UNIVERSIDADE FEDERAL DE PERNAMBUCO

CENTRO DE BIOCIÊNCIAS

DEPARTAMENTO DE GENÉTICA

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

CAMILLA ALBERTINA DANTAS DE LIMA

ANÁLISE DO PERFIL GENÉTICO E FUNCIONAL DAS CITOCINAS IL-23, IL-17, IL-12 E IFN-γ E SUAS RELAÇÕES COM A OSTEOPOROSE PRIMÁRIA PÓS-MENOPAUSA

Recife

2017

CAMILLA ALBERTINA DANTAS DE LIMA

ANÁLISE DO PERFIL GENÉTICO E FUNCIONAL DAS CITOCINAS IL-23, IL-17, IL-12 E IFN-γ E SUAS RELAÇÕES COM A OSTEOPOROSE PRIMÁRIA PÓS-MENOPAUSA

Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutora em Genética.

Orientadora: Paula Sandrin Garcia

Co-orientadores: Jaqueline de Azevêdo Silva

Paulo Roberto Eleutério de Souza

Recife

2017

Catalogação na fonte

Elaine Barroso

CRB 1728

Lima, Camilla Albertina Dantas de

Análise do perfil genético e funcional das citocinas IL-23, IL-17,IL-12 e IFN-γ e suas relações com a osteoporose primária pós-menopausa / Camilla Albertina Dantas de Lima- Recife: O Autor, 2017.

137 folhas: il., fig., tab.
Orientadora: Paula Sandrin-Garcia
Coorientadores: Jaqueline de Azevedo Silva e Paulo Roberto Eleutério de Souza
Tese (doutorado) – Universidade Federal de Pernambuco. Centro de Biociências. Genética, 2017.
Inclui referências e anexo

1. **Osteoporose em mulheres 2. Polimorfismos (genética) 3. Genes I. Sandrin-Garcia, Paula (orientadora) II. Silva, Jaqueline de Azevedo (coorientadora) III. Souza, Paulo Roberto Eleutério de (coorientador) IV. Título**

616.716 CDD (22.ed.) UFPE/CB-2017-258

ANÁLISE DO PERFIL GENÉTICO E FUNCIONAL DAS CITOCINAS IL-23, IL-17, IL-12 E IFN- γ E SUAS RELAÇÕES COM A OSTEOPOROSE PRIMÁRIA PÓS-MENOPAUSA

Aprovado em 24/02/2017

Banca Examinadora

Dra. Paula Sandrin Garcia

Universidade Federal de Pernambuco - UFPE

Dr. Will Barros Pita

Universidade Federal de Pernambuco - UFPE

Dra. Michelly Cristiny Pereira

Universidade Federal de Pernambuco - UFPE

Dr. Ronaldo Celerino da Silva

Universidade Federal de Pernambuco - UFPE

Dr. Fabrício Oliveira Souto

Universidade Federal de Pernambuco - UFPE

Dr. Luydson Richardson Silva Vasconcelos

Centro de Pesquisas Aggeu Magalhães – CpqAM/Fiocruz

Aos pacientes que em meio a suas dores físicas se doam na esperança da cura para todos.

AGRADECIMENTOS

A Deus que através da sua bondade e amor tem permitido todas as descobertas que trazem alívio para as dores da humanidade. Porque é nEle que todas as coisas encontram um sentido. Por todo amor que me fez viver nessa longa jornada.

À minha orientadora Paula Sandrin pelos ensinamentos não só profissionais, mas também pessoais. Por todo seu carinho, cuidado e dedicação. Você é uma grade pesquisadora, mas acima de tudo uma grande pessoa com quem aprendi muitas coisas que levarei para a vida inteira.

À minha co-orientadora Jaqueline de Azevêdo Silva por todos os ensinamentos, carinho, dedicação e amizade. Toda essa pesquisa está cheia das suas ideias e intervenções. Sua assinatura. Sua vida e inteligência me inspiram a continuar nessa jornada.

Ao meu co-orientador Paulo Souza, por me acompanhar em toda essa caminhada acadêmica. Sua dedicação, humildade, inteligência e humanidade me fazem acreditar num futuro feliz para a ciência que vivemos. Mais uma etapa que se encerra e posso contar com sua força e amizade. Quando crescer, quero ser, pelo menos parecida com você.

Aos meus queridos companheiros de pesquisa em Osteoporose (a turma do terceiro turno das sextas-feiras): Anninha, pelos desafios que aceitou enfrentar comigo ao longo de todos esses dias. Por todas nossas placas, coletas, planilhas e reuniões, que nos uniram e fizeram nascer nossa amizade. Pela sua sinceridade, dedicação, inteligência e carinho. Você é única e agradeço a Deus ter sua companhia nessa jornada. Nat, por ter abraçado nossas ideias e executar com tanto cuidado e inteligência, vejo seu carinho em todos os passos desse trabalho e sei que tudo isso não seria possível sem você. Guaraná, porque não é fácil encontrar alguém para abraçar nossos projetos em horas difíceis, mas tivemos sorte por você ter chegado. Vejo feliz em você um potencial promissor para ciência brasileira. Continue!! Alexandre, por toda disponibilidade em nos atender diante dos grandes desafios da Reumatologia e por cuidar com tanto carinho das pacientes atendidas.

Essa atitudes nos fazem acreditar num mundo melhor. A Jorginho, que por muito pouco não é do grupo da sexta, mas que disponibiliza tanta inteligência, amizade e cuidado. Não tenho palavras para você.

Ao grupo de Autoimunidade, que tem me ensinado tanto sobre pesquisa, trabalho em equipe e dedicação. Vocês me inspiram. Em especial a Catarina pelo acolhimento e Eduardo pelas parcerias e todo empenho em colocar nossas ideias para frente.

A todo grupo PatGen por toda ajuda e aprofundamento de conhecimento. É uma honra para mim poder conviver com todos. Em especial a Ronaldo, Heitor, Antônio e Anselmo por toda paciência e ensinamentos compartilhados. Aprendi e aprendo muito com vocês.

Ao Prof. Sergio Crovella por sempre apoiar nossas ideias e acrescentar opiniões mais que relevantes a todas elas.

À minha querida amiga e parceira científica Ana Paula Valença, por ter dedicado tanto do seu tempo a compartilhar seus conhecimentos comigo. Minha amiga, agradecer é pouco lembrando de tudo que aprendi com você nesses dias. Obrigada por todo seu carinho.

A Cida Seabra, pela sua dedicação e amor à pesquisa, pelos fins de semana que abriu mão para abraçar nosso trabalho, pelas tardes de reunião e todos os lanches de comemoração de experimento. Nossa parceria é para sempre!

À minha mãe e a minha irmã Carol em quem encontro meu suporte e lugar seguro em todos os dias, nos fáceis, difíceis e até mesmo naqueles que parecem insuportáveis. Sem dúvidas estar aqui é crédito de vocês. Palavras não podem definir tudo o que vivemos juntas, mas nós bem sabemos e guardamos no coração. Vocês são tudo que mais amo nessa vida.

Ao meu marido Welton Simões, amigo, confidente e companheiro, que suportou pacientemente todo o meu estresse e inabilidade nas planilhas do Excel.rsrsrsrs Minha admiração e respeito por você tem aumentado ainda mais

nessa jornada. Obrigada pelos conselhos e pelo colo, por todo seu amor e cuidado. Tudo isso é seu também. Amo você para sempre.

Ao Prof Batista que me mostrou que um pesquisador é muito além de uma publicação e um Lattes. Um pesquisador se faz com uma mente curiosa e disposta somada a um coração que valoriza o que encontra pela frente. Obrigada por oferecer coisas que não tem preço.

A todos meus amigos do Doutorado, por todo companheirismo, amizade, cuidado e amor vividos nesses 4 anos. Pelas tarde de estudo, pelas apresentações, pelos trabalhos... Netinho, Bruna, Rafa, Carol, Ludi e Wagner esse desfecho é nosso.

Ao Prof. Alex Costa, meu chefe no Dept. de Oceanografia por toda sua compreensão e paciência, visando o avanço da ciência e meu crescimento pessoal e profissional. Pessoas como você me fazem ter esperança em um mundo melhor.

Aos meus amigos e companheiros de trabalho no Docean, em especial a minha companheira de sempre Luciana Dantas por toda sua amizade, paciência e sabedoria compartilhadas. Você é especial e que bom poder aproveitar dos dias na sua presença.

A todos os meus amigos e irmãos de fé, que sempre torceram, oraram, se alegraram e algumas vezes choraram comigo. Nossos encontros de redes e pequenos grupos foram essenciais para a continuidade dessa caminhada. Em especial a Diego, Rafa, Filipe, Sula, Carminha, Margareth, Neide, Daniel e todos da IBJSP e PIEB Torre com quem dividi esses longos dias. A Elizama, pelas reflexões e empenho em me fazer melhor a cada dia

Às agencias de fomento CNPq, CAPES e Facepe pelo suporte financeiro para os projetos aprovados e bolsas do grupo, sem os quais a pesquisa não poderia ter sido realizada.

E por fim a todos que caminham em prol da pesquisa, trabalhando com seriedade, dedicação e respeito, com quem tive o prazer de cruzar nessa caminhada. Essas atitudes tem forjado não só o meu caráter, mas o de muitos que cruzam seus caminhos. Vida longa a vocês. Vida longa à ciência!

“Se esse DNA registrar histórias sobre música, esportes ou micróbios maquiavélicos, as narrativas, juntas, contam uma história mais intrincada sobre o surgimento dos seres humanos na Terra: por que somos uma das criaturas mais absurdas da natureza, bem como sua maior glória.” Sam Kean

RESUMO

A Osteoporose (OP) é uma doença osteometabólica e multifatorial relacionada à fatores genéticos em mais da metade dos casos diagnosticados. Recentes estudos de associação genômica ampla tem confirmado a estreita relação entre homeostase óssea e sistema imune, confirmando a influência de diversas citocinas e suas vias relacionadas à fenótipos característicos da doença. Com objetivo de validar funcionalmente essas descobertas e esclarecer a participação desses genes no desenvolvimento da OP pós-menopausa, o presente trabalho estudou a relação entre polimorfismos de nucleotídeo único (SNP) nos genes IL17A, IL23R, IL12B e IFN-γ e a susceptibilidade à OP e resposta terapêutica, além de validar os genes RPLP0 e B2M como referência para análises funcionais na doença. Adicionalmente, foram realizados ensaios *in vitro* em células de osteocarcoma (SaOs-2) para estudo do perfil de expressão de citocinas e processos de calcificação. Nossos resultados mostraram os SNPs IL23R +2284 (A>C) (rs10889677), IL17A +672 (G>A) (rs7747909), IL12B +1188 (T>G) (rs3212227) e IFN-γ -1616 (G>A) (rs2069705) associados à alterações em marcadores de *turnover* ósseo em pacientes em terapia com bisfosfonatos, o IFN-γ rs2069705 associado à susceptibilidade à OP e a citocina IFN-γ aumentando o processo de calcificação em SaOs-2. Após essas análises, podemos demonstrar a importância dos SNPs estudados na susceptibilidade e tomadas de decisões terapêuticas em OP, além de ajudar a esclarecer o papel do IFN-γ no processo de calcificação em células ósseas.

Palavras-chave: SNPs. Osteoimunologia. Sistema Immune. Citocinas.

ABSTRACT

Osteoporosis (OP) is an osteometabolic and multifactorial disease related to genetic factors in more than half of the diagnosed cases. Recent genome-wide association study have confirmed the close relationship between bone homeostasis and the immune system, confirming the influence of several cytokines and their pathways associated with phenotypes OP-related. The aim of this study was to investigate the relationship between single nucleotide polymorphisms (SNPs) in the IL17A, IL23R, IL12 and IFN-γ genes, to validate these findings and to clarify the role of these genes in postmenopausal OP susceptibility and therapeutic response to OP, besides we validate the RPLP0 and B2M genes as reference to OP functional studies. In addition, *in vitro* assays were performed in osteocarcoma cells (SaOs-2) to study the cytokine expression profile and calcification processes. Our results showed the SNPs IL23R + 2284 (A> C) (rs10889677), IL17A + 672 (G> A) (rs7747909), IL12B +1188 (T> G) (rs3212227) and IFN-γ (Rs2069705) were associated with bone turnover markers in patients treated with bisphosphonate, IFN-γ rs2069705 was associated with susceptibility to OP and the cytokine IFN-γ with increase in the viability and calcification process of SaOs-2. After these analyzes, we can demonstrate the importance of SNPs studied in the susceptibility and therapeutic decision in OP besides showing the role of IFN-γ in the process of calcification in bone cells.

Keywords: SNPs. Osteoimmunology. Imune System. Cytokines.

LISTA DE ILUSTRAÇÕES

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE TABELAS

CAPÍTULO 1

CAPÍTULO 2

CAPÍTULO 3

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

Item	Definição
ALP	- Fosfatase alcalina
DEXA	- Absormetria de dupla emissão com raios-X
DMO	- Densidade mineral óssea
Fwd	- *Foward*
GRCh	- Genome Reference Consortium Human
GWAS	- Estudos de associação genômica ampla
IFN-γ	- Interferon gamma
IL	- Interleucina
OMS	- Organização Mundial de Saúde
OP	- Osteoporose
OPG	- Osteoprotegerina
PTH	- Hormônio paratireoide
RANK	- Receptor do fator nuclear *kappa* B
RANKL	- Ligante do fator nuclear *kappa* B
SaOs-2	- Células de osteosarcoma humano
Rev.	- *Reverse*
SERMS	- Moduladores seletivos do receptor de estrógeno
SNP	- Polimorfismo de um único nucleotídeo
SOST	- Esclerostina
Th	- T helper
TNF	- Fator de necrose tumoral
TNFSF11	- Membro 11 da superfamília de ligantes de TNF
TNFRSF11A	- Membro 11a da superfamília de Receptores de TNF
TNFRSF11B	- Membro 11b da superfamília de Receptores de TNF
TRAF6	- Fator 6 associado ao receptor TNF

SUMÁRIO

1. Introdução

A Osteoporose (OP) é uma doença osteometabólica degenerativa e multifatorial, caracterizada por redução da densidade mineral óssea (DMO) e deterioração da microarquitetura do osso. Os principais fenótipos recorrentes são o aumento da fragilidade esquelética e consequente susceptibilidade à fraturas. Dos diversos fatores sistêmicos e locais envolvidos na etiopatogenia da doença, a associação entre componentes hormonais, *background* genético e o sistema imune tem se mostrado como a peça principal na manutenção da homeostase do tecido ósseo. No entanto, apesar do reconhecimento da importância destes fatores e dos conhecimentos gerados a partir de estudos de associação genômica ampla (GWAS, do inglês *Genome-Wide Association Study*), os resultados a respeito da atuação de muitos desses componentes ainda são contraditórios. Adicionalmente, poucos estudos funcionais tem sido realizados para uma melhor compreensão dos processos envolvidos no estabelecimento da doença.

A partir do uso, pela primeira vez, do termo Osteoimunologia por Arron e Choi. (2000), vários estudos tem relacionado proteínas do sistema imune ao equilíbrio entre as atividades dos osteoclastos (células de reabsorção óssea) e osteoblastos (células de reposição óssea). Citocinas pró- e antiinflamatórias tem sido associadas à homeostase óssea, atuando na indução ou inibição da reabsorção tecidual através do controle da proliferação dessas células. Alterações em genes destas citocinas, como os polimorfismos de um único nucleotídeo (SNPs, do inglês *Single Nucleotide Polymorphism*), podem alterar tanto a estrutura quanto a funcionalidade dessas proteínas e consequentemente modificar mecanismos biológicos nos quais estão envolvidas.

A importância do conhecimento dos mecanismos de atuação dessas proteínas está, não só em adquirir uma nova visão sobre a patogênese da OP, mas também na possibilidade de suas aplicações clínicas. Certas citocinas podem servir como biomarcadores no monitoramento e prognóstico da doença, podendo, inclusive, tornarem-se alvos terapêuticos para o seu tratamento, tendo em vista os efeitos colaterais causados pelas atuais terapias. Algumas citocinas pró-inflamatórias, como o ligante do receptor do fator nuclear *kappa* B *(*RANKL), já tem sido relatadas como alvos em potencial para diagnóstico e terapêutica no combate à redução da DMO e ocorrência de fraturas. Desta forma, o presente estudo teve como objetivo investigar se SNPs nos genes das citocinas pró-inflamatórias IL-23, IL-17A, IL-12B e IFN-γ, estariam relacionados com resposta terapêutica aos bisfosfonatos, classe de anti-catabólitos de primeira escolha para o tratamento da OP e susceptibilidade à OP primária pós-menopausa. Para os estudos funcionais, validamos os mais estáveis e os não recomendados genes de referência para estudos da doença. Adicionalmente, verificamos a influência da citocina relacionada à susceptibilidade para OP nos processos de calcificação e viabilidade de células da linhagem osteoblástica.

SNPs nos genes *IL23R*, *IL17A*, *IL12B* e *IFNG* apresentaram associação com alterações em marcadores bioquímicos do metabolismo ósseo e a resposta terapêutica aos bisfosfonatos. O *IFNG* foi o único polimorfismo associado à variações de DMO durante esse tratamento e também à susceptibilidade à OP. Devido a esse fato, a citocina foi selecionada para estudos funcionais, onde apresentou diferença nos níveis de expressão entre os grupos de pacientes e mulheres saudáveis, além de alterar o processo de calcificação e viabilidade em células humanas de osteosarcoma (SaOs-2). Desta forma demonstramos a

importância dos SNPs dos genes estudados nas tomadas de decisões terapêuticas e susceptibilidade em OP, além de ajudar a esclarecer o papel do IFN-γ no processo de calcificação de células da linhagem osteoblástica.

2. Revisão da Literatura

2.1 Epidemiologia da Osteoporose

De acordo com o último censo do IBGE (2010) o Brasil possui cerca de 51 milhões de pessoas, acima dos 55 anos de idade, das quais 10 milhões são portadores de OP. Apenas no ano de 2010, 74 mil pacientes foram internados na rede pública de saúde por fraturas decorrentes da doença. O gasto hospitalar brasileiro com pacientes vítimas de fraturas custa cerca de 24 mil reais por paciente e os gastos do governo com a doença atingiram o valor de 80 milhões de reais apenas no ano de 2010. O valor aproximado de gastos anuais para 2015 nos Estados Unidos, de acordo com a Fundação Nacional de Osteoporose, foi de 20 bilhões de dólares (Roush, 2011; MS, 2014). Apesar de representar um grave problema de saúde pública no Brasil e no mundo, a OP é considerada uma doença negligenciada, tendo em vista que no Brasil apenas 24% dos pacientes diagnosticados recebem terapia específica, e cerca de 20% vão à óbito em até um ano após fratura por doenças secundárias às quedas e morbidade (Iolascon et al., 2013; MS, 2014).

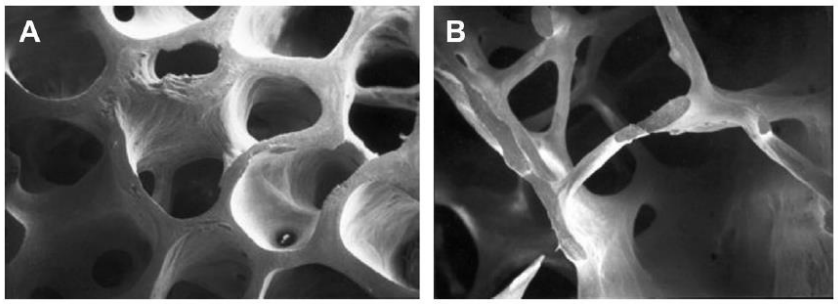
Neste contexto, o diagnóstico preciso e o tratamento específico são de extrema importância, tendo em vista que a adesão ao tratamento sugerida pelo clínico só pode ser avaliada em um prazo mínimo de 6 meses, tempo suficiente para risco de quedas e fraturas subsequentes (MS, 2014). Além das indicações de mudança de estilo de vida como prática de exercícios, ingestão de cálcio na dieta e diminuição da ingesta de álcool e consumo do cigarro, a terapia medicamentosa é indicada principalmente para mulheres pós-menopausa com valores DMO menor que -2,5 T-score; mulheres pós-menopausa com DMO entre -1 e -2,5 com previsão

de risco de 3% de fratura de quadril ou 25% em qualquer região nos próximos 10 anos e mulheres pós-menopausa com histórico de fraturas (Roush, 2011; Golob; Laya, 2015).

2.2 Caracterização da Osteoporose

De acordo com a Organização Mundial de Saúde (OMS) a OP é definida como uma doença sistêmica do esqueleto, caracterizada pela diminuição da densidade mineral óssea (DMO) e deterioração da microarquitetura do tecido. Disso decorre o aumento da fragilidade do osso e maior predisposição à fraturas (Kanis, 2008; Zaia, 2015; Rocha-Braz; Ferraz-de-Souza, 2016) (Figura 1). Apesar das técnicas de prognóstico, diagnóstico e monitoramento estarem intimamente relacionadas à DMO e aos níveis de estrógeno e calcemia, sabe-se que muitos são os fatores envolvidos na patogenia da doença, classificados como fatores clínicos de origem óssea e não óssea (Aspray, 2014).

Figura 1. Micrografia de osso. A: Osso normal. B: Osso osteoporótico.



Fonte: Golob; Laya, 2015.

Elementos como idade avançada, etnia caucasiana ou asiática, baixo peso corporal, histórico familiar da doença, fumo, consumo excessivo de álcool, vida sedentária, baixos níveis de vitamina D e ingesta insuficiente de cálcio compõem os principais

fatores de risco primário relacionados ao desenvolvimento da doença (Golob e Laya, 2015; Greenblatt et al., 2016). Nem sempre a OP tem início na alteração direta da composição e disposição do tecido ósseo, como na OP primária pós-menopausa. Em alguns casos ela pode ser secundária à outras patologias como síndromes hipogonadais, alterações na tireóide, diabetes mellitus, artrite reumatoide, síndromes de má absorção, doenças crônicas de fígado, rim e pulmão, desordens hematológicas, além do consumo excessivo de medicamentos, em especial os glicocorticoides (Pereira, 2009; Golob ; Laya, 2015).

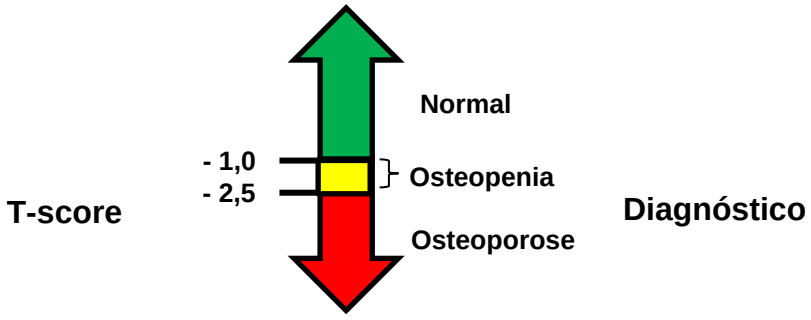
Além destes fatores clínicos citados, componentes de outros sistemas biológicos tem se mostrado relevantes em pesquisas recentes relacionadas à desordens esqueléticas, a exemplo do perfil genético dos pacientes e do comportamento do sistema imune e suas proteínas (Arron e Choi, 2000; Zupan et al., 2013; Brincat et al., 2014). Com o mapeamento do genoma completo, vários genes do sistema imune foram associados à diminuição da DMO e aumento da fragilidade óssea, apresentando possíveis novos marcadores prognósticos e terapêuticos da doença (Liu et al., 2014; Clark; Duncan, 2015; Qin et al., 2016).

A importância do aprofundamento nesses estudos de base fica ainda mais evidente diante do quadro de subdiagnósticos e subtratamentos da OP, o que agrava este problema na saúde pública (Roush, 2011; Golob; Laya, 2015). No grupo de doenças negligenciadas, a OP primária pós-menopausa é particularmente preocupante pelos seus altos índices de acometimento e diminuição significativa na qualidade de vida de mulheres mais velhas, podendo inclusive, além dos sintomas de dor intensa e aumento da morbidade, levar à morte (Iolascon et al., 2013; Baccaro et al., 2015). Outro fator a ser considerado é a heterogeneidade de

metodologia utilizada para diagnóstico, o que contribui para imprecisão dos números de prevalência da doença (Golob; Laya, 2015). Tomando como base alguns estudos populacionais feitos no Brasil e Estados Unidos, a taxa média de mulheres acometidas após os 50 anos de idade varia entre 15-35% (Pereira, 2009; Golob; Laya, 2015). De acordo com a OMS até o ano de 2050 é esperado um aumento de 400% no número de fraturas de quadril, na população de até 60 anos, e de 700% na população com idade superior a 65 anos, apenas para América Latina (Kanis, 2008).

Por ser uma patologia de causa multifatorial e, em grande parte das vezes assintomática, seu diagnóstico pode ser impreciso e de difícil predição. Inicialmente, esse diagnóstico se baseava apenas nos índices de DMO obtidos através da absormetria de dupla emissão com raios-X (DEXA), com resultado posteriormente classificado de acordo com o número de desvios padrão superior ou inferior do paciente em relação à DMO média de uma população jovem saudável de referência, o T-score (Golob; Laya, 2015; Zaia, 2015). Os resultados de T-score superiores a -1,0 são considerados como normais, entre -1,0 e -1,99 como osteopenia, entre -2,0 e 2,49 como osteopenia avançada, abaixo de -2,5 como OP e T-score abaixo de -2,5 associado a uma ou mais fraturas, são classificados como OP severa (Figura 2) (Baccaro et al., 2015; Golob; Laya, 2015).

Figura 2: Valores de T-scores resultantes dos exames DEXA e seus respectivos diagnósticos associados de acordo com a OMS.



Fonte: Autor

No entanto, outros estudos tem revelado que a DMO analisada isoladamente não possui alta eficácia diagnóstica, tendo em vista que não apenas a quantidade de tecido ósseo é importante na homeostase do sistema, mas também a sua microarquitetura, responsável por corrigir a fragilidade óssea decorrente da reabsorção da matriz tecidual (Aspray, 2014; Zaia, 2015).

Outros componentes que podem auxiliar nesse contexto são os marcadores bioquímicos do *turnover* osséo (Greenblatt et al., 2016). A variação dessas proteínas decorrentes da atividade das células ósseas pode ser detectada mais rapidamente que as mudanças na massa óssea (Chesnut et al., 1995; Greenblatt et al., 2016). Os principais marcadores utilizados na clínica e pesquisa em OP são a fosfatase alcalina (ALP), osteocalcina (OC), fosfatase ácida tartarato resistente (TRAP) e o telopeptídeo carboxiterminal do colágeno tipo I (CDX) (Seibel, 2005; Zhou et al., 2014; Zhou et al., 2015). Adicionalmente, níveis do hormônio paratireoide (PTH), 25 (OH) hidroxivitamina D e cálcio são monitorados nesse processo (Zhou et al., 2014; Zhou et al., 2015). No entanto, esses elementos geralmente estão estritos ao acompanhamento e não ao diagnóstico ou prognóstico da doença (Greenblatt et al., 2016). Na tentativa de preencher essas lacunas, o conhecimento dos constituintes celulares e inorgânicos e processos biológicos envolvidos na doença são relevantes, não apenas para o diagnóstico e prognóstico, mas também para alvos terapêuticos eficazes no combate à patologia.

2.2.1 O tecido ósseo

O osso é um tecido dinâmico de alto poder regenerativo constituído por componentes celulares (2%) e matriz óssea (98%) com homeostase garantida por

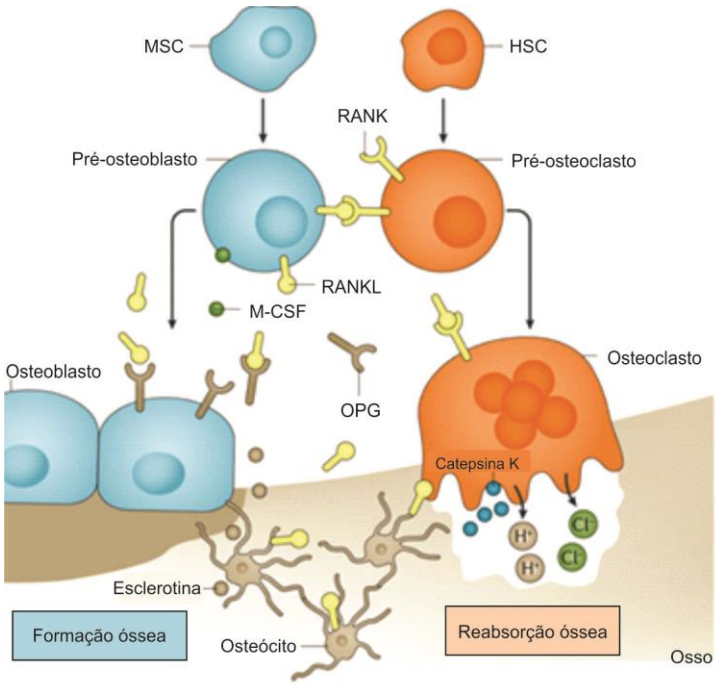
diversos fatores como hormônios, vitaminas, níveis de cálcio e citocinas, que coordenam a atividade dos três tipos celulares formadores do tecido: os osteoclastos, osteoblastos e osteócitos (Sathy et al., 2015.; Lee; Shin 2007; Lewiecki, 2011; Sims; Walsh, 2012; Faienza et al., 2013).

Os osteoclastos são grandes fagócitos multinucleados originados de células-tronco hematopoiéticas que reabsorvem o osso através da ação de enzimas ácidas secretadas na zona de vedação resultante da ligação dessas células na superfície óssea por intermédio de integrinas com longos domínio extracelulares (Lewiecki, 2011). Sua ativação é iniciada pela ação do RANKL secretada por osteoblastos que se liga ao receptor do fator nuclear *kappa* B (RANK) na superfície do osteoclasto, sendo todo processo mediado pelo fator estimulador de colônia de macrófago (M-CSF). Com a ativação, proliferação e aumento da sobrevivência dessas células, tem início todo processo de reabsorção óssea que é modulado pela ação do receptor solúvel osteoprotegerina (OPG), secretado também por osteoblastos, que inibe competitivamente a ligação entre o RANKL e o seu receptor RANK. Os níveis de expressão dessas citocinas e receptores pelas células ósseas é controlado e mediado por fatores de crescimento, hormônios, drogas e principalmente pela ação de outras citocinas (Figura 3) (Lewiecki, 2011; Drake et al., 2015).

A reabsorção é também mediada e compensada pelos osteoblastos, células originadas de células-tronco mesenquimais, através da secreção de colágeno tipo I e outras substâncias não colágenas, como a osteopontina, responsáveis por constituir a matriz óssea do tecido retirada pelos osteoclastos. Esse processo ocorre não apenas na patologia, mas também em processos fisiológicos normais (Seeman; Delmas, 2006; Lewiecki, 2011). A estimulação para diferenciação de osteoblastos é

induzida pela ativação da via canônica Wnt-β-catenina, através da ligação de proteínas de sinalização Wnt em receptores Frizzled associados às lipoproteínas LPR5 e LPR6 e inibida pelas proteínas Dickkopf-1 (DKK1) e esclerostina (SOST).

Figura 3: Remodelamento ósseo. As citocinas M-CSF, RANKL, seu receptor RANK e OPG atuando na diferenciação dos precursores das células ósseas.



Fonte: Adaptado de Eastell et al., 2016.

Outra via anabólica da osteoblastogênese é a do hormônio paratireoide (PTH) e seus homólogos (PTHrP), controlada pelos receptores sensores de cálcio (CaSR) nas glândulas paratireoides e que tem efeito direto na diferenciação dos osteoblastos a partir das células osteoprogenitoras (Lewiecki, 2011; Esbrit et al., 2015). Concluída sua função de reposição da matriz, os osteoblastos seguem para apoptose ou, se absorvidos pela matriz óssea, transformam-se em osteócitos, que regulam a reabsorção e a síntese do tecido ósseo (Lewiecki, 2011).

Os osteócitos se comunicam entre si e com as células da superfície através de prolongamentos nervosos em canalículos do osso, respondendo à microfissuras e pressões mecânicas (*mechanosensation*) e outras condições como alteração dos níveis de hormônios e glicocorticoides. Essa regulação na remodelação óssea pode favorecer não só a reabsorção mas também a reposição do tecido (Lewiecki, 2011; Asada et al., 2013). Essas células produzem tanto substancias inibitórias da via Wnt e estimuladoras da proliferação de osteoclastos, como a esclerostina, quanto produtos anabólicos que atuam na sobrevivência dos osteoblastos, a exemplo das prostaglandinas e óxido nítrico (Asada et al., 2013). Além dos componentes celulares, os osteócitos também regulam a liberação de minerais da matriz óssea, neste caso impedindo sua reabsorção (Pereira, 2009).

A matriz óssea possui uma composição mineral (70%), constituída de cristais de cálcio e fosfato (hidroxipatita) e orgânica (30%), constituída de colágeno tipo I e outras proteínas não colágenas. As glicoproteínas e os proteoglicanos formam a substância amorfa sobre a qual os cristais de hidroxiapatita se ajustam às fibras colágenas (Pereira, 2009).

O tecido ósseo mantem a estrutura do corpo humano, protege órgãos vitais, confere movimentos às contrações musculares, além de ser uma reserva de íons essenciais para a vida do indivíduo, como fósforo e cálcio. A desregulação entre formação e reabsorção óssea provoca patologias decorrentes da diminuição de DMO e perda da microarquitetura do tecido, a exemplo da OP. Apesar de todo esse sistema ser multifatorialmente influenciado, a íntima relação do sistema imunológico com a homeostase do tecido tornou-se destaque, principalmente após os estudos

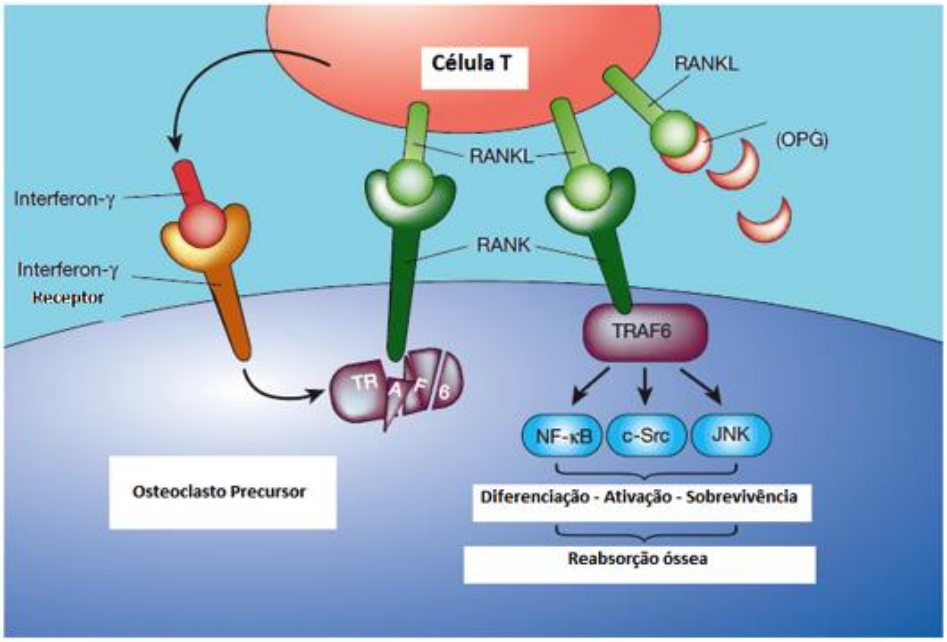
envolvendo genes de citocinas e remodelamento ósseo, na emergente Osteoimunologia (Arron; Choi, 2000; Pereira, 2009) .

2.2.2 Osteoimunologia

Apesar de várias citocinas já estarem comprovadamente relacionadas às variações de DMO e a relação osso e células do sistema imune estar clara devido à sua localização de origem, o termo Osteoimunologia foi utilizado pela primeira vez por Arron e Choi no ano de 2000, em uma revisão onde os autores ressaltam que a relação entre ambos vai além das células imunes se formarem na medula óssea. Os linfócitos T e muitas de suas proteínas teriam relação direta com o remodelamento ósseo, através do controle da ação dos osteoclastos, osteoblastos e osteócitos (Takayanagi et al., 2000).

A via mais conhecida e abordada na osteoimunologia é a RANKL/RANK/OPG, onde o RANKL é expresso nos linfócitos T ativados por inflamação ou queda de estrógeno sérico, e se liga ao RANK nos osteoclastos, ativando o Fator 6 associado ao receptor TNF (TRAF6), que por sua vez irá ativar o fator nuclear *kappa* B (NF*k*B), Jun-c kinase N-terminal (JNK) e c-Src, responsáveis pela diferenciação, ativação e sobrevivência dos osteoclastos (Figura 4).

Figura 4: Sistema Imune e Osso. A via RANKL/RANK/OPG atua na osteoclastogênese a partir da interação do RANKL de Células T com RANK no precursor de osteoclastos. OPG e IFN-γ atuam inibindo osteoclastogênese.



Fonte: Adaptado de Arron; Choi, 2015.

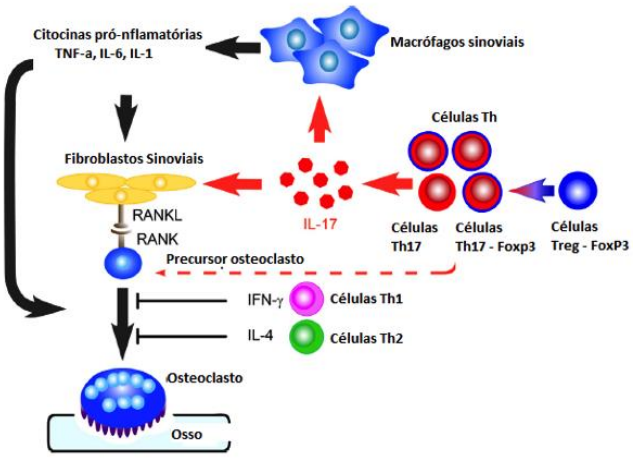
Ao mesmo tempo, em um mecanismo de *feedback* negativo, os linfócitos T secretam IFN-γ, que induz a quebra da proteína TRAF6 ou o receptor solúvel OPG, secretado por osteoblastos, que se ligam ao RANKL inibindo competitivamente sua ligação ao RANK, garantindo o balanço da DMO. No entanto, nos estados patológicos em que essa via é ativada, a reabsorção provocada pela ativação dos osteoclastos se sobrepõe à ação do IFN-γ e OPG, provocando diminuição da DMO e aumento do risco de fragilidade óssea (Arron; Choi, 2000; Takayanagi et al., 2000).

Como as respostas Th1 e Th2, através das citocinas IFN-γ e IL-4, são também reconhecidas por inibir a osteoclastogênese, e a atuação de outras respostas, a exemplo da Th17, pode ser a explicação para este quadro aparentemente

contraditório de atuação dos linfócitos T. As células Th17 induzem a expressão de RANKL em fibroblastos sinovais de pacientes com artrite reumatoide, através de IL-17 ou esta citocina pode induzir osteoclastogênese de forma indireta através da indução de um quadro inflamatório com aumento da expressão de TNF-α, IL-6 e IL-1, sugerindo que as citocinas relacionadas a esta resposta podem ter um papel fundamental na prevenção da perda óssea (Takayanagi, 2012) (Figura 5).

As células T *naïve* se diferenciam em Th17 na presença de IL-23, TGF-β, IL-1 e IL-6, produzindo principalmente a IL-17, acompanhada das IL-21, IL-22, IL-25 e IL-26 estimulando uma potente inflamação, ao mesmo tempo que produzem IL-10, citocina de ação anti-inflamatória como um mecanismo de *feedback* negativo (Takayanagi, 2012; Raphael et al., 2014). No entanto, de acordo com alguns autores, a depender do tecido em questão e estágio da patologia, o eixo IL23/IL17 participa da ativação da resposta Th1 de forma sinérgica, através da indução de IL-12 e posteriormente IFN-γ (Chen et al., 2009; Tartour et al., 2009; Damsker et al., 2010; Khader; Gopal, 2010).

Figura 5: Citocinas pró-inflamatórias e diferenciação de osteoclastos. Os macrófagos sinoviais ativados por IL-17 secretam citocinas pró-inflamatórias que estimulam fibroblastos sinoviais. As citocinas das respostas Th1 e Th2 atuam na inibição da osteoclastogênese.



Fonte: Adaptado de Guerrini; Takayakagi, 2014.

Pela reconhecida atuação das citocinas na diferenciação, proliferação, ativação e sobrevivência das células ósseas de forma direta ou indireta, essas proteínas representam um potencial não apenas prognóstico, impedindo tratamentos desnecessários e negligenciando intervenções necessárias, mas também terapêutico, tendo em vista as diversas dificuldades encontradas nas diretrizes terapêuticas atuais (Takayanagi, 2012; Guerrini; Takayanagi, 2014; Makras et al., 2015).

2.3 Tratamento da Osteoporose

Os medicamentos indicados para OP são classificados em três grupos de acordo com seu mecanismo de ação: os anti-catabólicos ou anti-reabsortivos, representados pelo cálcio, vitamina D, bisfosfonatos, estrógeno, moduladores seletivos do receptor de estrógeno, calcitonina e denosumab; os anabólicos, representados pela teriparatida e hormônio paratireoide e, por último, os de ação incerta, que provavelmente possuem ambas as atividades, como o ranelato de estrôncio (Riancho; Hernández, 2012).

A administração de cálcio é indicada para todos os tipos de tratamentos, em especial para riscos de fraturas vertebrais. No entanto, as mulheres com risco de doença cardíaca e renal devem dar preferência à obtenção de cálcio na dieta, já que alguns estudos sugerem que o mesmo eleva o risco de doenças nesses órgãos (Baccaro et al., 2015; Golob; Laya, 2015). A primeira linha de medicamento de escolha é a classe dos bisfosfonatos, representada pelo alendronato, risendronato, ibandronato e ácido zolendrônico, que atuam impedindo a osteoclastogênese através do acúmulo de análogos do ATP no citoplasma dos osteoclastos (não-

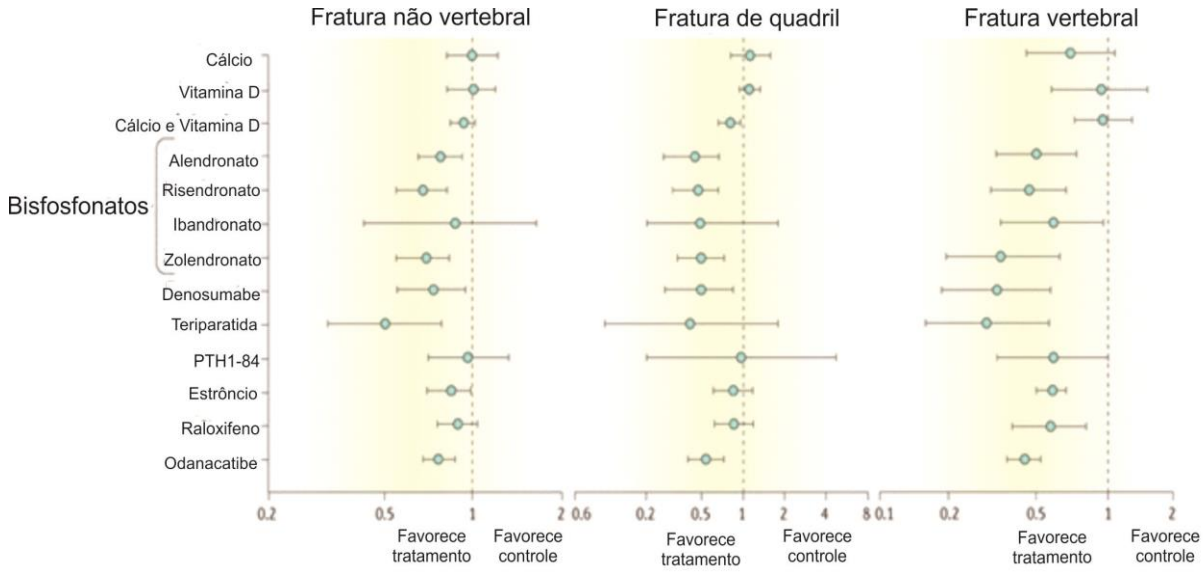
nitrogenados) ou interrompendo a via do mevalonato (nitrogenados) induzindo apoptose e modulando a reabsorção óssea (Riancho e Hernández, 2012; MS, 2014). O mecanismo de ação do estrógeno e dos moduladores seletivos do receptor de estrógeno (SERMS), este último com menos efeitos colaterais, ainda não está bem definido, mas acredita-se que não tenha ação direta na contenção da osteoclatogênese, mas sim na modulação através da alteração dos níveis de citocinas com consequente diminuição dos níveis de reabsorção óssea. Já a calcitonina age diretamente nos osteoclastos, através de uma ligação via receptores de membrana e consequente aumento do cAMP (Riancho; Hernandez, 2012). O denosumabe, aprovado em 2010 para OP de alto risco, é um anticorpo humano anti-RANKL que impede a ligação da proteína ao seu receptor e inibe a ativação e proliferação de osteoclastos, diminuindo a reabsorção óssea (Cummings et al., 2009; Lewiecki, 2011; Riancho; Hernández, 2012).

A classe dos anabólicos (Teriparatida ou PTH) atuam de forma diferente dos antirreabsortivos, estimulando a remodelação óssea, aumentando não apenas a quantidade de osso, mas promovendo a reestruturação da microarquitetura do tecido através da melhora da conectividade trabecular (Esbrit et al., 2015; Yoshiki et al., 2016). Seu mecanismo de ação é amplo indo desde estímulos diretos à proliferação de osteoblastos quanto modulação da via Wnt e estimulo de expressão de citocinas (Esbrit et al., 2015).

Em 2016, Eastell e cols. compilaram resultados de metanálises do efeito das drogas utilizadas com a incidência de fraturas de quadril, vertebrais e não-vertebrais (Figura 6). De acordo com a análise, dentre as terapias mais utilizadas, o uso apenas de cálcio e vitamina D apresentaram riscos de fraturas em todos os sítios

analisados. No grupo dos bisfosfonatos apenas o ibandronato apresentou riscos para fraturas não-vertebrais e de quadril. A teriparatida apresentou risco apenas para o quadril, embora nas outras áreas tenha apresentado melhora superior a todos os outros tratamentos. Por fim, o PTH mostrou risco para todas as áreas estudadas.

Figura 6: Metanálises relativas à incidência de fraturas durante os diversos tipos de terapia utilizadas em Osteoporose.



Fonte: Adaptado de Eastell et al., 2016.

Por ser um problema de saúde pública emergente em um período relativamente recente, as pesquisas tem voltado o interesse para os mecanismos de base do desenvolvimento da OP, seus agentes e mecanismos moleculares (Eastell et al., 2016). A consolidação de conhecimento desses fatores representa não apenas subsídios para um prognóstico preciso, mas principalmente para uma terapia eficiente, tendo em vista os índices de morbi-mortalidade envolvidos no processo. Resultados contraditórios na atuação desses componentes e escassez de estudos

funcionais envolvendo estes elementos fornecem ainda mais subsídios para estes estudos (Blumenfeld et al., 2014).

2.4 Genética da Osteoporose

Um dos principais fatores envolvidos no estabelecimento da OP é a hereditariedade, especialmente para variações de DMO, risco de fraturas e a área de ocorrência desses eventos (Rivadeneira et al., 2009; Eastell et al. 2016; Karasik et al., 2016; Rocha-Braz; Ferraz-de-Souza, 2016). Nos últimos anos a tecnologia *array* e os sequenciamentos de nova geração tem permitido a ampliação do número de genes estudados e sua relação com aspectos clínicos da OP, sugerindo assim possíveis vias atuantes no processo de remodelamento do tecido ósseo (Riancho; Hernández, 2012; Karasik et al., 2016). O reconhecimento do componente genético na doença cresceu de tal forma que 2 consórcios internacionais foram criados ainda na primeira década do séc XXI com objetivo de identificar variantes genéticas de risco para a doença: o Consórcio de Marcadores Genéticos para OP (GENOMOS, do inglês *Genetic Markers for Osteoporosis*), em 2003 e o Consórcio de Fatores Genéticos para OP (GEFOS do inglês *Genetic Factors for Osteoporosis Consortium*), em 2008. O GEFOS conduziu 3 estudos de metanálises a partir de GWAS nos quais 36.016 SNPs em mais de 150 genes foram avaliados. Nesses estudos um total de mais de 240 SNPs localizados em 23 diferentes genes apresentaram associação com variações de DMO, dentre eles os genes do RANKL (TNFSF11), RANK (TNFRSF11A) e OPG (TNFRSF11B). Em relação à fraturas, foi reportado um total de 60 SNPs em 5 genes, dentre eles o RANKL (TNFSF11) e RANK (TNFRSF11A).

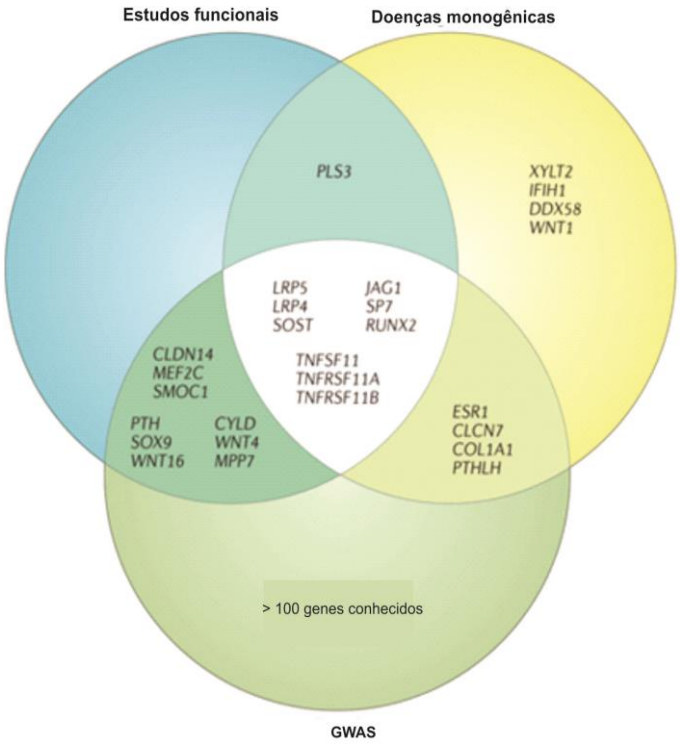
Esta iniciativa tem sido essencial para os avanços nos conhecimentos dos fatores genéticos relacionados à doença, pois apesar de variantes raras ou genes relacionados à doenças autossômicas como SOST e Wnt terem sido associados à OP, a patologia sendo uma doença complexa, parece ser mais influenciada por variantes comuns que contribuem conjuntamente para a susceptibilidade e alterações fenótipicas (Riancho e Hernández, 2012; Karasik et al., 2016). A DMO, por exemplo, é controlada em 60 a 80% dos casos por diferentes genes atuando em conjunto, de acordo com estudos realizados em gêmeos idênticos (Flicker et al. 1995; Zhou et al., 2014).

Em 2014, Liu e cols. conduziram um *update* dos GWAS realizados em OP, avaliando 19 estudos dos quais 5 foram metanálises e dentre essas, 2 realizadas pelo GEFOS. Os GWAS identificaram vários polimorfismos genéticos em mais de 60 genes associados à variações de DMO, em especial em genes de receptores de vitamina D (VDR), receptores de estrógeno (ESR-α e ESR-β) e de componentes das vias de osteoclasto e osteoblastogênese, como as vias RANKL/RANK/OPG, Wnt e MVK (Riancho e Hernández, 2012; Urano e Inoue, 2014; Clark e Duncan, 2015; Karasik et al., 2016). Além disso, cerca de 20 genes foram identificados como relacionados ao risco de fraturas e mais de 100 à susceptibilidade à OP (Liu et al., 2014; Clark; Duncan, 2015; Karasik et al., 2016).

Dentre esses genes relacionados à doença, pode-se destacar os genes do sistema imune. O gene do receptor da IL-21 (IL21R) e receptor do fator de transformação do crescimento beta III (TGFBR3) foram associados à mudanças de DMO em mulheres caucasianas chinesas, enquanto o genes do TNFSF11, TNFRSF11A e TNFRSF11B foram relacionados com o processo de

osteoclastogênese em mulheres caucasianas chinesas e europeias (Liu et al., 2014; Karasik et al., 2016; Rocha-Braz e Ferraz-de-Souza, 2016). Em meio à essa grande quantidade de genes relatados, Karasik e cols. (2016) sugeriram uma seleção dos mais intimamente ligados à doença a partir de análises de GWAS e estudos funcionais. Nessa seleção novamente são citados os genes da via das citocinas RANKL/RANK/OPG (Figura 7).

Figura 7: Seleção dos principais genes associados com a Osteoporose a partir de GWAS e estudos funcionais. .

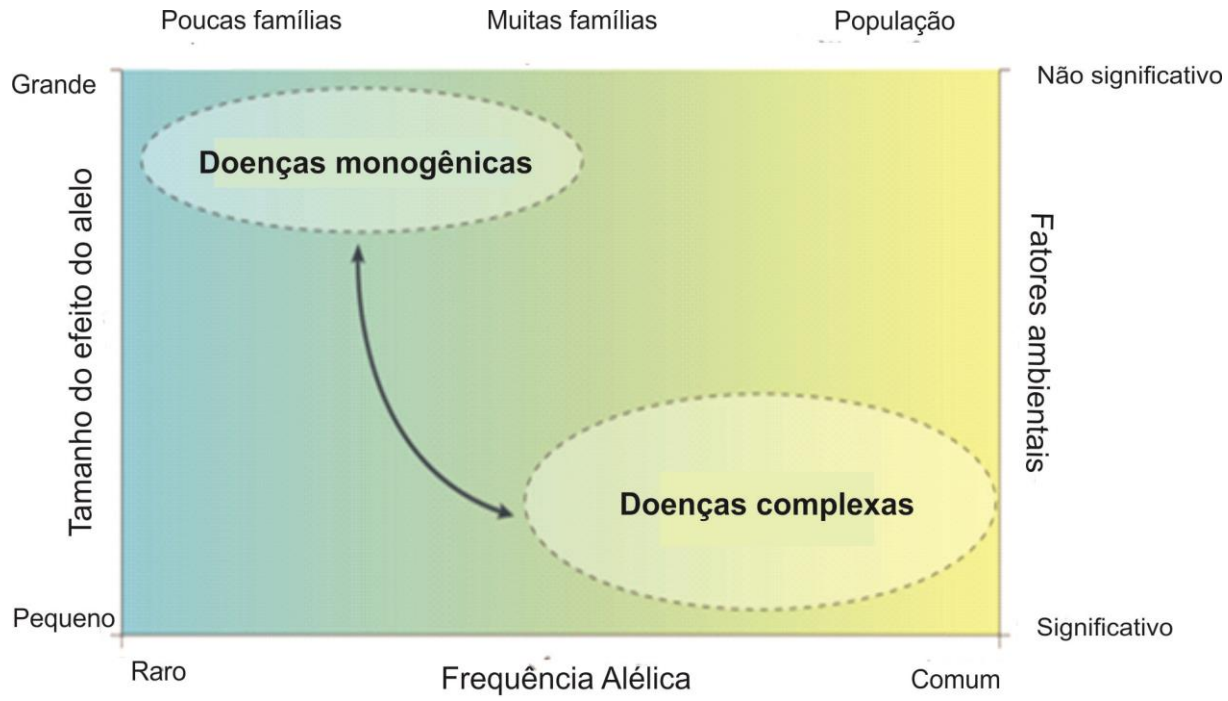


Fonte: Adaptado de Karasit et al., 2016.

Apesar do alto poder estatístico destes estudos, os SNPs identificados parecem contribuir apenas com uma pequena fração de alterações nos fenótipos e susceptibilidade à doença,. Karasik e cols. (2016) relataram bem a influência de vários genes de pequenos efeitos e a alta contribuição de fatores ambientais em

doenças complexas, como a OP, quando comparadas às doenças monogênicas,com perfil oposto (Figura 8).

Figura 8: Influência dos alelos em doenças monogênicas e complexas.



Fonte: Adaptado de Karasit et al., 2016.

Sendo assim, deve-se atribuir à essas análises a identificação de possíveis vias atuantes no processo de remodelamento do tecido ósseo como informação principal, não se limitando apenas aos marcadores estatisticamente significativos de forma isolada (Riancho; Hernández, 2012; Karasik et al., 2016). Muitos elementos do sistema imune, como algumas citocinas, não foram citados nas análises prévias de GWAS, no entanto são reconhecidas por atuarem em muitas vias relacionadas à doença, como MVK, SOST e RANKL/RANK/OPG.

Concordando com essa afirmativa, Rivanadeira e cols (2009) indicaram 13 SNPs que nunca haviam sido citados em GWAS anteriores, mas já haviam sido

reportados em estudos de associação comuns. Esses dados juntamente com as descobertas em Osteoimunologia encorajam as pesquisas com SNPs em genes de citocinas associadas a essas vias, mesmo que estas não tenham sido citados em GWAS prévios. Estudos recentes tem descrito citocinas como o IFN-γ inibindo a via RANKL/RANK/OPG bem como a IL-17 induzindo essa mesma via, ambas afetando indiretamente os processos de homeostase óssea (Guerrini; Takayanagi, 2014; Talaat et al., 2015). SNPs funcionais em genes dessas citocinas, bem como nas suas principais indutoras, a IL-23 e a IL-12, respectivamente, tem sido reportados em diversas doenças, inclusive em desordens ósseas (Wong et al., 2012; Emami et al., 2015; Marques et al., 2015; Zhang et al., 2015; Leng et al., 2016).

Embora seja reconhecido a importância dessas proteínas do sistema imune no estabelecimento da OP, poucos estudos tem sido feitos relacionando funcionalmente esses SNPs e a susceptibilidade à OP pós-menopausa e sua resposta terapêutica.

2.4.1. SNPs em genes de citocinas

As citocinas são proteínas com papeis essenciais não apenas na imunidade inata e adaptativa, mas também influenciando e sendo influenciada por diversos sistemas e órgãos como o osso, além de estarem associadas ao desenvolvimento de diversas patologias (Bhushan; Perumal, 2012; Takayanagi, 2012). SNPs podem alterar tanto a estrutura (éxon e íntrons em regiões de *splincing*) quanto os níveis dessas proteínas (regiões regulatórias: 5’ e 3’ UTR e promotoras) e muitos estudos, inclusive os de GWAS anteriormente citados, tem identificado marcadores importantes na susceptibilidade e manifestações de diversas doenças multifatoriais, como a OP (Richards et al., 2008; Rivadeneira et al., 2009; Mah et al., 2011; Qin et

al., 2016). Por outro lado, por não apresentar associação com patologias em outra parcela de estudos, muitos questionamentos são feitos em relação ao papel desses SNPs e as interpretações decorrentes dos estudos com associações estatisticamente significativas (Bhushan; Perumal 2012).

Com intuito de derivar parâmetros para relação de causalidade entre SNPs e doenças, foi criado em 2012 o DACS-DB, do inglês *Disease Associated Cytokine SNP Database* (http://www.iupui.edu/~cytosnp). Nesse banco público de dados constam as informações de 453 genes de citocinas, 63.000 SNPs e 853 doenças associadas à esses SNPs. Outros bancos de dados mais gerais que podem ser consultado para estudos são o 1000 genomes, Ensembl Genome Browser e a seção dbSNSP do *National Center for Biotechnology Information* (NCBI). Os sites de livre acesso fornecem informações de localização dos polimorfismos, frequência do alelo menor de acordo com as diferentes populações e estudos científicos publicados. Os quatro SNPs avaliados nesse estudo, *IL23R* +2284 (C>A, rs10889677), *IL17A* +672 (G>A, rs7747909), *IL12B* +1188 (T>G, rs3212227) e *IFNG* -1616 (G>A, rs2069705), estão cadastrados em todos os bancos de dados citados.

O *IL23R* +2284 (C>A, rs10889677) está localizado na região regulatória 3'UTR do gene, no cromossomo 1, já foi citado em 98 estudos e mostrou associação com o aumento do risco de doença de Crohn, espondilite anquilosante e Doença de Graves. O *IL17A* +672 (G>A, rs7747909), também localizado na região regulatória 3'UTR do gene no cromossomo, foi citado em 8 estudos, dentre os quais conferiu aumento de risco para arterite, panuveíte e degeneração macular. O *IL12B* +1188 (T>G, rs3212227), também da 3'UTR do gene no cromossomo 5, foi citado em 147 estudos e mostrou associação com a psoríase e artrite psoriática. E por fim o *IFNG* -

1616 (G>A, rs2069705), que embora seja citado em estudos prévios como localizado na região promotora do gene, está atualmente registrado com localização intrônica no cromossomo 12 e foi mencionado em 41 estudos publicados. O SNP foi reportado como associado à desfechos de mortalidade em pacientes norte-americanos (1000 Genomes - http://www.internationalgenome.org).

Lima e cols. (2017) relataram pela primeira vez a relação entre esses SNPs e marcadores bioquímicos de remodelagem óssea e variações de DMO em pacientes sob tratamento de bisfosfonatos. No estudo, os SNPs *IL17A* rs7747909 juntamente com *IL12* rs3212227 estiveram associados à variações nos níveis de vitamina D, e *IL23R* rs10889677 com alterações na calcemia. O SNP *IFNG* rs2069705 foi o que apresentou maior relação com a resposta terapêutica, estando associado à alterações nos valores de PTH e DMO durante os 4 anos de tratamento. Esses dados sugerem um importante papel desses SNPs nas decisões terapêuticas em OP.

Algumas ferramentas computacionais também tem sido uma abordagem utilizada para medida dos efeitos dos SNPs nas suas proteínas correspondentes, além de simulações de respostas terapêuticas. Essas análises *in silico* representam uma saída rápida e relativamente de fácil execução, comparadas aos experimentos que precisariam ser executados para a predição de efeitos fenotípicos da quantidade crescente de genes previamente associados à doenças (Mah et al., 2011; Singh; Mistry, 2016). Esses algoritmos mensuram as diferenças de energia livre, solubilidade e dinâmica das proteínas originadas dos diferentes SNPs e suas variantes selvagens e mutantes (Singh; Mistry, 2016). As sequencias a serem estudas podem ser obtidas a partir dos bancos de dados disponíveis e as

simulações feitas em softwares escolhidos de acordo com a análise desejada. Para análises inicias, programas com I-Mutant e SNP&Go podem predizer o efeito dos SNPs na estabilidade da proteína, enquanto ferramentas como o Swiss-PdbViewer mensuram o efeito dessas variantes a partir da análise da energia de forças de campo (Force Field Energies) e energia de minimização (Minimization Energies). Valores mais altos de energia correspondem a proteínas mais instáveis (Singh; Mistry, 2016; Swiss-PdbViewer tutorial).

Todo esse avanço tecnológico para determinação de potenciais SNPs relacionados à patologias e respostas terapêuticas tem permitido a identificação de forma mais rápida e precisa de potenciais marcadores para doenças complexas. No entanto é importante ressaltar a importância da validação funcional desses estudos para comprovar as associações estatísticas dos GWAS e das análises computacionais. Desta forma retornamos para os cenários de estudos de expressão e análises *in vivo*, utilizando das informações geradas pelas novas tecnologias para direcionar ou até mesmo ratificar os resultados obtidos.

Com base em todas as informações descritas sobre os fatores imunogenéticos envolvidos no estabelecimento da OP, o aprofundamento no estudo de genes em proteínas do sistema imune se mostra como uma alternativa promissora para melhor compreensão desta doença multifatorial e seu tratamento, tendo em vista a atuação dos componentes genéticos e imunológicos no equilíbrio do remodelamento ósseo.

3. Objetivos

3.1 Objetivo geral

Investigar a influência das citocinas pró-inflamatórias IL-23, IL-17, IL-12 e IFN-γ com a susceptibilidade e resposta terapêutica à Osteoporose primária pós-menopausa

3.2 Objetivos específicos

1. Determinar a frequência dos SNPs *IL23R* +2284 (C>A) (rs10889677), *IL17A* +672 (G>A) (rs7747909), *IL12B* +1188 (T>G) (rs3212227) e *IFNG* -1616 (G>A) (rs2069705) em pacientes com Osteoporose primária pós-menopausa e indivíduos saudáveis do estado de Pernambuco;

2. Avaliar o grau de associação desses SNPs com a susceptibilidade à OP primária pós-menopausa e com a resposta terapêutica aos bifosfonatos em pacientes de Osteoporose primária pós-menopausa;

3. Validar genes de referência para estudos funcionais em Osteoporose;

4. Avaliar comparativamente a expressão, em amostras de sangue periférico, da(s) citocina(s) associada à susceptibilidade, se existente, em pacientes com Osteoporose primária pós-menopausa e indivíduos saudáveis;

5. Avaliar a atuação da(s) citocina(s) associada(s) ao desenvolvimento da OP primária pós-menopausa, se existente, na atividade de células humanas de formação óssea.

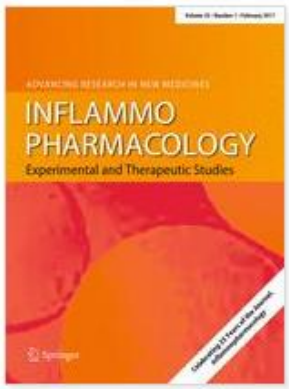
4. Capítulo I

Artigo aceito para publicação na revista Inflammopharmacology

Fator de Impacto: 2,3

Qualis CB1: B1

Guia para autores: Anexo A



Your Submission IPHM-D-16-00117R1 Entrada x

Inflammopharmacology (IPHM) <em@editorialmanager.com> 28 de jan

para mim

You are being carbon copied ("cc:'d") on an e-mail "To" "Camilla Lima" camilladelima@gmail.com;camilla.lima@ufpe.br
CC: javorski.natassia13@gmail.com, anna.paula.sl@hotmail.com, domingues.reumato@gmail.com, ana.mcvalenca@gmail.com, crovelser@gmail.com, prsouza30@gmail.com, j.azvedo@gmail.com, paulasandrin27@gmail.com

Dear Mrs. Lima,

Thank you for re-submitting the revised version your paper for consideration as a publication in Inflammopharmacology.
I am glad to inform you that the referees have recommended acceptance of your manuscript, and accordingly your paper will be published in the forthcoming issue of the journal. You should receive proofs in electronic pdf format shortly from the publishers, Springer Basel AG (Basel) Switzerland, through the editing and publications service, Spr Corrections. After checking or correcting these proofs they should be sent back to the publishers within 5 days of receipt. The corrected paper will then be immediately placed on the site for the journal which can be found at the publisher's website www.Springer.com/10787.
It is important that you should disclose if you have any Conflicts of Interest concerning this publication, and these should be stated at the end of the main text of the paper in proofstage.
The publication can then be cited as e-publication ahead of print with its own unique Dol identifier. The printed version of the paper will then appear in the Journal and a copy of this will be sent to you.
The publisher will send you copyright assignment and order forms for either electronic or paper copies of your paper of you choice.
All communications regarding the state of publication of your paper should now be directed to the production editor.

With best wishes,
Professor KD Rainsford, PhD, FRCPEdin, FRCPath, FRSC, FSB, FIBMS, Dr(hc)
Emeritus Professor of Biomedical Sciences
Editor-in-Chief, Inflammopharmacology
Biomedical Research Centre

Polymorphisms in key bone modulator cytokines genes influence bisphosphonates therapy in postmenopausal women

Lima, CAD*[1,2]; Javorski, NR[1,2]; Souza, APO[2]; Barbosa, AD[2,3]; Valença, APMC[4]; Crovella, S[1,2]; Souza, PRE[2,5]; De Azevêdo Silva, J[1,2] and Sandrin-Garcia, P[1,2]

1 – Department of Genetics - Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.
2 – Laboratory of Immunopathology Keizo Asami – Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.
3 - Division of Rheumatology, Clinical Hospital, Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.
4 – Department of Oceanography - Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.
5 – Department of Genetics – Rural Federal University of Pernambuco (UFRPE), Recife, Pernambuco, Brazil.

*Corresponding author: Camilla AD Lima

Email address: camilladelima@gmail.com

Accepted for publication in Inflammopharmacology Journal. DOI: 10.1007/s10787-017-0322-7

Abstract

Osteoporosis is a multifactorial and debilitating disease resulting from decreased bone mineral density (BMD) and loss of tissue microarchitecture. Ineffective therapies may lead to bone fractures and subsequent death. Single nucleotide polymorphisms (SNPs) in key immune regulator genes have been associated with therapeutic response to bisphosphonates, which are the first therapeutic line of choice for osteoporosis. However, cytokine pathways and their relation with therapeutic adhesion remain to be fully elucidated. Aimed at better understanding these processes, we investigated the response to bisphosphonate therapy in postmenopausal women and four SNPs in key proinflammatory cytokines genes: IL23R +2284 (C>A) (rs10889677), IL17A +672 (G>A) (rs7747909), IL12B +1188 (T>G) (rs3212227) and IFNG -1616 (G>A) (rs2069705). A total of 69 patients treated with bisphosphonate were followed for a period of 1 up to 4 years, genotyped and compared according their changes in bone mineral density (BMD) and level of biochemical markers during their treatment. The IFNG -1616 G/G associated with increased BMD values in femoral neck (GG/AA, p=0.016) and decreased BMD values in total hip (GG/GA, p= 0.019; GG/AA, p= 0.011). In relation to biochemical markers, IFNG -1616 SNP associated with increased alkaline phosphatase (GG/AA; p< 0.0001) and parathyroid hormone levels (AA/GA; p= 0.017). Vitamin D values changes were related to IL17A +672 (GG/GA, p=0.034) and to IL12B +1188 (TT/TG, p= 0.046) SNPs. Besides, significant differences in changes of calcium levels correlated with IL23R +2284 (CC/CA, p= 0.016) genotypes. Altogether, we suggest that these polymorphisms may play an important role for therapeutic decisions in osteoporosis treatment.

Keywords: Osteoimmunology, Osteoporosis Therapy, Bone Mineral Density, Bone Biochemical Markers, Genetics of osteoporosis**.**

1. Introduction

Osteoporosis (OP) is a systemic disease characterized by the decrease of bone mineral density (BMD) and by loss of tissue microarchitecture. OP main clinical manifestations are fragility fracture and injuries related to bone break (Sun et al. 2014; Tella and Gallagher 2014; Drake et al. 2015; Tastan et al. 2016). According to World Health Organization (WHO), OP diagnosis is determined by BMD measures with T-scores less than -2.5 (Kanis 2008). Despite being a major problem for global public health, osteoporosis is considered under diagnosed and, consequently, an undertreated disorder by health care systems (Kerschan-Schindl 2016; Miller 2016). About half of fractures diagnosed in OP patients are due to unspecific or inefficient drug therapies, increasing morbidity and mortality rates, since about 20% of patients die during the first year of the treatment due to bone fractures (Roush 2011; Iolascon et al. 2013; Drake et al. 2015; Miller 2016).

The bisphosphonates (BPs) are a class of drugs able to decrease bone loss, known as the anticatabolic therapy of choice for postmenopausal women, mainly due to its low cost (Zhou et al. 2014; Baccaro et al. 2015). The idea of "one fits all" is increasing morbidity, severity and overall costs with this disease and can even lead patient's death due to non-specific and general treatment (Hiligsmann et al. 2015; Abrahamsen and Prieto-Alhambra 2016). The fracture location and genetic information from patients are pivotal in treating these women since the phenotypic features in OP are modulated by genetic factors in about 50-80% from all cases (Riancho and Hernández 2012b).

The influence of genetic polymorphisms in genes involved in the main pathways of BPs action mechanisms have been well characterized, pointing out the importance of specific genes such as *Wnt*, *MVK* and *RANK* in the modulation of BPs therapeutic response (Marini

and Brandi 2014; Zhou et al. 2014; Wang et al. 2015a; Zheng et al. 2015; Zhou et al. 2015). Even though genetic polymorphisms within the above-mentioned genes are known to influence BPs therapeutic response, there are important pathways remaining to be elucidated, particularly the immune related ones. Noteworthy, cytokines and their network display several essential functions in the bone remodeling and are closely related to osteoporosis treatment (Yuan et al. 2012; Marini and Brandi 2014; Talaat et al. 2015). Recently, cytokines such as IFN-γ have been described as inhibitor of RANKL/RANK/OPG pathway through TRAF 6 protein degradation and IL-17 as inducer of RANK-L and Th17 cells production, both affecting bone homeostasis processes (Guerrini and Takayanagi 2014; Talaat et al. 2015).

Therefore, we investigated whether there is an association between single nucleotide polymorphisms (SNPs) within IL23R, IL17A, IL12B and IFN-γ genes from Th1 and Th17 pathways and the response to BPs therapy in a population-based sample of postmenopausal women from Northeast Brazil treated over a period of 1- 4 years.

2. Materials and methods

2.1 Subjects

In the present study we collected data from 240 postmenopausal osteoporotic women from the Division of Rheumatology of Clinical Hospital in Federal University of Pernambuco, Brazil enrolled within 2011 and 2015. The patients were diagnosed according WHO criteria (Kanis 2008). All therapies included in this study followed the clinical guidelines in health supplement from National Agency of Health of Brazil (Cunha et al. 2011). Dosage guidelines and oral doses administration recommended were alendronate (70 mg/week), risendronate (35 mg/week), ibandronate (150 mg/week) or intravenous infusion of zolendronic acid (5 mg/year). Additionally, all women were supplemented with 600 mg/day

of elemental calcium and 400 IU/day of vitamin D_3. Patients received one of these above-mentioned treatments for at least 1 year, and only BPs' class were included in all subjects in this study. The exclusion criteria in patients' group were osteopenia, inflammatory disease, autoimmune diseases, cancer, use of anti-inflammatories and other drugs for bone disorders such as anabolic agents, hormone replacement therapy or selective estrogen receptor modulators at any moment of the treatment period.

All the participants provided a written informed consent. This study was approved by the Research Ethics Committee of the Center for Health Sciences, Federal University of Pernambuco (CEP/CCS/UFPE nº 513/11) and is in accordance with the Declaration of Helsinki.

2.2 SNPs selection and genotyping

We selected four SNPs from IL23R, IL17A, IL12B and IFN-γ genes: IL23R +2284 (C>A, rs10889677), IL17A +672 (G>A, rs7747909), IL12B +1188 (T>G, rs3212227) and IFN-γ -1616 (G>A, rs2069705). SNPs information was obtained from SNP Browser software 4.0 (Applied Biosystems, Foster City, CA, USA) and 1000 Genomes (http://browser.1000genomes.org/index.html). The selection criteria were minor allele frequency (MAF) higher than 10% in African (YRI subpopulation) and European (CEU subpopulation) populations and SNPs positions in regulatory regions such as promoter regions, 5' or 3' UTR.

Genomic DNA extraction was performed from peripheral leukocytes using the rapid salting out method (Bignon and Viña 1995). Samples were genotyped with specific fluorogenic probes (Taqman Probes, Applied Biosystems, Foster City, CA, USA), using Real-

Time PCR performed with ABI 7500 detection system (Applied Biosystems, Foster City, CA, USA).

2.3 Assessment of BMD changes in response to BPs Therapy

The BMD areas assessed were: lumbar spine (LS: L1-L4), femoral neck (FN) and total hip (TH) at the beginning of treatment and after 1, 2, 3 and 4 years. The BPs' effects in BMD values were measured by percentage changes of BMD, calculated according to Zhou et al. (2015) following the formula: % changes of BMD = $[(BMD_{after\ treatment} - BMD_{baseline}) / BMD_{baseline}] \times 100\%$.

BMD values were measured by dual-energy x-ray absorptiometry (Hologic or Lunar). The standardization method for values correction from different machines was described by Genant et al. (1993) enabling the comparison between the measures from both x-rays (Genant et al. 1993; Zhou et al. 2015). For BMD values in lumbar spine, the formula applied was: Hologic BMD (g/cm2) = (Lunar BMD - 0.054)/1.074 ($r = 0.987$, $p < 0.05$, standard error of the estimate = ± 0.030 g/cm2) and for BMD values in both femoral neck and total hip: Hologic BMD (g/cm2) = (Lunar BMD - 0.142)/1.013 ($r = 0.920$, $p < 0.05$, standard error = ± 0.051 g/cm2).

2.4 Assessment of biochemical markers of bone turnover changes

The levels of alkaline phosphatase (ALP) in peripheral blood and calciuria in urine sample (24-hour collection) were used as indicators of bone activity. The vitamin D, calcium and parathyroid hormone (PTH) levels in peripheral blood were also analyzed. All measures were performed on AU680 (Beckman Coulter) and BT 3000 plus (Winer Lab.) equipments using specific kits. The BPs' effects in these biomarkers were assessed by percentage changes

of biomarkers, calculated according to Zhou et al. (2015) in the following formula (e.g. Vitamin D): % changes of Vitamin D = [(Vitamin $D_{after\ treatment}$ – Vitamin $D_{baseline}$) / Vitamin $D_{baseline}$] × 100%.

2.5 Statistical analyses

Allelic and genotypic frequencies and Hardy–Weinberg equilibrium were performed using the SNPStats tool (http://bioinfo.iconcologia.net/SNPstats). Comparisons among the genotypes related to BMD and biochemical markers changes were performed using Analyses of Variance (ANOVA) or Kruskal-Wallis tests as appropriate. When a significant difference was observed, Tukey test was applied for pairwise comparisons of genotypes. All statistical analyses were conducted using the SPSS 18.0 statistical package (SPSS Inc., Chicago, IL, USA). Differences were accepted as significant at *p-values* < 0.05.

3. Results

3.1 Patients characteristics

A total of 69 patients were analyzed in this study after the inclusion and exclusion criteria (Figure 1). Demographic characteristics (mean ±standard deviation) of these patients included age at the start of treatment (59.00 ±8.06 years old); age after 4 years of treatment (64.00 ±5.23 years old); years since menarche - YSM (14 ±2.11 years old) and body mass index - BMI (25.67 ±4.57 kg/m²). Among the patients, fractures occurred in 6 subjects, in the wrist (n=3), femoral (n=1), spine (n=1) and foot (n=1) areas, but with no significant differences related to frequencies distribution of IL23R +2284 (C>A), IL17A +672 (G>A), IL12B +1188 (T>G) and IFN-γ -1616 (G>A) genotypes.

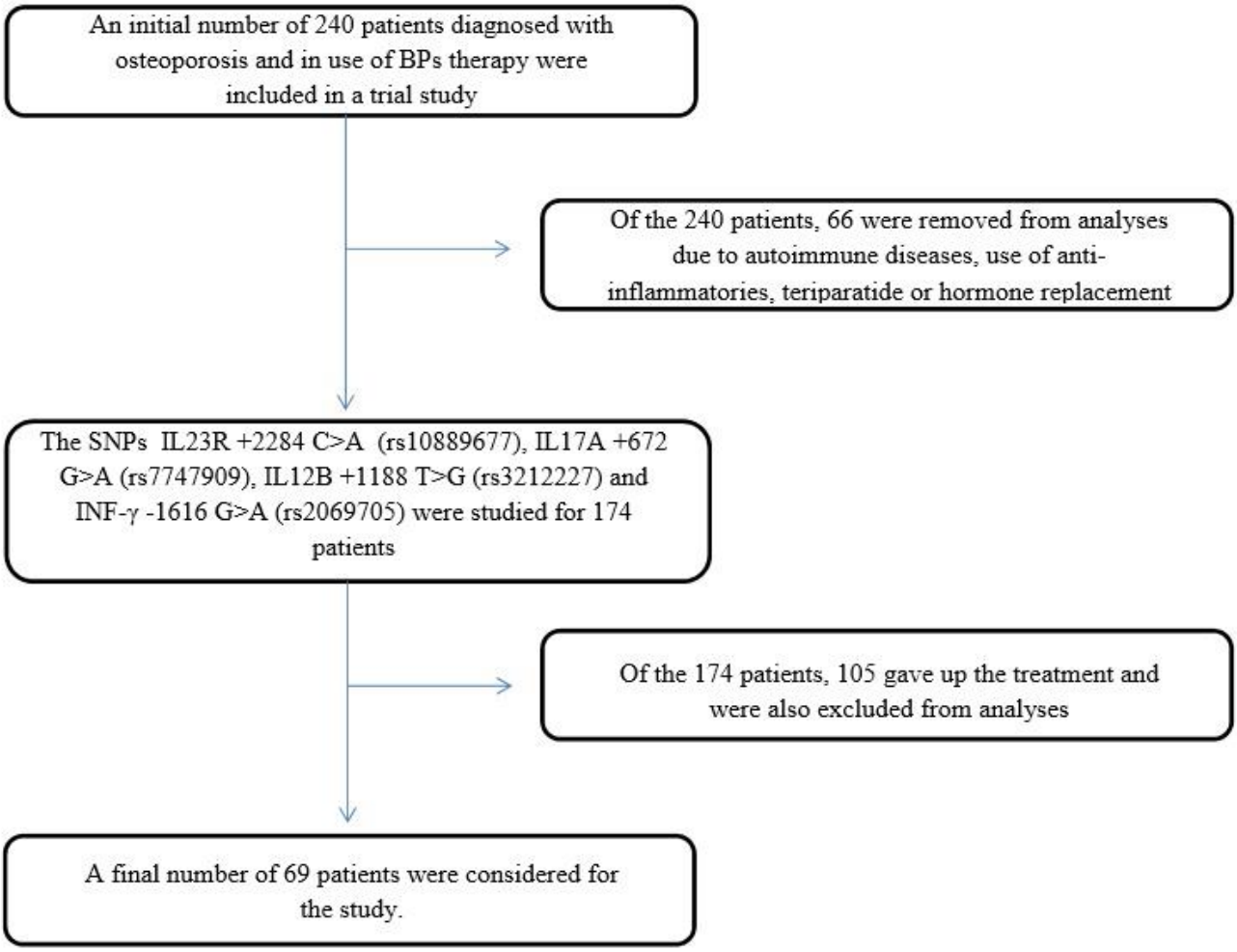


Figure 1. The flow chart describes patients' selection process

In relation to BPs therapy, the majority (77% of patients) used alendronate, followed by risendronate (16%) and ibandronate (7%). Four patients switched the drug used at the start of treatment (3 patients with alendronate and 1 patient with risendronate) to intravenous infusion of zolendronic acid (5mg/year), at different periods of treatment. Allelic and genotypic frequency distributions of all SNPs were in Hardy-Weinberg equilibrium in all assessed groups.

3.2 BMD and bone biochemical markers from baseline measures stratified according to SNPs genotypes

The highest BMD baseline values in lumbar spine (LS) and total hip (TH) areas were associated with IL17A +672 G/A, IL12B +1188 T/T and IFN-γ -1616 G/G genotypes. In relation to BMD values in femoral neck (FN) area, the highest values were exhibited in the presence of IL17A +672 A/A, IL12B +1188T/G and IFN-γ -1616 G/A genotypes. Among the studied SNPs only IL23R +2284 C/A showed the highest BMD values for all studied bone areas (Table 1). Despite that, no significant differences were detected among the genotypes variants in relation to BMD values.

When assessing the biochemical markers vitamin D and calcium, we identified higher baseline values associated with IL23R +2284 A/A and IFN-γ -1616 G/G genotypes. The highest vitamin D and calcium values were exhibited in the presence of IL17A +672 G/A and A/A genotypes, respectively. The IL12B +1188 showed significant differences among genotypes (GG/TG, p= 0.042), with T/G genotypes correlating with the highest vitamin D values. Higher baseline values of alkaline phosphatase (ALP) were exhibited in carriers of IL23R +2284 C/A, IL17A +672 G/G, IL12B +1188 T/T and IFN-γ -1616 A/A genotypes. Additionally, higher values of parathyroid hormone (PTH) were observed in subjects with IL23R +2284 A/A, IL17A +672 G/A, IL12B +1188 G/G and IFN-γ -1616 G/G genotypes and higher calciuria values were found for IL23R +2284 A/A, IL17A +672 A/A, IL12B +1188 T/T and IFN-γ -1616 A/A genotypes. Despite that, biochemical markers were not statistically different among genotypes' variants, except for IL12B +1188 and vitamin D levels (Table 1).

Table 1. BMD and biochemical markers baseline values from assessed patients.

Genotypes	BMD (g/cm2)			Bone biochemical markers				
	LS	FN	TH	Vitamin D (ng/mL)	Calcium (mg/dL)	ALP (U/L)	PTH (pg/mL)	Calciuria (mg/24h)
IL23R +2284 (rs10889677)								
CC	0.688±0.07	0.623±0.09	0.692±0.13	29.28±7.26	9.33±0.65	90.18±50.42	64.24±27.57	105.84±46.14
CA	0.701±0.13	0.664±0.07	0.739±0.10	30.94±8.66	9.34±0.72	96.07±40.35	68.97±31.97	133.63±97.35
AA	0.694±0.94	0.641±0.13	0.657±0.05	31.33±9.75	9.83±0.29	84.33±18.01	73.07±45.41	154.37±52.65
p	0.449	0.218	0.267	0.778	0.457	0.887	0.825	0.385
IL17A +672 (rs7747909)								
GG	0.686±0.10	0.632±0.09	0.699±0.12	29.93±7.08	9.38±0.62	93.84±48.81	62.98±28.47	129.73±73.99
GA	0.715±0.08	0.643±0.09	0.733±0.09	30.17±10.38	9.34±0.81	89.25±36.74	78.59±33.28	90.18±59.22
AA	0.704±0.09	0.700±0.12	0.662±0.15	30.15±1.91	9.4±0.85	66†	55.9±21.35	160.3†
p	0.558	0.429	0.515	0.996	0.987	0.814	0.292	0.312
IL12B +1188 (rs3212227)								
TT	0.715±0.10	0.633±0.09	0.716±0.14	26.64±5.59	9.30±0.57	98.33±54.93	62.43±26.40	132.49±66.76
TG	0.682±0.09	0.643±0.09	0.697±0.10	32.05±8.07	9.41±0.77	86.89±38.10	67.98±33.17	119.05±81.76
GG	0.607±0.10	0.533±0.02	0.580±0.09	22.3†	9.8†	71†	72.6†	48.3†
p	0.168	0.249	0.297	0.042 (GG/TG)	0.700	0.693	0.833	0.527
IFN-γ -1616 (rs2069705)								
GG	0.704±0.09	0.634±0.08	0.719±0.09	30.80±8.32	9.44±0.39	74.66±37.18	84.14±28.31	114.46±44.31
GA	0.702±0.09	0.640±0.08	0.707±0.11	30.09±6.90	9.38±0.60	89.63±46.70	59.19±23.33	116.69±74.60
AA	0.669±0.10	0.637±0.11	0.698±0.15	29.22±9.38	9.30±0.91	103.67±43.98	67.5±37.69	132.19±78.89
p	0.454	0.979	0.895	0.901	0.879	0.45	0.10	0.841

† Standard deviation was not calculated since there was only one sample of variant genotype

3.3 BMD and bone biochemical markers changes in response to BPs therapy stratified according to SNPs genotypes

The IFN-γ -1616 G/G genotype was associated with increased BMD values in FN area (GG/AA, p= 0.016) and decreased BMD values in TH area (GG/GA, p= 0.019; GG/AA, p= 0.011) after 3 and 4 years, respectively (Figure 2 and Table 2). Additionally, IL12B +1188 T/T genotype showed a trend of association (TT/TG; p= 0.058), with increased BMD values in TH area after 1 year of treatment (Figure 2 and Table 2). For the remaining comparisons, no significant differences were observed among the SNPs and BMD changes during the period of treatment. All BMD values in LS, FN and TH areas during the period of treatment (4 years) are shown in Figure 3.

Among the biochemical markers for bone resorption, significant associations were found between increased vitamin D levels and IL17A +672 GA genotype (GG/GA, p= 0.034) after 1 year of treatment as well as with IL12B +1188 T/G genotype (TT/TG, p= 0.046) after 4 years of treatment. An increase in calcium values was related to IL23R +2284 C/C genotype (CC/CA, p= 0.016) after 3 years of treatment. The IFN-γ -1616 G/G genotype was associated with increased ALP levels (GG/AA; p< 0.0001) and IFN-γ -1616 A/A genotype, with increased PTH levels (AA/GA; p= 0.017), after 1 and 2 years of treatment, respectively. These results are shown in Table 2.

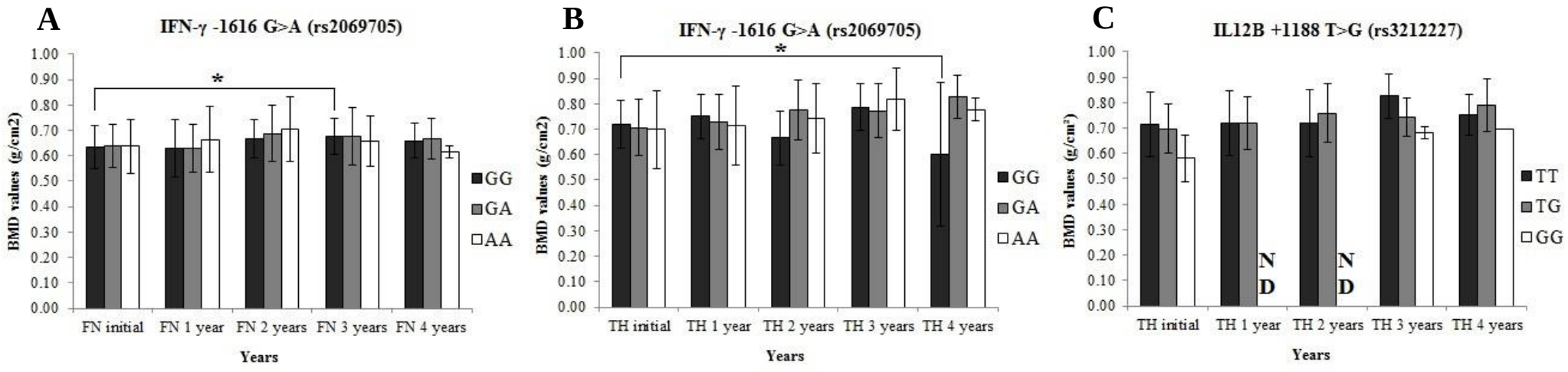


Figure 2. Changes in the BMD measures according to patient's genotype. 2A: IFN-γ -1616 G>A showed association between G/G genotypes and increased BMD values in FN (3 years) (GG/AA, *p*=0.016). 2B: IFN-γ -1616 G>A showed association between G/G genotypes and decreased BMD values in TH (4 years) (GG/GA, *p*=0.019; GG/AA, *p*=0.011). 2C: IL12B +1188 T>G showed a tendency of association between T/T genotype and increase of BMD values in TH (1 year) (TT/TG, p=0.058). BMD: bone mineral density, LS: lumbar spine, FN: femoral neck, TH: total hip. *$p < 0.05$. The absence of the standard deviation (SD) bar was due to the presence of only one sample for each variant genotype. No Data (ND): The presence of variant genotype has not been found.

Table 2: Significant correlation among baseline values and percentage changes in BMD and biochemical markers related to variants genotypes.

Genotype	FN (3 years)	TH (4 years)	ALP (1 year)	PTH (2 years)	**Genotype**	PTH (1year)	Vitamin D (4 years)
IFN-γ -1616 (rs2069705)					**IL12B +1188 (rs3212227)**		
GG	1.16±0.16	0.79±0.34	4.84	‡	**TT**	1.11±0.13	1.32±0.09
GA	1.02±0.06	1.10±0.08	0.87±0.30	0.99±0.39	**TG**	1.01±0.11	2.90†
AA	1.04±0.09	1.15±0.08	0.79±0.26	1.23±0.67	**GG**	‡	‡
p	0.016 (GG/AA)	0.019 (GG/GA) 0.011 (GG/AA)	$<10^{-4}$ (GG/AA)	0.017 (AA/GA)	***p***	0.058 (TT/TG)	0.046 (TT/TG)

Genotype	Calcium (3 years)	**Genotype**	Vitamin D (1 year)
IL23R +2284 (rs10889677)		**IL17A +672 (rs7747909)**	
CC	1.02±0.64	**GG**	1.00±0.17
CA	0.93±0.57	**GA**	1.42±0.40
AA	‡	**AA**	1.03±0.35
P	0.016 (CC/CA)	***p***	0.034 (GG/GA)

† Standard deviation was not calculated since there was only one sample of variant genotype

‡There was not representative of variant genotype

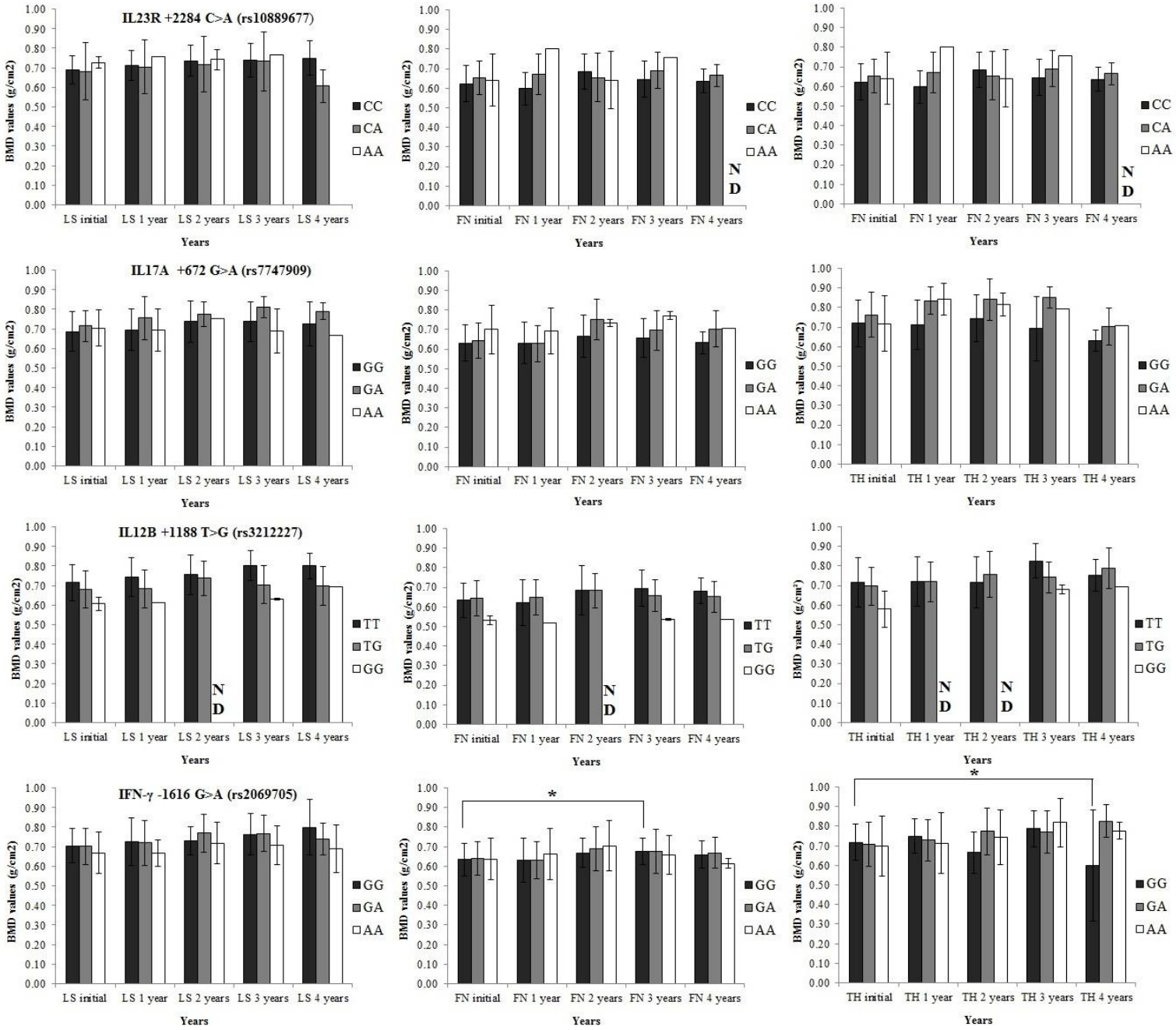


Figure 3. Changes in BMD values during 4 years of treatment stratified according to IL23R +2284 C>A; IL17A +672 G>A; IL12B +1188 T>G and IFN-γ -1616 G>A genotypes. BMD: bone mineral density, LS: lumbar spine, FN: femoral neck, TH: total hip. *p< 0.05. The absence of the standard deviation (SD) bar was due to the presence of only one sample for each variant genotype. No Data (ND): The presence of variant genotype has not been found.

4. Discussion

In our study we showed that cytokine genes SNPs within were associated with BMD changes in response to BPs therapy. To our knowledge, this is the first study showing these polymorphisms as related to BPs response and maybe shed some influence upon further OP therapy strategies.

Our results are consistent with previously reported studies which related SNPs in cytokine genes to BMD changes and metabolic bone disorders such as psoriatic arthritis and gout (Czerny et al. 2010; Jadon et al. 2013; Liu et al. 2015). Additionally, polymorphisms in genes related to bone disorders immune response and to variations in BMD levels showed different results according to the studied population (Swanberg et al. 2012; Boroń et al. 2014; Louahchi et al. 2016). In our population all SNPs analyzed showed differences related to any biochemical markers changes whereas only IFN-γ -1616 exhibited association regarding BMD changes.

In relation to the biochemical markers, our results showed that IL17A +672 G/A and IL12B +1188 T/G genotypes were associated with higher vitamin D levels. Vitamin D presents pleiotropic actions in bone environment: in osteoprogenitors cells it is responsible for increasing RANKL levels and in mature osteoblasts acts decreasing RANKL expression (Christakos et al. 2016). Therefore, it is possible that IL-17A and IL-12B may be related to induction of bone formation or resorption upon osteoblast maturation stage. Additionally, IL23R +2284 C/C genotype showed statistically significant correlation with higher calcium levels, a key element for mineralization in bone matrix and tissue homeostasis (Carmeliet et al. 2015). The relation between IL-23R and calcium mineralization has been cited by Tu et al. (2015) in osteogenic differentiation assays, which suggested that IL-23/IL-23R/β-catenin

pathway induces bone formation. These authors observed that IL-23R silencing suppressed calcium mineralization and modified the expression of osteogenic markers such as Runt-related transcription factor 2 (RUNX2), ALP, Osteocalcin (OCN) and Dickkopf-related protein 1 (DKK1) in mesenchymal stem cells in osteoblasts differentiation process. Besides, IFN-γ -1616 A/A genotype was also related to higher PTH levels, indicating bone resorption, and IFN-γ G/G genotype to higher ALP levels, denoting bone formation. PTH acts regulating bone resorption through matrix calcium release whereas alkaline phosphatase is a bioproduct of osteoblast activities related to bone calcification (Sharma et al. 2014; Carmeliet et al. 2015; Christakos et al. 2016).

Herein we also identified changes in BMD values. After 3 years of treatment IFN-γ G/G genotype continued associated with indicators of bone formation in FN area. Conversely, individuals with this genotype showed decreased BMD values in TH area. Therefore, a possible pleiotropic action of this cytokine is hypothesized, dependent on time/period of treatment and/or body-area assessed. Furthermore, TH area showed higher BMD values than FN area (t-test= -6.60; $p< 0.001$) suggesting that IFN-γ may act in BMD value-dependent as an anabolic or catabolic factor, depending on the number of cells (low or high quantities, respectively). The impact of this cytokine in the bone homeostasis has been widely recognized in the context of osteoimmunology (Arron and Choi 2000). Some authors suggest that lower levels of IFN-γ cytokine may increase the chances of survival, activation and differentiation in osteoclasts (Arron and Choi 2000; Nasr et al. 2014). According to Arron and Choi (2000) the binding of IFN-γ and its receptor induces TNF receptor-associated factor 6 (TRAF6) degradation, impairing RANKL action. The role of RANKL/RANK/OPG pathway has been established in the bone homeostasis: RANKL is secreted by osteoblast and bind to its receptor RANK in osteoclast, acting in osteoclastogenesis, whereas OPG act as

competitive antagonist for RANKL (Riancho and Hernández 2012; Makras et al. 2015). Furthermore, IFN-γ increases the bone morphogenetic protein 2 (BMP-2), which participates of *Wnt* pathway inducing bone mineralization and osteoblast differentiation (Raisz 2005). SNPs within genes from *Wnt* pathway have been associated with antireabsortive therapies' response as alendronate, risendronate and tamoxifen (Kruk et al. 2009; Hartmaier et al. 2012; Zhou et al. 2014; López-Delgado et al. 2016). Nevertheless, it is worth mentioning that some studies indicate that IFN-γ present contradictory functions in the bone environment when comparing their *in vivo* and *in vitro* actions (Takayanagi et al. 2005; Gao et al. 2007; Zhang et al. 2015a). Despite the *in vivo* impact described above, *in vitro* IFN-γ present a pleiotropic action by inducing both the increase and decrease of the osteoclastogenesis by different pathways (Gao et al. 2007; Zhang et al. 2015a). An important physiologic role for IFN-γ in terms of bone homeostasis was explored by Duque et al. (2011) with ovariectomized mice, which suggested this cytokine as a new anabolic agent against osteoporosis. On the other hand, Gao et al. (2007) showed that IFN-γ promotes the osteoclastogenesis by inducing specific cytokines and T cell activation. Our results suggest that IFN-γ may act as both anabolic and catabolic agent in bone environment depending on period of treatment or body-area assessed. Therefore the IFN-γ function may be influenced by these parameters as well as by BMD values. It is difficult to predict which major pathway is involved in the process taking into consideration that this cytokine acts in several routes. The IFN-γ is the main cytokine induced by IL-12 in the Th1 response (Raphael et al. 2014; Annunziato et al. 2015). Some authors pointed out that IL-12/ IFN-γ pathway from Th1 response is related to Th17 response since it is likely that IL-17 can induce IL-12 (Damsker et al. 2010; Khader and Gopal 2010). There is a general consensus about IL-17 being stimulated by IL-23 in the presence of TGF-β and IL-6, initiating the Th17 response (Iwakura and Ishigame 2006;

Sutton et al. 2009). The SNPs selection in genes of IL-23, IL-17, IL-12 and IFN-γ cytokines was performed in our study and analyzed in both pathways, Th1 and Th17, in therapeutic adhesion of OP patients. However, due to different responses in BMD and biochemical markers changes after BPs treatment, we suggest that cytokines from both pathways may have different roles in terms of bone homeostasis.

Additionally, we propose possible pathways involved in the bone resorption, as showed in Figure 4. In the JAK-STAT – dependent pathway, STAT dimers translocate to the nucleus being able to regulate Gamma Activated Sequences (GAS) and IFN Stimulated Regulatory Elements (ISRE), inducing the expression of Interferon regulatory factor 1 (IRF1) and other regulatory proteins from cell cycle and apoptosis (Ramana et al. 2002; Wong et al. 2002). Other JAK-STAT – independent pathway, studied in mice, showed that osteoclasts activate the expression of IRF9 gene through mitogen activated protein kinase kinase kinase 1 (Map3K1 in humans and MEKK1 in murine), mitogen activated protein kinase kinase 1 (Map2K1 in humans and MEK1), mitogen activated protein kinases 1 and 2 (ERK1/2) and C/Ebpbeta cascade, also involved in cell cycle activities (Roy et al. 2002; Haynes 2006; Pham et al.2014). Consequently, it is possible that the IFN-γ participates in bone remodeling by directly inducing osteoclasts and osteoblasts apoptosis during treatment.

The aminobisphosphonates (aminoBPs) used in the treatment of OP patients also induce apoptosis in osteoclasts by inhibiting the mevalonate kinase (MVK) pathway (Riancho and Hernández 2012b). Furthermore, it is important to highlight that aminoBPs may prevent apoptosis in osteoblasts by a cell survival mechanism through extracellular signal-regulated kinases that affects the production of bone mass (Riancho and Hernández 2012b).

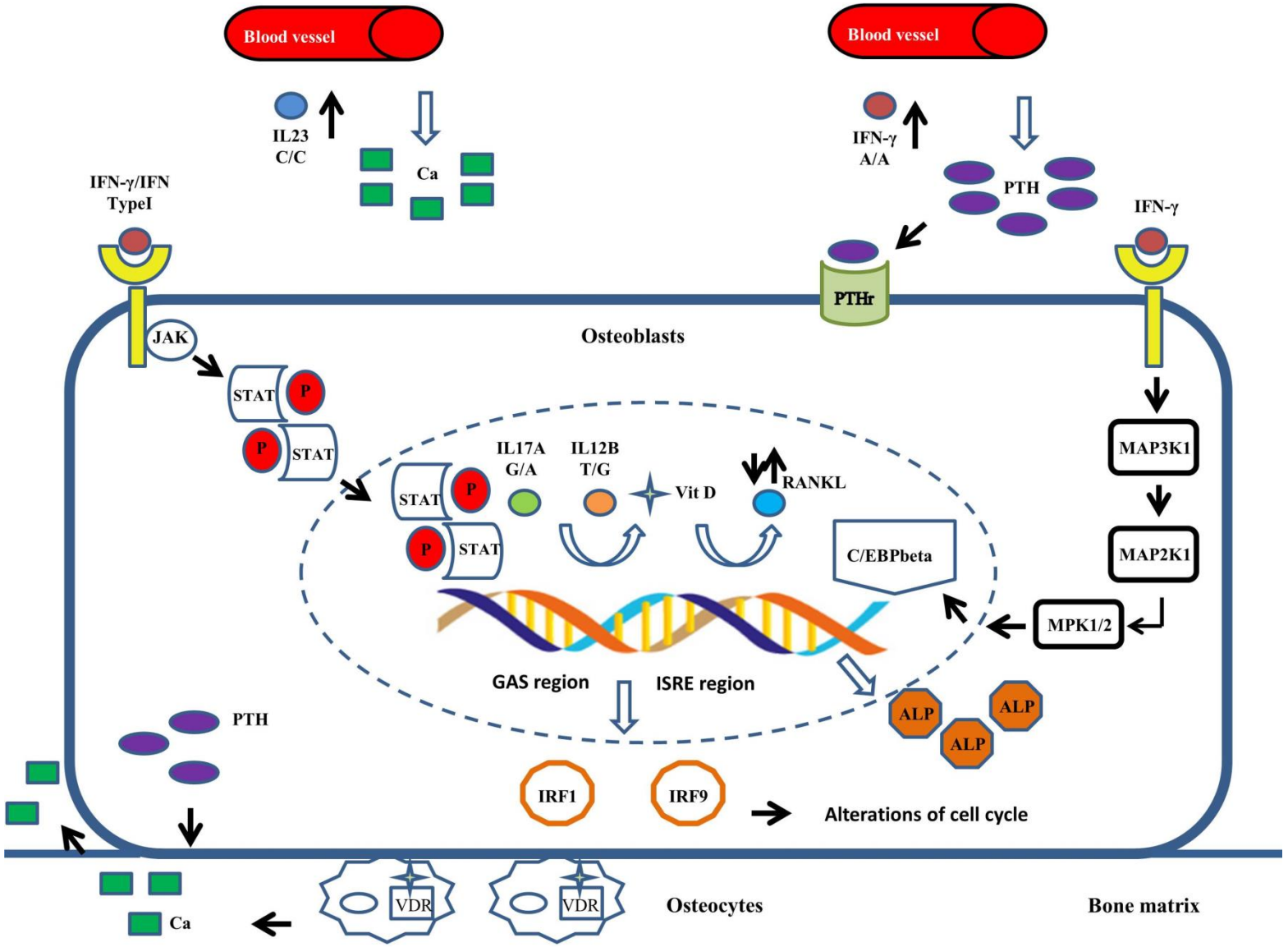


Figure 4. Possible pathways involved in bone homeostasis. It is possible that IFN-γ pathways occur in osteoclast and osteoblasts cells. The JAK/STAT and JAK/STAT-independent pathways activate GAS and ISRE gene transcription. These genes express IRF1 and IRF9 able to alter cell cycle inducing apoptosis. The IL23 C/C stimulates increased calcium levels in the bone, while IFN- γ A/A stimulates the increase in PTH levels and IFN- γ G/G stimulates the increase of ALP levels. Low levels of calcium serum can stimulate PTH and Vitamin D to liberate calcium of bone matrix. IL17A G/A and IL12B T/G stimulate increase of vitamin D level. The vitamin D participates to the control of RANKL levels, showing pleiotropic actions dependents of stage of osteoblast maturation.

Some cytokines such as RANKL and Nuclear Factor kappa B (NFk-B) cannot only influence osteoclastogenesis and osteoblastogenesis, but also act directly in MVK pathway. RANKL and NFk-B play a role in differentiation, activation and survival osteoclasts and are regulated by cytokines such as IFN-γ and IL-17 (Arron and Choi 2000; Riancho and Hernández 2012b).

We analyze simultaneously the association between IL23R, IL17A, IL12B and IFN-γ SNPs and BPs therapy. The SNPs in cytokine genes may change their levels and protein structure and this may interfere in the bone disease environment and response to BPs treatment. Understanding the genetic contribution in multifactorial diseases, such as the osteoporosis, is essential for providing specific therapeutic and diagnostic procedures, allowing customized treatments based on different patients' genetic profiles. Besides, our findings highlight the importance of other factors different than RANKL/RANK/OPG pathway associated with OP. It is acknowledged that other molecules as leptin or leptin receptors may activate the secretion of proinflammatory cytokines and regulate via Janus kinases downstream events (Legiran and Brandi 2012; Ye and Lu 2013; Lee et al. 2014). Additionally, SNPs in other genes related to immune systems such as IL-1, IL-6, IL-4, TNF-α, STATs, ERAP, HLA and TRAF proteins are already recognized as influencing bone environment (Takayanagi et al. 2005; Yuan et al. 2012; Jadon et al. 2013), possibly presenting potential action in OP therapeutic adhesion.

Conclusions

A relationship among SNPs in IL23R, IL17A, IL12B, IFN-γ genes and changes in BMD values or biomarkers bone turnover in BPs treatment are described for the first time in this study. The IFN-γ -1616 (G>A) genotype was associated with BMD changes while IL23R

+2284 (C>A), IL17A +672 (G>A) and IL12B +1188 (T>G) genotypes were associated with bone turnover, based on biochemical markers changes. This suggests that these SNPs may act as potential indicators in therapeutic decisions used in the treatment of osteoporosis, related to choice of BPs as first-line treatment, its therapy period and the use of supplements, such as vitamin D and calcium. SNPs in cytokines genes may have an important role in therapy's success and in paving the way for personalized medicine in osteoporosis.

Acknowledgments

We thank the following Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco) for financial support.

Conflict of interest

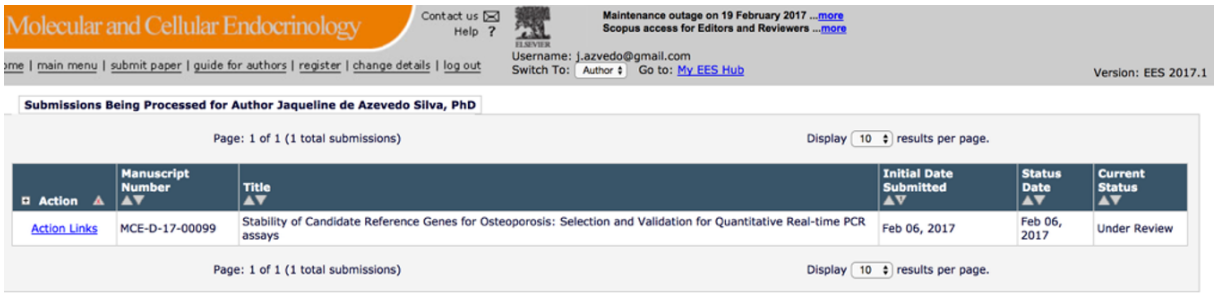
None of the authors declared any conflict of interest related to this manuscript.

5. Capítulo II

Artigo submetido na revista Molecular and Celular Endocrinology

Fator de Impacto: 3,8

Guia para autores: Anexo A


Molecular and Cellular Endocrinology

Contact us
Help ?

Maintenance outage on 19 February 2017 ...more
Scopus access for Editors and Reviewers ...more

ome | main menu | submit paper | guide for authors | register | change details | log out

Username: j.azvedo@gmail.com
Switch To: Author Go to: My EES Hub

Version: EES 2017.1

Submissions Being Processed for Author Jaqueline de Azevedo Silva, PhD

Page: 1 of 1 (1 total submissions) Display 10 results per page.

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	MCE-D-17-00099	Stability of Candidate Reference Genes for Osteoporosis: Selection and Validation for Quantitative Real-time PCR assays	Feb 06, 2017	Feb 06, 2017	Under Review

Page: 1 of 1 (1 total submissions) Display 10 results per page.

Stability of Candidate Reference Genes for Osteoporosis: Selection and Validation for Quantitative Real-time PCR assays

Lima, CAD[1,2*]; Lima, SC[2]; Barbosa, AD[2,3]; Crovella, S[1,2]; Sandrin-Garcia, P[1,2]; Pita, WB[4]; and De Azevêdo Silva, J[1,2]

1 – Department of Genetics - Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.

2 – Laboratory of Immunopathology Keizo Asami – Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.

3 - Division of Rheumatology, Clinical Hospital, Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.

4 – Department of Antibiotics - Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.

*Corresponding author: Camilla AD Lima

Email address: camilladelima@gmail.com

Submitted in Molecular and Cellular Endocrinology Journal

Abstract

Osteoporosis (OP) is a disease influenced by genetic factors in more than half of the cases. In spite of the efforts to clarify the relationship among these factors and OP development, many discoveries need to be further functionally validated. Real-time reverse transcription quantitative PCR (RT-qPCR) is the main used method for quantification of gene expression studies. However, some limitations as the choice of stably expressed reference genes (RG) are required to ensure the efficiency and quality of gene expression assays. By our knowledge, validation study for RG in OP is still missing. Therefore, this study compared the expression levels of 10 RG (G6PD, B2M, GUSB, HSP90, EF1A, RPLP0, GAPDH, ACTB, 18S and HPRT1) to assess their suitability in OP analysis by using of GeNorm, Normfinder, BestKeeper and RefFinder programs. A minimal number of two RG was recommended by GeNorm to obtain a reliable normalization. RPLP0 and B2M were identified as the most stable genes in OP studies while ACTB, 18S and HPRT1 were inadequate for normalization in our data. Moreover, we showed the dramatic effects of suboptimal RG choice on the quantification of a target gene highlighting the importance in identificating the most appropriate reference gene to specific diseases. The used algorithms showed different results among them in relation to top ranked genes, thus suggesting an individualized and carefully choice in the used packages and reference genes selection.

Keywords: Reference Genes, RT-qPCR, Genetics of Osteoporosis, Gene Expression Analysis.

1. Introduction

Osteoporosis (OP) is a multifactorial and complex-traits disease, characterized by low bone mineral density (BMD) and loss of tissue microarchitecture (Clark and Duncan 2015; Rocha-Braz and Ferraz-de-Souza 2016). The disease is influenced by genetic factors around 50-85% of cases, especially in early or idiopathic osteoporosis (Riancho and Hernández 2012b; Karasik et al. 2016; Rocha-Braz and Ferraz-de-Souza 2016). The occurrence of fractures, the most important clinical manifestation of OP, shows heritability estimated in the range of 25-68%, presenting the highest values for younger patients and fractures that occur before 70 years old (Albagha and Ralston 2006; Rocha-Braz and Ferraz-de-Souza 2016). Recent Genome-Wide Association Studies (GWAS) already have identified over 60 genes associated with BMD variations and 14 genes related to fractures risk, however these discoveries remain to be further functionally validated (Clark and Duncan 2015; Karasik et al. 2016).

Gene expression analyses performed by quantitative real-time polymerase chain reaction (qPCR) is a sensitive, accurate and commonly used approach in molecular biological studies (Wang et al. 2015b; Wang et al. 2016). In 2009, in view of resolving technical challenges and to standardize qPCR experiments, the Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) guidelines was published (Bustin et al. 2009). One of the points highlighted by those guidelines were the need for validating reference genes for specific tissues, thereby ensuring the efficiency and quality of gene expression assays (Bustin et al. 2009). Regardless of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Beta-actin (ACTB) being traditionally used as reference genes, it is necessary to choose constitutive and stably expressed genes for different tissues and different experimental conditions (Wang et al. 2015b; Normann et al. 2016).

In spite of the efforts of Genetic Markers for Osteoporosis (GENOMOS) and Genetic Factors of Osteoporosis (GEFOS) consortiums to clarify the genetic factors involving in OP development, to our knowledge, none of the studies and efforts brought out specific reference genes in OP gene expression assays were performed up until now. Therefore, we conducted an evaluation of 10 candidate reference genes commonly used in previous studies of rheumatic diseases to establish a set of specific and validated reference genes for expression analysis in OP-related genes contributing to the advance in the field of bone research.

2. Material and Methods

2.1. Subjects

Peripheral blood samples of ten osteoporosis patients diagnosed according WHO criteria and ten healthy controls in postmenopausal period and without osteometabolic and/or inflammatory and autoimmune diseases and cancer were selected for this study. The present study was approved by the Research Ethics Committee of the Center for Health Sciences, Federal University of Pernambuco (CEP/CCS/UFPE nº 513/11), performed according the Declaration of Helsinki and all the participants who accepted to participate of this research provided a written informed consent.

2.2. RNA Extraction and cDNA Synthesis

Total RNA was extracted by TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The integrity of RNA was verified by 1.5% agarose gel electrophoresis and the quantification and RNA quality was checked by Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc, Delaware, USA). The samples that showed integrity of 23S and 16S fragments and the absorbance ratio OD260/280 values from 1.8 to 2.0 were considered able to proceed to the analysis. The cDNA synthesis was performed from

each RNA sample (500 ng) using GoScript™ Reverse Transcription System (Promega, USA) following the manufacturer's instructions.

2.3. Selection of Candidate Reference Genes and PCR Primer Design

The selection of candidate reference genes was performed by using panels of the mainly used reference genes from researchers in humans (Applied Biosystems 2008; Roche Applied Science 2009). Besides, reference genes commonly used in rheumatic diseases were added to the analysis. A total of 10 genes were selected: glucose-6-phosphate dehydrogenase (G6PD), β2-microglobulin (B2M), β-glucuronidase (GUSB), heat shock protein 90 (HSP90), elongation factor 1 alpha (EF1A), ribosomal protein P0 (RPLP0), GAPDH, ACTB, 18S ribosomal RNA (18S) and hypoxanthine phosphoribosyltransferase 1 (HPRT1). All gene sequences were obtained from GenBank (www.ncbi.nlm.nih.gov/genbank/) and all primers were designed by NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). For ACTB and 18S were used Taqman ® probes. Sequence details are showed in Table 1. The optimal annealing temperature and amplicon size were tested by gradient PCR and 1.5% agarose gel electrophoresis.

2.4. Amplification Efficiency Testing

The efficiency value for each primer (E) was determined by slopes of standard curves from five 10-fold serial dilution points for each cDNA sample. The acceptable values were defined as between 95% and 105%. These data and the correlation coefficients (R^2) were calculated by ABI 7500 system software. For ACTB and 18S (Taqman® probes), it was considered a reaction efficiency of 100% ensured by manufactures' information.

2.5. Quantitative Real-Time PCR Assay

The qPCR was performed in the ABI 7500 (Applied Biosystems, Foster City, CA, USA) using 1 µL diluted cDNA (1:5) from cDNA synthesis previous experiments , 10 µM of

each primer, 5 μL SYBR® Green Master Mix (1X) (Thermo Fischer Scientific; former Savant, MA, USA) and ultrapure water in sufficient quantity to a final volume of 10 μL. The Taqman® assays were performed following the manufactures' instructions. PCR amplifications were performed in technical triplicate and negative controls without templates were added for each reaction. Thermo cycling conditions used were: initial denaturing at 50ºC for 2 min and 95°C for 10 minutes followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. The melting curve was analyzed to confirm the specificities of the amplification reactions.

2.6. Data analysis of gene expression stability

Three statistical algorithms from Excel based free software packages were used to evaluate the expression stability of 10 candidate reference genes to OP: GeNorm (Version v3.5) (Vandesompele et al. 2002), NormFinder (Version 20) (Andersen et al. 2004) and BestKeeper (Version 1) (Pfaffl et al. 2004). Additionally, the web-based online tool RefFinder (Xie et al. 2012) and GeNorm were used to assist in the ranking and to calculate the optimal combination and minimal number of the candidate reference genes, respectively. The relative quantities used to GeNorm and Normfinder input were calculated and corrected to amplifications efficiencies according the equation suggested by Spiegelaere et al. (2015): $RQ = E^{-(minCq-sampleCq)}$.

2.6.1. GeNorm

The GeNorm uses the geometric means to determine gene expression normalization factor and stability value (M) for each gene (Vandesompele et al. 2002). M values less than 1.5 are acceptable and the lowest values are considered most stable. Then, the software performs a pairwise comparison (Vn/n+1) adding genes, one by one, in order to set the most stable reference genes until a cut-off less than 0.15 for determining the minimal number of the

candidate reference genes (Vandesompele et al. 2002; Liu et al. 2012; Wang et al. 2015b; Normann et al. 2016).

2.6.2. NormFinder

The NormFinder uses the standard curve or the delta-Ct method from the transformation of Ct values in a linear scale (Andersen et al. 2004). The program estimates the expression variation of the candidate genes, providing a stability value (M) for each gene, and the variation between sample subgroups of the sample e.g. patient and healthy groups (Andersen et al. 2004). It is required at least 8 sample per analysis and the algorithm recommends the best combination of two reference genes, providing the M values of this combination. Similar to GeNorm, the lowest M values are considered most stable (Andersen et al. 2004; De Spiegelaere et al. 2015; Normann et al. 2016).

2.6.3. BestKeeper

The BestKeeper uses the geometric mean of the candidate reference genes and the software provides a correlation coefficient (*r*) of each gene besides to calculate standard deviation (SD) and coefficient of variation (CV) from the samples Ct-values (Pfaffl et al. 2004). It is recommended SD to be [± CP] < 1, SD [± x-fold] <2 and correlation coefficient to be as higher as possible, close to the value 1 (Pfaffl et al. 2004; Liu et al. 2012). Low SD and CV associated to high correlation coefficient (*r*) values indicate a stable reference gene, however the program does not perform a ranking order of the analyzed genes (Pfaffl et al. 2004; Normann et al. 2016). Spiegelaere et al. (2015) highlighted that correlation coefficient (*r*) is a better parameter to assess the most stable genes than the standard deviation because the first one is able to analyze the correlation of each gene with the BestKeeper Index from the geometric mean from the studied reference genes. Thus we chose this measurement to evaluate and to perform the rank of reference genes in BestKeeper analysis.

2.6.4. RefFinder

The RefFinder is a web-based platform (http://fulxie.0fees.us/?type=reference) which integrates the three software packages abovementioned and additionally performs the comparative ΔΔCt method (Xie et al. 2012; De Spiegelaere et al. 2015; Ma et al. 2015). The RefFinder only uses Cq to perform the reference genes rank through of geometric mean values (GM), without any possibility to include PCR efficiency as the Genorm and NormFinder (De Spiegelaere et al. 2015). Due to the comparison among algorithms to be impossible, the RefFinder platform was used as complementary tool to assess reference gene stability and its result interpreted from particularities of each program.

2.7. Reference and target genes analysis

After the determination of optimal combination and minimal number of the candidate reference genes by software analysis, the most stable combination and the recommended candidate reference genes were used to perform the normalization factor from geometric media and ΔΔCt analysis. To validate these potential reference genes, the IFNG was used as target gene (Table 1, SM1). This gene codes the interferon gamma protein recognized to playing an essential role in bone environmental homeostasis.

3. Results

3.1. Specificity and amplification efficiencies

The specificity of the primers was verified by using agarose gel electrophoresis, in which a single band was detected, followed by a single peak in the melting curve from qPCR analysis. The melting curves of all tested primers are showed in Supplementary material 1. The R^2 and E values of the 10 candidate reference genes ranged from 0.999 – 1.000 and 95.12% - 104.79%, respectively (Table 1). These values were in accordance to the MIQE guidelines instructions.

Table 1. Primers and PCR efficiencies of the candidate reference and target genes used for OP samples.

Name	Gene	Protein function	Primer sequences / Taqman ® probe reference	Product size/bp	R^2	E/%
G6PD	Glucose-6-phosphate 1-dehydrogenase	fatty acid and nucleic acid synthesis	F: CCGTGATGAGAAGGTCAAGGT R: TACTGGCCCAGGACCACATT	72	0.999	95.12
B2M	Beta-2-microglobulin	small subunit of the MHC1	F: TGAGTGGCATGAAGAAGGTGT R: GGCAGTTCTTTGCCCTCTCT	77	0.999	104.79
GUSB	Beta-glucuronidase	degradation of dermatan and keratan sulfates	F: CACTGTGGCTGTCACCAAGA R: TCCGCATCCTCATGCTTGTT	84	1	100.51
HSP90	Heat shock protein HSP 90-beta	regulation of proteins in cell cycle control and signal transduction.	F: GCCTACTTGGTGGCAGAGAA R: CAGCAGAAGACTCCCAAGCA	79	0.999	98.38
EF1A	Elongation factor 1-alpha 1	protein synthesis	F: GAGGCTGCTGAGATGGGAAA R: CGTTCACGCTCAGCTTTCAG	74	1	102.37
RPLP0	60S acidic ribosomal protein P0	ribosomal protein lateral stalk subunit P0	F: GCGACCTGGAAGTCCAACTA R: TCTGCTTGGAGCCCACATTG	100	0.999	102.02
GAPDH	Glyceraldehyde- 3-phosphate dehydrogenase	glycolytic enzyme	F: CTGATGCCCCCATGTTCGT R: GCAGGAGGCATTGCTGATGA	80	0.999	96.81
ACTB	Actin, cytoplasmic 1	cytoskeleton	Hs 99999903_m1	171	1	100
18S	18S ribosomal	ribosomal subunit	Hs 03003631_g1	187	1	100
HPRT1	Hypoxanthine-guanine phosphoribosyltransfe-rase	generation of purine nucleotides	F: ACAGGACTGAACGTCTTGCT R: GAGCACACAGAGGGCTACAA	74	0.99	101.43
IFNG	Interferon gamma	immunoregulation	F: TCCAAGTGATGGCTGAACTGT R: TCGACCTCGAAACAGCATCT	77	1	99.45

3.2. Expression profile of candidate reference genes

The quantification cycle (Cq) was used to determine the expression level of candidate genes (Table 2). Among the OP patients group, the EF1A exhibited the highest expression levels (ranging from 18.620 to 18.908 Cq values) while HSP90 exhibited the highest expression levels in healthy control group (ranging from 22.087 to 24.254 Cq values). In both groups, ACTB gene showed the lowest expression levels. Regarding to standard deviations (SD) G6PD exhibited the lowest values to OP group (ranging from 0.874 to 6.233 SD values), while HPRT1 exhibited the lowest values to healthy control group (ranging from 0.324 to

2.585) SD values. ACTB gene showed the highest SD values to both groups. Cq values and their variations can be explored in details in Figure 1.

Table 2. Ct values means of the candidate reference genes expressed in OP patients and healthy group.

	G6PD	B2M	GUSB	HSP90	EF1A	RPLP0	GAPDH	ACTB	18S	HPRT1
OP Patients	30.110	23.160	22.628	21.246	18.991	21.769	21.361	30.567	21.877	23.798
Healthy Control	33.741	27.142	26.305	22.871	23.639	25.732	25.320	37.285	25.656	28.868

Regarding to standard deviations (SD) G6PD exhibited the lowest values to OP group (ranging from 0.874 to 6.233 SD values), while HPRT1 exhibited the lowest values to healthy

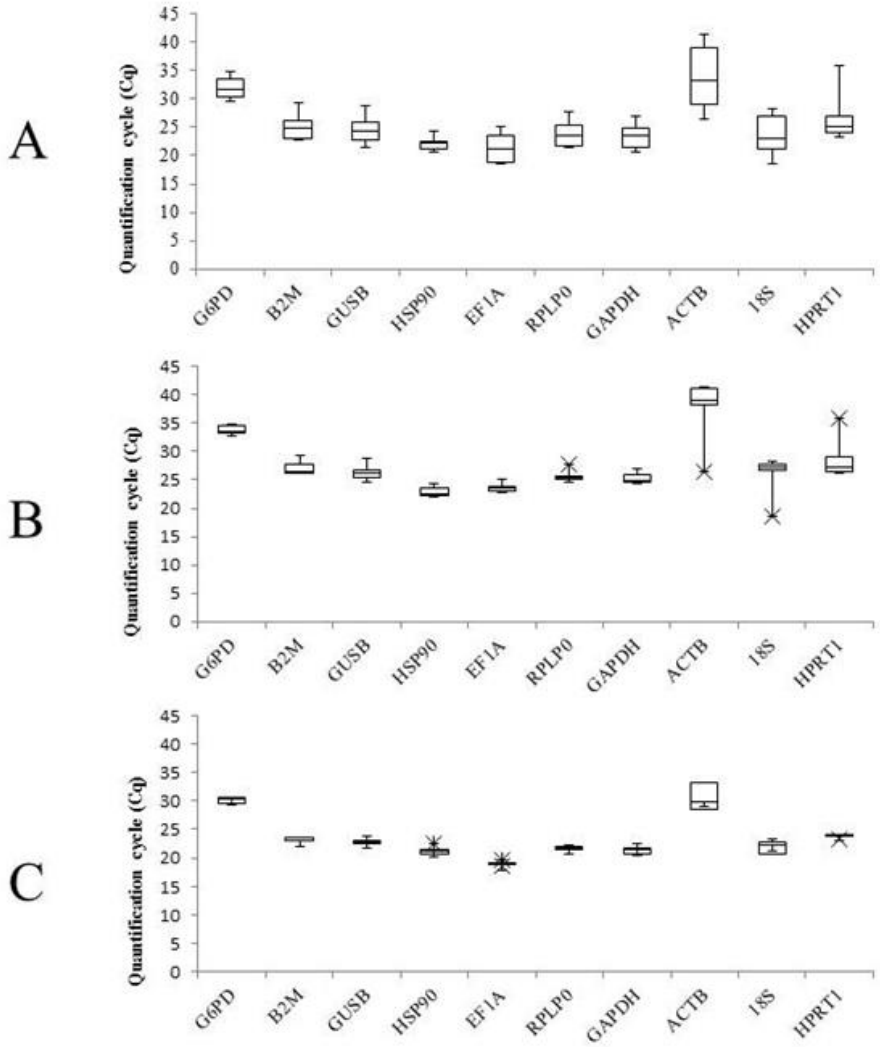


Figure 1. Expression levels of the candidate reference genes from all samples (A), healthy group (B) and patients' group (C) presented as the Cq mean. The boxes show the medians values (lines across the boxes), the one-quarter (Q1) and the three-quarters (Q3) and the whisker caps indicating the minimum and maximum Cq values. The (X) represent the outliers values.

control group (ranging from 0.324 to 2.585) SD values. ACTB gene showed the highest SD values to both groups. Cq values and their variations can be explored in details in Figure 1.

3.3. GeNorm analysis

In accordance to GeNorm ranking generated from M values, RPLP0 and B2M were the most stable reference genes (M = 0.823) for the present study (Figure 2).

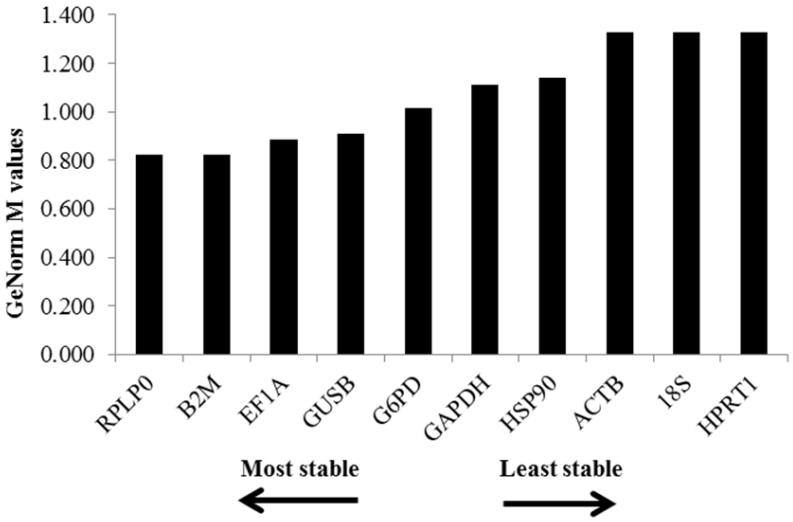


Figure 2. Candidate reference gene stability analyzed using GeNorm. Low M values predicts high stability while high M values indicate low stability

The pairwise variations (Vn/n+1) showed values below the cut-off (0.15) to V2/V3, V3/V4, V4/V5, V5/V6, V6/V7 and V9/V10 values (Figure 3).

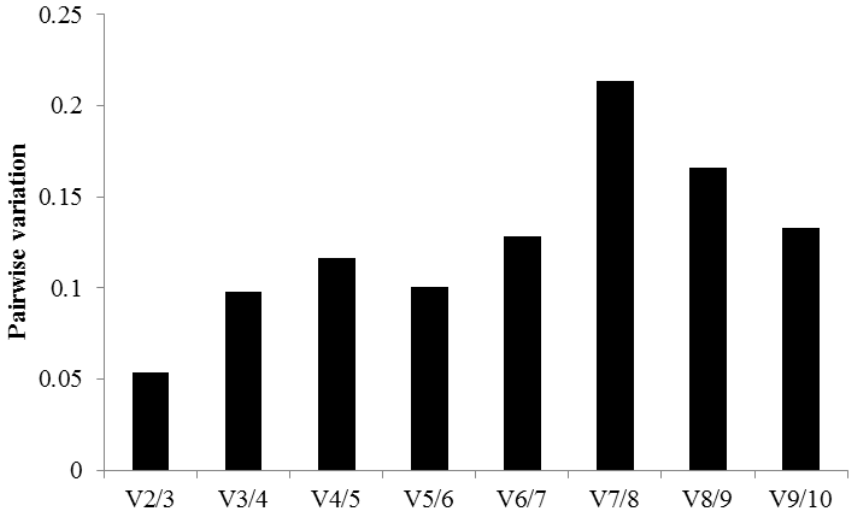


Figure 3. Pairwise variation (Vn/Vn+1) to determine the optimal number of reference genes required for accurate normalization by GeNorm. In this OP study, the pairwise variation value less than the cut-off (0.15) is reached with two reference genes.

Among these, V2/V3 exhibited the lowest pairwise value (0.053). Thus, the minimal number of reference genes calculated by the algorithm and recommended to obtain a reliable normalization was two. In the present study, these genes are RPLP0 and B2M. The ACTB, HPRT1 and 18S exhibited the highest M values (M = 1.329).

3.4. NormFinder analysis

The NormFinder software generated a ranking, in which RPLP0 (M = 0.460) exhibited the lowest variation values, being considered the most stably expressed gene, followed by EF1A (M = 0.599) (Figure 4).

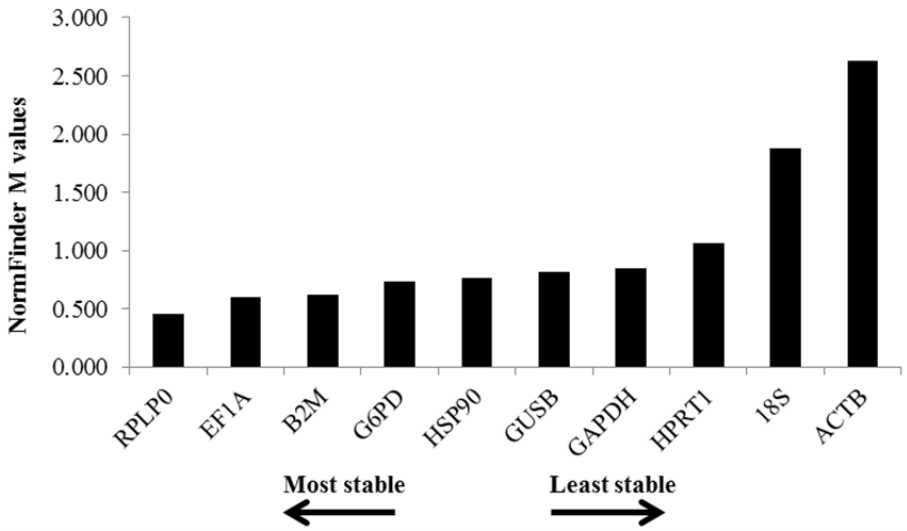


Figure 4. Candidate reference gene stability analyzed using NormFinder. Low M values predicts higher stability.

The most stable combination of genes was between both genes abovementioned (M = 0.562). The candidate reference genes that showed the highest variation values were ACTB (M = 2.631), 18S (M = 1.874) and HPRT1 (M = 1.065) (Figure 4).

3.5. BestKeeper analysis

Based on SD, HSP90 showed the lowest variation value (SD [± CP] = 0.91; SD [± x-fold] = 1.87), while ACTB exhibited de highest variation value (SD [± CP] = 4.87; SD [± x-fold] = 29.15). Based on the correlation coefficient (*r*), the better reference gene was RPLP0

(r = 0.987; *p* = 0.001) followed by EF1A (*r* = 0.975; *p* = 0.001). The lowest correlation coefficient (r) was observed to HPRT1 (*r* = 0.606; *p* = 0.064) followed by ACTB (r = 0.695; *p* = 0.026) and 18S (*r* = 0.721; *p* = 0.019) (Figure 5). When both the correlation coefficient (*r)* and the SD were considered, the HSP90 was not one of the top-ranked genes due to its lowest correlation coefficient (*r* = 0.797; p = 0.006).

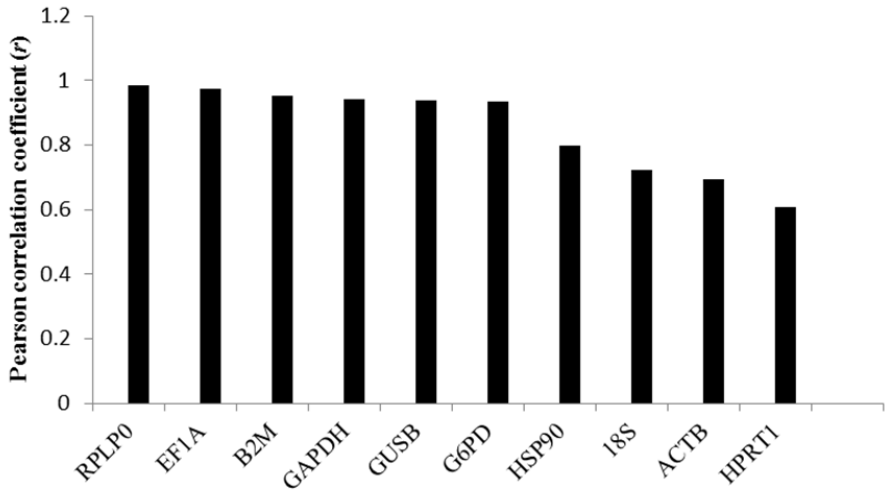


Figure 5. Candidate reference gene stability analyzed using RefFinder. High Pearson correlation coefficient (r)

3.6. RefFinder analysis

The ranking performed by RefFinder and by other analyses are showed in Table 3. According to RefFinder algorithm, RPLP0 (GM = 1.495) followed by B2M (GM = 2.449) were the better reference genes for OP studies. Similarly to other utilized analyses the ACTB (GM = 10), HPRT1 (GM = 8.485) and 18S (GM = 8.485) were the least stable reference genes. In spite of the final ranking being similar to other studied algorithms, the individual results performed by RefFinder were substantially different from the original output provided by each program (SM1).

Table 3: Reference genes rank from GeNorm, NormFinder, BestKeeper and RefFinder

GeNorm	NormFinder	Bestkeeper	RefFinder
RPLPO/B2M	RPLPO	RPLPO	RPLPO
EF1A	EF1A	EF1A	B2M
GUSB	B2M	B2M	G6PD
G6PD	G6PD	GAPDH	EF1A
GAPDH	GUSB	GUSB	GUSB
HSP90	HSP90	G6PD	HSP90
ACTB/HPRT1/18S	HPRT1	HSP90	GAPDH
	18S	18S	HPRT1
	ACTB	ACTB	18S
		HPRT1	ACTB

3.7. Validation of candidate reference genes

In accordance to results from GeNorm analysis, two reference genes are enough to perform the studies in OP. Thus, we chose the two most stable genes (RPLP0 and B2M; RPLP0 and EF1A) to compare with the least stable (ACTB, 18S and HPRT1) genes through IFNG expression analysis. When RPLP0 and B2M were used for normalization, the analysis showed that IFN-γ was up-regulated (5.06 fold change) in OP patients than healthy control group. When RPLP0 and EF1A were used for normalization, the analysis showed that IFN-γ was up-regulated (4.02 fold change) in OP patients than healthy control group. On the other hand, when ACTB, 18S and HPRT1 were used, the IFN-γ was up-regulated by only 1.23 fold change (FC) in OP patients than control healthy group. In the analysis for each gene individually, the group of patients presented higher expression of IFN-γ than healthy control group when using the following reference genes: RPLP0 (5.09 FC), B2M (5.02 FC), EF1A (3.17 FC) and 18S (5.79 FC). Conversely, when using either ACTB or HPRT1 as reference gene individually, the expression of IFN-γ was lower in the group of patients than in control group (-1.32 and -2.29 FC, respectively) (Figure 6).

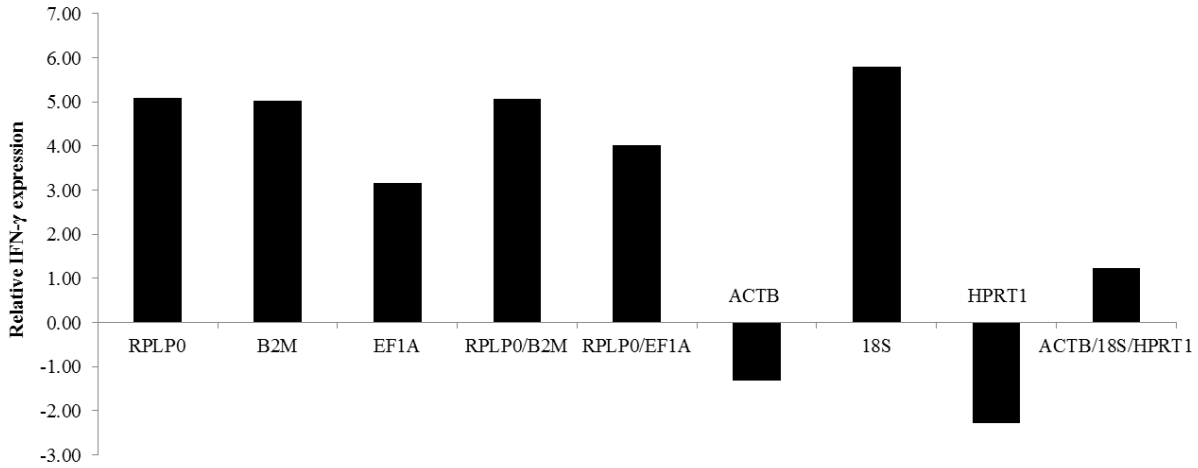


Figure 6. Relative quantification of IFN-γ expression using the most (RPLP0, B2M and EF1A) and the least (ACTB, 18S and HPRT1) reference genes for normalization.

4. Discussion

In this study, we investigated the expression stability of the 10 most commonly used reference genes in rheumatic diseases using four statistical algorithms. To our knowledge, this is the first study to select the optimal reference genes for reliable expression analysis in OP. The softwares used were GeNorm, NormFinder, BestKeeper and RefFinder, which are recognized algorithms for reference gene evaluation.

In spite of some differences exhibited among the software's ranking from the four used programs, the results are relatively similar, especially in relation to the most and the least stable reference genes suggested. RPLP0 was the most stable reference gene according to all used algorithms, followed by B2M (GeNorm and RefFinder) or EF1A (NormFinder and BestKepper). In accordance to the ranking abovementioned, the GeNorm showed a small difference in the most stable reference genes ranking in relation to NormFinder and BestKeeper. The latest two programs are recognized to generate more reliable data as they are less sensitive towards co-regulation and differences among the primers efficiencies (De Spiegelaere et al. 2015; Normann et al. 2016). However, in our study the relative quantitation were corrected by each primer efficiency, additionally the candidate reference genes belonged

to different functional groups, which helped to correct this deficiency from GeNorm. Similarly, the RefFinder also showed in its final rank RPLP0 and B2M as the best combination of reference genes. However the calculated ranking by RefFinder to each program were very discrepant in relation to original results calculated by programs individually, which probably occurred due to the fact that the program uses raw Cq values and did not accept corrections to reactions efficiencies (SM2) (De Spiegelaere et al. 2015). Because of that we do not recommend this software as a sole tool in order to validate reference genes as the output may be biased. On the other hand, the ΔΔCt and normalization analysis showed more similar values between RPLP0 and B2M than RPLP0 and EF1A. Thus, based in GeNorm output and these last two analyses, we suggest the combination RPLP0 and B2M as the most stable for OP gene expression studies.

RPLP0 encodes one out of approximately 80 ribossomal proteins in human, which are involved in protein synthesis and induction of proliferation and apoptosis processes (Artero-Castro et al. 2011). Previously studies already have demonstrated RPLP0 as one of the most stable reference genes among a set of candidate genes in inflammatory bowel disease (Bamias et al. 2013), ovarian (Kolkova et al. 2013) and gastrointestinal stromal tumors (Fassunke et al. 2010). In addition, Ragni et al. (2013) classified the RPLP0 in the most stable reference gene in assays of mesenchymal stem cell differentiation, the precursor cells of osteoblasts. The second most stable gene, B2M, encodes β2-microglobulin, which has not been cited by GWAS as a potential gene involved in OP, however, previous studies related its association with bone metabolism in tumor processes (Josson et al. 2011; Hsu and Kiel 2012; Levy et al. 2015). In spite of that, similarly to our study, Stephens et al. (2011) and Li et al. (2015) showed B2M as the one of the most stable reference genes in expression analysis in mouse bone cells and mesenchymal stem cells cultures, respectively. It appears that the relation

between bone system and B2M variation is more related to cancer cells than health bone ones (Josson et al. 2011; Rienzo et al. 2013).

In contrast, for the low stability analysis, all the programs showed the ACTB, HPRT1 and 18S as the three least stable reference genes. ACTB, as well as GAPDH, are used as internal controls in more than 70% of expression analyses performed by RT-qPCR (Wang et al. 2015b; Klenke et al. 2016). However, due to the very low stability in all used programs, ACTB was not recommended as reference gene for our analysis. ACTB gene encodes for β-actin, one of six different actin isoforms in vertebrates and ubiquitously expressed in cell cytoplasm (Rubenstein 1990; Bunnell et al. 2011). Actins compose the cytoskeleton, which plays critical roles in cell motility, structure, and integrity, besides to act in the regulation of gene expression (Bunnell et al. 2011). Tai et al. (2015) showed for the first time that the factor osteoinductive simvastatin acts in the bone regeneration by increasing actin filament organization and cell rigidity. Similarly, Elsafadi et al. (2016) related in regenerative medicine studies the involvement of distribution of the actin filament and changes in cytoskeletal organization in the osteoblastic and adipocytic differentiation of stem cells (hMSC) *in vitro*. This new approach of actin beta actin mechanism is a possible explanation to variation of ACTB gene expression in OP samples, as the disease is strictly related to the balance between bone formation and resorption.

HPRT1 gene encodes for hypoxanthine-guanine phosphoribosyl transferase (HPRT), recognized by transferase activity, able to catalyze purine bases guanine and hypoxanthine into their respective monophosphate nucleoside (Kelley and Andersson 2014). In spite of being acceptably stable in osteoblasts and osteoclasts (Stephens et al. 2011) and osteosarcoma (Rienzo et al. 2013) cell culture studies, Yan et al. (2015) suggested that HPRT1 might regulate bone metabolism. This study suggested that this protein might be involved in the

development of osteoporosis though the process of the transferase activity, which may contribute to the generation of free radical species and oxidative stress affecting the bone metabolism. Additionally, Isomura et al. (Isomura et al. 2004) also showed in postmenopausal rats studies that oxidative stress could be involved in the OP pathogenesis.

18S gene encodes for ribosome 18S rRNA subunit and to our knowledge there is no study report of alteration in expression levels of this gene or its protein in bone cells or diseases related to changes of bone mineral density. Nevertheless, studies have described several difficulties associated, for instance, with its expression levels being higher than the target gene (Paolacci et al. 2009; Kozera and Rapacz 2013) and also the regulation of their transcription by biological and chemical agents (Nicot et al. 2005). These alterations, similarly to the other candidate genes in our analysis, are not in accordance to criteria of constant level of the expression and the no influence by environmental factors required for an acceptable reference gene (Kozera and Rapacz 2013). Besides, Ragni et al. (2013) and Stephens et al. (2011) considered the 18S inadequate for normalization in osteogenic and chondrogenic differentiation and mouse osteoblasts and osteoclasts analysis, respectively.

The impact of using these reference genes might be visualized in the expression levels of the target gene, which ranged greatly when different reference genes combinations were used. The ACTB and HPRT1 showed that IFN-γ was down regulated in OP patients group, while according to all the other analyzed genes the IFN-γ was up regulated in the same group. These results highlight the importance of the validation of reference genes to specific studies and diseases and showed the aggravating effect of ACTB to be used in most expression analysis, which suggests that its validation has been often disregarded in expression studies (Bustin et al. 2009; Wang et al. 2015b). The qPCR is a technique recognized by high sensitivity and sequence-specificity, however, the conclusions about the mRNA expression

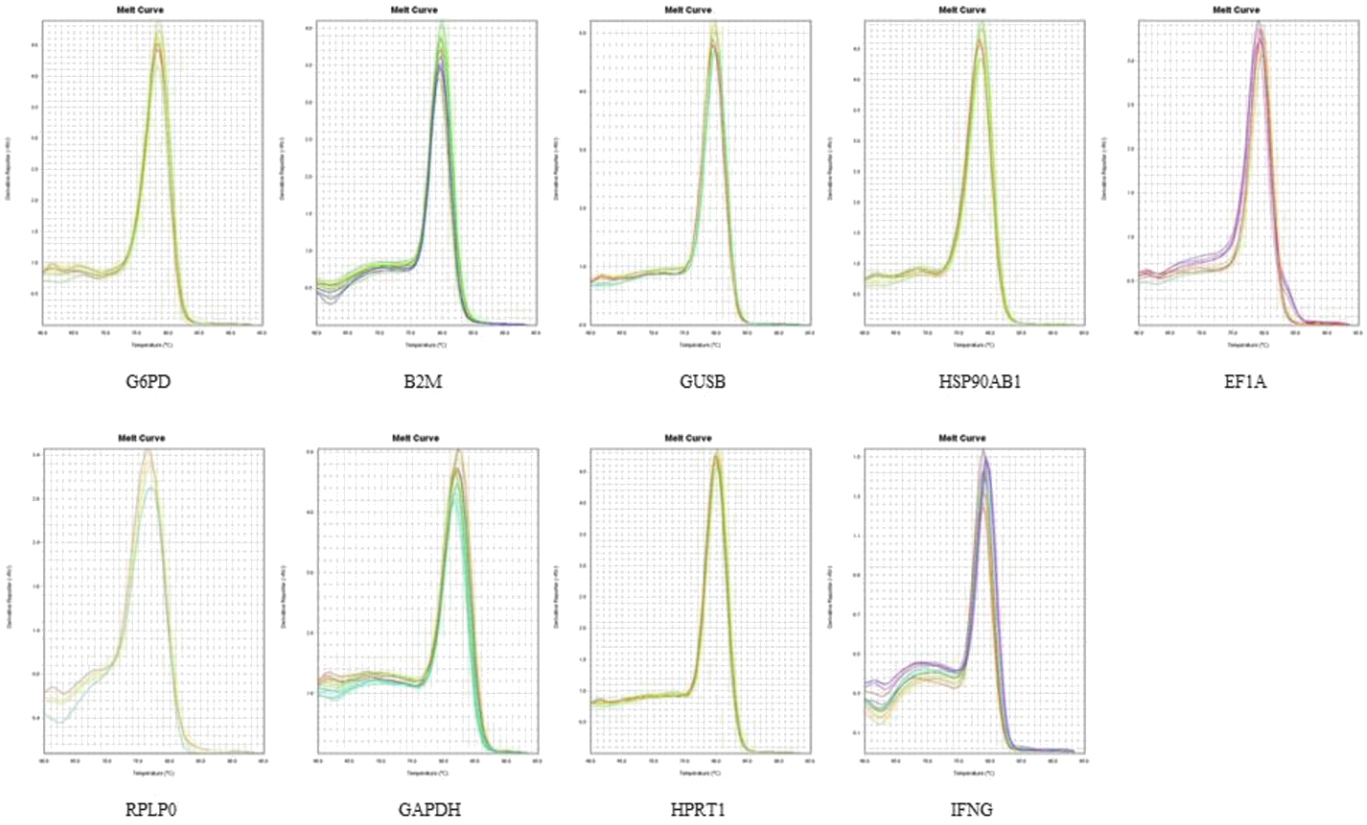
analysis may be performed only after the appropriate reference gene selection (Wong and Medrano 2005; Bustin et al. 2009; Klenke et al. 2016).

Therefore, according to our results, we recommend the use of RPLP0 and B2M as the most stable reference genes to OP studies, as we showed their lower variation impact and influence on the evaluation of a target gene expression. On the other hand, we do not recommend the use of the least stable reference genes (ACTB, HPRT1 and 18S) in OP expression assays. Finally, we emphasize a carefully choice of software packages used in reference gene selection.

Acknowledgments

We thank the following Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco) for financial support.

Supplementary Material



SM.1. Melting curves of the candidate reference and target gene.

SM.2. Ranking Order (Better--Good--Average) showing the individual values of each software calculated by ReFinder

Method	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Delta CT	RPLP0	B2M	GUSB	EF1A	G6PD	GAPDH	HSP90	18S	HPRT1	ACTB
BestKeeper	HSP90	G6PD	RPLP0	GUSB	GAPDH	B2M	EF1A	HPRT1	18S	ACTB
NormFinder	B2M	RPLP0	EF1A	GAPDH	GUSB	G6PD	HSP90	18S	HPRT1	ACTB
GeNorm	GUSB/ RPLP0		B2M	EF1A	G6PD	GAPDH	HSP90	HPRT1	18S	ACTB
Recommended comprehensive ranking	**RPLP0**	**B2M**	**GUSB**	**G6PD**	**EF1A**	**HSP90**	**GAPDH**	**HPRT1**	**18S**	**ACTB**

6. Capítulo III

Artigo a ser submetido na revista European Journal of Human Genetics

Fator de Impacto: 4,5

Guia para autores: Anexo A

Interferon-γ plays a role in postmenopausal osteoporosis and in human bone cells calcification: A genetic and functional study

LIMA, CAD[1,2]; SOUZA, APO[2]; SEABRA, MALB[2]; SILVA, HHSA[2]; GUARANÁ, W[2]; JAVORSKI, NR[1,2]; BARBOSA, AD[2,3]; LORENA, MVB[4]; SANTOS, PEA[2]; CROVELLA, S[1,2]; De AZEVÊDO SILVA, J[1,2]; SANDRIN-GARCIA; P[1,2].

1 – Department of Genetics - Federal University of Pernambuco (UFPE), Recife, Pernambuco, Brazil.

2 – Laboratory of Immunopathology Keizo Asami – Federal University of Pernambuco (LIKA - UFPE), Recife, Pernambuco, Brazil.

3 - Division of Rheumatology, Clinical Hospital, Federal University of Pernambuco (HC - UFPE), Recife, Pernambuco, Brazil.

4 - Aggeu Magalhães Research Center (CPqAM - Fiocruz) Department of Immunology, Pernambuco, Brazil.

*Corresponding author: Camilla AD Lima

Email address: camilladelima@gmail.com

To be submitted for publication in European Journal of Human Genetics

Abstract

Osteoporosis (OP) is a complex disease in which several genes from immune system are recognized to contribute in the susceptibility to disease's establishment. In spite of the efforts to be functionally validated, many of these genes, as interferon-γ (IFNG), remain with unrevealed mechanisms. The aim of the present study was to examine whether IFN-γ -1616 (G>A, rs2069705) polymorphism was associated to postmenopausal OP. A total of 366 patients were genotyped, followed by gene expression and IFN-γ levels measured. Furthermore, the IFN-γ action in calcifications and viability of osteoblast-line cells were analyzed in human bone osteosarcoma (SaOs-2) cell culture. We found that IFNG rs2069705 G allele is able to decrease the IFN-γ protein stability and thus increase risk for OP (OR = 1.45, 95% CI = (1.03 – 2.05), p = 0.03). Furthermore, IFN-γ stimulus in SaOs-2 cell culture seems to favor the calcification and viability in dose dependent manner. These findings suggested that IFNG rs2069705 G SNP may increase OP risk and IFN-γ might favor the bone formation. However, we suggest that further functional studies are required to confirm the action of this cytokine in osteoblasts-like cells.

Keywords: SNP, rs2069705, Bone environment, SaOs-2 cells.

1. Introduction

Osteoporosis (OP) is a multifactorial metabolic bone disease influenced by genetic factors in around 50 to 85% of all cases (Clark and Duncan 2015; Rocha-Braz and Ferraz-de-Souza 2016). Similarly to other complex diseases many genes, in a smaller effect, contribute to the overall phenotype such as bone mineral density (BMD) variation, fracture risk, bone turnover rate and bone geometry (Clark and Duncan 2015). In 2008, Genome-Wide Association Studies (GWAS) confirmed a close relationship between bone and immune system proposed in the basis of Osteoimmunology by Arron and Choi (2000), showing cytokines and its pathways involved in BMD, fractures and OP processes (Richards et al. 2008; Styrkarsdottir et al. 2008).

Although technological advances have revealed novel candidate genes and numerous polymorphisms associated to osteoporosis, biological function of many cytokines with a recognized role in the bone remodeling remain with unrevealed mechanisms (Karasik et al. 2016; Rocha-Braz and Ferraz-de-Souza 2016). Interferon-γ (IFN-γ), one of the main example, presents contradictory functions in bone environment (Takayanagi et al. 2005; Gao et al. 2007; Zhang et al. 2015) and its action as anabolic or catabolic agent yet raises many questions.

IFN-γ is recognized to influence in the survival, activation and differentiation of the osteoclasts by Tumor Necrosis Factor receptor-associated factor 6 (TRAF6) degradation and blockade of RANK/RANKL pathway (Arron and Choi 2000). On the other hand, previous studies showed that IFN-γ is able to promote the osteoclastogenesis by inducing specific cytokines and T cell activation (Gao et al. 2007; Yun gang et al. 2016). In spite of GWAS discoveries about immune systems and bone remodeling, a few functional studies with IFN-γ polymorphisms in osteoporosis were performed up to this date.

Thus, in the present study aimed to assess the role of IFN-γ by studying of IFN-γ -1616 (G>A, rs2069705) polymorphism, previously classified as a single nucleotide polymorphism (SNP) in the promoter region and its impact in the cytokine serum levels and consequent susceptibility to OP. Furthermore, the effects of this cytokine in the bone mineralization were assessed by calcifying human bone osteosarcoma (SaOs-2) cell line.

2. Materials and methods

2.1 Subjects

In the present study 366 patients from the Division of Rheumatology of Clinical Hospital in Federal University of Pernambuco, Brazil were analyzed. All the women were submitted to BMD measurements by dual energy X-ray absorptiometry (Hologic or Lunar) for OP diagnosis at lumbar spine (vertebrae L1-L4), femoral neck (FN) and total femur (TF). The women classified as osteoporotics according to World Health Organization criteria were included in patients group while the classified as healthy were included in healthy control group.

The patients group was evaluated for secondary diseases according health assessment questionnaire. Patients with osteopenia, inflammatory disease, autoimmune diseases, cancer, under the use of anti-inflammatories and other rheumatic diseases were excluded from this study. This study was approved by the Research Ethics Committee of the Center for Health Sciences, Federal University of Pernambuco (CEP/CCS/UFPE nº 513/11) and all the participants signed a written informed consent.

2.2 SNP prediction

SNP IFN-γ -1616 (G>A, rs2069705) have been cited in previous studies as localized in a promoter region (Kumar and Ghosh 2008; Kanchan et al. 2015; Leng et al. 2016). To

confirm this information we conducted a research in 1000 Genomes (http://browser.1000genomes.org/index.html) to analyze the SNP position. The Genome Reference Consortium (GRC) was used to analyze the SNP position in the CRCh38.p7 and CRCh37.p13 (for the 2016 and 2013 sequencing, respectively). Protein structure was analyzed by the forward orientation of the SNP (FwdSNP) (C>T, R160Q, rs201359065). The Three-Dimensional structure of the IFN-γ was downloaded from the Protein Database (RSCB-PDB, 1EKU). Finally Swiss-PdbViewer v4.1.0 (Guex, N. and Peitsch, M.C. 1997) software was used to visualize the protein structure, calculate their force field energy and its minimization energy caused by the polymorphism. The software is also capable to change a specific residue in FwdSNP, in this case, the residue of the FwdSNP, R160Q. More positive values indicate low stability.

2.3 DNA isolation and determination of IFN-γ genotypes

Genomic DNA extraction was performed from human peripheral blood monocyte cells (PBMCs) according to the rapid salting out method (Bignon and Viña 1995). Genotyping of extracted DNA were performed by Real-Time PCR ABI 7500 detection system (Applied Biosystems, Foster City, CA, USA) using specific fluorogenic probes (Taqman Probes, Applied Biosystems, Foster City, CA, USA).

2.4 RNA isolation and gene expression analysis

Total RNA from OP patients and healthy women was extracted from human PBMCs with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA integrity, concentration and purity were determined by agarose gel (1.5%) and Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc, Delaware, USA), respectively. Only

samples that exhibited 23S and 16S fragments and the absorbance ratio OD260/280 values from 1.9 to 2.0 were included in the analysis

cDNA synthesis was performed by GoScript™ Reverse Transcription System kit (Promega, USA) according to the manufacturer's instructions. β2-microglobulin (B2M), ribosomal protein P0 (RPLP0) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as reference genes to data normalization. Gene expression was determined, in triplicate, by Real-Time PCR ABI 7500 detection system (Applied Biosystems, Foster City, CA, USA) using specific primers designed by NCBI/Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) from gene sequences obtained in GenBank (www.ncbi.nlm.nih.gov/genbank/) (S1).

2.5 Measurement of IFN-γ serum levels from OP patients and Th1/Th2 cytokines from cell culture

IFN-γ serum levels from OP patients and healthy women as well Th1 and Th2 cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α) supernatants from SaOs-2 cell line were measured by Cytometric Bead Array (CBA) using CBA Human Th1/Th2 Kit, according to the manufacturer's protocol. All data were obtained in a Becton Dickinson FACScalibur flow cytometer and analyses were performed by BD CBA software (BD Biosciences, USA). The results are showed by mean intensity of fluorescence (MIF).

2.6 *In vitro* assays

SaOs-2 cell line derived from an osteosarcoma of 11-year old Caucasian female patient from the public tissue bank at the Federal University of Rio de Janeiro (UFRJ) and maintained in a humidified incubator at 37°C with 5% CO_2. This cell line were used as *in vitro* model for bone mineralization assay as it is recognized to calcify matrix *in vivo* besides expressing cytokines greatly similar to human osteoblastic cells (Querques F and Colella G

2015). For experiments, cells were cultured in 6-well plates and maintained for 15 days in hMSC Osteogenic Differentiation BulletKit™ Medium to induce calcification. The media changes were performed every 3 days.

Subsequently to this calcifying process, SaOs-2 cells confluent cultures were seeded at 2 $\times 10^5$ cells/well and then were treated in presence or absence of recombinant human IFN-γ (Peprotech, Germany). The maximum (1000 U/ml) and minimum (20 U/ml) concentrations were used based on Pedersen et al. (2015) (Pedersen et al. 2004) with modifications. After IFN-γ stimulation, calcification and viability analysis were performed with 24 hours, while the cytokines levels were measured after 6, 8 and 24 hours.

2.7Alizarin red staining and quantification

Calcified SaOs-2 cells were plated in 24-well plates and incubated at 37 ºC and 5 % CO_2 for 24 h in presence or absence of IFN-γ (20U/mL and 1000U/mL) for 24 h. Cells were stained with Alizarin Red (pH 4.2, Sigma cat# A5533) for 20 minutes then washed 5 times for 5 minutes with deionized water. 5% of Cetylpyridinium Chloride (Sigma cat# C0732) solution was used to dissolve the alizarin red bound to calcium deposits and their concentration was measured by using a spectrophotometer at a λ=570 nm wavelength (Varioskan Flash - Thermo Scientific, Rockford, IL,USA).

2.8 Cell viability assay

Calcified cells were stimulated for 24 h in presence of IFN-γ in 24-well plates (20U/mL and 1000U/mL). Mitochondrial activity assay was measured by methyl-tetrazolium bromide (MTT) according to Yao et al. (2017) (Yao et al. 2017). Solution of MTT reagent (Sigma Chemical Co, St Louis, Missouri) (5 mg/mL) was added and incubated at 37 ºC and 5 % CO_2 (under inspection of color change to brown). After 3 hours, the medium was removed

carefully and DMSO was used to dissolve all crystals. The plate was incubated for 5 min at 37ºC and 5% CO_2 and the absorbance was measured at λ=550 nm with a benchmark microplate reader (Dynex MRX Revelation Plate Reader, USA).

2.9 Statistical analysis

SNPStats tool (http://bioinfo.iconcologia.net/SNPstats) was used to perform the comparison between the allele and genotype frequencies of healthy controls and OP patients and to calculate the Hardy-Weinberg equilibrium. Student-test and analysis of variance (ANOVA) with Tukey test as post-test were performed to comparison among media values. These analyses were managed by SPSS software version 18.0. A p value less than 0.05 was considered statistically significant.

3. Results

3.1 SNP study

Using the CRCh38.p7 and CRCh37.p13 sequencing, available on 1000 genomes, we found that the FwdSNP are localized in exon 4 in the IFN-γ gene region. The Swiss-PdbViewer analysis showed that IFN-γ rs2069705 CC variant, corresponding to GG genotype, is able to alter the protein structure and to reduce the protein stability (Figure 1; Table 1). The results from minimization energy analysis confirmed this reduction in IFN-γ stability (Table 1).

3.2 Studied population's characteristics

From a total of 366 postmenopausal women selected to this study, 251 (age ± SD) (62.15 ±12.04) were classified as osteoporotic while 115 (61.22 ±5.13) were taken as healthy controls according to WHO criteria for OP diagnosis. The clinical and demographic characteristics of osteoporotic group are summarized in Table 2.

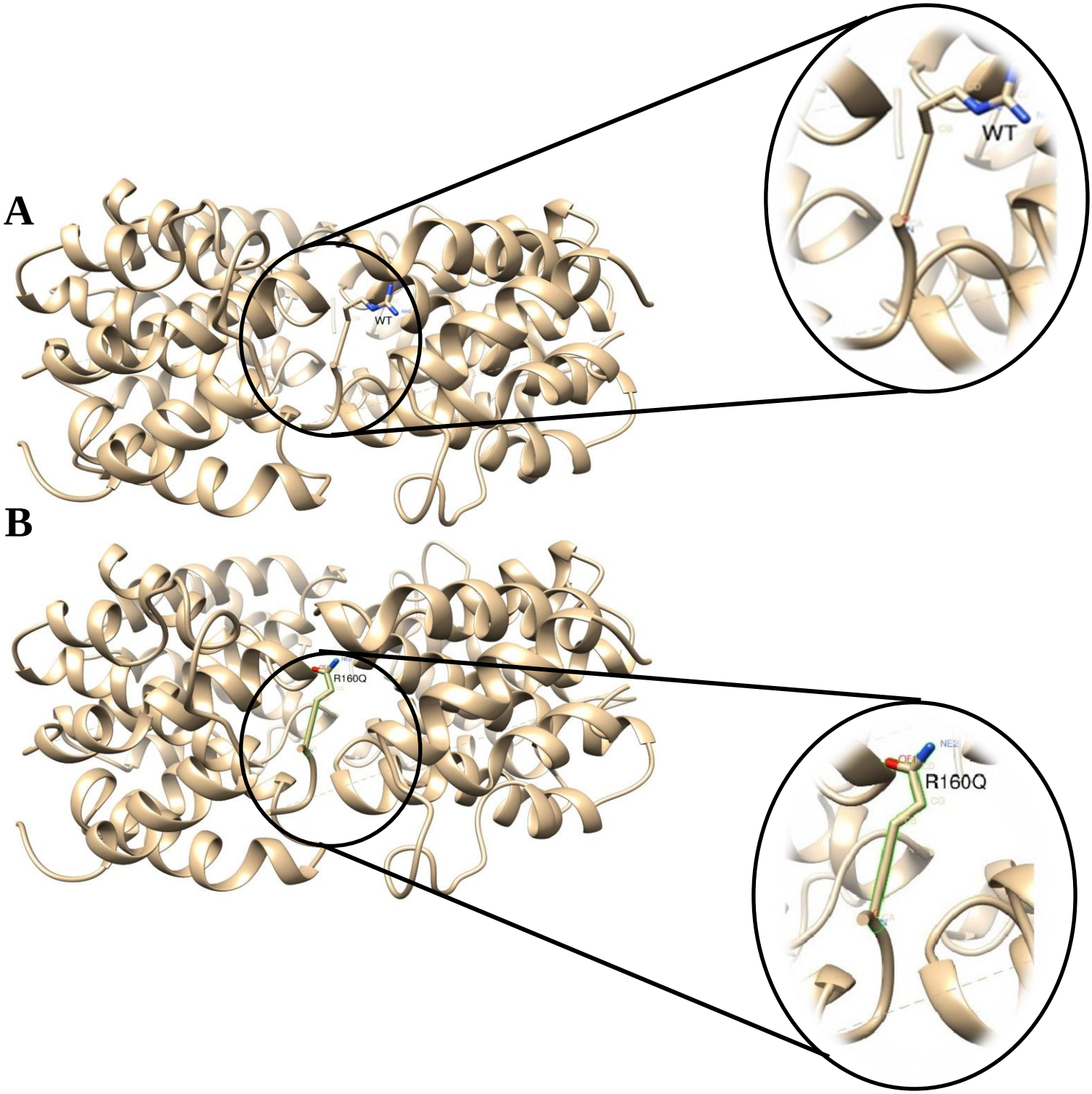


Figure 1. Change in IFN-γ structure caused by IFN-γ rs2069705 Forward SNP. A: IFN- IFN-γ rs2069705 AA protein. B: IFN-γ rs2069705 GG protein

Table 1. Force field Energies and minimization energies in IFN-γ rs2069705 AA (WT) and GG (R160Q).

Force Field Energies	Residue (KJ/mol)	System (KJ/mol)
IFN-γ (WT)	-168.583	-2003.34
IFN-γ (R160Q)	369.493	-1898.71
Minimization Energies	**Residue (KJ/mol)**	**System (KJ/mol)**
IFN-γ (WT)	-181.487	-28643.500
IFN-γ (R160Q)	-39.455	-27872.805

Table 2. Clinical and demographic characteristics of OP patients

Characteristics	OP patients N = 251
Age (years ±SD)	62.15 ± 12.04
Age at menarche (years ±SD)	13.59 ± 1.90
Age at menopause (years ±SD)	45.66 ± 6.81
Disease duration (years ±SD)	1.64 ± 2.30
BMI (kg/m^2 ±SD)	25.90 ± 4.38

Characteristics	Presence (%)	Absence (%)
Smoking	8.28	91.72
Alcoholism	5.94	94.06
Fragility facture	13.58	86.42
Family osteoporosis	38.58	61.41

OP, Osteoporosis; BMI, Body mass index

3.3 Distributions of IFN-γ genotypes and risk of osteoporosis

The genotype and allele frequencies of the IFN-γ -1616 (G>A, rs2069705) polymorphisms for all the studied groups are shown in Table 3. All genotype frequencies of OP patients and healthy control groups were found to be in the Hardy–Weinberg equilibrium. There were significant differences in the allele frequencies of IFN-γ rs2069705 between osteoporosis cases and healthy controls. Compared with the healthy control group, the OP patients exhibited a higher frequency of allele G, showing this allele significantly associated with increased risk for osteoporosis (OR = 1.45, 95% CI = (1.03 – 2.05), p = 0.03).

In spite of the OP patients group have showed higher IFN-γ rs2069705 GG genotype frequency than healthy control (22.4% vs 14.6%) only the dominant model exhibited a trend of statistically significant association (OR = 0.62, 95% CI = (0.38–1.01), p = 0.056) as shown in Table 3.

3.4 Expression levels of IFN-γ in OP patients and healthy controls

IFN-γ expression analyses using the normalization factor as quantification method showed cytokine levels increased significantly by 3.38 fold in OP patients when compared with healthy control group (p = 0.002). No statistically significant differences were found among studied genotypes and IFN-γ mRNA levels (Figure 2).

Table 3. Distribution of genotypes and allele frequencies of IFN-γ rs2069705 SNPs among OP patients and healthy

Model	Genotypes	OP n (%)	Controls n (%)	OR (95% CI)	p
Codominat	AA	65 (26.5)	38 (36.9)	1	
	GA	125 (51)	50 (48.5)	0.68 (0.41 – 1.15)	0.082
	GG	55 (22.4)	15 (14.6)	0.47 (0.23 – 0.94)	
Dominant	AA	65 (26.5)	38 (36.9)	1	0.056
	GA+GG	180 (73.5)	65 (63.1)	0.62 (0.38 – 1.01)	
Recessive	AA+GA	190 (77.5%)	88 (85.4%)	1	0.086
	GG	55 (22.4%)	15 (14.6%)	0.59 (0.32-1.10)	
Overdominant	AA+GG	120 (49)	53 (51.5)	1	
	GA	125 (51)	50 (48.5)	0.91 (0.57 – 1.44)	0.067
	Alleles				
	A	255 (52)	126 (61)	1	
	G	235 (48)	80 (39)	1.45 (1.03–2.05)	0.03

OP, Osteoporosis; p, pvalue; OR, odds ratio; CI confidence interval

3.5 Serum levels of IFN-γ in OP patients and healthy controls

Based on detected levels of IFN-γ, 52.5% of healthy controls were classified as positive to cytokine detection (> 0 pg/mL) and 32.35% of OP patients group exhibited detected levels of cytokine. Serum positive average (media (ranges)) cytokine levels was higher in healthy controls (2.12 (1.47 – 9.52) pg/mL) than OP patients group (1.03 (0.82 – 12.27) pg/mL) (Figure 3).

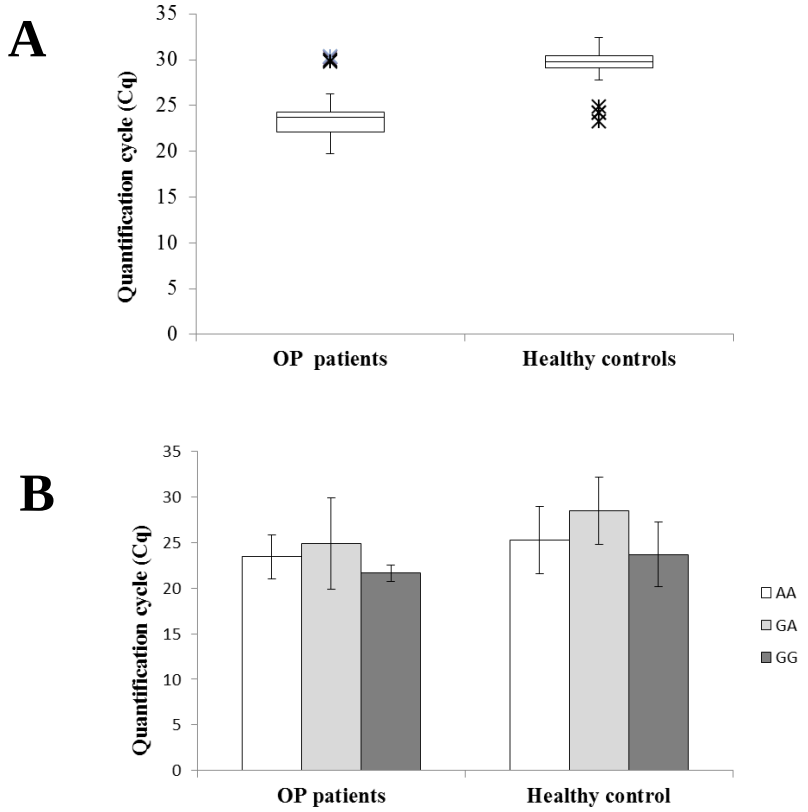


Figure 2. IFN-γ mRNA levels in OP patients healthy control group. A: Expression levels of IFN-γ in OP patients and healthy control group presented as the Cq mean. The boxes show the medians values (lines across the boxes), the one-quarter (Q1) and the three-quarters (Q3) and the whisker caps indicating the minimum and maximum Cq values. The (X) represent the outliers values. B: Correlations of IFN-γ mRNA levels with genotype in OP patients and healthy control group, presented as the mean ±SD.

In the OP group, the IFN-γ rs2069705 GG showed the highest levels (2.60 (2.00 – 12.27) pg/mL) compared with IFN-γ rs2069705 AA (1.80 (1.28 – 2.32) pg/mL) and IFN-γ rs2069705 GA (0.97 (0.61 – 5.81) pg/mL) (Figure 2). On the other hand, the healthy control group exhibited a reverse pattern compared to OP patients. IFN-γ rs2069705 AA showed the highest levels (5.51 (1.86 – 7.36) pg/mL) followed by IFN-γ rs206970 GA (3.64 (1.47 – 7.69) pg/mL) and IFN-γ rs206970 GG (2.78 (1.56 – 9.42) pg/mL) (Figure 3). In spite of all the observed differences among the IFN-γ levels and OP and healthy group and studied polymorphism, the values were not statistically significant.

3.6. Cytokines levels from Saos-2 cells after IFN-γ stimulation

Measurable levels of IL-2, IL-4, IL-6, IL-10, TNF-α cytokines were not detected in supernatants from SaOs-2 cells stimulated or unstimulated with IFN-γ. Thus, no changes were found between the different stimuli as well as time of treatment.

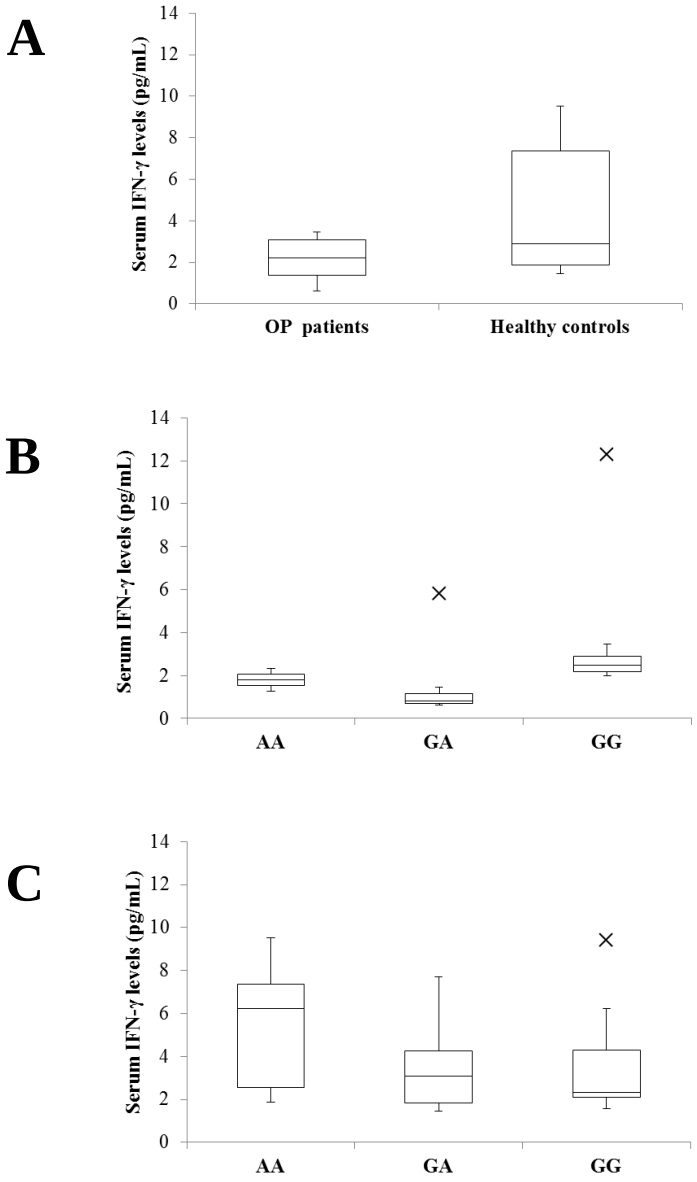


Figure 3. Serum IFN-γ levels in OP patients healthy control group. A: IFN-γ levels in serum from OP patients and healthy control group presented as mean (pg/mL). The boxes show the medians values (lines across the boxes), the one-quarter (Q1) and the three-quarters (Q3) and the whisker caps indicating the minimum and maximum Cq values. The (X) represent the outliers values. B: Correlations of serum IFN-γ levels with genotype in OP patients, presented as the mean ±SD. C: Correlations of serum IFN-γ levels with genotype in healthy control group, presented as the mean ±SD.

3.7 SaOs-2 calcification analyze

Calcification of stimulated or unstimulated cells was measured by Alizarin Red staining (Figure 4). IFN-γ increased calcification *in vitro* in both doses compared with unstimulated controls. Between stimulated group, 20 U/mL IFN-γ exhibited higher calcifications levels than 1000 U/mL IFN-γ (69.14% and 19.15%, greater than healthy control respectively).

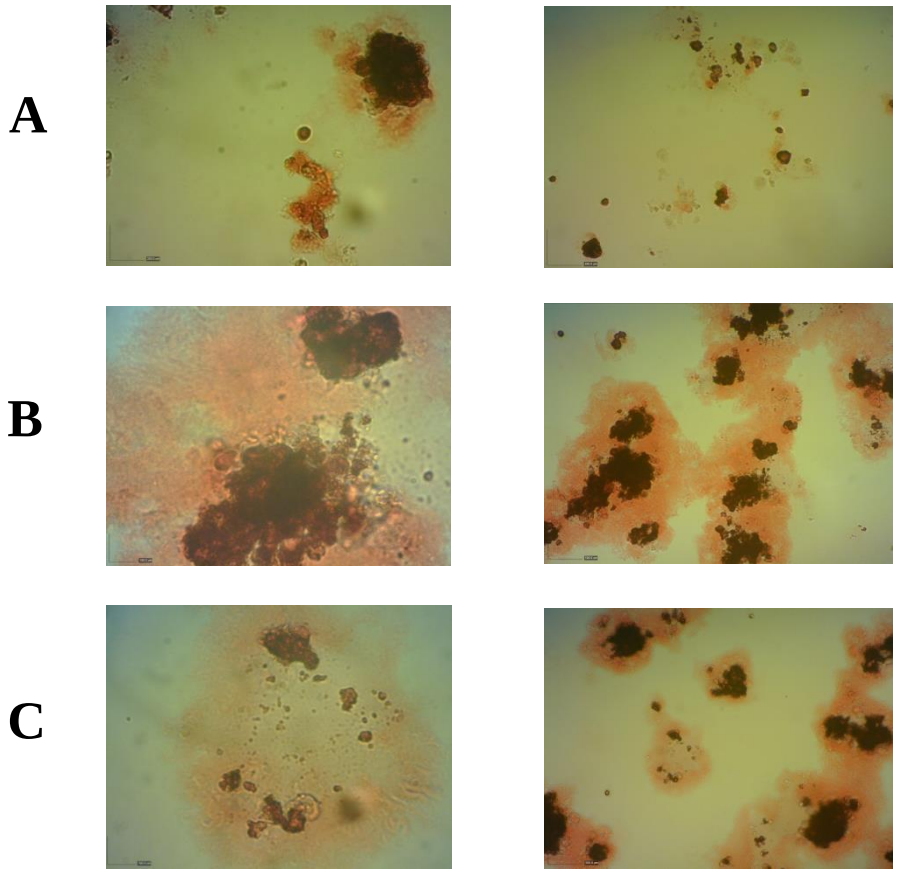


Figure 4. IFN-γ increases calcification in vitro. SaOs-2 cells were stimulated with IFN-γ for 24 h and before calcification was quantified by Alizarin Red staining followed by spectrophotometry. (A) Unstimulated controls. (B) Stimulated cells with 20 U/mL IFN-γ. (C) Stimulated cells with 1000 U/mL IFN-γ.

3.9 Viability assay

SaOs-2 cells viability was measured by MTT analysis and it is found that, compared with unstimulated control, the 20 U/mL and 1000 U/mL IFN-γ increased cell viability (7.23%

and 13.61% greater than healthy control, respectively) (Figure 5). However, only 20 U/mL showed significantly increased relative to unstimulated control group ($p = 0.017$).

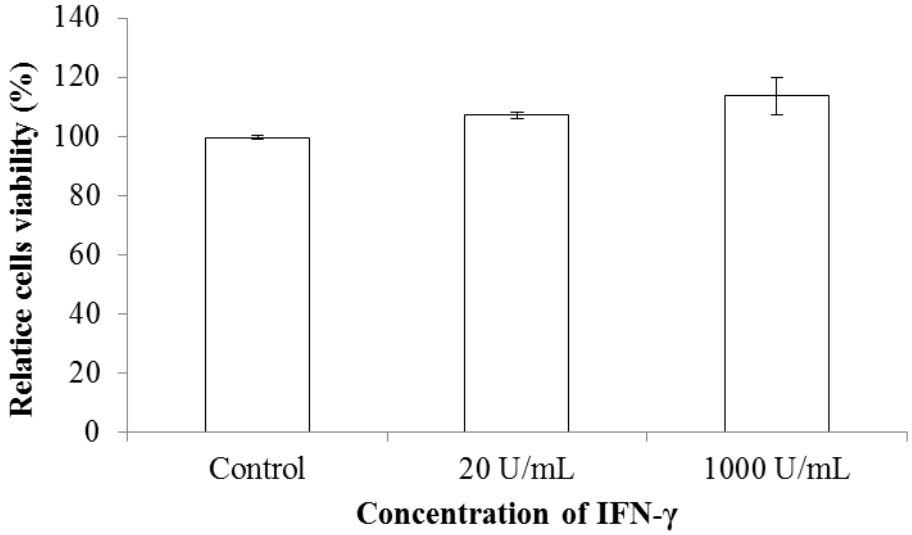


Figure 5. The effects of IFN-γ on cell viability measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay after stimulation for 24 h. Data were presented as means ± SD.

4.0 Discussion

IFN-γ -1616 (G>A, rs2069705) SNP have been described as localized in promoter region of IFN-γ gene, although previous studies have already related the absence of relation among this particulate SNP and IFN-γ levels (Erdei et al. 2010; Kim et al. 2010a; Huang et al. 2016). Nevertheless, similar to our results, previous researches have associated the IFN-γ rs2069705 with susceptibility in cancers (Erdei et al. 2010; Su et al. 2012), autoimmunity (Kim et al. 2010b) and infectious (Stappers et al., 2014) diseases. According to more recent sequencing updates, the 1000 Genomes Project has localized the FwdSNP within gene region. According to our analysis, the FwdSNP IFN-γ rs2069705 CC, corresponding to RevSNP IFN-γ rs2069705 GG, change the protein stability. Consequently, the SNP would influence in the susceptibility to diseases by change in protein interactions and not by protein levels, as described by previous studies. Perhaps it may be the justification to the absence of different

protein levels among genotypes although there is a relationship among these variants and susceptibility for several diseases.

In functional analysis, we found that OP patients groups exhibited 3-fold higher of mRNA IFN-γ levels than healthy control groups. This alteration appears to be an increasing protein levels compensatory mechanism in OP patients group, which showed the highest frequency of the more instable allelic variant IFN-γ rs2069705 G. Taken all these data together, it was observed that IFN-γ stimuli favored the calcification and viability of SaOs-2 cells and appeared to be involved to bone mass increasing. Similar results related to IFN-γ action in bone formation were found by Duque et al. (2011) (Duque et al. 2011) in *in vivo* studies with ovariectomized mice, which suggested that alterations in IFN-γ signaling pathway may be used advantageously to improve bone strength. Zhang et al (2015) (Zhang et al. 2015a) also associated the cytokine with inhibition of bone resorption in postmenopausal women. However, the IFN-γ dual role in bone environmental is already recognized (Ferrari-Lacraz and Ferrari 2007; Gao et al. 2007; Duque et al. 2011). Previous studies highlighted the direct anti-resorptive action of IFN-γ through inhibition of the RANK/RANKL pathway and blocks osteoclast formation. At the same time its indirect pro-resorptive action stimuli T cells and cytokines such as TNF-α and RANKL (Takayanagi et al. 2002; Cenci et al. 2003; Takayanagi et al. 2005; Ferrari-Lacraz and Ferrari 2007; Gao et al. 2007; Guerrini and Takayanagi 2014). Nevertheless, it is important to mention that the most of these results are related to studies with osteoclasts and murine cells and a few studies have been performed to clarify this role in human bone formation cells and their pathway. Among these studies with osteoblast-like cells, Wimbauer et al. (2012) (Wimbauer et al. 2012) described the IFN-γ associated with 2-methoxyestradiol treatment showed anti-proliferative effects in osteosarcoma cells by IFN-stimulated response element (ISRE) sequence-dependent

transcription and gamma-activated sequence (GAS)-dependent transcription. However, our results showed that IFN-γ increased calcification and viability in SaOs-2 cells. It is important to emphasize that the lowest concentration of IFN-γ was more favorable to osteoblasts activity than highest concentration. Additionally, we did not verify alterations in other cytokines levels, the main indirect action pro-resorptive associated with IFN-γ. We highlight that further studies should be conducted for confirmation of relationship between osteoblasts activity and cytokines levels. On the other hand, it is possible that other pathways different of observed in osteoclasts are involved in human osteoblasts survival process.

The direct effect of IFN-γ in osteoblastogenesis have been already reported by Urizar et al. (2015) (Ibarra Urizar et al. 2015) which related that 5 ng/mL (50 U/mL) IFN-γ was able to stimulate bone morphogenetic proteins 2 (BMP-2) expression in pancreatic beta cells, although had verified that this cytokine did not affect the expression of protein in rats islets. BMP-2 is recognized by stimulating the differentiation of progenitor cells in pre-osteoblasts besides to have a critical role for osteoblast function together with Wnt pathway and its inhibitors (Stein and Lian 1993; Raisz 2005; Bunnell et al. 2011). Additionally, previous studies have related IFN-γ together with interleukin 1 (IL-1) stimulates nitric oxid (NO) and indirectly induce increasing bone mass (Ralston et al. 1994; Van't Hof and Ralston 2001; Duque et al. 2011). The endothelial isoform of nitric oxide synthase (eNOS) and its NO derived acts as a mediator of the effects of estrogen in bone and associated with prostaglandins promote bone formation and suppress bone resorption (Van't Hof and Ralston 2001). In accordance to Duque et al. (2011) (Duque et al. 2011), we suggested that this IFN-γ anabolic activity is dose-dependent. High IFN-γ doses seems to contribute to bone loss (Mann et al. 1994; Tohkin et al. 1994; Duque et al. 2011). IFN-γ variable effects in bone homeostasis also appears to be dependents on the experimental model and other conditions used in the

analysis (Ferrari-Lacraz and Ferrari 2007; Duque et al. 2011). We verified that human bone cells and *in vivo* studies have showed different results when compared with cells murine analysis (Ferrari-Lacraz and Ferrari 2007; Gao et al. 2007; Duque et al. 2011).

To the best of our knowledge, this is the first time the IFN-γ -1616 (G>A, rs2069705) was analyzed in OP patients. In our study, we found a significantly increased risk for OP associated with the IFN-γ rs2069705 G allele. The FwdSNP is localized in exon region and according to our analysis is able to decrease the protein stability, but not its levels. Consequently, no significant differences have been observed in genotype variants. The high levels observed in mRNA IFN-γ levels seems to be associated with a compensatory response since IFN-γ favored the calcification and viability in the SaOs-2 study related to used doses. We also observed that previous studies showed differences according to their used cells and models. Therefore we strongly indicated that this cytokine concentration and the choice of a model *in vivo* and *in vitro* must be into consideration for future analysis. Finally, we suggest that further functional studies are required to examine the IFN-γ activity in osteoblast-like cells bearing in mind that this is one of the few studies that performs this approach.

Acknowledgments

We thank the following Brazilian funding agencies: CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FACEPE (Fundação de Amparo à Ciência e Tecnologia de Pernambuco) for financial support. We are also grateful to Dr. João Ricardo de Oliveira for SaOs-2 cells donation.

Supplementary material

SM.1. Primers and PCR efficiencies of the IFN-γ and reference genes

Name	Primer sequences / Taqman ® probe reference	Product size/bp	R^2	E/%
B2M	F: TGAGTGGCATGAAGAAGGTGT R: GGCAGTTCTTTGCCCTCTCT	77	0.999	104.79
RPLP0	F: GCGACCTGGAAGTCCAACTA R: TCTGCTTGGAGCCCACATTG	100	0.999	102.02
GAPDH	F: CTGATGCCCCCATGTTCGT R: GCAGGAGGCATTGCTGATGA	80	0.999	96.81
IFN-γ	F: TCCAAGTGATGGCTGAACTGT R: TCGACCTCGAAACAGCATCT	77	1	99.45

7. Discussão geral

Para o nosso conhecimento, este é o primeiro estudo que verificou a relação entre os SNPs IL23R +2284 (C>A) (rs10889677), IL17A +672 (G>A) (rs7747909), IL12B +1188 (T>G) (rs3212227) e IFN-γ -1616 (G>A) (rs2069705) e a OP primária pós-menopausa. Na análise de associação entre os SNPs e a resposta terapêutica aos bisfosfonatos encontramos os genótipos IL17A rs7747909 G/A e IL12B rs3212227 T/G associados a altos níveis de vitamina D, IL23R rs10889677 C/C associado a altos níveis de cálcio, IFN-γ rs2069705 A/A associado a altos níveis de PTH e o IFN-γ rs2069705 G/G a altos níveis de ALP. Adicionalmente, verificamos relação entre o genótipo IFN-γ rs2069705 G/G e o aumento da DMO na cabeça do fêmur e diminuição no quadril total, após 3 e 4 anos de tratamento, respectivamente. Por fim selecionamos para nosso terceiro estudo o SNP associado à susceptibilidade a OP, o IFN-γ -1616 (G>A) (rs2069705). Na confirmação da localização do SNP verificamos que o FwdSNP correspondente ao nosso polimorfismo de estudo se encontra no éxon 4 e não na região promotora do gene, como relatado por estudos anteriores. Nessa análise encontramos que a variante G, associada à susceptibilidade à OP no nosso estudo, confere maior instabilidade à proteína. Esses resultados foram reforçados pela análise *in vitro* com células SaOs-2 , que sugeriram o IFN-γ como proteína pró-absortiva, embora o grupo de pacientes tenha apresentando maiores níveis de mRNA da citocina comparados ao grupo controle saudável.

Os SNPs aqui estudados foram analisados pela primeira vez em OP, mas já haviam sido relacionados à outras doenças reumatológicas como artrite reumatoide (Faragó et al., 2008; Shen et al., 2015); espondilite anquilosante (Wong et al., 2012),

arterite (Márquez et al., 2014) e lúpus eritematoso sistêmico (LES) (Kim et al., 2010). Semelhante às nossas análises, muitos desses estudos não encontraram alterações nos níveis do IFN-γ em relação aos diferentes genótipos genótipo (Erdei et al. 2010; Kim et al. 2010; Huang et al. 2016). As citocinas estudadas também já mostraram influências nas vias da homeostase óssea. A via IL-23/IL-23R/β-catenina já foi citada por induzir formação óssea (Tu et al., 2015), a IL-17A por estimular RANKL, a IL-12B por inibir a formação óssea *in vivo* (Xu et al., 2016) e o IFN-γ com relatos contraditórios sobre sua participação na formação e na reabsorção óssea (Gao et al., 2007; Duque et al., 2011). O nosso trabalho também mostrou essa ação pleiotrópica da citocina na resposta terapêutica aos bisfosfonatos, com o mesmo genótipo IFN-γ rs2069705 G/G associado à diferentes respostas na DMO. Além disso, encontramos concentrações menores da citocina mais favoráveis à calcificação e viabilidade em células da linhagem osteoblástica que concentrações maiores induzidas no mesmo ensaio. Esses dados associados aos estudos anteriores, nos possibilitaram sugerir uma ação da citocina dependente dos valores de DMO, local de atuação e quantidade da proteína disponibilizada. Adicionalmente, levantamos a discussão sobre os processos de apoptose como fator importante nessa atuação antagônica do IFN-γ. Um outro componente importante que verificamos em estudos anteriores é o modelo selecionado para essas avaliações. O resultado das análises parece estar também relacionado a esses modelos *in vivo* e *in vitro* utilizados (Ferrari-Lacraz e Ferrari, 2007; Duque et al., 2011). E por fim, uma das contribuições que deve ser destacada no nosso estudo é a validação dos genes de referência que conduziu nossa posterior análise funcional e deve ser levada em consideração para os próximos ensaios de expressão em OP. Nesse estudo pioneiro, além de definirmos

os genes mais estáveis para análises de expressão, RPLP0 e B2M, enumeramos o 18S, ACTB e HPRT1 como não recomendados para estudos na doença. Reportamos a instabilidade do ACTB para nossa análise em OP, embora ele seja usado em mais de 70% das quantificações de mRNA.

A despeito do fato de que muito ainda tenha que ser feito para um completo esclarecimento dos fatores genéticos e osteoimunológicos envolvidos no estabelecimento da OP, acreditamos que os estudos avaliando SNPs e conduzindo suas validações funcionais são de grande importância na identificação de fatores de risco e marcadores precoces para diagnóstico e adesão terapêutica. Desta forma, além de tratamentos preventivos e personalizados baseados na susceptibilidade genética, poderíamos também evitar o óbito dos 20% pacientes de OP que não aderem ao tratamento ainda no primeiro ano de terapia (Roush, 2011; Iolascon et al., 2013; Drake et al., 2015; Miller, 2016). Além disso, nós acreditamos que essas contribuições em conjunto encorajam outras pesquisas proeminentes como a influência desses polimorfismos localizados em regiões 3’UTR na ligação de miRNAs e consequente susceptibilidade à OP (Coronnello et al., 2012; Qin et al., 2016).

8. Conclusões

Após os estudos realizados neste trabalho, alguns pontos em relação à Osteoporose e seus fatores genéticos e funcionais foram esclarecidos, de acordo com os objetivos inicialmente propostos.

Verificamos que SNPs nos genes das citocinas estudadas podem contribuir para decisões terapêuticas em OP. SNPs nos genes IL23R +2284 (C>A) (rs10889677), IL17A +672 (G>A) (rs7747909), IL12B +1188 (T>G) (rs3212227) e IFN-γ -1616 (G>A) (rs2069705) exibiram associação estatisticamente significativa com mudanças nos marcadores bioquímicos do metabolismo ósseo em pacientes submetidos à terapia com bisfofonatos. Além disso, o IFN-γ rs2069705 G/G mostrou associação com mudanças de DMO nesses pacientes. Adicionalmente sugerimos que um mecanismo de apoptose em osteoblastos relacionado ao IFN-γ pode explicar alguns casos em que ele está, contraditoriamente, associado à diminuição de DMO.

Para os estudos funcionais, validamos os genes de referência RPLP0 e B2M como os mais estáveis para nosso estudo de expressão em OP. Nesse estudo também verificamos que o ACTB, 18S e HPRT1 são inadequados para normalização dos dados de expressão na doença.

Por fim concluímos que O SNP IFN-γ -1616 (G>A) (rs2069705) parece alterar a estabilidade da proteína e não seus níveis. Esta informação está de acordo com o último sequenciamento de 2016 (CRCh38.p7) que comprova sua posição fora da região promotora e seu FwdSNP correspondente localizado no éxon 4. Além disso,o alelo IFN-γ rs2069705 G, que confere maior instabilidade à proteína esteve associado com susceptibilidade à OP na nossa população de estudo. Esses dados

nos fazem sugerir que o IFN-γ tenha um papel protetor na OP. Concordando com esses resultados, a proteína aumentou calcificação e viabilidade em células SaOs-2. No entanto, acreditamos que essa indução é dose-dependente, tendo em vista que a maior dose (1000 U/mL) utilizada para os estímulos celulares apresentaram níveis de calcificação e viabilidade menores que o valor mínimo utilizado da citocina (20 U/mL).

Além de responder à alguns dos nossos questionamentos iniciais, esses resultados encorajam pesquisas funcionais em Osteoimunologia, em busca de marcadores diagnósticos e terapêuticos, além de modelos de células ósseas e concentrações ótimas de estímulos com citocinas, a fim de nos aproximarmos cada vez mais do entendimentos do papel dessas proteínas na OP pós-menopausa.

Referências Bibliográficas

Abrahamsen B and Prieto-Alhambra D (2016) Patients with Atypical Femur Fractures have the Same Mortality as the Background Population - Drug-Channelling Bias, Bisphosphonate Effects and Public Health Implications. J Bone Miner Res 31: 31(3):488-490. doi: 10.1002/jbmr.2801

Albagha OME and Ralston SH (2006) Genetics and Osteoporosis. Rheum Dis Clin North Am 32:659–680. doi: 10.1016/j.rdc.2006.08.001

Andersen CL, Jensen JL and Ørntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250. doi: 10.1158/0008-5472.CAN-04-0496

Annunziato F, Romagnani C and Romagnani S (2015) The 3 major types of innate and adaptive cell-mediated effector immunity. J Allergy Clin Immunol 135:626–635. doi: 10.1016/j.jaci.2014.11.001

Applied Biosystems (2008) Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR. Gene Expr 2009:1–60. doi: 10.1007/978-3-642-31107-9

Arron JR and Choi Y (2000) Bone versus immune system. Nature 408:535–536. doi: 10.1038/35046196

Artero-Castro A, Castellvi J, García A, Hernández J, Cajal SRY and Lleonart ME (2011) Expression of the ribosomal proteins Rplp0, Rplp1, and Rplp2 in gynecologic tumors. Hum Pathol 42:194–203. doi: 10.1016/j.humpath.2010.04.020

Asada N, Katayama Y, Sato M, Minagawa K, Wakahashi K, Kawano H, Kawano Y, Sada A, Ikeda K, Matsui T et al. (2013) Matrix-embedded osteocytes regulate mobilization of hematopoietic stem/progenitor cells. Cell Stem Cell 12:737–747. doi: 10.1016/j.stem.2013.05.001

Aspray TJ (2014) Fragility fracture: recent developments in risk assessment. Ther Adv Musculoskelet Dis 7:17–25. doi: 10.1177/1759720X14564562

Baccaro LF, Conde DM, Costa-Paiva L and Pinto-Neto AM (2015) The epidemiology and management of postmenopausal osteoporosis: A viewpoint from Brazil. Clin Interv Aging 10:583–591. doi: 10.2147/CIA.S54614

Bamias G, Goukos D, Laoudi E, Balla IG, Siakavellas SI, Daikos GL and Ladas SD (2013) Comparative study of candidate housekeeping genes for quantification of target gene messenger RNA expression by real-time PCR in patients with inflammatory bowel disease. Inflamm Bowel Dis 19:2840–2847. doi: 10.1097/01.MIB.0000435440.22484.e8

Bhushan S and Perumal NB (2012) Disease associated cytokine SNPs database: An annotation and dissemination model. Cytokine 57:107–112. doi: 10.1016/j.cyto.2011.10.009

Bignon JD, Viña MF (1995) 12th IHWC HLA Class II Reference Protocol. In: Fauchet R and Charron D (ed) Technical Handbook of the 12th International Histocompatibility Workshop. Paris, HLA et Médecine.

Blumenfeld O, Williams FMK, Valdes A, Hart DJ, Malkin I, Spector TD and Livshits G (2014) Association of interleukin-6 gene polymorphisms with hand osteoarthritis and hand osteoporosis. Cytokine 69:94–101. doi: 10.1016/j.cyto.2014.05.012

Boroń D, Author E, Agnieszka S, Daniel K, Anna B and Adam K (2014) Polymorphism of interleukin-17 and its relation to mineral density of bones in perimenopausal women. Eur J Med Res. doi: 10.1186/s40001-014-0069-1

Bunnell TM, Burbach BJ, Shimizu Y and Ervasti JM (2011) β-Actin specifically controls cell growth, migration, and the G-actin pool. Mol Biol Cell 22:4047–58. doi: 10.1091/mbc.E11-06-0582

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL et al. (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem 55:611–622.

Carmeliet G, Dermauw V and Bouillon R (2015) Vitamin D signaling in calcium and bone homeostasis: A delicate balance. Best Pract Res Clin Endocrinol Metab 29:621–631. doi: 10.1016/j.beem.2015.06.001

Cenci S, Toraldo G, Weitzmann MN, Roggia C, Gao Y, Qian WP, Sierra O and Pacifici R (2003) Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-gamma-induced class II transactivator. Proc Natl Acad Sci U S A 100:10405–10410. doi: 10.1073/pnas.1533207100

Chen B, Zeng Z, Xu L, Wu X, Yu J, Xue L, Hao Y, Wang Y, Sung J, Chen M et al. (2009) IL23R +2199A/C polymorphism is associated with decreased risk of certain subtypes of gastric cancer in Chinese: A case-control study. Cancer Epidemiol. doi: 10.1016/j.canep.2010.08.006

Chesnut CH, McClung MR, Ensrud KE, Bell NH, Genant HK, Harris ST, Singer FR, Stock JL, Yood RA, Delmas PD et al. (1995) Alendronate treatment of the postmenopausal osteoporotic woman: Effect of multiple dosages on bone mass and bone remodeling. Am J Med 99:144–152. doi: 10.1016/S0002-9343(99)80134-X

Christakos S, Dhawan P, Verstuyf A, Verlinden L and Carmeliet G (2016) Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. Physiol Rev 96:365–408. doi: 10.1152/physrev.00014.2015

Clark GR and Duncan EL (2015) The genetics of osteoporosis. Br Med Bull 113:73–81. doi: 10.1093/bmb/ldu042

Coronnello C, Hartmaier R, Arora A, Huleihel L, Pandit K V., Bais AS, Butterworth M, Kaminski N, Stormo GD, Oesterreich S et al. (2012) Novel Modeling of Combinatorial miRNA Targeting Identifies SNP with Potential Role in Bone Density. PLoS Comput Biol. doi: 10.1371/journal.pcbi.1002830

Cummings SR, San Martin J, McClung MR, Siris ES, Eastell R, Reid IR, Delmas P, Zoog HB, Austin M, Wang A et al. (2009) Denosumab for prevention of fractures in postmenopausal women with osteoporosis. N Engl J Med 361:756–765. doi: 10.1056/NEJMoa0809493

Cunha, EP, Steiner MI, Strufaldi R, Fernades C, Laurindo IMM, Pereira RMR, Simões R (2011) Osteoporose: Tratamento. Diretrizes Clínicas Na Saúde Suplementar. Associaçiação Médica Brasileira E Agência Nacional de Saúde Suplementar. http://diretrizes.amb.org.br/ans/osteoporose-tratamento.pdf. Accessed 01 August 2016.

Czerny B, Kaminski A, Kurzawski M, Kotrych D, Safranow K, Dziedziejko V, Bohatyrewicz A and Pawlik A (2010) The association of IL-1beta, IL-2, and IL-6 gene polymorphisms with bone mineral density and osteoporosis in postmenopausal women. Eur J Obs Gynecol Reprod Biol 149:82–85. doi: 10.1016/j.ejogrb.2009.12.010.

Damsker JM, Hansen AM and Caspi RR (2010) Th1 and Th17 cells. Ann N Y Acad Sci 211–221. doi: 10.1111/j.1749-6632.2009.05133.x.Th1

De Spiegelaere W, Dern-Wieloch J, Weigel R, Schumacher V, Schorle H, Nettersheim D, Bergmann M, Brehm R, Kliesch S, Vandekerckhove L et al. (2015) Reference gene validation for RT-qPCR, a note on different available software packages. PLoS One 10:1–13. doi: 10.1371/journal.pone.0122515

Drake M, Clarke B and Lewiecki E (2015) The Pathophysiology and Treatment of Osteoporosis. Clin Ther 37:1837–1850. doi: 10.1016/j.clinthera.2015.06.006.

Duque G, Huang DC, Dion N, Macoritto M, Rivas D, Li W, Yang XF, Li J, Lian J, Marino FT et al. (2011) Interferon- γ Plays a Role in Bone Formation In Vivo and Rescues Osteoporosis in Ovariectomized Mice. 26:1472–1483. doi: 10.1002/jbmr.350

Eastell R, O'Neill TW, Hofbauer LC, Langdahl B, Reid IR, Gold DT and Cummings SR (2016) Postmenopausal osteoporosis. Nat Rev Dis Prim 29;2:16069. doi: 10.1038/nrdp.2016.69

Elsafadi M, Manikandan M, Dawud RA, Alajez NM, Hamam R, Alfayez M, Kassem M, Aldahmash A and Mahmood A (2016) Transgelin is a TGFβ-inducible gene that regulates osteoblastic and adipogenic differentiation of human skeletal stem cells through actin cytoskeleston organization. Cell Death Dis 7:e2321. doi: 10.1038/cddis.2016.196

Emami S, Ziaee V, Rezaei A, Sadr M, Maddah M, Amirzargar AA and Rezaei N (2015) IL23R gene polymorphism with juvenile idiopathic arthritis and its association with serum IL-17A. Int J Rheum Dis 1–8. doi: 10.1111/1756-185X.12674

Erdei E, Kang H, Meisner A, White K, Pickett G, Baca C, Royce M and Berwick M (2010) Polymorphisms in cytokine genes and serum cytokine levels among New Mexican women with and without breast cancer. Cytokine 51:18–24. doi: 10.1016/j.cyto.2010.03.014

Esbrit P, Herrera S, Portal-Núñez S, Nogués X and Díez-Pérez A (2015) Parathyroid Hormone-Related Protein Analogs as Osteoporosis Therapies. Calcif Tissue Int. doi: 10.1007/s00223-015-0050-1

Faienza MF, Ventura A, Marzano F and Cavallo L (2013) Review Article Postmenopausal Osteoporosis : The Role of Immune System Cells. Clin Dev Immunol. 2013;2013:575936. doi: 10.1155/2013/575936.

Faragó B, Magyari L, Sáfrány E, Csöngei V, Járomi L, Horvatovich K, Sipeky C, Maász a, Radics J, Gyetvai a et al. (2008) Functional variants of interleukin-23 receptor gene confer risk for rheumatoid arthritis but not for systemic sclerosis. Ann Rheum Dis 67:248–250. doi: 10.1136/ard.2007.072819

Fassunke J, Blum M-C, Schildhaus H-U, Zapatka M, Brors B, Kunstlinger H, Buttner R, Wardelmann E, Merkelbach-Bruse S, Künstlinger H et al. (2010) qPCR in gastrointestinal stromal tumors: Evaluation of reference genes and expression analysis of KIT and the alternative receptor tyrosine kinases FLT3, CSF1-R, PDGFRB, MET and AXL. BMC Mol Biol 11:100. doi: 10.1186/1471-2199-11-100

Ferrari-Lacraz S and Ferrari S (2007) Is IFN-γ involved in bone loss or protection? nothing is simple with cytokines. Bonekey-Osteovision 4:83–87. doi: 10.1138/20060251

Flicker L, Hopper JL, Rodgers L, Kaymakci B, Green RM and Wark JD (1995) Bone density determinants in elderly women: A twin study. J Bone Miner Res 10:1607–1613. doi: 10.1002/jbmr.5650101102

Gao Y, Grassi F, Ryan MR, Terauchi M, Page K, Yang X, Weitzmann MN and Pacifici R (2007) IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. J Clin Invest 117:122–132. doi: 10.1172/JCI30074

Genant H, Wu C, van Kuijk C and Nevitt M (1993) Vertebral fracture assessment using a semiquantitative technique. J Bone Min Res 8:1137–48.

Golob AL and Laya MB (2015) Osteoporosis. Med Clin North Am 99:587–606. doi: 10.1016/j.mcna.2015.01.010

Greenblatt MB, Tsai JN and Wein MN (2016) Bone Turnover Markers in the Diagnosis and Monitoring of Metabolic Bone Disease. Clin Chem. doi: 10.1373/clinchem.2016.259085

Guerrini MM and Takayanagi H (2014) The immune system, bone and RANKL. Arch Biochem Biophys 561:118–123. doi: 10.1016/j.abb.2014.06.003

Hartmaier RJ, Richter a S, Gillihan RM, Sallit JZ, McGuire SE, Wang J, Lee a V, Osborne CK, O'Malley BW, Brown PH et al. (2012) A SNP in steroid receptor coactivator-1 disrupts a GSK3β phosphorylation site and is associated with altered tamoxifen response in bone. Mol Endocrinol 26:220–227. doi: 10.1210/me.2011-1032

Haynes DR (2006) Emerging and future therapies for the treatment of bone loss associated with chronic inflammation. Inflammopharmacology 14:193–197. doi: 10.1007/s10787-006-0006-1

Hiligsmann MM, Bours SPG and Boonen A (2015) A Review of Patient Preferences for Osteoporosis Drug Treatment. Curr Rheumatol Rep 17:61. doi: 10.1007/s11926-015-0533-0

Hsu YH and Kiel DP (2012) Genome-wide association studies of skeletal phenotypes: What we have learned and where we are headed. J Clin Endocrinol Metab 97:1958–1977. doi: 10.1210/jc.2012-1890

Huang Y-H, Hsu Y-W, Lu H-F, Sung H, Wong -Ching, Yu H-R, Kuo H-C, Huang F-C, Chang W-C and Kuo H-C (2016) Interferon-gamma Genetic Polymorphism and Expression in Kawasaki Disease. Medicine (Baltimore) 95(17):e3501. doi: 10.1097/MD.0000000000003501

Ibarra Urizar A, Friberg J, Christensen DP, Lund Christensen G and Billestrup N (2015) Inflammatory Cytokines Stimulate Bone Morphogenetic Protein-2 Expression and Release from Pancreatic Beta Cells. J Interf Cytokine Res 0:150826113459005. doi: 10.1089/jir.2014.0199

Iolascon G, Napolano R, Gioia M, Moretti A, Riccio I and Gimigliano F (2013) The contribution of cortical and trabecular tissues to bone strength: Insights from denosumab studies. Clin Cases Miner Bone Metab 10:47–51. doi: 10.11138/ccmbm/2013.10.1.047

Isomura H, Fujie K, Shibata K, Inoue N, Iizuka T, Takebe G, Takahashi K, Nishihira J, Izumi H and Sakamoto W (2004) Bone metabolism and oxidative stress in postmenopausal rats with iron overload. Toxicology 197:93–100. doi: 10.1016/j.tox.2003.12.006

Iwakura Y and Ishigame H (2006) The IL-23 / IL-17 axis in inflammation. J Clin Invest 116:1218–1222. doi: 10.1172/JCI25982.8.

Jadon D, Tillett W, Wallis D, Cavill C, Bowes J, Waldron N, Dixon A, Sengupta R, Barton A, Korendowych E et al. (2013) Exploring ankylosing spondylitis-associated ERAP1, IL23R and IL12B gene polymorphisms in subphenotypes of psoriatic arthritis. Rheumatol (United Kingdom) 52:261–266. doi: 10.1093/rheumatology/kes254

Josson S, Nomura T, Lin JT, Huang WC, Wu D, Zhau HE, Zayzafoon M, Neale Weizmann M, Gururajan M and Chung LWK (2011) B2-Microglobulin Induces Epithelial To Mesenchymal Transition and Confers Cancer Lethality and Bone Metastasis in Human Cancer Cells. Cancer Res 71:2600–2610. doi: 10.1158/0008-5472.CAN-10-3382

Kanchan K, Jha P, Pati SS, Mohanty S, Mishra SK, Sharma SK, Awasthi S, Venkatesh V and Habib S (2015) Interferon-γ (IFNG) microsatellite repeat and single nucleotide polymorphism haplotypes of IFN-γ receptor (IFNAR1) associated with enhanced malaria susceptibility in Indian populations. Infect Genet Evol 29:6–14. doi:

10.1016/j.meegid.2014.10.030

Kanis JA (2008) Assessment of Osteoporosis at the Primary Health Care Level. Brussels: WHO Collaborating Centre for Metabolic Bone Diseases, University of Sheffield Medical School.

Karasik D, Rivadeneira F and Johnson ML (2016) The genetics of bone mass and susceptibility to bone diseases. Nat Rev Rheumatol 12:323–334. doi: 10.1038/nrrheum.2016.48

Kelley RE and Andersson HC (2014) Disorders of purines and pyrimidines, 1st ed. Handb Clin Neurol. doi: 10.1016/B978-0-7020-4087-0.00055-3

Kerschan-Schindl K (2016) Prevention and rehabilitation of osteoporosis. Wien Med Wochenschr 166:22–27.

Khader S a and Gopal R (2010) IL-17 in protective immunity to intracellular pathogens. Virulence 1:423–427. doi: 10.4161/viru.1.5.12862

Kim DH, Kong JH, Byeun JY, Jung CW, Xu W, Liu X, Kamel-Reid S, Kim YK, Kim HJ and Lipton JH (2010a) The IFNG (IFN-γ) genotype predicts cytogenetic and molecular response to imatinib therapy in chronic myeloid leukemia. Clin Cancer Res 16:5339–5350. doi: 10.1158/1078-0432.CCR-10-1638

Kim K, Cho S-K, Sestak A, Namjou B, Kang C and Bae S-C (2010b) Interferon-gamma gene polymorphisms associated with susceptibility to systemic lupus erythematosus. Ann Rheum Dis 69:1247–50. doi: 10.1136/ard.2009.117572

Klenke S, Renckhoff K, Engler A, Peters J and Frey UH (2016) Easy-to-use strategy for reference gene selection in quantitative real-time PCR experiments. Naunyn Schmiedebergs Arch Pharmacol. 389(12):1353-1366. doi: 10.1007/s00210-016-1305-8

Kolkova Z, Arakelyan A, Casslén B, Hansson S and Kriegova E (2013) Normalizing to GADPH jeopardises correct quantification of gene expression in ovarian tumours - IPO8 and RPL4 are reliable reference genes. J Ovarian Res 6:60. doi: 10.1186/1757-2215-6-60

Kozera B and Rapacz M (2013) Reference genes in real-time PCR. J Appl Genet 54:391–406. doi: 10.1007/s13353-013-0173-x

Kruk M, Ralston SH and Albagha OME (2009) LRP5 polymorphisms and response to risedronate treatment in osteoporotic men. Calcif Tissue Int 84:171–179. doi: 10.1007/s00223-008-9207-5

Kumar A and Ghosh B (2008) A single nucleotide polymorphism (A --> G) in intron 3 of IFNgamma gene is associated with asthma. Genes Immun 9:294–301. doi: 10.1038/gene.2008.17

Lee HJ, Kim H, Ku S-Y, Choi YM and Kim JH (2014) Association between polymorphisms in leptin, leptin receptor, and beta-adrenergic receptor genes and bone mineral density in postmenopausal Korean women. Menopause 21:67–73. doi:

10.1097/GME.0b013e31829366ed

Lee SH and Shin H (2007) Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. Adv Drug Deliv Rev 59:339–359. doi: 10.1016/j.addr.2007.03.016

Legiran S and Brandi ML (2012) Bone mass regulation of leptin and postmenopausal osteoporosis with obesity. Clin Cases Miner Bone Metab 9:145–149.

Leng RX, Pan HF, Liu J, Yang XK, Zhang C, Tao SS, Wang DG, Li XM, Li XP, Yang W et al. (2016) Evidence for genetic association of TBX21 and IFNG with systemic lupus erythematosus in a Chinese Han population. Sci Rep 6:22081. doi: 10.1038/srep22081

Levy R, Mott RF, Iraqi FA and Gabet Y (2015) Collaborative cross mice in a genetic association study reveal new candidate genes for bone microarchitecture. BMC Genomics 16:1013. doi: 10.1186/s12864-015-2213-x

Lewiecki EM (2011) New targets for intervention in the treatment of postmenopausal osteoporosis. Nat Rev Rheumatol 7:631–638. doi: 10.1038/nrrheum.2011.130

Li X, Yang Q, Bai J, Xuan Y and Wang Y (2015) Evaluation of eight reference genes for quantitative polymerase chain reaction analysis in human T lymphocytes co-cultured with mesenchymal stem cells. Mol Med Rep 7721–7727. doi: 10.3892/mmr.2015.4396

Lima CAD, Javorski NR, Souza APO, Barbosa AD, Valença APMC, Crovella S, Souza PRE, de Azevêdo Silva J, Sandrin-Garcia P. Polymorphisms in key bone modulator cytokines genes influence bisphosphonates therapy in postmenopausal women. Inflammopharmacology. In press. doi : 10.1007/s10787-017-0322-7

Liu D, Shi L, Han C, Yu J, Li D and Zhang Y (2012) Validation of Reference Genes for Gene Expression Studies in Virus-Infected Nicotiana benthamiana Using Quantitative Real-Time PCR. PLoS One 7(9):e46451. doi: 10.1371/journal.pone.0046451

Liu S, Zhou Z, Wang C, Guo M, Chu N and Li C (2015) Associations between interleukin and interleukin receptor gene polymorphisms and risk of gout. Sci Rep 5:13887. doi: 10.1038/srep13887

Liu Y-J, Zhang L, Papasian CJ and Deng H-W (2014) Genome-wide Association Studies for Osteoporosis: A 2013 Update. J Bone Metab 21:99–116. doi: 10.11005/jbm.2014.21.2.99

López-Delgado L, Riancho-Zarrabeitia L and Riancho J (2016) Genetic and acquired factors influencing the effectiveness and toxicity of drug therapy in osteoporosis. Expert Opin Drug Metab Toxicol 12:389–398. doi: 10.1517/17425255.2016.1154533

Louahchi S, Allam I, Berkani L, Boucharef A, Abdesemed A, Khaldoun N, Nebbab A, Ladjouze A and Djidjik R (2016) Association study of single nucleotide polymorphisms of IL23R and IL17 in rheumatoid arthritis in the Algerian population.

Acta Reum. Port. 2016 Apr-Jun;41(2):151-7.

Ma Q, Zhuang Z, Feng W, Liu S and Tang Q (2015) Evaluation of reference genes for quantitative real-time PCR analysis of gene expression during early development processes of the tongue sole (Cynoglossus semilaevis). Acta Oceanol Sin 34:90–97. doi: 10.1007/s13131-015-0725-5

Mah JTL, Low ESH and Lee E (2011) In silico SNP analysis and bioinformatics tools: A review of the state of the art to aid drug discovery. Drug Discov Today 16:800–809. doi: 10.1016/j.drudis.2011.07.005

Makras P, Delaroudis S and Anastasilakis AD (2015) Novel therapies for osteoporosis. Metabolism 64:1199–1214. doi: 10.1016/j.metabol.2015.07.011

Mann G, Jacobs T, Buchinsky F, Armstrong E, Li M, Ke H, Ma Y, Jee W and Epstein S (1994) Interferon-y Causes Loss of Bone Volume in Viva and Fails to Ameliorate Cyclosporin A-Induced Osteopenia. Endocrinology 135(3):1077-1083. doi: 10.1210/endo.135.3.8070349

Marini F and Brandi ML (2014) Pharmacogenetics of osteoporosis. Best Pract Res Clin Endocrinol Metab 28:783–793. doi: 10.1016/j.beem.2014.07.004

Marques A, Ferreira RJO, Santos E, Loza E, Carmona L and da Silva JAP (2015) The accuracy of osteoporotic fracture risk prediction tools: a systematic review and meta-analysis. Ann Rheum Dis annrheumdis-2015-207907. 74(11):1958-1967 doi: 10.1136/annrheumdis-2015-207907

Márquez A, Hernández-Rodríguez J, Cid MC, Solans R, Castañeda S, Fernández-Contreras ME, Ramentol M, Morado IC, Narváez J, Gómez-Vaquero C et al. (2014) Influence of the IL17A locus in giant cell arteritis susceptibility. Ann Rheum Dis 73(9):1742-1745. doi: 10.1136/annrheumdis-2014-205261

Miller PD (2016) Underdiagnoses and undertreatment of osteoporosis: The battle to be won. J Clin Endocrinol Metab 101:852–859. doi: 10.1210/jc.2015-3156

Ministério da Saúde, Brasil (2014). Protocolo Clínico e Diretrizes Terapêuticas: Osteoporose. Portaria SAS/MS n. 451 de 09 de junho de 2014, retificada em 18 de junho de 2014. Disponível em: http://portalsaude.saude.gov.br/images/pdf/2014/dezembro/15/Osteoporose.pdf. Acessado em julho, 2015.

Nasr A, Allam G, Hamid O and Al-Ghamdi A (2014) IFN-gamma and TNF associated with severe falciparum malaria infection in Saudi pregnant women. Malar J 13:314. doi: 10.1186/1475-2875-13-314

Nicot N, Hausman JF, Hoffmann L and Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907–2914. doi: 10.1093/jxb/eri285

Normann KR, Øystese KAB, Berg JP, Lekva T, Berg-Johnsen J, Bollerslev J and Olarescu NC (2016) Selection and validation of reliable reference genes for RT-

qPCR analysis in a large cohort of pituitary adenomas. Mol Cell Endocrinol 437:183–189. doi: 10.1016/j.mce.2016.08.030

Paolacci AR, Tanzarella OA, Porceddu E and Ciaffi M (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 10:11. doi: 10.1186/1471-2199-10-11

Pedersen KB, Andersen K, Fodstad Ø and Maelandsmo GM (2004) Sensitization of interferon-gamma induced apoptosis in human osteosarcoma cells by extracellular S100A4. BMC Cancer 4:52. doi: 10.1186/1471-2407-4-52

Pereira, F B. (2009) Análise da correlação da força muscular com a densidade mineral óssea em homens com idade igual ou superior a 60 anos, residentes em São Sebastião – Distrito Federal. Tese. Universidade de Brasiília, Centro de Ciências da Saúde.

Pfaffl MW, Tichopad A, Prgomet C and Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515. doi: 10.1023/B:BILE.0000019559.84305.47

Pham TT, Angus SP and Johnson GL (2014) MAP3K1: Genomic Alterations in Cancer and Function in Promoting Cell Survival or Apoptosis. Genes and Cancer 4:419–426. doi: 10.1177/1947601913513950

Qin L, Liu Y, Wang Y, Wu G, Chen J, Ye W, Yang J and Huang Q (2016) Computational characterization of osteoporosis associated SNPs and genes identified by genome-wide association studies. PLoS One 1;11(3):e0150070. doi: 10.1371/journal.pone.0150070

Querques F RC and Colella G GF (2015) Cellular and Animal Models for the Identification of Osteoporosis Determinants Increasing Vertebral Compression Fractures Risk. J Osteoporos Phys Act. 3:159. doi: 10.4172/2329-9509.1000159

Ragni E, Viganò M, Rebulla P, Giordano R and Lazzari L (2013) What is beyond a qRT-PCR study on mesenchymal stem cell differentiation properties: How to choose the most reliable housekeeping genes. J Cell Mol Med 17:168–180. doi: 10.1111/j.1582-4934.2012.01660.x

Raisz LG (2005) Science in medicine Pathogenesis of osteoporosis : concepts, conflicts , and prospects 115(12):3318-3325. doi: 10.1172/JCI27071.3318

Ralston SH, Todd D, Helfrich M, Benjamin N and Grabowski PS (1994) Human osteoblast-like cells produce nitric oxide and express inducible nitric oxide synthase. Endocrinology 135:330–336. doi: 10.1210/endo.135.1.7516867

Ramana C, Gil M, Schreiber R and Stark G (2002) Stat1-dependent and -independent pathways in IFN-gamma-dependent signaling. Trends Immunol 23:96–101.

Raphael I, Nalawade S, Eagar TN and Forsthuber TG (2014) T cell subsets and their

signature cytokines in autoimmune and inflammatory diseases. Cytokine 74(1):5-17. doi: 10.1016/j.cyto.2014.09.011

Riancho J a and Hernández JL (2012a) Pharmacogenomics of osteoporosis: a pathway approach. Pharmacogenomics 13:815–829. doi: 10.2217/pgs.12.50

Riancho J and Hernández J (2012b) Pharmacogenomics of osteoporosis: a pathway approach. Pharmacogenomics 13:815–829.

Richards J, Rivadeneira F, Inouye M, Pastinen T, Soranzo N, Wilson S, Andrew T, Falchi M, Gwilliam R, Ahmadi K et al. (2008) Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet 371:1505–1512. doi: 10.1016/S0140-6736(08)60599-1

Rienzo M, Schiano C, Casamassimi A, Grimaldi V, Infante T and Napoli C (2013) Identification of valid reference housekeeping genes for gene expression analysis in tumor neovascularization studies. Clin Transl Oncol 15:211–218. doi: 10.1007/s12094-012-0904-1

Rivadeneira F, Styrkársdottir U, Estrada K, Halldórsson B V, Aulchenko YS, Cupples LA, Deloukas P, Demissie S, Grundberg E, Hofman A et al. (2009) Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet. 41(11):1199-1206. doi: 10.1038/ng.446

Rocha-Braz MGM and Ferraz-de-Souza B (2016) Genetics of osteoporosis: searching for candidate genes for bone fragility. Arch Endocrinol Metab 60:391–401. doi: 10.1590/2359-3997000000178

Roche Applied Science, 2009. Human Reference Gene Panel, 384. https://lifescience.roche.com/documents/RTR-Plate-Layout-Human-Reference-Gene-Panel-384.pdf (accessed 05.12.16).

Roush K (2011) Prevention and treatment of osteoporosis in postmenopausal women: a review. Ajn 111:26–35. doi: 10.1007/s10354-015-0417-y.

Roy SK, Hu J, Meng Q, Xia Y, Shapiro PS, Reddy SPM, Platanias LC, Lindner DJ, Johnson PF, Pritchard C et al. (2002) MEKK1 plays a critical role in activating the transcription factor C/EBP-beta-dependent gene expression in response to IFN-gamma. Proc Natl Acad Sci U S A 99:7945–50. doi: 10.1073/pnas.122075799

Rubenstein PA (1990) The functional importance of multiple actin isoforms. BioEssays 12:309–315. doi: 10.1002/bies.950120702

Sathy BN, Watson BM, Kinard LA and Spicer PP Tissue Eng Part A (2015) Oct;21(19-20):2495-2503. doi: 10.1089/ten.TEA.2015.0099.

Seeman E and Delmas PD (2006) Bone quality--the material and structural basis of bone strength and fragility. N Engl J Med 354:2250–2261. doi: 10.1056/NEJMra053077

Seibel MJ (2005) Biochemical markers of bone turnover: Part I: biochemistry and variability. Clin Biochem Rev 26:97–122.

Sharma U, Pal D and Prasad R (2014) Alkaline phosphatase: An overview. Indian J Clin Biochem 29:269–278. doi: 10.1007/s12291-013-0408-y

Shen L, Zhang H, Zhou X and Liu R (2015) Association between polymorphisms of interleukin 12 and rheumatoid arthritis associated biomarkers in a Chinese population. Cytokine 76:363–367. doi: 10.1016/j.cyto.2015.09.007

Sims N a. and Walsh NC (2012) Intercellular cross-talk among bone cells: New factors and pathways. Curr Osteoporos Rep 10:109–117. doi: 10.1007/s11914-012-0096-1

Singh PK and Mistry KN (2016) A computational approach to determine susceptibility to cancer by evaluating the deleterious effect of nsSNP in XRCC1 gene on binding interaction of XRCC1 protein with ligase III. Gene 576:141–149. doi: 10.1016/j.gene.2015.09.084

Stein GS and Lian JB (1993) Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. Endocr Rev 14:424–442. doi: 10.1210/er.14.4.424

Stephens AS, Stephens SR, Morrison NA, Karsenty G, Brazier H, Stephens S, Ory S, Fort P, Morrison N, Blangy A et al. (2011) Internal control genes for quantitative RT-PCR expression analysis in mouse osteoblasts, osteoclasts and macrophages. BMC Res Notes 4:410. doi: 10.1186/1756-0500-4-410

Styrkarsdottir U, Halldorsson B V., Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, Jonsdottir T, Saemundsdottir J, Center JR, Nguyen T V. et al. (2008) Multiple Genetic Loci for Bone Mineral Density and Fractures. N Engl J Med 358:2355–2365. doi: 10.1056/NEJMoa0801197

Su Y, Tang LY, Chen LJ, He JR, Su FX, Lin Y, Chen WQ, Xie XM and Ren ZF (2012) Joint effects of febrile acute infection and an interferon-γ polymorphism on breast cancer risk. PLoS One 7(5):e37275. doi: 10.1371/journal.pone.0037275

Sun T, Chen M, Lin X, Yu R, Zhao Y and Wang J (2014) The influence of osteoprotegerin genetic polymorphisms on bone mineral density and osteoporosis in Chinese postmenopausal women. Int Immunopharmacol 22:200–203. doi: 10.1016/j.intimp.2014.06.023

Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC and Mills KHG (2009) Interleukin-1 and IL-23 Induce Innate IL-17 Production from γδ T Cells, Amplifying Th17 Responses and Autoimmunity. Immunity 31:331–341. doi: 10.1016/j.immuni.2009.08.001

Swanberg M, McGuigan FE, Ivaska KK, Gerdhem P and Åkesson K (2012) Polymorphisms in the Inflammatory Genes CIITA, CLEC16A and IFNG Influence BMD, Bone Loss and Fracture in Elderly Women. PLoS One 7(10):e47964. doi: 10.1371/journal.pone.0047964

Tai IC, Wang YH, Chen CH, Chuang SC, Chang JK and Ho ML (2015) Simvastatin enhances Rho/actin/cell rigidity pathway contributing to mesenchymal stem cells'

osteogenic differentiation. Int J Nanomedicine 10:5881–5894. doi: 10.2147/IJN.S84273

Takayanagi H (2012) New developments in osteoimmunology. Nat Rev Rheumatol 8:684–689. doi: 10.1038/nrrheum.2012.167

Takayanagi H, Kim S and Taniguchi T (2002) Signaling crosstalk between RANKL and interferons in osteoclast differentiation. Arthritis Res 4 Suppl 3:S227-32. doi: 10.1186/ar581

Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K et al. (2000) T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. Nature 408:600–605. doi: 10.1038/35046102

Takayanagi H, Sato K, Takaoka A and Taniguchi T (2005) Interplay between interferon and other cytokine systems in bone metabolism. Immunol Rev 208:181–193. doi: 10.1111/j.0105-2896.2005.00337.x

Talaat RM, Sidek A, Mosalem A and Kholief A (2015) Effect of bisphosphonates treatment on cytokine imbalance between TH17 and Treg in osteoporosis. Inflammopharmacology 23:119–125. doi: 10.1007/s10787-015-0233-4

Tartour E, Fossiez F, Joyeux I, Galinha A, Gey A, Claret E, Sastre-Garau X, Couturier J, Mosseri V, Vives V et al. (2009) Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. Cancer Res. 1999 Aug 1;59(15):3698-3704.

Tastan Y, Kann PH, Tinneberg H-R, Hadji P, Müller-Ladner U and Lange U (2016) Low bone mineral density and vitamin d deficiency correlated with genetics and other bone markers in female Turkish immigrants in Germany. Clin Rheumatol 35(11):2789-2795. doi: 10.1007/s10067-016-3237-6

Tella S and Gallagher J (2014) Prevention and treatment of postmenopausal osteoporosis. J Steroid Biochem Mol Biol 142:155–170.

Tohkin M, Kakudo S, Kasai H and Arita H (1994) Comparative study of inhibitory effects by murine interferon γ and a new bisphosphonate (alendronate) in hypercalcemic, nude mice bearing human tumor (LJC-1-JCK). Cancer Immunol Immunother 39:155–160. doi: 10.1007/BF01533380

Tu B, Liu S, Liu G, Yan W, Wang Y, Li Z and Fan C (2015) Macrophages derived from THP-1 promote the osteogenic differentiation of mesenchymal stem cells through the IL-23/IL-23R/β-catenin pathway. Exp Cell Res 339:81–89. doi: 10.1016/j.yexcr.2015.10.015

Urano T and Inoue S (2014) Genetics of osteoporosis. Biochem Biophys Res Commun 452:287–293. doi: 10.1016/j.bbrc.2014.07.141

Van't Hof R and Ralston S (2001) Nitric Oxide and. doi: 10.1046/j.1365-2567.2001.01261.x

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034. doi: 10.1186/gb-2002-3-7-research0034

Wang C, Zheng H, He J, Zhang H, Yue H, Hu W, Gu J, Shao C, Fu W, Hu Y et al. (2015a) Genetic polymorphisms in the mevalonate pathway affect the therapeutic response to alendronate treatment in postmenopausal Chinese women with low bone mineral density. Pharmacogenomics J 15(2)158-164 15:158–164.

Wang H, Yang B, Geng T, Li B, Dai P and Chen C (2015b) Tissue-specific selection of optimal reference genes for expression analysis of anti-cancer drug-related genes in tumor samples using quantitative real-time RT-PCR. Exp Mol Pathol 98:375–381. doi: 10.1016/j.yexmp.2014.10.014

Wang P, Xiong A, Gao Z, Yu X, Li M, Hou Y, Sun C and Qu S (2016) Selection of Suitable Reference Genes for RT-qPCR Normalization under Abiotic Stresses and Hormone Stimulation in Persimmon (Diospyros kaki Thunb). PLoS One 11:e0160885. doi: 10.1371/journal.pone.0160885

Wimbauer F, Yang C, Shogren KL, Zhang M, Goyal R, Riester SM, Yaszemski MJ and Maran A (2012) Regulation of interferon pathway in 2-methoxyestradiol-treated osteosarcoma cells. BMC Cancer 12:93. doi: 10.1186/1471-2407-12-93

Wong L, Sim H, Chatterjee-Kishore, M Hatzinisiriou I, Devenish R, Stark G and Ralph S (2002) Isolation and characterization of a human STAT1 gene regulatory element. Inducibility by interferon (IFN) types I and II and role of IFN regulatory factor-1. J Biol Chem 277:19408–19417.

Wong ML and Medrano JF (2005) Real-time PCR for mRNA quantitation. Biotechniques 39:75–85. doi: 10.2144/05391RV01

Wong R, Wei J, Huang C, Lee H, Chiou S, Lin S, Cai Y, Hung P, Wang M and Yang S (2012) Association of IL-12B genetic polymorphism with the susceptibility and disease severity of ankylosing spondylitis. J Rheumatol 39:135–140. doi: 10.3899/jrheum.110613

Xie F, Xiao P, Chen D, Xu L and Zhang B (2012) miRDeepFinder: A miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80:75–84. doi: 10.1007/s11103-012-9885-2

Xu J, Wang Y, Li J, Zhang X, Geng Y, Huang Y, Dai K and Zhang X (2016) IL-12p40 impairs mesenchymal stem cell-mediated bone regeneration via CD4+ T cells. Cell Death Differ 23:1941–1951. doi: 10.1038/cdd.2016.72

Yan B, Li J and Zhang L (2015) Identification of B cells participated in the mechanism of postmenopausal women osteoporosis using microarray analysis. 8:1027–1034.

Yao Y, Jiang C-S, Sun N, Li W-Q, Niu Y, Han H-Q, Miao Z-H, Zhao X-X, Zhao J and Li J (2017) Tamaractam, a New Bioactive Lactam from Tamarix ramosissima, Induces Apoptosis in Rheumatoid Arthritis Fibroblast-Like Synoviocytes. Molecules

22:96. doi: 10.3390/molecules22010096

Ye XL and Lu CF (2013) Association of polymorphisms in the leptin and leptin receptor genes with inflammatory mediators in patients with osteoporosis. Endocrine 44:481–488. doi: 10.1007/s12020-013-9899-9

Yoshiki F, Nishikawa A, Taketsuna M, Kajimoto K and Enomoto H (2016) Efficacy and safety of teriparatide in bisphosphonate-pretreated and treatment-naive patients with osteoporosis at high risk of fracture: Post hoc analysis of a prospective observational study. J Orthop Sci In press. doi: 10.1016/j.jos.2016.11.022

Yuan FL, Li X, Lu WG, Zhao YQ, Li CW, Li JP, Sun JM and Xu RS (2012) Type 17 T-helper cells might be a promising therapeutic target for osteoporosis. Mol Biol Rep 39:771–774. doi: 10.1007/s11033-011-0797-z

Yun gang L, Kang L and Wu G (2016) Fluorosis increases the risk of postmenopausal osteoporosis by stimulating interferon ?? Biochem Biophys Res Commun 479:372–379. doi: 10.1016/j.bbrc.2016.09.083

Zaia A (2015) Fractal lacunarity of trabecular bone and magnetic resonance imaging: New perspectives for osteoporotic fracture risk assessment. World J Orthop 6:221. doi: 10.5312/wjo.v6.i2.221

Zhang J, Fu Q, Ren Z, Wang Y, Wang C, Shen T, Wang G and Wu L (2015a) Changes of serum cytokines-related Th1/Th2/Th17 concentration in patients with postmenopausal osteoporosis. Gynecol Endocrinol 31:183–190. doi: 10.3109/09513590.2014.975683

Zhang L, Fan D, Liu L, Yang T, Ding N, Hu Y, Cai G, Wang L, Xin L, Xia Q et al. (2015b) Association study of IL-12B polymorphisms susceptibility with ankylosing spondylitis in mainland Han population. PLoS One 23;10(6):e0130982. doi: 10.1371/journal.pone.0130982

Zheng H, Wang C, He J-W, Fu W-Z and Zhang Z-L (2015) OPG, RANKL, and RANK gene polymorphisms and the bone mineral density response to alendronate therapy in postmenopausal Chinese women with osteoporosis or osteopenia. Pharmacogenet Genomics 26:2–3. doi: 10.1097/FPC.0000000000000181

Zhou P, Liu H, Liao E, Zhang Z, Chen de C, Liu J, Wu W, Xing X, Xia W, Xu L et al. (2014) LRP5 polymorphisms and response to alendronate treatment in Chinese postmenopausal women with osteoporosis. Pharmacogenomics 15:821–31. doi: 10.2217/pgs.14.12

Zhou P, Xu X, Zhang Z, Liao E, Chen D, Liu J, Wu W, Jiang Y, Wang O, Xia W et al. (2015) SOST polymorphisms and response to alendronate treatment in postmenopausal Chinese women with osteoporosis. Pharmacogenomics 16:1077–1088.

Zupan J, Jeras M and Marc J (2013) Osteoimmunology and the influence of pro-inflammatory cytokines on osteoclasts. Biochem Medica 23:43–63. doi: 10.11613/BM.2013.007

Anexo 1:

Guias de Submissão para os Autores

1-Infammopharmacology.

Disponível em:

https://www.springer.com/biomed/pharmacology+%26+toxicology/journal/10787?detailsPage=pltci_1924431

2-Molecular and Celular Endocrinology.

Disponível em:

https://www.elsevier.com/journals/molecular-and-cellular-endocrinology/0303-7207/guide-for-authors

3- European Journal of Human Genetics.

Disponível em:

http://www.nature.com/ejhg/ejhg_new_gta.pdf

Curriculum vitae (Lattes)

- Formação complementar:
  - Normas da ABNT para teses e dissertações. (Carga horária: 3h). Universidade Federal de Pernambuco, UFPE, Brasil.
  - Extensão universitária em PCR em Tempo Real: Princípios Básicos e Apl. (Carga horária: 60h). Universidade Federal de Pernambuco, UFPE, Brasil.
  - Gerenciamento de Resíduos Químicos e Biológicos de. (Carga horária: 16h). Rede Metrológica de Pernambuco, REMEPE, Brasil.
  - I Curso Teórico-Prático de Cultura de Células. (Carga horária: 40h). Universidade Estadual do Ceará, UECE, Brasil.
  - Curso Geral sobre Propriedade Intelectual. Instituto Nacional da Propriedade Industrial de Portugal, INPI, Portugal.

- Atuação profissional
  - Docente do I Curso de Especialização em Genética Humana (Início em 2017).

- Projetos de pesquisa
  - Avaliação do perfil da resposta pró - inflamatória na fisiopatologia da osteoporose in vitro. Financiador(es): Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco . (2015 - Atual)

- Artigos completos publicados em periódicos
  - TAVARES, M.; **DE LIMA, C**.; FERNANDES, W.; MARTINELLI, V.; DE LUCENA, M.; LIMA, F.; TELLES, A.; BRANDÃO, L.; DE MELO JÚNIOR, M. Tumour necrosis factor-alpha (-308G/A) promoter polymorphism is associated with ulcerative colitis in Brazilian patients. International Journal of Immunogenetics (Print), v. 2016, p. 1-7, 2016.

- Artigos aceitos para publicação
  - **LIMA, C.A.D**; JAVORSKI, N.R; SOUZA, A.P.O.; BARBOSA, A.D.; VALENÇA, A.P.M.C.; CROVELLA, S.; SOUZA, P.R.E.; De AZEVÊDO SILVA, J.; SANDRIN-GARCIA, P. Polymorphisms in key bone modulator cytokines genes influence bisphosphonates therapy in postmenopausal women. Inflammopharmacology, 2017.

- Apresentações de Trabalho
  - **LIMA, C.A.D**.; SOUZA, A.P.O.; JAVORSKI, N. R.; SILVA, H.H.S.A.; ARAGÃO, M.A.L.; GUARANÁ, W.; BARBOSA, A.D.; S; SOUZA, PRE; De AZEVÊDO SILVA, J; SANDRIN-GARCIA, P. Papel do polimorfismo de nucleotídeo único do IFN-γ (rs2069705) na susceptibilidade e progressão da Osteoporose primária pós-menopausa. 2016. Engene.
  - **LIMA, C.A.D**.; SOUZA, A.P.O.; JAVORSKI, N. R.; BARBOSA, A.D; SOUZA, P.E.A.; SOUZA, P.R.E; De AZEVÊDO SILVA, J.; SANDRIN-GARCIA, P. Th1 cytokines profiles in patients with Osteoporosis during anti-catabolic therapy. VIII International Symposium on Diagnostics and Therapeutics. Sinater 2016.

- **LIMA, C.A.D**.; LIMA, S.C.; De AZEVÊDO SILVA, J.A.; SOUZA, A.P.O.; SANDRIN-GARCIA, P. Polimorfismos de um único nucleotídeo e a terapia medicamentosa em pacientes com Osteoporose pós-menopausa V Jornada de Pós-Graduação em Genética PPGG/ UFPE 2015.
- **LIMA, C.A.D**.; CARMO, R.F.; FERNANDES, M.C.M. ; De AZEVÊDO SILVA, J.A. ; SOUZA, P.R.E. ; SANDRIN-GARCIA, P. Evolution of interleukin receptors homologous genes: IL28RA and IL23R. VI International Symposium on Diagnostics and Therapeutics. Sinater 2014.
- **LIMA, C.A.D**.; FERNANDES, M.C.M. ; MARTINELLI, V.F. ; LUCENA, M.T. ; LIMA, F.E.A. ; TELLES, A.M S. ; BRANDÃO, L.A.C. ; MELO JUNIOR, M. R. . Interleukin 10 (-1082G/A) promoter polymorphism is not associated with inflammatory bowel disease. VI International Symposium on Diagnostics and Therapeutics. Sinater 2014.

- Resumos publicados em anais de congresso
  - JAVORSKI, N.R. ; **LIMA, C.A.D.** ; SOUZA, A.P.O.; SANDRIN-GARCIA, P.; De AZEVÊDO SILVA, J. Analysis of candidate polymorphism in MYO9b gene in type 1 diabetes lack of association between MY09b gene polymorphism and susceptibility to type 1 diabetes disease in Pernambuco, Brazil. VIII International Symposium on Diagnostics and Therapeutics. Sinater 2016.
  - SOUZA, A.P.O.; **LIMA, C.A.D**.; JAVORSKI, N. R.; De AZEVÊDO SILVA, J. Interleukin-23 receptor gene polymorphism confers increased susceptibility to autoimmune type 1 diabetes mellitus in brazilian individuals. VIII International Symposium on Diagnostics and Therapeutics. Sinater 2016.

- SOUZA, A.P.O.; **LIMA, C.A.D.**; De AZEVÊDO SILVA, J.; BARBOSA, A.D; RUSHANSKY, E.; SANDRIN-GARCIA, P. Th1 and Th2 cytokine profile levels in patients with rheumatic disease. XLI Congress of the Brazilian Society of Immunology 2016.

- Cursos Ministrados
  - **LIMA, C. A. D.** Cultivo celular em Pesquisa. 2016. (Palestra – I Curso de Inverno de Genética Humana).
  - **LIMA, C. A. D.** Cultivo de Células. 2016. (Curso de curta duração ministrado - I Curso de Inverno de Genética Humana).
  - **LIMA, C. A. D.** Conceitos e procedimentos básicos em cultivo e ensaios celulares. 2015. (Curso de curta duração ministrado – V Jornada de Pós-Graduação em Genética PPGG/ UFPE).

- Participação em bancas de trabalhos de conclusão
  - Monografias de cursos de aperfeiçoamento/especialização
    - De AZEVÊDO SILVA, J. A.; **LIMA, C. A. D**.; SILVA NETO, J. C.. Participação em banca de Ana Carolina de Souza Leitão Arruda Falcão. Análise técnica experimental de DNA a partir de amostras biológicas de células da mucosa jugal e saliva. 2015. Monografia (Aperfeiçoamento/Especialização em Especialização em Biologia Molecular) - Universidade de Pernambuco.

- Trabalhos de conclusão de curso de graduação
    - **LIMA, C.A.D**. Participação em banca de Jorge José de Souza Pereira.Perfil fitoquímico e potencial antioxidante de Commiphora leptophloeos. 2016. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Fundação de Apoio ao Desenvolvimento da Universidade Federal de Pernambuco.
    - **LIMA, C.A.D.; SILVA; R.C**. Participação em banca de José Leandro de Andrade Santos. Análise das frequências do polimorfismo SDF1-3'A e sua possível associação com a susceptibilidade à infecção pelo HIV-1 e a falha imunológica em pacientes vivendo com o HIV-1 em terapia antiretroviral. 2016. Trabalho de Conclusão de Curso (Graduação em Biomedicina) - Universidade Federal de Pernambuco.

- Participação em eventos, congressos, exposições e feiras
    - Avaliador de pôsteres - I Simpósio de Genética e Biotecnologia. 2014. (Simpósio).
    - Ministrante do Minicurso "Biologia Molecular no Diagnóstico" – I Simpósio de Genética e Biotecnologia. 2014. (Simpósio).
    - Avaliador de Trabalhos Científicos - XIV Encontro de Extensão.Universidade/Sociedade em diálogo: Ensino/ Pesquisa/ Extensão: Indissociabilidade possível e necessária. 2014. (Encontro).
    - Avaliador de Trabalhos Científicos - XXII Congresso de Iniciação Científica da UFPE. Análise de trabalhos de Iniciação Científica. 2014. (Congresso).

- Avaliador de Trabalhos Científicos - Universidade/Sociedade em Diálogo: Tradição e Inovação na Extensão Universitária XIII Encontro de Extensão/ Enext. 2013. (Encontro).

- Orientações e supervisões concluídas
  - Natássia Javorski Rodrigues. Polimorfismos no gene receptor de vitamina D (VDR) como fatores de risco para o parto prematuro. 2016. Trabalho de Conclusão de Curso. (Graduação em Ciências Biológicas) - Universidade Federal de Pernambuco. Co-orientador: Camilla Albertina Dantas de Lima.
  - Anna Paula de Oliveira Souza. Análise de polimorfismos de base única (SNPs) nos genes codificantes das citocinas pró-inflamatórias IL-1B, IL-17E e IL-23R no diabetes mellitus tipo 1 infantil. 2016. Trabalho de Conclusão de Curso. (Graduação em Biomedicina) - Universidade Federal de Pernambuco. Co-orientador: Camilla Albertina Dantas de Lima.
  - Thiago Henrique Fragôso de Moura. Comparação da expressão dos genes da enzima conversora de angiotensina e da α-actina-3, do consumo de oxigênio e da velocidade entre atletas treinados e destreinados da categoria sub-17 de futebol do Santa Cruz – PE. (Graduação em Ed. Física) - Universidade Federal de Pernambuco. Co-orientador: Camilla Albertina Dantas de Lima.

- Orientações e supervisões em andamento
  - Natássia Javorski Rodrigues. Análise do efeito da glicose no nível de expressão do receptor DC-SIGN (CD209) in vitro em pacientes com

Diabetes Mellitus tipo 1. 2016. (Mestrado em Genética) - Universidade Federal de Pernambuco. Co-orientador: Camilla Albertina Dantas de Lima.

- Anna Paula de Oliveira Souza. Avaliação do perfil de expressão de genes da via imunomodulatoria do FOXP3 em Diabetes Mellitus Tipo I 2016. (Mestrado em Biologia Aplicada à Saúde) - Universidade Federal de Pernambuco. Co-orientador: Camilla Albertina Dantas de Lima.
- Werbson Lima Guarana. Expressão dos genes LPR5, DKK1 e SOST e sua relação com o tratamento e prevenção de Osteoporose. 2016. (Graduação em andamento). Co-orientador: Camilla Albertina Dantas de Lima.

- Outras informações relevantes
  - Avaliador Ad hoc de ações extensionistas
  - Artigos submetidos:
    - De AZEVÊDO SILVA, J.; BARBOSA, A.D.; **LIMA, C.A.D**; FRAGOSO; T.S.; DUARTE; A.L.B.P.; CROVELLA, S; SANDRIN-GARCIA, P. Vitamin D receptor gene polymorphisms influence on Anti-Osteoporotic therapy, Clinical Profile and Bone Mineral Density at different skeletal sites in Postmenopausal Osteoporotic Women. Submetido em: *Lupus Journal*.
    - **LIMA, C.A.D.**; De AZEVÊDO SILVA, J.; CARVALHO NETO; F.G.; TAVARES, N.A.C.; CROVELLA, S; SOUZA, P.R.E; SANDRIN-GARCIA, P. Comparative methodology for DNA isolation from challenging human biological samples

using Chelex-100®. Submetido em: *Biochemical Genetics Journal.*

- Trabalhos realizados
  - JAVORSKI, N.R.; **LIMA, C.A.D**; SILVA, L.V.C.; CROVELLA, S; SANDRIN-GARCIA, P.; De AZEVÊDO SILVA, J. Vitamin D receptor (VDR) polymorphisms are associated to spontaneous preterm birth and maternal aspects. – Artigo em Revisão para submissão
  - RUSHANSKY, E.; **LIMA, C.A.D.;** ADELINO, J.E.; SOUZA, A.P.O.; SANTOS, P.E.A.; MARIANO, M.H.Q.A.; CROVELLA, S.; De AZEVÊDO SILVA, J.; SANDRIN-GARCIA, P. Genetic variants in promoter regions of IL10, IL6 and IFN-γ genes and their effects in cytokine levels and clinical severity status in rheumatoid arthritis patients – Artigo em Revisão para submissão.