado pelo fato de a melanina agir como um filtro solar natural, podendo reduzir significativamente sua síntese (CLEMENS et al., 1982). Isto pode ser agravado por uma dieta mais deficitária nesta vitamina em populações menos favorecidas.

Os mecanismos pelos quais os níveis de vitamina D podem influenciar o risco cardiovascular ainda não estão de todo esclarecidos, sendo postulada a sua participação na patogenia da aterosclerose. Assim, alguns estudos têm demonstrado envolvimento da vitamina D no mecanismo de resistência insulínica (CHIU et al., 2004), na indução da secreção de prostaciclina, a qual tem papel importante na redução da trombogenicidade, adesão celular e proliferação de células musculares

lisas (WAKASUGI et al., 1991), na regulação da expressão de várias proteínas com ação vascular (ZITTERMANN; SCHLEITHOFF; KOERFER, 2005) e, ainda, na supressão de citocinas pró-inflamatórias, incluindo a interleucina-6 e o fator de necrose tumoral-α in vitro and in vivo (ZITTERMANN; KOERFER, 2008a). Além disso, em ratos, a vitamina D funciona como um inibidor do sistema renina-angiotensina (LI et al., 2002) e previne o desenvolvimento de hipertrofia miocárdica (XIANG et al., 2005).

No entanto, os poucos estudos que têm procurado correlacionar níveis de vitamina D com sinais precoces de aterosclerose são, em geral, de pequeno porte, envolvem populações específicas e têm reportado achados conflitantes (ARAD et al., 1998; TARGHER et al., 2006; REIS et al., 2009; JOERGENSEN et al., 2011; ZANG et al., 2012; YADAV et al., 2012), inexistindo pesquisas dessa natureza na população latino-americana. Em populações afro-descendentes, em especial em descendentes quilombolas, população objeto desta pesquisa, não apenas não foram encontradas pesquisas correlacionando hipovitaminose D com aterosclerose como é escassa a informação sobre o status da vitamina D especificamente neste perfil populacional (MIlJKOVIC et al., 2011), inexistindo estudos brasileiros.

Assim, no presente estudo, buscando-se explorar a possível existência de associação entre hipovitaminose D e aterosclerose, procurou-se correlacionar os níveis séricos de 25-hidroxivitamina D, a forma circulante estável da vitamina D, com a medida da espessura íntimo-medial carotídea (EMIC), um marcador de aterosclerose subclínica amplamente validado como preditor independente de eventos cardiovasculares (O'LEARY et al., 1999; RUNDEK et al., 2008), e outros fatores de risco cardiovascular, em indivíduos afro-descendentes participantes de uma coorte de base populacional envolvendo habitantes de comunidades quilombolas, em Alcântara, no litoral oeste do Estado do Maranhão.

2 REFERENCIAL TEÓRICO

2.1 Afrodescendentes residentes em comunidades quilombolas

Os grupos étnicos conhecidos como “comunidades remanescentes de quilombos”, “quilombolas” ou “comunidades negras rurais” são constituídos pelos descendentes dos escravos negros que, no processo de resistência à escravidão, originaram grupos sociais que passaram a ocupar um território comum e compartilham características culturais até os dias de hoje. O termo quilombola vem do tupi-guarani *cañybó* e significa “aquele que foge muito” (SILVA et al., 2008).

O Brasil é a segunda maior nação negra do mundo, atrás somente da Nigéria. Segundo dados oficiais, a proporção de pretos e pardos corresponde à metade da população brasileira (BEZERRA et al., 2013).

Os territórios ocupados por remanescentes de quilombolas estão distribuídos em todas as regiões do país, geralmente em áreas rurais (SILVA et al., 2008). Estima-se que haja no Brasil atualmente cerca de 1,17 milhões de afrodescendentes quilombolas, distribuídos em 1948 comunidades, sendo o maior contingente concentrado na região Nordeste (BRASIL, 2011).

No Estado do Maranhão, segundo a Comissão Pró-Índio de São Paulo, existem 527 comunidades quilombolas, distribuídas em 134 municípios. Essas comunidades se concentram principalmente nas regiões da Baixada Ocidental, Baixada Oriental, Munim, Itapecuru, Mearim, Gurupi e Baixo Parnaíba.

As comunidades quilombolas de Alcântara estão situadas na zona rural do município, a 22 quilômetros de São Luís, capital do Maranhão, no litoral oeste do Estado. Segundo a Rede Social de Justiça e Direitos Humanos, vivem em Alcântara cerca de 19 mil habitantes, na sua maioria descendentes de quilombolas e índios (CRISTALDO, 2012).

As atividades econômicas predominantes nos quilombos são a agricultura para subsistência, a pecuária tradicional e o artesanato. Os afrodescendentes residentes em quilombos vivem em condições de vulnerabilidade, em decorrência de sua baixa escolaridade, condições socioeconômicas desfavoráveis e dificuldade de acesso ao sistema de saúde. Devido a estes fatores, estes indivíduos apresentam maior

incidência de doenças e morrem mais precocemente em todas as idades (BEZERRA et al., 2013).

Apesar de seu papel marcante na História do Brasil, só recentemente as comunidades quilombolas foram reconhecidas pela Constituição Brasileira, sendo auto-definidas a partir das relações com a terra, parentesco, práticas culturais e presunção de ancestralidade negra, representando o resgate de uma dívida histórica com a população afrodescendente (SILVA et al., 2008).

2.2 Fisiologia e metabolismo da vitamina D

Em condições normais, apenas cerca de 10% da vitamina D necessária ao organismo são obtidos através da ingestão de alimentos, sob a forma tanto de vitamina D2 (ergocalciferol) como de vitamina D3 (colecalciferol). Assim, a principal fonte da vitamina D é representada por sua síntese no próprio organismo, que se inicia na pele. Quando exposto à radiação ultravioleta, o precursor cutâneo da vitamina D, o 7-dehidrocolesterol, sofre uma clivagem fotoquímica originando a pré-vitamina D3. Essa molécula termolábil, em um período de 48 horas, sofre um rearranjo molecular dependente da temperatura, o que resulta na formação da vitamina D3. Tanto a vitamina D originária da dieta, via absorção intestinal, quanto a formada a partir da pré-vitamina D3 na pele ligam-se a proteínas circulantes e são transportadas até o fígado onde são hidroxiladas no carbono 25, originando a chamada 25-hidroxivitamina D3 [25(OH)D]. Esta é, em seguida, novamente hidroxilada, desta vez no carbono 1, a nível mitocondrial nas células dos túbulos contorcidos proximais, bem como possivelmente em vários outros tecidos, sob ação da enzima 1α-hidroxilase (1α-OHase), originando finalmente a 1,25-dihidroxivitamina D3 [$1,25(OH)_2D$], sua forma biologicamente ativa. Esta fase de ativação é estritamente regulada pelos níveis séricos do PTH, cálcio e fósforo. Embora biologicamente inerte, no entanto, é a 25(OH)D, a forma circulante em maior quantidade e mais estável, que é dosada laboratorialmente e adotada na prática clínica para se avaliar a reserva de vitamina D no organismo (REIS et al., 2009; MARQUES et al., 2010). O PTH, por outro lado, é um peptídeo secretado em resposta a baixos níveis de cálcio e fósforo circulantes que estimula a reabsorção de cálcio nos rins e sua remoção do esqueleto, além de aumentar a produção renal de 1,25(OH)2D, como mencionado (REIS et al., 2009).

Já está bem estabelecido o complexo mecanismo de ação da vitamina D a nível intracelular. A sua forma ativa atravessa a membrana celular, penetra na célula-alvo e liga-se ao receptor específico, o receptor de vitamina D (VDR), que está presente no citoplasma. Este complexo, $1,25(OH)_2D$-VDR, sofre translocação para o núcleo celular e heterodimeriza com o receptor do ácido retinóico (RXR). Na sequência, o complexo 1,25(OH)2D-VDR-RXR liga-se aos elementos de resposta da vitamina D (VDRE) no DNA aumentando a transcrição para o RNA dos genes responsáveis pela expressão das proteínas envolvidas na absorção intestinal de cálcio e fósforo (PIKE et al., 2007).

Atualmente se sabe que receptores tipo VDR estão presentes em inúmeros tipos celulares, incluindo células hematopoiéticas, linfócitos, células epidérmicas, células pancreáticas, miócitos, neurônios, cardiomiócitos, células do músculo liso vascular, endotélio e tecido placentário, além de osteoblastos, osteoclastos, epitélio do intestino delgado e células tubulares renais, o que vem explicar a multiplicidade de ações não calcêmicas exercidas pela vitamina D sobre sistemas tão variados como o endócrino, o imunológico, o reprodutor, o sistema nervoso central e o aparelho cardiovascular, bem como as inúmeras implicações da sua deficiência para saúde (MARQUES et al., 2010). Estima-se que a vitamina D controle, direta ou indiretamente, cerca de 3% de todo o genoma humano. Assim, acredita-se que uma ampla variedade de processos fisiológicos, incluindo regulação de citocinas, inflamação, fibrogênese, sistema renina-angiotensina, resposta imune e crescimento e diferenciação celular, possam sofrer influências diretas ou indiretas da vitamina D (BOUILLON et al., 2008).

2.3 Prevalência e fatores determinantes da hipovitaminose D

A definição de hipovitaminose D tem sido ainda motivo de debate. No entanto, a maioria concorda que níveis séricos de 25(OH)D < 20 ng/mL (ou 50 nmol/l) sejam indicativos de deficiência, na faixa de 20 a 30 ng/ml (ou 50 a 75 nmol/l) correspondam a insuficiência e > 30 ng/ml (ou 75 nmol/l) representem suficiência de vitamina D.

Vários fatores podem interferir na prevalência de hipovitaminose D. A exposição cada vez menor à luz solar, característica da vida moderna em cidades, aliada ao uso disseminado de filtro solar, estimulado pelo receio de câncer de pele,

representa, sem dúvida, o fator mais importante para a prevalência crescente de hipovitaminose D em todo o mundo. Adicionalmente, em indivíduos de pele escura a síntese cutânea de vitamina D pode ser reduzida em até 50 a 90%, uma vez que a melanina funciona como um filtro solar natural. Fatores adicionais incluem baixa latitude, inverno, extremos de idade, sexo feminino, desnutrição, uso de vestes cobrindo a maior parte do corpo e obesidade (ARABI et al., 2010; DINI; BIANCHI, 2012).

Assim, segundo estudos realizados em vários continentes, a deficiência de vitamina D representa uma das condições mais comuns no mundo atual. Estima-se que mais da metade das crianças e adultos que vivem nos Estados Unidos da América, Canadá, México, Europa, Ásia, Nova Zelândia e Austrália sofram da hipovitaminose D (DINI; BIANCHI, 2012).

Inquérito populacional recente realizado nos Estados Unidos demonstrou que a prevalência de insuficiência de vitamina D duplicou nos últimos 10 anos, acometendo atualmente nada menos que 90% das populações pigmentadas (negros, hispânicos e asiáticos) e em torno de ¾ da população branca (ADAMS; HEWISON, 2010).

Vários estudos têm sido conduzidos na Índia, demonstrando em geral altas prevalências de deficiência de vitamina D, a despeito de se tratar de um país ensolarado na maior parte do ano, tanto nas áreas urbanas como rurais e em todos os grupos etários e ambos os sexos (GOSWAMI; MISHRA; KOCHUPILLAI, 2008; DINI; BIANCHI, 2012).

Em países do Oriente Médio e em outros países árabes, a hipovitaminose D também tem apresentado prevalência expressiva (DINI; BIANCHI, 2012). Estudo de base populacional realizado em Teerã, por exemplo, observou uma prevalência de 81,3% (HASHEMIPOUR et al., 2004).

Na China, em estudo transversal de base populacional realizado em duas grandes cidades, incluindo mais de 3.200 indivíduos, com idade variando de 50 a 70 anos, a prevalência de deficiência (< 20ng/ml) e insuficiência (20-30 ng/ml) de 25(OH)D foi de, respectivamente, 69,2 e 24,4% (LU et al., 2009).

Mesmo em habitantes da África subsaariana, que conta com ampla disponibilidade da luz solar ao longo do ano, nada menos que 1/3 a metade dos mesmos apresentam níveis séricos de 25(OH)D abaixo de 10 ng/ml (ARABI et al., 2010). Por outro lado, em estudo envolvendo 424 indivíduos afro-descendentes

idosos (>65 anos), participantes de uma coorte de base populacional em uma ilha do Caribe, onde a exposição solar da população era notadamente elevada, foi observada uma baixa prevalência de deficiência de vitamina D, de apenas 2,8%, com uma prevalência de insuficiência comparativamente modesta (24%), apesar de tratar-se de uma população de pele escura e idosa, o que reforça o papel preponderante da radiação ultravioleta na geração de vitamina D em seres humanos (MIlJKOVIC et al., 2011).

No Brasil, estudos realizados em várias regiões indicam valores subótimos de vitamina D, verificando-se alta prevalência de hipovitaminose D em diversas faixas etárias, inclusive em adolescentes (MAEDA et al., 2014). Em geral, são estudos pequenos, utilizando amostras de conveniência, em vários perfis populacionais (MAEDA et al., 2014). Nos dois maiores identificados (KUCHUK et al., 2009; ARANTES et al., 2013), ambos abordando mulheres na pós-menopausa, envolvendo, respectivamente, 1.933 e 1.486 participantes, a prevalência de hipovitaminose D foi superior a 60%. Merece destaque ainda o estudo de Unger et al. (2010), que encontrou prevalência acima de 70% em 603 funcionários e estudantes voluntários saudáveis da Universidade de São Paulo, com idade variando de 18 a 90 anos. Não foram identificados estudos envolvendo especificamente populações afro-descendentes no Brasil.

2.4 Associação entre deficiência de vitamina D e marcadores de aterosclerose subclínica e inflamação

Alguns estudos, predominantemente transversais e de pequeno porte, têm evidenciado associação entre níveis séricos de 25(OH)D e marcadores de aterosclerose subclínica e inflamatórios, embora persistam alguns achados conflitantes.

Em análise transversal de 119 pacientes diabéticos tipo 2 de meia idade, Bonakdaran e Varasteh (2009) observaram associação significativa entre deficiência de 25(OH)D e tanto proteína C-reativa ultra-sensível (PCR-US) elevada como a presença de microalbuminúria. Já em estudo observacional prospectivo envolvendo 227 diabéticos tipo 1, Joergensen et al. (2011) verificaram que a deficiência severa de 25(OH)D (<15.5 nmol/l) na avaliação basal não foi preditiva de desenvolvimento de microalbuminúria, embora tenha se associado a maior mortalidade total.

Amer e Qayyum (2012), analisando os dados de adultos assintomáticos incluídos no National Health and Nutrition Examination Survey (NHANES) 2001-2006, verificaram também uma correlação inversa significativa entre níveis séricos de 25(OH)D e PCR-US, independente dos fatores de risco convencionais, mas apenas naqueles com níveis de 25(OH)D ≤21 ng/ml, notando, de forma aparentemente paradoxal, uma correlação positiva entre estas duas variáveis quando consideraram apenas o subgrupo com níveis de 25(OH)D > 21 ng/ml.

Em estudo envolvendo 100 pacientes submetidos a angiografia coronária, observou-se uma correlação positiva entre níveis séricos de 25(OH)D e a dilatação mediada por fluxo (DMF) de artéria braquial, ou seja, quanto mais baixos os níveis de 25(OH)D, maior o grau de disfunção endotelial (SYAL et al., 2012). Tarcin et al. (2009), comparando 23 indivíduos assintomáticos com deficiência de 25(OH)D (níveis séricos < 25 nmol/l) com um grupo com níveis normais da vitamina (média de 75 nmol/L), também verificaram que a taxa de DMF era significativamente menor no grupo deficiente em vitamina D.

Em estudo envolvendo 63 indivíduos adolescentes obesos, Bacha e Arslanian (2016) encontraram inicialmente associação inversa significativa entre níveis de 25(OH)D e EMIC, mas tal associação desapareceu após inclusão da variável raça no modelo multivariado, demonstrando associação independente entre a etnia afro-americana e maior EMIC.

Em análise transversal de uma coorte de base populacional, o Rancho Bernardo Study, abordando 654 indivíduos saudáveis, predominantemente idosos (média de idade de 75,5 anos), Reis et al. (2009) observaram, após análise multivariada, correlação inversa entre os níveis séricos de 25(OH)D e a EMIC interna.

Associação inversa significativa, independente de fatores de risco convencionais, entre níveis séricos de 25(OH)D e EMIC (carótida comum) também foi verificada em diabéticos tipo 2 (TARGHER et al., 2006; LIU et al., 2012). No estudo de Liu et al. (2012), observou-se ainda que indivíduos com placas ateromatosas carotídeas apresentavam níveis séricos de 25(OH)D significativamente mais baixos do que aqueles sem este achado. No estudo de Targher et al. (2006), foi verificado também que aqueles com hipovitaminose D, quando comparados com o subgrupo com níveis normais de 25(OH)D,

apresentavam concentrações significativamente mais elevadas de fibrinogênio e PCR-US.

Analisando transversalmente 415 indivíduos, também diabéticos tipo 2, com média de idade de 60±9 anos, participantes de uma coorte dinamarquesa, Winckler et al. (2015), no entanto, não encontraram nenhuma associação entre níveis de 25(OH)D e a EMIC, nem rigidez arterial, avaliada pelo cálculo do coeficiente de distensibilidade carotídea e módulo elástico de Young, por ultrassonografia.

Em estudo envolvendo 1.193 pacientes com diabetes mellitus tipo 1, participantes do *Diabetes Control and Complications Trial* (DCCT), Sachs et al. (2013) verificaram associação significativa entre deficiência de 25(OH)D e escore de cálcio coronário, avaliado por tomografia computadorizada, mas também nenhuma correlação com a EMIC.

Em análise transversal de 926 mulheres chinesas climatéricas saudáveis, com hipovitaminose D, Hao et al. (2015) verificaram uma associação inversa significativa independente entre níveis de 25(OH)D e a EMIC.

Em pacientes com doença renal crônica, correlação inversa entre os níveis séricos de 25(OH)D e a medida da EMIC foi observada por Yadav et al. (2012), em indivíduos em estágios 4 e 5 não dialíticos, mas não por Zang et al. (2012), que abordaram apenas nefropatas diabéticos, nem por Ng et al. (2016). No estudo de Yadav et al. (2012) foi observada também correlação inversa com os níveis séricos da PCR-US, e no de Zang et al. (2012), com o escore de cálcio coronariano.

Ross et al. (2011), analisando 149 pacientes HIV-positivos, não encontraram nenhuma associação entre níveis séricos de 25(OH)D e nem marcadores inflamatórios (fator de necrose tumoral α, interleucina 6 e PCR-US) nem marcadores endoteliais (molécula de adesão intercelular-1 e molécula de adesão celular vascular-1).

Por fim, em coorte multicêntrica europeia, envolvendo 3.430 indivíduos de alto risco cardiovascular, de meia idade e idosos, Deleskog et al. (2013) observaram múltiplas associações entre níveis de 25(OH)D e fatores de risco cardiovascular já conhecidos, mas nenhuma associação consistente, independente, com medidas da EMIC.

2.5 Implicações cardiometabólicas e vasculares da deficiência de vitamina D: estudos epidemiológicos e clínicos

Resultados de várias grandes coortes e estudos tipo caso-controle, publicados nos últimos anos, têm trazido importantes evidências associando deficiência de vitamina D com maior risco cardiovascular.

A evidência da importância da vitamina D na homeostase do sistema cardiovascular foi inicialmente sugerida, experimentalmente, pela observação de que ratos knockout, desprovidos dos receptores VDR, apresentavam mineralização óssea deficiente, fibras musculares pequenas, sofriam de HAS e morriam de insuficiência cardíaca (IC) (BOUILLON et al., 2008).

Em coorte prospectiva do Third National Health and Nutrition Examination Survey (NHANES III), envolvendo 3.408 americanos idosos (≥ 65 anos), acompanhados durante cerca de sete anos, verificou-se, após análise multivariada, que níveis basais de 25(OH)D estiveram inversamente associados com o risco de mortalidade por todas as causas, mas a associação mostrou-se ainda mais forte para a mortalidade cardiovascular (GINDE et al., 2009).

No estudo Framingham Offspring, envolvendo 1.739 indivíduos saudáveis na avaliação basal, com média de idade de 59 anos, seguidos por cinco anos, verificou-se que aqueles com deficiência de vitamina D [25(OH)D < 15 ng/ml] apresentaram uma razão de risco para ocorrência de eventos cardiovasculares de 1,62 quando comparados com aqueles com níveis ≥ 15 ng/ml, mesmo após ajuste para fatores de risco convencionais (WANG et al., 2008).

Na coorte Health Professionals Follow-up Study, que acompanhou 18.225 indivíduos do sexo masculino, com idade entre 40 e 75 anos, por 10 anos, os autores observaram que os participantes com deficiência de vitamina D [25(OH)D ≤15 ng/mL] apresentaram uma incidência de infarto do miocárdio significativamente maior do que aqueles com níveis considerados suficientes (≥30 ng/mL), com um risco relativo de 2,42, após análise multivariada (GIOVANNUCCI et al., 2008). Estudo recente, envolvendo 1.259 indivíduos, demonstra que o status da vitamina D pode influenciar também o prognóstico de indivíduos já acometidos pelo infarto do miocárdico (NG et al., 2013).

No estudo *Ludwigshafen Risk and Cardiovscular Health* (LURIC), em que 3.316 pacientes, referenciados para realizar uma angiografia coronária, foram

acompanhados por sete anos, evidenciou-se que baixos níveis tanto de 25(OH)D quanto de 1,25$(OH)_2$D foram preditores independentes de acidente vascular cerebral (AVC) fatal (PILZ et al., 2008). Resultado semelhante foi obtido no Copenhagen City Heart Study, uma coorte que acompanhou 10.170 indivíduos de uma população geral durante 21 anos, onde se evidenciou também uma clara associação inversa independente entre níveis séricos de 25(OH)D e incidência de AVC isquêmico (BRØNDUM-JACOBSEN et al., 2013).

Analisando transversalmente dados de 4.839 participantes do National Health and Nutrition Examination Survey 2001–2004, Melamed et al. (2008) verificaram que indivíduos no quartil inferior de níveis séricos de 25(OH)D, comparativamente àqueles do quartil superior, apresentavam uma razão de risco de prevalência de doença arterial periférica de 1,8, após análise multivariada.

Associação entre deficiência de vitamina D e presença de aneurisma de aorta abdominal foi relatada por Wong et al. (2013) em estudo observacional envolvendo 4.233 homens idosos (70-88 anos), sendo 311 diagnosticados como portadores de aneurisma de aorta abdominal. Após análise multivariada, os autores observaram que participantes pertencentes ao quartil inferior quando comparados com aqueles do quartil superior de 25(OH)D apresentavam uma razão de risco de apresentar um aneurisma de aorta abdominal variando de 1,23, para aneurismas ≥ 30 mm, a 5,42, para aneurismas ≥40 mm.

Analisando 90 pacientes idosos (idade ≥60 anos) com insuficiência cardíaca estável e 31 controles, Ameri et al. (2010) observaram concentrações de 25(OH)D significativamente mais baixas naqueles com insuficiência cardíaca, verificando entre estes uma prevalência de hipovitaminose D (25(OH)D < 30ng/ml) de quase 100%. Observaram ainda que indivíduos com níveis <10 ng/ml apresentavam diâmetros e volumes tanto telediastólicos como telessistólicos do ventrículo esquerdo maiores e fração de ejeção mais baixa comparativamente àqueles com níveis ≥10 ng/ml.

A associação entre deficiência de vitamina D e hipertensão arterial sistêmica (HAS), síndrome metabólica (SM) e diabetes mellitus (DM) também tem sido pesquisada. Um estudo realizado na África e publicado no início da década de 1990, onde foram observadas diferenças nos níveis de pressão arterial de acordo com a área geográfica analisada, registrando-se níveis mais elevados em indivíduos residindo em áreas mais distantes da linha do equador, já chamava a atenção dos

pesquisadores para a possibilidade de os níveis de vitamina D estarem implicados nesta associação (COOPER; ROTIMI, 1994). Mais recentemente, várias análises da população do *Third National Health and Nutrition Examination Survey* (NHANES III), resumidas em revisão de Ullah et al. (2010), têm demonstrado uma clara associação inversa entre níveis de 25(OH)D e pressão arterial, de forma independente de inúmeras variáveis potencialmente confundidoras.

Em participantes de duas coortes prospectivas, incluindo 613 homens do *Health Professionals' Follow-Up Study* e 1.198 mulheres do Nurses' Health Study, durante quatro anos de seguimento, o risco relativo de desenvolvimento de HAS em homens com deficiência de 25(OH)D (níveis séricos <15 ng/mL), após análise multivariada, foi de 6,13 em comparação com aqueles com níveis ≥ 30 ng/ml. Entre as mulheres, a mesma comparação evidenciou um risco relativo de 2,67 (FORMAN et al., 2007).

Em estudo transversal de base populacional realizado em duas grandes cidades chinesas, incluindo mais de 3.200 indivíduos, com idade variando de 50 a 70 anos, verificou-se que níveis mais baixos de 25(OH)D se associaram de forma significativa não só com a presença de SM como de qualquer de seus componentes, após análise multivariada. O estudo evidenciou ainda, em indivíduos com sobrepeso ou obesidade, uma significativa associação inversa entre níveis séricos de 25(OH)D e tanto a insulinemia de jejum como o índice de resistência insulínica avaliado pelo HOMA (homeostasis model assessment of insulin resistance) (LU et al., 2009). Associação entre hipovitaminose D e presença de SM, em estudos menores, também foi observada em obesos (MIÑAMBRES et al., 2012) e crianças (PACIFICO et al., 2011).

Em análise transversal de 6.228 participantes do NHANES III, com idade ≥ 20 anos, a presença de DM associou-se significativamente com níveis mais baixos de 25(OH)D, após análise multivariada, em brancos não-hispânicos e indivíduos de origem mexicana, notando-se, no entanto, ausência de tal associação entre negros não-hispânicos (SCRAGG; SOWERS; BELL, 2004).

Em meta-análise realizada por Pittas et al. (2007), observou-se, com base em estudos observacionais, uma associação consistente entre baixos níveis de 25(OH)D e prevalência tanto de SM como de DM tipo 2. Baixos níveis de vitamina D também se associaram a maior incidência destas condições.

Uma outra associação que tem sido explorada é entre deficiência de vitamina D e ocorrência de pré-eclâmpsia. O fato de gestantes negras, em comparação às brancas, apresentarem maior incidência de pré-eclâmpsia e maior prevalência de hipovitaminose D levantou a hipótese de que a deficiência da vitamina pudesse ter alguma implicação com a patogenia da doença (BODNAR et al., 2007). Um estudo tipo caso-controle desenvolvido pelos autores, em que gestantes nulíparas foram acompanhadas desde o primeiro trimestre até o parto, reforçou a existência desta associação. Na pesquisa, uma concentração basal < 15 ng/l associou-se, de forma independente, a um risco cinco vezes mais elevado de ocorrência da doença.

Por fim, meta-análise publicada recentemente por Wang et al. (2012), que analisou 19 estudos prospectivos envolvendo ao todo 65.994 participantes, confirma a associação inversa e geralmente linear entre os níveis circulantes de 25(OH)D e o risco de DCV. Nesta análise, o risco relativo da categoria de níveis mais elevados de 25(OH)D em relação à de níveis mais baixos foi de 1,52 para todas as doenças cardiovasculares, 1,42 para mortalidade cardiovascular, 1,38 para doença arterial coronariana (DAC) e 1,64 para AVC.

2.6 Possíveis mecanismos envolvidos na associação entre vitamina D e doença cardiovascular

Os mecanismos pelos quais a vitamina D exerceria seus efeitos cardio e vasculoprotetores ainda não estão completamente esclarecidos, sendo apontados os seus efeitos regulatórios sobre o Sistema Renina-Angiotensina (SRA), o controle glicêmico, as citocinas inflamatórias, os níveis do Exame do hormônio da paratireoide (PTH) e a deposição de cálcio no músculo liso vascular, além de ações vasculares diretas.

2.6.1 Vitamina D e sistema renina-angiotensina

O SRA tem sabidamente um papel crítico na homeostase da volemia e eletrólitos, na regulação da pressão arterial e na patogenia da aterosclerose, já estando bem estabelecido que a estimulação inapropriada deste sistema se associa com HAS e maior incidência de doenças cardiovasculares (DCV) (ULLAH et al., 2010).

Uma boa evidência favorecendo o papel da vitamina D na regulação do SRA provém de estudos experimentais. Em estudo envolvendo ratos knockout, desprovidos dos VDR, demonstrou-se produção elevada de renina e angiotensina II, causando HAS, hipertrofia cardíaca e aumento da ingestão de água. Estas anomalias puderam ser evitadas com o tratamento de um inibidor da enzima conversora da angiotensina ou de um antagonista do receptor da angiotensina II. Com base nestes achados, pôde-se concluir que a vitamina D é um potente supressor endócrino da biossíntese da renina. Por outro lado, em ratos normais, demonstrou-se que a deficiência de vitamina D estimula a expressão de renina, enquanto que, se for administrada a $1,25(OH)_2D$, ocorre uma redução da síntese de renina. E ainda, em culturas de células, a $1,25(OH)_2D$ foi capaz de suprimir diretamente a transcrição do gene da renina por um mecanismo dependente do VDR (LI et al., 2004). Por outro lado, há evidências de que o PTH, cujos níveis séricos podem aumentar secundariamente à hipovitaminose D, pode também ter um efeito estimulador direto sobre a secreção de renina (ULLAH et al., 2010). Assim, as evidências sugerem fortemente que a deficiência de vitamina D possa estar implicada na patogenia da HAS via ativação do SRA.

2.6.2 Vitamina D e sensibilidade à insulina

A associação entre obesidade, especialmente a abdominal, e diminuição da sensibilidade à insulina já está bem estabelecida. Por outro lado, tem sido demonstrado que obesos têm prevalência aumentada de deficiência de vitamina D, o que tem sido explicado tanto pela eventual menor exposição à luz solar, já que estes indivíduos têm menor chance de realizar atividades físicas ao ar livre, como pelo maior sequestramento da mesma no tecido adiposo, devido à sua conhecida lipossolubilidade, com diminuição de sua biodisponibilidade (ULLAH et al., 2010). A demonstração de uma forte correlação inversa independente entre glicemia e níveis séricos de 25(OH)D (BAYNES et al., 1997; LIU et al., 2009) aliada aos estudos epidemiológicos associando deficiência de vitamina D com maior incidência de DM tipo 2 (SCRAGG; SOWERS; BELL, 2004; PITTAS et al., 2007) tem sugerido um papel direto importante da deficiência desta vitamina na patogenia da doença. Parece que níveis adequados de vitamina D no organismo são essenciais no processo de sensibilidade à insulina, como apontam algumas pesquisas. No estudo

de Chiu et al. (2004), por exemplo, envolvendo 126 adultos saudáveis, ficou evidente a correlação positiva entre as concentrações séricas de 25(OH)D e a sensibilidade à insulina. Por outro lado, existe também evidência de que a hipovitaminose D está associada com diminuição da secreção da insulina, via disfunção da célula β, como demonstrado em ratos (NORMAN et al., 1980), o que tem sido corroborado por sua associação também com a incidência de DM tipo 1 (THE EURODIAB SUBSTUDY 2 STUDY GROUP, 1999).

Um mecanismo que tem sido proposto para explicar a associação entre deficiência de vitamina D e obesidade, HAS, DM tipo 2 e outras manifestações da SM envolve o metabolismo do cálcio (RESNICK, 1991). Baixos níveis séricos de cálcio, resultantes da deficiência de vitamina D, podem acarretar, como já bem sabido, elevação secundária do PTH, o qual, por sua vez, promove aumento deste íon a nível intracelular. O aumento do cálcio intracelular pode levar tanto a maior diferenciação de pré-adipócitos em adipócitos como inibir a função da GLUT-4, enzima envolvida na captação celular de glicose mediada pela insulina. Há também evidência de que o cálcio intracelular aumentado pode ter um efeito estimulante sobre a atividade da enzima 11-beta hidroxiesteróide desidrogenase tipo 1, semelhante ao da angiotensina II, determinando aumento da produção de cortisol nos adipócitos (ZEMEL; SOBHANI, 2003).

2.6.3 Ações vasculares e cardíacas da vitamina D

Estudos experimentais têm demonstrado várias ações da vitamina D diretamente sobre o coração e vasos sanguíneos, podendo exercer, assim, importante papel protetor contra o desenvolvimento da DCV.

Em cultura de células de músculo liso vascular de coelho, Wakasugi et al. (1991) demonstraram que a síntese da PGI2, a qual tem papel importante na redução da trombogenicidade, adesão celular e proliferação de células musculares lisas, aumentou significativamente na presença de $1,25(OH)_2D$. É possível, pois, que a vitamina D funcione como um importante agente vasoativo e possa, assim, exercer um papel protetor contra o desenvolvimento da aterosclerose.

Além disso, tem sido demonstrada a sua participação na regulação da expressão de várias proteínas com ação vascular, como fator de crescimento endotelial, metaloproteinase tipo 9, miosina, elastina, colágeno tipo 1 e ácido γ-

carboxiglutâmico, esta última uma proteína que protege o vaso contra a calcificação parietal e, ainda, na supressão de citocinas pró-inflamatórias, incluindo a interleucina-6 e o fator de necrose tumoral-α in vitro e in vivo (ZITTERMANN; KOERFER, 2008a).

Há ainda evidências experimentais de que sua ação antiaterogênica envolve efeitos específicos sobre o sistema imunológico, incluindo um efeito direto sobre as células CD4 naive, induzindo o desenvolvimento de linfócitos Th2, que produzem interleucina-10 (IL-10), a qual inibe a ativação de macrófagos, etapa-chave no processo aterogênico (BOONSTRA et al., 2001). Além disso, inibe a transcrição do interferon- γ (IFN-γ), secretado pelas células Th1, que é, ao contrário, um potente ativador dos macrófagos e supressor dos linfócitos Th2 (STAEVA-VIEIRA; FREEDMAN, 2002).

Em relação ao coração, há evidências de que a vitamina D tem um importante papel na modulação e manutenção da sua estrutura e função celular. O tratamento com $1,25(OH)_2D$ aumenta a expressão da miotrofina, uma proteína muscular cardíaca, e diminui a expressão do peptídeo natriurético atrial, um marcador bioquímico de risco, que está inversamente relacionado com a função cardíaca. Além disso, o tratamento com $1,25(OH)_2D$ aumenta a expressão e localização nuclear do VDR nas células cardíacas (ZITTERMANN; KOERFER, 2008b). Assim, a supressão dos efeitos da vitamina D justifica o desenvolvimento de hipertrofia miocárdica e insuficiência cardíaca no modelo experimental já citado de ratos knockout, desprovidos de VDR (BOUILLON et al., 2008).

2.7 Efeitos da reposição de vitamina D na doença cardiovascular

Dados de estudos randomizados controlados para avaliar o impacto da suplementação de vitamina D no risco cardiovascular são limitados, principalmente em relação aos desfechos pesados, e têm reportado, por vezes, resultados conflitantes. Em geral as evidências são oriundas de estudos que abordaram populações específicas.

Em mulheres pós-menopáusicas, os achados têm sido bastante variados e em geral desapontadores. Em um estudo envolvendo 305 mulheres saudáveis, com idades variando de 60 a 70 anos, que receberam de forma randômica 400 ou 1.000 UI de vitamina D3 ou placebo, objetivando avaliar o efeito da vitamina sobre o perfil

lipídico, resistência insulínica, biomarcadores inflamatórios e pressão arterial, os autores observaram apenas pequenas alterações nos níveis séricos de apolipoproteína B100 (-1,0 mg/dl no grupo 400 UI, -1,0 mg/dl no grupo 1000 UI e +0,02 mg/dl no grupo placebo), que, apesar de significativas, foram consideradas clinicamente irrelevantes (WOOD et al., 2012). No entanto, há que frisar-se, como limitações, que, neste estudo, a deficiência de vitamina D não constituiu critério de inclusão e, ainda, as doses de suplementação utilizadas podem ser consideradas baixas.

Da mesma forma, Gannagé-Yared et al. (2003), avaliando um grupo de apenas 47 mulheres pós-menopáusicas saudáveis, quanto aos efeitos de um curso de 12 semanas de 800 UI de vitamina D associada a 1.000 mg de cálcio por dia sobre o perfil inflamatório, função pancreática e parâmetros lipídicos, não observaram nenhuma alteração significativa nos níveis séricos de interleucina 6, fator de necrose tumoral alfa, PCR-US, insulina, triglicérides, HDL ou LDL-colesterol. No entanto, como limitações deste estudo, além do pequeno tamanho amostral, podem ser apontados os baixos níveis basais de citocinas pró-inflamatórias nestas mulheres saudáveis, o curto período de tratamento, a dose relativamente baixa de vitamina D administrada e, principalmente, o fato de a deficiência de vitamina D não ter sido considerada um critério de inclusão.

Gepner et al. (2012), em um estudo prospectivo, randomizado, duplo-cego, envolvendo 114 mulheres com média de idade de 64 anos, com níveis séricos de 25(OH)D entre 10 e 60 ng/ml, em que os efeitos administração oral de 2.500 UI de vitamina D3 por dia durante quatro meses sobre marcadores de risco cardiovascular foram comparados com placebo, também não evidenciaram nenhuma diferença significativa entre os dois grupos em relação às alterações da DMF, velocidade da onda de pulso carotídeo-femoral, *augmentation index* de aorta nem dos níveis séricos da PCR-US.

No clássico estudo *Women's Health Initiative* (WHI), que envolveu 36.282 mulheres pós-menopáusicas saudáveis, com idade variando de 50 a 79 anos, randomizadas para tomar 1.000 mg de cálcio + 400 UI de vitamina D por dia ou placebo e acompanhadas por sete anos, também não foi observada nenhuma diferença significativa entre os grupos, tratamento e placebo, em relação à incidência de eventos coronarianos e cerebrovasculares (HSIA et al., 2007). Convém frisar, no entanto, que os níveis basais de 25(OH)D no WHI não eram conhecidos e,

portanto, deficiência de vitamina D não constituiu critério de inclusão no estudo. Além disso, no estudo, utilizou-se baixa dose de vitamina D. Chama a atenção, no entanto, no WHI, o fato de mulheres com maior índice de massa corpórea e múltiplos fatores de risco para DAC terem apresentado menor incidência de eventos cardiovasculares no grupo tratamento ativo.

No entanto, em estudo envolvendo 148 mulheres idosas (média de idade de 74 anos) com hipovitaminose D documentada [níveis séricos de 25(OH)D < 20 ng/ml], randomizadas para receber, durante oito semanas, suplementação oral de 800 UI de vitamina D3 + 1200 mg cálcio ou apenas 1200 mg cálcio por dia, Pfeifer et al. (2001) reportaram quedas significativas na pressão arterial sistólica, frequência cardíaca e níveis séricos do PTH nos participantes do primeiro grupo.

Efeitos favoráveis da suplementação oral de vitamina D sobre a pressão arterial também foram relatados por Forman et al. (2013), que abordaram indivíduos negros saudáveis, uma população que costuma apresentar níveis circulantes de 25(OH)D mais baixos. Neste estudo, 283 indivíduos com média de idade de 51 anos (30 a 80 anos), dos quais 41,7% faziam uso de fármacos anti-hipertensivos, foram randomizados de forma duplo-cega para receber por via oral vitamina D3 em diferentes doses (1.000, 2.000 ou 4.000 UI por dia) ou placebo durante três meses. Na avaliação de três meses, os autores observaram alterações pequenas mas significativas na pressão arterial sistólica (em média, +1,7 mmHg no grupo placebo e -0,66, -3,5 e -4,0 mmHg, respectivamente, nos grupos que foram tratados com 1.000, 2.000 e 4.000 UI de vitamina D3).

No entanto, em estudo mais recente e de maior porte, o *DAYLIGHT*, no qual 534 indivíduos, pré-hipertensos ou hipertensos em estágio 1, com idades variando de 18 a 50 anos, todos com deficiência de vitamina D, foram randomizados para receber uma alta dose (4000 UI/dia) ou uma dose baixa (400 UI/dia) de vitamina D por via oral, durante seis meses, os autores não observaram nenhuma diferença significativa na pressão arterial, avaliada por meio de monitorização ambulatorial de 24 horas, entre os subgrupos (ARORA et al., 2015).

Alguns efeitos favoráveis da suplementação de vitamina D foram relatados em pacientes diabéticos e intolerantes à glicose. No estudo Calcium and Vitamin D for Diabetes Mellitus (CaDDM), conduzido por Mitri et al. (2011), em que 92 indivíduos adultos (média de idade de 57 anos) com intolerância à glicose foram randomizados de forma duplo-cega para receber 2.000 UI de vitamina D ou 800 mg de cálcio por

dia durante 16 semanas, os autores observaram aumento da secreção de insulina no grupo vitamina D e sua diminuição no grupo controle, com diferença estatisticamente significante, bem como um aumento menor na hemoglobina glicada no grupo vitamina D, em relação ao grupo controle, com significância estatística limítrofe.

Com o objetivo de pesquisar os efeitos de altas doses de vitamina D3 por via oral sobre a saúde vascular e controle glicêmico de pacientes diabéticos tipo 2, Witham et al. (2012) randomizaram, de forma duplo-cega, 61 indivíduos com níveis séricos de 25(OH)D < 40 ng/ml para receber uma dose única de placebo ou diferentes doses de vitamina D3 (100.000 ou 200.000 UI). Nas análises de oito e 16 semanas, os autores não observaram diferenças significativas entre os grupos quanto à função endotelial, avaliada pela DMF, resistência insulínica e níveis de hemoglobina glicada. No entanto, evidenciaram reduções significativas, em relação ao grupo placebo, tanto da pressão arterial sistólica em ambos os grupos que receberam vitamina D, à análise de oito semanas, quanto do peptídeo natriurético tipo B no grupo que recebeu 200.000 UI, à análise de 16 semanas.

Em estudo retrospectivo envolvendo pacientes com doença renal crônica terminal, atendidos em cinco centros de diálise em Massachusetts, a suplementação oral de ergocalciferol (50.000 UI/semana durante 24 semanas) naqueles com níveis séricos de 25(OH)D < 40 ng/ml promoveu redução significativa dos níveis de hemoglobina glicada (BLAIR et al., 2008).

Já Yiu et al. (2013) não demonstraram nenhum efeito significativo da suplementação de vitamina D em alta dose sobre parâmetros de função vascular e inflamação em indivíduos diabéticos tipo 2. Neste estudo, 100 indivíduos foram randomizados, de forma duplo-cega, para tomarem vitamina D por via oral na dose de 5.000 UI/dia ou placebo, durante 12 semanas. Ao final, os autores não evidenciaram nenhum efeito significativo da suplementação de vitamina D sobre a função endotelial, avaliada pela DMF, níveis séricos de células endoteliais progenitoras e velocidade de onda de pulso tornozelo-braquial, nem sobre biomarcadores de inflamação e estresse oxidativo, perfil lipídico ou hemoglobina glicada.

Em outro pequeno estudo randomizado, Shaseb et al. (2016) testaram o efeito uma dose alta única de vitamina D sobre o *status* glicêmico e os níveis de PCR-US em indivíduos diabéticos tipo 2 com DAC. Nesta pesquisa, 95 pacientes foram

randomizados para receber uma injeção IM de 300.000 UI ou placebo. Ao cabo de oito semanas, os autores observaram reduções significativas nos níveis de glicemia de jejum e hemoglobina glicada, mas não dos níveis de PCR-US, no subgrupo que recebeu suplementação, sem mudanças significativas no grupo placebo.

Alguns benefícios da suplementação oral de vitamina D foram demonstrados em indivíduos obesos. Em 200 indivíduos saudáveis com sobrepeso e baixos níveis séricos de 25(OH)D (média de 12 ng/ml), participantes de um programa de controle de peso, randomizados de maneira duplo-cega para receber vitamina D (83 microgramas/dia) ou placebo durante 12 meses, Zittermann et al. (2009), verificaram que, além de não interferir significativamente na perda de peso, a suplementação da vitamina se associou a reduções significativas dos níveis séricos de triglicérides e do marcador inflamatório fator de necrose tumoral alfa. No entanto, os autores observaram também uma associação com um aumento pequeno mas significativo dos níveis de Lipoproteína de baixa densidade (LDL)-colesterol.

Em alguns estudos, demonstrou-se um efeito favorável da suplementação de vitamina D sobre a função endotelial. No ensaio conduzido por Tarcin et al. (2009), envolvendo apenas 23 indivíduos assintomáticos com deficiência severa de 25(OH)D (níveis séricos abaixo de 10 ng/ml), observou-se, após a reposição vitamina D, sob a forma de 300.000 UI por via intramuscular mensalmente por três meses, melhora da função endotelial, refletida por aumento da DMF, além de redução da peroxidação lipídica, caracterizada por diminuição dos níveis séricos das substâncias reativas ao ácido tiobarbitúrico, as *thiobarbituric acid reactive substances* (TBARS) e aumento dos níveis de leptina.

Harris et al. (2011), em estudo randomizado envolvendo 57 afro-americanos adultos, verificaram também aumento significativo da DMF no grupo que recebeu suplementação oral de 60.000 UI de vitamina D3 por mês durante 16 semanas e ausência de mudança significativa deste parâmetro no grupo placebo.

Witham et al. (2010a) avaliaram os efeitos da reposição de vitamina D sobre a pressão arterial e função endotelial em pacientes acometidos por Acidente Vascular Cerebral (AVC). Ao todo, 58 pacientes, com média de idade de 67 anos e níveis de 25(OH)D < 30 ng/ml, foram randomizados para receber por via oral, de forma duplo-cega, 100.000 UI de vitamina D2 ou placebo em dose única. À análise de oito semanas, nenhuma diferença foi observada na pressão arterial, tanto sistólica quanto diastólica, entre os grupos, porém verificou-se uma função endotelial

significativamente melhor no grupo vitamina D, traduzida por maior DMF. Convém frisar que, no estudo, os pacientes já se apresentavam com pressão arterial controlada na avaliação basal.

Os efeitos da suplementação de vitamina D sobre a capacidade funcional e qualidade vida em pacientes com insuficiência cardíaca foram avaliados por Witham et al. (2010b). No estudo, 105 idosos (≥ 70 anos) com insuficiência cardíaca sistólica e deficiência de 25(OH)D (<20 ng/ml) foram randomizados de forma duplo-cega para receber por via oral 100.000 UI de vitamina D2 ou placebo no início e 10 semanas depois, não sendo observado qualquer benefício a favor do grupo tratamento ativo, nas análises de 10 e 20 semanas, no que tange aos vários parâmetros avaliados, como distância caminhada em seis minutos, qualidade de vida, segundo o escore de Minnesota, e níveis séricos do fator de necrose tumoral alfa. No entanto, os autores observaram, à análise de 10 semanas, diminuição do peptídeo natriurético atrial, um marcador prognóstico reconhecido, no grupo vitamina D, e aumento do mesmo no grupo placebo, com diferença significativa entre os grupos.

Os efeitos da suplementação oral de vitamina D foram testados também em 123 pacientes com insuficiência cardíaca randomizados para receber 50 microgramas de vitamina D (2.000 UI) + 500 mg de cálcio ou placebo + 500 mg de cálcio por dia, durante nove meses. A taxa de sobrevida não diferiu entre os dois grupos, após seguimento de 15 meses, mas foi observado um perfil inflamatório mais favorável no grupo que recebeu vitamina D, traduzido por níveis mais elevados da citocina anti-inflamatória interleucina 10 e mais baixos do fator de necrose tumoral alfa, que tem ação pró-inflamatória (SCHLEITHOFF et al., 2006).

Resultados ainda mais favoráveis com a suplementação oral de vitamina D em pacientes com insuficiência cardíaca foram relatados recentemente por Amin et al (2013). No estudo, 94 indivíduos apresentando níveis séricos de 25(OH)D abaixo do normal (<30 ng/ml) receberam suplementação oral de vitamina D3 por quatro meses, sendo 50.000 UI por semana por oito semanas e, a seguir, 50.000 UI por mês durante os dois meses subsequentes. Na avaliação final, os autores observaram redução significativa dos níveis séricos do peptídeo natriurético cerebral (pro-BNP) e da PCR-US, além de melhora significativa da classe funcional e aumento da distância caminhada em seis minutos.

Em um subestudo do Eurodiab Study Group tipo caso-controle, foi pesquisado o impacto da suplementação de vitamina D na infância sobre o risco de desenvolver

DM tipo 1 no futuro. Analisando os dados de 820 casos e 2.335 controles, os autores verificaram que a suplementação de vitamina D na infância se associou a diminuição significativa da incidência de DM tipo 1, mesmo após ajuste para vários fatores potencialmente confundidores. sendo estimada uma razão de chance de 0,67, o que reforçaria o possível papel imunomodulador protetor da vitamina contra o desenvolvimento da doença em indivíduos susceptíveis (THE EURODIAB SUBSTUDY 2 STUDY GROUP, 1999).

Por fim, alguns efeitos benéficos da suplementação de vitamina D sobre fatores de risco cardiovascular foram demonstrados em mulheres com síndrome do ovário policístico. No estudo, 50 mulheres, com idades entre 20 e 40 anos, com deficiência de vitamina D, foram randomizadas de forma duplo-cega para receber três cápsulas de 50.000 UI de vitamina D3 por via oral ou placebo a cada 20 dias durante dois meses. Os autores observaram, ao final do estudo, no grupo tratamento, reduções significativas nos níveis de colesterol total, triglicérides e lipoproteína de densidade muito baixa (VLDL)-colesterol, sem alterações no grupo controle, embora não tenha havido mudanças nos níveis de Lipoproteína de alta densidade (HDL)-colesterol, LDL-colesterol, apo-AI e PCR-US (RAHIMI-ARDABILI et al., 2013).

Infelizmente o grande estudo multicêntrico *Thiazolidinedione Intervention with vitamin D Evaluation* (TIDE), randomizado, duplo-cego e controlado com placebo, projetado para incluir 16.000 indivíduos diabéticos em 33 países a serem acompanhados por mais de cinco anos, em que 1.221 indivíduos chegaram a ser alocados para receber 1.000 UI por dia de vitamina D ou placebo, com o objetivo primário de avaliar os efeitos da vitamina sobre a mortalidade por qualquer causa e incidência de câncer, teve que ser interrompido precocemente por motivos regulatórios internos, sem atingir os objetivos, observando-se, no entanto, incidência comparável de eventos adversos nos dois grupos (PUNTHAKEE et al., 2012).

Em resumo, estudos observacionais têm evidenciado consistentemente uma associação inversa entre baixas concentrações séricas de vitamina D e a ocorrência de vários tipos de doenças extra-esqueléticas, incluindo-se a doença cardiovascular. No entanto, em contraste com estes estudos observacionais, os resultados de estudos randomizados, utilizando suplementação de vitamina D com o propósito de prevenir estas doenças, têm sido conflitantes. Mas várias limitações observadas nestes estudos randomizados podem justificar tais resultados, de modo que a questão permanece não resolvida. Baixo poder estatístico, suplementação

inadequada, seguimento insuficiente, além da variabilidade nos níveis basais e finais de vitamina D, nos hábitos dietéticos e no grau de exposição solar entre participantes, constituem algumas destas limitações. Além disso, muitos estudos randomizados têm medido a 25 (OH) D com precisão variável e não se asseguraram da adequação da suplementação, ou seja, para elevar os níveis séricos do hormônio para, pelo menos, 30 ng/ml. Acresce-se ainda que muitos deles abordaram populações específicas, não estabeleceram deficiência de vitamina D como critério de entrada e utilizaram suplementos de cálcio de forma combinada, o que poderia, por si só, influenciar desfechos, de forma independente da vitamina D. Como se não bastasse, há ainda a variabilidade genética na resposta dos receptores de vitamina D, o que poderia afetar a eficácia da suplementação em alguns indivíduos. A evidência disponível, portanto, não preenche critério para o estabelecimento de uma relação de causalidade entre deficiência de vitamina D e doença, em razão das limitações dos estudos (AL NOZHA, 2016).

A discrepância entre os estudos observacionais e os de intervenção tem levantado a possibilidade de que baixos níveis séricos de 25 (OH) D representem apenas um marcador de estado inflamatório. Sob este ponto de vista, os processos inflamatórios envolvidos nas doenças é que acarretariam a redução da biodisponibilidade da 25 (OH) D, explicando assim o baixo *status* de vitamina D que tem sido descrito em uma ampla variedade de doenças (AL NOZHA, 2016).

Assim, espera-se que apenas após a conclusão dos grandes estudos randomizados em curso, iniciados nos últimos cinco anos, como o americano *Vitamin D and Omega-3 Trial* (VITAL), o australiano *D-Health*, o finlandês *Finnish Vitamin D trial* (FIND) e o britânico *Vitamin D and Longevity* (VIDAL), os quais deverão incluir coletivamente perto de 100.000 participantes, poderá finalmente ser esclarecido o papel da suplementação de vitamina D na prevenção da DCV, assim como do câncer (MANSON et al., 2012; MANSON; BASSUK, 2015).

Vale frisar, por fim, que a suplementação de vitamina D parece segura: em condições naturais, uma exposição total do corpo à luz solar, por exemplo, é capaz de induzir rapidamente (<20 minutos) à síntese do equivalente a mais de 10.000 UI, sem qualquer efeito adverso conhecido, afora os possíveis malefícios à pele, ou seja, não causa intoxicação, pois o excesso de vitamina D3 é simplesmente fotolisado em produtos inativos. De fato, evidência acumulada tem demonstrado que a ingestão prolongada de 10.000 UI/dia (ou 250 microgramas) de vitamina D3 não

acarreta em geral nenhum risco de efeitos adversos, sendo esta dose considerada totalmente segura. Assim, a intoxicação por vitamina D é extremamente rara, só ocorrendo com a ingestão inadvertida ou intencional de doses excessivamente elevadas, acima de 50.000 UI por dia, situação em que os níveis séricos de 25(OH)D podem ultrapassar 150 ng/ml e causar hipercalcemia e hiperfosfatemia (HOLICK, 2007).

No entanto, a dose da suplementação oral de vitamina D3 (colecalciferol) necessária, em indivíduos que vivem sem exposição adequada à luz solar, ainda tem sido motivo de debate. O Institute of Medicine recomenda como adequadas doses diárias de 200 UI para crianças e adultos de até 50 anos, 400 UI para adultos de 51 a 70 anos e 600 UI para aqueles com mais de 70 anos. Entretanto, a maioria dos experts concorda que, sem exposição solar adequada, crianças e adultos requerem aproximadamente 800 a 1.000 UI por dia. Uma vez que a eficácia da vitamina D2 para manter níveis adequados de 25(OH)D corresponde a apenas 30% da da vitamina D3, as doses de vitamina D2 (ergocalciferol) necessárias, para serem obtidos os mesmos efeitos, devem corresponder a três vezes esses valores. Em indivíduos com deficiência comprovada de vitamina D, Holick et al. (2007) tem preconizado doses ainda maiores: 50.000 UI de vitamina D2 por semana durante oito semanas, seguindo-se metade desta dose por tempo indeterminado.

Por fim, muitos autores ainda ponderam que, a menos que mais evidências de efeitos clinicamente significativos se tornem disponíveis, pode ser ainda muito precoce para tanto recomendar quanto contra-indicar a suplementação de vitamina D, em doses mais elevadas, com o objetivo de manter a saúde extra-esquelética e prevenir doenças.

2.8 Deficiência de vitamina D: um novo fator de risco cardiovascular?

Em suma, a vitamina D parece desempenhar um importante papel na saúde cardiovascular. Inúmeros estudos têm demonstrado, como exposto, forte associação independente entre hipovitaminose D e risco cardiometabólico. Evidências crescentes sugerem que os seus efeitos sobre o sistema cardiovascular podem decorrer tanto de ações indiretas, via modulação de fatores de risco conhecidos, como diretas sobre as células cardíacas e vasculares. Embora ainda haja necessidade de mais estudos, é inegável a importância potencial da hipovitaminose

D como um enorme problema de saúde pública emergente a nível global, com importantes implicações para a morbimortalidade cardiovascular. Especificamente em relação à DCV, a hipovitaminose D pode se revestir de uma importância especial, considerando-se de um lado a elevada prevalência de ambas as condições em todo o mundo e de outro, a possibilidade de sua prevenção e correção de maneira simples.

No entanto, ainda carecemos de evidências cabais, baseadas em grandes estudos randomizados e controlados com placebo, utilizando doses maiores de vitamina D, com seguimento de mais longo prazo e com poder estatístico adequado para avaliação de desfechos pesados, para que se possa estabelecer em definitivo o papel da suplementação de vitamina D, especialmente em sua forma oral, na prevenção e tratamento da DCV. Como visto, muitos estudos já publicados envolveram amostras pequenas, populações específicas e não há uniformidade quanto aos critérios de inclusão nem à dose de vitamina D administrada e tempo de tratamento, bem como muitas vezes a vitamina D tem sido administrada conjuntamente com cálcio, sendo necessários mais estudos para avaliar primariamente o papel da vitamina D e estabelecer a dose adequada neste contexto.

3 OBJETIVOS

3.1 Geral

Correlacionar os níveis séricos de vitamina D com marcadores de aterosclerose subclínica e fatores de risco cardiovascular convencionais em afrodescendentes remanescentes de quilombos.

3.2 Específicos

a) Caracterizar a população em estudo quanto aos aspectos sócio-demográficos, antropométricos e clínico-laboratoriais;
b) Determinar a prevalência de hipovitaminose D e fatores associados nesta população;
c) Determinar a prevalência de aterosclerose carotídea e fatores associados;
d) Correlacionar os níveis séricos de 25-hidroxivitamina D com:
   - Espessura médio-intimal carotídeo
   - Razão albumina/creatinina em amostra isolada de urina;
   - Proteína C-reativa ultrassensível;
   - Fatores de risco cardiovascular convencionais.

4 MÉTODOS

4.1 Tipo de estudo

Transversal, descritivo e analítico.

4.2 População

Foram objeto de análise indivíduos afro-descendentes, com idade ≥ 18 anos, habitantes de comunidades quilombolas, localizadas no município de Alcântara, Maranhão, incluídos em um estudo de prevalência de doença renal crônica e seus principais fatores de risco, o PREVRENAL. Todos os participantes do PREVRENAL, selecionados dentre os habitantes de 32 comunidades por um processo de amostragem probabilística, em número de 1539, foram examinados na própria comunidade, entre agosto de 2012 e abril de 2013, após receberem os esclarecimentos devidos sobre os objetivos da pesquisa e assinarem o termo de consentimento. Foram coletados dados sócio-demográficos, sobre estilo de vida (atividade física, tabagismo, consumo de bebidas alcoólicas e atividade laboral), antropométricos (peso e altura) e clínicos (história patológica pregressa e pressão arterial).

Amostras sanguíneas, após jejum de 12 horas, e de urina foram colhidas para análises bioquímicas e pesquisa da excreção urinária de albumina. Após a coleta, o sangue era processado, com separação das alíquotas, e acondicionado em bolsa térmica, com controle de temperatura, e, posteriormente, encaminhado para o laboratório de referência, para as devidas análises e armazenamento em freezer a -80º C.

Os indivíduos identificados como apresentando pelo menos um fator de risco cardiovascular (hipertensão arterial sistêmica, diabetes mellitus, proteinúria e/ou taxa de filtração glomerular estimada reduzida) foram encaminhados para o Hospital Universitário da Universidade Federal do Maranhão para realização de exames especializados e constituíram a amostra objeto da presente pesquisa, composta de 382 participantes. Uma vez que o presente estudo se propõe a correlacionar os níveis de vitamina D com a presença de aterosclerose subclínica, os indivíduos com

história e/ou evidência clínica de doença cardiovascular aterosclerótica manifesta (angina do peito, infarto do miocárdio, revascularização miocárdica, acidente vascular cerebral, aneurisma e/ou doença arterial periférica) foram excluídos da análise.

4.3 Avaliação clínica

Questionários padronizados foram utilizados na avaliação inicial para registro de informações demográficas, história clínica e hábitos de vida. Dados sobre o consumo de álcool (doses / semana), tabagismo (corrente, passado, nunca) e prática de atividade física três ou mais vezes por semana (sim / não) foram obtidos. Foi pesquisado também o uso corrente de medicamentos anti-diabéticos e anti-hipertensivos.

A pressão arterial foi definida como a média das duas últimas de três medidas, realizadas com intervalos ≥ 2 minutos utilizando-se um esfigmomanômetro de coluna de mercúrio, após repouso na posição sentada por pelo menos 5 minutos. Hipertensão arterial foi definida pelo achado de qualquer dos seguintes 4 critérios: pressão arterial sistólica ≥140 mmHg, pressão arterial diastólica ≥90 mmHg, diagnóstico prévio feito por um médico ou uso de medicação anti-hipertensiva.

O diagnóstico de diabetes mellitus foi realizado de acordo com os seguintes critérios: uso de hipoglicemiantes, glicemia de jejum ≥ 126 mg/dL ou um teste oral de tolerância à glicose ≥ 200 mg/dL.

O índice de massa corpórea (IMC) foi calculado utilizando-se a fórmula: IMC=peso/altura2 (Kg/m2). Para cálculo do IMC, o peso foi aferido em balança eletrônica com capacidade de até 150 kg e precisão de 50 g e a estatura, em duplicata por meio de antropômetro portátil com precisão de 0,1 cm. A aferição da circunferência da cintura foi realizada com o paciente em pé, em expiração, utilizando-se uma fita métrica não extensível circundando o abdômen no ponto médio entre a costela e a crista ilíaca.

4.4 Avaliação laboratorial

As dosagens bioquímicas foram realizadas de forma automatizada no Laboratório do Centro de Prevenção de Doenças Renais. Concentração sérica de

creatinina elevada foi definida como ≥ 1,5 mg/dL em homens e ≥ 1,3 mg/dL em mulheres e foi considerada baixa uma taxa de filtração glomerular < 60 ml/min/1,73m^2 com base na definição de doença renal crônica do *National Kidney Foundation* (LEVEY et al., 2003).

A dosagem da 25(OH)D foi realizada por meio do ensaio de fixação por eletroquimioluminescência (Elecsys, Roche Diagnóstica). Foi considerado normal um nível sérico > 30 ng/ml. Valores ≤ 30 ng/ml foram subclassificados como insuficientes (20-30 ng/ml) e deficientes (< 20 ng/ml) (ARAD et al., 1998).

As dosagens da albumina na urina e da proteína C-reativa ultra-sensível no soro foram realizadas por imunoturbidimetria. A albuminúria foi avaliada por meio da razão albumina/creatinina (RAC) em amostra isolada de urina. Microalbuminúria foi definida como valores de excreção urinária de albumina entre 30 e 300 mg/g de creatinina.

4.5 Medida da espessura íntimo-medial carotídea

O exame ultra-sonográfico de artéria carótida foi realizado com transdutor linear de 7,5 MHz em seção longitudinal no modo B, por um único examinador experiente, em aparelho da marca GE modelo *Vivid 3*. Após repouso de pelo menos 10 minutos na posição supina, com o pescoço em discreta hiperextensão, era feita uma avaliação panorâmica do sistema carotídeo bilateralmente. A EMIC foi medida na parede distal (mais afastada do transdutor) da artéria carótida comum, 1cm proximalmente à sua bifurcação, conforme recomendações vigentes (MONTEIRO JUNIOR et al., 2012). A medida consiste na distância entre duas linhas ecogênicas representadas pelas interfaces lúmen-íntima e média-adventícia da parede arterial, sendo considerada normal quando ≤ 1 mm.

4.6 Análise estatística

O teste de Kolmogorov Smirnov foi utilizado para avaliar a normalidade das variáveis contínuas. As variáveis qualitativas foram representadas pela freqüência relativa (%) e as variáveis contínuas, como média e desvio padrão (SD). O teste do qui-quadrado foi utilizado para comparar as proporções. Para comparação de médiass, utilizou-se o teste t de Student. Para analisar o grau de correlação linear

entre variáveis contínuas, o coeficiente de correlação de Pearson foi calculado. As associações apresentando uma p <0,20 à análise bivariada foram submetidas a análise de regressão linear múltipla. No modelo de regressão linear múltipla, sexo (masculino = 0 e feminino = 1) e tabagismo (ausente = 0 e presente = 1) foram incluídos como variáveis dummy. A dicotomização dos níveis séricos de 25 (OH) D de acordo com o percentil 50 foi adotada por causa da baixa prevalência de hipovitaminose D, de acordo com o ponto de corte convencional. Os dados foram processados e submetidos à análise estatística usando-se o programa SPSS 18.0 para Windows, adotando-se como significante um p <0,05 (5%).

4.7 Considerações éticas

Esta pesquisa, por envolver seres humanos, foi realizada de acordo com a Resolução CNS nº 196/96 e suas complementares em vigor em todo o território nacional. A inclusão dos participantes se fez mediante a assinatura de Termo de Consentimento Livre e Esclarecido, onde havia a informação de que os dados obtidos seriam confidenciais e utilizados apenas nesta pesquisa. O projeto PREVRENAL contou com pareceres consubstanciados dos comitês de ética tanto da Universidade Federal do Maranhão (UFMA) quanto da Universidade Federal de São Paulo (UNIFESP).

5 RESULTADOS

5.1 Artigo 1

Is Vitamin D Deficiency a New Risk Factor for Cardiovascular Disease?

(Publicado na Revista The Open Cardiovascular Medicine Journal)

Is Vitamin D Deficiency A New Risk Factor For Cardiovascular Disease?

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Abstract

The role of vitamin D in the regulation of bone metabolism has been well established. However, in recent years, many studies have demonstrated that its role extends far beyond bone health. Growing evidence has shown a strong association between vitamin D deficiency and hypertension, metabolic syndrome, diabetes mellitus and atherosclerosis. The mechanisms by which vitamin D exerts its cardiovascular protective effects are still not completely understood, but there is evidence that it participates in the regulation of renin-angiotensin system and the mechanisms of insulin sensitivity and activity of inflammatory cytokines, besides its direct cardiovascular actions. In this review, several studies linking vitamin D deficiency with cardiometabolic risk as well as small randomized trials that have evaluated the cardiovascular effects of its supplementation are presented. However, large randomized placebo-controlled studies are still needed before we can definitively establish the role of vitamin D supplementation in the prevention and control of cardiovascular disease.

Keywords: Atherosclerosis, Cardiometabolic risk, Cardiovascular disease, Risk factor, Supplementation, Vitamin D deficiency.

1. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the world [1]. Although the role of traditional risk factors is already consolidated, it is known that they cannot fully explain the development of CVD, which has caused continuous search for new risk factors. Growing evidence, obtained in recent years, has suggested that vitamin D deficiency may be associated with an increased risk of CVD [2].

Vitamin D is actually a steroid hormone whose primary function is the regulation of calcium and phosphorus homeostasis, through its interaction with parathyreoid gland, the kidneys and intestines. Although it can be obtained through food intake, the main source of vitamin D is represented by its synthesis in the body itself [2].

The role of vitamin D in regulating bone metabolism is well established for decades. In this function, it promotes increased intestinal absorption and renal reabsorption of calcium, acting also in its mobilization from the bone, in the presence of parathyroid hormone (PTH). Thus, diseases such as rickets and osteomalacia have been classically attributed to prolonged deficiency of vitamin D [2]. However, in recent years several studies have shown that the function of vitamin D in the body extends far beyond bone health, including the regulation of the immune system and anti-proliferative effects on cells, and may play an important role in the physiology of the cardiovascular system. Thus, vitamin D deficiency has been associated with disorders as varied as autoimmune diseases, infections, adverse maternal and fetal outcomes and various types of cancer, besides CVD [3]. However, whether vitamin D deficiency represents a new cardiovascular risk factor and also whether its oral supplementation can reduce the incidence of cardiovascular events is still under debate.

Thus, in this review, besides presenting the basic aspects of the metabolism and physiology of vitamin D in the body, we discuss the association between its deficiency and the occurrence of CVD, as well as the possible mechanisms involved in this association and the impact of its replacement on the prevention and control of CVD.

2. PHYSIOLOGY AND METABOLISM OF VITAMIN D

Under normal conditions only about 10% of the vitamin D needed by the body is achieved by ingestion of food, both in the form of vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). Thus, the main source of vitamin D is represented by its synthesis in the body itself, which begins in the skin. When exposed to ultraviolet radiation, the skin vitamin D precursor 7-dehydrocholesterol undergoes a photochemical cleavage, yielding the previtamin D3. Such labile molecule, in a 48 hour period, undergoes a molecular rearrangement resulting in the formation of vitamin D3. Both the vitamin D coming from the diet, via intestinal absorption, and that formed from the previtamin D3 in the skin bind to circulating proteins and are transported to the liver where it is hydroxylated at the carbon 25, giving the 25-hydroxyvitamin D3 [25(OH)D]. This is then hydroxylated again, this time at carbon 1, at mitochondrial level in cells of the proximal convoluted tubules, as well as possibly in many other tissues, under the action of 1α-hydroxylase enzyme (1α-OHase), finally yielding the 1,25-dihydroxyvitamin D3 [1,25(OH)2D], its biologically active form (Fig. 1). This phase activation is tightly regulated by the serum levels of PTH, calcium and phosphorus. Although biologically inert, however, is the 25(OH)D, the circulating form in a larger quantity and more stable, which is the measured and adopted in clinical practice to assess the vitamin D in the body [2, 3]. PTH, by the other way, is a peptide secreted in response to low circulating levels of calcium and phosphorus which stimulates calcium reabsorption in the kidneys and its removal from the skeleton and increases renal production of 1,25(OH)2D, as mentioned above [2].

The complex mechanism of action of vitamin D inside the cells is already well established. Its active form crosses the cell membrane, enters the target cell and binds to a specific receptor, the VDR, which is present in the cytoplasm. This complex, 1,25(OH)2D-VDR, undergoes translocation to the nucleus and heterodimerizes with retinoic acid receptor (RXR). Next, the complex 1,25(OH)2D-VDR-RXR binds to the response elements of vitamin D (VDRE) in the deoxyribonucleic acid (DNA) increasing transcription to RNA of the genes responsible for the expression of proteins involved in the actions of vitamin D (Fig. 2) [4].

It is now known that the VDR type receptors are present in many cell types, including hematopoietic cells, lymphocytes, epidermal cells, pancreatic cells,

myocytes, neurons, cardiomyocytes, vascular smooth muscle cells, endothelium and placental tissue, besides osteoblasts, osteoclasts, epithelial small intestine and renal tubular cells, which explains the multiplicity of non-calcemic actions taken by vitamin D for various tissues such as the endocrine, immune, central nervous and cardiovascular systems, as well as the numerous implications of their deficiency to health [3]. It is estimated that vitamin D controls, directly or indirectly, about 3% of the entire human genome. Thus, it is believed that a wide variety of physiological processes, including regulation of cytokines, inflammation, fibrogenesis, the renin-angiotensin system, immune response and cell growth and differentiation, may have direct or indirect influence of vitamin D [5].

3. PREVALENCE AND DETERMINANTS OF HYPOVITAMINOSIS D

The definition of vitamin D deficiency has also been a matter of debate. However, most agree that serum 25 (OH) D <20 ng/ml (or 50 nmol/l) is indicative of deficiency, in the range 20-30 ng/ml (or 50 to 75 nmol/l) match insufficiency and >30 ng/ml (or 75 nmol/l) represents vitamin D sufficiency [6].

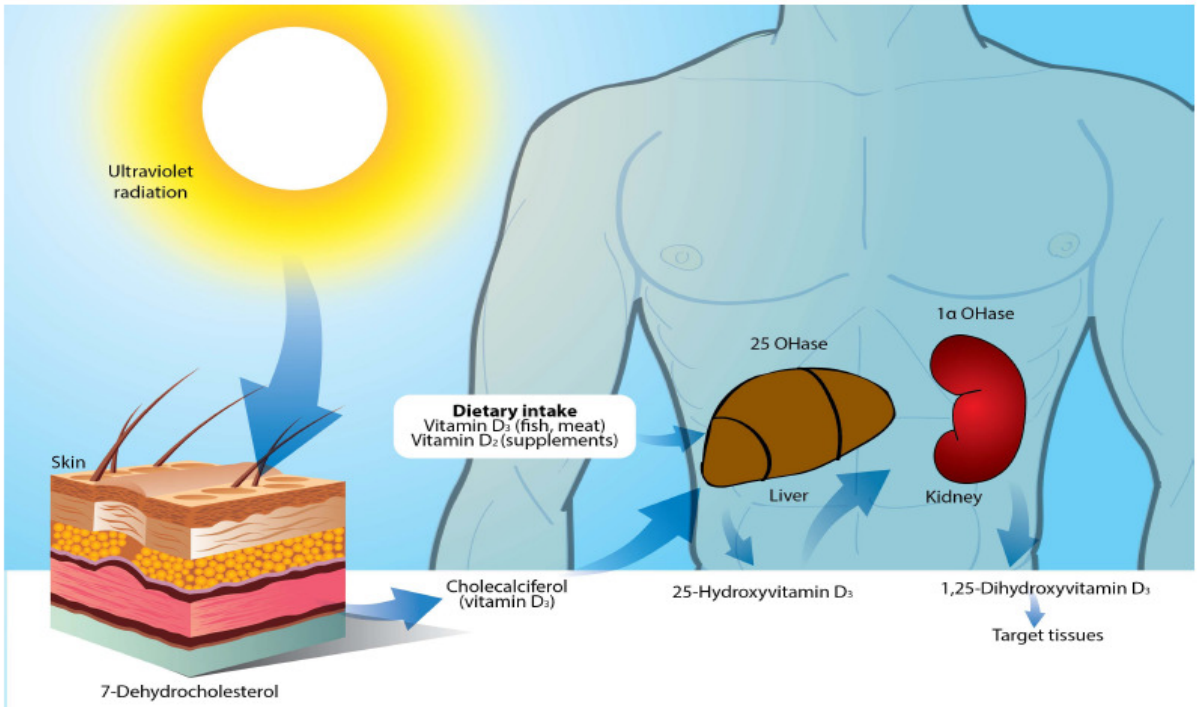

Fig. (1). Schematic representation of the biosynthesis of vitamin D.

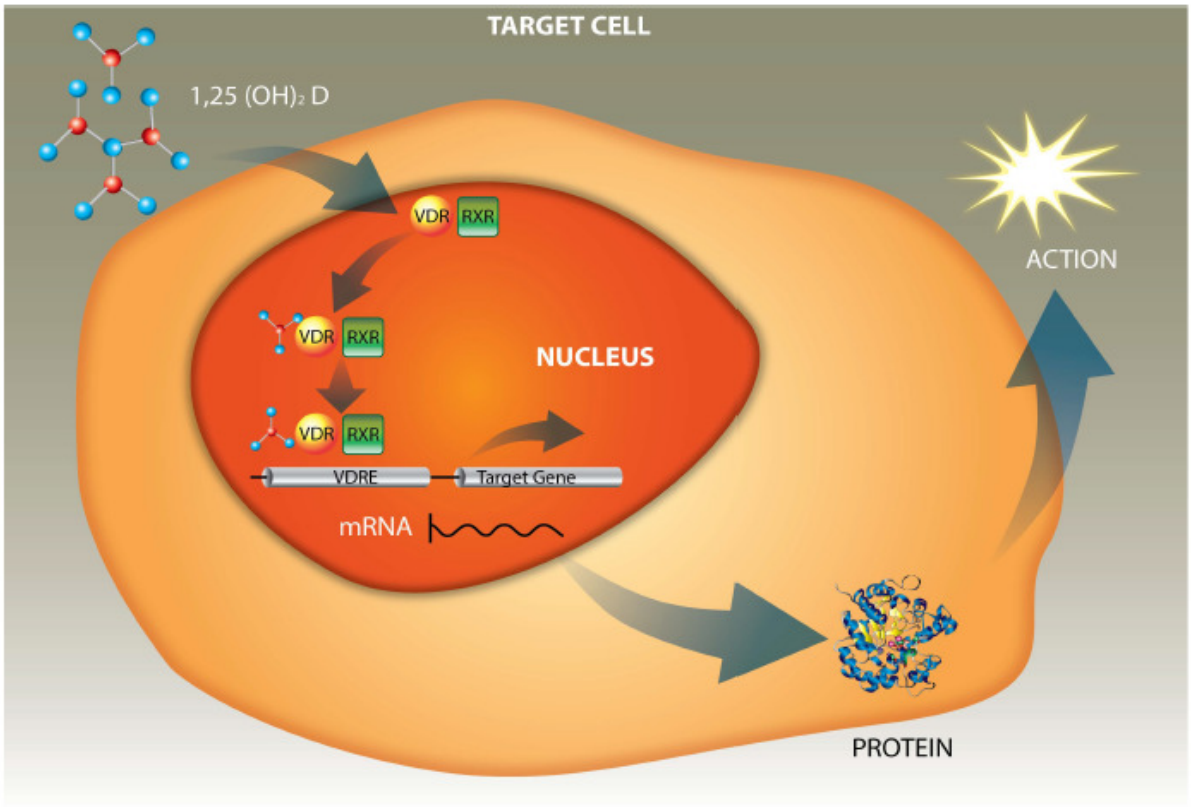

Fig. (2). Schematic representation of the transcriptional control of gene expression by 1,25(OH)2D *via* VDR.

According to studies conducted in several continents, vitamin D deficiency is one of the most common conditions in the world today. It is estimated that more than half of children and adults living in the United States, Canada, Mexico, Europe, Asia, New Zealand and Australia suffer from hypovitaminosis D [6].

Recent population survey in the U.S. showed that the prevalence of vitamin D insufficiency has doubled in the last 10 years, currently affecting not less than 90% of pigmented populations (blacks, Hispanics and Asians) and around ¾ of the caucasian population [7].

Several factors can interfere with the prevalence of hypovitaminosis D. Less sunlight exposure, that characterizes modern life in cities, coupled with the widespread use of sunscreen, stimulated by the fear of skin cancer, is undoubtedly the most important factor to the increasing prevalence of vitamin D deficiency in the world. In addition, in dark-skinned individuals cutaneous synthesis of vitamin D can be reduced by 50 to 90%, since melanin acts as a natural sunscreen. Additional factors include low latitude, winter, extremes of age,

female gender, malnutrition, use of robes covering most of the body and obesity [6, 8].

4. ASSOCIATION BETWEEN VITAMIN DEFICIENCY AND MARKERS OF SUBCLINICAL ATHEROSCLEROSIS

Some studies, predominantly cross-sectional and small, have shown an association between serum 25(OH)D and markers of subclinical atherosclerosis and inflammation, although there remain some conflicting findings.

In a cross-sectional analysis of 119 type 2 diabetic patients of middle age, Bonakdaran and Varasteh [9] observed a significant association between deficiency of 25 (OH)D and both increased high sensitive C-reactive protein (hs-CRP) and the presence of microalbuminuria. However in a prospective observational study involving 227 type 1 diabetics, Joergensen et al. [10] reported that severe deficiency of 25(OH)D (<15.5 nmol/l) at baseline was not predictive of the development of microalbuminuria, although has been associated with higher total mortality. Amer et al. [11], analyzing data from asymptomatic adults included in the National Health and Nutrition Examination Survey (NHANES) from 2001 to 2006, also found a significant inverse correlation between serum 25(OH)D and hs-CRP, independent of conventional risk factors, but only in those with levels of 25(OH)D ≤ 21 ng/ml, noting paradoxically a positive correlation between these two variables when they considered only the subgroup with levels of 25(OH)D > 21 ng/ml.

In a study involving 100 patients undergoing coronary angiography, a positive correlation between serum 25(OH)D and flow-mediated dilatation of the brachial artery (FMD) was observed, i.e., the lower the levels of 25(OH)D, the greater the degree of endotelial dysfunction [12]. Tarcin et al. [13], comparing 23 asymptomatic individuals with vitamin D deficiency (serum levels of 25(OH)D < 25 nmol/l) with a group with normal levels of the vitamin (average of 75 nmol/L) also found that the rate of FMD was significantly lower in the group with vitamin D deficiency.

In a cross-sectional analysis of a population-based cohort, the Rancho Bernardo Study, involving 654 healthy subjects, predominantly elderly (mean age 75.5 years), Reis et al. [2] observed, after multivariate analysis, an inverse

correlation between serum levels of 25 (OH)D and intima-medial thickness (IMT) of the internal carotid artery. Significant inverse association, independent of conventional risk factors, between serum 25(OH)D and common carotid IMT was also observed in type 2 diabetics [14, 15].

In patients with chronic kidney disease, inverse correlation between serum 25(OH) D and measurement of carotid IMT was observed by Yadav et al. [16] in individuals at stages 4 and 5 not on dialysis, but not by Zang et al. [17], who included only patients with diabetic nephropathy.

And finally, Ross et al. [18], analyzing 149 HIV-positive patients, observed no association between serum 25(OH)D and inflammatory markers (tumor necrosis factor α, interleukin 6 and hs-CRP) nor endothelial markers (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1).

5. CARDIOMETABOLIC AND VASCULAR IMPLICATIONS OF VITAMIN D DEFICIENCY

Results of several observational studies published in recent years have brought important evidence linking vitamin D deficiency with increased cardiovascular risk.

Evidence of the importance of vitamin D in the homeostasis of the cardiovascular system was initially suggested experimentally by the observation that knockout mice, lacking the VDR receptors, had impaired bone mineralization, small muscle fibers, suffered from high blood pressure (hypertension) and died of heart failure (HF) [5].

In the prospective cohort Third National Health and Nutrition Examination Survey (NHANES III), involving 3,408 elderly (≥ 65 years), followed for seven years, it was found, after multivariate analysis, that baseline levels of 25(OH)D were inversely associated with the risk of mortality from all causes, with an even stronger association for cardiovascular mortality [19].

In the Framingham Offspring study, involving 1,739 subjects with a mean age of 59 years, followed for five years, it was found that those with vitamin D deficiency [25 (OH)D<15 ng/ml] had a relative risk for the occurrence of cardiovascular events of 1.62 compared with those with levels ≥15 ng/ml, even after adjustment for conventional risk factors [20].

In the cohort Health Professionals Follow-up Study, which followed 18,225 male subjects, aged 40 to 75 years, for ten years, the authors found that participants with vitamin D deficiency [25 (OH) D ≤ 15 ng/ml] presented an incidence of myocardial infarction significantly higher than those with levels considered sufficient (≥ 30 ng/ml), with a relative risk of 2.42, after multivariate analysis [21]. A recent study involving 1,259 subjects showed that vitamin D status may also influence the prognosis of individuals already affected by myocardial infarction [22].

In the study Ludwigshafen Cardiovscular Health and Risk (LURIC), where 3,316 patients referred for a coronary angiogram were followed for seven years, it was evidenced that lower levels of both 25(OH)D and 1,25(OH)2D were independent predictors of fatal cerebrovascular accident (CVA) [23]. A similar result was obtained in the Copenhagen City Heart Study, a cohort that followed 10,170 individuals in a general population over 21 years, which also showed a clear independent inverse association between serum 25(OH)D and incidence of ischemic stroke [24].

Analyzing 90 elderly patients with stable HF and 31 controls, Ameri et al. [25] observed concentrations of 25(OH)D significantly lower in those with HF and found among them a prevalence of hypovitaminosis D (25 (OH)D <30 ng/ml) of almost 100%. They also observed that subjects with levels <10 ng/ml had left ventricle end-systolic and end-diastolic diameters and volumes greater and ejection fraction lower compared to those with levels ≥10 ng/ml. Low levels of 25(OH)D were also associated with greater prevalence of peripheral arterial disease [26] and abdominal aortic aneurysm [27].

The association between vitamin D deficiency and hypertension, metabolic syndrome (MS) and diabetes mellitus (DM) has also been investigated. Several analyses of the Third National Health and Nutrition Examination Survey (NHANES III) population, summarized in a review of Ullah et al. [28] have shown a clear inverse association between levels of 25(OH)D and blood pressure independently of numerous potentially confounding variables. In participants of two prospective cohorts, 613 men from the Health Professionals' Follow-Up Study and 1,198 women from the Nurses' Health Study followed for four years, the relative risk of developing hypertension in men with deficiency of 25 (OH)D (serum levels <15 ng/ml), after multivariate analysis, was 6.13 compared with those with

levels ≥30 ng/ml. Among women, the same comparison revealed a relative risk of 2.67 [29].

In a population-based cross-sectional study carried out in two major Chinese cities, including more than 3,200 individuals, aged 50 to 70 years, it was found that lower levels of 25(OH)D were significantly associated not only with the presence of MS but with any of its individual components after multivariate analysis. The study also showed, in overweight and obese individuals, a significant inverse association between serum 25(OH)D and both fasting insulin and the insulin resistance index assessed by homeostasis model assessment of insulin resistance (HOMA) [30]. Association between vitamin D deficiency and MS, in smaller studies, was also observed in obese patients [31]and children [32].

In a cross-sectional analysis of 6,228 participants in the Third National Health and Nutrition Examination Survey (NHANES III), aged ≥20 years, the presence of DM was significantly associated with lower levels of 25(OH)D, after multivariate analysis, in non-Hispanic whites and individuals of Mexican origin, being observed, however, no such association among non-hispanic blacks [33].

Association between vitamin D deficiency and the occurrence of preeclampsia was also reported [34]. In a case-control study, in which women were followed from the first trimester until delivery, a basal concentration of 25(OH)D<15 ng/l was associated, independently, to a five times higher risk of occurrence of the disease [34].

Finally, in a meta-analysis published recently, which included 19 prospective studies and 65,994 individuals, Wang et al. [35] reported that the relative risk of the category of lower levels of 25(OH)D in relation to that of higher levels was 1.52 for all cardiovascular diseases, 1.42 for cardiovascular mortality, 1.38 for coronary artery disease (CAD) and 1.64 for stroke.

6. POSSIBLE MECHANISMS INVOLVED IN THE ASSOCIATION BETWEEN VITAMIN D DEFICI-ENCY AND CARDIOVASCULAR DISEASE

The mechanisms by which vitamin D exerts its cardio and vasculoprotective effects are not yet fully understood, being considered its regulatory effects on the renin-angiotensin system (RAS), glycemic control,

inflammatory cytokines, the levels of PTH and calcium deposition in vascular smooth muscle, in addition to its direct vascular actions.

6.1. Vitamin D and Renin-Angiotensin System

It is well established that inappropriate stimulation of the RAS is associated with higher incidence of hypertension and CVD [28]. A good evidence favoring the role of vitamin D in the regulation of the RAS comes from experimental studies. In a study involving knockout mice, lacking the VDR, it was demonstrated an elevated production of renin and angiotensin II, causing hypertension, cardiac hypertrophy and increased water intake. These anomalies could be prevented by treating the mice with an angiotensin converting enzyme inhibitor or an angiotensin II receptor blocker. Based on these findings, it was concluded that vitamin D is a potent endocrine suppressor of renin biosynthesis [36]. On the other hand, in normal rats, it was demonstrated that vitamin D deficiency stimulates the expression of renin, while, if 1,25(OH)2D is administered, a reduction of renin synthesis occurs [36]. There is also evidence that PTH, whose serum levels may increase secondary to vitamin D deficiency, may also have a direct stimulatory effect on renin secretion [28]. Thus, the evidence strongly suggests that vitamin D deficiency may be implicated in the pathogenesis of hypertension via activation of the RAS.

6.2. Vitamin D and Insulin Sensitivity

The association between obesity, especially abdominal, and decreased insulin sensitivity is well established. On the other hand, it has been shown that obese patients have increased prevalence of vitamin D deficiency, which may be attributed to both a possible lower exposure to sunlight and its lower bioavailability, due to its greater sequestration by adipose tissue [28]. The demonstration of a strong independent inverse correlation between blood glucose and serum 25(OH)D [37, 38] coupled with epidemiological studies linking vitamin D deficiency with increased incidence of type 2 DM [33, 39] has suggested a major direct role of this vitamin deficiency in the pathogenesis of the disease. It

seems that adequate levels of vitamin D in the body are essential in the process of insulin sensitivity, as indicated by some research. In the study by Chiu et al. [40], for example, involving 126 healthy adults, a clear positive correlation between serum concentrations of 25(OH)D and insulin sensitivity was demonstrated. On the other hand, there is also evidence that vitamin D deficiency is associated with impaired insulin secretion, via β cell dysfunction, as demonstrated in mice [41], which has been confirmed also by its association with the incidence of type 1 DM [42].

One mechanism that has been proposed to explain the association between vitamin D deficiency and obesity, hypertension, type 2 DM and other manifestations of MS involves the metabolism of calcium [43]. Low serum calcium levels, resulting from vitamin D deficiency, as we know, can lead to secondary elevation of PTH, which in turn promotes increased intracellular levels of this ion. The increase of intracellular calcium can lead to both increased differentiation of preadipocytes into adipocytes and inhibition of the function of GLUT4, an enzyme involved in cellular glucose uptake mediated by insulin [43].

6.3. Vascular and Cardiac Effects of Vitamin D

Experimental studies have demonstrated various protective actions of vitamin D directly on the heart and blood vessels. In cultured vascular smooth muscle cells from rabbit, Wakasugi et al. [44] demonstrated that the synthesis of PGI2, which plays an important role in reducing thrombogenicity, cell adhesion and proliferation of smooth muscle cells, increased significantly in the presence of 1,25(OH)2D. It is possible, therefore, that vitamin D acts as an important vasoactive agent and can thus exert a protective role against the development of atherosclerosis.

Moreover, it has shown its participation in regulating the expression of many proteins with vascular action, such as vascular endothelial growth factor, type 9 metalloproteinase, myosin, elastin, type 1 collagen and γ-carboxyglutamic acid, the latter a protein that protects the vessel against parietal calcification, and also in the suppression of pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor-α in vitro and in vivo [45].

There is also experimental evidence that its antiatherogenic action involves specific effects on the immune system, including a direct effect on naive CD4 T cells, inducing the development of T helper type 2 (Th2) lymphocytes, responsible for the production of interleukin-10 (IL-10), which inhibits macrophage activation, a key step in the process of atherogenesis [46]. Furthermore, inhibits the transcription of interferon-γ (IFN-γ), secreted by T helper type (Th1) cells, that is, on the contrary, a potent activator of macrophages and suppressor of Th2 lymphocytes [47].

In relation to the heart, there is evidence that vitamin D plays an important role in the modulation and maintenance of cell structure and function. The treatment with 1,25(OH)2D increases expression of myotrophin, a cardiac muscle protein, and decreases expression of atrial natriuretic peptide, which is inversely related to cardiac function. Furthermore, the treatment with 1,25(OH)2D increases the expression and nuclear localization of the VDR in cardiac cells [48]. Thus, the suppression of the effects of vitamin D fully justifies the development of myocardial hypertrophy and heart failure in the aforementioned experimental model of knockout mice, lacking VDR [5].

7. EFFECTS OF VITAMIN D REPLACEMENT ON CARDIOVASCULAR DISEASE

Data from randomized controlled trials to assess the impact of vitamin D supplementation on cardiovascular risk are limited, particularly in relation to heavy outcomes, and have reported sometimes conflicting results. Overall, the evidence is derived from studies that addressed specific populations. In addition, some studies were limited by the small sample size, lack of uniformity in the inclusion criteria, short observation period and use of relatively low doses of vitamin D. Table 1 presents the randomized placebo-controlled trials that evaluated the effects of vitamin D supplementation on cardiovascular outcomes.

Table 1. Randomized placebo-controlled trials evaluating the effects of vitamin D supplementation over cardiovascular outcomes.

Author	N	Study population	Follow-up	Intervention	Main results
Hsia *et al.* [49]	36,282	Healthy postmenopausal women (50-79 years)	7 years	Calcium 1.000 mg + vitamin D 400 IU *vs* placebo	No effect on coronary and cerebrovascular events
Wood *et al.* [50]	305	Healthy women (60-70 years)	1 year	Vitamin D 400 or 1,000 IU *vs* placebo	Small changes in apolipoprotein B 100 levels (-1,0 mg/dl in 400 IU subgroup, -1,0 mg/dl in 1,000 IU subgroup and +0,02 mg/dl in placebo subgroup)
Gepner*et al.* [52]	114	Healthy women with a mean age of 64 years and serum levels of 25(OH)D between 10 and 60 ng/ml	4 months	Vitamin D 2,500 IU *vs* placebo	No effect on FMD, carotid-femoral pulse wave velocity, aortic augmentation index and serum levels of hs-CRP
Pfeifer *et al.* [53]	148	Elderly women (mean age 74 years) with serum levels of 25(OH)D < 20 ng/ml	8 weeks	Vitamin D 800 IU + calcium 1,200 mg vs only calcium 1,200 mg	Significant decreases in systolic blood pressure, heart rate and serum levels of PTH
Forman *et al.* [54]	283	Healthy black individuals with a mean age of 51 years	3 months	Vitamin D in different dosages (1,000, 2,000 or 4,000 IU) *vs* placebo	Small but significant decreases in systolic blood pressure in all vitamin D subgroups
Mitri *et al.* [55]	92	Adult individuals (mean age 57 years) with glucose intolerance	16 weeks	Vitamin D 2,000 IU *vs* calcium 800 mg	Significant increase in insulin secretion in vitamin D group
Witham *et al.* [56]	61	Type 2 diabetics with serum levels of 25(OH)D < 40 ng/ml	16 weeks	Vitamin D in different single doses (100.000 ou 200.000 IU) *vs* placebo	Significant decreases in systolic blood pressure in both vitamin D subgroups at eight weeks analysis as well as in type B natriuretic peptide in the subgroup that was treated with vitamin D 200.000 IU at 16 weeks analysis
Yiu *et al.* [57]	100	Type 2 diabetics	12 weeks	Vitamin D 5,000 IU or placebo	No significant effect on FMD neither on markers of inflammation and oxidative stress, lipid profile and glycaded hemoglobin
Zittermann *et al.* [58]	200	Healthy obese individuals with low serum levels of 25(OH)D (mean 12 ng/ml)	12 months	VitaminD 83 microgram *vs* placebo	Significant decrease in serum levels of triglycerides and TNF-α and a small but significant increase in LDL-cholesterol levels
Witham *et al.* [61]	105	Elderly (≥70 years) with systolic heart failure and serum levels of 25(OH)D <20 ng/ml	20 weeks	Vitamin D 100,000 IU *vs* placebo at the beginning and ten weeks after	No benefits in quality of life (Minnesota score) and serum levels of TNF-α but significant decrease in type B natriuretic peptide

In postmenopausal women, the findings have been quite varied and generally disappointing. In the classic Women's Health Initiative (WHI) study, which

involved 36,282 postmenopausal healthy women, aged 50-79 years, randomly assigned to take 1000 mg of calcium plus 400 IU of vitamin D daily or placebo and followed for seven years, no significant difference between the treatment and placebo groups was observed for the incidence of coronary and cerebrovascular events [49]. It should be stressed, however, that besides the low doses used, deficiency of vitamin D did not constitute a criterion for inclusion in the study. In the WHI, the fact that women with higher body mass index and multiple coronary risk factors have shown a lower incidence of cardiovascular events in the active treatment group draws attention.

In a study involving 305 healthy women aged 60-70 years who received randomly 400 or 1,000 IU of vitamin D3 or placebo daily for one year to evaluate the effects of the vitamin on lipid profile, insulin resistance, inflammatory biomarkers and blood pressure, the authors observed only minor changes in the levels of apolipoprotein B100 (-1.0 mg/dl in the 400 IU group, -1.0 mg/dl in the 1,000 IU group and +0.02 mg/dl in the placebo group), that, although significant, were considered clinically irrelevant [50]. However, it must be stressed, as a limitation, that, in this study, vitamin D deficiency also did not constitute a criterion for inclusion.

Likewise, Gannagé-Yared et al. [51], evaluating a set of only 47 healthy postmenopausal women, regarding the effects of a 12-week course of 800 IU of vitamin D associated to 1,000 mg of calcium per day on the inflammatory profile, pancreatic function and lipid parameters, did not observe any significant change in serum levels of interleukin 6, tumor necrosis factor-α, hs-CRP, insulin, triglycerides, HDL and LDL-cholesterol. We emphasize that in this study, vitamin D deficiency was also considered an inclusion criterion.

Gepner et al. [52], in prospective, randomized, double-blind study involving 114 women with a mean age of 64 years, with serum 25(OH)D between 10 and 60 ng/ml, in which the effects of oral administration of 2,500 IU of vitamin D3 per day for four months on cardiovascular risk markers were compared with placebo, also did not show any significant difference between the two groups regarding changes in FMD, carotid-femoral pulse wave velocity, aortic augmentation index or serum levels of hs-CRP.

However, in a study involving 148 elderly women (mean age 74 years) with documented hypovitaminosis D [serum 25(OH)D<20 ng/ml], randomized to receive for eight weeks oral supplementation of 800 IU of vitamin D3 plus 1,200 mg

of calcium or just 1,200 mg of calcium daily, Pfeifer et al. [53] reported significant decreases in systolic blood pressure, heart rate and serum levels of PTH in the participants of the first group.

Favorable effects of oral vitamin D supplementation on blood pressure were also reported by Forman et al. [54] that evaluated healthy blacks, a population with a known high prevalence of hypovitaminosis D. In this study, 283 individuals with a mean age of 51 years, almost half in use of antihypertensive drugs, were randomized in a double-blind fashion to receive oral vitamin D3 in different doses (1,000, 2,000 or 4,000 IU per day) or placebo for three months. In the analysis of three months, the authors found small but significant changes in systolic blood pressure (on average, +1.7 mmHg in the placebo subgroup and -0.66, -3.5 and -4.0 mmHg in the three active treatment subgroups, respectively).

Some beneficial effects of vitamin D have also been reported in diabetic and glucose intolerant patients. In the Calcium and Vitamin D for Diabetes Mellitus (CaDDM) study, conducted by Mitri et al. [55], in which 92 adults (mean age 57 years) with glucose intolerance were randomized in a double-blind fashion to receive 2,000 IU of vitamin D or 800 mg calcium per day for 16 weeks, the authors observed an increased insulin secretion in the vitamin D group and a decrease in the control group, with statistically significant difference, as well as a smaller increase in glycated hemoglobin in the vitamin D group, compared to the control group, with borderline statistical significance.

In order to investigate the effects of high doses of vitamin D3 orally on vascular health and glycemic control in type 2 diabetic patients, Witham et al. [56] randomized, in a double-blind fashion, 61 subjects with serum 25(OH)D<40 ng/ml to receive a single dose of placebo or different doses of vitamin D3 (100,000 or 200,000 IU). In the analyses of eight and 16 weeks, the authors observed no significant differences between the groups regarding endothelial function assessed by FMD, insulin resistance and glycated hemoglobin levels. However, they found significant reductions, compared to placebo, in systolic blood pressure in both groups that received vitamin D, in the analysis of eight weeks, as well as in B-type natriuretic peptide in the group that received 200,000 IU, in the analysis of 16 weeks.

And also in type 2 diabetics, Yiu et al. [57] showed no significant effect of high-dose vitamin D supplementation on vascular function and inflammatory parameters. In this study, 100 subjects were randomized in a double-blind fashion to

take vitamin D orally at a dose of 5000 IU/day or placebo for 12 weeks. In the end, the authors did not show any significant effect of vitamin D supplementation on endothelial function, assessed by FMD, serum levels of endothelial progenitor cells and brachial-ankle pulse wave velocity, or on biomarkers of inflammation and oxidative stress, lipid profile and glycated hemoglobin.

Some benefits of oral supplementation of vitamin D have been demonstrated in obese individuals. In 200 healthy overweight individuals with low serum 25(OH)D (mean 12 ng/ml) participants in a weight control program, randomized in a double-blind fashion to receive vitamin D (83 micrograms/day) or placebo for 12 months, Zittermann et al. [58] found that vitamin supplementation was associated with significant reductions in serum triglyceride levels and the inflammatory marker tumor necrosis factor-α, although it has promoted a small but significant increase in LDL-cholesterol.

In some studies, a favorable effect of vitamin D supplementation on endothelial function was demonstrated. In the trial conducted by Tarcin et al. [13], involving 23 asymptomatic individuals with severe deficiency of 25(OH)D (serum levels below 10 ng/ml), it was observed, after vitamin D replacement in the form of 300,000 IU intramuscularly monthly for three months, a significant improvement of endothelial function, reflected by an increase in FMD. Similar results, also using high doses of vitamin D, were observed by Harris et al. [59] in African Americans and by Witham et al. [60] in stroke patients.

The effects of vitamin D supplementation on functional capacity and quality of life in patients with HF were assessed by Witham et al. [61]. In the study, in which 105 elderly patients (≥ 70 years) with systolic heart failure and vitamin D deficiency [25(OH)D<20 ng/ml] were randomized in a double-blind fashion to receive orally 100,000 IU of vitamin D2 or placebo at the beginning and 10 weeks later, no benefit in favor of the active treatment group was observed in the analyses of 10 and 20 weeks, with respect to various parameters evaluated such as six-minute walk test, quality of life according to Minnesota Living with Heart Failure Questionnaire score and serum levels of tumor necrosis factor-α. However, the authors noted, at 10 weeks, a decrease in serum atrial natriuretic peptide in the vitamin D group and an increase in the placebo group, with significant difference between groups.

The effects of oral supplementation of vitamin D have also been tested on 123 heart failure patients randomized to receive 50 micrograms of vitamin D (2000

IU) plus 500 mg of calcium or placebo plus 500 mg of calcium per day for nine months. The survival rate was not different between the two groups after 15 months of follow-up but a more favorable inflammatory profile was observed in the group receiving vitamin D, translated by higher levels of interleukin 10, an anti-inflammatory cytokine, and lower levels of tumor necrosis factor-α, which has an inflammatory action [62].

Results even more favorable with oral vitamin D supplementation in patients with HF were recently reported by Amin et al. [63]. In the study, 94 subjects with serum levels of 25(OH)D below normal (<30 ng/ml) were given oral supplementation of vitamin D3 for four months, being 50,000 IU per week for eight weeks and then 50,000 IU monthly for the following two months. In the final evaluation, the authors observed a significant reduction in serum levels of brain natriuretic peptide (pro-BNP) and hs-CRP, as well as significant improvement in functional class and increase in 6-minute walk distance.

Through a case-control evaluation of participants of the EURODIAB Study [42], the impact of vitamin D supplementation during infancy on the risk of developing type 1 diabetes in the future was investigated. Analyzing data from 820 cases and 2,335 controls, the authors found that vitamin D supplementation in infancy was associated with a significant decrease in the incidences of type 1 diabetes, even after adjustment of several potential confounders, with an estimated odd ratio of 0.67, which reinforces the possible immunomodulatory effect and protective role of the vitamin against the development of the disease in susceptible individuals.

Unfortunately, the large multicenter study Thiazolidinedione Intervention with Vitamin D Evaluation (TIDE), randomized, double-blind, placebo-controlled, designed to include 16,000 diabetics in 33 countries to be followed for more than five years, in which 1,221 individuals came to be assigned to receive 1,000 IU per day of vitamin D or placebo, with the primary objective of evaluating the effects of the vitamin on all-cause mortality and incidence of cancer, had to be stopped early for internal regulatory reasons, without reaching the goals, being observed, however, comparable incidence of adverse events in the two groups [64].

Finally, it is worth noting that vitamin D supplementation is considered safe: under natural conditions, a full body exposure to sunlight, for example, is able to induce rapidly (<20 minutes) to the synthesis of the equivalent of more than 10,000 UI without any known adverse effects, apart from the possible harm to the skin, i.e.,

does not cause intoxication, because excess vitamin D3 is simply converted into inactive products [65]. Indeed, accumulating evidence has shown that prolonged intake of 10,000 IU/day (or 250 micrograms) of vitamin D3 causes, in general, no risk of adverse effects, so this dose can be considered totally safe. Thus, vitamin D intoxication is extremely rare, occurring only upon accidental or intentional ingestion of excessively high doses, more than 50,000 IU per day, in which serum levels of 25(OH)D may exceed 150 ng/ml and cause hypercalcemia and hyperphosphatemia [65].

However, the dose of oral supplementation of vitamin D3 (cholecalciferol) required in individuals who live without adequate sunlight exposure also has been a matter of debate. The Institute of Medicine recommends, as an adequate intake, daily doses of 200 IU for children and adults up to 50,400 IU for adults 51-70 years and 600 IU for those over 70 years. However, most experts agree that, without adequate sun exposure, children and adults require approximately 800 to 1,000 IU per day. In individuals with proven vitamin D deficiency, Holick [65] has recommended doses even higher: 50,000 IU per week for eight weeks, followed by half this dose indefinitely.

8. FINAL CONSIDERATIONS

Vitamin D appears to play an important role in cardiovascular health. Numerous studies have shown, as demonstrated above, strong independent association between hypovitaminosis D and cardiometabolic risk. Increasing evidence suggests that the effects on the cardiovascular system may stem from both indirect actions, via modulation of known risk factors, and direct actions on cardiac and vascular cells. Although there is still need for further studies, the potential importance of vitamin D deficiency as an emerging major public health problem of global proportions, with important implications for cardiovascular morbidity and mortality is undeniable. Specifically in relation to CVD, hypovitaminosis D may be of particular importance, considering, on one hand, the high prevalence of both conditions around the world and, on the other, the possibility of its prevention and correction in a simple way.

However, we still lack thorough evidence, based on large randomized placebo-controlled studies using higher doses of vitamin D, with longer-term follow-

up and with adequate statistical power to assess heavy outcomes, so you can establish definitively the role of vitamin D, especially in its oral form, in the prevention and treatment of CVD.As seen, many published studies have involved small samples, specific populations and there is no uniformity regarding the inclusion criteria or the dose of vitamin D administered and duration of treatment, and often vitamin D has been administered together with calcium. So, further studies to evaluate primarily the role of vitamin D and to establish its appropriate dose in this context are needed. We believe, however, that the already published data linking vitamin D deficiency and numerous health problems, not just cardiovascular disease, and pointing to the multiple benefits of its replacement, which can be made in a simple and safe way, argue strongly in favor of prevention and treatment of hypovitaminosis D in order to reduce cardiovascular morbidity and mortality, despite the lack of definitive studies in this area, as already explained.

CONFLICT OF INTEREST

The authors state that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors thank Ribamar Martins and Hudson Santos, from the Open University of Brazilian Health System (UNA-SUS), São Luís, Maranhão, Brazil, for the illustrations presented in this article.

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5.2 Artigo 2

Correlation Between Serum 25-Hydroxyvitamin D Levels And Carotid Intima-Media Thickness In A Brazilian Population Descended From African Slaves

(Submetido à Revista Brazilian Journal of Medical and Biological Research)

Correlation between serum 25-hydroxyvitamin D levels and carotid intima-media thickness in a Brazilian population descended from African slaves

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Running title: Serum 25(OH)Dand C-IMT in A*frodescendants*

ABSTRACT

Hypovitaminosis D has been identified as a possible new cardiovascular risk factor. However, the results of studies correlating serum vitamin D levels with markers of subclinical atherosclerosis have been conflicting. The aim of this study was to correlate serum levels of 25-hydroxyvitamin D [25(OH)D] with carotid intima-media thickness (C-IMT) and conventional cardiovascular risk factors in Afro-descendants. A cross-sectional analysis was performed on a sample of 382 individuals, from a cohort of *descendants o*f *African slaves,* inhabitants of "Quilombola" communities, with a mean age of 57.79 (±15.3) years, 54.5% of whom were women. Socio-demographic and clinical data were collected and biochemical tests were performed, including serum levels of 25(OH)D by electrochemiluminescence and urinary albumin excretion, evaluated by the albumin/creatinine ratio in a spot urine sample (ACR). All participants underwent high-resolution ultrasonography for C-IMT measurement. Hypovitaminosis D was defined as serum 25(OH)D levels<30 ng/ mL. The mean serum 25(OH)D levels were 50.4 (±13.5) ng/mL, with a low prevalence of hypovitaminosis D (4,86%). By simple linear correlation, a significant inverse association between 25(OH)D levels and C-IMT (r=-0.174, P=0.001) was observed. However, after multiple linear regression analysis, the significance of the association between serum levels of 25 (OH) D and C-IMT measurement was lost (β=-0.039, P=0.318) and only male sex, age, smoking, systolic blood pressure, glucose and LDL-cholesterol remained significantly associated with C-IMT. Levels of 25(OH)D were independently and positively associated with HDL-cholesterol and inversely associated with age and ACR. In conclusion, no independent association between 25(OH)D levels and C-IMT was observed in this population. On the other hand, there was an inverse association with albuminuria, a marker of endothelial lesion.

Keywords: Vitamin D deficiency, Carotid intima-media thickness (C-IMT), Atherosclerosis, African continental ancestry group

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death and disability worldwide (1). Although the role of traditional risk factors has already been established, it is known that they may not fully explain the development of CVD. Recent evidence suggests that vitamin D deficiency, currently very prevalent, affecting around one billion individuals worldwide, according to estimates (2), may be associated with an increased risk of CVD (3).

Vitamin D, in fact a steroid hormone, has as its primary function the regulation of calcium and phosphorus homeostasis, in interaction with parathyroids, kidneys and intestines. Under normal conditions, only about 10% of the vitamin required is obtained by food intake, the rest being synthesized in the body, starting with the activation of a cutaneous precursor by solar ultraviolet radiation and involving successive hydroxylation processes at the hepatic and renal levels (4,5).

The role of vitamin D in the regulation of bone metabolism is already well established. Thus, diseases such as rickets and osteomalacia have been classically attributed to hypovitaminosis D (5). However, in recent years, several studies have demonstrated that the vitamin D function extends well beyond bone health, being exerted on specific receptors, the VDRs, present in innumerable cell types, modulating more than 200 genes (5). Thus, hypovitaminosis D has been associated with disorders as varied as autoimmune diseases, infections and cancer. In the cardiovascular area, it has been independently associated with the occurrence of myocardial infarction (6), stroke (7), peripheral arterial disease (8) and cardiovascular death (3).

The mechanisms by which vitamin D levels may influence cardiovascular risk are still not fully understood and their participation in atherogenesis has been postulated. However, studies attempting to correlate hypovitaminosis D with early signs of atherosclerosis in different population profiles have reported conflicting findings (4,9-17), and no research of this nature has been identified in the Latin American population.

Thus, in the present study, we sought to correlate serum levels of 25-hydroxyvitamin D (25 (OH) D), the stable circulating form of the vitamin, with a marker of subclinical atherosclerosis, the measure of carotid intima-media thickness (C-IMT), and conventional risk factors in Afro-descendant individuals, inhabitants of quilombola communities in the western coast of the State of Maranhão, Brazil.

METHODS

Study population

This cross-sectional study evaluated individuals aged ≥ 18 years, inhabitants of quilombola communities, located in the municipality of Alcântara, west coast of the State of Maranhão, Brazil, included in a prevalence study of chronic kidney disease, the PREVRENAL (18). All PREVRENAL participants, selected from among the inhabitants of 32 communities by a probabilistic sampling process, numbered 1539, were examined in the community itself, after receiving clarifications about the research objectives and signing the consent form. Socio-demographic, lifestyle (smoking, alcohol consumption and work activity), anthropometric (weight, height and waist circumference) and clinical (previous pathological history and blood pressure) data were collected. Blood samples, after a 12-hour fast, and urine were collected for biochemical analysis and measure of urine albumin excretion.

All the individuals identified as having systemic arterial hypertension (SAH), diabetes mellitus (DM), albuminuria and/or reduced estimated glomerular filtration rate (GFR) were referred to a University Hospital for specialized exams and considered eligible for the present study. Thus, out of a total of 416 participants who fulfilled this initial inclusion criterion, 25 were excluded because they presented an overt cardiovascular disease and nine did not attend to perform the complementary exams, so that, at the end, 382 individuals (91,8% of those initially eligible) constituted the sample object of the present research.

Clinical evaluation

Standardized questionnaires were used in the initial evaluation to record information. Data on alcohol consumption (yes / no) and smoking (yes / no) were obtained. Blood pressure was defined as the mean of the last two of three measurements, performed at intervals ≥ 2 minutes, using a mercury column sphygmomanometer after sitting in the sitting position for at least five minutes. SAH was defined as the finding of any of the following four criteria: systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, previous diagnosis by a physician

or use of antihypertensive medication. The diagnosis of DM was performed according to the following criteria: use of hypoglycemic agents, fasting glycemia ≥ 126 mg / dL or an oral glucose tolerance test ≥ 200 mg / dL. The body mass index (BMI) was calculated using the formula: BMI = weight / height2 (kg / m2). The waist circumference was measured with the patient standing at expiration at the midpoint between the last rib and the iliac crest.

Biochemical evaluation

The biochemical measurements were performed in an automated way in a single laboratory. The GFR was estimated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula, being considered low a GFR <60 ml / min / 1.73m^2, based on the definition of chronic kidney disease (CKD) from the National Kidney Foundation (19).

The 25(OH)D dosage was performed by means of the electrochemiluminescence fixation assay (Elecsys, Roche Diagnóstica). A serum level ≥ 30 ng / ml was considered normal. Values <30 ng / ml were subclassed as insufficient (20-29 ng / ml) and deficient (<20 ng / ml) (20).

Urine albumin and ultra-sensitive serum C-reactive protein assays were performed by immunoturbidimetry. Albuminuria was evaluated by means of the albumin / creatinine ratio (ACR) in an isolated urine sample. Microalbuminuria was defined as urinary albumin excretion values between 30 and 300 mg / g.

Measurement of carotid media-intima thickness (C-IMT)

The carotid artery ultrasound examination was performed with a 7.5 MHz linear transducer in longitudinal section in mode B, by a single experienced examiner, blinded to the participants' clinical and laboratory information, in a GE model Vivid 3 device. The C-IMT was measured at the distal wall (farthest from the transducer) of the common carotid artery, 10 mm proximally to its bifurcation, on both sides, according to current recommendations (21). The measurement consists of the distance between two echogenic lines represented by the lumen-intima and medium-adventitia interfaces of the arterial wall, being considered normal when <0.9 mm. In the present study, the arithmetic mean of the C-IMT measurements obtained in the

two carotid arteries was considered for analysis. A thickening ≥ 1.5 mm was categorized as atheromatous plaque.

Statistical analysis

The Kolmogorov Smirnov test was used to evaluate the normality of the continuous variables. Qualitative variables were represented by relative frequency (%) and continuous variables, such as mean and standard deviation (SD). The chi-square test was used to compare proportions. For comparison of means, we used the Student t test. In order to analyze the degree of linear correlation between continuous variables, Pearson's correlation coefficient was calculated. Associations presenting a $p < 0.20$ at the bivariate analysis were submitted to multiple linear regression analysis. In the multiple linear regression model, sex (male = 0 and female = 1) and smoking (absent = 0 and present = 1) were included as dummy variables. The dichotomization of 25(OH)D serum levels according to the 50th percentile was chosen because of the very low prevalence of hypovitaminosis D, according to the conventional cut-off point. Data were processed and submitted to statistical analysis using the program SPSS 18.0 for Windows, adopting as significant a $p < 0.05$ (5%).

Ethical Considerations

This research, because it involved human beings, was carried out in accordance with the Brazilian National Health Council Resolution 196/96, in force throughout the national territory. The inclusion of the participants was done by signing a Term of Free and Informed Consent, where there was the information that the data obtained would be confidential and used only in this research. The PREVRENAL project was approved by the Ethics Committee
of the university institution involved in the study.

RESULTS

The analyzed sample consisted of 382 individuals, with a mean age of 57.79 (±15.39) years, being almost half with age ≥ 60 years, with a slight predominance of females. As expected, almost 90% of participants were non-white, and most had low income and low level of schooling. Farmers and fishermen made up half the sample. The expressive prevalence of SAH, DM and albuminuria are justified by the inclusion criteria of study participants. The mean serum levels of 25(OH)D were within the range considered healthy (≥30 ng/mL), with a low prevalence of hypovitaminosis D (4,86%). Slightly more than half of the participants had increased C-IMT and almost half had carotid atheromatous plaques. These and other characteristics of the study population are presented in Table 1.

Table 2 presents the characteristics of the participants according to the 50th percentile of serum 25(OH)D levels. Lower levels of vitamin D were observed in women, older individuals, hypertensive individuals, diabetics, smokers, those with higher blood pressure and higher BMI. Regarding the biochemical parameters, lower levels of 25(OH)D were associated with lower levels of creatinine, HDL-cholesterol and hemoglobin, and higher glycemia, LDL-cholesterol and albuminuria. Higher mean C-IMT was also observed among those with lower levels of 25(OH)D. There was no significant difference in the prevalence of carotid atheromatous plaques between the two subgroups of 25(OH)D levels.

After multiple linear regression analysis (Table 3), lower levels of 25(OH)D remained significantly associated with the female sex. In addition, the variables creatinine and HDL-cholesterol were positively associated with 25(OH)D levels, and age, BMI and ACR were inversely associated.

As seen in Table 4, bivariate analysis showed that an increased C-IMT was significantly associated with male sex, older age, presence of SAH, DM and smoking, as well as higher BMI, systolic blood pressure, creatinine, glycemia and LDL-cholesterol and lower levels of 25(OH)D. In a simple linear correlation analysis (Pearson), there was a significant inverse association between serum 25(OH)D levels and C-IMT ($r=-0.174$, $P=0.001$). However, after multiple regression analysis, this association lost significance when the variable age was added to the model ($\beta=-0.039$, $P=0.318$), so that, besides age, only male gender, smoking and, in the positive

direction, systolic blood pressure, glycemia and LDL-cholesterol levels remained significantly associated with C-IMT (Table 5).

DISCUSSION

The present study analyzed adult individuals of both sexes, Afro-descendant, with cardiovascular risk factors, inhabitants of Quilombola remnant communities, and found no evidence of an independent association between serum levels of 25(OH)D and C-IMT, as well as the presence of carotid atheromatous plaques. On the other hand, an independent inverse association between 25(OH)D levels and urinary albumin excretion, assessed by ACR in an isolated urine sample, was observed.

A low prevalence of hypovitaminosis D (< 5%), with only three cases of deficiency (0.81%), was observed in this population, with a mean serum level of 25(OH)D of 50 ng/mL.This finding differs substantially from what has been described in most studies from around the world, including tropical countries, which show that it affects in general more than half of the individuals (22). Our finding is even more striking considering that it was found in a predominantly dark-skinned population, since melanin is considered a potent natural sun blocker (22). Factors related to the lifestyle of this population, including professional activities such as farming and fishing, characterized by extensive exposure to solar radiation, probably explain this characteristic.

C-IMT is a marker of subclinical atherosclerosis widely validated as an independent predictor of cardiovascular events (23). The inverse association between serum levels of 25(OH)D and C-IMT, initially observed in this study, lost its significance when the variable age was included in the multivariate analysis model. The study corroborated, on the other hand, the role of traditional risk factors for atherosclerosis, with independent associations of the variables age, male sex, smoking, systolic blood pressure, fasting glucose and LDL cholesterol with C-IMT.

The independent inverse association between 25(OH)D levels and the urinary albumin excretion observed in this study should be highlighted. Albuminuria is considered a sensitive marker of endothelial lesion and an independent predictor of cardiovascular events (24,25). This association has also been reported in type 2 diabetics (26), in the Third National Health and Nutrition Examination Survey

(NHANES III) population (27) and in individuals with CKD (28). In addition to its possible antiatherosclerotic effects (29), a nephroprotective role of vitamin D has also been suggested, and there is evidence that vitamin D therapy can reduce albuminuria (24) and delay the progression of renal disease (25).

The participation of vitamin D deficiency in the pathogenesis of atherosclerosis has been evoked in numerous studies, based on the finding that low levels of 25(OH)D correlate with a higher incidence of deaths and cardiovascular events, particularly of atherosclerotic nature (3,6,7,30). This hypothesis is reinforced by the fact that a higher prevalence of obesity, dyslipidemia, metabolic syndrome, SAH and DM, which are recognized as important factors in the pathophysiology of atherosclerosis, has also been observed in individuals with hypovitaminosis D (22).

In addition, there is biological plausibility to justify the antiatherosclerotic role of hypovitaminosis D, which has been explained by several possible mechanisms (29). The active form of vitamin D, the 1,25-dihydroxyvitamin D, through its binding to VDR, regulates numerous genes involved in processes of fundamental importance in cardiovascular physiology, such as the renin-angiotensin system activity, proliferation, cell migration and differentiation, membrane transport, cell adhesion, immune response and cytokine expression. Vitamin D receptors have been found in all major cellular types of the cardiovascular system, including cardiomyocytes and especially cells involved directly in the atherosclerotic process, such as arterial wall cells (endothelial and smooth muscle cells) and immune cells. Thus, vitamin D deficiency, according to several studies, some of which experimental, may play a determining role in processes such as apoptosis, oxidative stress, smooth muscle cell proliferation, inflammation and thrombosis. Vitamin D also has a role in the mechanisms of insulin synthesis, secretion and resistance, which would justify the association between its deficiency and a higher incidence of DM (29,31,32). On the other hand, hyperparathyroidism secondary to chronic hypovitaminosis D has also been reported as an additional cardiovascular risk factor in this condition (31).

Thus, studies attempting to correlate hypovitaminosis D with subclinical atherosclerosis markers have attracted considerable interest from several researchers in recent years, with variable results. Vitamin D deficiency has been associated with endothelial dysfunction (9,10) and higher coronary calcium score (13). In the elderly, Reis et al. (4) verified an independent inverse association between serum 25(OH)D levels and internal C-IMT. In type 2 diabetics, an inverse

association was also observed by Targher et al. (11) but not by Winkler et al. (12). In patients with CKD, inverse correlation was observed by Yadav et al. (14) but not by Zang et al. (15). Finally, the two largest studies identified, one involving 926 climacteric women (16), and the other, 3,430 individuals at high cardiovascular risk (17), also found divergent results, as only in the former there was an association between low levels of 25(OH)D and greater C-IMT. Population differences and methodological limitations, such as the small sample size of some of them and the lack of adequate adjustment for all potential confounding factors, probably justify such discrepancies.

The positive association between 25(OH)D and HDL-cholesterol levels, which is classically linked to lower cardiovascular risk, observed in this study and also by others (17,29, 33,34), deserves to be emphasized. Other favorable effects of vitamin D on the lipid profile, such as reduction of triglycerides and LDL-cholesterol, have also been described (29). The mechanisms by which vitamin D may favorably affect lipid metabolism have not yet been fully elucidated. In vitro, studies have shown that the incubation of adipocyte culture with calcitriol promoted an increased expression and activity of lipoprotein lipase, an enzyme related to the reduction of triglyceride levels and increase in HDL-cholesterol (29).

The results of the present study, therefore, do not support the hypothesis of the antiatherosclerotic, vasculoprotective role of vitamin D, since the inverse association between levels of 25(OH)D and C-IMT did not persist in the multivariate analysis. However, our study may have had limited statistical power because of the very low prevalence of hypovitaminosis D. With a variation of 25(OH)D levels almost entirely within the normal range, it would be unlikely to find a relevant role of the vitamin in the pathogenesis of atherosclerosis in these individuals. On the other hand, its inverse association with urinary excretion of albumin should be valued and may indicate a role for lower levels of vitamin D, even though currently classified as "normal", at an earlier stage of atherosclerosis in this population. Therefore, based on our findings, the association of hypovitaminosis D with the pathogenesis of atherosclerosis in this population should not be totally discarded.

The inconsistency of results in the literature on the association between vitamin D deficiency and early markers of atherosclerosis, coupled with the divergence between observational and intervention studies, have estimulated an intense debate on the causality of hypovitaminosis D in CVD in the last decade

(22,31,35). Many scholars argue that hypovitaminosis D would not play a determinant role in the pathophysiology of diseases, but would be only a marker of inflammation, hence its association with CVD. However, the remarkable limitations of previous interventional studies, such as small sample size, short period of observation, varied doses of vitamin D used, and joint administration with calcium, among others, should be emphasized. Thus, such a debate should persist until results from large, randomized ongoing trials assessing the impact of supplementation on hard outcomes, are available in the coming years.

The present study has some limitations. Its cross sectional design does not allow establishing a causal relationship in the associations found, as well as the selection of a sample from a specific population does not allow the extrapolation of our results to other populations. In addition, the limited statistical power of the study, due to its relative small sample size and the low proportion of individuals with hypovitaminosis D observed, should be considered. On the other hand, some strengths should be highlighted. It is a study approaching a population profile not previously studied, that correlated serum levels of 25(OH)D with C-IMT and albuminuria, two established early markers of atherosclerosis, in addition to several traditional cardiovascular risk factors / markers. Finally, this seems to be the first Latin American study of this nature.

In conclusion, no independent association between serum levels of 25(OH)D and C-IMT was observed in this population. On the other hand, there was an inverse association with albuminuria, a marker of endothelial injury and also an independent predictor of cardiovascular events.

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Table 1 - Socio-demographic, clinical-anthropometric and laboratory characteristics of the study population (n = 382).

Age, mean (±SD), years	57,79 (± 15,39)
Age ≥ 60 years, n (%)	181 (47,38)
Female sex, n (%)	208 (54,5)
Non-whites, n (%)	328 (86)
Occupation, n (%)	
Farmers/ fishermen	194 (50,9)
Retired	133 (34,9)
Other	54 (14,2)
Income up to a minimum wage, n (%)	302 (79,06)
Up to four years of schooling, n (%)	328 (85,86)
Smokers, n (%)	41 (10,7)
Alcoholics, n (%)	109 (28,5)
Weight, mean (±SD), Kg	62,95 (± 12,79)
Height, mean (±SD), cm	155 (± 8,30)
Body mass index, mean (±SD), kg/m^2	26,23 (± 5,42)
Waist circumference, mean (±SD), cm	68,64 (± 32,50)
Systemic arterial hypertension, n (%)	241 (66,5)
Systolic blood pression, mean (±SD), mmHg	148,58 (± 28,18)
Diastolic blood pressure, mean (±SD), mmHg	84,64 (± 14,41)
Diabetes mellitus, n (%)	59 (17,70)
Fasting glycemia, mean (±SD), mg/dl	119,36 (± 54,60)
Creatinine, mean (±SD), mg/dl	0,86 (± 0,31)
HDL-cholesterol, mean (±SD), mg/dl	47,87 (± 13,57)
Men	45,81 (± 14,01)
Women	49,55 (± 13,52)
Triglycerides, mean (±SD), mg/dl	171,77 (± 114,21)
Albumin-creatinine ratio, mean (±SD), mg/g	38,8 (± 16,19)
Albumin-creatinine ratio (isolated sample of urine), mg/g, n (%)	
<30	284 (75,53)
30 – 300	84 (22,34)
> 300	8 (2,12)
25(OH)D, mean (±SD), ng/mL	50,4 (± 13,5)
< 30ng/mL, n (%)	18 (4,86)
< 20 ng/mL, n (%)	3 (0,81)
> 100 ng/mL, n (%)	3 (0,81)
Carotid intima-media thickness, mean (±SD), mm	0,92 (± 0,21)
Carotidintima-mediathickness≥ 0,9 mm, n (%)	200 (53,50)

Carotidatheromatous plaque, n (%)	168 (44,2)

SD – standard deviation; 25(OH)D – 25-hydroxyvitamin D.

Table 2. Characteristics of the participants according to the 50th percentile of serum 25(OH)D levels.

	< 50,7 ng/L	≥ 50,7 ng/L	P value
Sex			
Male, n, (%)	56 (33,5)	111 (66,5)	
Female, n, (%)	133 (65,5)	70 (34,5)	< 0,001
Age, mean ± SD, years	60,8 ± 14,4	54,4 ± 15,8	< 0,001
SAH, n (%)	139 (57,7)	95 (39,4)	0,001
DM, n (%)	36 (61,0)	23 (39)	0,002
Smokers, n (%)	16 (39,0)	23 (56)	0,006
SBP, mean ± SD, mmHg	152,5 ± 29,6	144,9 ± 26,7	0,010
DBP, mean ± SD, mmHg	85,6 ± 14,0	83,7 ± 14,9	0,197
BMI, mean ± SD, kg/m2	26,7 ± 5,4	24,4 ± 4,2	<0,001
WC, mean ± SD, cm	65,7 ± 35,4	72,2 ± 28,4	0,054
Creatinine, mean ± SD, mg/dL	0,86 ± 0,2	0,95 ± 0,2	<0,001
Cystatin C, mean ± SD, mg/dL	0,35 ± 0,18	0,38 ± 0,18	0,671
ACR, mean ± SD, mg/g	13 (4,2)	4 (3,8)	0,043
Fasting glycemia, mean ± SD, mg/dL	125,7 ± 60,2	114,1 ± 49,2	0,043
HDL-cholesterol, mean ± SD, mg/dL	46,6 ± 11,4	49,6 ± 15,2	0,038
LDL-cholesterol, mean ± SD, mg/dL	140,0 ± 39,2	124,5 ± 45,3	0,001
Triglycerides, mean ± SD, mg/dL	159,6 ± 97,8	141,9 ± 88,0	0,069
Uric acid, mean ± SD, mg/dL	4,78 ± 1,38	4,73 ± 1,34	0,796
Ferritin, mean ± SD, mg/dL	123,6 ± 167,0	128,4 ± 133,5	0,760
Hemoglobin, mean ± SD, g/dL	13,4 ± 1,4	14,0 ± 1,6	<0.001
Hs-CRP, mean ± SD, mg/dL	0,55 ± 1,3	0,84 ± 4,5	0,406
C-IMT, mean ± SD, mm	0,96 ± 0,2	0,88 ± 0,2	<0,001
C-IMT ≥ 0,9 mm, n (%)	112 (56)	81 (40,5)	<0,001
Carotid atheromatous plaque, n (%)	88 (46,56)	76 (42,0)	0,506

25(OH)D: 25-hydroxyivitamin D; SD – standard deviation; SAH – systemic arterial hypertension; DM – diabetes mellitus; SBP – systolic blood pressure; DBP – diastolic blood pressure; BMI – body mass index; WC – waist circumference; ACR - albumin / creatinine ratio in an isolated sample of urine; hs-CRP - high-sensitivity C-reactive protein; C-IMT – carotid intima-media thickness.

Table 3. Variables independently associated with serum 25(OH)D levels after multiple linear regression analysis.

Variable	β	P value
Sex	-0,246	<0,001
Age	-0,235	<0,001
BMI	-0,179	0,001
Creatinine	0,159	0,004
HDL-cholesterol	0,104	0,030
ACR	-0,235	<0,001

25(OH)D - 25-hydroxyivitamin D; BMI – body mass index; ACR - albumin / creatinine ratio in an isolated sample of urine

Table 4. Correlation between the various clinical-laboratory variables of the participants and the categorized C-IMT.

	C-IMT		
	< 0,9 mm	≥ 0,9 mm	P value
Sex, n (%)			
Male	77 (45,57)	92 (54,43)	
Female	97 (47,32)	108 (52,68)	< 0,001
Age, mean ± SD, years	47,8 ± 13,5	66,1 ± 11,2	< 0,001
SAH, n (%)	101 (42,44)	137 (57,56)	< 0,001
DM, n (%)	19 (33,93)	37 (66,07)	0,015
Smokers, n (%)	18 (47,37)	20 (52,63)	< 0,001
SBP, mean ± SD, mmHg	140,2 ± 23,8	156,5 ± 29,3	< 0,001
DBP, mean ± SD, mmHg	84,2 ± 13,7	85,5 ± 14,6	0,362
BMI, mean ± SD, kg/m^2	26,2 ± 4,6	25,0 ± 5,2	0,019
WC, mean ± SD, cm	70,9 ± 31,5	66,6 ± 33,1	0,197
Creatinine, mean ± SD, mg/dL	0,87 ± 0,1	0,95 ± 0,3	0,002
ACR, mean ± SD, mg/g	5,0 (3,8)	12,5 (6,4)	< 0,001
Fasting glycemia, mean ± SD, mg/dL	112,3 ± 48,4	124,9 ± 59,3	0,025
HDL-cholesterol, mean ± SD, mg/dL	47,3 ± 13,7	48,6 ± 13,4	0,354
LDL-cholesterol, mean ± SD, mg/dL	121,9 ± 35,3	142,2 ± 46,7	< 0,001
Triglycerides, mean ± SD, mg/dL	151,3 ± 108,1	154,2 ± 87,9	0,499
Uric acid, mean ± SD, mg/dL	4,66 ± 1,25	4,76 ± 1,48	0,596
Ferritin, mean ± SD, mg/dL	126,4 ± 195,2	122,8 ± 91,8	0,825
Hemoglobin, mean ± SD, g/dL	13,9 ± 1,5	13,5 ± 1,4	0,011
hs-CRP, mean ± SD, mg/dL	0,81 ± 4,5	0,57 ± 1,2	0,499
25(OH)D, mean ± SD, ng/mL	52,2 ± 14,2	48,9 ± 12,6	0,020

C-IMT – carotid intima-media thickness; SD – standard deviation; SAH – systemic arterial hypertension; DM – diabetes mellitus; PAS – pressão arterial sistólica; SBP – systolic blood pressure; DBP – diastolic blood pressure; BMI – body mass index; WC – waist circumference; ACR - albumin / creatinine ratio in an isolated sample of urine; hs-CRP - high-sensitivity C-reactive protein; 25(OH)D - 25-hydroxyivitamin D.

Table 5. Variables independently associated with mean C-IMT, after multiple linear regression, stepwise mode.

	β	P value
Age	0,618	<0,001
Sex	-0,148	0,001
Smoking	0,080	0,026
Systolic blood pressure	0,191	<0,001
Fasting glycemia	0,127	<0,001
LDL-cholesterol	0,141	<0,001

C-IMT – carotid intima-media thickness.

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ANEXO A - INSTRUCTIONS FOR AUTHORS - THE OPEN CARDIOVASCULAR MEDICINE JOURNAL

1.1 Instructions for Authors

The Open Cardiovascular Medicine Journal is an Open Access online journal, which publishes Research articles, Reviews and Letters in the field of cardiovascular medicine, aiming at providing the most complete and reliable source of information on current developments in the field.
Manuscripts may be submitted directly to tocmj@benthamopen.org. Each peer-reviewed article that is published in a *Bentham OPEN*Journal is universally and freely accessible *via* the Internet in an easily readable and printable PDF format.

1.1.1 ONLINE MANUSCRIPT SUBMISSION:

An *online* submission and tracking service *via* Internet facilitates a speedy and cost-effective submission of manuscripts. The full manuscript has to be submitted online *via* Bentham's Content Management System (CMS) at http://www.bentham-editorial.org/ **View Instructions**
Alternatively, you may also submit your full manuscript by e-mail to tocmj@benthamopen.org

Manuscripts must be submitted by one of the authors of the manuscript, and should not be submitted by anyone on their behalf. The principal/corresponding author will be required to submit a Copyright Letter along with the manuscript, on behalf of all the co-authors (if any). The author(s) will confirm that the manuscript (or any part of it) has not been published previously or is not under consideration for publication elsewhere. Furthermore, any illustration, structure or table that has been published elsewhere must be reported, and copyright permission for reproduction must be obtained.
For all online submissions, please provide your complete manuscript in the form of a single zipped folder containing soft copies of all the materials (main text in MS Word or Tex/LaTeX), figures/illustrations in TIFF, PDF or JPEG, and chemical structures drawn in ChemDraw (CDX)/ISISDraw (TGF) as separate files, while a PDF version of the entire manuscript must also be included, embedded with all the figures/illustrations/tables/chemical structures etc.

A successful electronic submission of a manuscript will be followed by a system-generated acknowledgement to the principal/corresponding author within **72** hours of the dispatch of the manuscript. Any questions with regards to the preparation of and submission of your manuscript to the journal should be addressed to tocmj@benthamopen.org and copied to managingeditor@benthamopen.org

NOTE: **Any queries therein should be addressed to oa@benthamscience.org and copied to Jalil@benthamscience.org**

1.1.1.1. Editorial Policies:

The editorial policies of Bentham Open on publication ethics, peer-review, plagiarism, copyrights/ licenses, errata/corrections and article retraction/ withdrawal can be viewed at Policy Page. Articles are licensed under the terms of the Creative Commons Attribution non-commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided that the work is properly cited.

1.1.1.2. Manuscript Preparation:

The manuscript should be written in English in a clear, direct and active style. All pages must be numbered sequentially, facilitating in the reviewing and editing of the manuscript.

For further convenience, the customer support team available at Eureka Science can provide assistance to authors for the preparation of manuscripts.

1.1.1.3. Manuscript Length:

1.1.1.3.1 Research Articles:

The total number of words for a published research article is from 4000 to 8000 words.

1.1.1.3.2 Review Articles:

The total number of words for a published comprehensive review article is from 8000 to 40000 words, and for mini-review articles from 3000 to 6000 words.

1.1.1.3.3 Letter Articles:

The total number of words for a published letter/short communication article is from 3000 to 6000 words.

There is no restriction on the number of figures, tables or additional files e.g. video clips, animation and datasets, that can be included with each article online. Authors should include all relevant supporting data with each article.

1.1.1.4. Manuscripts Published:

The journal accepts original research articles, letters and review articles written in English. Supplements, proceedings of conferences and open access book reviews may also be considered for publication.

1.1.1.5. Supplements/Thematic Issue:

The journal also considers Supplements/Thematic Issue for publication. A Supplement/Single Thematic Issue will be a collection of review articles (minimum of 6, maximum of 20 articles) based on a contemporary theme or topic of great importance to the field. Mini-supplements consisting of between 3 to 5 articles are also welcome. The Guest Editors' main editorial task is to invite the contributors to the Supplement and to manage the peer review of submitted manuscripts. A short summary or proposal for editing a supplement should be submitted to the Editor-in-Chief at e-mail to tocmj@benthamopen.org with a copy to specialissue@benthamopen.org

1.1.1.6. Conference Proceedings:

For proposals to publish conference proceedings in this journal, please contact us at email: proceedings@benthamscience.org.

1.1.1.7. Book Reviews:

This journal publishes open access reviews on recently published books (both print and electronic) relevant to the journal. Publishers and authors of books are invited to contact our book reviews editor at tocmj@benthamopen.org with book review requests. All submitted books will be reviewed by an independent expert in the field. No page charges will be levied to authors for the publication of book reviews.

1.1.2 MANUSCRIPT SECTIONS FOR PAPERS:

Manuscripts for research articles and letters submitted to the respective journals should be divided into the following sections; however, there can be an extension in the number of sections in review articles in accordance with the requirements of the topic.

- Copyright letter
- Title
- Title page
- Structured Abstract
- Keywords

- Text organization
- List of abbreviations (if any)
- Conflict of interest
- Acknowledgements
- References
- Appendices
- Figures/illustrations (if any)
- Chemical structures (if any)
- Tables (if any)
- Supportive/supplementary Material (if any)

1.1.2.1. Copyright Letter:

It is a mandatory requirement that a signed copyright letter also be submitted along with the manuscript by the author to whom correspondence is to be addressed, delineating the scope of the submitted article declaring the potential competing interests, acknowledging contributions from authors and funding agencies, and certifying that the paper is prepared according to the '***Instructions for Authors***'. All inconsistencies in the text and in the reference section, and any typographical errors must be carefully checked and corrected before the submission of the manuscript. The article should not contains such material or information that may be unlawful, defamatory, fabricated, plagiarized, or which would, if published, in any way whatsoever, violate the terms and conditions as laid down in the agreement. The authors acknowledge that the publishers have the legal right to take appropriate action against the authors for any such violation of the terms and conditions as laid down in the agreement. Download the Copyright letter

1.1.2.2. Title:

The title should be precise and brief and must not be more than 120 characters. Authors should avoid the use of non-standard abbreviations. The title must be written in title case except for articles, conjunctions and prepositions.

1.1.2.3. Title Page:

Title page should include paper title, author(s) full name and affiliation, corresponding author(s) names complete affiliation/address, along with phone, fax and email.

Authors should also provide a short 'running title'.

1.1.2.4. Structured Abstract:

The abstract of an article should be its clear, concise and accurate summary, having no more than 250 words, and including the explicit sub-headings (as in-line or run-in headings in bold). Use of abbreviations should be avoided and the references should not be cited in the abstract. Ideally, each abstract should include the following sub-headings, but these may vary according to requirements of the article.

- Background
- Objective
- Method
- Results
- Conclusion

1.1.2.5. Keywords:

Provide 6 to 8 keywords in alphabetical order.

1.1.2.6. Text Organization:

The main text should begin on a separate page and should be divided into separate sections. For Research articles, the preparation of the main text must be structured into separate sections as **Introduction, Materials and Methodology, Results, Discussion, Conclusion and Trial Registration**. For Review and Letter articles, the manuscript should be divided into title page, abstract and the main text. The text may be subdivided further according to the areas to be discussed, which should be followed by the Acknowledgements and Reference sections. The review article should mention any previous important reviews in the field and contain a comprehensive discussion starting with the general background of the field. It should then go on to discuss the salient features of recent developments. The authors should avoid presenting material which has already been published in a previous review.

1.1.2.6.1 Randomized Drug Clinical Trial Studies:

Randomized drug clinical trial studies are biomedical or health-related interventional and/or observational research studies conducted in phases in human beings who are randomly allocated to receive or not receive a preventive, therapeutic, or diagnostic intervention that follows a pre-defined protocol. The study is intended to determine the safety and efficacy of approaches to disease prevention, diagnosis and treatment.

Authors of randomized controlled trials are encouraged to submit trial protocols along with their manuscripts. All clinical trials must be registered (before recruitment of the first participant) at an appropriate online public trial registry that must be independent of for-profit interest (e.g.,www.clinicaltrials.gov). If you wish the editor(s) to consider an unregistered trial, please explain briefly why the trial has not been registered.

- All randomized clinical trials should include a flow diagram and authors should provide a completed randomized trial checklist (see CONSORT Flow Diagram and Checklist; www.consort-statement.org) and a trial protocol.
- Studies of diagnostic accuracy must be reported according to STARD guidelines; (www.stard-statement.org)
- Observational studies (cohort, case-control, or cross-sectional designs) must be reported according to the STROBE statement, and should be submitted with their protocols; (www.strobe-statement.org).
- Genetic association studies must be reported according to STREGA guidelines; (www.medicine.uottawa.ca)
- Systematic reviews and meta-analyses must be reported according to PRISMA guidelines; (www.prisma-statement.org)
- To find the reporting guidelines see (www.equator-network.org)

Important points to remember while submitting clinical trials:

- Each manuscript should clearly state an objective or hypothesis; the design and methods (including the study setting and dates, patients or participants with inclusion and exclusion criteria, or data sources, and how these were selected for the study); the essential features of any interventions; the main outcome measures; the main results of the study; a comment section placing the results in context with the published literature and addressing study limitations; and the conclusions. Data included in research reports must be original.
- Trial registry name, registration identification number, and the URL for the registry should be included at the end of the abstract and also in the space provided on the online manuscript submission form. If your research article reports the results of a controlled health care intervention, list the trial registry, along with the unique identifying number (Please note that there should be no space between the letters and numbers of your trial registration number). Studies designed for other purposes, such as to study pharmacokinetics or major toxicity (e.g., phase 1 trials), are exempted.

- All reports of randomized trials should include a section entitled “Randomization and Masking”, within the Methods section.
- The manuscript must include a statement identifying the institutional and/or licensing committee that has approved the experiments, including any relevant details.
- The SI system of units and the recommended international non-proprietary name (rINN) for drug names must be used. Kindly ensure that the dose, route, and frequency of administration of any drug you mention are correct.
- Please ensure that the clinical trials sponsored by pharmaceutical companies follow the guidelines on good publication practice: (www.gpp-guidelines.org)

The editors reserve the right to reject manuscripts that do not comply with the above-mentioned requirements. The author will be held responsible for false statements or failure to fulfill the above-mentioned requirements.

1.1.2.6.2 Greek Symbols and Special Characters:

Greek symbols and special characters often undergo formatting changes and get corrupted or lost during preparation of manuscript for publication. To ensure that all special characters used are embedded in the text, these special characters should be inserted as a symbol but should not be a result of any format styling (*Symbol* font face) otherwise they will be lost during conversion to PDF/XML.

Authors are encouraged to consult reporting guidelines. These guidelines provide a set of recommendations comprising a list of items relevant to their specific research design. All kinds of measurements should be reported only in International System of Units (SI). Chemical equations, chemical names, mathematical usage, unit of measurements, chemical and physical quantity & units must conform to SI and Chemical Abstracts or IUPAC.

1.1.2.7. List Of Abbreviations:

If abbreviations are used in the text either they should be defined in the text where first used, or a list of abbreviations can be provided.

1.1.2.8. Conflict Of Interest:

Financial contributions to the work being reported should be clearly acknowledged, as should any potential conflict of interest.

1.1.2.9. Acknowledgements:

Please acknowledge anyone (individual/company/institution) who has contributed to the study by making substantial contributions to conception, design, acquisition of data, or analysis and interpretation of data, or who was involved in drafting the manuscript or revising it critically for important intellectual content. Please list the source(s) of funding for the study, for each author, and for the manuscript preparation in the acknowledgements section.

This journal complies with the International Committee of Medical Journal Editors' Uniform Requirements for Manuscripts Submitted to Biomedical Journals www.icmje.org and the FDA's Good Reprint Practices for the Distribution of Medical Journal Articles and Medical or Scientific Reference Publications on Unapproved New Uses of Approved Drugs and Approved or Cleared Medical Deviceshttp://www.fda.gov/oc/op/goodreprint.html

1.1.2.10. References:

References must be listed in the numerical system (Vancouver). All references should be numbered sequentially [in square brackets] in the text and listed in the same numerical order in the reference section. The reference numbers must be finalized and the bibliography must be fully formatted before submission.

See below few examples of references listed in the correct Vancouver style:

1.1.2.10.1 Typical Paper Reference:

- [1] Boehm M, Nabel EG. Angiotensin-converting enzyme 2-a new cardiac regulator. N Engl J Med 2002; 347: 1795-7.
- [2] Moses RG, Luebcke M, Davis WS, *et al*. Effect of a low-glycemic-index diet during pregnancy on obstetric outcomes. Am J Clin Nutr 2006; 84: 807-12.

1.1.2.10.2 Typical Chapter Reference:

- [3] Stevenson WG, Friedman PL. In: Hennekens CH, Ed. Clinical trials in cardiovascular disease. Philadelphia, WB Saunders Co. 1999; 217-30.

1.1.2.10.3 Book Reference:

- [4] Carlson BM. Human embryology and developmental biology. 3rd ed. St. Louis: Mosby; 2004.

1.1.2.10.4 Edited Book:

- [5] Brown AM, Stubbs DW, editors. Medical physiology. New York: Wiley; 1983.

1.1.2.10.5 Conference Proceedings:

- [6] Harris AH, editor. Economics and health: 1997: Proceedings of the 19th Australian Conference of Health Economists; 1997 Sep 13-14; Sydney, Australia. Kensington, N.S.W.: School of Health Services Management, University of New South Wales; 1998.

1.1.2.10.6 Journal Article on the Internet:

- [7] Aylin P, Bottle A, Jarman B, Elliott, P. Paediatric cardiac surgical mortality in England after Bristol: descriptive analysis of hospital episode statistics 1991-2002. BMJ [serial on the Internet]. 2004 Oct 9; [cited 2004 October 15]; 329: [about 10 screens]. Available from: http://bmj.bmjjournals.com/cgi/content/full/329/7470/825

1.1.2.10.7 Patent:

- [8] Kimura K, Lipeles A. Fuzzy controller component. U. S. Patent 14,860,040, December 14, 1996.

1.1.2.10.8 E-citations:

- [9] Citations for articles/material published exclusively online or in open access (free-to-view) , must contain the exact Web addresses (URLs) at the end of the reference(s), except those posted on an author's Web site unless editorially essential, e.g. 'Reference: Available from: URL'.

Some important points to remember:

- All references must be complete and accurate.
- If the number of authors exceeds six then *et al* will be used after three names (the term "*et al* " should be in italics).
- Online citations should include the date of access.
- Journal abbreviations should follow the *Index Medicus*/MEDLINE.
- Take special care of the punctuation convention as described in the above-mentioned examples.
- Avoid using superscript in the in-text citations and reference section.

- Abstracts, unpublished data and personal communications (which can only be included if prior permission has been obtained) should not be given in the reference section but they may be mentioned in the text and details provided as footnotes.
- The authors are encouraged to use a recent version of EndNote (version 5 and above) or Reference Manager (version 10) when formatting their reference list, as this allows references to be automatically extracted.

1.1.2.11. Appendices:

In case there is a need to present lengthy, but essential methodological details, use appendices, which can be a part of the article. An appendix must not exceed three pages (Times New Roman, 12 point fonts, 900 max. words per page).The information should be provided in a condensed form, ruling out the need of full sentences. A single appendix should be titled APPENDIX, while more than one can be titled APPENDIX A, APPENDIX B, and so on.

1.1.2.12. Figures/Illustrations:

The authors should provide the illustrations as separate files, as well as embedded in the text file, numbered consecutively in the order of their appearance. Each figure should include a single illustration. No charges will be levied on the use of color figures except in the reprints. Each figure should be closely cropped to minimize the amount of white space surrounding the illustration.

If a figure consists of separate parts, it is important that a single composite illustration file be submitted, containing all parts of the figure.

Photographs should be provided with a scale bar if appropriate, as well as high-resolution component files.

1.1.2.12.1 Scaling/Resolution:

For Line Art image type, which is generally an image based on lines and text and does not contain tonal or shaded areas, the preferred file format is TIFF or EPS, with colour mode being Monochrome 1-bit or RGB, in a resolution of 900-1200 dpi.

For Halftone image type, which is generally a continuous tone photograph and contains no text, the preferred file format is TIFF, with colour mode being RGB or Grayscale, with a minimum resolution of 300 dpi.

For Combination image type, which is generally an image containing halftone in addition to text or line art elements, the preferred file format is TIFF, with colour mode being RGB or Grayscale, in a resolution of 500-900 dpi.

1.1.2.12.2 Formats:

For illustrations, the following file formats are acceptable:

- **Illustrator**
- **EPS** (preferred format for diagrams)
- **PDF** (also especially suitable for diagrams)
- **PNG** (preferred format for photos or images)
- **Microsoft Word** (version 5 and above; figures must be a single page)
- **PowerPoint** (figures must be a single page)
- **TIFF**
- **JPEG** (conversion should be done using the original file)
- **BMP**
- **CDX (ChemDraw)**
- **TGF (ISISDraw)**

Bentham OPEN does not process figures submitted in GIF format.

If the large size of TIFF or EPS figures acts as an obstacle to online submission, authors may find that conversion to JPEG format before submission results in significantly reduced file size and upload time, while retaining acceptable quality. In order to maintain acceptable image quality, it is recommended that JPEG files are saved at High or Maximum quality.

Files should not be compressed with tools such as Zipit or Stuffit prior to submission as these tools will in any case produce negligible file-size savings for JPEGs and TIFFs, which are already compressed.

Please do not:

1. Supply embedded graphics in your word processor (spreadsheet, presentation) document.
2. Supply files that are optimized for screen use (like GIF, BMP, PICT, WPG); the resolution is too low.
3. Supply files that are too low in resolution.
4. Submit graphics that are disproportionately large for the content.

1.1.2.12.3 Image Conversion Tools:

There are many software packages, many of them freeware or shareware, capable of converting to and from different graphics formats, including PNG.

Good general tools for image conversion include GraphicConverter on the Macintosh, PaintShop Pro, for Windows, and ImageMagick, which is available on Macintosh, Windows and UNIX platforms.

Note that bitmap images (e.g. screenshots) should not be converted to EPS, since this will result in a much larger file size than the equivalent JPEG, TIFF, PNG or BMP, with no increase in quality. EPS should only be used for images produced by vector-drawing applications such as Adobe Illustrator or CorelDraw. Most vector-drawing applications can be saved in, or exported as, EPS format. In case the images have been originally prepared in an Office application, such as Word or PowerPoint, then the original Office files should be directly uploaded to the site, instead of being converted to JPEG or another format that may be of low quality.

1.1.2.13. Chemical Structures:

Chemical structures MUST be prepared according to the guidelines below.

Structures should be prepared in ChemDraw and provided as separate file, submitted both on disk and in printed formats.

1.1.2.13.1 Structure Drawing Preferences:

[As according to the ACS style sheet]

Drawing Settings:	
Chain angle	120°
Bond spacing	18% of width
Fixed length	14.4 pt (0.500cm, 0.2in)
Bold width	2.0 pt (0.071cm, 0.0278in)
Line width	0.6 pt (0.021cm, 0.0084in)
Margin width	1.6 pt (0.096cm)
Hash spacing	2.5 pt (0.088cm, 0.0347in)
Text Settings:	
Font	Times New Roman
Size	8 pt
Under the Preference Choose:	
Units	points

Tolerances	3 pixels
Under Page Setup Use:	
Paper	US letter
Scale	100%

1.1.2.14. Tables:

- Data Tables should be submitted in Microsoft Word table format.
- Each table should include a title/caption being explanatory in itself with respect to the details discussed in the table. Detailed legends may then follow.
- Table number in bold font i.e. Table 1, should follow a title. The title should be in small case with the first letter in caps. A full stop should be placed at the end of the title.
- Tables should be embedded in the text exactly according to their appropriate placement in the submitted manuscript.
- Columns and rows of data should be made visibly distinct by ensuring that the borders of each cell are displayed as black lines.
- Tables should be numbered in Arabic numerals sequentially in order of their citation in the body of the text.
- If a reference is cited in both the table and text, please insert a lettered footnote in the table to refer to the numbered reference in the text.
- Tabular data provided as additional files can be submitted as an Excel spreadsheet.

1.1.2.15. Supportive/Supplementary Material:

We do encourage to append supportive material, for example a PowerPoint file containing a talk about the study, a PowerPoint file containing additional screenshots, a Word, RTF, or PDF document showing the original instrument(s) used, a video, or the original data (SAS/SPSS files, Excel files, Access Db files etc.) provided it is inevitable or endorsed by the journal's Editor.

Supportive/Supplementary material intended for publication must be numbered and referred to in the manuscript but should not be a part of the submitted paper. In-text citations as well as a section with the heading "Supportive/Supplementary Material" before the "References" section should be provided. Here, list all Supportive/Supplementary Material and include a brief caption line for each file describing its contents.

Any additional files will be linked into the final published article in the form supplied by the author, but will not be displayed within the paper. They will be made available in exactly the same form as originally provided only on our Web site. Please also make sure that each additional file is a single table, figure or movie (please do not upload linked worksheets or PDF files larger than one sheet). Supportive/ Supplementary material must be provided in a single zipped file not larger than 4 MB.

Authors must clearly indicate if these files are not for publication but meant for the reviewers'/editors' perusal only.

1.1.3 PERMISSION FOR REPRODUCTION:

Published/reproduced material should not be included unless you have obtained written permission from the copyright holder, which should be forwarded to the Editorial Office in case of acceptance of your article for publication.

For obtaining permission for reproducing any material published in an article by Bentham Open, please fill in the request **FORM** and send totocmj@benthamopen.org for consideration.

1.1.4 AUTHORS AND INSTITUTIONAL AFFILIATIONS:

The author will be required to provide their full names, the institutional affiliations and the location, with an asterisk in front of the name of the principal/corresponding author. The corresponding author(s) should be designated and their complete address, business telephone and fax numbers and e-mail address must be stated to receive correspondence and galley proofs.

1.1.5 REVIEWING AND PROMPTNESS OF PUBLICATION:

All manuscripts submitted for publication will be immediately subjected to peer-reviewing, usually in consultation with the members of the Editorial Advisory Board and a number of external referees. Authors may, however, provide in their Copyright Letter the contact details (including e-mail addresses) of four potential peer reviewers for their paper. Any peer reviewers suggested should not have recently published with any of the authors of the submitted manuscript and should not be members of the same research institution.

All peer-reviewing will be conducted *via* the Internet to facilitate rapid reviewing of the submitted manuscripts. Every possible effort will be made to assess the manuscripts quickly with the decision being conveyed to the authors in due course. Papers which are delayed by authors in revision for more than 30 days will have to be re-submitted as a new submission.

1.1.6 LANGUAGE AND EDITING:

Manuscripts submitted containing language inconsistencies will not be published. Authors must seek professional assistance for correction of grammatical, scientific and typographical errors. Professional team available at Eureka Science may assist you in the English language editing of your article. Please contact Eureka Science for a language editing quote at e-mail: info@eureka-science.com stating the total number of words of the article to be edited.

1.1.6.1. 언어 및 편집:

영문 오타가 많은 원고는 출판되지 않을 것입니다. 영문 오타를 없애겠다는 조건으로 받은 원고는 영어 편집 전문회사인 유럽 공동 기술개발 기구로부터 가격 견적서가 보내 질 것입니다. 영어 작문에 어려움이 있는 비영어권 국가의 저자들은 원고를 학술지에 제출하기 전에 영어 편집회사와 접촉할 것을 권합니다. 영어 편집 견적서를 받기 위해서 교정될 원고의 단어수를 적은 메일을 유럽 공동 기술개발 기구 메일인 info@eureka-science.com 로 보내시기 바랍니다.

1.1.6.2. 语言和编辑:

含有很多英文印刷错误的提交稿将不予发表。接受发表的稿件其英文写作应是正确的；专业的语言编辑公司（尤里卡科学），可对稿件的英文润色提供报价。建议非英语国家、且英文写作欠佳的作者在投稿前先与语言编辑公司联系。请与尤里卡科学联系info@eureka-science.com.

1.1.7 EDITION ET LANGUE:

Les manuscrits soumis avec plusieurs erreurs typographiques en Anglais ne seront pas publiés en l'état. Les manuscrits sont acceptés pour publication à la condition que l'anglais utilisé soit corrigé après la soumission et seront envoyés pour examen à Eureka Science, une société d'édition de langue professionnelle. Les auteurs en provenance de pays où la langue est différente de l'anglais et qui ont de médiocres compétences en anglais écrit, sont priés de contacter la société d'édition de langue avant de soumettre leur manuscrit à la revue. Merci de contacter Eureka Science à info@eureka-science.com pour un devis en indiquant le nombre total de mot de l'article à éditer.

1.1.8 PROOF CORRECTIONS:

Authors are required to proofread the PDF versions of their manuscripts before submission. To avoid delays in publication, proofs should be checked immediately for typographical errors and returned within **48** hours. Major changes are not acceptable at the proof stage. If unable to send corrections within **48** hours due to some reason, the author(s) must at least send an acknowledgement on receiving the galley proofs or the article will be published exactly as received and the publishers will not be responsible for any error occurring in the manuscript in this regard.

The corresponding author will be solely responsible for ensuring that the revised version of the manuscript incorporating all the submitted corrections receives the approval of all the authors of the manuscript.

1.1.9 COPYRIGHT LICENSE:

The Corresponding Author retains ownership of the copyright in the published work in accordance with the terms of the Creative Commons Attribution 4.0 International Public License.

1.1.10 PLAGIARISM PREVENTION:

Bentham Open uses the iThenticate software to detect instances of overlapping and similar text in submitted manuscripts. iThenticate software checks content against a database of periodicals, the Internet, and a comprehensive article database. It generates a similarity report, highlighting the percentage overlap between the uploaded article and the published material. Any instance of content overlap is further scrutinized for suspected plagiarism according to the publisher's Editorial Policies. Bentham OPEN allows an overall similarity of 20% for a manuscript to be considered for publication. The similarity percentage is further checked keeping the following important points in view:

1.1.10.1. Low Text Similarity:

The text of every submitted manuscript is checked using the Content Tracking mode in iThenticate. The Content Tracking mode ensures that manuscripts with an overall low percentage similarity (but which may have a higher similarity from a single source) are not overlooked. The acceptable limit for similarity of text from a single source is 5%. If the similarity level is above 5%, the manuscript is returned to the author for paraphrasing the text and citing the original source of the copied material.

It is important to mention that the text taken from different sources with an overall low similarity percentage will be considered as aplagiarized content if the majority of the article is a combination of copied material.

1.1.10.2. High Text Similarity:

There may be some manuscripts with an overall low similarity percentage, but a higher percentage from a single source. A manuscript may have less than 20% overall similarity but there may be 15 % similar text taken from a single article. The similarity index in such cases is higher than the approved limit for a single source. Authors are advised to thoroughly rephrase the similar text and properly cite the original source to avoid plagiarism and copyright violation.

1.1.11 TYPES OF PLAGIARISM:

We all know that scholarly manuscripts are written after thorough review of previously published articles. It is therefore not easy to draw a clear boundary between legitimate representation and plagiarism. However, the following important features can assist in identifying different kinds of plagiarized content.

These are:

- Reproduction of others words, sentences, ideas or findings as one's own without proper acknowledgement.
- Text recycling, also known as self-plagiarism. It is an author's use of a previous publication in another paper without proper citation and acknowledgement of the original source.
- Paraphrasing poorly: Copying complete paragraphs and modifying a few words without changing the structure of original sentences or changing the sentence structure but not the words.
- Verbatim copying of text without putting quotation marks and not acknowledging the work of the original author.

- Properly citing a work but poorly paraphrasing the original text is considered as unintentional plagiarism. Similarly, manuscripts with language somewhere between paraphrasing and quoting are not acceptable. Authors should either paraphrase properly or quote and in both cases, cite the original source.
- Higher similarity in the abstract, introduction, materials and methods, and discussion and conclusion sections indicates that the manuscript may contain plagiarized text. Authors can easily explain these parts of the manuscript in many ways. However, technical terms and sometimes standard procedures cannot be rephrased; therefore Editors must review these sections carefully before making a decision.

1.1.12 PLAGIARISM IN PUBLISHED MANUSCRIPTS:

Published manuscripts which are found to contain plagiarized text are retracted from the journal website after careful investigation and approval by the Editor-in-Chief of the journal. A 'Retraction Note' as well as a link to the original article is published on the electronic version of the plagiarized manuscript and an addendum with retraction notification in the journal concerned.

1.1.13 PUBLICATION FEES:

The publication fee details for each article published in the journal are given below:

1.1.13.1. Letters:

The publication fee for each published Letter article submitted is US $600.

1.1.13.2. Research Articles:

The publication fee for each published Research article is US $800.

1.1.13.3. Mini-Review Articles:

The publication fee for each published Mini-Review article is US $600.

1.1.13.4. Review Articles:

The publication fee for each published Review article is US $900.

The publication fee includes the professional copy editing charges. Once the paper is accepted for publication, the author will receive an electronic invoice via email. Subsequent submissions from the Bentham Open Authors will receive a discount of **US$ 100** on the total publication charges providing their previous submission did not avail any discount off the listed full author open access fee rate. The fee form is also available on the Web site at Fee Form

1.1.13.5. Special Fee Waiver:

Bentham *OPEN* allows Special FEE Waiver to authors from 79 countries which are classified by the World Bank as low-income economies or lower-middle-income economies as of September 2012 (reference - World Bank 1st September 2012). Refer to the complete list of these countries click here.

1.1.14MEMBERSHIP:

Join as a member of Bentham OPEN today to obtain great discounts on your article publication fees! For details click here.

1.1.15 REPRINTS:

High quality printed reprints of published articles are available for purchase, if ordered, with a minimum number of 100 reprints.

ANEXO B - INSTRUCTIONS TO AUTHORS – BRAZILIAN JOURNAL OF MEDICAL AND BIOLOGICAL RESEARCH

Brazilian Journal of medical and biological research

ISSN 1414-431X *online version*

INSTRUCTIONS TO AUTHORS

Since 1997 the Journal is published only as Online Version
2015 Impact Factor 1.146
Qualis 2014 - Medicine B2; Biological Sciences B4
Average time for submission until publication online on SciELO: 6 months

Scope and policy

The purpose of the *Brazilian Journal of Medical and Biological Research* is to publish the results of original experimental research that contribute significantly to knowledge in medical and biological sciences. Major criteria for acceptance are scientific quality, originality, and conciseness. Preference will be given to manuscripts that develop new concepts or experimental approaches and are not merely repositories of data. Papers that report negative results require special justification for publication. Methodological papers shall be considered for publication provided they describe new principles or a significant improvement of an existing method.

The following papers will not be accepted for publication

- Studies on people not approved by an accredited Ethics Committee or without written informed consent from the subject or legal guardian.
- Studies on animals not approved by an accredited Ethics and Animal Care Committee.
- Manuscripts that report preliminary results or only confirm previously reported results.
- Manuscripts that describe the pharmacokinetics, bioavailability and toxicity of drugs in people or animals.
- Manuscripts that deal with transcultural adaptation and validation of instruments of measurements.
- Manuscripts that translate a text published in another language and validate it on local patients.
- Manuscripts that use questionnaires translated from the language of another country and their validation in local patients.
- Manuscripts that present only *in silico* analysis.
- Manuscripts from the area of veterinary medicine.
- The Journal does not publish toxicological studies.

Publication charges

- The authors are responsible for "publication charges" of all accepted papers. Publication charges will be billed to the Corresponding Author when the paper is accepted.
- The charge is R$3.300,00/paper for Brazilian authors and US$1.600,00/paper for authors outside Brazil and is independent of the length of the paper.

Preparation of Research Manuscripts

The ***Brazilian Journal of Medical and Biological Research*** publishes original research articles of outstanding scientific significance. Manuscripts must be submitted in English. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a limited number of experiments. The key criteria are that the research clearly demonstrates its novelty, its importance to a particular field as well as its interest to those outside that discipline, and conclusions that are justified by the data.

Authorship requirements

Only those persons who contributed directly to the intellectual content of the paper should be listed as authors. Authors should meet all of the following criteria, thereby allowing persons named as authors to take public responsibility for the content of the paper.

- Conceived, planned and carried out the experiments that led to the paper or interpreted the data it presents, or both.
- Wrote the paper, or reviewed successive versions.
- Approved the final version.

  Holding positions of administrative leadership, contributing patients, and collecting and assembling data, however important to the research, are not by themselves criteria for authorship. Other persons who have made substantial, direct contributions to the work but cannot be considered authors should be cited in the Acknowledgment section, with their permission, and a description of their specific contributions to the research should be given.

Cover Letter

It is important that you include a cover letter with your manuscript. Take the time to consider why this manuscript is suitable for publication in the *Brazilian Journal of Medical and Biological Research*. Why will your paper inspire the other members of your field, and how will it drive research forward? Please explain this in your cover letter.

The cover letter should also contain the following information:

- Title of article.
- Name(s) of all author(s).
- Name, complete mailing address, including zip code, telephone number, fax number and e-mail of author to whom correspondence should be sent.
- If a version of the manuscript has been previously submitted for publication to another journal, include comments from the peer reviewers and indicate how the authors have responded to these comments.
- Papers in the area of Clinical Investigation should include a statement indicating that the protocol has been approved by the Hospital Ethics Committee (Hospital with which at least one of the authors is associated) and that written informed consent was obtained from all participants. This information must also be cited in the Material and Methods section of the manuscript.

Text format

- The text of a manuscript can only be accepted as a Microsoft Word file created with MS Word as a "doc", "docx" or "rtf" document.
- Each page should contain the page number in the upper right-hand corner starting with the title page as page 1.
- Report all measurements in Système International, SI (http://physics.nist.gov/cuu/Units) and standard units where applicable (see below).
- Do not use abbreviations in the title and limit their use in the abstract and text.
- The length of the manuscript and the number of tables and figures must be kept to a minimum.
- Ensure that all references are cited in the text.
- Generic names must be used for all drugs. Instruments may be referred to by proprietary name; the name and country or electronic address of the manufacturer should be given in parentheses in the text.

Guidance on grammar, punctuation, and scientific writing can be found in the following sources:

- Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers. 8th edn. Rockefeller University Press, Reston, 2006. http://www.scientificstyleandformat.org/Home.html

- Medical Style and Format. Huth EJ (Editor). ISI Press, Philadelphia, 1987, Marketed by Williams & Wilkins, Baltimore, MD.
- Writing scientific articles like a native English speaker: top ten tips for Portuguese speakers. Clinics (Sao Paulo). Mar 2014; 69(3): 153-157. doi: 10.6061/clinics/2014(03)01. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3935133/

The *Brazilian Journal of Medical and Biological Research* follows the reference format of the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, which can be found on the website of the National Library of Medicine (http://www.icmje.org/).

The writing style should be concise and accessible. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

Although we encourage submissions from around the globe, we require that manuscripts be submitted in American English. As a step towards overcoming language barriers, we encourage authors to seek the assistance of professional services available on the homepage of the journal/Service and Information.

Footnotes

Text footnotes, if unavoidable, should be numbered consecutively in superscript in the manuscript and written on a separate page following the abstract.

Abbreviations

Abbreviations should be kept to a minimum. Define all abbreviations upon first use in the abstract and the text. Non-standard abbreviations should not be used unless they appear at least three times in the text.

- Explain all abbreviations in the abstract, text, figure and table legends **when they first appear**. Keep the number of abbreviations to a minimum.
- Do not explain abbreviations for units of measurement [3 mL, not 3 milliliters (mL)] or standard scientific symbols [Na, not sodium (Na)].
- Abbreviate long names of chemical substances and terms for therapeutic combinations. Abbreviate names of tests and procedures that are better known by their abbreviations than by the full name (VDRL test, SMA-12).
- Use abbreviations in figures and tables to save space, but they **must be defined in the legend**.

Nomenclature

The use of standardized nomenclature in all fields of science and medicine is an essential step toward the integration and linking of scientific information reported in published literature. We will enforce the use of correct and established nomenclature wherever possible: We strongly encourage the use of SI units.

- s for second
- min for minute
- h for hour
- L for liter
- m for meter
- kDa for mass in kilodaltons
- 5 mM rather than ^{5} x 10-3 M or 0.005 M

Species names (e.g., *Homo sapiens*), genes, mutations, genotypes, and alleles should be italicized. Use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. It is sometimes advisable to indicate the synonyms for the gene the first time it appears in the text.

The Recommended International Non-Proprietary Name (rINN) of drugs should be provided.

Manuscript categories

Authors should state in the cover letter that the manuscript is intended to be a Full-length Paper, Short Communication, Review Article, Overview, Concepts and Comments, or Case Report.

Full-length paper

Each manuscript should clearly state its objective or hypothesis; the experimental design and methods used (including the study setting and time period, patients or participants with inclusion and exclusion criteria, or data sources and how these were selected for the study);

the essential features of any interventions; the main outcome measures; the main results of the study; and a discussion placing the results in the context of published literature.
The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text should be divided into separate sections (Introduction, Material and Methods, Results, Discussion), without a separate section for conclusions
- no more than 40 references (without exceptions)

Short communication
A short communication is **a report on a single subject**, which should be concise but definitive. The scope of this section is intended to be wide and to encompass methodology and experimental data on subjects of interest to the readers of the Journal.
The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text should be divided into separate sections (Introduction, Material and Methods, Results, Discussion), without a separate section for conclusions
- no more than 20 references (without exceptions)
- no more than three illustrations (figures and/or tables)

Review article
A review article should provide a synthetic and critical analysis of a relevant area and should not be merely a chronological description of the literature. A review article by investigators who have made substantial contributions to a specific area in medical and biological sciences will be published by invitation of the Editors. However, an outline of a review article may be submitted to the Editors without prior consultation. If it is judged appropriate for the Journal, the author(s) will be invited to prepare the article for peer review. A minireview is focused on a restricted part of a subject normally covered in a review article.
The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text should be divided into sections with appropriate titles and subtitles
- no more than 90 references (without exceptions)

Overview
An overview does not contain unpublished data. It presents the point of view of the author(s) in a less rigorous form than in a regular review or minireview and is of interest to the general reader.
The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text may be divided into sections with appropriate titles and subtitles
- no more than 90 references (without exceptions)

Concepts and Comments
The Concepts and Comments section provides a platform for readers to present ideas, theories and views.
The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text may be divided into sections with appropriate titles and subtitles

- no more than 40 references (without exceptions)

Case report

A case report should have at least one of the following characteristics to be published in the Journal:

- special interest to the clinical research community
- a rare case that is particularly useful to demonstrate a mechanism or a difficulty in diagnosis
- new diagnostic method
- new or modified treatment
- a text that demonstrates relevant findings and is well documented and without ambiguity

The manuscript should contain:

- abstract of no more than 250 words
- no more than 6 key words
- a running title to be used as a page heading, which should not exceed 60 letters and spaces
- the text may be divided into sections with appropriate titles and subtitles
- no more than 20 references (without exceptions)
- no more than three illustrations (figures and/or tables)

Organization of the Manuscript

Most articles published in the *Brazilian Journal of Medical and Biological Research* will be organized into the following sections:

Title, Authors, Affiliations, Abstract, Key words, Running Title, Author for Correspondence and email address
Introduction
Material and Methods
Results
Discussion
Acknowledgments
References
Tables with a short descriptive title and footnote legends
Figures with a short descriptive title, descriptive legends and uniformity in format

Continuous page numbers are required for all pages including figures. There are no specific length restrictions for the overall manuscript or individual sections. However, we urge authors to present and discuss their findings concisely. We recognize that some articles will not be best presented in our research article format. If you have a manuscript that would benefit from a different format, please contact the editors to discuss this further.

Title Page

Title - The title should be as short and informative as possible, should not contain non-standard acronyms or abbreviations, and should not exceed two printed lines.

Example: **Single-step purification of crotapotin and crotactine from *Crotalus durissus terrificus* venom using preparative isoelectric focusing**

Authors and Affiliations

Initials and last name(s) of author(s) (matched with superscript numbers identifying institutions). Institution(s) (Department, Faculty, University, City, State, Country) of each author (in Portuguese if authors are from Brazil).

Example:

A.S. Aguiar[1], A.R. Melgarejo[1], C.R. Alves[2] and S. Giovanni-De-Simone[2,3]
[1]Divisão de Animais Peçonhentos, Instituto Vital Brazil, Niterói, RJ, Brasil
[2]Laboratório de Microsequenciamento de Proteínas, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil
[3]Departamento de Biologia Celular e Molecular, Universidade Federal Fluminense, Niterói, RJ, Brasil

Abstract

Since abstracts are published separately by Information Services, they should contain sufficient hard data, to be appreciated by the reader. The *Brazilian Journal* publishes **unstructured abstracts** in a single paragraph. The abstract should not exceed 250 words.

The abstract should briefly and clearly present the objective, experimental approach, new results

as quantitative data if possible, and conclusions. It should mention the techniques used without going into methodological detail and mention the most important results.
Abbreviations should be kept to a minimum and should be defined in both the Abstract and text. Please do not include any reference citations in the abstract. If the use of a reference is unavoidable, the full citation should be given within the abstract.
Please see <http://www.bjournal.com.br/writing_a_good_abstract.html> for suggestions on writing a good abstract

Key Words

A list of key words or indexing terms (no more than 6) should be included. A capital letter should be used for the first letter of each key word, separated by a semicolon. The Journal recommends the use of medical subject headings of Index Medicus for key words to avoid the use of several synonyms as entry terms in the index for different papers on the same subject. Remember, key words are used by the Scielo Database (see http://www.scielo.br/bjmbr;articles search/subject) to index published articles.

Running title

This short title, to be used as a page heading, should not exceed 60 letters and spaces.

Corresponding author

One of the authors should be designated as the corresponding author. It is the corresponding authors responsibility to ensure that the author list is accurate and complete. If the article has been submitted on behalf of a consortium, all consortium members and affiliations should be listed in the Acknowledgments section. Provide the name and email address of the author to whom correspondence should be sent.

Introduction

The Introduction should put the focus of the manuscript into a broader context. As you compose the Introduction, think of readers who are not experts in this field. This should state the purpose of the investigation and justification for undertaking the research and relationship to other work in the field. An extensive listing or review of the literature should not be used. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The Introduction should conclude with a brief statement of the overall aim of the experiments and a comment about what was achieved.

Material and Methods

Sufficient information should be provided in the text or by referring to papers in generally available journals to permit the work to be repeated.
This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. We encourage authors to submit, as separate files, detailed protocols for newer or less well-established methods. These will be linked to the article and will be fully accessable.

Results

The results should be presented clearly and concisely. Tables and figures should be used only when necessary for effective comprehension of the data. The Results section should provide results of all of the experiments that are required to support the conclusions of the paper. There is no specific word limit for this section, but a description of experiments that are peripheral to the main message of the article and that detract from the focus of the article should not be included. The section may be divided into subsections, each with a concise subheading. Large datasets, including raw data, should be submitted as supplementary files; these are published online linked to the article. The Results section should be written in past tense. In some situations, it may be desirable to combine Results and Discussion into a single section.

Discussion

The purpose of the Discussion is to identify new and relevant results and relate them to existing knowledge. Information given elsewhere in the text, especially in Results, may be cited but all of the results should not be repeated in detail in the Discussion. The Discussion should spell out the major conclusions and interpretations of the work including some explanation of the significance of these conclusions. How do the conclusions affect the existing assumptions and models in the field? How can future research build on these observations? What are the key experiments that must be done? The Discussion should be concise and tightly argued. If warranted, the Results and Discussion may be combined into one section.

Acknowledgments

When appropriate, briefly acknowledge technical assistance, advice and contributions from colleagues. People who contributed to the work, but do not fit the criteria for authors should be listed in the Acknowledgments section, along with their contributions. Donations of animals, cells,

or reagents should also be acknowledged You must also ensure that anyone named in the Acknowledgments agrees to being so named. Financial support for the research and fellowships should be acknowledged in this section (agency and grant number).

Figures

Figures must be submitted in high-resolution version (600 dpi).

Please ensure that the files conform to our Guidelines for Figure Preparation when preparing your figures for production and/or "Image Quality Specifications". The link contains important information about image quality from PubMed Central where the Brazilian Journal of Medical and Biological Research is indexed.

The Brazilian Journal of Medical and Biological Research requires the same quality as PubMed Central. Please follow these instructions when you submit figures to the Brazilian Journal.

Preparing figure files for submission

The *Brazilian Journal of Medical and Biological Research* encourages authors to use figures where this will increase the clarity of an article. The use of color figures in articles is free of charge. The following guidelines must be observed when preparing figures. Failure to do so is likely to delay acceptance and publication of the article.

- Each figure of a manuscript should be submitted as a single file.
- Figures should be numbered in the order they are first mentioned in the text, and uploaded in this order.
- Figure titles and legends should be provided in the main manuscript, not in the graphic file.
- The aim of the figure legend should be to describe the key messages of the figure, but the figure should also be discussed in the text.
- An enlarged version of the figure and its full legend will often be viewed in a separate window online, and it should be possible for a reader to understand the figure without moving back and forth between this window and the relevant parts of the text.
- Each legend should have a concise title of no more than 15 words. The legend itself should be succinct, while still explaining all symbols and abbreviations. Avoid lengthy descriptions of methods. Statistical information should be given as well as the statistical tests used.
- Arrows or letters should be used in the figure and explained in the legend to identify important structures.
- Figures with multiple panels should use capital letters A, B, C, etc. to identify the panels.
- Each figure should be closely cropped to minimize the amount of white space surrounding the illustration. Cropping figures improves accuracy when placing the figure in combination with other elements, when the accepted manuscript is prepared for publication.
- Individual figure files should not exceed 5 MB. If a suitable format is chosen, this file size is adequate for extremely high quality figures.

Please note that it is the responsibility of the author(s) to obtain permission from the copyright holder to reproduce figures (or tables) that have previously been published elsewhere. In order for all figures to be open-access, authors must have permission from the rights holder if they wish to include images that have been published elsewhere in non-open-access journals. Permission should be indicated in the figure legend, and the original source included in the reference list.

Supported file type

The following file format can be accepted: TIFF (suitable for images) with 600 dpi.

Micrographs should be treated like photographs with the following additional guidelines

 Electron micrographs **must contain a magnification bar** with its equivalence in micrometers. This information can be found on all micrographs together with the magnification size.

 Details of any stains used and the method of preparation the sample should be given in the figure legend or in the Material and Methods section.

 Detailed information about the microscope used should be included in the Material and Methods section.

 The type of camera, photographic software and details of any subsequent image manipulation should be included in the Material and Methods section.

Tables

 Tables must be submitted in Word (.doc) or Excel (.xls), not as an image.

 Tables must be numbered consecutively with Arabic numerals in the text.

 Tables must have a concise and descriptive title.
 All explanatory information should be given in a footnote below the table. Footnotes should be used to explain abbreviations and provide statistical information, including statistical tests used.
 All abbreviations must be defined in this footnote, even if they are explained in the text.
 Tables must be understandable without referring to the text.
 Tables occupying more than one printed page should be avoided, if possible.
 Vertical and diagonal lines should not be used in tables; instead, indentation and vertical or horizontal space should be used to group data.
 Tables in Excel must be cell-based; do not use picture elements, text boxes, tabs, or returns in tables.

References

Only published or accepted manuscripts should be included in the reference list. Meeting abstracts, conference talks, or papers that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only. All personal communications should be supported by a letter from the relevant authors. Authors are responsible for the accuracy and completeness of their references and for correct text citation. When possible, references which are easily available in English should be cited.

The BJMBR uses the numbered citation (citation-sequence) method. References are listed and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in parentheses. Multiple citations within a single set of parentheses should be separated by commas without a space (1,5,7). Where there are 3 or more sequential citations, they should be given as a range (4-9).

Because all references will be linked electronically (doi), if possible, to the papers they cite, proper formatting of the references is crucial. For all references, **list the first 6 authors** followed by et al., Title of article, Journal (abbreviation), Year, Volume, Complete Pages, The *Brazilian Journal of Medical and Biological Research* follows the reference format of the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, which can be found on the website of the National Library of Medicine (http://www.nlm.nih.gov/bsd/uniform_requirements.html). Use the Medline journal abbreviations and follow the reference style shown on the Website noted above, with several exceptions. See below for details. If the author uses the program "Reference Manager", copy the file containing the style of the Brazilian Journal of Medical and Biological Research and place it in the folder of "Styles". When submiting the manuscript, send the file produced in Reference Manager (".rmd" and ".rmx") as an attachment.

Please use the following style for the reference list:

Published Papers. First 6 authors followed by et al., Title, Journal (abbreviation in italics), Year, Volume, Complete Pages. Lammers AE, Hislop AA, Flynn Y, Haworth SG. The 6-minute walk test: normal values for children of 4-11 years of age. *Arch Dis Child* 2008; 93: 464-468. Zhang Q, Malik P, Pandey D, Gupta S, Jagnandan D, Belin de CE, et al. Paradoxical activation of endothelial nitric oxide synthase by NADPH oxidase. *Arterioscler Thromb Vasc Biol* 2008; 28: 1627-1633.

Article accepted for publication but not yet published. First 6 authors followed by et al., Title, Journal (abbreviation), Year of expected publication, (in press) at the end of the citation. Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CXC, et al. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. *J Biol Chem* 2005 (in press).

Internet Communication. Ensure that URLs are active and available. Provide DOI, if available. Developmental toxicology. http://www.devtox.org/nomenclature/organ.php. Accessed April 10, 2015.

CAPES Statistics. http://www.capes.gov.br/capes/portal. Accessed March 16, 2006.

CNPq Plataforma Lattes, "Investimentos do CNPq em CT&I". http://fomentonacional.cnpq.br/dmfomento/home/index.jsp. Accessed March 16, 2006.

Audiovisual Material

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