UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA, METABOLISMO E NUTRIÇÃO

DISSERTAÇÃO DE MESTRADO

ASSOCIAÇÃO ENTRE NÍVEIS SÉRICOS DE VITAMINA D, ESTILO DE VIDA E PRESSÃO ARTERIAL EM PACIENTES COM DIABETE MELITO TIPO 2 E HIPERTENSÃO ARTERIAL SISTÊMICA

Juliano Soares Rabello Moreira

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências Médicas: Endocrinologia da Universidade Federal do Rio Grande do Sul (UFRGS) como requisito parcial para obtenção do título de Mestre em Endocrinologia.

Orientadora: Profa. Dra. Luciana Verçoza Viana

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APRESENTAÇÃO

Essa Dissertação de Mestrado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição. Serão apresentados dois capítulos, sendo o primeiro composto por uma introdução sobre tema em questão e o segundo é apresentado como manuscrito com dados originais e descreve o trabalho de pesquisa propriamente dito. A formatação diferenciada dos capítulos é justificada pela escolha de envio para revista científica do segundo texto, sendo elaborado conforme normas desta publicação.

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DEDICATÓRIA

“Suba o primeiro degrau com fé. Não é necessário que você veja toda a escada. Apenas dê o primeiro passo.”

Martin Luther King

À minha esposa, Bárbara, companheira de todas as horas.

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LISTA GERAL DE ABREVIATURAS

Inrodução

DM *Diabete Melito*

HAS *Hipertensão Arterial Sistêmica*

PA *Pressão Arterial*

UI *Unidades Internacionais*

VDR *Vitamin D Receptor*

Artigo Original

ABPM *Ambulatory Blood Pressure Monitoring*

AUC *Area Under the Curve*

ARB *Angiotensin Receptor Blocker*

ACE *Angiotensin-converting Enzyme*

BMI *Body Mass Index*

BP *Blood Pressure*

CAPES *Comissão de Aperfeiçoamento de Pessoal do Nível Superior (Coordination for the Improvement of Higher Education Personnel*)

CI *Confidence Interval*

CNPq *Conselho Nacional de Desenvolvimento Científico e Tecnológico (National Council for Scientific and Technological Development)*

CKD *Chronic Kidney Disease*

DXA *Dual-energy x-Ray Absorptiometry*

FAPEGS *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul*

FIPE *Fundo de Incentivo à Pesquisa e Eventos*

HCPA *Hospital de Clínicas de Porto Alegre*

HDL *High-density Lipoprotein Cholesterol*

LDL *Low-density Lipoprotein Cholesterol*

OR *Odds Ratio*

PNPD *Programa Nacional de Pós-Doutorado*

ROC *Receiver Operating Characteristic Curve*

UAE *Urinary Albumin Excretion*

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SUMÁRIO

RESUMO

Introdução e Objetivos: Valores séricos de vitamina D têm sido inversamente associados com índice de massa corporal (IMC) e pressão arterial (PA) na população em geral. Sendo a obesidade um fator de risco para hipovitaminose D e, considerando o fato de a maior parte dos pacientes com diabete melito (DM) tipo 2 serem obesos e hipertensos, o objetivo deste estudo foi avaliar a deficiência de vitamina D com parâmetros nutricionais, metabólicos e níveis de pressão arterial nessa população.

Métodos: Neste estudo transversal, pacientes ambulatoriais hipertensos com DM tipo 2 realizaram avaliação clínica, nutricional e laboratorial. PA foi avaliada por medidas em consultório (Omron HEM-705CP) e Monitorização Ambulatorial de 24 horas (MAPA; Spacelabs®). Atividade física foi avaliada por contagem diária de passos (Pedômetro-Yamax Digi-Walker®), composição corporal por Densitometria por emissão de raios X de dupla energia (DXA Lunar Prodigy®) e dieta por questionário alimentar de frequência. Resultados: Um total de 116 pacientes com idade média de 65,0±8,9 anos, 43% masculino, com IMC 30,3±4,1kg/m², duração do DM 11,5 (5-19) anos, HbA1c 7,2 (6,5-8,3)%, e PA de consultório 150,7±20,9/83,5±11,0mmHg foram incluídos. A 25(OH)D média foi 21,0 (16,0-26,9)ng/ml e 43% dos pacientes foram considerados deficientes [25(OH)D<20 ng/ml]. Na MAPA, pacientes deficientes mostraram maior PA sistólica em 24h (135,7±10,2 vs. 130,2±13,3mmHg; P=0,016) e em vigilia (138,1±11,3 vs. 132,8±13,4mmHg; P=0,026) comparado aos não deficientes. Menor contagem de passos [4350,0 (2647,8-6598,0) vs. 6390,6 (4706,9-8081,1)passos/dia], menor cálcio urinário em 24 horas [47,0 (32,0-141,2) vs. 89,5 (67,7-152,5)mEq] e maior massa de gordura (30,8±8,2 vs. 27,2±6,6Kg) ocorreram em deficientes quando comparado aos não deficientes. Consumo de leite (35,6 vs. 64,4%; P=0,009) e peixe (31,2 vs. 68,8%; P<0,001) foi menor em pacientes com do que sem deficiência de vitamina D. Em análise multivariada, ajustada para massa de gordura e coleta dos dados no inverno, <5000 passos/dia (OR=3,30; IC95% 1,34-8,12), não consumo de leite e peixe (OR=6,56; IC95% 2,52-17,17) e ambos número <5000 passos/dia e não consumo de leite/peixe (OR=7,24; IC95% 2,19-23,90) permaneceram associados com deficiência de vitamina D.

Conclusão: Deficiência de vitamina D é altamente prevalente em pacientes hipertensos e com DM tipo 2 e foi associada com elevada PA sistólica (MAPA), pouca atividade física e ausência de consumo de peixe e leite na dieta.

CAPÍTULO I - INTRODUÇÃO

Data do início do século passado, fruto dos trabalhos realizados por McCollum *et al.* (1922), a descoberta de uma nova substância com potencial terapêutico para cura do raquitismo, doença altamente prevalente na época.[1] Esse novo fator foi denominado vitamina D. Atualmente, a vitamina D é considerada um pró-hormônio esteroide, estando presente em alimentos e em suplementos vitamínicos nas suas duas formas moleculares: a vitamina D2 (ergocalciferol), de origem vegetal, e vitamina D3 (colecalciferol), de origem animal.[2-4]

A recomendação diária de ingestão postulada para manutenção da saúde óssea é de 400 unidades internacionais (UI) para crianças até 1 ano de idade, 600 UI para pessoas entre 1 e 70 anos (incluindo gestantes e lactantes) e 800 UI para idosos acima de 71 anos.[5] Após exposição solar, a síntese cutânea representa 80% da fonte desse micronutriente, sendo o restante obtido através de alimentos ricos em vitamina D, como os peixes, os ovos e os produtos lácteos. A descrição da rota de síntese, desde sua forma inativa até a ativa final, é demonstrada na Figura 1.[3]

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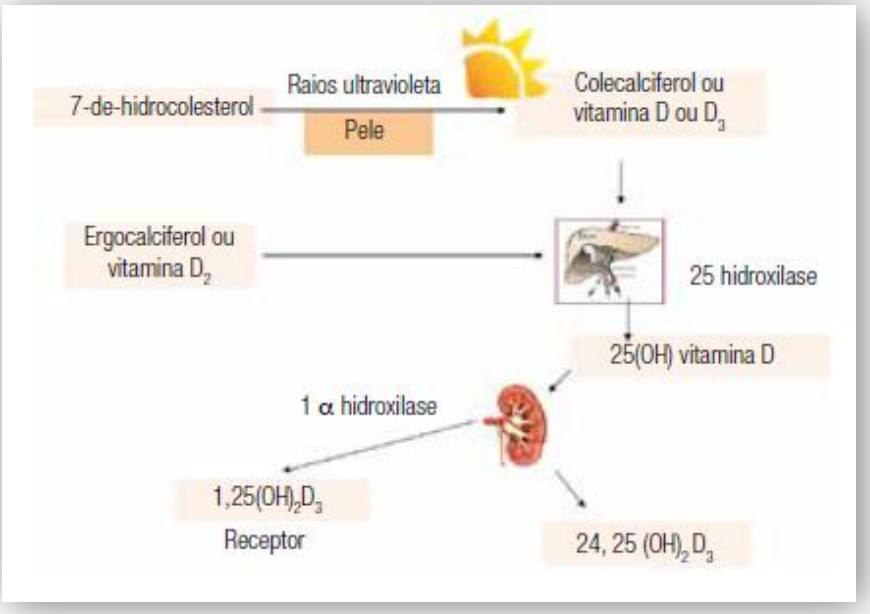
Figura 1 – Rota de síntese da vitamina D



Fonte: Recomendações da Sociedade Brasileira de Endocrinologia e Metabologia para o diagnóstico e tratamento da hipovitaminose D.[3]

O calcitriol [1-25 (OH) vitamina D] é a forma ativa da vitamina D e é resultante da 1-alfa hidroxilação que ocorre no rim a partir da 25(OH)D, que por sua vez resultou da 25-hidroxilação da vitamina D2 e D3 no fígado. Fisiologicamente, os efeitos da forma ativa da vitamina D ocorrem nas células-alvo nucleadas que apresentam seu receptor, denominado VDR (Vitamin D Receptor). Considerados receptores esteróides ligados ao fator de transcrição celular, os VDRs, uma vez ligados ao calcitriol, regulam a expressão genética celular, estimando-se que cerca de 3% do genoma humano esteja sob influência desse mecanismo.[6-8] São identificados VDRs em diversos locais, como: tireóide, paratireóide, cérebro, pâncreas, coração, vasos sanguíneos, intestino, adrenal, células alveolares, miócitos, placenta, próstata, hepatócitos, tecido mamário, macrófagos e as células dendríticas.[8-12] Associações entre hipovitaminose D e aumento de risco cardiometabólico, infecções, neoplasias, doenças autoimunes, câncer, fraturas e depressão são exemplos dos desfechos em investigação.[13]

A dosagem dos níveis séricos de vitamina D é realizada através da determinação laboratorial da 25(OH)D, o calcidiol, forma inativa, porém mais estável, com meia-vida de 3 a 4 semanas. Valores acima de 30ng/ml (75nmol/L) são estabelecidos para suficiência, níveis entre 20-29ng/ml (50-75nmol/L) para insuficiência e inferiores à 20ng/ml (50nmol/L) para deficiência.[3] Ressalta-se que os valores recomendados basearam-se em estudos histopatológicos de saúde óssea (quantificação de osteóide).[14] Níveis ideais de 25(OH)D para potenciais desfechos não ósseos ainda estão em discussão na literatura.

Os fatores de risco para hipovitaminose D incluem pouca exposição solar (elevadas latitudes, estações frias do ano e uso de protetores solares), pele negra, obesidade, tabagismo, síndromes de má absorção, hepatopatias ou nefropatias, idade avançada e institucionalização.[3,15] Dados norte-americanos apontam para prevalência de hipovitaminose D de 41,6% em adultos maiores de 20 anos.[16] No Brasil, os dados são escassos; entretanto, em estudo envolvendo mulheres pós-menopáusicas, a prevalência de deficiência de vitamina D foi de 17%, sendo de 24% a prevalência estimada em Porto Alegre, cidade localizada na latitude $30^{0}01'59''S$.[17] Outro estudo também realizado em Porto Alegre mostrou uma prevalência de hipovitaminose D de 57,4% entre médicos residentes.[18]

Como a obesidade é fator de risco conhecido para hipovitaminose D e, considerando que obesidade e diabetes melito (DM) tipo 2 coexistem em 90% dos pacientes, estima-se que prevalência de hipovitaminose D neste grupo possa ser mais expressiva do que na população em geral.[19] Pesquisa realizada na cidade de Nova Iorque em pacientes com DM tipo 1 e tipo 2 demonstrou uma taxa de hipovitaminose D de 31% e 63,5%, respectivamente. Neste trabalho, os autores também encontraram uma associação inversa entre índice de massa corporal e valores de vitamina D.[20]

No DM, a vitamina D pode atuar de forma direta ou indireta na resposta insulínica à glicose na célula β pancreática.[13,21] Em relação ao controle glicêmico, estudo observacional realizado na Grécia demonstrou uma relação inversa entre HbA1c e níveis de 25(OH)D em pacientes com DM tipo 2. Além disso, nesse mesmo estudo, pacientes com DM tipo 2 apresentavam níveis mais baixos deste hormônio quando comparados a indivíduos saudáveis.[22] A hipovitaminose D também foi associada a complicações microvasculares do DM, como retinopatia e nefropatias diabéticas.[23]

Setenta e três por cento dos pacientes com DM tipo 2 atendidos em ambulatórios de hospitais gerais no Rio Grande do Sul têm hipertensão arterial sistêmica (HAS).[24] A HAS é um importante fator de risco para o desenvolvimento de complicações crônicas micro e macrovasculares em pacientes com DM, e o tratamento da HAS nesses pacientes está associado à redução de desfechos clínicos negativos.[25,26] No entanto, apesar das diversas drogas disponíveis, muitos pacientes não atingem os alvos pressóricos preconizados.[27]

A deficiência da vitamina D também tem sido associada a maiores valores de pressão arterial (PA).[28,29] O mecanismo proposto para associação entre esse micronutriente e homeostase pressórica estaria ligado à inibição do sistema renina-angiotensina-aldosterona, além de ações no endotélio, no músculo liso vascular e nas células musculares cardíacas.[30-32] Como mecanismo alternativo, estaria envolvido o hiperparatireoidismo secundário, com seus conhecidos efeitos de aumento de rigidez vascular e aumento de massa ventricular.[31]

Estudo prévio demonstrou o potencial efeito determinante da irradiação ultravioleta em relação aos níveis de pressão sistolico e diastólico. Nesta

investigação, correlação positiva foi demonstrada com relação à latitude ao norte e sul do equador e valores tensionais sistólicos (R = 0,34; P < 0,0001) e diastólicos (R = 0,33; P < 0,0001). Os autores propuseram como mecanismo a seguinte cascata: uma reduzida irradiação ultravioleta desencadearia menor produção de vitamina D, com consequente aumento nos níveis de paratormônio, o qual poderia estimular crescimento da parede vascular e maior rigidez arterial.[33]

Investigação em modelo animal também demonstrou potencial efeito modulador da vitamina D no sistema renina-angiotensina-aldosterona. Ratos selvagens apresentam essa cascata de produção hormonal de forma fisiológica. Já animais desprovidos dos VDRs demonstram um consequente aumento na produção dessas substâncias. Por outro lado, a administração de vitamina D ativa na forma de calcitriol provocou uma diminuição na síntese desses hormônios. Esses achados apontam para um potencial efeito hipotensor desse nutriente. [34]

No entanto, estudos com suplementação de vitamina D para potencial controle pressórico têm mostrado resultados conflitantes. Meta-análise de ensaios clínicos envolvendo a suplementação de vitamina D em pacientes hipertensos e não hipertensos não demonstrou efeito significativo da suplementação na redução da PA.[35] Deve-se ressaltar que os pacientes avaliados nestes estudos possuíam tanto pressão normal quanto elevada, eram de etnias diversas, e as doses e apresentações de vitamina D eram diferentes. Especificamente em pacientes com DM tipo 2, meta-análise recentemente publicada pelo nosso grupo demonstrou que a suplementação oral de vitamina D em doses variadas resultou em uma redução de 4,56 mmHg na PA sistólica, e 2,44 mmHg na PA diastólica.[35]

De fato, a real prevalência de hipovitaminose D em pacientes com HAS e DM tipo 2 não está bem estabelecida. Além disso, permanece em aberto a questão desse nutriente ser apenas um marcador de estilo de vida ou um real desfecho a ser corrigido. Pacientes com DM são conhecidamente mais sedentários e existe elevada associação de obesidade e DM, ambos determinantes que poderiam contribuir para menores valores séricos de vitamina D.[36] Nesse contexto, estudos com enfoque nos fatores associados à deficiência de vitamina D, qualidade alimentar e tempo de exposição solar ainda permanecem escassos na literatura.

Políticas públicas voltadas para o cuidado de pacientes com doenças crônicas vêm sendo uma constante nas últimas décadas. O custo social e econômico do DM tipo 2 e da HAS são altos em todo mundo. De fato, estatísticas internacionais apontam para o DM como a sexta condição de saúde causadora de incapacidade em 2015, e dados do Ministério da Saúde do Brasil indicam um gasto superior a um bilhão de reais em medicamentos anti-hipertensivos no ano de 2014.[37,38] Estratégias nutricionais e de baixo custo são cada vez mais bem-vindas no sentido de reduzir a morbidade relacionada a essas patologias.

Neste contexto, o tema da hipovitaminose D e possíveis desfechos clínicos associados encontram grande relevância clinica. A identificação de fatores de risco específicos em pacientes com DM tipo 2 e HAS, bem como a potencial associação entre níveis pressóricos e 25(OH)D podem levar ao desenvolvimento de táticas de controle pressórico e redução de complicações do DM no futuro.

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CAPÍTULO II – ARTIGO ORIGINAL

ASSOCIATION BETWEEN PLASMA VITAMIN D STATUS WITH LIFESTYLE PATTERNS AND BLOOD PRESSURE IN PATIENTS WITH TYPE 2 DIABETES AND HYPERTENSION

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2.1 ABSTRACT

Background and Aims: Plasma vitamin D values have been inversely correlated with body mass index (BMI) and blood pressure (BP) in general population. Considering that most patients with type 2 diabetes are obese and hypertensive, the aim of the present study was to evaluate vitamin D deficiency with nutritional and metabolic parameters, and blood pressure levels in these population.

Methods and Results: In this cross-sectional study, outpatients with type 2 diabetes and hypertension underwent clinical, nutritional, and laboratory evaluation. BP was assessed by office measurements (Omron HEM-705CP) and 24h ambulatory BP monitoring (ABPM; Spacelabs®). Physical activity was evaluated by daily steps count (pedometer-Yamax Digi-Walker®), body composition by Dual-energy X-ray Absorptiometry (DXA Lunar Prodigy®), and diet by food frequency questionnaire. A total of 116 patients aged 65.0±8.9 years, 43% males, with BMI 30.3±4.1kg/m², diabetes duration 11.5 (5-19) years, HbA1c 7.2 (6.5-8.3)%, and office BP of 150.7±20.9/83.5±11.0mmHg were included. 25(OH)D was 21.0 (16.0-26.9)ng/ml and 43% of patients were vitamin D deficient [25(OH)D<20 ng/ml]. On ABPM, deficient patients had higher systolic BP at 24h (135.7±10.2 vs. 130.2±13.3mmHg; P=0.016) and daytime (138.1±11.3 vs. 132.8±13.4mmHg; P=0.026) than nondeficient. Lower steps count [4350.0(2647.8-6598.0) vs. 6390.6(4706.9-8081.1)steps/day], lower 24h urinary calcium [47.0(32.0-141.2) vs. 89.5(67.7-152.5)mEq], and higher fat mass (30.8±8.2 vs. 27.2±6.6Kg) occurred in deficient as compared to nondeficient patients. Consumption of milk (35.6 vs. 64.4%; P=0.009) and fish (31.2 vs. 68.8%; P<0.001) was lower in patients with than without vitamin D deficiency. In multivariate analysis, adjusted for fat mass and data collection in colder seasons, <5000 steps/day (OR=3.30; CI95% 1.34-8.12), no consumption of milk and fish (OR=6.56; CI95% 2.52-17.17), and both step counts <5000 steps/day and no milk/fish consumption (OR=7.24; IC95% 2.19-23.90) remained associated with vitamin D deficiency.

Conclusion: Vitamin D deficiency D is highly prevalent in hypertensive and type 2 diabetic patients, and was associated with higher systolic ABPM, little physical activity, and no consumption of milk and fish.

2.2 INTRODUCTION

Vitamin D deficiency, defined as serum 25-hydroxyvitamin D [25(OH)D] lower than 20ng/ml, is a growing worldwide health condition.[1] In specific clinical settings, up to 90% of subjects may have vitamin D below the optimal levels.[2] Sources of vitamin D in humans are diet and vitamin supplements, but sunlight exposure to ultraviolet B radiation is the main plasma vitamin D determinant.[3-6] The most relevant risk factors for vitamin D deficiency include reduced skin synthesis, decreased vitamin D bioavailability, malabsorption syndromes, obesity, liver and kidney failure, and specific medications.[7,8]

Originally, vitamin D deficiency studies pertained exclusively to bone metabolism effects (e.g. rickets, osteomalacia, and osteoporosis). However, evidence of extraskeletal ubiquitously expression of vitamin D receptors and its activation enzyme (1-α-hydroxylase) have prompted investigations in alternative metabolic routes and outcomes.[9-11]

Data from observational and ecological studies demonstrated an inverse association between circulating vitamin D levels and conditions like obesity, hypertension, and type 2 diabetes.[12] Moreover, the association of 25(OH)D plasma concentration with all-cause and cause-specific mortality was already demonstrated.[13] Potential proposed mechanisms involved in these associations comprise fat tissue vitamin D sequestration, endothelial dysfunction, inflammation, parathyroid hormone vascular effects, renin-angiotensin system regulation, cardiac and vascular muscle cells proliferation, insulin resistance, and control of insulin synthesis by the pancreas.[14-17]

Up to 80% of patients with type 2 diabetes are obese and have hypertension. Since high wieght is recognized as risk factor for low vitamin D levels and poor blood pressure (BP) control is associated with this nutritional condition, a higher prevalence of vitamin D deficiency in type 2 diabetic patients could at least partially be explained by these observations. [18;19]

The aim of the present study was to identify possible associated factors with vitamin D deficiency in patients with type 2 diabetes and hypertension, including

metabolic, body composition and lifestyle characteristics, such as dietary habits and physical activity.

2.3 METHODS

This cross-sectional study was performed between March 2015 and October 2016. Outpatients with type 2 diabetes and hypertension consecutively attending Primary Care and Endocrine Divisions of the Hospital de Clínicas de Porto Alegre, Brazil ($30^0$0'S latitude) were recruited. Type 2 diabetes was defined as a history of diabetes starting after the age of 30 and no insulin use before 5 years of the diagnosed disease.[20] Hypertension was defined as blood pressure (BP) ≥140/90 mmHg in office setting and/or current use of anti-hypertensive drugs.[21] Exclusion criteria were: vitamin supplementation, pregnancy, breastfeeding, night shift work, current use of glucocorticoids or anticonvulsant therapy, illicit drug abuse, serum creatinine ≥2.0 mg/dl, liver failure, dementia, current malignancy, gastroparesis or enteropathy, body mass index ≥ 40Kg/m^2 or weight ≥100 Kg, physical disability that prevents patients from walking, and presence of secondary causes of hypertension or uncontrolled BP (>180/120mmHg).

Clinical, demographic, and lifestyle characteristics were collected based on standard protocols. Records included: known duration of type 2 diabetes and hypertension, smoking habits, alcohol intake, ethnic self-classification, current medications, and usual sunlight exposure in routine days and calculated through sun exposure questionnaire.[22] Cardiovascular outcome were diagnosed in the presence of previous cardiac event [ischemic heart disease, positive cardiovascular event history, intermittent claudication, stroke confirmed by imaging tests or disability, positive current screening symptoms of intermittent claudication (Edinburgh Claudication and Self-administration Questionnaire on Chest Pain and Intermittent Claudication) or myocardial ischemia (Minnesota codes 1-1 to 1-3 or 7-1 on a resting ECG)].[23-26]

BP was measured twice at first visit, with at least 2-minute interval, in the sitting position after 10 minutes of resting and mean value was used for analysis (Automatic BP Monitor HEM-720, Omron Corporation, Kyoto, Japan). Ambulatory

Blood Pressure Monitoring (ABPM) was evaluated by oscillometry using a Spacelabs device (90 207; Spacelabs Healthcare, Snoqualmie, WA) with a validated certificate at 15-minute intervals during the day and 20-minute intervals overnight, on an ordinary working day.[27] Sleep time was recorded as the period between the time when the patient went to bed and the time when the patient woke up in the following morning.

Physical activity was assessed using of a pedometer device (Digi-Walker CW200, Yamax, Tokyo, Japan). Participants used the dispositive for seven days attached to the waistband of their clothing during waking hours, except when bathing or swimming. While using the pedometer, patients were also instructed to maintain their usual physical activity and record data in a diary at least three days during a week period.

Body weight and height were obtained using an anthropometric scale with measurements recorded to the nearest 100 g for weight and to the nearest 0.1 cm for height. Waist circumference was measured midway between the lowest rib margin and the iliac crest, near the umbilicus, using flexible nonstretch fiberglass tape. Body composition (total skeletal muscle and fat mass, trunk fat, percent overall body fat, and bone mineral density) were evaluated by Dual-energy x-Ray Absorptiometry (DXA – Lunar Prodigy® - GE Healthcare Madison, WI, United States of America). Due to technical limitations, only patients lower than 100Kg weigh underwent DXA evaluation.

The patient's usual diet was assessed through a food frequency questionnaire applied by a nutritionist and validated in patients with diabetes. An adapted version of this questionnaire focused on specific foodstuff naturally containing vitamin D was used.[28,29] Food frequency intake were described as the number of times a specific aliment was consumed: daily, weekly, monthly, or yearly.

Blood samples were collected after a 12-hour fast and the respective season of the year was then registered. Plasma glucose was determined by a glucose oxidase method, parathyroid hormone by electrochemiluminescence, serum albumin by bromocresol green**,** blood and 24-hour urinary creatinine by Jaffe's reaction, and glycated hemoglobin by ion-exchange high-performance liquid chromatography (reference range, 4.0-6.0%). Plasma calcium, total cholesterol, high-density

lipoprotein (HDL-cholesterol), triglycerides, and urinary 24-h calcium were determinate by enzymatic colorimetric methods. Low-density lipoprotein cholesterol (LDL - cholesterol) was calculated using Friedewald's formula [LDL-cholesterol = total Cholesterol – HDL-cholesterol – (triglyceride/5)] only for patients with triglycerides values <400 mg/dl.[30] Blood and 24-h urine potassium and sodium were analyzed by selective ion electrode. Urinary albumin was measured by immunoturbidimetry in a random spot urine sample and classified as normal (<14mg/l) or increased (≥14mg/l).[31] 25(OH)D vitamin was determined by chemiluminescence technique (Abbott®) and patients were classified into two groups according to its levels: vitamin D deficient [25(OH)D <20ng/ml] and vitamin D nondeficient [25(OH)D ≥20ng/ml].[1]

The present study was conducted according to the Declaration of Helsinki and it was approved by the research ethics committee of the Hospital de Clínicas de Porto Alegre. Written informed consent was obtained from all patients.[32]

2.3.1 Statistical Analysis

Results were expressed according Gaussian distribution as mean (± standard deviation), and median (P25–P75). Categorial variables were expressed as number of patients with the characteristic and percentage. Student t test and Mann–Whitney U test were used to compare ordinal variables, and the Pearson χ 2 test to compare categorical variables.

Univariate and multivariate logistic regression analysis were performed with vitamin D deficiency as the dependent variable. Covariates were selected based on univariate analysis results and biological plausibility. Based on dietary and physical activity (independent variables) three lifestyle patterns were constructed: Model 1 – only inclusion of walking >5000 or <5000 steps/day; Model 2 - only inclusion of consumption (yes/no) of both fish and milk and Model 3 - walking >5000 or <5000 steps/day and consumption of both fish (yes/no) and milk (yes/no). Model 4 describes specific food consuption in diet, while model 5 included all variables in the

same model. Data analysis was performed using the statistical software IBM SPSS Version 20.0. The type I error rate was fixed at $P<0.05$ (two-tailed).

The Receiver Operating Characteristic (ROC) curve approach, generating values of area under the curve (AUC) with 95% CI, was used to analyze the performance of the dietary intake of specific foods and physical activity (as continuous variables) to identify vitamin D deficiency in type 2 diabetic patients. The presence or absence of vitamin D deficiency was considered as the reference standard. The optimal cutoffs for the evaluated variables to predict vitamin D deficiency were established by the Youden Index (Sensitivity + Specificity – 1).[33] Using these calculated values we could establish the cutoff point for fish and milk daily intake (whole and low fat milk), and the number of steps to identify vitamin D deficiency. For ROC curve generation and evaluation of the differences between AUCs, analysis and calculations were done using R version 2.15.3 via the Rstudio platform, version 0.97.320 and the R package "OptimalCutpoints".[34,35]

2.4 RESULTS

From a total of 275 screened patients, 148 were excluded due to: refused to participate (n=19), clinical conditions (n=83) such as severe obesity, walking disability, chronic kidney disease, and use of medications (n=36). After inclusion in the study, 11 patients did not complete the protocol due to lack of laboratory evaluation (n=4), missing protocol's appointment (n=4), and inconclusive ABPM (n=3) Final analyses was performed per protocol and included 116 patients (**Figure 1**).

2.4.1 General Characteristics of Patients

The mean 25(OH)D of 116 studied patients was 21.0 (16.0-26.9) ng/ml and 50 patients (43%) were classified as vitamin D deficients. Most patients were white, 43% were males, 6% were current smokers. Their age was 65.0 ± 8.9 years, their known diabetes duration was 11.5 (5-19) years, and they had 15 (10-25) years of

hypertension duration. The majority of patients were obese (BMI 30.3 ± 4.1 kg/m², waist circumference 103.3 ± 9.7 cm for females and 106.7 ± 12.3 cm for males). All patients included in study were currently users of anti-hypertensive medications. Office systolic BP was 150.7 ± 20.9 and office diastolic BP was 83.5 ± 11.0 mmHg. Systolic 24h BP was 132.6 ± 12.3 mmHg and diastolic 75.6 ± 8.7 mmHg. Pedometer analysis revealed median 5595 (3207-7663) steps/day. Most laboratory parameters were in accordance to current guidelines for diabetes control: fasting plasma glucose 137.0 (114-178) mg/dl, HbA1c 7.2 (6.5-8.3) %, and LDL-C 93.1 (71-122) mg/dl.[36]

2.4.2 Characteristics of Patients According to Vitamin D Status

Demographic, clinical, anthropometric and laboratory characteristics of the patients according to 25(OH)D status are shown in **Table 1.** Deficient patients had their data collected mostly in cold seasons (autumn and winter) as compared to vitamin D nondeficient patients. Also, they were less physically active than nondeficient patients. Regarding BP homeostasis, systolic ABPM at 24-h, as well as in daytime, were higher in deficient than in nondeficient patients Corretation index between 25(OH)D and 24-h Systolic BP was R = -0.26 (P=0.004) and daytime BP was R = -0.273 (P=0.003). Neverthless, in ajusted models, BP revealed no association with vitamin D deficiency. There were no differences in patients with and without vitamin D deficiency regarding medications in use. On laboratory evaluation, the daily urinary calcium excretion was lower in the presence of vitamin D deficiency as compared to nondeficiency state. Body composition evaluation revealed that vitamin D deficient patients had a greater amount of total body fat mass and a higher percentage of trunk fat than patients without vitamin D deficiency. There was no difference between the two groups in bone mineral density.

2.4.3 Dietary Assessment According to Vitamin D Status

Dietary intake of specific foodstuffs based on vitamin D status are shown in **Table 2**. In general, fish consumption was higher in vitamin D nondeficiency patients

than in deficients (68.8% vs. 31.2; P<0.001). Consumption of milk was more frequent in non-deficient patients (64.4% vs. 35.6; P=0.009), particularly for whole and low-fat milk, in comparison to fat-free ones. Milk-based products with elevated fat proportion were less frequently consumed by vitamin D deficient patients.

2.4.4 Roc Curves Analysis

ROC curves were constructed using the presence of vitamin D deficiency as the reference criteria. The continuous predictor variables were whole milk (ml/day), fat-free milk (ml/day), fish (g/month), and daily steps count **(Supplemental Figure 1)**. **Table 3** shows the AUC values for steps count and fish, whole milk, and fat-free milk consumption. The respective cutoff values for identify vitamin D deficiency with the correspondent sensibility and specificity are also described.

2.4.5 Logistic Regression Analysis

In univariate analysis the chance of having vitamin D deficiency in cold seasons (winter/fall) was much higher (OR = 3.5; IC 95% 1.3-9.0) as compared to spring or summer seasons altogether.

In multivariate logistic regression models **(Table 4)**, all evaluated lifestyle patterns were associated with vitamin D deficiency: <5000 steps/day (OR = 3.3 CI; 95% 1.34–8.12); no consumption of milk and fish (OR = 6.56; IC95% 2.52-17.17); and both, <5000 steps/day and no milk and fish consumption (OR=7.24; IC95% 2.19–23.90). All regression models were adjusted for fat mass (kg) and data collection in colder seasons. The lifestyle pattern with highest OR for vitamin D deficiency was model 3, in which the lifestyle variable represents no consumption of milk and fish plus less physical activity. After BP inclusion as covartiate in regression models, the results remained unaffected (data not shown).

2.5 DISCUSSION

In the present study we demonstrated that in a sample of hypertensive patients with type 2 diabetes, deficiency of vitamin D was associated with systolic BP and with non-healthy lifestyle behaviors, namely low physical activity and no consumption of milk and fish. Vitamin D deficient patients presented higher levels of systolic BP in ABPM at 24h (5.5 mmHg) and daytime (5.3 mmHg) than nondeficients. Based on step count parameters, the vitamin D deficient patients walked approximately 2040 steps/day less as compared to nondeficient patients. Additionally, body composition analysis indicated an increased body fat mass (about 3.6 kg higher) with central adiposity pattern in patients with vitamin D deficiency. To our knowledge, this is the first study that concurrently evaluated vitamin D decifiency with BP homeostasis, physical activity, and dietary intake in patients with type 2 diabetes and hypertension.

In clinical settings, poor BP control is a major risk factor for microvascular and macrovascular chronic diabetic complications in patients with type 2 diabetes.[37,38] Current recommendations postulate target systolic BP of 140 mmHg.[39-41] Previous cross-sectional investigations in general population demonstrated an inverse association between vitamin D deficiency and office BP.[42,43] Similarly, this type of association was previously described in a sample of patients with resistant hypertension but not in our study (data not shown).[12] In the present study, both 24-h and daytime systolic BP were inversely correlated with vitamin D plasma values, but we could not demonstrate an association between office BP and vitamin D status. Actually, ABPM seems to have the best accuracy for BP evaluation in patients with diabetes.[44] Physiologic 24h BP changing may be altered in up to 30% of patients with type 2 diabetes.[45] Furthermore, evidences attribute a more consistent predictive value for cardiovascular outcomes and target organ damage to ABPM than to office BP.[46,47]

In the current study we evaluated physical activity using a pedometer, an stablished device to estimate the patient's routine activity.[48] It was previously demonstrated that patients with type 2 diabetes used to take less steps per day than nondiabetic subjects.[49] Physical activity seems to be a relevant determinant of

vitamin D status. Indeed, we demonstrated an association of steps count with vitamin D deficiency even considering that our patients walked less than the recommended goal of 10 000 steps per day.[50] In fact, previous data from cross-sectional study revealed that 25(OH)D was 1.54ng/ml (95% CI 1.09-1.98) higher per hour increase in moderate-vigorous activity (p=0.001).[51]

In our sample, although the intake of dairy products did not differed between patients with and without vitamin D deficiency, deficient patients consumed less milk, as suggested by a lower daily urinary calcium excretion in this group. A hypothetical influence of salt intake masking results of calcium excretion did not occur, since 24h urinary sodium was not different between patients with and without vitamin D deficiency. Furthermore, deficient patients consumed more free-fat milk instead of whole or low-fat milk. Considering that vitamin D is fat-soluble and that the milk consumed by patients did not contain vitamin D supplements, this aspect (less fat in milk) could also had influence in our results. Actually, the fat content in dairy products are important to maintain 25(OH)D concentration in blood.[29] It is well known that milk and milk products are important contributors to micronutrients status, representing 65% of vitamin D dietary intake in the USA and 34 to 42% in Brazil.[52,53] Regarding fish consumption, the duration and type of fish influence directly the 25(OH)D levels as demonstrated by a meta-analysis of randomized clinical trials.[54] The general recommendation of eating fish at least two times a week was not followed by South Brazilian type 2 diabetics.[55,56] The current study confirmed this observation and, although 66% of our patients referred consuming some fish, the monthly consumption is not relevant. Then, we included fish intake together with milk consumption in regression models.

A higher fat mass associated with low circulating vitamin D levels as demonstrated in our study was already described.[16] Hypothetically, the deposition of this nutrient in adipose tissue could result in a worse vitamin D status.[57] In a bi-directional Mendelian Randomization research analyzing over 42.000 patients a one-directional causal relationship was demonstrated, indicating that obesity leads to low vitamin D levels and constitutes a risk factor in approximately one third of deficient patients.[58] Wintertime was also associated with lower levels of vitamin D in Canadian healtly women. In fact, the prevalence of vitamin D insufficiency was less than 2.5% in August (summer) and almost 30% in February (winter).[59] Taking into account

these two possible confounding factors, we have adjusted all constructed multivariated regression models by fat mass and by the seasons of the year in which patients data were collected.

Comparison of regression models constructed to evaluate which lifestyle pattern conferred more predisposition for the presence of vitamin D showed that both diet (no consumption of milk and fish) and less physical activity can increase the chance of a patient being vitamin D deficient more than seven times. This result demonstrated an additive effect of diet and physical activity on vitamin D levels.

Using a frequency questionnaire to evaluate dietary intake instead of weighed diet records could be a limitation.[60] However, that questionnaire was validated in patients with diabetes.[28] One expected limitation is the cross-sectional design of our study that precluded any inference about causes and effects. Whether vitamin D is a risk factor for high blood levels or a simple health status marker remains to be established.

In conclusion, the present study demonstrated that vitamin D deficiency is highly prevalent in hypertensive patients with type 2 diabetes and that it was associated with high systolic ABPM, little physical activity, and no consumption of milk and fish. However, these results must be confirmed in cohort studies and randomized clinical trials conducted in type 2 diabetic patients.

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2.7 CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1 - Characteristics of patients with type 2 diabetes according to the presence of vitamin D deficiency

	Nondeficient	Deficient	*P Value*
N	66	50	-
25 (OH) Vitamin D (ng/ml)	25.4 (22.8 - 29.8)	14.3 (9.9 -18.0)	-
Data collected in cold season – n (%)	42 (63.6)	43 (86.0)	0.013[a]
Sex (Male) –n (%)	31 (46.9)	19 (38.0)	0.437[a]
Age (y)	65.0 ± 8.2	64.9 ± 9.7	0.926[b]
Sunscreen use - n (%)	14 (21.2)	14 (28.0)	0.531[a]
White ethnicity - n (%)	56 (84.8)	41 (82.0)	0.875[a]
Current smoking - n (%)	2 (3.0)	5 (10.0)	0.236[a]
Diabetes duration (y)	12.0 (5.0 - 19.2)	11.0 (5.0 - 18.2)	0.692[c]
Hypertension duration (y)	15.0 (10.0 - 21.2)	14.0 (9.5 - 25.2)	0.651[c]
Years of study	10.0 (5.0 - 11.0)	10.0 (8.0 - 11.0)	0.527[c]
Body mass index (kg/m^2)	30.0 ± 3.8	30.6 ± 4.2	0.421[b]
Waist circumference (cm)	103.0 (96.5 - 110.5)	104.9 (99.5 - 108.5)	0.580[c]
Mean of 3 days daily steps	6390.6 (4706.9 - 8081.1)	4350.0 (2647.8 - 6598.0)	0.004[c]
Cardiovascular outcomes - n (%)[1]	19 (28.8)	9 (18.0)	0.260[a]
Office Blood Pressure (mm Hg)			
Systolic BP	151.0 ± 21.9	150.0 ± 19.5	0.797[b]
Diastolic BP	81.7 (75.0 - 89.2)	83.5 (78.7 - 90.0)	0.307[c]
ABPM (mm Hg)			
24-h Mean BP	94.5 ± 9.5	96.0 ± 8.4	0.376[b]
24-h Pulse BP	55.9 ± 10.3	59.4 ± 8.6	0.057[b]
24-h systolic	130.2 ± 13.3	135.7 ± 10.2	0.016[b]
24-h diastolic	75.0 ± 9.2	76.2 ± 8.0	0.465[b]
Daytime systolic	132.8 ± 13.4	138.1 ± 11.3	0.026[b]
Daytime diastolic	77.7 ± 9.6	79.2 ± 8.8	0.373[b]
Nighttime systolic	123.7 ± 13.9	128.2 ± 11.2	0.064[b]
Nighttime diastolic	68.0 (63.0 - 73.0)	70.5 (64.5 - 76.0)	0.172[c]
Use of aspirin - n (%)	34 (51.5)	34 (68.0)	0.111[a]
Diabetes medication - n (%)			
Oral antidiabetics	65 (98.5)	49 (98.0)	>0.99[a]
Insulin	21 (31.8)	14 (28.0)	0.811[a]
Other	7 (10.6)	1 (2.0)	0.135[a]
Hypertension medication			
B-blockers - n (%)	24 (36.4)	17 (34.0)	0.946[a]
ARB - n (%)	21 (31.8)	18 (36.0)	0.784[a]
Diuretics - n (%)	42 (63.6)	37 (74.0)	0.325[a]
ACE inhibitors - n (%)	35 (53.0)	27 (54.0)	>0.99[a]
Calcium channel blockers - n (%)	20 (30.3)	15 (30.0)	>0.99[a]
Others - n (%)	3 (4.5)	3 (6.0)	>0.99[a]
Laboratorial parameters			
UAE ≥14 mg/L	8.2 (3.0 - 22.9)	14.4 (3.8 - 27.3)	0.219[c]
Urinary calcium (mEq/24h)	89.5 (67.7 - 152.5)	47.0 (32.0 - 141.2)	0.037[c]
Urinary sodium (mEq/24h)	179.9 ± 83.2	195.1 ± 69.2	0.311[b]
Glycated hemoglobin (%)	7.1 (6.4 - 7.9)	7.5 (6.6 - 8.8)	0.075[c]
Fasting glucose (mg/dl)	137.0 (111.5 - 170.5)	132.5 (115.0 - 190.5)	0.733[c]
Total cholesterol (mg/dl)	167.5 (145.7 - 197.0)	175.0 (154.5 - 210.5)	0.420[c]
HDL cholesterol (mg/dl)	45.0 (36.7 - 52.5)	48.0 (41.0 - 57.5)	0.064[c]
Triglycerides (mg/dl)	139.0 (102.0 - 210.0)	134.5 (103.0 - 203.7)	0.830[c]
LDL cholesterol (mg/dl)	90.6 (73.0 - 117.2)	97.0 (68.6 - 128.2)	0.683[c]
Body composition by DXA[2]			
Fat mass (%)	37.2 ± 6.8	40.2 ± 9.1	0.069[b]
Fat Mass (Kg)	27.2 ± 6.6	30.8 ± 8.2	0.017[b]
Muscle Mass (Kg)	45.6 ± 9.3	46.5 ± 7.7	0.649[b]
Trunk Fat (%)	42.5 (36.1 - 45.1)	45.6 (39.5 - 48.8)	0.019[c]
Bone Mineral Density (g/cm^2)	1.1 ± 0.1	1.2 ± 0.1	0.144[b]

Data expressed as mean ± standard deviation), median (P25-P75), or number of patients with the analyzed characteristic (%)
Abbreviations: y, years; ABPM, ambulatory blood pressure monitoring; ACE, angiotensin-converting enzyme; ARB angiotensin receptor blocker; BP, blood pressure; UAE, urinary albumin excretion; DXA, Dual-energy X-ray absorptiometry
a - Pearson's χ 2; b - Student t test; c - Mann-Whitney U test
1 - Cardiovascular outcomes: ischemic heart disease, positive cardiovascular event history, intermittent claudication, strok positive current screening symptoms questionnarie.; 2 - 97 patients evaluated: 41 deficients, 56 nondeficients

Table 2 - Dietary Intake of specific foodstuffs according to the presence of vitamin D deficiency

	Nondeficient n = 66	**Deficient n = 50**	***P Value***
Leguminous (yes): n (%)	63 (95.5)	46 (92.0)	0.462[a]
Vegetables (yes): n (%)	62 (93.9)	43 (86.0)	0.203[a]
Vegetables (g/day)	71.4 ± 45.0	74.8 ± 48.6	0.421[b]
Fish (yes): n (%)	53 (80.3)	24 (48.0)	<0.001[a]
Total Fish (g/month)	381.9 ± 392.2	155.3 ± 224.6	<0.001[b]
Sardine (g/month)	111.3 ± 184.3	46.5 ± 112.1	0.009[b]
Tuna fish (g/month)	50.2 ± 122.5	18.5 ± 56.0	0.155[b]
Others (g/month)	227.3 ± 252.8	92.0 ± 161.5	<0.001[b]
Eggs			
yes: n (%)	43 (65.2)	32 (64.0)	>0.99[a]
units/week	1 (0-3)	1 (0-2)	0.836[a]
Dairy Products (yes): n (%)	64 (97.0)	47 (94.0)	0.651[a]
Milk (yes**):** n (%)	56 (84.8)	31 (62.0)	0.009[a]
Whole milk (ml/day)	124.7 ± 212.5	32.0 ± 109.6	<0.001[b]
Low-fat milk (ml/day)	153.0 ± 229.5	9.7 ± 45.7	<0.001[b]
Free-fat milk (ml/day)	23.15 ± 89.9	119.8 ± 175.6	<0.001[b]
Milk-based products (yes): n (%)	59 (89.4)	41 (82.0)	0.383[a]
Yogurt	23 (34.8)	20 (40.0)	0.708[a]
Cream	24 (36.4)	3 (6.0)	<0.001[a]
Cheese	50 (75.8)	32 (64.0)	0.241[a]
Alcohol consumption (yes): n (%)	28 (42.4)	28 (56.0)	0.207[a]
Alcohol, ml/week	278.2 ± 568.1	737.6 ± 1420.9	0.074[b]

Data are expressed as mean ± standard deviation or number of individuals with the analyzed variable

[a] Pearson's $\chi 2$; [b] Mann-Whitney U test

Table 3 - Receiving Operating Characteristics (ROC) curve for physical activity and dietary consumption of specific foods for identification of vitamin D deficiency

Parameter	AUC (95% CI)	Cutt-off Value	Sensitivity	Specificity
Daily steps count (pedometer)	0.65 (0.55 - 0.76)	4880	0.60	0.73
Fish (g/month)	0.71 (0.61 - 0.80)	30	0.81	0.54
Whole Milk (ml/day)	0.61 (0.54 - 0.68)	42.8	0.90	0.33
Fat-free Milk (ml/day)	0.68 (0.60 - 0.76)	28.5	0.46	0.92

Abbreviations: AUC - Area Under the Curve; CI - confidence interval.

Table 4 - Multivariate logistic regression models: lifestyle patterns associations with Vitamin D deficiency (dependent variable)

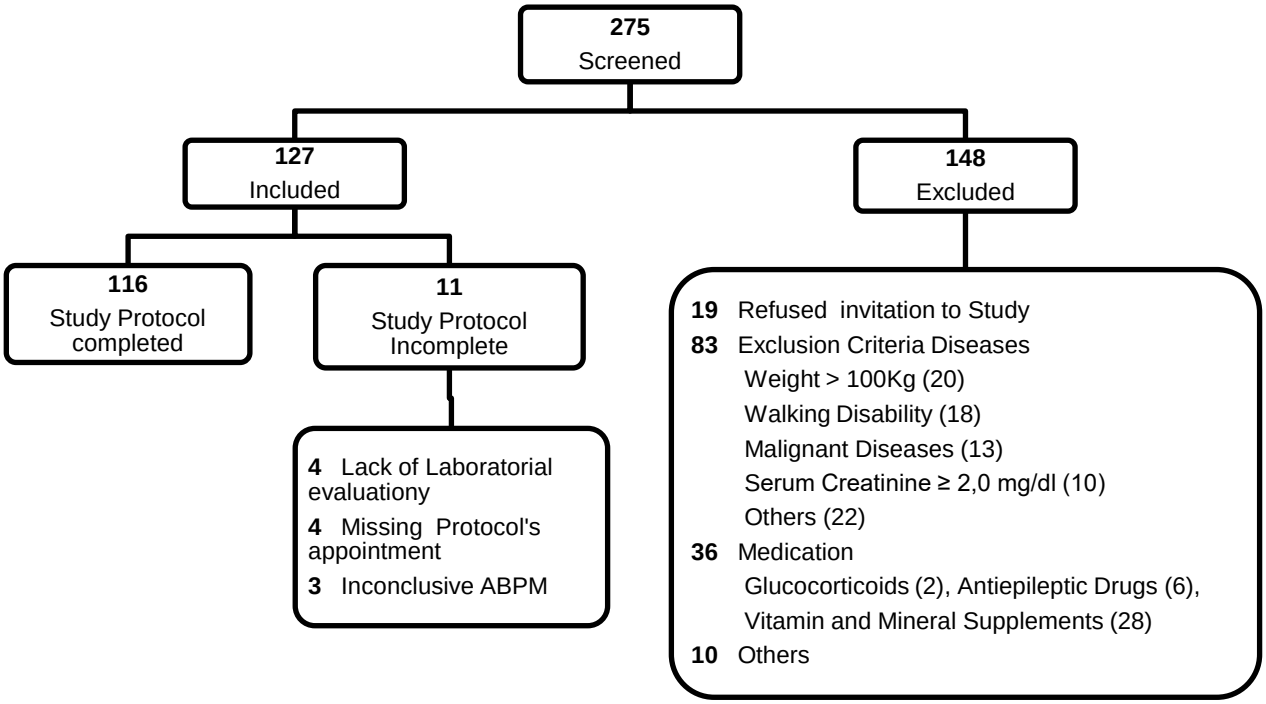
Lifestyle Patthern	n (%)	OR	IC 95%	P
Model 1 - (Physical Activity)	49 (43.4)	3.30	1.34 – 8.12	0.009
Model 2 - (Diet)	56 (48.3)	6.56	2.52 – 17.17	<0.001
Model 3 - (Diet + Physical Activity)	81 (69.8)	7.24	2.19 – 23.90	0.001
Model 4				
No Fish consuption	-	3.60	1.34 – 9.63	0.011
No Milk consuption	-	4.25	1.43 – 12.64	0.009
Model 5				
Physical Activity	-	3.89	1.42 – 10.67	0.008
No Fish consuption	-	3.80	1.33 – 10.84	0.013
No Milk consuption	-	4.56	1.46 – 14.27	0.009

Abbreviations: OR - Odds Ratio, CI: Confiance Interval

Diet: No consumption of milk and fish; Physical Activity: <5000 steps/day

Ajusted for fat mass (kg) and data collection on colder seasons

Figure 1 - Diagram of Patients Selection



Supplemental Figure 1 - Area under the curve for dietary intake of specific foods and physical activity in patients with vitamin D deficiency