AVALIAÇÃO DOS NÍVEIS PLASMÁTICOS E URINÁRIOS DAS CITOCINAS EM CRIANÇAS E ADOLESCENTES PORTADORES DE HIPERCALCIÚRIA IDIOPÁTICA

Augusto César Soares dos Santos Junior

Universidade Federal de Minas Gerais

Belo Horizonte

2011

Augusto César Soares dos Santos Junior

AVALIAÇÃO DOS NÍVEIS PLASMÁTICOS E URINÁRIOS DAS CITOCINAS EM CRIANÇAS E ADOLESCENTES PORTADORES DE HIPERCALCIÚRIA IDIOPÁTICA

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde - Área de Concentração Saúde da Criança e do Adolescente - da Faculdade de Medicina da Universidade Federal de Minas Gerais sob a orientação da Prof^{a}. Dr^{a}. Ana Cristina Simões e Silva.

Belo Horizonte

Faculdade de Medicina da UFMG

2011

UNIVERSIDADE FEDERAL DE MINAS GERAIS
FACULDADE DE MEDICINA

AVALIAÇÃO DOS NÍVEIS PLASMÁTICOS E URINÁRIOS DAS CITOCINAS EM CRIANÇAS E ADOLESCENTES PORTADORES DE HIPERCALCIÚRIA IDIOPÁTICA

Augusto César Soares dos Santos Junior

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde - Área de Concentração Saúde da Criança e do Adolescente - da Faculdade de Medicina da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do grau Mestre.

Orientadora: Profª. Ana Cristina Simões e Silva
Professora Titular do Departamento de Pediatria
Faculdade de Medicina da Universidade Federal de Minas Gerais

Co-Orientadora: Profª. Maria Goretti Moreira Guimarães Penido
Professora Adjunta do Departamento de Pediatria
Faculdade de Medicina da Universidade Federal de Minas Gerais

Belo Horizonte
2011

Santos Junior, Augusto César Soares dos.
S237a Avaliação dos níveis plasmáticos e urinários das citocinas em crianças e adolescentes portadores de hipercalciúria idiopática [manuscrito]. / Augusto César Soares dos Santos Junior. - - Belo Horizonte: 2011.
103f.: il.
Orientadora: Ana Cristina Simões e Silva.
Co-Orientadora: Maria Goretti Moreira Guimarães Penido.
Área de concentração: Saúde da Criança e do Adolescente.
Dissertação (mestrado): Universidade Federal de Minas Gerais, Faculdade de Medicina.

1. Hipercalciúria. 2. Citocinas. 3. Biomarcadores Farmacológicos. 4. Doenças Ósseas Metabólicas. 5. Quimiotaxia de Leucócito. 6. Dissertações Acadêmicas. I. Silva, Ana Cristina Simões e. II. Penido, Maria Goretti Moreira Guimarães. III. Universidade Federal de Minas Gerais, Faculdade de Medicina. IV. Título.

NLM: WS 270

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

FACULDADE DE MEDICINA
CENTRO DE PÓS-GRADUAÇÃO
Av. Prof. Alfredo Balena 190 / sala 533
Belo Horizonte - MG - CEP 30.130-100
Fone: (031) 3409.9641 FAX: (31) 3409.9640
cpg@medicina.ufmg.br
UFMG

ATA DA DEFESA DE DISSERTAÇÃO DE MESTRADO de **AUGUSTO CÉSAR SOARES DOS SANTOS JÚNIOR** nº de registro 2009654735. Às nove horas, do dia **vinte e quatro de fevereiro de dois mil e onze**, reuniu-se na Faculdade de Medicina da UFMG, a Comissão Examinadora de dissertação indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho intitulado: **"AVALIAÇÃO DOS NÍVEIS PLÁSMÁTICOS E URINÁRIOS DAS CITOCINAS EM CRIANÇAS E ADOLESCENTES PORTADORES DE HIPERCALCIÚRIA IDIOPÁTICA""**, requisito final para a obtenção do Grau de Mestre em Ciências da Saúde: Saúde da Criança e do Adolescente, pelo Programa de Pós-Graduação em Ciências da Saúde: Saúde da Criança e do Adolescente. Abrindo a sessão, a Presidente da Comissão, Profa. Ana Cristina Simões e Silva, após dar a conhecer aos presentes o teor das Normas Regulamentares do trabalho final, passou a palavra ao candidato para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu sem a presença do candidato e do público para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações:

Profa. Ana Cristina Simões e Silva/Orientador	Instituição: UFMG	Indicação: APROVADO
Profa. Rosa Maria Affonso Moysés	Instituição: USP	Indicação: APROVADO
Prof. Eduardo Araújo de Oliveira	Instituição: UFMG	Indicação: APROVADO

Pelas indicações o candidato foi considerado APROVADO

O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a sessão e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 24 de fevereiro de 2011.

Profa. Ana Cristina Simões e Silva /Orientador _____

Profa. Rosa Maria Affonso Moysés _____

Prof. Eduardo Araújo de Oliveira _____

Profa. Ana Cristina Simões e Silva/Coordenadora _____

Obs.: Este documento não terá validade sem a assinatura e carimbo do Coordenador.

Profa. Ana Cristina Simões e Silva
Coordenadora do Programa de Pos-Graduação em
Ciências da Saúde: Saúde da Criança e do Adolescente
Faculdade de Medicina/UFMG

CONFERE COM O ORIGINAL
Centro de Pós-Graduação

FACULDADE DE MEDICINA
CENTRO DE PÓS-GRADUAÇÃO
Av. Prof. Alfredo Balena 190 / sala 533
Belo Horizonte - MG - CEP 30.130-100
Fone: (031) 3409.9641 FAX: (31) 3409.9640
cpg@medicina.ufmg.br

UFMG

DECLARAÇÃO

A Comissão Examinadora, abaixo assinada, composta pelos Professores Doutores: Ana Cristina Simões e Silva, Rosa Maria Affonso Moysés, e Eduardo Araújo de Oliveira, aprovou a defesa da dissertação intitulada: **"AVALIAÇÃO DOS NÍVEIS PLÁSMÁTICOS E URINÁRIOS DAS CITOCINAS EM CRIANÇAS E ADOLESCENTES PORTADORES DE HIPERCALCIÚRIA IDIOPÁTICA"**, apresentado pelo mestrando **AUGUSTO CÉSAR SOARES DOS SANTOS JÚNIOR,** para obtenção do título de Mestre, pelo Programa de Pós-Graduação em Ciências da Saúde: Saúde da Criança e do Adolescente da Faculdade de Medicina da Universidade Federal de Minas Gerais, realizada em 24 de fevereiro de 2011.

Profa. Ana Cristina Simões e Silva
Orientador

Profa. Rosa Maria Affonso Moysés

Prof. Eduardo Araújo de Oliveira

UNIVERSIDADE FEDERAL DE MINAS GERAIS

Reitor: Prof. Clélio Campolina Diniz

Vice-Reitor: Profª. Rocksane de Carvalho Norton

Pró-Reitor de Pós Graduação: Prof. Ricardo Santiago Gomez

Pró-Reitor de Pesquisa: Prof. Renato de Lima dos Santos

FACULDADE DE MEDICINA

Diretor: Prof. Francisco José Penna

Vice-Diretor: Prof. Tarcizo Afonso Nunes

Coordenador do Centro de Pós-Graduação:

Prof. Manoel Otávio da Costa Rocha

Sub-coordenadora do Centro de Pós-Graduação:

Profª. Teresa Cristina de Abreu Ferrari

Chefe do Departamento de Pediatria: Profª. Maria Aparecida Martins

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE – ÁREA DE CONCENTRAÇÃO SAÚDE DA CRIANÇA E DO ADOLESCENTE

Coordenadora: Profª. Ana Cristina Simões e Silva

Sub-coordenador: Prof. Eduardo Araújo Oliveira

COLEGIADO DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE – ÁREA DE CONCENTRAÇÃO EM SAÚDE DA CRIANÇA E DO ADOLESCENTE

Profª. Ana Cristina Simões e Silva

Prof. Cassio da Cunha Ibiapina

Prof. Eduardo Araújo Oliveira

Prof. Francisco José Penna

Profª. Ivani Novato Silva

Prof. Jorge Andrade Pinto

Prof. Marcos José Burle de Aguiara

Profª. Maria Cândida Ferrarez Bouzada Viana

Representante discente: Michelle Ralil da Costa

À minha esposa, Ana Carolina, pelo estímulo, apoio e presença.

À minha querida filha, Ana Luísa, que participou comigo dessa jornada desde a sua gestação.

A Deus, presente em todos os momentos.

AGRADECIMENTOS

À Prof[a]. Dra. Ana Cristina Simões e Silva, presente desde os meus primeiros passos na iniciação científica, pela amizade, ensinamentos e confiança.

À Prof[a]. Dra. Maria Goretti Moreira Guimarães Penido, pela amizade, convivência e colaborações que fizeram despertar em mim o gosto pela nefrologia.

Aos meus familiares por compreenderem meus momentos de ausência.

Ao Prof. Mauro Martins Teixeira pela grande colaboração na execução do trabalho.

À Kátia Daniela da Silveira pelo apoio no árduo trabalho de laboratório.

Meus pais, Augusto e Maria da Conceição, pelo amor, confiança, dedicação, e por estarem sempre ao meu lado, investindo na minha formação mesmo nos momentos de dificuldade.

À minha irmã Rosana, companheira de todos os momentos.

Enfim, a todos aqueles que contribuíram de alguma forma para a conclusão deste trabalho, o meu carinho.

“Viver sem filosofar é o que se chama ter os olhos fechados

sem nunca os haver tentado abrir”

René Descartes

NOTA EXPLICATIVA

A apresentação da presente dissertação foi organizada sob a forma de artigos científicos, de acordo com a resolução 03/2010, aprovada pelo Programa de Pós-graduação em Ciências da Saúde, Área de concentração Saúde da Criança e do Adolescente, da Faculdade de Medicina da Universidade Federal de Minas Gerais, disponível em http://www.medicina.ufmg.br/cpg/programas/saude_crianca/arquivos/2010/Resolucao03-2010.pdf.

O primeiro artigo consiste em uma revisão da literatura, na qual são discutidos os principais aspectos, achados recentes e controvérsias sobre o papel das citocinas no remodelamento ósseo e na hipercalciúria idiopática (HI). O segundo artigo avalia os níveis plasmáticos e urinários de citocinas e quimiocinas associadas à regulação do metabolismo ósseo em crianças e adolescentes com HI em acompanhamento ambulatorial na Unidade de Nefrologia Pediátrica do Hospital das Clínicas da Universidade Federal de Minas Gerais, entre 2009 e 2010.

As referências bibliográficas estão dispostas ao final de cada artigo ou seção, conforme as normas de Vancouver (Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication - www.ICMJE.org).

LISTA DE ABREVIATURAS E SIGLAS

COEP – Comitê de Ética em Pesquisa

DMO – Densidade mineral óssea

DP – Desvio padrão

ELISA – Enzyme-linked immunosorbent assay

GM-CSF - Granulocyte-macrophage colony-stimulating factor

HI – Hipercalciúria idiopática

IL-1β – Interleucina 1 beta

IL-6 - Interleucina 6

IL-8 - Interleucina 8

MCP-1 - Proteína de quimiotaxia de monócitos-1

M-CSF - Macrophage colony-stimulating factor

OPG - Osteoprotegerina

RANK - Receptor activator of NF-κβ

RANKL - Receptor activator of NF-κβ ligand

TGF-β - Fator de crescimento e transformação β

TNF-α - Fator de necrose tumoral do tipo alfa

UFMG – Universidade Federal de Minas Gerais

SUMÁRIO

1. INTRODUÇÃO

A hipercalciúria idiopática (HI), descrita por Albright et al (1), é a principal alteração metabólica responsável pela formação de cálculos urinários (2-4). Acomete todas as faixas etárias (5) e caracteriza-se pela hiperexcreção urinária de cálcio na ausência de estados hipercalcêmicos ou de qualquer outra doença primária (6-8).

A hipercalciúria é definida como excreção de cálcio igual ou maior a 4mg/kg/24h em crianças e adolescentes. Em adultos, entende-se por hipercalciúria, excreção urinária de cálcio igual ou maior que 300 mg/24h e 250mg/24h, para homens e mulheres respectivamente. (9-10)

Apesar de muito estudada, a fisiopatologia da HI permanece obscura. Atualmente, se sabe que a HI é uma entidade complexa associada a modificações em fatores regulatórios envolvidos na absorção intestinal, no remodelamento ósseo e na excreção urinária de cálcio. (11)

Nos últimos anos, tem sido proposto que alterações no perfil de citocinas e quimiocinas podem estar envolvidas no processo que determina o aparecimento da hipercalciúria e da osteopenia em pacientes com HI (12-13). As citocinas são um grupo de proteínas produzidas por células de vários tecidos, com propriedades sinalizadoras, auxiliando na comunicação intercelular (14). Já as

quimiocinas constituem um grupo de citocinas de baixo peso molecular cuja principal ação é o recrutamento e ativação de leucócitos em vários modelos de inflamação (15).

Diversos autores relataram redução da densidade mineral óssea em adultos e crianças portadores de HI, sugerindo a necessidade de uma investigação mais aprofundada sobre os mecanismos regulatórios do remodelamento ósseo na HI (16-26). Estudos clínicos e experimentais demonstraram a participação das citocinas e quimiocinas no processo que regula o remodelamento ósseo, controlando tanto a formação quanto a reabsorção óssea (25, 27-28). No entanto, a participação dessas substâncias na fisiopatologia da HI em crianças e adolescentes ainda não foi esclarecida.

Nesse contexto, o presente estudo tem por escopo avaliar, em crianças e adolescentes portadores de HI, os níveis plasmáticos e urinários da interleucina 1 beta (IL-1β), interleucina 6 (IL-6), interleucina 8 (IL-8), fator de necrose tumoral do tipo alfa (TNF-α), fator de crescimento e transformação β (TGF-β) e proteína de quimiotaxia de monócitos-1 (MCP-1).

REFERÊNCIAS BIBLIOGRÁFICAS

1. Albright F, Henneman P, Benedict PH, Forbes AP. Idiopathic hypercalciuria: a preliminary report. Proc R Soc Med. 1953 Dec;46(12):1077-81.

2. Stapleton FB. Idiopathic hypercalciuria: association with isolated hematuria and risk for urolithiasis in children. The Southwest Pediatric Nephrology Study Group. Kidney Int. 1990 Feb;37(2):807-11.

3. Stapleton FB, Miller LA. Renal function in children with idiopathic hypercalciuria. Pediatr Nephrol. 1988 Apr;2(2):229-35.

4. Coe FL, Parks JH, Asplin JR. The pathogenesis and treatment of kidney stones. N Engl J Med. 1992 Oct 15;327(16):1141-52.

5. Vezzoli G, Soldati L, Gambaro G. Hypercalciuria revisited: one or many conditions? Pediatr Nephrol. 2008 Apr;23(4):503-6.

6. Ammenti A, Neri E, Agistri R, Beseghi U, Bacchini E. Idiopathic hypercalciuria in infants with renal stones. Pediatr Nephrol. 2006 Dec;21(12):1901-3.

7. Moore ES, Coe FL, McMann BJ, Favus MJ. Idiopathic hypercalciuria in children: prevalence and metabolic characteristics. J Pediatr. 1978 Jun;92(6):906-10.

8. Spivacow FR, Negri AL, del Valle EE, Calvino I, Zanchetta JR. Clinical and metabolic risk factor evaluation in young adults with kidney stones. Int Urol Nephrol. 2010 Jun;42(2):471-5.

9. Coe FL, Evan A, Worcester E. Kidney stone disease. J Clin Invest. 2005 Oct;115(10):2598-608.

10. Srivastava T, Schwaderer A. Diagnosis and management of hypercalciuria in children. Curr Opin Pediatr. 2009 Apr;21(2):214-9.

11. Srivastava T, Alon US. Pathophysiology of hypercalciuria in children. Pediatr Nephrol. 2007 Oct;22(10):1659-73.

12. Tasca A, Dalle Carbonare L, Nigro F, Giannini S. Bone disease in patients with primary hypercalciuria and calcium nephrolithiasis. Urology. 2009 Jul;74(1):22-7.

13. Pacifici R. The immune system and bone. Arch Biochem Biophys. 2010 Nov 1;503(1):41-53.

14. Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol. 2003 Feb;111(2 Suppl):S460-75.

15. Segerer S, Alpers CE. Chemokines and chemokine receptors in renal pathology. Curr Opin Nephrol Hypertens. 2003 May;12(3):243-9.

16. Penido MG, Lima EM, Marino VS, Tupinamba AL, Franca A, Souto MF. Bone alterations in children with idiopathic hypercalciuria at the time of diagnosis. Pediatr Nephrol. 2003 Feb;18(2):133-9.

17. Penido MG, Lima EM, Souto MF, Marino VS, Tupinamba AL, Franca A. Hypocitraturia: a risk factor for reduced bone mineral density in idiopathic hypercalciuria? Pediatr Nephrol. 2006 Jan;21(1):74-8.

18. Vezzoli G, Rubinacci A, Bianchin C, Arcidiacono T, Giambona S, Mignogna G, et al. Intestinal calcium absorption is associated with bone mass in stone-forming women with idiopathic hypercalciuria. Am J Kidney Dis. 2003 Dec;42(6):1177-83.

19. Polito C, Iolascon G, Nappi B, Andreoli S, La Manna A. Growth and bone mineral density in long-lasting idiopathic hypercalciuria. Pediatr Nephrol. 2003 Jun;18(6):545-7.

20. Garcia-Nieto V, Ferrandez C, Monge M, de Sequera M, Rodrigo MD. Bone mineral density in pediatric patients with idiopathic hypercalciuria. Pediatr Nephrol. 1997 Oct;11(5):578-83.

21. Tasca A, Cacciola A, Ferrarese P, Ioverno E, Visona E, Bernardi C, et al. Bone alterations in patients with idiopathic hypercalciuria and calcium nephrolithiasis. Urology. 2002 Jun;59(6):865-9; discussion 9.

22. Freundlich M, Alonzo E, Bellorin-Font E, Weisinger JR. Reduced bone mass in children with idiopathic hypercalciuria and in their asymptomatic mothers. Nephrol Dial Transplant. 2002 Aug;17(8):1396-401.

23. Skalova S, Palicka V, Kutilek S. Bone mineral density and urinary N-acetyl-beta-D-glucosaminidase activity in paediatric

patients with idiopathic hypercalciuria. Nephrology (Carlton). 2005 Apr;10(2):99-102.

24. Giannini S, Nobile M, Sartori L, Calo L, Tasca A, Dalle Carbonare L, et al. Bone density and skeletal metabolism are altered in idiopathic hypercalciuria. Clin Nephrol. 1998 Aug;50(2):94-100.

25. Weisinger JR, Alonzo E, Bellorin-Font E, Blasini AM, Rodriguez MA, Paz-Martinez V, et al. Possible role of cytokines on the bone mineral loss in idiopathic hypercalciuria. Kidney Int. 1996 Jan;49(1):244-50.

26. Jaeger P, Lippuner K, Casez JP, Hess B, Ackermann D, Hug C. Low bone mass in idiopathic renal stone formers: magnitude and significance. J Bone Miner Res. 1994 Oct;9(10):1525-32.

27. Ghazali A, Fuentes V, Desaint C, Bataille P, Westeel A, Brazier M, et al. Low bone mineral density and peripheral blood monocyte activation profile in calcium stone formers with idiopathic hypercalciuria. J Clin Endocrinol Metab. 1997 Jan;82(1):32-8.

28. Pacifici R, Rothstein M, Rifas L, Lau KH, Baylink DJ, Avioli LV, et al. Increased monocyte interleukin-1 activity and decreased vertebral bone density in patients with fasting idiopathic hypercalciuria. J Clin Endocrinol Metab. 1990 Jul;71(1):138-45.

2. REVISÃO DA LITERATURA

BONE DISEASE AND CYTOKINES IN IDIOPATHIC HYPERCALCIURIA: A REVIEW

Augusto C. S. Santos Jr[1], Ana Cristina Simões e Silva[1]

[1]Pediatric Nephrology Unit, Department of Pediatrics, Faculty of Medicine, Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil

Key words: biomarkers, cytokines, MCP-1, idiopathic hypercalciuria, mineral bone disease

Conflicts of interest: none

Correspondence: Ana Cristina Simões e Silva, MD, PhD
Avenida Bernardo Monteiro 1300 / 1104
Belo Horizonte - Minas Gerais
Postal Code: 30150-281
Email: acssilva@hotmail.com

2.1. ABSTRACT

Bone remodeling is a continuous and dynamic process of skeletal destruction and renewal. A complex regulatory mechanism with the participation of several cytokines precisely defines the role of osteoclasts in the chain of events leading to bone resorption. There are multiple mechanisms underlying the regulation of bone resorption, which may involve increased calcium excretion and decreased bone density in patients with IH. However, the pathogenesis of bone mass reduction in IH remains uncertain. The purpose of this review is to summarize the recent published evidence on the possible mechanisms by which cytokines may be associated to the pathogenesis of IH.

2.2. INTRODUCTION

Idiopathic hypercalciuria (IH) was first described by Albright et al. and is characterized by normal serum calcium levels and excessive urine calcium loss. It is the most common metabolic abnormality in patients with nephrolithiasis accounting for 30-50% of calcium-oxalate stone formers. (1-4)

IH is defined by a daily urinary calcium excretion equal or superior to 4mg/kg or >300 mg Ca/d (7.5 mmol) in men and >250mg Ca/d (6.25 mmol) in women. (5) The diagnosis of IH depends on the exclusion of other causes of hypercalciuria such as sarcoidosis, malignancy, Paget's disease, high calcium or vitamin D intake, renal tubular acidosis and thyrotoxicosis. The most frequent clinical findings in IH are hematuria, abdominal and flank pain, urinary tract infections, nephrolithiasis, dysuria, urinary frequency, nocturnal enuresis and osteopenia. (6, 7)

The pathogenesis of IH is not yet fully understood. It is generally considered that IH is due to an alteration in calcium homeostasis at sites where large amounts of calcium must be precisely controlled. Immune mediators, which stimulate bone resorption, seem to be critically involved in the pathogenesis of IH. Experimental studies have demonstrated the importance of cytokines in the regulation of resorption and calcium release from bones. (8-14)

The purpose of this review is to summarize the recent published evidence on the possible mechanisms by which cytokines may be associated to the pathogenesis of IH.

2.3. BRIEF OVERVIEW OF CYTOKINE FUNCTION IN BONE METABOLISM

Cytokines are redundant secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of immune responses and control the immune cell trafficking and the cellular arrangement of immune organs. These mediators are involved in virtually every facet of immunity and inflammation, including innate immunity, antigen presentation, bone marrow differentiation, cellular recruitment and activation, and adhesion molecule expression. A cascade of responses is triggered in response to cytokines, and several cytokines acting together are required to express their optimal function. Numerous cytokines have both inflammatory and anti-inflammatory properties (15). Chemokines constitute a large family of low molecular-weight cytokines whose main action is the recruitment and activation of leukocyte subsets in various models of inflammation—the word “chemokine” is a contraction of the terms “chemoattractant” and “cytokine” (16).

Bone remodeling is a continuous and dynamic process of skeletal destruction and renewal. It consists of two distinct stages: resorption and formation. A complex regulatory mechanism with the participation of several cytokines precisely defines the role of osteoclasts or osteoblasts in the chain of events leading to bone resorption or formation (17, 18).

The receptor activator of NF-κβ ligand (RANKL) is a member of the tumor necrosis factor (TNF) superfamily critically important in the differentiation of osteoclast precursor cells. It exists in membrane-bound and soluble forms. The interaction between RANKL and its receptor, the receptor activator of NF-κβ (RANK), induce bone-resorbing activity in mature osteoclasts and the formation of osteoclasts from precursor cells. (17-19) *In vitro* studies have demonstrated that low levels or absence of either or both RANKL and RANK cause osteopetrosis and reduce mature osteoclast concentrations. On the other hand, an excess of either or both RANKL and RANK results in osteoporosis and rapid bone loss as a consequence of increased osteoclastic activity.(20)

In addition to RANKL, the macrophage colony-stimulating factor (M-CSF) is also required for osteoclast formation. M-CSF is a potent stimulator of RANK expression in osteoclast precursor cells. (21) Experimental studies by Felix et al showed that the injection of

M-CSF corrected the defect in osteoclast formation and bone resorption. (22) The granulocyte-macrophage colony-stimulating factor (GM-CSF) is also implicated in bone loss, but this factor can stimulate human osteoblastic cells as an autocrine proliferative factor. (23)

Osteoprotegerin (OPG), a soluble secreted receptor of the TNF superfamily, acts as a decoy receptor for RANKL by preventing RANK activation (24). Therefore, OPG is a potent inhibitor of osteoclast formation. (19, 25) Overexpression of OPG in transgenic mice results in severe osteopetrosis, characterized by increased bone turnover and the inhibition of osteoclastogenesis. (24) On the other hand, OPG-deficient mice develop osteoporosis due to unopposed RANKL activity. (26)

Interleukin 1 (IL-1) refers to two different polypeptides: IL-1α and IL-1β. IL-1 is a potent bone resorbing factor, which is generally involved in bone loss. (27) This cytokine promotes osteoclastogenesis both directly by stimulating osteoclasts to bone resorption (28) and also indirectly by increasing RANKL production (29). IL-1 prolongs the survival of osteoclasts by preventing apoptosis and induces the multinucleation and bone-resorbing activity of osteoclasts even in the absence of osteoblasts/stromal cells. (28)

Interleukin 6 (IL-6) is also known to induce osteoclast formation and bone resorption. IL-6 directly induces increased expression of RANKL and OPG in osteoblasts and regulates osteoclast progenitor cell differentiation into mature osteoclasts in states of increased bone turnover. (30-32)

Interleukin 8 (IL-8) is a chemokine produced by osteoclasts and serve as an important mediator in bone remodeling. The mechanism of action of this chemokine is independent of the RANKL pathway. It involves the expression and activation of the specific IL-8 receptor (CXCR1) on the surface of osteoclasts and their precursors. (33, 34)

The monocyte-derived TNF, as IL-1, also refers to two polypeptides with potent bone resorption induction capacity: TNF-α and TNF-β. TNF directly stimulates bone marrow osteoclastogenesis by increasing the expression of c-fms, the receptor for M-CSF. As a consequence of M-CSF stimulation, the differentiation and proliferation of progenitor osteoclasts cells occur.(35) TNF also acts directly on the osteoclast precursor by enhancing RANK signaling mechanisms, even in the absence of elevated levels of RANKL. (36)

Transforming growth factor β (TGF-β) is known to stimulate bone formation, mineralization, and inhibiting bone resorption through a proapoptotic effect on mature osteoclasts and by the

inhibition of osteoclast differentiation. TGF-β strongly decreases messenger RNA (mRNA) expression for RANKL in cultured osteoblasts. Low TGF-β levels stimulate osteoclast differentiation by changing the RANKL/OPG ratio, while high TGF-β levels suppress osteoclast differentiation by alternative pathways independent of the RANKL/OPG ratio or M-CSF expression regulation.(37, 38)

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine mainly involved with the recruitment of monocytes to areas of both bone formation and resorption during bone remodeling. Monocyte products are potential regulators of bone cell activity, since growth factors produced by these cells may stimulate bone formation (39, 40). *In vitro* and *in vivo* studies indicated that MCP-1 induces the recruitment of monocytes to bone, which, in turn, is associated with an increase in osteoblast number (39, 40). This is likely to occur via indirect mechanisms, because MCP-1 did not directly enhance DNA synthesis in osteoblastic cells *in vitro* (40). Thus, activated mononuclear phagocytes may play an important role in bone metabolism by stimulating proliferation of osteoblastic cells. MCP-1 is typically not expressed in normal bone or by normal osteoblasts *in vitro*. Upon stimulation by inflammatory mediators, MCP-1 is up-regulated (41-43). This expression is temporally and spatially associated with the recruitment of monocytes in both osseous

inflammation and during developmentally regulated bone remodeling (40). Indeed, monocytes seem to have different functional roles in areas of bone formation and resorption. The recruitment of monocytes in areas of bone formation was associated with a decrease in the number of osteoclasts, while in bone-resorbing areas, recruitment of cells of the monocytic lineage is associated with formation of osteoclasts (44). The receptor activator of NF-κβ ligand (RANKL) seems to be a key variable in this process, once MCP-1 stimulates the formation of osteoclasts in the presence of RANKL. MCP-1 is also induced by RANKL during osteoclast differentiation (41). Receptors for MCP-1 (CCR2 and CCR4) are induced by RANKL, providing evidence for an autocrine loop for MCP-1 in human osteoclasts (45).

2.4. BONE DISEASE AND CYTOKINES IN IDIOPATHIC HYPERCALCIURIA

In IH, the perfect balance between intestinal absorption, bone metabolism and urinary calcium excretion is disrupted. Theoretically, a negative balance between the net intestinal absorption and the total urinary calcium excretion could be the result of an increased bone resorption, as shown in Figure 1.

Insert Figure 1

To date, several studies have been performed to evaluate bone mass density (BMD) in patients with IH, as summarized in Table 1. (4, 13, 14, 46-49) These studies reported significant bone loss in patients with IH regardless of age. Despite the increased risk for reduced BMD, children with long-lasting IH tend to have normal growth curves. (4) This bone loss in patients with IH mainly involves areas of trabecular bone in the axial skeleton, such as vertebral bodies. (50) Malluche et al reported an increased osteoid volume and surface in line with a reduced osteoblastic activity. (51) Steiniche et al. showed decreased bone formation, increased mineralization time and resorption surfaces in a large series of patients with IH. (52)

Insert table 1

This progressive decrease in calcium bone mineral content suggest that bone cells involved in bone formation and resorption could play a key role in the chain of events leading to hypercalciuria. In IH, cytokines may be responsible to trigger specific alterations on bone metabolism, which in turn contribute to the development of excessive bone remodeling, with the possible predominance of bone mass resorption over formation (Figure 2).

Insert Figure 2

There are multiple mechanisms underlying the regulation of bone remodeling, which may be involved in the pathogenesis of IH. In fact, decreased bone formation or excessive bone resorption or both are possible mechanisms to explain the decreased BMD in patients with IH. Experimental studies demonstrated the importance of RANKL, OPG, TGF-β, IL-1α, IL-1β, IL-6, TNF-α and GM-CSF in the regulation of resorption and calcium release from bones in IH, as summarized in Table 2. (8-10, 48)

Insert table 2

Gomes et al. performed immunohistochemical analysis in undecalcified bone samples from transiliac bone biopsies of patients with IH (10). The authors reported a higher expression of RANKL in bone tissue of patients with IH, suggesting that increased bone resorption in IH is mediated by RANKL. In this study, OPG was also increased possibly as an attempt to counteract the bone resorption triggered by RANKL. TGF-β was reduced, thus justifying a delayed mineralization process observed in these patients. The levels of IL-1α did not differ from controls. (10)

Pacifici et al described an association, but not a cause-effect relationship, between IL-1 activity and bone resorption (8). An increased production of IL-1 by cultured peripheral blood monocytes in line with a decreased vertebral BMD was described in patients with fasting hypercalciuria. The authors also found an association between IL-1 and urinary calcium excretion. (8)

Likewise, Weisinger et al detected an increased expression of IL-1α, IL-6 and TNF-α mRNA in unstimulated blood monocytes obtained from hypercalciuric patients when compared with normal subjects (9). It was found a correlation between basal production of IL-1α, but not IL-1β, and decreased trabecular bone density. The authors suggested a link between the high mRNA expression for IL-1α from unstimulated peripheral blood mononuclear cells with spinal bone loss in IH. The results indicated that cytokines could play an important role in bone resorption in IH either by direct activation of osteoclasts or by cell recruitment. (9)

Ghazali et al. showed bone mineral decrease and peripheral blood monocytes activation in calcium stone formers with IH (48). This monocyte activation was characterized by a spontaneously increased synthesis of IL-1β, TNF-α and GM-CSF. This study showed that IL-6 and GM-CSF are correlated with vertebral BMD.

The authors hypothesized that monocyte activation is directly involved in the bone loss of calcium stone formers with IH (48).

Taken together, the findings of these studies allow assuming a hypothetical mechanism for cytokine mediated bone involvement in IH (8-10, 39, 48). Indeed, several control pathways might simultaneously interact during bone remodeling process in IH. The chemokine MCP-1 is involved with the recruitment of monocytes to areas of both bone formation and resorption (39). The inflammatory cytokines, IL-1, IL-6 and TNF, stimulate osteoclast differentiation by increasing the synthesis of two critical substances in the osteoclastogenesis: RANKL and M-CSF (28-31, 35, 36). Concurrently, these cytokines exert direct effect on osteoclast and others osteoclatogenic substances. M-CFS up-regulates the expression of RANK favoring its interaction with RANKL (21, 22). This process will activate osteoclast progenitors and will stimulate proliferation and differentiation of these cells. In parallel, the inhibition of osteoclasts apoptosis also occurs (28). To counter-regulate bone resorption, growth factors such as OPG and TGF-β are released (25, 37). The release these products will inhibit osteoclast differentiation and will induce bone formation (19, 24, 38). An imbalance in bone remodeling process, favoring osteoclast production, will stimulate bone resorption. Consequently, increased urinary calcium excretion

and decreased bone mineral density could be the clinical expression of this phenomenon in patients with IH. Figure 3 displays the hypothetical mechanism by which cytokines release induce osteoclasts differentiation.

Insert Figure 3

2.5. CONCLUDING REMARKS

Despite the evidence that cytokines play an important role in modulating osteoclasts function towards bone resorption, the pathogenesis of bone mass reduction in IH remains uncertain. More studies are needed to elucidate the possible mechanisms involving increased calcium urinary excretion and bone mass reduction in IH. Serum and urine bone remodeling biomarkers should be more extensively studied to enable a better understanding of the possible physiopathology of IH.

2.6. ABBREVIATIONS

BMD - Bone mass density

GM-CSF - Granulocyte-macrophage colony-stimulating factor

IH - Idiopathic hypercalciuria

IL-1 - Interleukin 1

IL-6 - Interleukin 6

IL-8 - Interleukin 8

MCP-1 - Monocyte chemoattractant protein-1

M-CSF - Macrophage colony-stimulating factor

OPG - Osteoprotegerin

RANK - Receptor activator of NF-κβ

RANKL - Receptor activator of NF-κβ ligand

TGF-β - Transforming growth factor β

TNF - Monocyte-derived tumor necrosis factor

BIBLIOGRAPHY

1. Stapleton FB. Idiopathic hypercalciuria: association with isolated hematuria and risk for urolithiasis in children. The Southwest Pediatric Nephrology Study Group. Kidney Int. 1990 Feb;37(2):807-11.

2. Vachvanichsanong P, Malagon M, Moore ES. Recurrent abdominal and flank pain in children with idiopathic hypercalciuria. Acta Paediatr. 2001 Jun;90(6):643-8.

3. Stojanovic VD, Milosevic BO, Djapic MB, Bubalo JD. Idiopathic hypercalciuria associated with urinary tract infection in children. Pediatr Nephrol. 2007 Sep;22(9):1291-5.

4. Polito C, Iolascon G, Nappi B, Andreoli S, La Manna A. Growth and bone mineral density in long-lasting idiopathic hypercalciuria. Pediatr Nephrol. 2003 Jun;18(6):545-7.

5. Coe FL, Evan A, Worcester E. Kidney stone disease. J Clin Invest. 2005 Oct;115(10):2598-608.

6. Ammenti A, Neri E, Agistri R, Beseghi U, Bacchini E. Idiopathic hypercalciuria in infants with renal stones. Pediatr Nephrol. 2006 Dec;21(12):1901-3.

7. Moore ES, Coe FL, McMann BJ, Favus MJ. Idiopathic hypercalciuria in children: prevalence and metabolic characteristics. J Pediatr. 1978 Jun;92(6):906-10.

8. Pacifici R, Rothstein M, Rifas L, Lau KH, Baylink DJ, Avioli LV, et al. Increased monocyte interleukin-1 activity and decreased vertebral bone density in patients with fasting idiopathic hypercalciuria. J Clin Endocrinol Metab. 1990 Jul;71(1):138-45.

9. Weisinger JR, Alonzo E, Bellorin-Font E, Blasini AM, Rodriguez MA, Paz-Martinez V, et al. Possible role of cytokines on the bone mineral loss in idiopathic hypercalciuria. Kidney Int. 1996 Jan;49(1):244-50.

10. Gomes SA, dos Reis LM, Noronha IL, Jorgetti V, Heilberg IP. RANKL is a mediator of bone resorption in idiopathic hypercalciuria. Clin J Am Soc Nephrol. 2008 Sep;3(5):1446-52.

11. Freundlich M, Alonzo E, Bellorin-Font E, Weisinger JR. Reduced bone mass in children with idiopathic hypercalciuria and in their asymptomatic mothers. Nephrol Dial Transplant. 2002 Aug;17(8):1396-401.

12. Misael da Silva AM, dos Reis LM, Pereira RC, Futata E, Branco-Martins CT, Noronha IL, et al. Bone involvement in idiopathic hypercalciuria. Clin Nephrol. 2002 Mar;57(3):183-91.

13. Penido MG, Lima EM, Marino VS, Tupinamba AL, Franca A, Souto MF. Bone alterations in children with idiopathic hypercalciuria at the time of diagnosis. Pediatr Nephrol. 2003 Feb;18(2):133-9.

14. Penido MG, Lima EM, Souto MF, Marino VS, Tupinamba AL, Franca A. Hypocitraturia: a risk factor for reduced bone mineral density in idiopathic hypercalciuria? Pediatr Nephrol. 2006 Jan;21(1):74-8.

15. Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol. 2003 Feb;111(2 Suppl):S460-75.

16. Segerer S, Alpers CE. Chemokines and chemokine receptors in renal pathology. Curr Opin Nephrol Hypertens. 2003 May;12(3):243-9.

17. Lorenzo J, Horowitz M, Choi Y. Osteoimmunology: interactions of the bone and immune system. Endocr Rev. 2008 Jun;29(4):403-40.

18. Pacifici R. The immune system and bone. Arch Biochem Biophys. 2010 Nov 1;503(1):41-53.

19. Perez-Sayans M, Somoza-Martin JM, Barros-Angueira F, Rey JM, Garcia-Garcia A. RANK/RANKL/OPG role in distraction osteogenesis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010 May;109(5):679-86.

20. Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, et al. Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. Am J Pathol. 2003 Nov;163(5):2021-31.

21. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, et al. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear

factor kappaB (RANK) receptors. J Exp Med. 1999 Dec 20;190(12):1741-54.

22. Felix R, Cecchini MG, Fleisch H. Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. Endocrinology. 1990 Nov;127(5):2592-4.

23. Takahashi N, Udagawa N, Akatsu T, Tanaka H, Shionome M, Suda T. Role of colony-stimulating factors in osteoclast development. J Bone Miner Res. 1991 Sep;6(9):977-85.

24. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell. 1997 Apr 18;89(2):309-19.

25. Hofbauer LC, Neubauer A, Heufelder AE. Receptor activator of nuclear factor-kappaB ligand and osteoprotegerin: potential implications for the pathogenesis and treatment of malignant bone diseases. Cancer. 2001 Aug 1;92(3):460-70.

26. Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, et al. Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Commun. 1998 Jun 29;247(3):610-5.

27. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. Blood. 1991 Apr 15;77(8):1627-52.

28. Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, et al. Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. Exp Cell Res. 1999 Feb 25;247(1):84-93.

29. Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. Interleukin-1beta and tumor necrosis factor-alpha, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone. 1999 Sep;25(3):255-9.

30. Palmqvist P, Persson E, Conaway HH, Lerner UH. IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. J Immunol. 2002 Sep 15;169(6):3353-62.

31. Franchimont N, Wertz S, Malaise M. Interleukin-6: An osteotropic factor influencing bone formation? Bone. 2005 Nov;37(5):601-6.

32. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, et al. IL-6 is produced by osteoblasts and induces bone resorption. J Immunol. 1990 Nov 15;145(10):3297-303.

33. Rothe L, Collin-Osdoby P, Chen Y, Sunyer T, Chaudhary L, Tsay A, et al. Human osteoclasts and osteoclast-like cells synthesize and release high basal and inflammatory stimulated levels of the potent chemokine interleukin-8. Endocrinology. 1998 Oct;139(10):4353-63.

34. Bendre MS, Margulies AG, Walser B, Akel NS, Bhattacharrya S, Skinner RA, et al. Tumor-derived interleukin-8 stimulates osteolysis independent of the receptor activator of nuclear factor-kappaB ligand pathway. Cancer Res. 2005 Dec 1;65(23):11001-9.

35. Yao Z, Li P, Zhang Q, Schwarz EM, Keng P, Arbini A, et al. Tumor necrosis factor-alpha increases circulating osteoclast precursor numbers by promoting their proliferation and differentiation in the bone marrow through up-regulation of c-Fms expression. J Biol Chem. 2006 Apr 28;281(17):11846-55.

36. Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest. 2000 Dec;106(12):1481-8.

37. Quinn JM, Itoh K, Udagawa N, Hausler K, Yasuda H, Shima N, et al. Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. J Bone Miner Res. 2001 Oct;16(10):1787-94.

38. Karst M, Gorny G, Galvin RJ, Oursler MJ. Roles of stromal cell RANKL, OPG, and M-CSF expression in biphasic TGF-beta regulation of osteoclast differentiation. J Cell Physiol. 2004 Jul;200(1):99-106.

39. Posner LJ, Miligkos T, Gilles JA, Carnes DL, Taddeo DR, Graves DT. Monocyte chemoattractant protein-1 induces monocyte recruitment that is associated with an increase in numbers of osteoblasts. Bone. 1997 Oct;21(4):321-7.

40. Graves DT, Jiang Y, Valente AJ. Regulated expression of MCP-1 by osteoblastic cells in vitro and in vivo. Histol Histopathol. 1999 Oct;14(4):1347-54.

41. Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. J Immunol. 1998 Oct 1;161(7):3719-28.

42. Rahimi P, Wang CY, Stashenko P, Lee SK, Lorenzo JA, Graves DT. Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse. Endocrinology. 1995 Jun;136(6):2752-9.

43. Williams SR, Jiang Y, Cochran D, Dorsam G, Graves DT. Regulated expression of monocyte chemoattractant protein-1 in normal human osteoblastic cells. Am J Physiol. 1992 Jul;263(1 Pt 1):C194-9.

44. Volejnikova S, Laskari M, Marks SC, Jr., Graves DT. Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. Am J Pathol. 1997 May;150(5):1711-21.

45. Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor-{kappa}B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. J Biol Chem. 2005 Apr 22;280(16):16163-9.

46. Schwaderer AL, Cronin R, Mahan JD, Bates CM. Low bone density in children with hypercalciuria and/or nephrolithiasis. Pediatr Nephrol. 2008 Dec;23(12):2209-14.

47. Garcia-Nieto V, Ferrandez C, Monge M, de Sequera M, Rodrigo MD. Bone mineral density in pediatric patients with idiopathic hypercalciuria. Pediatr Nephrol. 1997 Oct;11(5):578-83.

48. Ghazali A, Fuentes V, Desaint C, Bataille P, Westeel A, Brazier M, et al. Low bone mineral density and peripheral blood monocyte activation profile in calcium stone formers with idiopathic hypercalciuria. J Clin Endocrinol Metab. 1997 Jan;82(1):32-8.

49. Asplin JR, Bauer KA, Kinder J, Muller G, Coe BJ, Parks JH, et al. Bone mineral density and urine calcium excretion among subjects with and without nephrolithiasis. Kidney Int. 2003 Feb;63(2):662-9.

50. Vezzoli G, Rubinacci A, Bianchin C, Arcidiacono T, Giambona S, Mignogna G, et al. Intestinal calcium absorption is associated with bone mass in stone-forming women with idiopathic hypercalciuria. Am J Kidney Dis. 2003 Dec;42(6):1177-83.

51. Malluche HH, Tschoepe W, Ritz E, Meyer-Sabellek W, Massry SG. Abnormal bone histology in idiopathic hypercalciuria. J Clin Endocrinol Metab. 1980 Apr;50(4):654-8.

52. Steiniche T, Mosekilde L, Christensen MS, Melsen F. A histomorphometric determination of iliac bone remodeling in patients with recurrent renal stone formation and idiopathic hypercalciuria. APMIS. 1989 Apr;97(4):309-16.

53. Tasca A, Cacciola A, Ferrarese P, Ioverno E, Visona E, Bernardi C, et al. Bone alterations in patients with idiopathic hypercalciuria and calcium nephrolithiasis. Urology. 2002 Jun;59(6):865-9; discussion 9.

54. Skalova S, Palicka V, Kutilek S. Bone mineral density and urinary N-acetyl-beta-D-glucosaminidase activity in paediatric patients with idiopathic hypercalciuria. Nephrology (Carlton). 2005 Apr;10(2):99-102.

55. Giannini S, Nobile M, Sartori L, Calo L, Tasca A, Dalle Carbonare L, et al. Bone density and skeletal metabolism are altered in idiopathic hypercalciuria. Clin Nephrol. 1998 Aug;50(2):94-100.

56. Jaeger P, Lippuner K, Casez JP, Hess B, Ackermann D, Hug C. Low bone mass in idiopathic renal stone formers: magnitude and significance. J Bone Miner Res. 1994 Oct;9(10):1525-32.

TABLE 1 - STUDIES ON BONE MINERAL DENSITY (BMD) IN PATIENTS WITH IDIOPATHIC HYPERCALCIURIA

Author	Year	Number of patients with IH	Method	BMD result
Penido et al (44)	2006	88	DXA*	↓[#]
Vezzoli et al (49)	2003	106	DXA	↓
Polito et al (4)	2003	26	DXA	↓
Penido et al (45)	2003	88	DXA	↓
Garcia-Nieto et al (46)	1997	73	DXA	↓
Tasca et al (52)	2002	70	DXA	↓
Freundlich et al (11)	2002	21	DXA	↓
Skalova et al (53)	2005	15	DXA	↓
Giannini et al (54)	1998	49	DXA	↓
Weisinger et al (9)	1996	29	DXA	↓
Jaeger et al (55)	1994	49	DXA	↓

* DXA = dual energy X-ray absorptiometry

[#] ↓= reduced

TABLE 2 - STUDIES ON CYTOKINES IN PATIENTS WITH IDIOPATHIC HYPERCALCIURIA

Author	Year	Number of patients with IH included in the study	Cytokine	Material used for cytokine analysis
Gomes et al (10)	2008	36	RANKL, OPG, IL-1α, TGF-β, bFGF	Transiliac bone biopsies
Ghazali et al (47)	1997	25	IL-1β, IL-6, TNF-α, GM-CSF	Plasma
Weisinger et al (9)	1996	29	IL-1α, IL-6, TNF-α	Plasma
Pacifici et al (8)	1990	74	IL-1	Plasma

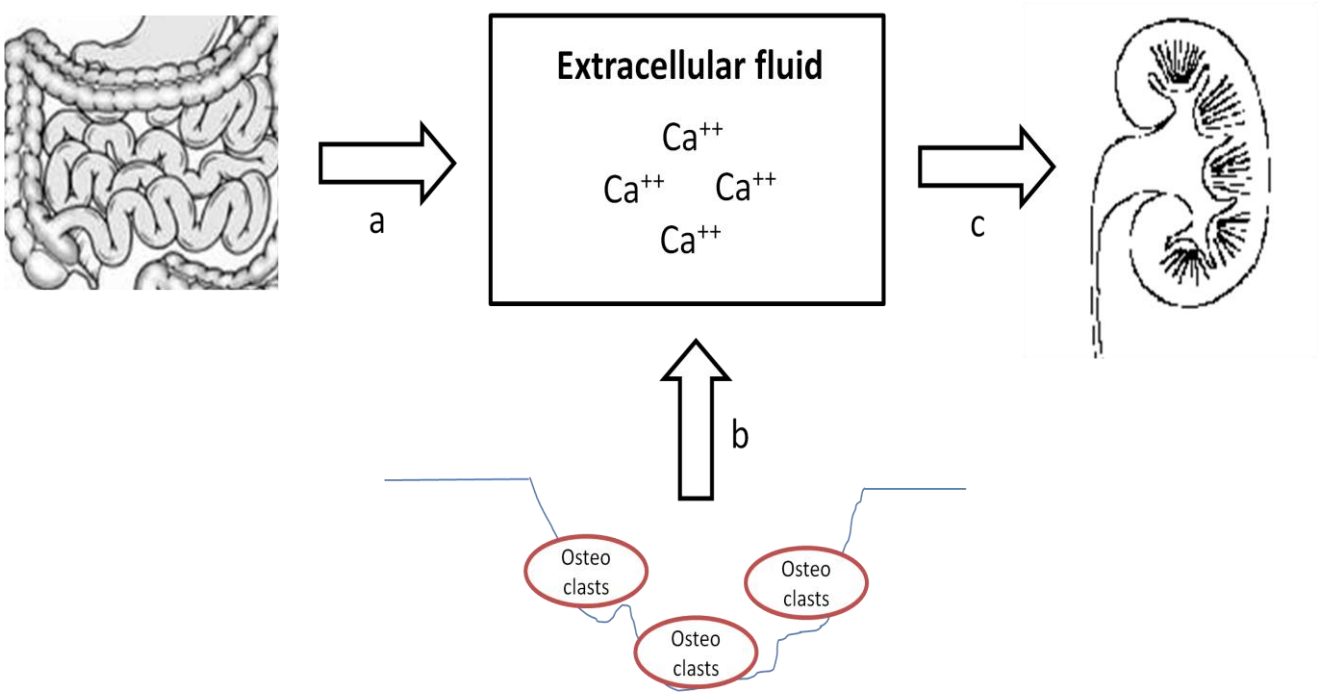
FIGURE LEGENDS

FIGURE 1 – Hypothetical mechanism by which increased bone resorption may contribute to hypercalciuria.

FIGURE 2 – Hypothetical mechanism by which increased bone resorbing cytokines trigger calcium release from bones.

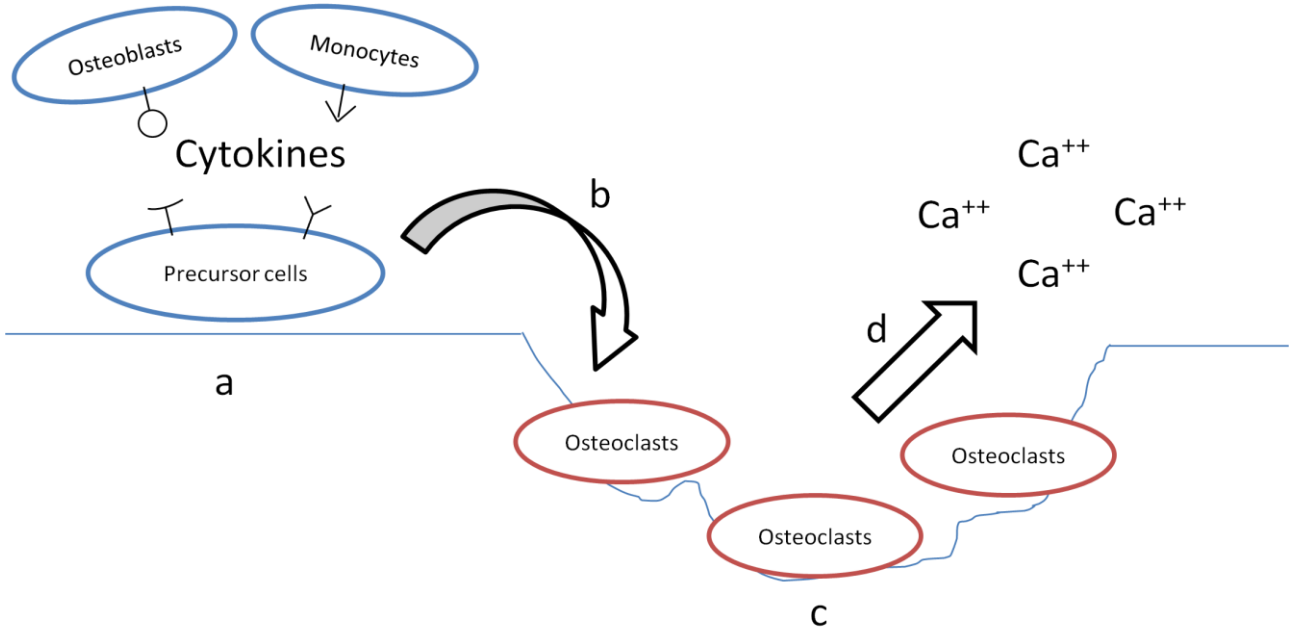
FIGURE 3 - Hypothetical mechanism by which cytokines release induce osteoclasts differentiation.

FIGURE 1



(a) Calcium absorption from the intestine (b) Calcium release from bone (c) Excessive urinary calcium excretion in idiopathic hypercalciuria

FIGURE 2



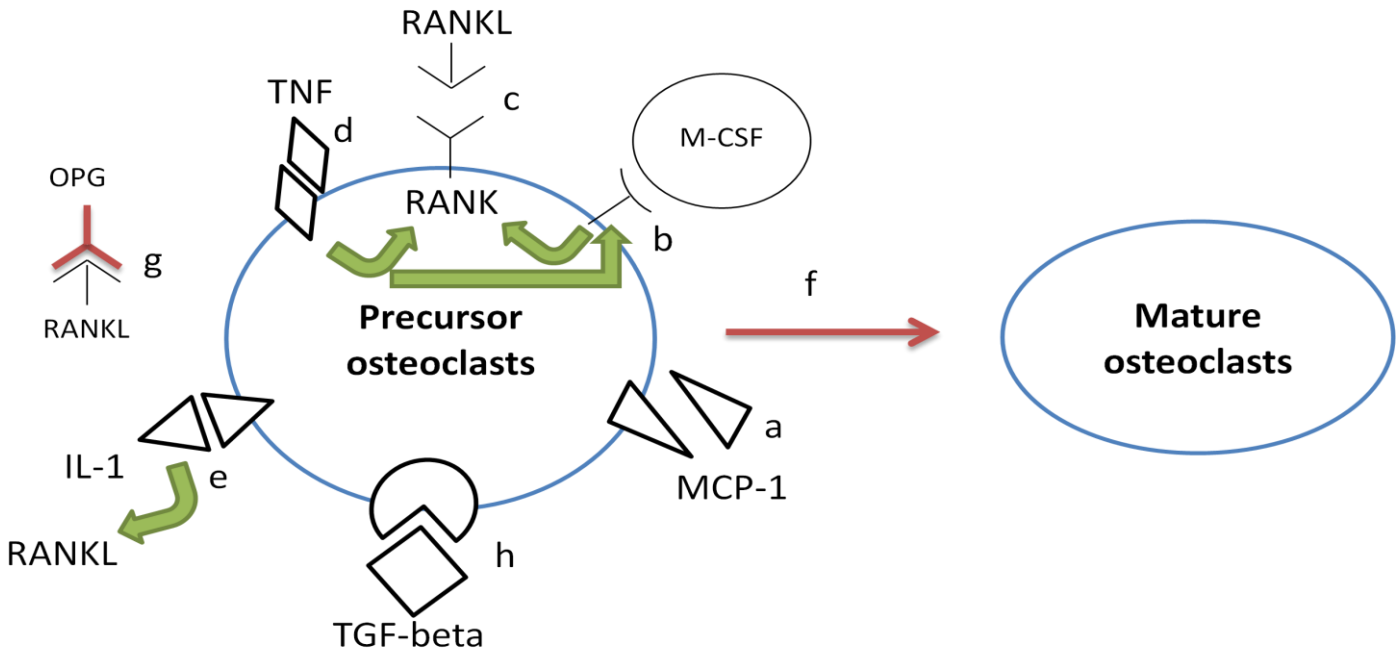
(a) Resting bone;

(b) Differentiation and activation;

(c) Osteoclasts performing bone resorption;

(d) Calcium release from bones

FIGURE 3



(a) MCP-1 recruiting monocytes to areas of bone remodeling; (b) M-CSF stimulating the expression of RANK receptors; (c) RANKL binding to RANK and triggering the differentiation of mature osteoclasts; (d) TNF increasing the expression of RANK and M-CSF receptors; (e) IL-1 increasing the expression of RANKL; (f) Differentiation to mature osteoclasts; (g) OPG acting as a decoy receptor for RANKL; (h) TGF-beta reducing the expression of RANKL.

3. OBJETIVOS

O objetivo principal desse trabalho foi avaliar, em crianças e adolescentes com HI, os níveis plasmáticos e urinários de citocinas e quimiocinas associadas à regulação do metabolismo ósseo.

Objetivos específicos:

a. Verificar se há diferença entre as concentrações das citocinas após dividir os pacientes em grupos estratificados de acordo com a excreção urinária de cálcio (< 4 mg/Kg/dia versus ≥ 4 mg/Kg/dia);
b. Verificar se há diferença entre as concentrações das citocinas após dividir os pacientes em grupos estratificados de acordo com a densidade mineral óssea (Z-score ≤ -1 desvio padrão versus Z-score > - 1 desvio padrão);
c. Verificar se há diferença entre as concentrações das citocinas após dividir os pacientes em grupos estratificados de acordo com a idade dos pacientes (≤12 anos versus > 12 anos);
d. Verificar se há correlação entre os níveis urinários e plasmáticos das citocinas e quimiocinas avaliadas;

e. Verificar se há correlação entre as medidas de densitometria óssea e os níveis urinários e plasmáticos das citocinas e quimiocinas avaliadas.

4. PACIENTES E MÉTODOS

4.1. CRITÉRIOS DE INCLUSÃO

Foram inicialmente incluídas 81 crianças e adolescentes com diagnóstico estabelecido de HI e que estavam em acompanhamento regular na Unidade de Nefrologia Pediátrica do Hospital das Clínicas da UFMG, durante o período de coleta (2009 a 2010). Desses 81 pacientes, 11 se recusaram em participar do estudo, totalizando, portanto, 70 pacientes com IH avaliados.

O diagnóstico de HI foi realizado de acordo com critérios diagnósticos estabelecidos pela literatura internacional (1-2), que consideram portadores de HI os pacientes que apresentam aumento persistente da excreção urinária de cálcio na ausência de estados hipercalcêmicos ou de qualquer outra doença primária. De acordo com os valores de referência estabelecidos para excreção urinária de cálcio em crianças e adolescentes (3-4), a hipercalciúria foi definida como excreção de cálcio igual ou maior a 4 mg/Kg/dia.

4.2. CRITÉRIOS DE EXCLUSÃO

Foram excluídos os pacientes que apresentavam doenças ou utilizavam medicamentos que poderiam interferir na excreção urinária de cálcio ou na regulação do remodelamento ósseo tais

como: hiperparatireoidismo, hipertireoidismo, sarcoidose, doenças oncológicas, acidose tubular renal, doença inflamatória aguda, estados febris, tratamento com estrogênio, progesterona, corticosteróides, anticonvulsivantes, bifosfonados, calcitonina ou vitamina D. (5)

4.3. ASPECTOS ÉTICOS

Comitê de Ética em Pesquisa (COEP) da UFMG aprovou o estudo através do Parecer ETIC 036/00, anexo 1. Todos os pacientes e seus responsáveis foram esclarecidos sobre a natureza do estudo por meio da leitura e análise do termo de consentimento livre e esclarecido, anexo 2. Os pacientes portadores de hipercalciúria idiopática e as crianças e adolescentes saudáveis foram incluídos no estudo somente mediante concordância e assinatura do termo de consentimento por parte do responsável e do próprio paciente conforme a idade. O protocolo de pesquisa não interferiu com qualquer recomendação ou prescrição médica. Ressalta-se ainda que o seguimento clínico-laboratorial e a abordagem terapêutica dos pacientes foram assegurados, mesmo no caso de recusa em participar do estudo.

4.4. PROTOCOLO DO ESTUDO

Trata-se de um estudo transversal, com amostra de conveniência, reunindo um total de 70 pacientes.

Os pacientes foram avaliados quanto à calciúria em 24h, Z-score e conteúdo mineral ósseo da densitometria óssea.

Os pacientes foram divididos em 2 grupos de acordo com os níveis de calciúria na data da coleta das amostras: calciúria descompensada (n=23) e calciúria compensada (n=47). A calciúria descompensada foi definida como excreção de cálcio igual ou superior a 4mg/kg/24h.

Os pacientes também foram estratificados levando-se em consideração a idade (≤ 12 anos, n=18; > 12 anos, n=52) e o Z-score da densidade mineral óssea (Z-score >-1 desvio padrão, n=28; Z-score ≤-1 desvio padrão, n=18), quando disponível.

Os pacientes foram submetidos a exame clínico e avaliação laboratorial de acordo com o protocolo utilizado pela Unidade de Nefrologia Pediátrica do Hospital das Clínicas da UFMG. Todos os pacientes realizaram hemograma, gasometria venosa, dosagens séricas de uréia, creatinina, ácido úrico, sódio, potássio, cloreto, cálcio, fosfato e magnésio, PTH, TSH, T4 livre, exame de urina rotina, determinação de pH urinário em urina recém emitida, urocultura e, em urina de 24 horas, dosagens de cálcio, fósforo,

citrato, magnésio, ácido úrico, cistina, oxalato e creatinina. Os exames foram realizados para afastar causas secundárias de hipercalciúria (4, 6).

Após confirmação do diagnóstico de HI e assinatura do termo de consentimento, os pacientes foram submetidos, em única ocasião, a coletas simultâneas de amostras de sangue e urina para determinação de citocinas e quimiocinas conforme detalhado a seguir.

4.5. COLETA E PROCESSAMENTO DE AMOSTRAS

A coleta de sangue foi realizada em veia periférica, entre 7 e 9 horas da manhã, em tubo estéril contendo citrato como anti-coagulante. As amostras foram, então, transportadas em embalagem com gelo e processadas em até 30 minutos após a coleta. Foi utilizada centrífuga refrigerada (Jouan BR4i) a 4ºC, de acordo com o seguinte protocolo: foi feito inicialmente um ciclo a 700 g por 10 minutos; o plasma sobrenadante foi, a seguir, transferido para tubo estéril, que, por sua vez, foi centrifugado a 1300 g por 20 minutos para sedimentar plaquetas (4). Depois desta etapa, o plasma foi aliquotado em amostras de 0,5 ml, que foram armazenadas em freezer -80ºC até a data do ensaio.

As amostras de urina foram coletadas no mesmo momento em que foi realizada a coleta das amostras de sangue. Após homogeneização, 10 ml de urina foram recondicionados em tubo estéril. As amostras foram, então, centrifugadas a 1300 g por 20 minutos. Alíquotas de 1,5 ml foram também conservadas em freezer a -80ºC.

4.6. ENSAIOS IMUNOENZIMÁTICOS

Os níveis plasmáticos e urinários de IL-1β, IL-6, IL-8, TNF-α, TGF-β1 e MCP-1 foram medidos usando kits de ensaio imuno-enzimático (enzyme-linked immunoassay - ELISA) produzidos pelo laboratório R&D Systems (Minneapolis, MN, EUA).

Foram seguidas as instruções do fabricante para cada kit. As amostras foram analisadas em duplicata. O protocolo de ELISA utilizou um anticorpo monoclonal específico para determinação de cada citocina ou quimiocina estudada, fornecido pelo fabricante.

Em linhas gerais, os anticorpos de captura, específicos para cada citocina (fornecidos pelo kit), foram diluídos em tampão fosfato (PBS) e adicionados a cada poço de placas de poliestireno (96 poços). As placas foram, então, incubadas a 4ºC por 12 horas. Em seguida, foram submetidas a quatro ciclos de lavagem com PBS e Tween 20 a 0,05% (Sigma). As placas foram então bloqueadas com albumina sérica bovina (BSA) 1% e PBS, e incubadas por uma hora

em temperatura ambiente. Um novo procedimento de lavagem foi feito, como descrito acima. Em seguida, as amostras foram adicionadas às placas e incubadas por 12 horas a 4ºC, sendo depois submetidas a novos ciclos de lavagem. Os anticorpos de detecção específicos para cada citocina, diluídos em PBS, foram adicionados, e foi feita incubação por duas horas em temperatura ambiente, seguida de novo procedimento de lavagem. A seguir, o reagente de cor (fenilenediamina) foi adicionado a cada poço e as placas deixadas no escuro por 15 minutos. A reação foi parada com a adição de 1M H_2SO_4 aos poços. A absorbância foi lida em um leitor de placas (Emax, Molecular Devices, MN, EUA), ajustado no comprimento de onda de 492nm.

Especificamente para a determinação das concentrações de TGF-β1, foi utilizado kit do tipo Quantikine® (R&D Systems). O kit fornece placas de poliestireno já preenchidas com anticorpo monoclonal anti-TGF-β1. As amostras de plasma e de urina foram submetidas inicialmente ao procedimento específico de ativação do TGF-β1, da seguinte forma: foi adicionado a cada amostra 20μL HCl e incubado por 10 minutos em temperatura ambiente. Em seguida, a amostra acidificada foi neutralizada usando 20μL de NaOH/HEPES. As amostras foram, então, adicionadas aos poços, apropriadamente diluídas, com diluente fornecido no kit, e depois incubadas em

temperatura ambiente por duas horas. Em seguida, os poços foram submetidos a quatro ciclos de lavagem com tampão de lavagem. O conjugado específico, que consiste de anticorpo anti-TGF-β1 conjugado a peroxidase, foi adicionado a cada poço, sendo a placa incubada por uma hora em temperatura ambiente. Foi realizado, em seguida, novo ciclo de lavagens. O próximo passo foi adicionar aos poços a solução de substrato, contendo peróxido de hidrogênio e tetra-metil-benzidina. As placas foram, então, deixadas por 15 minutos em temperatura ambiente, protegidas da luz. Em seguida, foi adicionada a cada poço a solução de parada, e foi medida a densidade óptica usando leitor de placas, ajustado no comprimento de onda de 450nm (Emax, Molecular Devices, MN, EUA).

As concentrações plasmáticas de citocinas foram expressas em pg/ml. Em relação às dosagens urinárias, os resultados foram expressos em valores absolutos das concentrações (pg/ml) e valores relativos à creatinina urinária medida simultaneamente na mesma amostra de urina (pg/mg cr). Os limites de detecção para cada citocina foram de: 0,1 µg/mL (IL-1β), 0,039 pg/mL (IL-6), 6 pg/mL (IL-8), 0,106 pg/mL (TNF-α), 6 pg/mL (TGF-β1), 8 pg/mL (MCP-1). As determinações de IL-1β, IL-6, IL-8, TNF-α, TGF-β1 e MCP-1 foram realizadas em um único ensaio para eliminar variações inter-ensaio. A variação intra-ensaio foi inferior a 3%.

4.7. ANÁLISE ESTATÍSTICA

Os dados foram expressos como mediana e intervalo interquartílico (percentil 25, percentil 75) ou média ± desvio padrão, quando apropriado. O teste de Mann-Whitney foi utilizado para comparar medidas não paramétricas, enquanto que o teste exato de Fisher foi utilizado para variáveis categóricas. As comparações de médias foram feitas utilizando o Teste T de Student. O teste de Spearman foi utilizado para avaliação de correlações. O nível de significância considerado foi $p<0,05$. A análise dos dados foi feita utilizando o software Stata versão 11.0.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Ammenti A, Neri E, Agistri R, Beseghi U, Bacchini E. Idiopathic hypercalciuria in infants with renal stones. Pediatr Nephrol. 2006 Dec;21(12):1901-3.

2. Moore ES, Coe FL, McMann BJ, Favus MJ. Idiopathic hypercalciuria in children: prevalence and metabolic characteristics. J Pediatr. 1978 Jun;92(6):906-10.

3. Srivastava T, Schwaderer A. Diagnosis and management of hypercalciuria in children. Curr Opin Pediatr. 2009 Apr;21(2):214-9.

4. Butani L, Kalia A. Idiopathic hypercalciuria in children--how valid are the existing diagnostic criteria? Pediatr Nephrol. 2004 Jun;19(6):577-82.

5. Zerwekh JE. Bone disease and hypercalciuria in children. Pediatr Nephrol. 2010 Mar;25(3):395-401.

6. Hughes P. The CARI guidelines. Kidney stones: metabolic evaluation. Nephrology (Carlton). 2007 Feb;12 Suppl 1:S31-3.

5. RESULTADOS E DISCUSSÃO

PLASMA AND URINARY LEVELS OF CYTOKINES IN PATIENTS WITH IDIOPATHIC HYPERCALCIURIA

Augusto C. S. Santos Jr[1], Katia D. Silveira[2], Maria Goretti M. G. Penido[1], Eleonora Moreira Lima[1], Mauro M. Teixeira[2], Eduardo A. Oliveira[1], Ana Cristina Simões e Silva[1]

[1]Pediatric Nephrology Unit, Department of Pediatrics, Faculty of Medicine, Federal University of Minas Gerais (UFMG), Belo Horizonte, MG, Brazil

[2]Laboratory of Immunopharmacology, Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, Belo Horizonte, MG, Brazil

Acknowledgements: This study was partially supported by CNPq and FAPEMIG

Key words: biomarkers, cytokines, MCP-1, idiopathic hypercalciuria, mineral bone disease

Conflicts of interest: none

Correspondence: Ana Cristina Simões e Silva, MD, PhD
Avenida Bernardo Monteiro 1300 / 1104
Belo Horizonte - Minas Gerais
Postal Code: 30150-281
Email: acssilva@hotmail.com

5.1. ABSTRACT

Several studies suggest that cells involved in bone formation and resorption take part in the mechanisms of increased urinary calcium excretion. Cytokines have been implicated in this process by modulating osteoclast function towards bone resorption. Therefore, this study aimed to identify noninvasive biomarkers in patients with uncontrolled idiopathic hypercalciuria (IH). Plasma and spot-urine levels of interleukin (IL) 1β, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), transforming growth factor beta1 (TGF-β1) and monocyte chemoattractant protein (MCP-1) were measured in 70 children and adolescents with IH. Patients were divided in two groups according to their calciuria levels at the time of sample collection: equal or superior to 4mg/kg/day (uncontrolled IH, n=27) and below 4mg/kg/day (controlled IH, n=43). Cytokines were determined by specific enzyme-linked immunoassay kits. Plasma and urinary levels of MCP-1 and TGF-β1 were detected in patients IH, but without differences between controlled and uncontrolled hypercalciuria. Plasma and urinary concentrations of IL-1β, IL-6, IL-8, TNF-α were under detection limits. Urinary MCP-1 levels were positively correlated to the bone mineral content (BMC) (p=0.013). In conclusion, bone turnover cytokine measurements were not useful to differentiate uncontrolled and controlled IH.

5.2. INTRODUCTION

Idiopathic hypercalciuria (IH) was first described by Albright et al (1), which defined as an excessive urinary calcium loss accompanied by normal serum calcium levels. IH is the most common metabolic abnormality in patients with nephrolithiasis, accounting for 30-50% of calcium-oxalate stone formers (2-4).

Although the pathogenesis of IH is not fully understood, it is generally due to an alteration in calcium homeostasis at sites where large amounts of calcium must be precisely controlled (5). Several studies have shown decreased bone mineral density (BMD) in patients with IH (6-18). The progressive decrease in bone mineral content suggest that cells involved in bone formation and resorption could play a key role in the chain of events leading to hypercalciuria. Recent studies indicate that cytokines are key factors in the pathogenesis of IH, mostly by the modulation of the balance between bone formation/resorption (19-24).

Biomarkers are potentially useful diagnostic tools, which have recently become a focus of clinical research (10). In the setting of IH, we hypothesized that the measurement of cytokines could help in the detection of uncontrolled urinary calcium excretion probably associated to changes in bone remodeling mechanisms. Therefore, as an attempt to identify noninvasive markers associated to

increased calcium excretion we evaluated plasma and urinary levels of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α), transforming grow factor β1 (TGF-β1) and monocyte chemoattractant protein (MCP-1) in children and adolescents with IH.

5.3. PATIENTS AND METHODS

5.3.1. PATIENTS

This cross-sectional study consisted of a sample of patients with confirmed diagnosis of IH followed at the Pediatric Nephrology Unit of our institution from 2009 to 2010.

In pediatric patients, hypercalciuria was defined by serum calcium within normal limits and 24 hour urinary excretion of calcium equal or higher than 4 mg/kg per day for both genders in two nonconsecutive samples, under unrestricted diet. (25, 26) In order to define the diagnosis of IH, all patients were submitted to a systematic protocol to investigate diseases and conditions that might affect urinary calcium excretion. Briefly, the protocol included: blood gas analysis (pH and bicarbonate), serum electrolytes (sodium, potassium, chloride, calcium, phosphate, magnesium), eritrogram, urea, creatinine, uric acid, PTH, TSH, T4, spot urine (pH, abnormal elements, microscopy and culture), and 24 hour urinary

concentrations of calcium, citrate, uric acid, oxalate, cystine and creatinine (25, 27).

Therefore, patients with known diseases or use of medication that could affect calcium excretion, bone remodeling or monocyte function such as hyperparathyroidism, hyperthyroidism, sarcoidosis, malignancy, Paget's disease, renal tubular acidosis, acute inflammatory disease, febrile infections, treatment with estrogen, progesterone, corticosteroids, anticonvulsants, bisphosphonate, calcitonin or vitamin D were excluded. (6)

A total of 81 patients with confirmed IH were invited to participate in the study. However, 11 patients refused to participate. The remaining 70 patients were then divided in two subgroups according to their urinary calcium excretion at the time of urine and blood sample collection. Patients with calcium excretion equal or superior to 4mg/kg/day were allocated to the uncontrolled IH group (n=27). Patients with calcium excretion below 4mg/kg/day were allocated at the controlled group (n=43) (25). Figure 1 shows a flow diagram of eligible, excluded and included patients.

5.3.2.STUDY PROTOCOL

All participants were interviewed and underwent physical examination. Blood and urine samples were obtained simultaneously. Age, gender, race, weight, height, body mass index, systolic and

diastolic blood pressure, serum creatinine, calciuria, citraturia, phosphaturia, magnesuria, use of potassium citrate and hydrochlorothiazide, bone mineral density (BMD), familiar history for nephrolithiasis, presence of calculus, past history for extracorporeal shock lithotripsy and symptoms were analyzed.

BMD was assessed by dual energy x-ray absorptiometry at lumbar spine (L1-L4) using a Lunar Prodigy Primo DXA System (GE Healthcare Lunar Corp., Madison, WI, USA). Bone density was stratified as Z-score >-1 SD and ≤-1 SD (12, 17, 18, 28, 29).

5.3.3. BLOOD SAMPLING

After informed consent, all subjects were submitted to blood collection. Blood sampling occurred at only one occasion, simultaneously to other routine exams. The samples were collected into sterile citrate tubes, which were immediately immersed in ice, and processed within 30 min after collection. Cells were sedimented by centrifugation at 700 g for 10 min at 4^{o}C. Then the supernatant was collected and re-spun for another 20 min at 1300 g to sediment platelets. (30) Cell-free plasma was aliquoted into 0.5 mL samples and stored at -80^{o}C until measurements.

5.3.4. URINE SAMPLING

A single urine sample was obtained from all patients at the same day of blood collection from 7.30 AM to 9.00 AM. After homogenization, 10 mL of the collected urine were centrifuged at 4^{o}C for 20 min at 1300 g. Cell-free urine was aliquoted into 0.5 mL tubes and stored at -80^{o}C until measurements.

5.3.5. CYTOKINES MEASUREMENT

Plasma and urinary levels of IL-1β, IL-6, IL-8, TNF-α, TGF-β1 and MCP-1 were measured by specific enzyme-linked immunoassay (ELISA) kits (R&D Systems, Minneapolis, MN), following the manufacturer's instructions, as described elsewhere (31). Urine cytokine levels were expressed as absolute concentrations (pg/ml) as well as concentrations standardized for urine creatinine measured in the same urine spot (pg/mg cr). All samples were assayed in duplicate in a single assay to avoid interassay variation. Our intra-assay variation for the ELISA measurements was below 3%. For measurement of TGF-β1, we used a Quantikine kit (R&D Systems, Minneapolis, MN), and the samples were activated before the assay. The detection limits were 0.1 μg/mL (IL-1β), 0.039 pg/mL (IL-6), 6 pg/mL (IL-8), 0.106 pg/mL (TNF-α), 6 pg/mL (TGF-β1), 8 pg/mL (MCP-1).

5.3.6.STATISTICAL ANALYSIS

The values are expressed as medians and interquartile range (percentile 25, percentile 75) or means and standard deviation (SD), when appropriate. The Mann-Whitney test was used to compare nonparametric continuous variables. Dichotomous variables were compared by the two-sided Fisher's exact test. Correlation between plasma cytokines, urinary cytokines and BMD was performed using a nonparametric test (Spearman rank correlation test). The level of significance was set at $p < 0.05$.

5.3.7.ETHICAL ASPECTS

The Ethics Committee of the Federal University of Minas Gerais approved the study. Informed consent was obtained from all parents and, when appropriate, also from the included patients. The research protocol did not interfere with any medical recommendations or prescriptions. Subject follow-up was guaranteed even in cases of refusal to participate in the study.

5.4. RESULTS

General clinical characteristics at baseline

A total of 70 patients were included in the analysis. The baseline clinical characteristics are summarized in Table 1. Except for the increased use of hydrochlorothiazide in the uncontrolled group ($p<0.05$), there were no differences between patients with uncontrolled and controlled IH. All patients were normotensive and had normal serum creatinine levels at the time of sample collections. The most common signs and symptoms at the time of diagnosis were recurrent abdominal pain (45.7%) and macroscopic hematuria (27.1%). Other findings at baseline were microscopic hematuria (14.3%), urinary tract infection (11.4%) and nephrolithiasis (1.4%).

Association of plasma and urinary cytokine concentrations with urinary calcium excretion

Both in uncontrolled and controlled IH groups, plasma and urinary concentrations of IL-1β, IL-6, IL-8, TNF-α were under the detection limits of the ELISA kits. On the other hand, plasma and urinary concentrations of TGF-β1 and MCP-1 were detectable and shown in Table 2. No significant differences were detected between controlled and uncontrolled IH patients. The same analysis by adopting cytokine levels standardized for urinary creatinine (pg/ mg

cr) also revealed similar values for urinary TGF-β1/cr and MCP-1/cr between the two studied groups (table 2).

There was also a trend toward a positive correlation between plasma and urinary levels of MCP-1 standardized to creatinine (r=0.24, p=0.08), as shown in Figure 2.

Association of plasma and urinary cytokine concentrations with bone mineral density (BMD)

Patients were also stratified according to their BMD Z-score in two groups: >-1 SD, n=28; ≤-1SD; n=18, as shown in Table 3. The comparison between these groups did not reveal differences in general clinical findings as well as in 24 hour urinary calcium excretion.

The comparison of plasma and urinary concentrations of MCP-1 and TGF-β1 in patients with BMD Z-score >-1 SD and ≤-1 SD did not reveal significant differences (Table 4). On the other hand, there was a positive correlation between urinary levels of MCP-1 pg/ml and bone mineral content (BMC) (r=0.379, p=0.013), as shown in Figure 3.

Association of plasma and urinary cytokine concentrations with age groups

In order to detect possible changes in cytokine levels related to age, the patients were stratified in the following age groups: school (age ≤ 12 year; n=18) and adolescent (age> 12 year; n=52). The absolute levels of MCP-1 (pg/ml) were significantly higher in adolescents than in school age children (p=0.02). However, this difference was not observed when values were standardized to creatinine (p=0.61, Table 6).

5.5. DISCUSSION

Recent studies have shown strong clinical and epidemiological evidence supporting an association of bone loss and IH (6, 8, 14, 16, 24, 32). New biomarkers that can improve the diagnostic capability or help determine the risk for episodes of uncontrolled hypercalciuria and bone mineral loss in children and adolescents with IH are needed. Therefore, in this study, we hypothesized the usefulness of plasma and spot-urine measurements of IL-1β, IL-6, IL-8, TNF-α, TGF-β1 and MCP-1 in indentifying uncontrolled IH patients. To our knowledge, this was the first study that measured urinary and plasma cytokines in children and adolescents with IH. Despite the theoretical evidence, our results showed that these biomarkers seem not to be useful in discriminating

patients with high urinary calcium excretion or reduced BMD Z-score. However, there was a significant positive correlation between urinary MCP-1 levels and the BMC.

There is very little information concerning the role of MCP-1 in bone metabolism. The principal function of MCP-1 is the recruitment of monocytes (33, 34). Monocyte products are potential regulators of bone cell activity, since growth factors produced by these cells may stimulate bone formation (35, 36). *In vitro* and *in vivo* studies indicated that MCP-1 induces the recruitment of monocytes to bone, which, in turn, is associated with an increase in osteoblast number (35, 36). This is likely to occur via indirect mechanisms, because MCP-1 did not directly enhance DNA synthesis in osteoblastic cells *in vitro* (36). Thus, activated mononuclear phagocytes may play an important role in bone metabolism by stimulating proliferation of osteoblastic cells. MCP-1 is typically not expressed in normal bone or by normal osteoblasts *in vitro*. Upon stimulation by inflammatory mediators, MCP-1 is up-regulated (37-39). This expression is temporally and spatially associated with the recruitment of monocytes in both osseous inflammation and during developmentally regulated bone remodeling (36). Indeed, monocytes seem to have different functional roles in areas of bone formation and resorption. The recruitment of monocytes in areas of bone

formation was associated with a decrease in the number of osteoclasts, while in bone-resorbing areas, recruitment of cells of the monocytic lineage is associated with formation of osteoclasts (33). The receptor activator of NF-κβ ligand (RANKL) seems to be a key variable in this process, once MCP-1 stimulates the formation of osteoclasts in the presence of RANKL. MCP-1 is also induced by RANKL during osteoclast differentiation. (37) Despite the absence of differences in MCP-1 levels according to urinary calcium excretion and according to the presence of lower BMD Z-score, the positive correlation between urinary MCP-1 and the BMC might indicate a role of this chemokine in bone remodeling in patients with IH. In IH, the mechanisms involved in bone formation and bone resorption were probably activated in spite of the levels of urinary calcium excretion. Therefore, MCP-1 could be locally produced by osteoblasts and signaling towards both events depending on the area of expression and on the interactions with other mediators.

TGF-β is known to stimulate bone formation, mineralization, and inhibiting bone resorption through a proapoptotic effect on mature osteoclasts and by the inhibition of RANKL expression in osteoblasts (22, 40-42). Our measurements showed low levels of TGF-β in patients with IH. Previously studies by Gomes et al

observed similar results with a significantly lower immunostaining for TGF-β in patients with IH when compared with control subjects (22).

In our study, plasma and urinary levels of IL-1β, IL-6, IL-8, TNF-α were under detectable limits. Other authors, by using different methodologies, were able to evaluate these cytokines in IH (20, 22, 43). Pacifici et al described an association, but not a cause-effect relationship, between IL-1β activity and bone resorption. In his study, cultured peripheral blood monocytes were used to show an increased production of IL-1β in IH. (43) Weisinger et al used unstimulated blood monocytes to show an increased expression of IL-1α, IL-6 and TNF-α mRNA in patients with IH. (18) These authors also described a correlation between basal production of IL-1α, but not IL-1β, and decreased trabecular bone (20)

There was no statistical difference in cytokines when patients with BMD Z-score >-1 SD and ≤-1 SD were compared. In addition, an association between reduced BMD and elevated urinary calcium excretion was not observed in our patients. Serial densitometries and concomitant cytokine measurements might be necessary to understand the sequence of events resulting in decreased BMD and increased urinary calcium excretion, once patients with IH probably decrease their calcium bone mineral content progressively as a result of prolonged resorption stimulus (44).

The main possible weakness of our study was that our patients were not on standard diets during sample collections. Diet rich in proteins and salt can significantly affect calciuria by different mechanisms from those involved in bone remodeling in IH. (45) Parathyroid hormone and vitamin D are also important variables not concomitantly measured with cytokines in our research protocol. (46) In this study, age was addressed as a possible confounder. Bone remodeling regulatory mechanisms may vary according to age, once children and adolescents experience different stages in the skeletal development. (47) In spite of that, no significant difference in cytokine measurements were found in the comparison between school age and adolescents with IH.

Nevertheless, some aspects of the study may increase the strength of our findings, such as the sample size, strict inclusion criteria, well-established protocols for cytokine measurements and the homogeneity between groups. Our sample size was considerably large if compared to previous studies on cytokines in IH. (20, 22, 43, 48). Except for calciuria and hydrochlorothiazide use, there was no difference between the controlled and uncontrolled IH groups. Indeed, the increased use of hydrochlorothiazide in the uncontrolled group seemed not to influence cytokine measurements.

In conclusion, bone turnover cytokine measurements were not useful to differentiate uncontrolled and controlled IH. However, we found a significant positive correlation between spot-urine MCP-1 and the BMC, suggesting that the chemokine MCP-1 could play a role in the balance between bone formation/resorption in childhood IH. Further studies are necessary to elucidate the role of MCP-1 in the physiopathology of IH.

BIBLIOGRAPHY

1. Albright F, Henneman P, Benedict PH, Forbes AP. Idiopathic hypercalciuria: a preliminary report. Proc R Soc Med. 1953 Dec;46(12):1077-81.

2. Ammenti A, Neri E, Agistri R, Beseghi U, Bacchini E. Idiopathic hypercalciuria in infants with renal stones. Pediatr Nephrol. 2006 Dec;21(12):1901-3.

3. Moore ES, Coe FL, McMann BJ, Favus MJ. Idiopathic hypercalciuria in children: prevalence and metabolic characteristics. J Pediatr. 1978 Jun;92(6):906-10.

4. Spivacow FR, Negri AL, del Valle EE, Calvino I, Zanchetta JR. Clinical and metabolic risk factor evaluation in young adults with kidney stones. Int Urol Nephrol. 2010 Jun;42(2):471-5.

5. Frick KK, Bushinsky DA. Molecular mechanisms of primary hypercalciuria. J Am Soc Nephrol. 2003 Apr;14(4):1082-95.

6. Zerwekh JE. Bone disease and hypercalciuria in children. Pediatr Nephrol. 2010 Mar;25(3):395-401.

7. Zerwekh JE. Bone disease and idiopathic hypercalciuria. Semin Nephrol. 2008 Mar;28(2):133-42.

8. Weisinger JR, Alonzo E, Carlini RG, Paz-Martinez V, Martinis R, Bellorin-Font E. Bone disease in hypercalciuria: a new form of osteodystrophy? Nephrol Dial Transplant. 1998;13 Suppl 3:88-90.

9. Weisinger JR. New insights into the pathogenesis of idiopathic hypercalciuria: the role of bone. Kidney Int. 1996 May;49(5):1507-18.

10. Tasca A, Dalle Carbonare L, Nigro F, Giannini S. Bone disease in patients with primary hypercalciuria and calcium nephrolithiasis. Urology. 2009 Jul;74(1):22-7.

11. Skalova S, Palicka V, Kutilek S. Bone mineral density and urinary N-acetyl-beta-D-glucosaminidase activity in paediatric patients with idiopathic hypercalciuria. Nephrology (Carlton). 2005 Apr;10(2):99-102.

12. Schwaderer AL, Cronin R, Mahan JD, Bates CM. Low bone density in children with hypercalciuria and/or nephrolithiasis. Pediatr Nephrol. 2008 Dec;23(12):2209-14.

13. Polito C, Iolascon G, Nappi B, Andreoli S, La Manna A. Growth and bone mineral density in long-lasting idiopathic hypercalciuria. Pediatr Nephrol. 2003 Jun;18(6):545-7.

14. Giannini S, Nobile M, Sella S, Dalle Carbonare L. Bone disease in primary hypercalciuria. Crit Rev Clin Lab Sci. 2005;42(3):229-48.

15. Garcia-Nieto V, Ferrandez C, Monge M, de Sequera M, Rodrigo MD. Bone mineral density in pediatric patients with idiopathic hypercalciuria. Pediatr Nephrol. 1997 Oct;11(5):578-83.

16. Garcia-Nieto V, Navarro JF, Ferrandez C. Bone loss in children with idiopathic hypercalciuria. Nephron. 1998;78(3):341-2.

17. Penido MG, Lima EM, Souto MF, Marino VS, Tupinamba AL, Franca A. Hypocitraturia: a risk factor for reduced bone mineral density in idiopathic hypercalciuria? Pediatr Nephrol. 2006 Jan;21(1):74-8.

18. Penido MG, Lima EM, Marino VS, Tupinamba AL, Franca A, Souto MF. Bone alterations in children with idiopathic hypercalciuria at the time of diagnosis. Pediatr Nephrol. 2003 Feb;18(2):133-9.

19. Pfeilschifter J, Chenu C, Bird A, Mundy GR, Roodman GD. Interleukin-1 and tumor necrosis factor stimulate the formation of human osteoclastlike cells in vitro. J Bone Miner Res. 1989 Feb;4(1):113-8.

20. Weisinger JR, Alonzo E, Bellorin-Font E, Blasini AM, Rodriguez MA, Paz-Martinez V, et al. Possible role of cytokines on the bone mineral loss in idiopathic hypercalciuria. Kidney Int. 1996 Jan;49(1):244-50.

21. Gowen M, Mundy GR. Actions of recombinant interleukin 1, interleukin 2, and interferon-gamma on bone resorption in vitro. J Immunol. 1986 Apr 1;136(7):2478-82.

22. Gomes SA, dos Reis LM, Noronha IL, Jorgetti V, Heilberg IP. RANKL is a mediator of bone resorption in idiopathic hypercalciuria. Clin J Am Soc Nephrol. 2008 Sep;3(5):1446-52.

23. Freundlich M, Alonzo E, Bellorin-Font E, Weisinger JR. Reduced bone mass in children with idiopathic hypercalciuria and in their asymptomatic mothers. Nephrol Dial Transplant. 2002 Aug;17(8):1396-401.

24. Misael da Silva AM, dos Reis LM, Pereira RC, Futata E, Branco-Martins CT, Noronha IL, et al. Bone involvement in idiopathic hypercalciuria. Clin Nephrol. 2002 Mar;57(3):183-91.

25. Butani L, Kalia A. Idiopathic hypercalciuria in children--how valid are the existing diagnostic criteria? Pediatr Nephrol. 2004 Jun;19(6):577-82.

26. Coe FL, Evan A, Worcester E. Kidney stone disease. J Clin Invest. 2005 Oct;115(10):2598-608.

27. Hughes P. The CARI guidelines. Kidney stones: metabolic evaluation. Nephrology (Carlton). 2007 Feb;12 Suppl 1:S31-3.

28. Lewiecki EM, Gordon CM, Baim S, Leonard MB, Bishop NJ, Bianchi ML, et al. International Society for Clinical Densitometry 2007 Adult and Pediatric Official Positions. Bone. 2008 Dec;43(6):1115-21.

29. Brandao CM, Camargos BM, Zerbini CA, Plapler PG, Mendonca LM, Albergaria BH, et al. [2008 official positions of the Brazilian Society for Clinical Densitometry--SBDens]. Arq Bras Endocrinol Metabol. 2009 Feb;53(1):107-12.

30. Reinhold D, Bank U, Buhling F, Junker U, Kekow J, Schleicher E, et al. A detailed protocol for the measurement of TGF-beta1 in human blood samples. J Immunol Methods. 1997 Dec 1;209(2):203-6.

31. Sousa-Pereira SR, Teixeira AL, Silva LC, Souza AL, Antunes CM, Teixeira MM, et al. Serum and cerebral spinal fluid levels of chemokines and Th2 cytokines in Schistosoma mansoni myeloradiculopathy. Parasite Immunol. 2006 Sep;28(9):473-8.

32. Heilberg IP, Weisinger JR. Bone disease in idiopathic hypercalciuria. Curr Opin Nephrol Hypertens. 2006 Jul;15(4):394-402.

33. Volejnikova S, Laskari M, Marks SC, Jr., Graves DT. Monocyte recruitment and expression of monocyte chemoattractant

protein-1 are developmentally regulated in remodeling bone in the mouse. Am J Pathol. 1997 May;150(5):1711-21.

34. Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. Clin Chim Acta. 2010 Nov 11;411(21-22):1570-9.

35. Posner LJ, Miligkos T, Gilles JA, Carnes DL, Taddeo DR, Graves DT. Monocyte chemoattractant protein-1 induces monocyte recruitment that is associated with an increase in numbers of osteoblasts. Bone. 1997 Oct;21(4):321-7.

36. Graves DT, Jiang Y, Valente AJ. Regulated expression of MCP-1 by osteoblastic cells in vitro and in vivo. Histol Histopathol. 1999 Oct;14(4):1347-54.

37. Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. J Immunol. 1998 Oct 1;161(7):3719-28.

38. Rahimi P, Wang CY, Stashenko P, Lee SK, Lorenzo JA, Graves DT. Monocyte chemoattractant protein-1 expression and monocyte recruitment in osseous inflammation in the mouse. Endocrinology. 1995 Jun;136(6):2752-9.

39. Williams SR, Jiang Y, Cochran D, Dorsam G, Graves DT. Regulated expression of monocyte chemoattractant protein-1 in normal human osteoblastic cells. Am J Physiol. 1992 Jul;263(1 Pt 1):C194-9.

40. Lorenzo J, Horowitz M, Choi Y. Osteoimmunology: interactions of the bone and immune system. Endocr Rev. 2008 Jun;29(4):403-40.

41. Quinn JM, Itoh K, Udagawa N, Hausler K, Yasuda H, Shima N, et al. Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. J Bone Miner Res. 2001 Oct;16(10):1787-94.

42. Pacifici R. The immune system and bone. Arch Biochem Biophys. 2010 Nov 1;503(1):41-53.

43. Pacifici R, Rothstein M, Rifas L, Lau KH, Baylink DJ, Avioli LV, et al. Increased monocyte interleukin-1 activity and decreased

vertebral bone density in patients with fasting idiopathic hypercalciuria. J Clin Endocrinol Metab. 1990 Jul;71(1):138-45.

44. Baroncelli GI, Bertelloni S, Sodini F, Saggese G. Osteoporosis in children and adolescents: etiology and management. Paediatr Drugs. 2005;7(5):295-323.

45. Giannini S, Nobile M, Sartori L, Dalle Carbonare L, Ciuffreda M, Corro P, et al. Acute effects of moderate dietary protein restriction in patients with idiopathic hypercalciuria and calcium nephrolithiasis. Am J Clin Nutr. 1999 Feb;69(2):267-71.

46. Soylemezoglu O, Ozkaya O, Gonen S, Misirlioglu M, Kalman S, Buyan N. Vitamin D receptor gene polymorphism in hypercalciuric children. Pediatr Nephrol. 2004 Jul;19(7):724-7.

47. Borges JL, Brandao CM. Low bone mass in children and adolescents. Arq Bras Endocrinol Metabol. 2006 Aug;50(4):775-82.

48. Ghazali A, Fuentes V, Desaint C, Bataille P, Westeel A, Brazier M, et al. Low bone mineral density and peripheral blood monocyte activation profile in calcium stone formers with idiopathic hypercalciuria. J Clin Endocrinol Metab. 1997 Jan;82(1):32-8.

TABLE 1 - Subject characteristics, casual measurements and clinical features in patients with idiopathic hypercalciuria (IH) divided according to the level of urinary calcium excretion into uncontrolled IH (≥4 mg/Kg/day) and controlled IH (<4 mg/kg/day).

Characteristics	**Uncontrolled IH (n=27)**	**Controlled IH (n=43)**	**P**
Age (years)	16.99 ± 5.24	14.63 ± 4.42	0.06
Follow up (years)	10.92 ± 6.36	8.60 ± 5.24	0.15
Gender Male (%)	15 (55.56)	25 (58.13)	1.00
Female (%)	12 (44.44)	18 (41.87)	
Race White (%)	16 (59.26)	28 (65.11)	0.80
Non-white (%)	11 (40.74)	15 (34.89)	
Weight (kg)	50.26 ± 17,05	44.21 ± 13,78	0.13
Height (cm)	157.87 ± 16.79	152.14 ±18.03	0.18
BMI (kg/m^2)	19.33 ± 4.32	18.67 ± 2.69	0.49
Systolic pressure (mmHg)	102.92 ± 10.22	97.97 ± 11.06	0.07
Diastolic pressure (mmHg)	62.00 ± 5.95	58.75 ± 9.31	0.09

BMI – body mass index. Student T-test compared continuous variables and Fisher's exact test for percentage comparisons.

TABLE 1 - Subject characteristics, casual measurements and clinical features in patients with idiopathic hypercalciuria (IH) divided according to the level of urinary calcium excretion into uncontrolled IH (≥4 mg/Kg/day) and controlled IH (<4 mg/kg/day). (Continued)

Characteristics	**Uncontrolled IH (n=27)**	**Controlled IH (n=43)**	**P**
Serum Creatinine (mg/dl)	0.73 ± 0.17	0.65 ± 0.18	0.08
Calciuria (mg/24h)	***259.24 ± 88.01***	***107.14 ± 47.33***	***<0.0001***
Calciuria (mg/kg/day)	***5.40 ± 1.53***	***2.34 ± 0.84***	***<0.0001***
Citraturia mg/24h	598.10 ± 295.53	482.34 ± 192.73	0.09
Fosfaturia mg/24h	686.70 ± 241.76	706.90 ± 196.14	0.83
Magnesiuria mg/24h	97.58 ± 37.47	97.73 ± 74.54	0.99
Use of Potassium Citrate (%)	23 (85.18)	32 (74.41)	0.38
Use of hydrochlorothiazide (%)	14 (51.85)	6 (13.95)	0.001
Low BMD L1-L4 (%)	9 (45.00)	9 (34.62)	0.55
Family history of nephrolithiasis (%)	21 (77.78)	31 (72.09)	0.77
Presence of calculus in US (%)	8 (29.63)	20 (46.51)	0.21
History of lithotripsy (%)	4 (14.81)	8 (18.60)	0.76

BMD – bone mineral density, US – ultrasound scan. Student T-test compared continuous variables and Fisher's exact test for percentage comparisons.

TABLE 2 – Median and interquartile range (percentile 25 - p25 and percentile 75 - p75) of plasma and urinary (absolute and standardized to creatinine) levels of MCP-1 and TGF-β1 in patients with uncontrolled and controlled idiopathic hypercalciuria

Cytokines	**Groups**	**Median (p25, p75)**	**P**
Plasma MCP-1 (pg/ml)	Uncontrolled	125.38 (86.90, 157.64)	0.31
	Controlled	129.52 (103.95, 168.48)	
Plasma TGF-β1 (pg/ml)	Uncontrolled	0 (0, 4.36)	0.12
	Controlled	0 (0, 20.04)	
Urinary MCP-1 (pg/mg cr)	Uncontrolled	2.83 (1.38, 4.80)	0.18
	Controlled	4.08 (1.85, 8.66)	
Urinary MCP-1 (pg/ml)	Uncontrolled	209.68 (95.26, 239.16)	0.63
	Controlled	213.28 (109.87, 278.29)	
Urinary TGF-β1 (pg/mg cr)	Uncontrolled	0 (0, 0.21)	0.70
	Controlled	0 (0, 0.20)	
Urinary TGF-β1 (pg/ml)	Uncontrolled	0 (0, 1.35)	0.75
	Controlled	0 (0, 7.70)	

Mann-Whitney U test were used in all comparisons

TABLE 3 - Patients characteristics according to BMD Z-score

Characteristics	**BMD Z-score ≤ -1 SD (n = 18)**	**BMD Z-score > -1 SD (n = 28)**	**P**
BMD Z-score	***-1.65 ± 0.58***	***-0.2 ± 0.68***	***<0.001***
Age (years)	15.15 ± 4.29	13.38 ± 4.96	0.20
Gender: Male (%)	10 (55.56)	15 (53.57)	1.00
Female (%)	9 (50.00)	12 (42.86)	
Uncontrolled IH (%)	9 (50.00)	9 (32.14)	0.23
BMI (kg/m^2)	19.19 ± 3.74	18.65 ± 3.95	0.66
Calciuria (mg/24h)	175.77 ± 93.75	161.78 ± 112.04	0.65

BMI – body mass index, BMD – bone mineral density. Student T-test compared continuous variables and Fisher's exact test for percentage comparisons.

TABLE 4 - Median and interquartile range (percentile 25 - p25 and percentile 75 - p75) of plasma and urinary (absolute and standardized to creatinine) levels of MCP-1 and TGF-β1 in patients according to BMD Z-score

Cytokines	**Groups ***	**Median (p25, p75)**	**P**
Plasma MCP-1 (pg/ml)	>-1	135.61 (87.15, 185.89)	0.88
	≤-1	145.96 (104.80, 165.58)	
Plasma TGF-β1 (pg/ml)	>-1	0 (0, 4.36)	0.06
	≤-1	8.95 (0, 27.20)	
Urinary MCP-1 (pg/mg cr)	>-1	5.14 (1.79, 8.58)	0.81
	≤-1	3.68 (2.67, 8.56)	
Urinary MCP-1 (pg/ml)	>-1	209.68 (121.51, 237.42)	0.67
	≤-1	225.16 (131.79, 296.24)	
Urinary TGF-β1 (pg/mg cr)	>-1	0 (0, 0.24)	0.36
	≤-1	0 (0, 0.21)	
Urinary TGF-β1 (pg/ml)	>-1	0 (0, 5.976)	0.48
	≤-1	0 (0, 8.87)	

* BMD Z-score SD

Median comparisons were made by Mann-Whitney test

TABLE 5 - Median and interquartile range (percentile 25 - p25 and percentile 75 - p75) of plasma and urinary (absolute and standardized to creatinine) levels of MCP-1 and TGF-β1 in patients equal or under 12 years old (school, n=18) and above 12 years old (adolescent, n=52).

Cytokines	**Groups**	**Median (p25, p75)**	**P**
Plasma MCP-1 (pg/ml)	School	134.39 (99.36, 187.23)	0.82
	Adolescent	129.15 (103.95, 163.12)	
Plasma TGF-β1 (pg/ml)	School	0 (0, 21.78)	0.69
	Adolescent	0 (0, 11.15)	
Urinary MCP-1 (pg/mg cr)	School	6.08 (1.31, 8.58)	0.61
	Adolescent	3.18 (1.65, 7.29)	
Urinary MCP-1 (pg/ml)	***School***	***121.51 (72.34, 203.74)***	***0.02***
	Adolescent	***220.95 (137.86, 296.37)***	
Urinary TGF-β1 (pg/mg cr)	School	0 (0, 0.20)	0.74
	Adolescent	0 (0, 0.21)	
Urinary TGF-β1 (pg/ml)	School	0 (0, 5.15)	0.56
	Adolescent	0 (0, 11.01)	

Median comparisons were made by Mann-Whitney test

FIGURE LEGENDS

Figure 1 - Flow diagram of the study. Uncontrolled idiopathic hypercalciuria (IH) was defined as a 24-hour urinary calcium excretion equal or superior to 4 mg/kg/day.

Figure 2 – Correlation between plasma (pg/ml) and urinary MCP-1 standardized to creatinine (pg/mg cr). p=0.089, r =0.213 (Spearman test).

Figure 3 - Correlation between absolute levels of urinary MCP-1 (pg/ml) and the bone mineral content (BMC). p=0.013, r =0.379 (Spearman test).

FIGURE 1

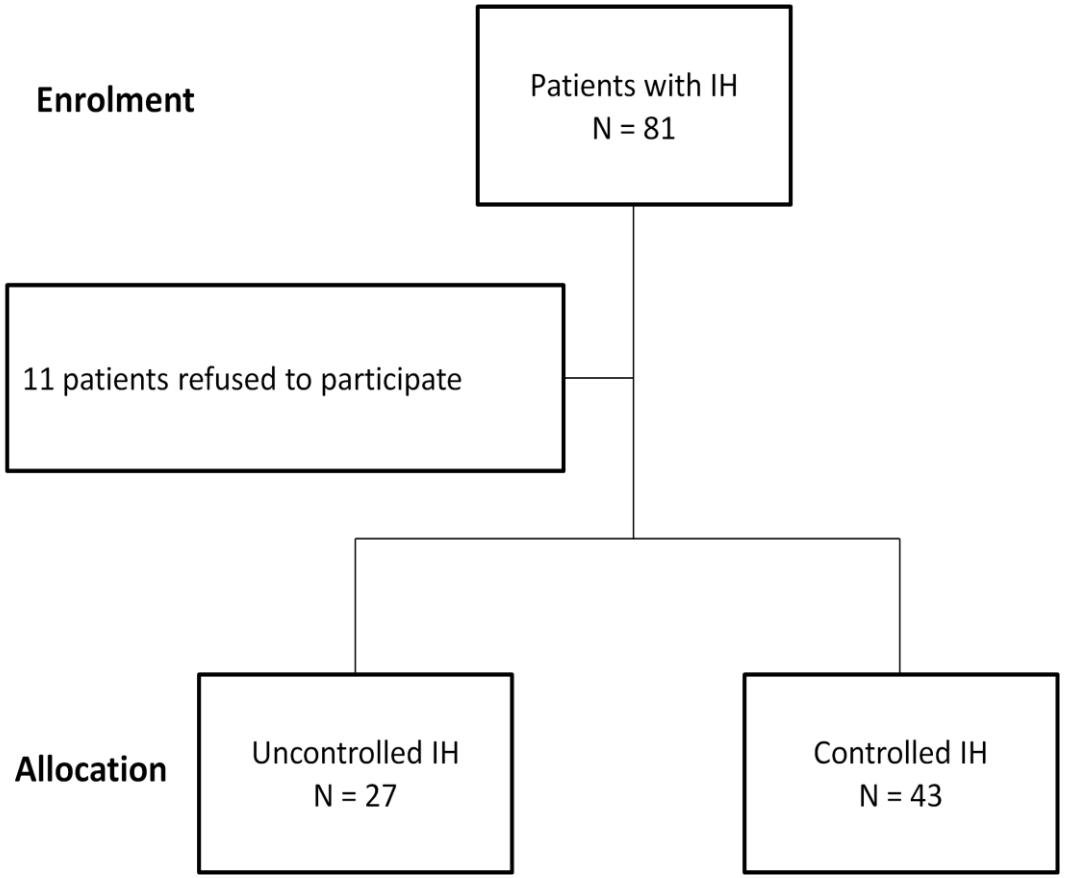

Enrolment
Patients with IH
N = 81
11 patients refused to participate
Allocation
Uncontrolled IH
N = 27
Controlled IH
N = 43

FIGURE 2

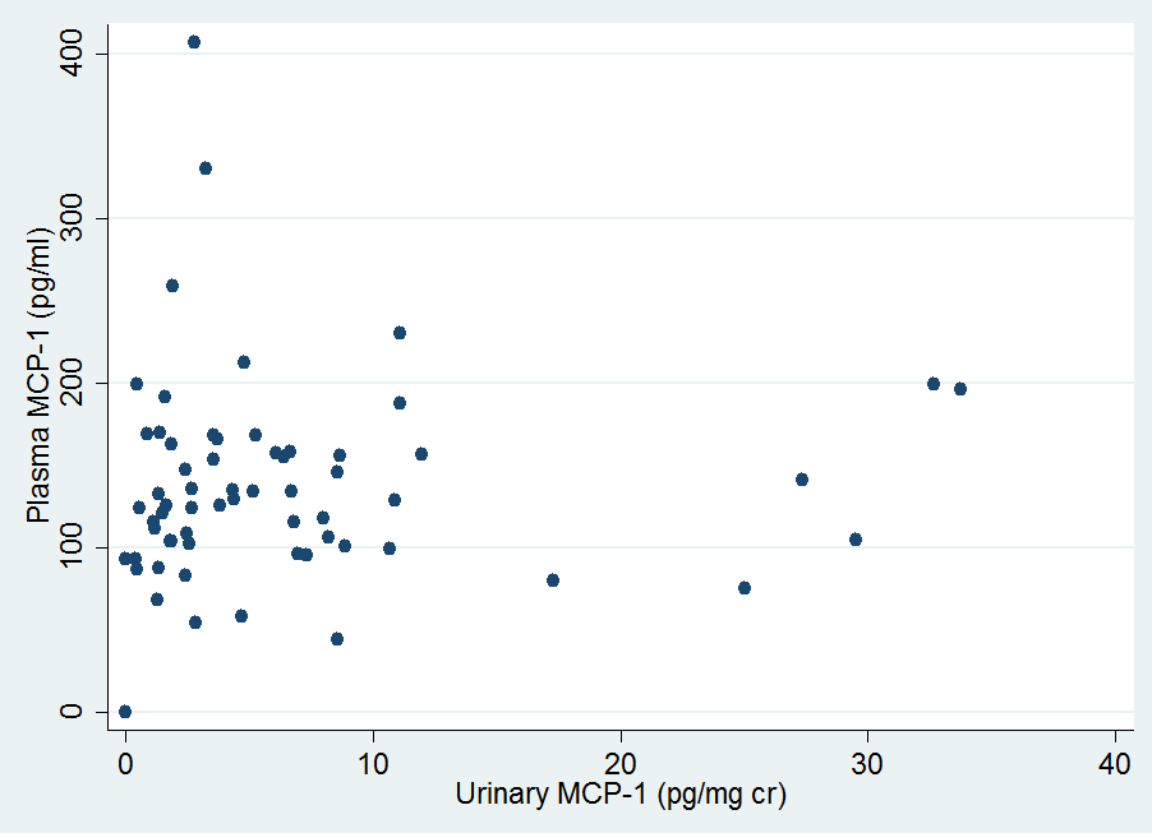
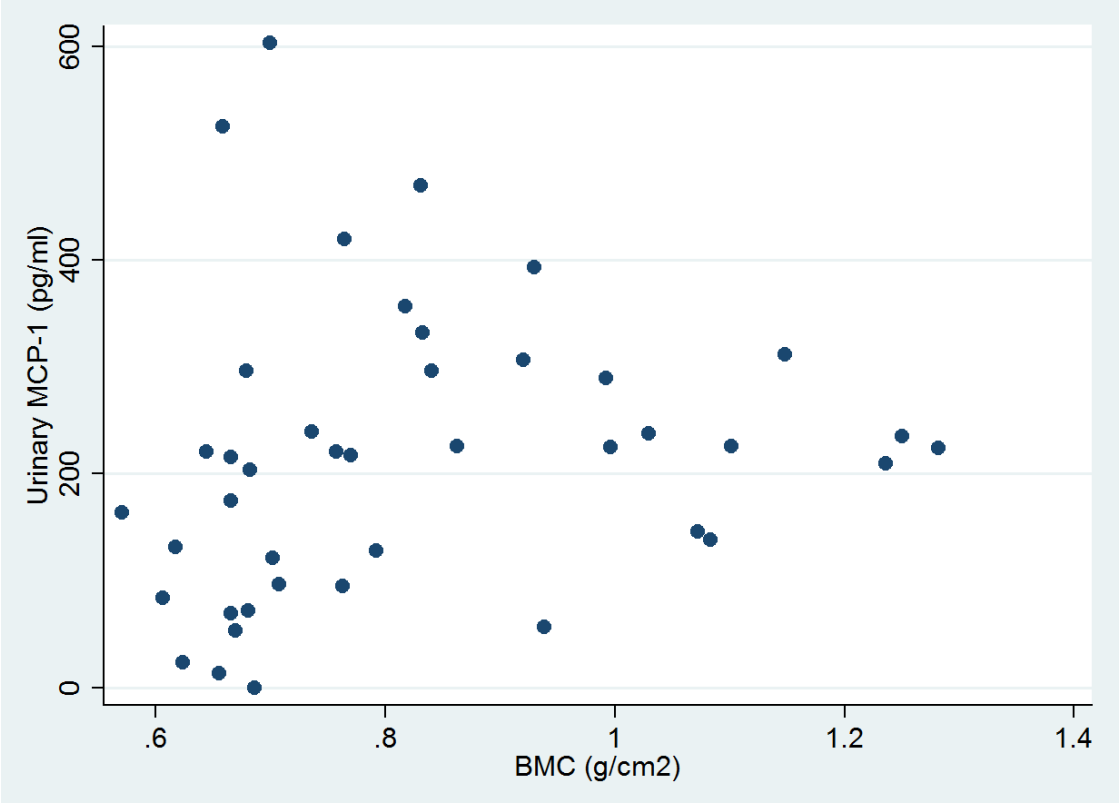

Plasma MCP-1 (pg/ml)
0
100
200
300
400
0
10
20
30
40
Urinary MCP-1 (pg/mg cr)

FIGURE 3



6. COMENTÁRIOS FINAIS

A HI é uma desordem metabólica freqüente em crianças e adolescentes (1). As alterações ósseas observadas em estudos clínicos em crianças e adolescentes portadores de HI sugerem que mecanismos relacionados à regulação do remodelamento ósseo estejam envolvidos na fisiopatologia da HI (2).

No entanto, a etiopatogenia das alterações ósseas na HI é complexa e pouco compreendida. Apesar dos estudos sugerirem a participação citocinas e quimiocinas na gênese da excreção aumentada de cálcio na urina, ainda não se sabe precisar se as alterações no metabolismo ósseo representam um fenômeno primário ou secundário na fisiopatologia da HI (3). Há, além disso, alguns fatores que dificultam a interpretação dos resultados de DMO em crianças e adolescentes. Ainda não existe consenso em relação aos ajustes da DMO para fatores como tamanho do osso, estadiamento puberal, maturidade esquelética e composição corporal em pacientes na faixa pediátrica (4). Por este motivo, nesses pacientes, o diagnóstico de osteoporose não pode ser feito fundamentando-se unicamente no critério densitométrico (4). Além disso, ainda não há dados suficientes para se estimar com precisão, por meio da DMO, o risco de fraturas em crianças e adolescentes (4). Recomenda-se, então, utilizar os termos “baixa massa óssea

para a idade cronológica" ou "abaixo da faixa esperada para a idade" em detrimento à osteopenia quando o Z-score for menor que −2 DP (4). Esses aspectos devem ser levados em consideração na elaboração de novos protocolos de estudos clínicos em crianças e adolescentes portadores de HI.

Na HI, estudos têm demonstrado aumento na atividade de citocinas e quimiocinas com potencial de desencadear a maturação de osteoclastos e, conseqüentemente, favorecer a reabsorção óssea (2). Nesse contexto, diversos estudos têm avaliado, principalmente *in vitro*, o papel de citocinas e quimiocinas no processo de remodelamento ósseo (5-8).

No presente estudo, avaliamos se citocinas e quimiocinas dosadas no plasma e na urina de pacientes com HI poderiam atuar como biomarcadores dessa alteração metabólica. No entanto, detectamos apenas duas das citocinas dosadas no plasma e urina de pacientes com HI: o MCP-1 e o TGF-β1. Os demais marcadores não foram detectados nas amostras coletadas de nossos pacientes. Ao compararmos os níveis urinários e plasmáticos destas duas citocinas de acordo com a excreção urinária de cálcio, a faixa etária e a estratificação pelo Z-score da DMO, não foram verificadas diferenças significativas. Por outro lado, observamos uma correlação positiva e estatisticamente significativa entre os níveis urinários de

MCP-1 e o conteúdo mineral ósseo (CMO), sugerindo um possível papel desta quimiciona no remodelamento ósseo.

O MCP-1 é uma quimiocina envolvida primordialmente no recrutamento de monócitos (9). Estudos *in vitro* e *in vivo* sugerem que o MCP-1 possa induzir o recrutamento de monócitos para os ossos, desencadeando o processo de remodelamento (9-10). Estes monócitos desempenham papéis distintos, conforme a área para onde são recrutados, podendo tanto ativar o processo de formação óssea quanto promover a reabsorção óssea. Estudos experimentais associaram o recrutamento de monócitos em áreas de formação óssea com uma redução no número de osteoclastos, enquanto que em áreas de reabsorção óssea houve associação com a maturação de osteoclastos (11). O RANKL parece ser o fator preponderante para definir o papel do monócito neste processo, uma vez que, na presença de RANKL, o MCP-1 estimula a maturação de osteoclastos (12-13).

O TGF-β1 está associado à formação, mineralização e inibição da reabsorção óssea. Níveis reduzidos de TGF-β estimulam a diferenciação do osteoclasto, levando ao aumento da reabsorção óssea (14-15). A detecção de valores baixos para o TGF-β1 em nossos pacientes, independente da calciúria e da faixa etária, pode indicar uma expressão diminuída dessa citocina no tecido ósseo,

que poderia favorecer os processos reabsorção óssea. Este resultado é compatível com estudos anteriores, que sugerem a preponderância do processo de reabsorção sobre o de formação óssea nestes pacientes (8).

Vale ressaltar que estamos cientes das limitações do presente estudo. Há evidencias de que dietas ricas em proteínas e sal podem alterar a excreção urinária de cálcio (16). No entanto, os pacientes envolvidos neste estudo não estavam em dieta padronizada previamente as coletas das amostras. Nosso protocolo de pesquisa não contemplou a análise do PTH e das dosagens de vitamina D em conjunto com as dosagens das citocinas (17). Pacientes de diferentes idades apresentam diferentes estágios de desenvolvimento ósseo (18). Mesmo assim, não encontramos diferenças nos valores das citocinas ao comparar grupos etários (<12 anos e ≥12 anos).

Entender a fisiopatologia da HI ainda é um desafio. Nosso estudo foi pioneiro na determinação de citocinas na urina e plasma de crianças e adolescentes portadores de HI. Conforme já mencionado, nosso resultado mais interessante foi a verificação de uma correlação positiva e estatisticamente significativa ($p=0.013$) entre as medidas de DMO e os níveis urinários absolutos de MCP-1. Obviamente, este resultado não permite estabelecer nenhum

mecanismo, mas sugere a participação do MCP-1 na fisiopatologia da HI. Além disso, houve um resultado próximo da significância estatística (p=0.08) para a correlação entre os níveis urinários e plasmáticos do MCP-1. Este resultado sugere a possibilidade de se utilizar dosagens de MCP-1 em amostras únicas de urina nas investigações futuras sobre o papel desta quimiocina na HI.

Ainda existe um longo caminho para desvendar os mecanismos fisiopatológicos da HI. O presente estudo foi apenas um passo inicial no sentido de avaliar possíveis biomarcadores desse processo em amostras biológicas de fácil obtenção. Novos estudos deverão ser realizados com o intuito de desvendar as alterações imunológicas presentes na HI e determinar o potencial dessas descobertas para a propedêutica e o tratamento da HI.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Srivastava T, Alon US. Pathophysiology of hypercalciuria in children. Pediatr Nephrol. 2007 Oct;22(10):1659-73.

2. Tasca A, Dalle Carbonare L, Nigro F, Giannini S. Bone disease in patients with primary hypercalciuria and calcium nephrolithiasis. Urology. 2009 Jul;74(1):22-7.

3. Heilberg IP, Weisinger JR. Bone disease in idiopathic hypercalciuria. Curr Opin Nephrol Hypertens. 2006 Jul;15(4):394-402.

4. Brandao CM, Camargos BM, Zerbini CA, Plapler PG, Mendonca LM, Albergaria BH, et al. [2008 official positions of the Brazilian Society for Clinical Densitometry--SBDens]. Arq Bras Endocrinol Metabol. 2009 Feb;53(1):107-12.

5. Ghazali A, Fuentes V, Desaint C, Bataille P, Westeel A, Brazier M, et al. Low bone mineral density and peripheral blood monocyte activation profile in calcium stone formers with idiopathic hypercalciuria. J Clin Endocrinol Metab. 1997 Jan;82(1):32-8.

6. Pacifici R, Rothstein M, Rifas L, Lau KH, Baylink DJ, Avioli LV, et al. Increased monocyte interleukin-1 activity and decreased vertebral bone density in patients with fasting idiopathic hypercalciuria. J Clin Endocrinol Metab. 1990 Jul;71(1):138-45.

7. Weisinger JR, Alonzo E, Bellorin-Font E, Blasini AM, Rodriguez MA, Paz-Martinez V, et al. Possible role of cytokines on the bone mineral loss in idiopathic hypercalciuria. Kidney Int. 1996 Jan;49(1):244-50.

8. Gomes SA, dos Reis LM, Noronha IL, Jorgetti V, Heilberg IP. RANKL is a mediator of bone resorption in idiopathic hypercalciuria. Clin J Am Soc Nephrol. 2008 Sep;3(5):1446-52.

9. Posner LJ, Miligkos T, Gilles JA, Carnes DL, Taddeo DR, Graves DT. Monocyte chemoattractant protein-1 induces monocyte recruitment that is associated with an increase in numbers of osteoblasts. Bone. 1997 Oct;21(4):321-7.

10. Graves DT, Jiang Y, Valente AJ. Regulated expression of MCP-1 by osteoblastic cells in vitro and in vivo. Histol Histopathol. 1999 Oct;14(4):1347-54.

11. Volejnikova S, Laskari M, Marks SC, Jr., Graves DT. Monocyte recruitment and expression of monocyte chemoattractant protein-1 are developmentally regulated in remodeling bone in the mouse. Am J Pathol. 1997 May;150(5):1711-21.

12. Valente AJ, Xie JF, Abramova MA, Wenzel UO, Abboud HE, Graves DT. A complex element regulates IFN-gamma-stimulated monocyte chemoattractant protein-1 gene transcription. J Immunol. 1998 Oct 1;161(7):3719-28.

13. Kim MS, Day CJ, Morrison NA. MCP-1 is induced by receptor activator of nuclear factor-{kappa}B ligand, promotes human osteoclast fusion, and rescues granulocyte macrophage colony-stimulating factor suppression of osteoclast formation. J Biol Chem. 2005 Apr 22;280(16):16163-9.

14. Quinn JM, Itoh K, Udagawa N, Hausler K, Yasuda H, Shima N, et al. Transforming growth factor beta affects osteoclast differentiation via direct and indirect actions. J Bone Miner Res. 2001 Oct;16(10):1787-94.

15. Karst M, Gorny G, Galvin RJ, Oursler MJ. Roles of stromal cell RANKL, OPG, and M-CSF expression in biphasic TGF-beta regulation of osteoclast differentiation. J Cell Physiol. 2004 Jul;200(1):99-106.

16. Giannini S, Nobile M, Sartori L, Dalle Carbonare L, Ciuffreda M, Corro P, et al. Acute effects of moderate dietary protein restriction in patients with idiopathic hypercalciuria and calcium nephrolithiasis. Am J Clin Nutr. 1999 Feb;69(2):267-71.

17. Soylemezoglu O, Ozkaya O, Gonen S, Misirlioglu M, Kalman S, Buyan N. Vitamin D receptor gene polymorphism in hypercalciuric children. Pediatr Nephrol. 2004 Jul;19(7):724-7.

18. Borges JL, Brandao CM. Low bone mass in children and adolescents. Arq Bras Endocrinol Metabol. 2006 Aug;50(4):775-82.

7. ANEXO 1- PARECER DO COEP

UFMG

Universidade Federal de Minas Gerais
Comitê de ética em pesquisa da UFMG - COEP

Parecer nº: ETIC 036/00
Interessada : Profa. Maria Goretti Moreira Guimarães Penido

VOTO:

O Comitê de Ética em Pesquisa da UFMG - COEP aprova definitivamente no dia 17.05.2000 o projeto de pesquisa intitulado: «***Hipercalciúria Idiopática e a Doença Óssea Metabólica: Avaliação, Marcadores, Evolução e Efeitos sobre o Crescimento Pondo-Estatural em Crianças e Adolescentes***» e o Termo de Consentimento do referido projeto, de interesse da Profa. Maria Goretti Moreira Guimarães Penido. O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Prof. Dr. Dirceu Bartolomeu Greco
Presidente do COEP

Av. Alfredo Balena, 110-1º andar
Bairro Santa Efigênia – Cep: 30.130-100 – Belo Horizonte -MG
Telefone: (031)– 248-9364
FAX: (031) 248 9380 – Telex: (031) 2544
e-mail: coep@reitoria.ufmg.br

8. ANEXO 2 - TERMO DE CONSENTIMENTO

TÍTULO DO PROJETO: "AVALIAÇÃO DOS NÍVEIS PLASMÁTICOS E URINÁRIOS DAS CITOCINAS EM CRIANÇAS E ADOLESCENTES PORTADORES DE HIPERCALCIÚRIA IDIOPÁTICA"

Investigadores
Coordenadora: Ana Cristina Simões e Silva
Pesquisadores: Maria Goretti Moreira Guimarães Penido
Augusto César Soares Dos Santos Junior

OBJETIVOS E JUSTIFICATIVA

Você está sendo convidado a participar de um estudo clínico sobre uma doença chamada hipercalciúria idiopática (HI) que é uma enfermidade muito comum no nosso meio. Essa doença pode levar a formação de cálculos urinários e aparece como a principal alteração metabólica responsável pela formação de cálculos urinários e pode também causar desmineralização óssea com risco de osteopenia e osteoporose. Acredita-se que também pode causar importante comprometimento da estatura final de crianças e adolescentes. Estudos têm demonstrado a participação de algumas substâncias chamadas citocinas, em particular o TNF-α, a IL-1 e a IL-6, no processo de reabsorção do osso nessa doença. Embora essas citocinas estejam envolvidas no processo de reabsorção óssea, ainda não se sabe qual o mecanismo exato que essas

citocinas agem nesses pacientes. Além disso, não existe nenhum estudo, na literatura, relacionando os níveis do sangue e urinários de citocinas à evolução clínica de crianças e adolescentes com essa doença. Este estudo pretende determinar os níveis dessas substâncias (fator de necrose tumoral do tipo alfa - TNF-α, interleucina 1 - IL-1 e interleucina 6 - IL-6) em crianças e adolescentes com Hipercalciúria Idiopática. Dessa forma, este projeto visa também o aprofundamento no entendimento de como a doença evolui e do seu tratamento.

PROCEDIMENTOS

Na primeira consulta o médico irá examiná-lo. Serão feitas algumas perguntas sobre a sua história médica, após o que será realizado o exame físico. Esses dados serão anotados no seu prontuário.

Posteriormente, você será submetido à coleta de sangue e urina, em uma única ocasião.

QUESTÕES

Nós, Doutores Ana Cristina Simões e Silva, Maria Goretti Moreira Guimarães Penido, e Augusto César Soares dos Santos Junior, médicos pesquisadores e responsáveis por esta avaliação, explicaremos claramente todos os procedimentos e buscaremos esclarecer todas as suas dúvidas. Caso você apresente qualquer

questão a respeito do estudo ou se algo acontecer neste intervalo, você poderá sempre entrar em contato conosco.

Dra. Ana Cristina Simões e Silva, Dra. Maria Goretti Moreira Guimarães Penido e Dr. Augusto César Soares dos Santos Junior no telefone 3248-9445 ou no endereço: Av. Alfredo Balena, 190, bairro Santa Efigênia – Ambulatório de Nefrologia Pediátrica (Hospital Bias Fortes – 4º andar – sala 420).

Telefone de contato do Comitê de Ética em Pesquisa da UFMG: 3248-9364

BENEFÍCIOS

Você pode não se beneficiar diretamente ao participar deste estudo. Por outro lado, o seguimento clínico de sua doença está assegurado em nosso serviço.

ALTERNATIVAS – DIREITO À RECUSA

Sua participação no estudo é voluntária e, caso se recuse a participar ou se retire do estudo, isso não afetará sua relação com o seu médico ou qualquer outro profissional que cuide de sua saúde. Além disso, as suas necessidades clínicas não serão comprometidas pelo fato de você não participar do estudo.

RISCOS

O estudo é praticamente isento de maiores riscos, no entanto, serão coletados sangue e urina e haverá os riscos relacionados a esses procedimentos.

CARÁTER CONFIDENCIAL

Todos os registros identificando você serão mantidos de modo confidencial e a sua identidade será conhecida apenas pelo seu médico e os responsáveis pela pesquisa. Sua identidade também será mantida de modo confidencial inclusive quando este estudo for publicado. Todas as informações obtidas neste estudo, além de confidenciais, serão utilizadas exclusivamente para a investigação científica.

Ao assinar este formulário, você autoriza o Comitê de Ética em Pesquisa da UFMG de Belo Horizonte e outras autoridades regulamentadoras a consultar seus registros médicos a fim de checar os dados coletados neste estudo com o que está escrito nos registros. Sua identidade não será revelada e as leis regulando tais procedimentos serão seguidas.

Este estudo seguirá as diretrizes e normas regulamentadoras de pesquisa envolvendo seres humanos – Resolução no 196/96 e 215/97 do Conselho Nacional de Saúde.

CONSENTIMENTO

Eu li e entendi o texto acima e da forma como foi descrita pelo meu médico. Eu recebi uma cópia deste termo de consentimento, tive chance de lê-lo e minhas dúvidas foram esclarecidas. Com a minha assinatura, concordo em participar do estudo descrito acima.

_____

Assinatura do paciente ou representante legal Data:

Eu, por este meio, confirmo que o voluntário deu seu livre consentimento em participar do estudo.

_____

Assinatura do investigador Data:

Eu, por meio deste, confirmo que testemunhei o voluntário recebendo estas informações e dando livremente seu consentimento em participar do estudo.

_____

Assinatura da testemunha Data:

9. ANEXO 3 - COMPROVANTE DE APROVAÇÃO PARA APRESENTAÇÃO EM CONGRESSO INTERNACIONAL (PÔSTER)

WCN Abstract Notifications

De: **Jennifer Spinks** (jspinks@marathonmultimedia.com)
Enviada: terça-feira, 18 de janeiro de 2011 17:48:58
Para: acssjunior@hotmail.com

Dear Dr. Santos Junior:
Abstract Number: 662
Abstract Title: ARE URINARY LEVELS OF MCP-1 ASSOCIATED WITH CALCIUM EXCRETION IN IDIOPATHIC HYPERCALCIURIA?
On behalf of the International Society of Nephrology, thank you for submitting an abstract for the World Congress of Nephrology on April 8-12, 2011 in Vancouver, Canada. We are pleased to inform you that your abstract listed above has been selected by the Scientific Program Committee for a Poster presentation.

WCN will honor the Early Bird fee for your registration to the WCN 2011 to present your abstract. In order to receive the Early Bird rate, you must click on the link below and follow the instructions to register for the Congress. You will receive a registration confirmation that must bring with you to the Congress to pick up your badge in the West Lobby Registration. Next step after registering is to secure your hotel rooms and block your rooms in the designated Congress hotels. After your registration is confirmed, you can proceed in the same steps to register for your hotel room.

For Registration to the WCN meeting, please click https://www.compusystems.com/servlet/ar?evt_uid=307&PromoCode=10511

For Poster Presentation

Posters are located in Exhibit West Hall B. The poster board display area is 4' high x 4' wide (1.22mx1.22m). The exhibit hall opens each day of the poster sessions at 09:00am. You can begin set up of your poster at 08:00am. Please have your poster ready for viewing by 10:30am on the day of your poster presentation. You can begin taking down your poster at 13:30pm and it must be completely removed by 17:00pm.

Poster Presentation Date: Monday, 4/11/2011
Publication/Poster #: MO250

For your convenience, a poster printing service (Call4Posters®) for WCN 2011 Vancouver will also be available. We are sure that you will find it a convenient and simple way to produce a professional poster.
You will be sent information on how to access the website after Friday 25 February 2011.

Congratulations on having your abstract selected for a presentation. We appreciate your willingness to participate and look forward to seeing you in Vancouver, Canada.
Sincerely,

Carol Pollock and Brenda Hemmelgarn
WCN Scientific Abstract Co-Chairs

8th Conference on Kidney Disease in Disadvantaged Populations
Disparities in Renal Disease - Moving Towards Solutions
12-14 April 2011, Harbour Towers Hotel, Victoria BC, Canada
An official satellite meeting of the World Congress of Nephrology 2011

To: Dr. Augusto Cesar Soares dos Santos Ju
Brazil
E-mail: acssjunior@hotmail.com

Subject: Abstract notification letter*

4 January 2011

Dear Dr. Soares dos Santos Ju,

We are pleased to inform you that your abstract (listed below) has been accepted for presentation at the above mentioned meeting.

Your abstract details:

Original Abstract submission # **150**

NEW ABSTRACT NUMBER: # 204

Abstract title: **ARE URINARY LEVELS OF MCP-1 ASSOCIATED WITH CALCIUM EXCRETION IN IDIOPATHIC HYPERCALCIURIA?**

Presenter: Augusto Cesar Soares dos Santos Ju
(if you are not the presenter, please send us a note with the name of the presenter)

Presentation method: poster

In case you need to cancel your participation, please inform us timely.

Sincerely yours,

Ilja Huang, CKHDP Manager
On behalf of the Conference Organizing Committee:
Dr. Guillermo Garcia-Garcia (Mexico), Chairman ISN GO (Global Outreach) Committee for Kidney Disease in Disadvantaged Populations.
Dr. Lawrence Agodoa (USA) and Dr. Karen Yeates (Canada), Chairmen Scientific Program Committee

Website: www.wcn2011satellite.com