

Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Ciências Farmacêuticas

**Influência dos marcadores inflamatórios no
metabolismo ósseo em pacientes infectados
pelo HIV em uso ou não da terapia
antirretroviral**

Erika Grasiela Marques de Menezes

Tese apresentada ao Programa de
Pós-graduação em Alimentos e
Nutrição para obtenção do título de
Doutor em Alimentos e Nutrição.

Área de Concentração: Ciências
Nutricionais

Orientador: Prof. Dr. Anderson
Marliere Navarro

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ERIKA GRASIELA MARQUES DE MENEZES

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Tese de Doutorado apresentada à Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista – UNESP, Campus de Araraquara como requisito para a obtenção do título de Doutor(a) em Alimentos e Nutrição.

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RESUMO

A história natural da infecção pelo HIV e a terapia antirretroviral (TARV) já estão bem esclarecidos, por proporcionarem aumento da contagem das células T CD4+, redução da viremia e diminuição de riscos para doenças oportunistas. Apesar da eficácia do tratamento antirretroviral, evidenciam-se os efeitos sistêmicos da inflamação mediados pela infecção crônica do HIV e a toxicidade dos antirretrovirais que contribuem para o aumento de riscos de complicações metabólicas. Nós hipotizamos que pacientes HIV positivos em uso ou não da terapia antirretroviral apresentam níveis aumentados nos marcadores inflamatórios, e isto afeta o metabolismo ósseo que leva à perda óssea, sendo mais acometidos os pacientes com maior tempo de exposição ao vírus e ao tratamento antirretroviral. **Objetivo:** Investigar a influência de citocinas pró-inflamatórias no metabolismo ósseo em pacientes HIV positivos crônicos em uso ou não da terapia antirretroviral. **Métodos:** Trata-se de um estudo do tipo transversal com 50 homens adultos HIV positivos, em tratamento ou não com drogas antirretrovirais. Os participantes foram subdivididos segundo o uso ou não da TARV, sendo grupo controle (GC): 10 participantes virgens de tratamento; grupo G<2 anos de TARV: 20 participantes abaixo de dois anos de tratamento antirretroviral; grupo G>2 anos de TARV: 20 participantes acima de dois anos de tratamento antirretroviral. Foi realizado *dual energy x-ray absorptiometry* (DXA) para avaliar a densidade mineral óssea e a composição corporal. Realizou-se a avaliação do consumo alimentar. Foram coletadas amostras de sangue periférico para as análises de interleucina-6 (IL-6), fator de necrose tumoral alfa (TNF- α), osteocalcina, paratormônio, 25 hidroxivitamina D, cálcio total e albumina. Amostras de urina foram coletadas para as análises de cálcio 24h e deoxipiridinolina urinária. Foram realizados testes estatísticos para testar a igualdade entre os grupos, testes paramétricos e/ou não paramétricos e correlação entre as variáveis quantitativas, considerando diferenças significativas o valor de $p < 0,05$. **Resultados:** Os participantes não tratados com TARV apresentaram valores mais elevados de IL-6 e TNF- α do que os participantes tratados com TARV por mais de dois anos. Os valores de TNF- α foram maiores nos participantes tratados com TARV por menos de dois anos do que nos participantes tratados com TARV por mais de dois anos ($p < 0,05$). Os valores aumentados de deoxipiridinolina urinária indicaram um aumento na atividade reabsortiva do tecido ósseo em todos os grupos, com uma diferença significativa entre os participantes não tratados com TARV e os participantes tratados com TARV por menos de dois anos. Através do DXA, nós encontramos uma redução da massa óssea em todos os sítios ósseos em cada grupo. **Conclusão:** O aumento da produção de citocinas pró-inflamatórias, mais notavelmente no grupo virêmico tem demonstrado estimular a atividade osteoclástica afetando a massa óssea. A redução da densidade mineral óssea foi observada em todos os sítios ósseos, principalmente para os grupos em tratamento com antirretrovirais. **Palavras-chaves:** HIV. TARV. Mediadores inflamatórios. Metabolismo ósseo. Osteopenia. Osteoporose.

ABSTRACT

The natural history of HIV infection and antiretroviral treatment (ART) are well evidenced by increase in the CD4+ T cell count, reduction of viral replication, and reduced risk for opportunistic diseases. Despite the efficacy of ART, the current repercussions are the systemic effects of inflammation mediated by chronic HIV infection and toxicity of the antiretroviral drugs that significantly contribute to increase the risk of metabolic complications. We hypothesized that HIV-infected patients treated or not with antiretroviral therapy have increased levels of inflammatory markers that can affect bone metabolism leading to bone loss, and the patients with longer exposure to HIV and ART are more affected. **Objective:** Investigate the influence of pro-inflammatory cytokines in bone metabolism in patients with chronic HIV infection treated or not with antiretroviral therapy. **Methods:** A cross-sectional study was conducted on 50 HIV-seropositive men treated or not with ART. The participants were divided, according to the use or not of ART, into the control group (CG): 10 participants not in treatment; the G<2 years of ART group: 20 participants treated with ART for less than 2 years; and the G>2 years of ART group: 20 participants treated with ART for more than 2 years. Dual energy x-ray absorptiometry (DXA) was performed to evaluate bone mineral density and body composition. The dietary intake was evaluated. Blood was collected for measurement of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), osteocalcin, parathyroid hormone, 25-hydroxyvitamin D, total calcium, albumin. Urine samples were collected for measurement of 24h urinary calcium and urinary deoxypyridinoline. Statistical tests were performed to test for equality between groups, parametric tests and or nonparametric and correlation between quantitative variables, considering the significant differences $p<0.05$. **Results:** The participants not treated with ART exhibited higher values of IL-6 and TNF- α than the participants treated with ART for more than 2 years. TNF- α values were higher in the participants treated with ART for less than 2 years than in participants treated with ART for more than 2 years ($p<0.05$). The increased values of urinary deoxypyridinoline indicated a high reabsorptive activity of bone tissue in all groups, with a significant difference between the participants not treated with ART and the participants treated with ART for less than 2 years. Through the DXA we found a bone mass reduction in all bone sites in each group. **Conclusion:** The increased production of proinflammatory cytokines associated osteoclast activity affecting bone mass was most notably elevated in the viremic group. The reduction of bone mineral density was observed in all bone sites principally for the groups in treatment with antiretroviral therapy. **Key-words:** HIV. ART. Inflammatory mediators. Bone metabolism. Osteopenia. Osteoporosis.

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Introdução

A Síndrome da Imunodeficiência Adquirida (aids) foi descrita pela primeira vez nos Estados Unidos em 1981. É uma doença provocada por um retrovírus, o vírus da imunodeficiência humana (HIV), que pertence ao membro da família dos lentivírus de retrovírus animais (1).

Foram identificados dois tipos de HIV, denominados de HIV-1 e HIV-2. O HIV-1 é responsável pela grande maioria da pandemia global. O HIV-2 é diferente na sua estrutura genômica e a progressão da doença é mais lenta do que a doença desencadeada pelo HIV-1 (2,3). Além disso, o HIV-2 é mais prevalente na África Ocidental, embora pequenas epidemias tenham sido relatadas em Portugal, França, Espanha e Brasil (4).

O HIV consiste em duas mono-fitas idênticas de RNA, com aproximadamente 9,2 quilobases (kb) de comprimento, acondicionadas dentro de um núcleo de proteínas virais e enzimas associadas, incluindo a transcriptase reversa, integrase e protease, e circundado por um envelope de membrana fosfolipídica derivado das células do hospedeiro (5-7).

A infecção das células pelo HIV se inicia com a ligação de glicoproteínas do envelope viral com as moléculas de CD4 e os co-receptores de quimiocinas que medeiam um processo de múltiplas etapas da fusão do envelope do vírion. Após a fusão, ocorre a liberação do genoma viral no citoplasma da célula e são ativadas as enzimas virais do complexo nucleoproteico. O genoma RNA do HIV é transcrito pela transcrição reversa para uma forma de DNA de cadeia dupla, e o DNA do vírus é integrado ao

genoma da célula hospedeira. O DNA do HIV integrado ao genoma do hospedeiro, conhecido como provírus, pode permanecer latente a infecção pelo HIV de uma célula individual durante meses ou anos. Em alternativa, o DNA do vírus é transcrito e produz novos genomas de RNA que são exportados para o citoplasma para produzir novas proteínas virais e vírus maduros (2).

O Programa Conjunto das Nações Unidas sobre HIV/aids – UNAIDS – estima que no final de 2015, haviam 36,7 milhões de pessoas vivendo com o HIV em todo mundo e houve uma diminuição de novas infecções pelo HIV entre os adultos nos últimos anos. As taxas de novas infecções pelo HIV foram relativamente estáticas na América Latina e Caribe, Europa Ocidental e Central. As maiores reduções de novas infecções ocorreram na África do sul e na região leste da África com um declínio de 4% em 2015 em relação a 2010, enquanto os números de novas infecções na Europa Oriental e na Ásia Central aumentaram em 57% (8).

No Brasil, desde 1980 até junho de 2016, foram registrados 842.710 mil casos de aids, sendo 65,1% casos de aids em homens e 34,9% em mulheres. As taxas de detecção de aids em homens nos últimos dez anos têm apresentado tendência de crescimento; em 2006, a taxa foi de 24,1 casos/100 mil habitantes, a qual passou para 27,9 em 2015, representando um aumento de 15,9%. Entre as mulheres, observa-se tendência de queda dessa taxa nos últimos dez anos, que passou de 15,8 casos/100 mil habitantes, em 2006, para 12,7 em 2015, representando uma redução de 19,6% (9).

A via de transmissão do HIV é realizada através de relações sexuais desprotegidas, por contato sanguíneo, reutilização de seringas e agulhas, feridas expostas, transfusão de sangue que não tenha sido rastreado, acidentes ocupacionais envolvendo agulhas contaminadas, de mãe para filho, transmissão pré-parto, intraparto, pós-parto ou via aleitamento materno (2,10).

Após a infecção pelo HIV, algumas pessoas não ficam doentes imediatamente, mas pode surgir um conjunto de sinais e sintomas semelhantes ao estado gripal. A evolução da doença causada pelo HIV pode ser acompanhada por meio da medição da quantidade de vírus no plasma e pela contagem de células T CD4+ no sangue.

A fase aguda da doença é o período de viremia caracterizada por sintomas inespecíficos da infecção, que não é facilmente identificada devido à sua semelhança com outras infecções virais. Nesta fase, os pacientes podem ser acometidos por algumas características clínicas, tais como: febre, dor de garganta com faringite, linfadenopatia e em muitos pacientes podem não apresentar sintomatologia (2,11-13).

No próximo estágio da infecção pelo HIV conhecida como a fase crônica da latência clínica, que pode durar muitos anos, os pacientes permanecem assintomáticos ou apresentam infecções secundárias. Eventualmente, com a destruição das células T CD4+, o sistema imune não pode mais combater infecções por outros organismos causadores de doenças e pacientes soropositivos para HIV desenvolvem uma ou mais

condições definidora da aids, incluindo infecções graves, como sarcoma de Kaposi, e linfoma não-Hodgkin (11,14).

O tempo de sobrevida dos pacientes vivendo com HIV/aids aumentou significativamente após a introdução da terapia antirretroviral (TARV), a partir de 1996. A TARV provou ser eficaz na redução do RNA viral plasmático para níveis não detectáveis na maioria dos pacientes tratados e na elevação dos linfócitos T CD4+, células-alvo do HIV (15-18).

Atualmente, 28 antirretrovirais (ARVs) estão licenciados pelo *Food and Drug Administration* (FDA), e incluem: os inibidores de transcriptase reversa análogo de nucleosídeos e nucleotídeos (ITRNs): abacavir, didanosina, emtricitabina, estavudina, lamivudina, tenofovir, zalcitabina e zidovudina; os inibidores de transcriptase reversa não análogo de nucleosídeos (ITRNNs): delavirdina, efavirenz, nevirapina, rilpivirina e etravirina; os inibidores de protease (IPs): amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir e tipranavir; o inibidor de fusão: enfuvirtida; o Antagonista CCR5: maraviroc; e o inibidor da integrase: dolutegravir, elvitegravir e raltegravir (19).

Apesar do tratamento eficaz dos antirretrovirais, as pessoas soropositivas para HIV têm um risco maior do que o esperado para doenças cardíacas, hepáticas, renais, neurológicas e ósseas (2,20,21). Estudos clínicos sugerem que os pacientes soropositivos para HIV apresentam manifestações de envelhecimento mais precoce do que na população geral. A fisiopatologia deste envelhecimento precoce pode ser multifatorial, incluindo a inflamação crônica do HIV que leva a ativação persistente do

sistema imune associado com uma deficiência imunológica grave e liberação de citocinas pró-inflamatórias, mesmo após o controle do vírus com a TARV (22-25).

Consequentemente, a ativação imunológica associada à condição inflamatória pode induzir perturbação na interface imuno-esquelético e estas interrupções entre os sistemas imunológico e esquelético impulsionam ao desequilíbrio do turnover ósseo, contribuindo para uma deterioração esquelética e aumento de taxas de fraturas ósseas (26).

Desta forma, nós hipotizamos que pacientes soropositivos para HIV em uso ou não da terapia antirretroviral apresentam níveis aumentados nos marcadores inflamatórios, e isto afeta o metabolismo ósseo que leva à perda da densidade mineral óssea, sendo mais acometidos os pacientes em maior tempo de exposição ao vírus e ao tratamento antirretroviral.

Portanto, o objetivo deste trabalho é investigar a influência de citocinas pró-inflamatórias no metabolismo ósseo em pacientes soropositivos para HIV crônicos em uso ou não da TARV, e determinar a associação entre a densidade mineral óssea, marcadores inflamatórios, marcadores do metabolismo ósseo, exposição à duração da infecção pelo HIV e ao tratamento antirretroviral.

Outro importante objetivo deste estudo é avaliar as causas secundárias que podem contribuir para a redução da densidade mineral óssea, tais como: deficiência de vitamina D, ingestão inadequada de cálcio, níveis aumentados de paratormônio (PTH) e hipocalcemia. Outros fatores associados para a perda óssea, como o baixo peso corpóreo, idade

avançada, hipogonadismo, hipofosfatemia, insuficiência renal e ou hepática foram considerados de exclusão neste trabalho.

Capítulo 1.

Osteoimmunology: How does HIV lead to alterations in the immuno-skeletal interface in the context of antiretroviral therapy?

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Abstract

In the past two decades the life expectancy of people living with HIV infection has significantly increased. Despite the efficacy of antiretroviral therapy, HIV causes immune abnormalities that persist even after virus replication is suppressed. These consistent changes in the immune system associated with inflammation in the context of HIV have been implicated in damage to lymphoid tissues and in the early onset of several diseases that often present later in life such as osteoporosis. Much evidence has been reported for the significantly higher prevalence of fractures in HIV-infected adults compared to uninfected people of the same age and sex, which suggests HIV leads to early aging. The field of osteoimmunology has expanded to better understand the interaction between immune and skeletal systems, and it has been made clear that the persistence of inflammatory mediators associated with HIV at the immuno-skeletal interface contributes to bone turnover imbalance and the loss of bone mass. This review seeks to examine how HIV leads to alterations in the immuno-skeletal interface, and how this affected by antiretroviral therapy.

Keywords: HIV. ART. Osteoimmunology. Inflammatory cytokines. Osteoporosis.

1. Introduction

The advances in antiretroviral therapy (ART) have had an impressive impact on the extension of life expectancy for patients infected with the human immunodeficiency virus (HIV), turning HIV infection into a long-term chronic disease for many patients [1].

The potential effect of HIV and the toxicity of ART have been well described and more clearly demonstrated by a series of immunologic alterations that persist despite the effective suppression of virus replication. One characteristic of HIV infection is the continuous stimulation of the immune system with resulting inflammation that accelerates T-cell senescence and causes extensive damage to multiple arms of the immune system [2-4]. Such immunologic changes predispose B cells and T cells to produce excessive pro-inflammatory mediators such as tumor necrosis factor *alpha* (TNF- α), interleukin-6 (IL-6), and interleukin-1 *beta* (IL-1 β) and lead to the early onset of several comorbidities, including osteoporosis [5].

New evidence keeps arising from the investigation process in the field of osteoimmunology for new challenges and strategies in the interaction between the immune and skeletal systems. This review seeks to examine the role of inflammation in bone loss associated with the immune deregulation that may influence bone turnover, and with the mechanisms by which HIV and antiretroviral drugs affect bone metabolism.

2. Bone Tissue

The bone is a complex and dynamic tissue that is under continuous renovation and plays important roles in the support of musculature, protection of vital organs and bone marrow, and continuous maintenance of mineral homeostasis and acid-base equilibrium. It is considered the largest reservoir of calcium (Ca), phosphorus (P), and magnesium (Mg) ions, as well as of growth factors and cytokines [7-11].

Bone composition consists of an organic matrix, or osteoid, and mainly collagen fibers onto which calcium and phosphate salts are deposited in combination with hydroxyl ions in hydroxyapatite crystals. The tensile capacity of collagen and the solidity of hydroxyapatite combine to provide the bone its sturdiness [12,13], with osteocalcin, osteopontin, and several other proteins being part of the bone matrix [8,12]. Cortical bone makes up approximately 80% of skeletal bone mass, while the remaining 20% is spongy bone. Cortical bone is denser and plays a role in protection and support of the skeleton, whereas trabecular bone has much lower density, higher metabolic activity, and much larger surface area [11,14,15].

The cells that make up bone tissue are osteoblasts from osteoprogenitor cells, responsible for the synthesis of organic compounds of the bone matrix and acting in regulating bone formation; osteocytes, which are derived from osteoblasts and are located within the network of gaps of the bone matrix; and osteoclasts, responsible for the bone resorption process and initiation of bone remodeling [11,12,16].

Osteoblasts originate from mesenchymal cells, and alkaline phosphatase is considered the main enzyme produced by osteoblasts and is

associated with bone formation [17,18]. Osteocalcin is also produced by osteoblasts and is the most abundant non-collagen protein in the bone matrix. Collagen type I is secreted by osteoblasts, responsible for 90% of the bone matrix, which is produced by fibroblasts in the conjunctive tissue and other tissues [11,15].

Bone formation is regulated by systemic and local factors, including sex steroids, glucocorticoids, calcitriol, parathyroid hormone (PTH), growth hormone, insulin, IL-1, and prostaglandins. After osteoblasts have completed the formation of the bone matrix and the organization of collagen and non-collagen proteins, the mineralization process starts, and they evolve into osteocytes or enter a cell apoptosis process [7,18].

Osteoclasts are pluripotent hematopoietic stem cells and are characterized by being large multinucleated cells with a large number of mitochondria, rich in lysosomal enzymes, free ribosomes, and long Golgi complexes [11,15]. Osteoclast formation, activation, and resorption are regulated by the relation of the receptor activator of nuclear factor kappa (RANK), RANKL (RANK-ligand), osteoprotegerin (OPG), IL-1, IL-6, colony stimulating factor-1 (CSF-1), PTH, 1,25-dihydroxyvitamin D, and calcitonin [7,14].

After skeletal growth is completed, the bone undergoes continuous remodeling as a response to stress on the skeleton, adaptation to changes in lifestyle, dietary intake, maintenance of calcium concentration in extracellular fluids, systemic inflammation, and bone diseases [12].

3. Bone Metabolism

Normal bone metabolism features constant bone remodeling. The remodeling process initiates with the activation phase followed by resorption, reversal, and formation, respectively. The complete remodeling cycle, from the beginning of resorption up to the mineralization of the newly formed matrix, usually takes from eight to 12 weeks [15]. Bone mineral content consists mainly of Ca, P, and Mg, which are absorbed in the intestine and regulated by the PTH, 1,25-dihydroxyvitamin D, and calcitonin. Intestinal absorption must meet the requirement of bone mass increase during the growth and turnover phases, as well as bone remodeling in adulthood [11,18,19].

PTH is a polypeptide hormone synthesized by parathyroid glands, and its primordial actions involve the release of calcium into the extracellular liquid, the increase in calcium resorption and decrease in phosphate resorption by kidney tubules, and conversion of 25-hydroxycholecalciferol into 1,25-dihydroxyvitamin D, which, in turn, increases calcium absorption by the intestines [20,21].

In the kidney, PTH determines the resorption of Ca and Mg and the excretion of phosphate and bicarbonate through specific receptors in the distal convoluted tubules, regulating the release of Ca and phosphate into the extracellular liquid and, when secreted continuously, favoring bone mass loss. In the bone, the actions of PTH are performed through osteoblasts, which increase the production of IL-6 and other cytokines that, in turn, stimulate bone resorption [19]. Calcitonin, a peptide hormone secreted by the

thyroid gland, lowers the plasma concentration of Ca and has opposite effects to PTH, directly inhibiting osteoclast activity [21-23].

Vitamin D is an essential nutrient for the mineralization of the skeleton and maintenance of bone mass in its active form. 1,25-dihydroxyvitamin D plays an essential role in the homeostasis of Ca and P and increases the calcium absorption in the intestinal tract, with significant effects on bone deposition and absorption [24,25]. The main role of vitamin D is to act along with PTH in the maintenance of extracellular calcium concentrations [21,25,26]. Through the liver and kidneys, vitamin D is converted into the final active product, 1,25-dihydroxyvitamin D, which, in turn, inhibits PTH production [24,25,27].

Calcium is associated with bone development and maintenance and plays countless physiological roles in the organism, such as vasoconstriction and vasodilation, transmission of nervous impulses, muscle contraction, cell metabolism, and as a second messenger to mediate the effects of membrane signaling to the release of substances and hormones [27,28]. Over 99% of the calcium in the body is found in the bones and teeth, mainly as hydroxyapatite, and 1% is present in the extracellular fluid.

The amount of calcium absorbed by the gastrointestinal tract is determined by the bioavailability of dietary calcium and by the intestinal capacity of absorption [27,29,30]. Ca absorption occurs through two processes: active saturable transcellular absorption, stimulated by 1,25-dihydroxyvitamin D, and non-saturable paracellular absorption, independent of the active form of vitamin D and determined by the Ca concentration in the

intestinal lumen [25,29]. Moreover, Ca may be transported in the blood bound to albumin or pre-albumin, complexed with sulfate, phosphate, or citrate, or in its free form [27].

Calcium and 1,25-dihydroxyvitamin D interact in a complex manner and may reduce Ca absorption when intake is high, when 1,25-dihydroxyvitamin D levels are low, or when the intestine is unable to respond to the action of 1,25-dihydroxyvitamin D [25,28]. The proper action of hormones and of intestinal absorption of calcium is key in the maintenance of bone mass and any factor, dietary or otherwise, that impacts this system will compromise bone mass [21].

4. The Immuno-Skeletal Interface

Over the last decade, evidence has suggested that the immune system and skeletal system are closely integrated, forming a connection called the “immuno-skeletal interface” (ISI) [4,6]. Numerous factors and mechanisms have been shown to regulate the interactions among mesenchymal stem cells (MSCs), hematopoietic stem cells, and immune cells. Those processes maintain skeletal and hematopoietic homeostasis and may be susceptible to alterations in many physiological processes and biochemical systems in the body [31].

The bone is a multifunctional and very metabolically active tissue that undergoes continuous renovation and remodeling, and it has been long understood that renovation is extremely sensitive to changes in multiple metabolic and biochemical systems in the body. Consequently, several

pathologies may impact bone turnover, such as endocrine disorders, rheumatologic and autoimmune diseases, hypogonadal status, gastrointestinal and hematological diseases, and infectious diseases [32,33].

It has been well established that osteoblasts develop from cells of mesenchymal origin, while osteoclasts, the cells that reabsorb the bone, are of myeloid origin and derived from precursors that circulate within the lineage of monocytes and macrophages. Those precursors may be distinguished from other monocytic cells through the expression of RANK on the surface of the membrane. RANKL, a cytokine identified as a mediator of T-cell growth and involved in functions of dendritic cells, is known to play important immunologic roles [34,35].

An *in vitro* study carried out in France investigated the expression of CD200 derived from MSCs and its receptor, CD200R, expressed in myeloid cells, as well as in monocytes and macrophages, assuming that CD200 might be a new molecule to control osteoclastogenesis and the MSC-osteoclast communication in humans. The findings showed that soluble CD200 did not modify the monocyte phenotype, but inhibited RANKL as well as its maturation in bone resorption cells *in vitro* [36].

Another study carried out in Germany investigated whether CTLA4 could affect the differentiation of monocytes into osteoclasts *in vitro* and *in vivo*. CTLA4 (cytotoxic T-lymphocyte-associated protein 4) is an inhibitor of CD28 activation, which, in turn, is considered another co-stimulating molecule involved in the activation of T cells. Interestingly, Axmann and colleagues showed *in vitro* that CTLA4 directly inhibited the formation of

osteoclasts induced by RANKL, classifying CTLA4 as an anti-osteoclastogenic molecule [37].

In the context of bone biology, RANKL is considered to interact with RANK on osteoclast precursors, which induces their differentiation into pre-osteoclasts. These pre-osteoclasts then merge into mature multinucleated cells, i.e., osteoclasts, responsible for bone resorption. The macrophage colony stimulating factor (M-CSF) contributes to the proliferation, survival, and differentiation of osteoclast precursors, as well as to bone resorption. RANKL and M-CSF are considered essential cytokines for osteoclastogenesis and are secreted by osteoblasts. Meanwhile, osteoprotegerin (OPG) is considered a potent inhibitor of osteoclastic differentiation and is a member of the superfamily of the TNF receptor. OPG is produced by osteoblast lineage cells, whose role is to block the interaction of RANK-RANKL to moderate RANKL-induced osteoclast formation [38-40].

4.1 T and B Lymphocytes: In the RANK-RANKL-OPG System

The cells in the innate and adaptive immune system are present as cells carried in blood and lymph and are found in virtually all tissues. B cells are essential components of the adaptive immune system and of humoral immunity since they produce antibodies against soluble antigens. B cells also present antigens to T cells, and the latter regulate the function of the antigen-presenting cells (APCs) to develop specific responses to antigens and retain memory of those antigens [41]. Besides those cells comprehending the entire adaptive immune system, B cells also differ from hematopoietic stem cells

(HSCs) on the surfaces of endosteal cells, showing a very close relation with bone cells such as in the basal regulation of the skeleton [58].

A study carried out in the United States investigated the involvement of T and B lymphocytes in the basal bone turnover process *in vivo* and focused mainly on the role of lymphocytes in the regulation of OPG. The results showed that both T and B cells play an important role in limiting basal bone resorption *in vivo*, implicating B cells as regulators of osteoclastogenesis and as suppressors of mineral bone density (BMD) through OPG production [42].

Under inflammatory conditions, activated B and T cells are a significant additional source of RANKL. The activation of lymphocytes has been implicated in bone loss associated with pathological conditions such as post-menopause osteoporosis, rheumatoid arthritis, bacterial osteomyelitis, and alveolar bone loss with periodontal infection [43]. Indeed, a recent study carried out in China compared 66 patients diagnosed with rheumatoid arthritis versus 20 healthy controls and showed higher RANKL levels in serum and expressed in T cells in peripheral blood of patients with rheumatoid arthritis than in the control group [44].

Studies have shown that permissive concentrations of M-CSF and RANKL act as final effectors of osteoclast formation, in conjunction with other inflammatory cytokines (IL-1, IL-6, IL-7, IL-17, TNF- α , IFN- γ , VEGF, and IGF-1) that play a significant role in BMD reduction. These inflammatory cytokines are able to upregulate osteoclastogenesis by stimulating RANKL production by lymphocytes and/or by osteoblast lineage cells and by

upregulating the RANK-RANKL receptor in osteoblast precursors [45-47]. The actions of other inflammatory cytokines on bone cells are summarized in Table 1.

Recently, Zhao and colleagues investigated the cellular expression of IL-17 by CD4⁺ T cells in postmenopausal women with normal BMD and in women with osteoporosis. The women with osteoporosis showed higher concentrations of IL-17, IL-6, and TNF- α than those with normal BMD. In that study, cellular expression of IL-17 by CD4⁺ T cells was higher in the group of women with osteoporosis.

An experimental study carried out in the United States assessed the direct effects of IFN- γ on osteoclastogenesis and subsequently the indirect pro-resorptive activities of IFN- γ in three different murine models, namely, postmenopausal osteoporosis, bacteria-induced infection, and inflammation, respectively. The three experimental models with bone turnover imbalance showed a balance between IFN- γ anti-resorptive activity and the pro-resorptive effects in favor of osteoclastogenesis and bone resorption [49].

Another study carried out in Japan showed that the production of IFN- γ by T cells induces rapid degradation of the RANK adaptive protein, TRAF6 (tumor necrosis factor receptor-associated factor 6). This indicates that TRAF6 is a critical target for IFN- γ action, which inhibits osteoclastogenesis by interfering in the RANKL-RANK signaling pathway [50]. However, IFN- γ may have an inhibitory action on osteoclastogenesis, as well as IFN- γ may be potentialized with the antigen presentation and drive the activation of T cells and RANK and TNF- α production [49,50].

4.2 Effects of HIV Infection on the Skeleton

It has been clearly established that HIV infection is an independent risk factor for osteopenia and osteoporosis. The prevalence of osteoporosis is 3-4 times higher in HIV-infected adults than in uninfected people of the same age, race, and sex [6,51,52]. HIV infection causes damages to lymphoid tissues and mucosae, leading to progressive immunodeficiency, microbial translocation, and chronic inflammation, as well as chronic activation of monocytes and macrophages, leading to accelerated aging [2].

Evidence suggests that two out of every three HIV-positive individuals not receiving antiretroviral treatment have low BMD and are three-fold more likely to develop osteoporosis [6,52,53]. Numerous studies have pointed out the high prevalence of bone mass loss, including a meta-analysis of 11 studies that showed that HIV-infected patients (n=884) had a decrease by 67% in BMD compared to control groups (n=654), and 15% of the HIV-infected patients had osteoporosis [54].

Recently, a study carried out in France tested whether HIV proteins such as Tat and/or Nef could induce the senescence of bone marrow MSCs and reduce their ability to differentiate into osteoblasts *in vitro*. MSCs chronically treated with Tat and/or Nef for 30 days had lower proliferative activity and experienced early senescence through the activation of nuclear factor-kappa B (NF- κ B), as well as an increase in oxidative stress and mitochondrial dysfunction compared to non-treated cells [55]. Another study carried out in Singapore tested the effect of the proteins Tat and Rev on the differentiation of osteoclast activity and on inducing the resorptive activity of

osteoclasts through an increase in reactive oxygen species and TNF- α production in osteoclast precursors *in vitro*. Those results showed that HIV proteins and the host's immune response contribute to changes in the balance of bone formation and resorption [56].

In a study carried out in the United States, bone mass loss was investigated in a genetically modified rat model that expressed the HIV gene associated with the increase in B-cell expression of RANKL and the decline in osteoprotegerin expression [4]. Based on this finding, Titanji and colleagues carried out a cross-sectional study assessing the immuno-skeletal profile of HIV-infected individuals not receiving antiretroviral treatment and of an HIV-negative control group. The intracellular expression of OPG and RANKL was quantified in circulating peripheral blood B cells derived from HIV-positive and HIV-negative individuals. In HIV positive subjects, the B-cell expression of RANKL was significantly increased, whereas the B-cell expression of OPG was significantly decreased, which favors the increase in bone resorption activity. The analyses of B-cell subsets from HIV-positive individuals showed significant increases in RANKL-expressing naïve, resting memory, and exhausted tissue-like memory B cells, while OPG showed a significant decline in resting memory B cells [57].

The important role of B cells as osteoclastogenesis regulators in the basal bone turnover process is noteworthy and extensively documented, such as the effect of B-cell dysfunction associated with HIV infection in increasing osteoclast activity and decreasing OPG production [42,43,57,58].

4.3 Effects of Antiretroviral Therapy on the Skeleton

Interestingly, after the initiation of ART, new dysfunction of the immuno-skeletal interface may occur, mediated by the subsequent immunologic reactivation and the repopulation of T cells established in nearly all types of antiretroviral regimes, which contributes to excessive production of osteoclastogenic cytokines and consequent bone mass loss [6,59]. Evidence suggests that the loss of 2 to 6% of BMD occurs at the beginning of antiretroviral treatment, typically within the first 48 weeks, which is higher than bone loss than expected from aging itself [60,61].

Our data showed that HIV infected individuals not receiving ART have higher serum values of inflammatory markers than those on suppressive treatment. That would mean the initial phase of the infection may be characterized by an increase in the production of pro-inflammatory cytokines and subsequent persistence through the natural course of the disease. At the same time, it was observed that the persistent HIV-mediated inflammation and the toxicity of extended ART contributed to the increase in bone resorption markers, directly affecting bone mass particularly in the group with greater exposure to the virus and antiretroviral drugs [62].

Nucleoside analog reverse-transcriptase inhibitors (NRTIs) have been associated with increased risk of fractures, and data from an experimental model have shown specifically that tenofovir (TDF, NRTI) affects bone mineralization [63]. Another recent study showed that the NRTI class results in disruption of osteoblast expression and that these changes imply

osteoblast dysfunction, leading to a decrease in bone formation and, consequently, to a reduction in bone mineral density [59,64].

In randomized controlled trials comparing BMD in HIV-positive patients treated with TDF versus non-TDF regimens, the authors found that TDF significantly reduced BMD in the hip and lumbar spine [64]. Briot and colleagues showed that patients initiating a monotherapy regime with a protease inhibitor (PI) or triple therapy with a protease inhibitor combined with two NRTIs had similar significant bone loss after 48 weeks of treatment [65]. McComsey and colleagues, in turn, compared the bone loss under three different schemes of ART classes such as tenofovir (NRTIs), efavirenz (NNRTIs), and atazanavir (PI). The three different classes led to BMD loss, which stabilized after 48 weeks, but tenofovir was significantly associated with the highest BMD loss in the hip and back [66]. A recent double-blind, placebo-controlled study carried out in the United States compared the antiretroviral treatment containing maraviroc (MVC) *versus* TDF in HIV-positive patients. The reduction in hip BMD in 48 weeks was lower for MVC than for TDF, as well as the decline in BMD of the lumbar spine was lower for those on MVC [67].

Another recent study carried out in the United States by Ofotokun and colleagues used an animal model to show the association between immune activation with the reconstitution of T cells and the increase in RANKL and TNF- α production and consequent bone loss [68]. In order to confirm those findings in humans, the authors carried out a 24-week prospective cohort study investigating the early kinetics of osteoclastogenesis regulator markers

on CD4⁺ T cells and of bone turnover markers in HIV-infected patients treated with lopinavir/ritonavir plus tenofovir disoproxil fumarate/emtricitabine at baseline and at 2, 8, 12, and 24 weeks after ART. Interestingly, the bone resorption marker rose by 200% above baseline at week 12 and remained elevated through week 24 which was associated with a significant increase in plasma levels of RANKL and TNF- α . Moreover, the recovery of CD4⁺ T cells was correlated with the bone resorption marker, suggesting ART-induced bone loss occurs early, associated with the initial events of immune reconstitution [69].

On the other hand, a longitudinal study carried out in the United States compared the levels of bone turnover markers, OPG, RANKL, and inflammatory cytokines in HIV-positive participants prior to ART and 6-12 months after ART initiation with a regime that did or did not contain TDF. Pre-ART, 35% and 1% of the participants showed bone resorption markers and bone formation above normal values, respectively. After treatment initiation the inflammatory cytokines OPG and RANKL significantly decreased for both treatment regimens [70].

Erlandson and colleagues carried out a cross-sectional study to evaluate the association between bone health, inflammatory markers, and immune activation of monocytes and lymphocytes in HIV-infected patients on stable ART. The results showed no significant correlations of BMD or bone turnover markers with T-cell activation markers or soluble monocyte activation markers. On the contrary, for the marker sVCAM-1 (soluble vascular cell adhesion molecule-1), a significant difference was found among

the participants with hip and lumbar spine osteopenia, while a strong correlation was found with BMD and all bone turnover markers [71].

In fact, another study carried out by Tanaka and colleagues showed that osteoblasts are regulated by the expression of sVCAM-1 and ICAM-1 and may induce osteoblast maturation and facilitate the production of bone-resorbing cytokines [72].

Such evidence contributes to the concept that the inflammatory state associated with T-cell reconstitution after the initiation of ART may significantly contribute to dysfunction in the immuno-skeletal interface regardless of the ART regime. Evidence in studies of clinical samples in the context of HIV suggests the loss of BMD may likely be multifactorial and that inflammatory events are a contributing factor to it [73-75].

5. Osteoporosis: Other Traditional Risk Factors

Osteoporosis is a systemic skeletal disease characterized by low bone mass and breakdown of the microarchitecture of the bone tissue, with consequent increase in bone fragility and susceptibility to fractures, particularly in the hip, lumbar spine, and wrist, although any bone may be affected [76-78]. Osteoporosis is classified as primary when it occurs as the result of any bone change related to age or idiopathic causes, and is classified as secondary when it is related to lifestyle, medication, and other diseases [77].

Osteopenia and osteoporosis are diagnosed using a double-energy x-ray absorptiometry (DXA) that provides an estimate of BMD (the amount of

bone per cm²) of the skeleton [79,80]. The bone status is defined through the measure of bone mass based on the following criteria for postmenopausal women and men 50 years old or more, as follows: a) normal: standard deviation up to -1.00 (*T-score* from 0 to -1); b) osteopenia: standard deviation from -1.00 to -2.50 (*T-score* between -1 and -2.5); and c) osteoporosis: standard deviation equal to or below -2.50 (*T-score* $\leq$ -2.50). In 2005, the International Society for Clinical Densitometry published a recommendation for premenopausal women and men below 50 years old, i.e., *Z-score* values, with the following classifications: a) below the estimate for the age group: *Z-score* $\geq$ -2.00 and b) within the estimate for the age group: *Z-score* $>$ -2.00 [81-82].

Associated with bone densitometry, the classic biochemical parameters assessed to detect the etiology responsible for the variation in bone mass are calcium, phosphorus, 25(OH) vitamin D, albumin, creatinine, urea, insulin-like growth factor I (IGF-I), thyroid stimulating hormone (TSH), PTH, and testosterone [33,83]. Furthermore, bone turnover markers are considered indispensable for the dynamic evaluation of the skeleton that complements the static measurement of BMD.

The main bone formation biomarkers are osteocalcin, bone-specific alkaline phosphatase (BALP), procollagen type-I carboxy-terminal peptide (PICP), procollagen type-I amino-terminal peptide (PINP), and the bone resorption markers tartrate-resistant acid phosphatase, hydroxyproline, calciuria, pyridinoline, deoxypyridinoline, and type-I collagen cross-linked telopeptides (N-Tx and C-Tx) [11,33,84,85].

Countless factors may impact osteoblast and osteoclast activities or calcium homeostasis and calciotropic hormones, such as PTH, 1,25-dihydroxyvitamin D, and calcitonin [18], as well as direct effects of inflammation and the effects of drug treatments [7]. Another factor that becomes harmful to bone health is inadequate calcium intake, leading to important implications to bone metabolism and bone mass loss [30]. Some conditions may impact intestinal calcium absorption, such as increased calcium absorption due to growth factors, pregnancy, lactation, estrogen replacement, and excessive salt intake, also associated with granulomatous diseases, hyperparathyroidism, and idiopathic hypercalciuria. Reduced calcium absorption conditions are associated with advanced age, hypoparathyroidism, chronic kidney failure, and malabsorption syndrome [12,86].

Other risk factors among people living with HIV as well as for the general population include, for example, low body mass index, vitamin D deficiency, kidney disease, diabetes mellitus, endocrine diseases, rheumatologic diseases, hypogonadism, inflammatory bowel disease, increase in serum concentration of parathormone, hypophosphatasia, anorexia nervosa, smoking, illicit drug use, family history, prior fracture, use of steroids and other medications (heparin, proton pump inhibitors, valproic acid, methotrexate, etc.), physical inactivity, advanced age, and menopause status [73,74,81,87,88].

6. Interventions to Prevent Bone Loss

Several interventions have been shown to reduce the rates of fractures and to preserve bone strength for the general population. The important considerations for bone mass quality include adequate calcium intake, exposure to sunlight for vitamin D synthesis and/or supplement intake, regular weight lifting and muscle strengthening exercises, cessation of tobacco use, and avoiding excessive alcohol consumption [33,81,89,90].

The adequate intake of Ca throughout life is required for achieving peak bone mass and for the maintenance of bone health. In 2011, the Institute of Medicine (IOM) published new reference dietary intakes for calcium. The estimated average requirement (EAR) of Ca for men and women between 19 and 70 years old is 800 mg/day and 1,000 mg/day above 70 years old. During pregnancy and lactation, the recommended intake is 1,100 mg/day between 14 and 18 years old and 900 mg/day between 19 and 50 years old. The EAR of vitamin D for both sexes between 9 and 70 years old or above 70 years old is 10 µg/day [91].

Dietary calcium is available mainly in milk and derivatives and in some dark green leafy vegetables and grains. The main sources of vitamin D are fish liver oils, dairy products such as butter and fatty cheeses, eggs, and enriched margarines. It must be pointed out that Ca absorption may be reduced in the presence of other dietary components such as phytic acid (present in soybean derivatives, seeds, and oily plants) and oxalate (present in spinach, rhubarb, raw beans, seeds, nuts, cocoa, and caffeine) [76,91].

However, it should be highlighted that a balanced diet rich in dairy products with low fat content, fruits, and vegetables provide numerous

nutrients required for bone quality and the therapeutic agents that contribute to increasing BMD [33,81].

7. Conclusion

The field of osteoimmunology continues to evolve, and it has been a great challenge to better understand the physiology of bone loss in the context of HIV and the different antiretroviral regimens. The activation of T and B lymphocytes and the inflammatory condition occur both in HIV-infected subjects and in the normal aging process of non-infected patients.

Indeed, since HIV leads to disturbances in the immuno-skeletal interface, it becomes extremely important to continue investigating the unexplained convergence of inflammation associated with HIV in the ART era and the immunosenescence that causes the early onset of other comorbidities, particularly osteoporosis/osteopenia.

Thus, it may be worth further exploring new therapeutic strategies or new molecular mechanisms in intracellular communication in the immuno-skeletal interface aiming to prevent or reverse immunologic alterations to better understand or to have new findings of diagnostic indicators of skeleton disorders, as well as to reach the crucially important goal of HIV-positive patients leading a normal life.

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Table 1: Effects of inflammatory cytokines on osteoblasts and osteoclasts

Inflammatory cytokines	Bone cells effect
RANKL	RANKL is a member of the TNF cytokine family that has role as a stimulator of osteoclasts formation. RANKL is the critical cytokine that induces osteoclastogenesis and is also considered the final effector of osteoclast differentiation and activity [6,92-94,97].
OPG	OPG is a member of the TNF receptor superfamily and a potent inhibitor of osteoclast formation that acts as a decoy receptor for RANKL and moderates RANKL activity [6,92,95-97].
M-CSF	M-CSF has an important role for normal osteoclast formation and is involved in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells. This cytokine is released by osteoblasts and alone is incapable of stimulating osteoclast differentiation in the absence of RANKL. M-CSF also is a potent stimulator of RANK expression in osteoclasts, expanding the osteoclast precursor pool. [6,92,98].
IFN- γ	IFN- γ has been reported to enhance osteoclastogenic cytokine production during inflammation and infection by stimulating antigen presenting cells and leading to bone loss [49]. Conversely, IFN- γ has been found to induce rapid degradation of the RANK adaptive protein, TRAF6, which results in inhibition of osteoclastogenesis [50].
TNF- α	TNF- α is an osteoclastogenic cytokine that promotes RANKL production by osteoblasts and increases osteoclastogenesis. Furthermore, this cytokine is involved in systemic inflammation and is a potent stimulator of bone resorption [6,92,100,101].
TGF- β	TGF- β is secreted by many cell types and plays an important role in T cell regulation and differentiation. TGF- β has anti-inflammatory properties that suppress T-cell activation as well as inhibit production of osteoclastogenic cytokines [103,107].
IL-1	IL-1 is a potent inflammatory cytokine stimulator of bone resorption and plays a role in the regulation of immune and inflammatory responses to infections. IL-1 enhances the activity of RANKL by osteoblasts to stimulate osteoclastogenesis [92,99].
IL-6	IL-6 is a multifunctional cytokine produced by T cells, dendritic cells and macrophages to regulate hematopoiesis, inflammation, immune responses, and bone homeostasis. This cytokine is a major product of osteoblasts and their progenitors, and has been shown to stimulate the proliferation of osteoclasts and induce RANKL expression [101-104].
IL-7	IL-7 has several effects on the immunological and hematopoietic systems and plays an important role in the regulation of bone homeostasis. Under inflammatory conditions, cytokines such as IL-1 and TNF- α induce an increase the production of IL-7 that stimulates osteoclast formation by up-regulation of T cell secretion of osteoclastogenic cytokines [102,105,106].
IL-11	IL-11 is produced in bone marrow stromal cells and induces the formation of osteoclasts and bone resorption. In mice deficient in the specific IL-11 receptor, bone turnover is decreased [102,108,109].

Note: RANKL= receptor activator of nuclear factor kappa ligand; OPG= osteoprotegerin; M-CSF= macrophage colony-stimulating factor IFN- γ = Interferon-gamma (IFN- γ); TNF- α = tumor necrosis factor- α ; TGF- β = transforming growth factor beta; IL-1= interleukin-1; IL-6= interleukin-6; IL-7= interleukin-7; IL-11= interleukin-11. (*Adapted from Ofotokun et al. [6]*).

Capítulo 2.

Bone metabolism dysfunction mediated by the increase of proinflammatory cytokines in chronic HIV infection

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ABSTRACT

Despite the efficacy of antiretroviral therapy (ART) on the control of viral replication, the current challenge is to decrease the chronic inflammatory status and toxicity of the antiretroviral drugs that contribute to increase the risk of metabolic complications. **Objective:** To verify the influence of proinflammatory cytokines on bone metabolism mediated by chronic HIV infection. **Materials and Methods:** A cross-sectional study was conducted with 50 HIV-infected adult men treated or not with ART. Dual energy x-ray absorptiometry (DXA) was performed to assess bone mineral density. Biochemical analysis were performed of IL-6, TNF- α , osteocalcin, PTH, 25-OH-D, total calcium, albumin, 24h urinary calcium and urinary deoxypyridinoline. **Results:** The participants not treated with ART exhibited higher values of IL-6 and TNF- α than the participants treated with ART for more than 2 years. The TNF- α values was higher in the participants treated with ART for less than 2 years than in participants treated with ART for more than 2 years ($p < 0.05$). The increased values of urinary deoxypyridinoline indicated a high reabsorptive activity of bone tissue in all groups, with a significant difference between the participants not treated with ART and the participants treated with ART for less than 2 years. Through the DXA we found a bone mass reduction in all bone sites in each group. **Conclusion:** The increase production of proinflammatory cytokines, most notably in the viremic group have demonstrated to stimulate osteoclast activity affecting bone mass and the reduction of bone mineral density was observed in all bone sites principally for the groups in treatment with antiretroviral.

Keywords: HIV. ART. Proinflammatory cytokines. Bone mineral density. Urinary deoxypyridinoline.

1. INTRODUCTION

The natural history of HIV infection and antiretroviral treatment are well evidenced by increase in the CD4+ T cell count, reduction of viral replication, and reduced risk for opportunistic infections. Despite the efficacy of ART, the current repercussions are the systemic effects of inflammation mediated by the chronic HIV infection and toxicity of the antiretroviral drugs that significantly contribute to increase the risk of metabolic complications. There is evidence that these complications are more common in HIV-infected adults than in uninfected people of the same age, suggesting that HIV leads to premature aging [1].

One of the aspects highlighted for premature aging is HIV mediated chronic inflammation, which leads to persistent activation of the innate and adaptive immune systems associated with the release of proinflammatory cytokines, even after controlling the virus with ART, leading to lymphoid tissue abnormal function and the emergence of various diseases that would manifest later in life [1,2]. The release of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), and their increased levels, may interfere in bone metabolism and contribute to increased bone resorption [3, 4].

Simultaneously, the cumulative effect of HIV infection and the toxicity of the antiretroviral drugs contributes to mitochondrial dysfunction, which in turn increases the production of proinflammatory cytokines leading to an increase in osteoclast maturation and a decrease in osteoprotegerin, resulting in a reduction in bone mineral density (BMD) [4,5]. Additionally,

other factors common in this group of patients can also contribute to bone loss, such as low body mass index, alcoholism, smoking, drug use, decreased calcium intake, physical inactivity, vitamin D deficiency, hypogonadism, and increased parathyroid hormone serum concentrations [6-9].

Importantly, the direct effects of chronic inflammation due to HIV may increase bone reabsorption, resulting to a negative bone balance [7]. Therefore, the elevation of inflammatory biomarkers mediated by chronic HIV infection and the toxicity of the antiretroviral drugs influence metabolic disorders and increase the risk of morbidity and mortality [8,9]. This study aimed to verify the influence of proinflammatory cytokines on bone metabolism mediated by chronic HIV infection in patients undergoing antiretroviral treatment or not.

2. MATERIALS AND METHODS

2.1 Recruitment, Ethics and General Study design

This cross-sectional study was performed with 50 adult men, seropositive for HIV, with different viral load measurements and under treatment or not with ART. The exclusion criteria considered were: use of calcium and vitamin D supplements, use of drugs active on bone and mineral metabolism, previous thyroidectomy, presence of renal and/or hepatic insufficiency, hypophosphatemia, hypogonadism, rheumatic diseases and the presence opportunistic infection. The recruitment of the volunteers was conducted at the Clinical Hospital of the Ribeirão Preto Medical School,

University of São Paulo (HCFMRP-USP). The study was approved by the Research Ethics Committee of HCFMRP- USP, process No. 2605/2010 and all the volunteers gave their free prior and written informed consent to participate in this study.

Data collection (duration of HIV infection, length of ART exposure, CD4+ T cell count, and viral load quantification) was performed by reviewing the patient records of HCFMRP- USP. The volunteers were divided, according to the use or not of different antiretroviral regimen, into the CG: 10 volunteers not in treatment; the G<2 group: 20 volunteers treated with ART for up to 2 years; and the G>2 group: 20 volunteers treated with ART for more than 2 years.

2.2 Anthropometric evaluation

Weight and height were measured and the body mass index (BMI) was calculated using the formula (weight/height²) [10]. The patients were classified according to the recommendation of the World Health Organization (WHO) [19,20]. Body composition (lean mass and fat mass) was assessed by dual energy x-ray absorptiometry (DXA).

2.3 Evaluation of bone mineral density

Dual energy x-ray absorptiometry was used to evaluate the following bone sites: whole body, total hip, and lumbar spine, and was performed in the Radiology Department of HCFMRP- USP using a Hologic QDR 4500A scanner; Hologic Inc., Waltham, MA, USA. The bone mineral density

evaluation was performed according to the classifications proposed by the WHO (1994) [11]. Standard procedures were adopted for the placement of the volunteers into the supine position during the examination phase, without metal objects or shoes. The examination was performed through a full body scan of the patient.

2.4 Biochemical Analyses

For the biochemical analyzes, peripheral venous blood samples were collected after 12 hours of fasting, 24 hour urine collections and collect of the 2nd morning urine sample. After collection, the blood samples were centrifuged and the serum obtained stored in a freezer at -80 °C until the time of analysis. The urine samples were stored in a freezer at -20 °C until the time of analysis.

The inflammatory markers, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α), were determined with Luminex® equipment using a Milliplex® MAP Kit. The osteocalcin and PTH concentration were measured using the immuno-chemiluminescence method (ICMA) with an IMMULITE® 1000 analyzer and the serum levels of 25-OH vitamin D were determined by chemiluminescence (CLIA) using the LIAISON® 25-OH vitamin D test. The serum albumin was determined by the colorimetric method, using Labtest® Kits. Quantification of CD4+ T lymphocytes was determined through flow cytometry using a Multitest® Kit, and quantification of viral load through chemiluminescence using a Versant® HIV-1 RNA 3.0 Kit.

To determine the serum and urinary calcium concentrations a mass spectrometer equipped with an inductively coupled plasma source was used (DRC-ICP-MS ELAN DRCII, Perkin Elmer, Sciex, Norwalk, CT, USA), operating with high purity argon (99.999%, Praxaair, Brazil).

The urinary deoxypyridinoline determination performed through the enzyme immunoassay method (ELISA) using a Quidel brand kit. The urinary creatinine analysis, used in the calculations for the correction of urinary deoxypyridinoline, was determined by the automated enzymatic method, using a WIENER[®] kinetic creatinine AA Kit in a BT 3000 Plus device.

The parameters of Young (1987) were used as a reference for serum calcium, and those of Hodgkinson and Pyrah (1958) for urinary calcium [16,21]. The reference value of 25-OH vitamin D (ng/mL) was provided by the Liaison[®] Kit for the North American population, considering >150 excessive; >30 sufficient; 10 to 30 insufficient; and <10 deficient. The osteocalcin and PTH were calculated according to the reference value indicated by the Immulite[®] Kit (<2.0 to 21 ng/mL) and (12 to 65 pg/ml) respectively. The urinary deoxypyridinoline according to the reference value indicated by the Quidel[®] kit (2.3 to 5.4 nmol/mmol), and albumin according to the reference value of the Labtest[®] Kit (3.5 to 5.5 g/dL). The reference values used for CD4+ T lymphocytes were those published by the Ministry of Health [17]. Regarding the viral load quantification, undetectable viremia was considered for values less than 50 copies/mL HIV RNA, as proposed by the Versant[®] Kit.

2.5 Dietary evaluation

The dietary evaluation was performed by recording food intake over three days. Participants were instructed to fill out the forms immediately after eating to minimize errors and avoid possible underreporting. The dietary data obtained in household measures were converted to grams and milliliters to enable the chemical analysis of the food consumption and the information was processed through the Dietpro[®] version 5i nutritional analysis program [12]. The foods that were not included in the program were subsequently added, according to the database tables: Brazilian Food Composition Table (TACO) [13] and National Nutrient Database for Standard Reference (USDA) [14].

Energy consumption recommendations were obtained through the Melchior equation [27], and for macronutrients and calcium, the concepts proposed by the Dietary Reference Intakes (DRIs) were considered [15]. In the dietary evaluation the mean intake of macronutrients and calcium was considered, obtained from the three food records.

2.5 Statistical analysis

To describe the results obtained in the study in relation to the quantitative variables the mean and standard deviation were calculated, considering a 95% confidence level. The Kruskal Wallis test was used to compare the groups in relation to the continuous variables and, in the cases where there was no difference between any pair of groups, Dunn's post test was performed. The Fisher exact test was applied to determine the

association between categorical variables and the groups. The Spearman correlation coefficient (r) was used to determine correlations.

To compare the groups, with respect to the variables: urinary deoxypyridinoline, BMD of the femur, BMD of the lumbar spine, and BMD of the whole body, a method for selecting variables from a set of independent variables was first performed. The variable selection method used was the adjusted R^2 , which selects a set of variables that present the highest adjusted R^2 for the regression model. From the set of selected variables, analysis of covariance (ANCOVA) was proposed. This methodology allows the ANOVA to incorporate one or more quantitative variables that present a linear relationship with the response variable. The results of the statistical analyzes were obtained using the SPSS 17.01 software program [18].

3. RESULTS

The mean age of the participants of the G>2 group was higher: 47.0 ± 10.6 years, and lower in those of the CG group: 30.8 ± 9.4 years. There was a significant difference in age between the CG and the G>2 group, and between the G<2 group and the G>2 group ($p < 0.05$). The body mass index (kg/m^2) was greater for the G<2 group: 23.2 ± 4.1 , and lower for the CG group: 20.4 ± 6.7 , and according to the WHO BMI classification the volunteers presented normal weight. The percentage of fat mass was higher in the CG group: 24.9 ± 7.5 (**Table 1**).

The G>2 group presented a longer duration of HIV infection, undetectable viremia (<50 copies/ml) and an increased amount of CD4+ T

cells (mm^3). There was a significant difference in the duration of HIV infection between the CG and G<2 groups and between the CG the G>2 groups ($p<0.05$). Furthermore, the G<2 and G>2 groups showed significant differences in the length of exposure to antiretroviral therapy treatment ($p<0.05$) (**Table 1**).

Osteoporosis was observed in lumbar spine bone site in the three groups and, according to the number of participants, the G>2 group presented a higher percentage (20%). Additionally, in total hip and whole body exams, only the participants of the G>2 group presented osteoporosis (10%). The percentage of osteopenia in the whole body was lower for the participants of the CG and G<2 groups (40%), whereas it was 55% in the G>2 group. A significant difference was also observed in total hip BMD between the two groups on antiretroviral treatment ($p<0.05$) (**Table 1**).

Regarding the laboratory parameters, the serum concentrations of PTH, calcium, albumin and the bone formation marker osteocalcin presented values within normal limits. Calcemia was within normal limits, except for in the CG group: 8.5 ± 0.6 mg/dL, which showed a value slightly below the normal reference, with a significant difference between the CG and G>2 groups, as well as between the G<2 and G>2 groups ($p<0.05$). The bone reabsorption marker, urinary deoxypyridinoline, presented increased values in all the groups, with a significant difference between the CG and G<2 groups ($p<0.05$). The 25-OH vitamin D levels were below the acceptable limits in all groups (**Table 2**). When considering all participants ($n=50$), 34%

presented insufficient vitamin D values and 22% deficiency, with the lowest vitamin D serum value being for the group with longer exposure to ART.

The proinflammatory markers, IL-6 and TNF- α , presented the highest mean in the HIV seropositive group (CG), with a lower mean for the group with longer exposure to the antiretroviral treatment (G>2). The TNF- α values presented a significant difference between the CG and G>2 groups, as well as between the G<2 and G>2 groups ($p<0.05$) (**Table 2**). Evaluating all the participants and grouping them by detectable versus undetectable viral load (data not shown in tables) mean IL-6 values of 0.9 ± 1.1 pg/mL and 0.7 ± 1.0 pg/mL and mean TNF- α values of 15.2 ± 6.5 pg/mL and 12.0 ± 6.3 pg/mL, respectively were found, with no significant association independent of viral load.

Regarding food consumption, the groups mostly presented inappropriate consumption, with the intake of energy, carbohydrates, and protein greater than the need, and fat intake for the G<2 group equal to the recommendation and for the G>2 group lower than the recommendation. All groups presented inadequate calcium intakes. There was a significant difference only for fat intake between the CG and G<2 groups ($p<0.05$) (**Table 3**). The significant correlations of BMD with BMI, 25-OH vitamin D, and calcium are shown in **Table 4**. When the urinary deoxypyridinoline values were correlated with the continuous variables (data not shown in table) significant correlations were found with IL-6 ($r=-0.29$, $p<0.05$), and serum calcium ($r=0.37$; $p<0.05$).

Table 5 presents the results of linear regression models that were adjusted between the groups to check the possible influence that the variables: age, BMI, fat mass, lean mass, duration of HIV infection, length of exposure to antiretroviral therapy, calcium and protein intake, and serum and urinary biochemical markers, had on the bone health indicators evaluated: urinary deoxypyridinoline, total hip BMD, lumbar spine BMD, and whole body BMD.

4. DISCUSSION

In the present study an interesting aspect was observed with the individuals infected with HIV and not using ART presenting higher serum levels of inflammatory markers than the individuals in treatment, affirming that the initial phase of infection may be characterized by an increased production of proinflammatory cytokines and their persistence throughout the natural course of the disease, even in the presence of an effective suppression of viral replication with antiretroviral therapy [1,22]. In this study it was noted that the group with shorter exposure time to ART and to HIV infection presented higher serum inflammatory marker values than the group with longer treatment time, most likely this could be due to detectable viremia that directly contributed to the activation of T cells and the release of proinflammatory cytokines [1].

Interestingly some cytokines anti and pro-inflammatory such as IL-3, IL-4, IL-10, TGF- β and IL-1, IL-6, IL-12, IL-17, IFN- γ , TNF- α , respectively are involved in the regulation of bone remodeling with a crucial role to inhibit and

stimulate the activity of bone cells [36] and in this study the proinflammatory cytokines (TNF- α , IL-6) evaluated have demonstrated to stimulate osteoclast activity by the results observed in the bone resorption marker, mainly viremic groups.

At the same time it was observed that the persistent inflammation mediated by HIV and the toxicity of long-term ART use contributed to the increase in the bone reabsorption markers, directly affecting bone mass, especially for the group with the longest exposure to the virus and antiretrovirals. When analyzing the coefficients of determination (R^2) of the linear regression models it was observed that the length of HIV infection exposure had significant influence over the bone reabsorption marker with a tendency for reduced urinary deoxypyridinoline levels.

Despite the evidence of the adverse effects of use of the antiretroviral drug regimens, principally on use of tenofovir (TDF) leading to reduced bone mineral density due to the alterations in metabolism of vitamin D and phosphate and in osteoblast function [35], this evidence was not associated in our study by limitation of patients in treatment with TDF.

In a cross-sectional study, high levels of IL-6 were detected in HIV-infected individuals compared with uninfected subjects, with a significant correlation between the quantification of the viral load in patients and advanced HIV disease [23]. Another recent study found higher levels of plasma IL-6 in HIV-infected subjects when their viral load quantification was elevated [22], which draws attention to the data presented here, which, despite presenting no significant correlation between IL-6 and the increase

of viremia, showed increased IL-6 values in HIV-infected participants with detectable viremia compared to the infected individuals with undetectable viremia.

Regarding TNF- α , a recent study showed a significant association between elevated TNF- α levels and increased risk of progression to AIDS, even after adjustment for antiretroviral treatment, CD4+ T lymphocyte count and viral load quantification. Furthermore, the study found that the increased levels of inflammatory markers were independently associated with an increased risk of AIDS events versus non-AIDS events [24]. In the present study an increase was observed in the amounts of TNF- α in the group with detectable viremia compared to the group with undetectable viremia, with this finding being similar to another study that showed increased viral load in acute HIV infection associated with elevations in plasma levels of several cytokines, including a rapid rise in TNF- α [25].

Over production of proinflammatory cytokines such as IL-6, TNF- α , and others, can stimulate the activity of bone-reabsorbing cells, affecting bone remodeling and leading to bone metabolism dysfunction [4]. Similar results were identified in the present study, with reduced bone mineral density at different skeletal sites and greater involvement in the whole body bone site. A preclinical model developed in Erlangen showed high levels of HIV viral proteins and inflammatory cytokines, such as IL-6 and TNF- α , associated with increased osteoclast activity, considered to be one of the factors implicated in reduced bone mineral density in HIV-infected patients [26].

However, in the linear regression models for the bone densitometry values there was a reduction in femoral BMD when the effect of age was analyzed ($p<0.001$), unlike the BMI, that showed a tendency to increase the femoral BMD values (1kg each/m^2 led to an increase of 0.013 g/cm^2 BMD of the femur, ($p<0.001$). For the lumbar BMD, a decrease in value was found when the effect of 25-OH vitamin D ($p<0.01$) and total calcium ($p<0.05$) were analyzed, while, for the whole body BMD, only the length of exposure to antiretroviral therapy showed a significant influence on bone mass ($p=0.05$).

A study carried out in Bologna showed that proinflammatory cytokines, such as IL-6 and TNF- α , promoted an increase of osteoclastogenesis and bone reabsorption, and that high concentrations of HIV RNA were associated with elevated concentrations of RANKL (receptor activator of nuclear factor kappa-B ligand) [28]. Other studies have shown that exposure to ART results in a disturbance of the metabolism of amino acids directly into osteoblasts and osteoclasts, altering bone homeostasis, as well as the presence of mitochondrial dysfunction, thus contributing to an inflammatory process and consequently to the loss of bone mass [29,30]. It is important to emphasize that many factors can influence the activities of osteoblasts and osteoclasts or calcium homeostasis and calciotropic hormone, such as parathyroid hormone and vitamin D [31].

In this study, despite PTH values being within normal limits in all groups, it was observed that the group with longer exposure to both the treatment and HIV infection had higher PTH and bone reabsorption marker values and lower levels of IL-6, which may be justified due to better viremic

control [1]. Furthermore, all groups of this study showed insufficient serum levels of vitamin D, even though it is known that a reduction in vitamin D affects its role as an inhibitor in the production of PTH and reduces absorption of calcium from the intestinal tract, favoring the reduction of bone mass [32].

It was not detected alterations in other factors (low body weight, advanced age, hypercalciuria, and low CD4⁺ T cell count) that could contribute to reduce bone mass. However, the mean values of calcium and protein intake were, respectively, low and high in the three groups. It is necessary to take into consideration that the age of the group with longer exposure to antiretroviral therapy was higher than the age of the other two groups [33]. Nonetheless, the mean age of the G>2 was less than 50 years and these subjects showed a high frequency of osteoporosis. Therefore, it is important to call attention to that even with control of viremia through the use of antiretrovirals, inflammation persists and its chronic effects may contribute to bone loss [34].

The present study showed an increase in the production of proinflammatory cytokines, most notably in the untreated group, due to their higher levels and greater number of copies of HIV RNA, and that the inflammation persisted throughout the antiretroviral treatment, as observed in the other groups. The increased levels of urinary deoxypyridinoline mainly observed in the group with longer ART resulted in greater loss of bone mineral density of the whole body.

However, even in the presence of a suppression of viral replication through highly active antiretroviral therapy, chronic activation of the innate immune system persists releasing proinflammatory cytokines and contributing to the increased risk of metabolic complications and the emergence of multiple morbidities. Thus, it is extremely important to conduct longitudinal studies to better detect damage in various tissues due to the persistence of inflammation over time, aiming to prevent harm and restore the health of HIV-infected individuals.

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CONFLICT OF INTERESTS

All authors have no conflicts of interest to declare.

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Table 1: General characteristics of HIV-seropositive patients according to study group.

Variables	Control group (CG)	Group <2 years of ART (G<2)	Group >2 years of ART (G>2)
n	10	20	20
Age (years)	30.8±9.4 ^a	34.2±10.6 ^a	47.0±10.6 ^b
BMI (kg/m ²)	20.4±6.7	23.2±4.1	22.9±2.6
Fat mass (%)	24.9±7.5	23.8±6.8	23.3±5.4
Lean mass (%)	69.4±7.0	70.2±6.1	71.6±5.2
Duration of infection in months	34.8±20.7 ^a	34.2±22.8 ^a	162.0±62.5 ^b
Time of exposure to ART in months	-	10±6.7 ^a	127.2±54.3 ^b
NRTIs+NNRTIs-based ART [n(%)]	-	10 (50)	10 (50)
PIs+NRTIs-based ART [n(%)]	-	10 (50)	10 (50)
TDF (NRTIs)-based ART [n(%)]	-	11 (55)	7 (35)
Undetectable VL (%)	12.5	70	100
CD4+ (cells/mm ³)	521.9±82.6	424.1±275.8 ^a	593.1±234.7 ^b
Lumbar spine <i>T score</i>	-0.7±0.9	-0.9±0.8	-1.1±0.8
Osteopenia [n (%)]	3 (30)	9 (45)	7 (35)
Osteoporosis [n (%)]	1 (10)	1 (5)	4 (20)
Hip total <i>T score</i>	-0.51±0.6	-0.33±0.9 ^a	-0.95±0.78 ^b
Osteopenia [n (%)]	2 (20)	4 (20)	6 (30)
Osteoporosis [n (%)]	-	-	2 (10)
Whole body <i>T score</i>	-0.7±0.8	-0.6±0.8	-0.9±0.7
Osteopenia [n (%)]	4 (40)	8 (40)	11 (55)
Osteoporosis [n (%)]	-	-	2 (10)

Note: Kruskal-Wallis Test. Values are reported as mean ± SD. ^{a,b} Different letters indicate p <0.05.

BMI=body mass index; ART=antiretroviral therapy; NRTIs=nucleoside analog reverse-transcriptase inhibitors; NNRTIs=non-nucleoside analog reverse-transcriptase inhibitors; PIs= protease inhibitors; TDF= tenofovir; VL = viral load; undetectable VL: < 50 copies/mL.

Table 2: Comparisons between serum and urinary biochemical concentrations according to study group

Variables	Control group (CG)	Group <2 years of ART (G<2)	Group >2 years of ART (G>2)
DPD (nmol/mmol)	7.5±2.3 ^a	9.7±2.9 ^b	8.5±3.0
Osteocalcin (ng/mL)	0.5±0.3	0.4±0.2	0.6±0.9
PTH (pg/mL)	18.8±13.6	16.1±10.9	24.9±19.7
25 OH vitamin D (ng/mL)	29.0±11.3	29.1±9.5	26.8±7.7
TNF-α (pg/mL)	16.8±8.8 ^a	14.0±4.3 ^a	10.0±5.9 ^b
IL-6 (pg/mL)	1.0±1.1	0.8±0.9	0.5±1.0
Total calcium (mg/dL)	8.5±0.6 ^a	8.7±0.7 ^a	9.3±1.0 ^b
Urinary calcium (mg/Kg/24 h)	1.3±0.6	1.1±0.8	1.3±0.8
Albumin (mg/dL)	4.0±0.3	4.1±0.6	3.9±0.6

Note: Kruskal-Wallis test. Values are reported as mean ± SD. ^{a,b} Different letters indicate p<0.05. ART=antiretroviral therapy; DPD=urinary deoxypyridinoline; PTH=parathormone intact molecule; TNF-α=tumor necrosis factor alpha; IL-6= interleukin-6.

Table 3: Food intake values energy, macronutrient and calcium intake according to study group

Variables	Control group (CG)	Group <2 years of ART (G<2)	Group >2 years of ART (G>2)
Energy (kcal)	2139.3±621.1	1843.0±474.2	2096.6±1027.7
Recommendation	1787.8±133.0	1766.8±268.9	1740.0±195.1
CHO (g)	290.8±113.2	260.8±88.9	295.8±160.2
Recommendation	245.8±18.3	242.9±37.0	293.3±26.8
Protein (g)	118.5±51.7	107.4±57.1	125.1±68.9
Recommendation	67.0±5.0	66.3±10.1	65.3±7.3
Lipids (g)	62.7 ± 17.1 ^a	48.7 ± 14.3 ^b	53.5±30.9
Recommendation	59.6±4.4	48.7±9.0	58.0±7.3
Calcium (mg)	642.9±218.3	684.6±315.3	786.7±400.2
Recommendation	800	800	800

Note: Kruskal-Wallis test. Values are reported as mean ± SD. ^{a,b} Different letters indicate

p<0.05. ART=antiretroviral therapy; CHO=carbohydrates in grams. Recommendation:

Dietary Reference Intakes.

Table 4: Spearman correlation between the quantitative variables and bone densitometry of the study groups.

Variables	Total Hip BMD	p*	Lumbar spine BMD	p*	Whole body BMD	p*
Age (years)	-0.24	0.08	-0.01	0.93	-0.24	0.08
BMI (kg/m ²)	0.42	<0.01	0.10	0.47	0.01	0.94
Fat mass (%)	-0.11	0.42	0.07	0.62	-0.20	0.15
Lean mass (%)	0.10	0.47	-0.06	0.65	0.18	0.19
DPD (nmol/mmol)	-0.04	0.75	-0.20	0.14	-0.01	0.97
PTH (pg/mL)	-0.01	0.94	0.15	0.28	-0.10	0.44
25 OH vitamin D (ng/mL)	-0.16	0.24	-0.37	<0.01	0.15	0.28
TNF- α (pg/mL)	-0.01	0.95	0.07	0.58	0.08	0.53
IL-6 (pg/mL)	-0.23	0.10	-0.01	0.89	0.07	0.58
Total calcium (mg/dL)	-0.19	0.17	-0.22	0.11	-0.12	0.37
Urinary calcium (mg/Kg/24 h)	-0.01	0.90	-0.27	0.05	0.14	0.29
CD4+ (cells/mm ³)	-0.02	0.87	<-0.01	0.99	0.07	0.60
PTN consumption (g)	0.14	0.31	0.13	0.33	0.22	0.11
Calcium consumption (mg)	0.23	0.10	-0.10	0.45	0.11	0.43
Duration of infection	-0.14	0.32	0.04	0.77	0.03	0.80
Time of exposure to ART	-0.12	0.37	-0.07	0.62	-0.24	0.08

Note: Significant values: $p < 0.05$. BMD= total bone mineral density in g/cm²; BMI = body mass index;

TNF- α = Tumor necrosis factor alpha; IL-6= interleukin-6; PTN=protein; duration of infection in months; time of exposure to ART in months; ART=antiretroviral therapy.

Table 5: Linear regression models for total hip bone densitometry values (model I), for bone densitometry values of the lumbar spine (model II), for whole body bone densitometry values (model III) and for urinary deoxypyridinoline biochemical values (model IV).

Model	Parameters	Estimate	p-value	95% CI		R ²
				LL	UL	
I	Age (years)	-0.005	<0.001	-0.007	-0.003	0.47
	BMI (kg/m ²)	0.013	<0.001	0.006	0.019	
	IL-6 (pg/mL)	-0.018	0.18	-0.045	0.009	
	Calcium consumption (mg)	<0.001	0.13	<0.001	<0.001	
II	25 OH vitamin D (ng/mL)	-0.004	<0.001	-0.007	-0.001	0.25
	Total calcium (mg/dL)	-0.032	<0.05	-0.061	-0.003	
	Urinary calcium (mg/Kg/24 h)	-0.013	0.41	-0.046	0.019	
III	Time of exposure to ART	0.0004	0.05	-0.0007	0.0002	0.13
	PTN consumption (g)	0.0004	0.07	-0.00004	0.001	
IV	Duration of infection	-0.012	<0.05	-0.023	-0.002	0.25
	IL-6 (pg/mL)	-0.502	0.21	-1.295	0.292	
	Urinary calcium (mg/Kg/24 h)	1.306	0.01	0.258	2.355	

Note: The linear regression model I; II and III were adjusted according to serum concentration variables. Statistically significant: $p < 0.05$. CI=confidence interval; LL=lower limit; UL=upper limit; R²=coefficient of variation; BMI=body mass index; IL-6=interleukin-6; Ca=calcium; PTN=protein; time of exposure to ART in months; duration of infection in months.

Considerações finais

O campo da osteoimunologia continua a evoluir e tem sido um grande desafio para melhor entender a fisiologia da perda óssea no contexto do HIV e aos diferentes regimes de antirretrovirais. De fato, como o HIV conduz para perturbações na interface imuno-esquelético torna-se extremamente importante para os contínuos processos investigativos para a inexplicada convergência da inflamação associada ao HIV na era TARV, e a imunossenescência implicada para o início prematuro de outras comorbidades, no qual se destaca a perda da massa óssea.

Nossos resultados colaboram para o conceito que o estado inflamatório associado com a ativação crônica do sistema imunológico pode contribuir significativamente para a disfunção na interface imuno-esquelético e independente do regime da terapia antirretroviral.

Vale ressaltar que a causalidade é certamente multifatorial, em geral inclui, a questão de estilo de vida, idade mais avançada, menopausa, baixo índice de massa corporal, deficiência de vitamina D, ingestão inadequada de cálcio, outros medicamentos, tabagismo, inatividade física em conjunto com a duração da infecção pelo HIV e tempo de uso da TARV.

Assim, novas estratégias terapêuticas destinadas a prevenir ou reverter alterações imunológicas ou novos mecanismos moleculares na comunicação intracelular na interface imuno-esquelético podem valer a pena explorar ainda mais para melhor compreensão ou para novos achados de indicadores de diagnósticos dos distúrbios do esqueleto a fim de que as pessoas soropositivas para HIV alcancem uma vida normal.

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**Anexo 1 – Autorização recebida pelo Journal Bone Mineral Metabolism
para incluir o artigo completo no capítulo 2**

From: "Kanako Nakakura" <nakakura@ac-square.co.jp>
Date: 19 January 2017 12:51:48 AM GMT-2
To: "Erika Grasiela Marques de Menezes" <erikagmm7@yahoo.com.br>
Subject: RE: Request permission - JBMM-D-15-00314R1

Dear Dr. Erika Grasiela Marques de Menezes, Ph.D

Thank you very much for your prompt reply.

If you have no relation with companies, you don't need any application to us, and we would like you just to clarify the detailed information, Vol., Issue, and page number of your originating paper of JBMM when you cite it in your thesis.

With best regards,

Kanako Nakakura
JBMM editorial secretary

From: Erika Grasiela Marques de Menezes [<mailto:erikagmm7@yahoo.com.br>]
Sent: Thursday, January 19, 2017 2:36 AM
To: jsbmr@ac-square.co.jp
Subject: Request permission - JBMM-D-15-00314R1

Dear JBMM Editorial Office

Please I'd like to request a permission to include my full article in my thesis that was published on March, 2016.

The article entitled is "Bone metabolism dysfunction mediates by the increase of proinflammatory cytokines in chronic HIV infection published in the Journal Bone and Mineral Metabolism (DOI 10.1007/s00774-016-0749-8).

Thank you.

Sincerely,
Erika Grasiela Marques de Menezes, Ph.D