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ESTUDO DOS MECANISMOS ENVOLVIDOS NA REABSORÇÃO ÓSSEA ALVEOLAR INDUZIDA PELA DEFICIÊNCIA DE ESTRÓGENO.

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RESUMO

INTRODUÇÃO: Durante a menopausa, a redução dos níveis de estradiol (E2) pode acarretar um processo de osteoporose. Embora esteja bem estabelecido que o E2 apresenta efeitos diretos sobre as células ósseas, seu mecanismo de ação não está completamente esclarecido. Considerando-se que o processo de formação/reabsorção óssea é também influenciado por citocinas e quimiocinas, fica clara a necessidade de avaliar a relação entre o E2 e estas moléculas no controle da homeostase óssea e em processos patológicos, como a osteoporose. **OBJETIVOS:** 1. Avaliar o efeito da redução do E2 na perda óssea alveolar e se a reposição com E2 leva à modificação do fenótipo; 2. Avaliar o efeito da deleção do receptor de estrogênio ERα na reabsorção óssea alveolar e mecanicamente induzida; 3. Avaliar o efeito da deleção do receptor de IL-33, ST2, na reabsorção óssea alveolar associada a ovariectomia (OVX) e mecanicamente induzida. **MÉTODOS:** *Objetivo 1*. Foi realizada OVX e reposição hormonal com 17β-estradiol (E2) em animais C57BL/6 e Balb/c. Animais intactos foram utilizados como controle. Foi realizada a eutanásia após períodos de 15 e 30 dias para coleta dos ossos maxilares para avaliação por microtomografia computadorizada (microCT) e ELISA e fêmures para análise histomorfométrica; *Objetivo 2*. O osso alveolar de animais fêmeas e machos homozigotos $ER\alpha^{+/+}$ (*wild type* - WT) and $ER\alpha^{-/-}$ (ERKOα - deficiente para o receptor de estrogênio alfa) submetidos ou não à força ortodôntica, foram analisados empregando-se microCT, RT-PCR e espectroscopia de energia dispersiva (EDS). Células de medula óssea (CMO) dos animais WT e ERKOα foram utilizadas para obtenção de culturas de osteoblastos e osteoclastos; *Objetivo 3*. OVX e reposição hormonal com mini-implantes contendo E2 foram realizadas em animais Balb/c (WT) e deficientes para o receptor de IL-33 ($ST2^{-/-}$). Ossos maxilares submetidos ou não à movimentação ortodôntica foram analisados por meio de análise histológica, histomorfométrica, RT-PCR e CMO foram obtidas para diferenciação em osteoclastos. **RESULTADOS:** *Objetivo 1*. As análises por microCT demonstraram que a OVX resultou em diminuição da espessura, densidade e volume ósseo, assim como aumento da separação do osso trabecular. Houve também redução da crista óssea alveolar associada a OVX. Estes efeitos foram associados à redução da porcentagem de osso trabeculado e espessura cortical do fêmur. A reposição hormonal com E2 reverteu o fenótipo ósseo observado nos ossos alveolares e fêmur após OVX. Verificamos ainda, aumento da expressão de TNF-α e da razão RANKL/OPG nos tecidos periodontais após OVX, o qual também foi revertido pela reposição com E2; *Objetivo 2*. Animais fêmeas e machos ERKOα demonstraram aumento da perda óssea alveolar e movimentação

ortodôntica dentária (OTM) associado com a diminuição da porcentagem de cálcio e aumento da expressão de IL-33 no periodonto. Ambos os sexos ERKOα demonstraram fenótipo ósseo osteoporótico nos fêmures e vértebras. Resultados *in vitro* mostraram aumento da diferenciação de osteoclastos e de osteoblastos em CMO obtidas dos animais ERKOα quando comparados aos WT; *Objetivo 3*. A deficiência do receptor de IL-33, ST2, resultou em perda óssea significativa no fêmur e maxila. Animais WT e deficientes do receptor de ST2, exibiram similar perda óssea no fêmur após OVX. Por outro lado, a estrutura do osso maxilar dos camundongos $ST2^{-/-}$ não foi afetada pela OVX. Além disso, em condições de estímulo mecânico, E2 e ST2 individualmente proporcionaram "osteoproteção", porém na ausência de ambos (camundongos $ST2^{-/-}$ submetidos a OVX) este efeito não foi observado. Os experimentos *in vitro* confirmaram os efeitos anti-osteoclastogênicos da IL-33 e E2, entretanto este resultado não foi observado quando o tratamento com IL-33 foi realizado em células provenientes de animais OVX. **CONCLUSÕES:** A falta de estrogênio acarreta perda óssea alveolar com aumento da expressão de TNF-α e da razão RANKL/OPG. Nossos resultados permitem sugerir que o efeito do E2 no osso alveolar ocorre via ERα e que a perda óssea alveolar, causada pela falta de estrogênio, pode estar relacionada ao aumento de IL-33. Os efeitos "osteoprotetores" de IL-33/ST2 no osso alveolar, por sua vez, não são observados em condições de redução de E2.

ABSTRACT

INTRODUCTION: Throughout the immediate postmenopausal first years, decreased estrogen levels lead to rapid bone loss that may lead to an osteoporosis process. Estradiol (E2) mechanism of action remains unclear, despite of it well-established effect of on bone cells. Bone remodeling/resorption also depends on cytokines and chemokines, thus it is clear the necessity to better understand the relationship between E2 and chemokines in pathologic condition as osteoporosis. **PURPOSE:** 1. To evaluate the effect of estrogen-deficiency and E2 replacement in the mice alveolar bone microarchitecture; 2. To evaluate the effect of estrogen receptor ERα in alveolar bone and mechanical loading-induced bone remodeling; 3. To determine the effect of ST2/IL-33 in alveolar bone loss by ovariectomy (OVX) and mechanical loading-induced bone remodeling. **METHODOS:** *Purpose 1*. C57BL6/J and Balb/c mice were OVX and implanted with oil- (OVX) or 17β-estradiol (E2)-containing (OVX+E2) capsules. Ovary-intact mice were used as controls. Euthanasia was achieved 15 and 30 days after OVX and maxillary bone were collected for micro computed tomography (microCT) analysis and ELISA and femur for histomorphometric analysis; *Purpose 2*. The alveolar bone and mechanical loading induced bone of females and males homozygote $ER\alpha^{+/+}$ (wild type - WT) and $ER\alpha^{-/-}$ (ERKOα - estrogen receptor α knockout) mice were submitted to microCT, RT-PCR and energy dispersive spectroscopy (EDS). WT and ERKOα mice bone marrow cells (BMC) were differentiated into osteoblasts and osteoclasts cell culture; *Purpose 3*. Balb/c (Wilde-type-WT) and $ST2^{-/-}$ mice were ovariectomized and implanted with oil- (OVX) or E2-containing capsules (OVX+E2). Maxillary bones submitted or not to mechanical loading were analyzed by histology and histomorphometric analysis, RT-PCR and bone marrow cells (BMC) were isolated to osteoclasts differentiation. **RESULTS:** *Purpose 1*. As determined by maxillary alveolar bone microCT analysis, OVX mice displayed decreased trabecular thickness, bone density and bone volume, and increased trabecular separation. A significant loss of alveolar bone crest was also associated with ovariectomy. These effects were associated with a reduction of trabecular bone percentage and cortical thickness in the femur. The E2 replacement fully prevented ovariectomy-induced alterations in the alveolar and femoral bones. Moreover, TNF-α levels and RANKL/OPG ratio were increased in the maxilla after OVX, and these responses were also reversed by E2. *Purpose 2*. Maxillay alveolar bone loss and orthodontic tooth movement (OTM) were augmented in female and male ERKOα mice and associated with decreased calcium percentage levels and increased expression IL-33 in periodontium. Both genders ERKOα

demonstrated an osteoporotic phenotype in the femur and vertebrae. *In vitro* results showed increased osteoclasts and osteoblasts differentiation from BMC in ERKOα mice compared to WT mice; *Purpose 3*. IL-33 receptor deficiency, ST2, caused bone loss in femur and maxillary bone. Similar effects of OVX (loss of bone mass) were observed in long bones of WT and ST2 deficient mice. However, the ovariectomized $ST2^{-/-}$ mice maxillary bone did not exhibit bone loss. Moreover, under mechanical loading-induced bone remodeling, E2 and ST2 individually yielded bone protection, but the phenotype was reversed by their complete absence ($ST2^{-/-}$ OVX mice). *In vitro* results confirmed that E2 and IL-33 were able to suppress osteoclasts formation. Nevertheless, when BMC were taken from OVX mice, IL-33 treatment did not affect osteoclasts differentiation. **CONCLUSION:** Estrogen lack will lead to alveolar bone loss in maxillary bone with TNF-α and RANKL/OPG ratio increase. Our results suggest that E2 acts through ERα in the alveolar bone and that maxillary alveolar bone loss, caused by estrogen lack, might be associated with increased IL-33 levels. However, the "osteoprotective" effect of IL-33/ST2 in alveolar bone is not observed under the condition of estrogen deficiency.

LISTA DE ABREVIATURAS

ABC - *Alveolar bone crest*

ALP - *Alkaline Phosphatase*

ANOVA - *One-way Analysis of Variance*

BMD - *Bone Mineral Density*

BV - *Bone Volume*

BV/TV% - *Percent Bone Volume*

Ca^{2+} - *Calcium*

Cbfa1 - *Core-binding factor α1*

CCL2 - *Monocyte chemotactic protein-1 (MCP-1)*

CCL3 - *Macrophage inflammatory protein-1α (MIP-1α)*

CCR2 - *C-C chemokine receptor type 2*

cDNA - *Complementary DNA*

CEJ - *Cementum-enamel-junction*

Col1A1 - *Collagen Type I, Alpha 1*

Cs.Th - *Cortical Thickness*

DMEM - *Dulbecco's Modified Eagle Medium*

E2 - *estradiol*

EDTA - *Ethylenediamine Tetraacetic Acid*

ELISA - *Enzyme-Linked Immunosorbent Assay*

ERα - *Estrogen receptor alpha*

ERKO - *Estrogen receptor alpha knockout mice*

FBS - *Fetal Bovine Serum*

g - *Gram*

IL-1 - *Interleukin-1*

IL-10 - *Interleukin-10*

IL-33 - *Interlukin-33*

LPS - *Lipopolysaccharides*

M-CSF - *Macrophage stimulating-colony factor*

MicroCT - *Microcomputed tomography*

N - *Newton*

NFATc1 - *Nuclear Factor of Activated T-cells Cytoplasmic Calcineurin-dependent 1*

Ni-Ti - *Nickel-Titanium*

OCN - *Osteocalcin*

OPG - *Osteoprotegerin*

OTM - *Orthodontic Tooth Movement*

PBS - *Phosphate Buffered Saline*

PCR - *Polymerase Chain Reaction*

R - *Root*

RANK - *Activator of Nuclear Factor Kappa-B*

RANKL - *Activator of Nuclear Factor Kappa-B ligand*

Rpm - *Rotation per minute*

RT-PCR - *Real Time-Polymerase Chain Reaction*

RUNX2 - *Runt-related transcription factor* 2

Sema 3A - *Semaphorin-3A*

S.C. - *Subcutaneous injection*

SMI - *Structure model index*

$ST2^{-/-}$ - *ST2 knockout mice*

Tb.N - *Trabecular Number*

Tb.Sp - *Trabecular Separation*

Tb.Th - *Trabecular Thickness*

TNF-α - *Tumor Necrosis Factor-alpha*

TRAF6 - *Tumor Necrosis Factor (TNF) receptor associated factor 6*

TRAP - *Tartrate-resistant Acid Phosphatase*

WT - *Wild-type*

SUMÁRIO

1. SÍNTESE BIBLIOGRÁFICA

Tecido ósseo e suas células

O osso é composto por aproximadamente 10% de células, 60% de matriz mineralizada (composta principalmente por cálcio e fósforo em forma de cristais de hidroxiapatita [Ca10(PO4)6(OH)2]) e 30% de matriz orgânica (fibras colágenas do tipo I, glicosaminoglicanas, lipídios e outras proteínas). O osso possui três funções vitais: (1) promover suporte e local de adesão aos músculos, (2) proteger órgãos vitais como medula óssea e cérebro, e (3) atuar como a maior reserva de cálcio e fósforo (Feng and McDonald, 2011).

O osso é um tecido altamente dinâmico que está em constante processo de remodelação para manutenção da saúde do esqueleto. O processo de remodelação é coordenado por diversos fatores locais e sistêmicos sendo assim de suma importância a compreensão do mecanismo envolvido na diferenciação, recrutamento e ativação das células ósseas que são os osteoclastos, osteoblastos e osteócitos (Eriksen, 2010; Henriksen *et al.*, 2011; Raggatt and Partridge, 2010; Rochefort *et al.*, 2010).

Os osteoclastos, células responsáveis pela reabsorção óssea, são de origem hematopoiética e se formam à partir da fusão de células mononucleares progenitoras da linhagem monócito-macrófago (Teitelbaum, 2000). Estas células expressam fosfatase ácida resistente ao tartarato (TRAP) (Faust *et al.*, 1999; Henriksen *et al.*, 2011; Liu *et al.*, 2003), catepsina K e metaloproteinases, que participam da degradação de colágeno tipo I da matriz óssea (Nakamura *et al.*, 2004). O osteoclasto maduro adere intimamente ao osso, selando completamente a superfície de contato membrana/osso. Quando ativado, enzimas hidrolíticas e ácido clorídrico são secretados para a dissolução da matriz mineralizada (Raggatt and Partridge, 2010).

Mecanismos de sinalização diretos ou indiretos dos osteoblastos regulam o processo de diferenciação, recrutamento e ativação dos osteoclastos. Duas citocinas que são o fator estimulador de colônia de macrófagos (M-CSF), expresso por osteoblastos e células estromais, e o ligante do receptor ativador de NF-kappa-B (RANKL), expresso por osteoblastos e linfócitos, são os principais reguladores da diferenciação de monócitos mononucleares em osteoclastos maduros (Tolar *et al.*, 2004). Este processo ocorre quando M-CSF e RANKL ligam-se aos seus respectivos receptores, receptor fator estimulador de colônia-1 (c-Fms) e receptor ativador do NF-kappa B (RANK), respectivamente, expressos nos precursores de osteoclastos (Boyce *et al.*, 2012).

O osteoblasto pode também enviar estímulos inibitórios a reabsorção óssea ao expressar a osteoprotegerina (OPG), que age como receptor solúvel de RANKL e, como consequência, interrompe o sinal pró-osteoclástico (Eriksen, 2010; Yamaguchi, 2009). Além disso, a OPG contribui para a inibição da reabsorção óssea por meio da inibição da fase terminal de diferenciação dos osteoclastos e induz sua apoptose (Theoleyre *et al.*, 2004a; Theoleyre *et al.*, 2004b). Portanto, pode-se afirmar que a interação RANK-RANKL-OPG é a principal via de remodelação óssea.

Os osteoblastos originam-se de células mesenquimais pluripotentes indiferenciadas da medula óssea e produzem diversas proteínas como o colágeno tipo I (COL-I), osteocalcina (OCN) e fosfatase alcalina (ALP), as quais são empregadas como marcadores de diferenciação e atividade destas células (Katagiri and Takahashi, 2002). Vários fatores de transcrição específicos são responsáveis pela diferenciação das células mesenquimais em osteoblastos. O Cbfa1 (*core-binding factor α1*) é um dos mais importantes para o processo de diferenciação, embora não seja suficiente para a completa maturação do osteoblasto. Outro fator de transcrição que está presente na fase inicial de diferenciação dos osteoblastos é o Runx-2 (*Runt-related transcription factor* 2), que está envolvido na síntese das proteínas da

matriz óssea e regula positivamente a expressão dos genes de colágeno tipo I, osteopontina, sialoproteinase óssea e osteocalcina guiando a formação óssea (Harada and Rodan, 2003; Neve *et al.*, 2011). O recrutamento, diferenciação e ativação dos osteoblastos são controlados por diversos fatores locais e sistêmicos como hormônios, a via canônica Wingless (Wnt)/ beta-catenina, o fator de crescimento semelhante à insulina tipo 1 (IGF-1) e forças mecânicas (Neve *et al.*, 2011).

O controle da diferenciação dos osteoblastos, via a expressão de Runx2, reflete diretamente na diferenciação dos osteoclastos e controle da reabsorção óssea (Baniwal *et al.*, 2012). Adicionalmente, estudos demonstram que osteoblastos, estimulados por interleucina-1 (IL-1) e fator de necrose tumoral-α (TNF-α), são fontes de quimiocinas como proteína quimiotática para monócitos–1 (MCP-1/CCL2), proteína inflamatória de macrófagos-1α (MIP-1α/CCL3) e quimiocina regulada sob ativação normalmente expressada e secretada por células T (RANTES/CCL5). Estas quimiocinas por sua vez, atuam no recrutamento dos precursores de osteoclastos para o sítio de reabsorção óssea, no qual irão se diferenciar em osteoclastos maduros (Kim *et al.*, 2006; Yano *et al.*, 2005; Yu *et al.*, 2004). Além disso, prostaglandinas (PGE2) e citocinas, tais como IL-1, IL-6, IL-8 e TNF-α, estimulam os osteoblastos a produzirem os principais reguladores da diferenciação de osteoclastos: o M-CSF e RANKL (Boyce *et al.*, 2012; Huang *et al.*, 2006).

Os osteócitos são as células mais numerosas no tecido ósseo e estão localizadas no interior da matriz óssea. No decorrer do processo de formação óssea, os osteoblastos aprisonam-se na matriz recém sintetizada, transformando-se assim em osteócitos (Rochefort *et al.*, 2010). A literatura é controversa em relação à função destas células porém, estudos indicam que (1) os osteócitos estão envolvidos no *turnover* ósseo, (2) participa na troca de íons por meio de sua vasta rede de células e (3) atua como células mecano-sensoriais realizando a transdução de sinais e assim apresentando papel fundamental no processo de

adaptação funcional e início da remodelação óssea (Atkins *et al.*, 2014). As vias de sinalização dos osteócitos ocorrem via geração de óxido nítrico e prostaglandinas assim como por meio de junções tipo *gap* (Heuck, 1970). Os osteócitos podem direcionar a remoção de danos teciduais por meio de mecanismos envolvendo sua apoptose ou por via de secreção de proteínas especializadas como a osteopontina (Aarden *et al.*, 1994). Receptores de hormônios da paratireóide e receptores de estrogênio alfa (α) e beta (β) são expressos nos osteócitos que contribuem para a modulação de sua via de sinalização (Aarden *et al.*, 1994; Atkins *et al.*, 2014).

Desta forma, podemos dizer que a reabsorção/formação óssea por osteoclastos, osteoblastos e osteócitos é normalmente um processo bem coordenado e regulado por fatores de crescimento, citocinas e hormônios que controlam a proliferação, recrutamento, diferenciação, atividade e sobrevivência das células precursoras. Entretanto, doenças inflamatórias dos ossos e outras doenças esqueléticas apresentam um desequilíbrio nesta regulação que leva à reabsorção óssea excessiva e destruição tecidual, como observado na osteoporose (Teitelbaum, 2000), artrite reumatóide (Danks *et al.*, 2002; Firestein and Zvaifler, 2002; Goldring, 2003; Katrib *et al.*, 2002), doença periodontal (Ejeil *et al.*, 2003; Nair *et al.*, 1996), osteomielite (Kumta *et al.*, 2003; O'Keefe *et al.*, 1997) e tumores ósseos (Kumta *et al.*, 2003; O'Keefe *et al.*, 1997; Yasko, 2002). O conhecimento do processo de remodelação pelas células ósseas é fundamental para avaliar a eficácia de terapias para inibir a perda óssea ou interferir com os estímulos osteoclastogênicos (Stepan *et al.*, 2003; Tolar *et al.*, 2004).

Remodelação óssea

Podemos classificar os ossos basicamente em dois tipos: cortical e trabeculado, sendo este último o local onde ocorre a maior parte das remodelações ósseas. A remodelação é um

processo fisiológico que cosiste na reabsorção óssea, promovida por osteoclastos, e formação óssea realizada por osteoblastos (Eriksen, 2010). Todos os ossos do esqueleto sofrem remodelação, incluindo o osso alveolar presente ao redor dos dentes da maxila e mandíbula. A remodelação do osso alveolar possibilita a erupção/movimentação dentária e regeneração tecidual após cirurgias e infecções periodontais (Melsen, 1999; Sodek and McKee, 2000).

O movimento dentário ortodôntico é realizado pelo remodelamento do osso alveolar em resposta à força mecânica (Krishnan and Davidovitch, 2006). Esta alteração na região do ligamento periodontal desencadeia uma resposta inflamatória aguda com a liberação de mediadores químicos, tais como: fatores de crescimento, citocinas, quimiocinas e metabólitos do ácido araquidônico (Garlet *et al.*, 2008). Estes fatores podem atuar conjuntamente para promover o recrutamento e diferenciação de osteoclastos e osteoblastos para áreas de compressão (reabsorção óssea) e tensão (formação óssea) geradas na movimentação dentária (Garlet *et al.*, 2008; Krishnan and Davidovitch, 2006).

A movimentação dentária induzida por aparelho ortodôntico é dividida didaticamente em três fases: inicial, platô e pós-platô (Smith and Burstone, 1984). A fase inicial é caracterizada pela rápida movimentação do dente após a aplicação da força, que pode ser atribuída pelo deslocamento dentário no espaço do ligamento periodontal (LP) (Wise and King, 2008). A seguir, o platô apresenta baixas taxas ou nenhuma movimentação dentária devido a formação de áreas de hialinização no LP no lado de compressão. A movimentação dental irá ocorrer somente após a completa remoção do tecido hialinizado (necrótico), caracterizando a terceira fase pós-platô (Krishnan and Davidovitch, 2006; Pilon *et al.*, 1996).

Na fase inicial da movimentação ortodôntica a compressão e estiramento das fibras e células do LP, além da mecanotransdução de sinais realizada pelos osteócitos, levam ao início das respostas celulares com recrutamento dos precursores de osteoclastos e osteoblastos assim como produção de citocinas inflamatórias. Durante a fase de platô a área

de compressão é facilmente reconhecida pela distorção da estrutura das fibras do LP. Esta alteração estrutural gera interrupção do fluxo sanguíneo local e formação de áreas hialinizadas que poderão permanecer por período de 4 a 20 dias (Pilon *et al.*, 1996; Sandy *et al.*, 1993). Os osteoclastos e macrófagos são os responsáveis pela reabsorção óssea e remoção do tecido hialinizado, respectivamente. Na área de tensão ocorre depósito de matriz óssea (tecido osteóide) pelos osteoblastos. A última fase, pós-platô, é caracterizada pela presença de superfície óssea irregular no lado de pressão, enquanto que no lado de tensão há formação de tecido ósseo (Krishnan and Davidovitch, 2006; Wise and King, 2008).

A movimentação dentária ortodôntica, assim como a remodelação óssea são realizadas por meio de uma estrutura anatômica e funcional denominada unidade básica multicelular (BMU) que requer a ação coordenada de todas as células ósseas (Henriksen *et al.*, 2011). A superfície óssea, é recoberta por uma camada única de células de revestimento chamada células de revestimento ósseo. A remodelação pode ser dividida em fases que se superpõe iniciada com (1) os osteócitos que são o pivô da reabsorção e início da transdução de sinais; (2) posteriormente ocorre o recrutamento dos precursores de osteoclastos, sua maturação e início da reabsorção; (3) osteoblastos diferenciados e maduros depositam a matriz orgânica (tecido osteóide); e (4) ocorre mineralização do osteóide e a superfície óssea é novamente recoberta pela camada de *bone-lining cells* finalizando o processo de remodelação (Boyce *et al.*, 2012; Eriksen, 2010; Feng and McDonald, 2011; Raggatt and Partridge, 2010).

Compreender a biologia básica da remodelação óssea é um fator importante para o esclarecimento dos mecanismos celulares e moleculares envolvidos em doenças associadas à desordens ósseas. No caso da osteoporose em mulheres no período pós-menopausa, ocorre aumento da frequência de ativação das BMUs, além dos efeitos de vários fatores sistêmicos e

locais que interferem na diferenciação, função e sobrevida das células ósseas (Imai *et al.*, 2010; Lee *et al.*, 2004; Lerner, 2006).

Na década de 40, Fuller Albright foi o primeiro autor a relatar que a falta de estrogênio em mulheres estava diretamente associado à perda óssea e desenvolvimento de osteoporose. À partir de então, muitos estudos realizados verificaram que o estrogênio é um importante regulador do metabolismo ósseo, não só em mulheres mas também em homens (Aguirre *et al.*, 2007; Almeida *et al.*, 2013; Cenci *et al.*, 2003; Imai *et al.*, 2010; Manolagas *et al.*, 2013; Nakamura *et al.*, 2007; Novack, 2007). Desta forma, há a necessidade de mais estudos para compreendermos melhor os processos moleculares envolvidos na osteoporose, principalmente em relação ao osso alveolar, o que poderá contribuir para novos alvos para intervenções terapêuticas.

Estrogênio

O estrogênio é um hormônio esteroidal, sintetizado à partir de andrógenos por meio da enzima aromatase. Nas mulheres, a síntese ocorre nos ovários de forma cíclica (Jones *et al.*, 2007; Meinhardt and Mullis, 2002). Nos homens sua produção ocorre em vários tecidos de forma localizada como exemplo os testículos (Gennari *et al.*, 2004; Pino *et al.*, 2006).

Estrogênio é um nome dado a um grupo de hormônios que são o 17β-estradiol (E2), estriol (E3) e estrona (E1), sendo que o estradiol é o mais potente e está presente na adolescência e em toda fase reprodutiva da mulher (Fang *et al.*, 2001). Para que ocorra a transformação dos andrógenos em estrogênio é necessário a atuação da enzima aromatase. Uma vez finalizada a síntese do estrogênio, este é liberado na corrente sanguínea e entrará de forma passiva nas células se ligando a seus receptores denominados receptor de estrogênio alfa e beta (ERα e ERβ). Estes receptores se translocam para o núcleo e funcionam como fatores de transcrição (Krum, 2011; Syed *et al.*, 2005; Weitzmann and Pacifici, 2006).

O estrogênio apresenta papel fundamental na manutenção da homeostase óssea. No osso os osteoblastos e osteócitos expressam a aromatase (Miki *et al.*, 2007; Sjogren *et al.*, 2009). Antes de 1987, as células ósseas não eram consideradas alvo direto do estrogênio. Entretanto, atualmente está bem definido que os osteoblastos (Komm *et al.*, 1988), osteócitos (Tomkinson *et al.*, 1998) e osteoclastos (Imai *et al.*, 2009; Imai *et al.*, 2010) expressam receptor para estrogênio (ERs). Estes receptores são também expressos nas células da medula óssea e nos precursores de osteoblastos, os quais proporcionam "suporte" para a formação de osteoclastos, células T, células B e muitas outras células na medula óssea de humanos e camundongos (Weitzmann and Pacifici, 2005a; b; 2006).

O estrogênio sinaliza por meio de dois subtipos de receptores: ERα e ERβ (Kuiper *et al.*, 1996). Ambos ERα e ERβ são expressos nas células da medula óssea (Bord *et al.*, 2001; Ramalho *et al.*, 2002), osteoblastos (Eriksen *et al.*, 1988; Komm *et al.*, 1988; Onoe *et al.*, 1997), osteócitos (Windahl *et al.*, 2013) e osteoclastos (Imai *et al.*, 2009; Imai *et al.*, 2010; Pensler *et al.*, 1990) e em seus precursores mononucleares (Huang *et al.*, 1998; Oreffo *et al.*, 1999a; Oreffo *et al.*, 1999b). Em humanos, Bord *et al.* (2001) verifiou que o ERα predomina no osso cortical, enquanto que o ERβ no osso trabecular. Em geral, considera-se que o ERα está envolvido na maioria das funções das células ósseas (Barkhem *et al.*, 1998; Hall and McDonnell, 1999).

Após se ligar a seus receptores o estrogênio atua na estimulação da atividade osteogênica (Qu *et al.*, 1998; Zhou *et al.*, 2001) e efeitos anti-apoptóticos nos osteoblastos (Gohel *et al.*, 1999; Kousteni *et al.*, 2001; Kousteni *et al.*, 2007; Zhou *et al.*, 2001), promovendo a sobrevida dos osteoblastos e a apoptose dos osteoclastos, favorecendo desse modo a formação óssea em detrimento da reabsorção. Também está bem determinado que a formação dos osteoclastos à partir de células mononuclares hematopoiéticas é inibida pelo estrogênio (Jilka *et al.*, 1992; Srivastava *et al.*, 1998; Srivastava *et al.*, 2001).

Adicionalmente, a atividade de reabsorção óssea de osteoclastos maduros é inibida pelo estrogênio (Parikka *et al.*, 2005), o qual proporciona redução da lacuna de reabsorção (Parikka *et al.*, 2001).

Embora esteja bem estabelecido que o estrogênio apresente efeitos diretos sobre as células ósseas, seu mecanismo de ação ainda não está completamente esclarecido. Aparentemente, o principal mecanismo pelo qual o estrogênio previne a perda óssea é pela inibição da osteoclastogênese (Manolagas *et al.*, 1993; Manolagas and Jilka, 1995; Nakamura *et al.*, 2007; Pacifici, 1996; 1998). A consequência mais relevante da deficiência do estrogênio devido à ovariectomia é a elevada formação de osteoclastos (Weitzmann and Pacifici, 2005a; 2006). Um dos mecanismos pelo qual o estrogênio atua reduzindo a osteoclastogênese é pela diminuição da resposta dos precursores de osteoclastos ao RANKL (Manolagas *et al.*, 2013; Srivastava *et al.*, 2001).

Em camundongos fêmeas ovariectomizadas (modelo de osteoporose pós-menopausa) ocorre aumento da expressão de CCR2 nos pré-osteoclastos, aumentando assim a expressão de RANK nestas células e seu potencial osteoclastogênico, enquanto que os camundongos deficientes de receptores CCR2 são resistentes à perda óssea induzida pela ovariectomia (Binder *et al.*, 2009).

Evidências consideráveis suportam a hipótese de que o estrogênio reduz a formação de osteoclastos pela diminuição da produção de citocinas IL-1, IL-6 e TNF-α (Ammann *et al.*, 1997; Lee *et al.*, 2006; Lorenzo *et al.*, 1998; Manolagas and Jilka, 1995; Pacifici, 1996; Poli *et al.*, 1994), as quais aumentam a produção de RANKL e M-CSF pelas células mesenquimais (Hofbauer *et al.*, 1999a; Hofbauer *et al.*, 1999b; Kimble *et al.*, 1996; Srivastava *et al.*, 1998; Taguchi *et al.*, 1998). Outros estudos demonstram que o estrogênio previne a perda óssea via receptor ERα e indução de apoptose via Fas ligante nos osteoclastos (Nakamura *et al.*, 2007). Mecanismos adicionais que podem explicar os efeitos

antiosteoclastogênicos do estrogênio incluem a habilidade dos hormônios esteroidais estimularem a produção de OPG (Hofbauer *et al.*, 1999a), e um efeito inibitório direto de ligação do M-CSF (Lea *et al.*, 1999; Sarma *et al.*, 1998).

Além disso, sabe-se que a deficiência de estrogênio acelera a movimentação ortodôntica (Tan *et al.*, 2009; Xu *et al.*, 2010; Yamashiro and Takano-Yamamoto, 2001) e tratamentos ortodônticos em mulheres e homens com idades mais avançadas necessitam maior atenção, pois a deficiencia de estrogênio nesta faixa etária poderá interferir com os resultados do tratamento (Miyajima *et al.*, 1996).

A perda óssea induzida pela deficiência de estrogênio é causada modificações no processo de remodelação óssea (Weitzmann and Pacifici, 2006). A deficiência de estrogênio leva ao aumento da expressão de IL-7 nos ossos, timo e fígado, mediada pela diminuição da síntese do fator de transformação do crescimento (TGF-β) e aumento de IGF-1 (Ryan *et al.*, 2005; Toraldo *et al.*, 2003). Consequentemente, ocorre a ativação dos linfócitos T que liberam interferon gama (IFN-γ) aumentando o recrutamento de macrófagos (Cenci *et al.*, 2003; Roggia *et al.*, 2001). A deficiência de estrogênio também amplifica a osteoclastogênese pela inibição de vias antioxidantes, gerando aumento da expressão de espécies reativas de oxigênio (ROS) (Jagger *et al.*, 2005; Muthusami *et al.*, 2005). Em virtude do aumento de ROS, osteoclastos maduros aumentam sua expressão de TNF-α, que estimulam a produção de RANKL e M-CSF, levando a formação de osteoclastos (Srivastava *et al.*, 2001; Zhang *et al.*, 2001). Além disso, os efeitos de IFN-γ e ROS associados amplificam a ativação dos linfócitos T com aumento da produção de RANKL (Gao *et al.*, 2007).

Sendo assim, dentro do contexto de remodelação óssea e moléculas inflamatórias, alguns estudos sugerem que a IL-1 (Salla *et al.*, 2012) e as quimiocinas CC apresentam funções importantes na remodelação do osso alveolar induzida por força ortodôntica em animais (Andrade *et al.*, 2007; Andrade *et al.*, 2009; Taddei *et al.*, 2012; Taddei *et al.*, 2013)

e em humanos (Garlet *et al.*, 2008). Entretanto, pouco se sabe sobre quais citocinas/interleucinas e seus receptores correspondentes estão envolvidos na reabsorção e formação óssea no osso alveolar em situações de deficiência de estrogênio.

IL-33 e seu receptor ST2

A interleucina-33 (IL-33), membro da família IL-1, é expressa por diversos tipos celulares decorrente de estímulos pró-inflamatórios e acredita-se que sua liberação ocorra durante a lise celular. O receptor de IL-33 que consiste na associação do receptor ST2 e proteína acessória para o receptor IL-1, é amplamente expresso por células T helper 2 (TH2) e mastócitos. IL-33 é protetor contra infecções por helmintos e reduz aterosclerose ativando a resposta imunológica TH2. Entretanto, a IL-33 é responsável por promover a patogênese da asma por meio da expansão das células TH2 e mediar inflamações articulares, dermatites atópicas e choques anafiláticos pela ativação dos mastócitos (Liew *et al.*, 2010).

Schulze *et al.* (2011) demonstraram que a IL-33 está expressa nos osteoblastos durante o processo de formação óssea sendo capaz de inibir a formação de osteoclastos. Estes e outros autores verificaram também que animais deficientes para o receptor ST2 apresentaram menor massa óssea trabecular com aumento da osteoclastogênese (Keller *et al.*, 2012; Schulze *et al.*, 2011). Além disso, a super expressão de IL-33 em osteoblastos transgênicos resulta na diminuição da osteoclastogênese (Keller *et al.*, 2012).

Dados na literatura demonstraram que os níveis de IL-33, em cultura de células primárias de osteoblastos, aumentaram na presença do hormônio da paratireóide (Saleh *et al.*, 2011). Este aumento levaria à inibição da osteoclastogênese e estímulo da formação de osteoblastos com a redução dos níveis de esclerotina, sendo esta inibidora da sinalização Wnt nos osteoblastos. A IL-33 também induz o aumento de IL-4, IL-13, IL-10 e GM-CSF, sendo

que somente a combinação das quatro levaria à ação inibitória dos osteoclastos (Saleh *et al.*, 2011; Zaiss *et al.*, 2011).

Estudo realizado em animais super expressando TNF e que apresentavam inflamação articular espontânea demonstraram que a administração de IL-33 reduziu a destruição da cartilagem, perda óssea na tíbia e diferenciação de osteoclastos (Zaiss *et al.*, 2011), demonstrando assim os efeitos protetores de IL-33 no osso articular.

No entanto, contradizendo os estudos anteriores, Mun *et al.* (2010) verificou que a IL-33 estimula a formação de osteoclastos mediada por TRAF6 e atua de forma independente da via RANKL/OPG, atuando assim como uma interleucina pró-reasorptiva.

Entretanto, não há estudos analisando o papel da IL-33 no tecido ósseo alveolar durante o processo de remodelação. Além disso, a necessidade de compreensão do efeito da IL-33 em condições de doença, como a osteoporose, foram os fatores que motivaram o início deste trabalho.

A necessidade de compreender se a falta de estrogênio acarreta perda óssea alveolar e se a mesma está relacionada ao receptor Erα e á produção de IL-33, motivaram a realização deste estudo.

2. OBJETIVOS

2.1. Geral:

Avaliar mecanismos associados a reabsorção óssea induzida pela deficiência de estrogênio.

2.2. Específicos:

1. Avaliar o efeito da redução de estrogênio, por meio da ovariectomia em camundongos, na perda óssea alveolar e se a reposição com E2 leva à reversão do fenótipo;
2. Avaliar o efeito da deleção do receptor de estrogênio ERα na reabsorção óssea alveolar e mecanicamente induzida;
3. Avaliar o efeito da deleção do receptor de IL-33, ST2, na reabsorção óssea alveolar associada a ovariectomia e mecanicamente induzida.

3. RESULTADOS E DISCUSSÃO

PARTE I

Durante o período pós-menopausa ocorre uma rápida diminuição dos níveis de estrogênio nas mulheres o que pode acarretar em osteoporose. Esta doença caracterizada pela fragilidade e perda de estrutura óssea é observada principalmente nos ossos longos, vértebras e punho (Kanis *et al.*, 2013). Embora haja falta de consenso entre os estudos, existem evidências que os ossos maxilares também podem ser afetados pela deficiência de estrogênio (Bonnet *et al.*, 2013; Streckfus *et al.*, 1997). Sabe-se também que ocorre aumento de moléculas inflamatórias na osteoporose pós-menopausa (Cenci *et al.*, 2003; Roggia *et al.*, 2001; Straub, 2007). Assim, nosso primeiro objetivo foi verificar se a deficiência de estrogênio, resultante de ovariectomia, acarreta perda óssea alveolar e aumento de mediadores inflamatórios. Os resultados e discussão referentes ao **objetivo 1** serão apresentados no formato do artigo científico 1 publicado no periódico *Archives of Oral Biology*.

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Oestrogen regulates bone resorption and cytokine production in the maxillae of female mice

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ABSTRACT

Oestrogen plays major role in bone metabolism/remodelling. Despite of well-established effect of oestrogen deficiency on long bones, it remains unclear whether alveolar bone is affected. We aimed to determine the effect of oestrogen-deficiency in the alveolar bone microarchitecture. C57BL6/J and Balb/c mice were ovariectomized and implanted with oil- (OVX) or 17β-estradiol (E2)-containing (OVX + E2) capsules. Ovary-intact mice were used as controls. The dose of E2 replacement was selected based on trophic effects on the uterus and femur bone loss. As determined by maxillary alveolar bone MicroCT analysis, both C57BL6/J and Balb/c OVX mice displayed decreased trabecular thickness, bone density and bone volume, and increased trabecular separation at 15 and 30 days after ovariectomy. These effects were associated with a reduction of trabecular bone percentage and cortical thickness in the femur. A significant loss of alveolar bone crest was also associated with ovariectomy in both mice strains. The E2 replacement fully prevented ovariectomy-induced alterations in the alveolar and femoral bones. Moreover, TNF-α (tumour necrosis factor-α) levels and RANKL/OPG (receptor activator of NF-κB ligand/osteoprotegerin) ratio were increased in the maxilla after OVX, and these responses were also reversed by E2. In conclusion, oestrogen deficiency causes maxillary alveolar bone loss, which is similar to the effects found in the femur. The release of inflammatory molecules like TNF-α, RANKL and OPG is the potential mechanism to the decrease of bone quality and alveolar bone crest.

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1. Introduction

Throughout the immediate postmenopausal first years, decreased oestrogen levels lead to rapid bone loss that may induce an osteoporosis process. Osteoporosis is defined as a systemic skeletal disorder characterized by a decrease in bone density, lower bone strength and micro architectural deterioration, which leads to increased bone fragility and risk of fracture.[1] The most common sites of osteoporosis are the proximal femur and the vertebral bodies, but there is evidence to believe that decrease of oestrogen may also affect the facial bones.[2] Accordingly, osteoporosis is associated with lower mandibular bone density[3] and higher incidence of periodontal disease and tooth loss in post-menopausal women,[4–8] however there is a lack of consensus among most published studies of osteoporosis and alveolar bone loss.

The pathogenesis of post-menopausal osteoporosis involves increased production of pro-inflammatory cytokines related to oestrogen withdrawal.[9,10] Accordingly, oestrogen deficiency-induced bone loss is associated with an increase of TNF-α expression and RANKL.[11,12] It has been shown that the alveolar bone loss in postmenopausal women is associated with increased salivary levels of IL-6.[2] Moreover, 17β-Estradiol (E2) is able to reduce lipopolysaccharide-induced expression of inflammatory cytokines such as tumour necrosis factor (TNF)-α and receptor activator of NF-κB ligand (RANKL) in human periodontal ligament cells.[13] However, with respect to the alveolar bone, the effects of oestrogen on bone resorption remain not well characterized.

The ovariectomized (OVX) rat has been established as a reliable animal model for oestrogen deficiency osteoporosis,[14] however the mice model and the ideal period after OVX when alveolar bone loss occurs should be better standardized once the literature is still controversial about the effects of oestrogen on mouse bone. Ovariectomy has been documented to exert deleterious effects on the alveolar bone of rodents. Long-term ovariectomy in rats is reported to cause loss of maxillary bone through increased osteoclastic activity.[15–19] Although Liu et al. (2014) have elucidated the bone loss and microarchitecture changes in OVX rat model, the time required for oestrogen-deficiency-induced alveolar bone loss and its relative effects in the long bones in mice are still unknown. Furthermore, the effects of oestrogen deficiency on the mouse alveolar bone remain elusive, with contradictory findings being reported. Although a reduction of mandibular bone mineral density has been documented after long-term ovariectomy in the mouse,[21,22] no difference in the loss of alveolar bone has been found between OVX and E2-treated OVX mice.[23] Moreover, different mice strains demonstrate distinct innate immune response on which C57BL6/J predominantly presents Th1-response whereas Balb/c is Th2-type.[24] Once bone remodelling is linked to immune-inflammatory process, variations in bone response after ovariectomy could be expected. Thus, we did analyze two different mice strains. In this context, we determined the temporal effects of ovariectomy and E2 replacement on the maxillary alveolar bone parameters. The production of inflammatory cytokines in the maxilla was also investigated, as a possible mechanism for oestrogen-deficiency-induced alveolar bone loss.

2. Materials and methods

2.1. Mice

Forty-five C57BL6/J and 25 Balb/c female mice (10 weeks of age) were utilized in the investigation. The animals were housed under conditions of controlled lighting (12 h light/12 h dark) and temperature (22 ± 2 °C), with food and water provided ad libitum. Experimental protocols were approved by the Ethics Committee on the Use of Experimental Animals of the Federal University of Minas Gerais (protocol 39/2011).

2.2. Ovariectomy and E2 replacement

Except by the Control group, all the female mice were bilaterally ovariectomized and immediately implanted with subcutaneous capsules containing corn oil (OVX) or E2 (17β-Estradiol, Sigma–Aldrich, St. Louis, MO; OVX + E2). Vaginal smears were routinely used to monitor the progression of oestrous cycle.

Firstly we aimed to determine the efficacy of ovariectomy and to select a dose for E2 replacement in C57BL6/J mice. The experimental groups were (n = 5 per group): Control, OVX (corn oil implants) and OVX + E2 (doses of 10, 60 or 120 μg of E2/implant). As a Control group, ovary-intact regularly cycling mice were euthanized on the day of proestrus of the estrous cycle, characterized by elevated serum E2 levels.[25] The OVX and OVX + E2 animals were euthanized by decapitation on days 15 (D15) and 30 (D30) after ovariectomy and uterus, maxilla and femur were collected for analysis. The uterine weight was determined.

2.3. Alveolar bone loss analysis

The effect of ovariectomy and E2 replacement in the alveolar bone was evaluated in C57BL6/J and Balb/c female mice which were kept as ovary-intact (Control), or evaluated as OVX (corn oil implants) or OVX + E2 (60 μg/implant/mouse of E2) groups on D15 and D30 after ovariectomy. The maxillary alveolar bones were scanned using a microCT system (Skyscan 1176 X-Ray microtomograph, Aartselaar, Belgium) and CT-Analyser software (Skyscan, Belgium). High-resolution scans with an isotropic voxel size of 18 μm were acquired (50 kV, 0.5 mm aluminium filter, 0.5° rotation angle). The furcation area of the first molar root was taken for analyses of the trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), bone mineral density (BMD), bone volume (BV) and percentage of bone volume/total volume (BV/TV%). The alveolar bone crest (ABC) loss was determined by the analysis of the area between the cemento-enamel junction (CEJ) and the ABC (CEJ-ABC) in three-dimensional images (Fiji – National Institutes of Health, USA) of the first, second and third molars.[22]

2.4. Femur histomorphometric analyses

For the haematoxylin and eosin staining, the femurs were fixed in 10% formaldehyde, decalcified in 14% EDTA and embedded in paraffin. Sagittal sections of 5 μm were stained

by haematoxylin and eosin. The BV/TV% of femur's proximal epiphysis and metaphysis were determined using a ocular containing a 25-point grid (Zeiss KLP 10×) at 40× magnification.[26] For the Von Kossa staining, the femurs were fixed in 10% formaldehyde, dehydrated and embedded in resin (Technovit 7200, Exakt, Kulzer GmbH, Wehrheim, Germany). The blocks were cut in the sagittal plane, stained by Von Kossa staining, and the proximal metaphysis were photographed at 5× magnification and the diaphysis at 10× magnification under a light microscope connected to a digital camera (PowerShot A620, Canon, Tokyo, Honshu, Japan). The BV/TV% and cortical bone thickness (Cs.Th) were measured using the Image J software (National Institutes of Health, USA).

2.5. ELISA

The maxillary bone was homogenized in phosphate-buffered saline (PBS) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA and 0.01 mg/mL aprotinin A) and 0.05% Tween 20, pH 7.4, using Power Gen 1000 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). Samples were then centrifuged (9000 × g, 10 min) and the supernatants used to determine cytokine concentrations using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The following kits were used to analyze serum and maxillae: TNF-α (DY410), RANKL (DY462), osteoprotegerin (OPG) (DY459) and macrophage inflammatory protein-1α (CCL3) (DY450). Results were expressed as pg/ml for the serum and pg/100 mg of tissue for the maxilla.

2.6. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The differences among groups for the time points D15 and D30 were analyzed by one-way ANOVA followed by the Newman–Keuls post hoc test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. E2 replacement dose selection

The uterine horns of OVX mice showed marked atrophy at D15 and D30 after ovariectomy compared with Control mice ($p < 0.05$). All doses of E2 in OVX + E2 mice reversed the uterine atrophy on D15, whereas on D30 the dose of 10 μg resulted in lower uterus weight compared with Control group and the doses of 60 and 120 μg of E2 ($p < 0.05$; Fig. 1A and B). The femur BV/TV% was lower in OVX mice compared with Control mice on D15 and D30 after ovariectomy ($p < 0.05$) and was increased in a dose dependent manner by the E2 replacement in OVX + E2 mice ($p < 0.05$; Fig. 1C and D). These results validate a condition of oestrogen deficiency in short-term OVX mice and demonstrate the effectiveness of different doses of E2 in restoring biological effects of E2. Because the dose of 60 μg of E2 consistently reversed all the effects of ovariectomy on D15 and D30, it was elected for the E2 replacement in the subsequent experiments.

3.2. Effect of ovariectomy and E2 on the bone of C57BL6/J mice

As determined by microCT analysis, there was a marked increase in Tb.Sp and decrease in Tb.Th, BMD, BV, and BV/TV% in the alveolar bone of OVX mice compared with Control on D15 and D30 after ovariectomy ($p < 0.05$; Fig. 2A and B). The CEJ-ABC area was increased in OVX mice on D30 compared with the Control group ($p < 0.05$), whereas no difference was found on D15 (Fig. 2C and D). The histomorphometric analysis of Von Kossa stained sections showed a consistent reduction of BV/TV% and Cs.Th in the femur of OVX mice compared with Control on D15 ad D30 after ovariectomy ($p < 0.05$; Fig. 2E and F). Moreover, the ovariectomy-induced relative loss of BV/TV% in the alveolar bone (D15: 20.45%; D30: 38%) was similar to that in the femur (OVX D15: 32.12%; D30: 42%). Notably, E2 replacement in OVX + E2 mice reversed all the effects of ovariectomy on the maxillary and femoral bones ($p < 0.05$).

3.3. Effect of ovariectomy and E2 on the bone of Balb/c mice

In order to determine if different mice strains would respond in the same manner to ovariectomy, Balb/c mice were evaluated on a similar experimental protocol to C57BL6/J mice. Indeed, the responses of maxillary alveolar bone (Fig. 3A and B), CEJ-ABC area (Fig. 3C and D) and femur bone (Fig. 3E and F) of Balb/c mice to ovariectomy and E2 treatment were comparable to those seen in C57BL6/J mice. Nevertheless, the loss of alveolar bone crest in Balb/c mice appeared to happen in earliest stages than in C57BL6/J mice once the CEJ-ABC area was significantly increased in OVX compared with OVX + E2 mice on D15 after ovariectomy ($p < 0.05$).

3.4. Alveolar bone and serum inflammatory cytokine levels of C57BL6/J mice

OVX mice displayed marked increase in RANKL/OPG ratio on both D15 and D30 (Fig. 4A) ($p < 0.05$), while significantly higher TNF-α (Fig. 4B) concentration in maxilla have been shown only on D15 ($p < 0.05$) but not on D30 after ovariectomy. These responses were also reversed in OVX + E2 mice. Although not reaching statistical significance, CCL3 (Fig. 4C) levels tended to be higher in OVX than in OVX + E2 mice on D15. Likewise, serum RANKL/OPG ratio was increased on D15 and D30 (Control 0.077 ± 0.029; OVX D15 0.142 ± 0.008; OVX + E2 D15 0.016 ± 0.008; OVX D30 0.170 ± 0.019 and OVX + E2 D30 0.048 ± 0.02 pg/ml) and CCL3 levels on D15 in OVX mice compared with Control and OVX + E2 groups ($p < 0.05$) (Control 3.308 ± 0.681; OVX D15 7.757 ± 1.113; OVX + E2 D15 4.538 ± 1.230; OVX D30 5.143 ± 0.977 and OVX + E2 D30 4.759 ± 0.987 pg/ml).

4. Discussion

In this study, we demonstrated for the first time that mouse alveolar bone microarchitecture is significantly affected by oestrogen-deficiency in a time-dependent manner. As expected, femur also exhibited bone loss dependent on oestrogen-deficiency. The changes in alveolar bone were

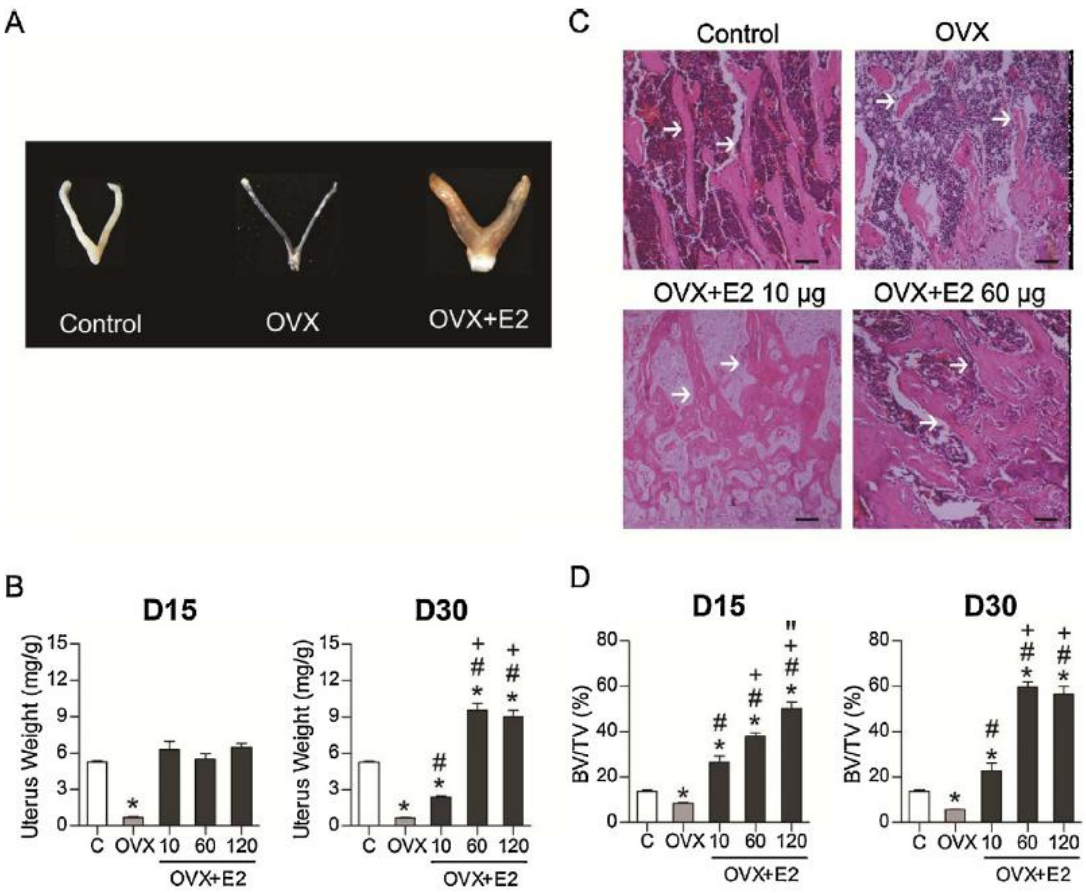

Fig. 1 – Efficacy of short-term ovariectomy and 17β-Estradiol (E2) replacement in C57BL6/J mice. The animals were divided in three experimental groups: Control, OVX and OVX + E2 (doses of 10, 60 or 120 µg/implant/mice of E2), evaluated on D15 and D30 after ovariectomy. A – Representative images of C57BL6/J mice uterus weight in Control, D30 OVX and D30 OVX + E2 mice (60 µg/implant/mice of E2). B – C57BL6/J uterus mice weight. C – Histomorphometric analysis of the femur trabecular bone (Haematoxylin and Eosin staining) Femur trabecular representative images of Control, D30 OVX and D30 OVX + E2 (doses of 10, 60 µg/implant/mice of E2) (Scale bar = 100 µm) (white arrows indicate the trabecular bone). D – The femur BV/TV% was lower in OVX mice compared with Control mice on D15 and D30 after ovariectomy and was increased in a dose dependent manner after the E2 replacement in OVX + E2 mice. Five mice were used for each time-point. Data were expressed as mean ± SEM. $^{*}p < 0.05$ – statistical difference from control group. $^{\#}p < 0.05$ – statistical difference from OVX group. $^{+}p < 0.05$ – statistical difference from 10 µg OVX + E2 group. $^{''}p < 0.05$ – statistical difference from 60 µg OVX + E2 group. One-way ANOVA with Newman–Keuls multiple comparison test.

associated with increased expression of TNF-α and RANKL/OPG ratio in maxilla indicating a possible mechanism for oestrogen-deficiency-induced alveolar bone resorption. The E2 replacement reversed bone phenotype and reduced cytokine production.

Oestrogen is important for bone health and its lack increases bone loss in spine, hips and forearms enhancing the risk of bone fracture.[1] In our study, animals were subjected to ovariectomy to cause oestrogen deficiency. Oestrogen-deficiency and E2 replacement effects were confirmed by assessing uterus weight which is consistent with other studies.[21,27] Under the conditions of oestrogen-deficiency, as observed in the ovariectomized mice, there was marked bone loss in the femur confirmed by the reduction of BV/TV% and Cs.Th which corroborates with Bartell et al.[27] and Bonnet et al.[22] Accordingly, bone phenotype was reversed by oestrogen in agreement with Bartell et al.[27]

We also evaluated alveolar bone microarchitecture by microCT. The results show clearly that there is bone loss represented by increased Tb.Sp and decreased Tb.Th, BMD, BV and BV/TV, that is in accordance with literature.[21,22] Liu et al.[20] using a rat model, found increased Tb.Sp in the maxillary bone 24 weeks after OVX and reduced Tb.Th in a period of 2, 12 and 24 weeks after OVX. In our mouse model we could detect alveolar microarchitecture bone loss in the maxilla on D15 and D30 in both mice strains, which could be an advantage using mice instead of rats.

In the same way, corroborating with Bonnet et al.[22] the ABC-CEJ area was increased 30 days after OVX. However, our results demonstrated that oestrogen-deficiency may cause a

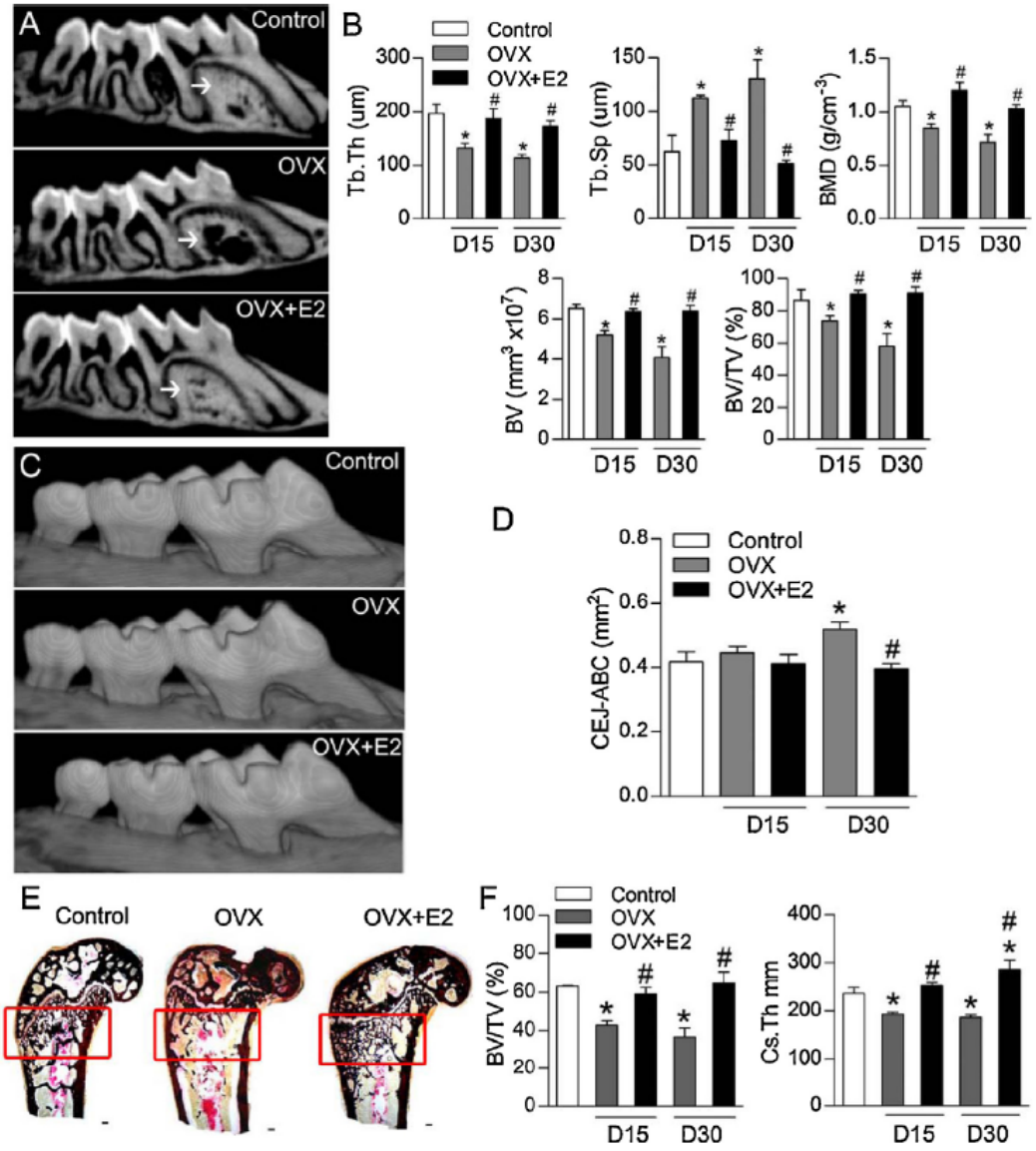

Fig. 2 – Effect of ovariectomy and 17β-estradiol (E2) on the alveolar bone, area between the cemento-enamel junction and the alveolar bone crest (CEJ-ABC) and femur in C57BL6/J mice. The mice were bilaterally ovariectomized and immediately implanted with subcutaneous capsules containing corn oil (OVX) or E2 (17β-Estradiol, OVX + E2) and euthanized by decapitation on days 15 (D15) and 30 (D30) after ovariectomy A – MicroCT representative images of Control, D30 OVX and D30 OVX + E2 mice (white arrows indicate the analyzed area). B – C57BL6/J mice maxillary microCT results in experimental period of D15 and D30: trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), bone mineral density (g/cm^{-3}), bone volume (BV, mm^3) and percent bone volume (BV/TV %). C – CEJ-ABC high quality 3D representative image of Control, D30 OVX and D30 OVX + E2 mice. D – Alveolar bone crest (ABC) loss quantification performed through the measurements of CEJ-ABC area (mm^2) in the palatal face of the first, second and third molars on days D15 and D30. E – Von Kossa staining representative images of Control, D30 OVX and D30 OVX + E2 mice (Scale bar = 100 μm), rectangle shows the proximal epiphysis and metaphysis investigated area. F – C57BL6/J mice Von Kossa staining histomorphometric analysis of the femur trabecular bone on days D15 and D30: trabecular bone (BV/TV%) and cortical thickness (Cs.Th – μm). Scale bar = 100 μm. Five mice were used for each time-point. Data were expressed as mean ± SEM. $^*p < 0.05$ – statistical difference from Control group. $^\#p < 0.05$ – statistical difference from OVX group. One-way ANOVA with Newman–Keuls multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

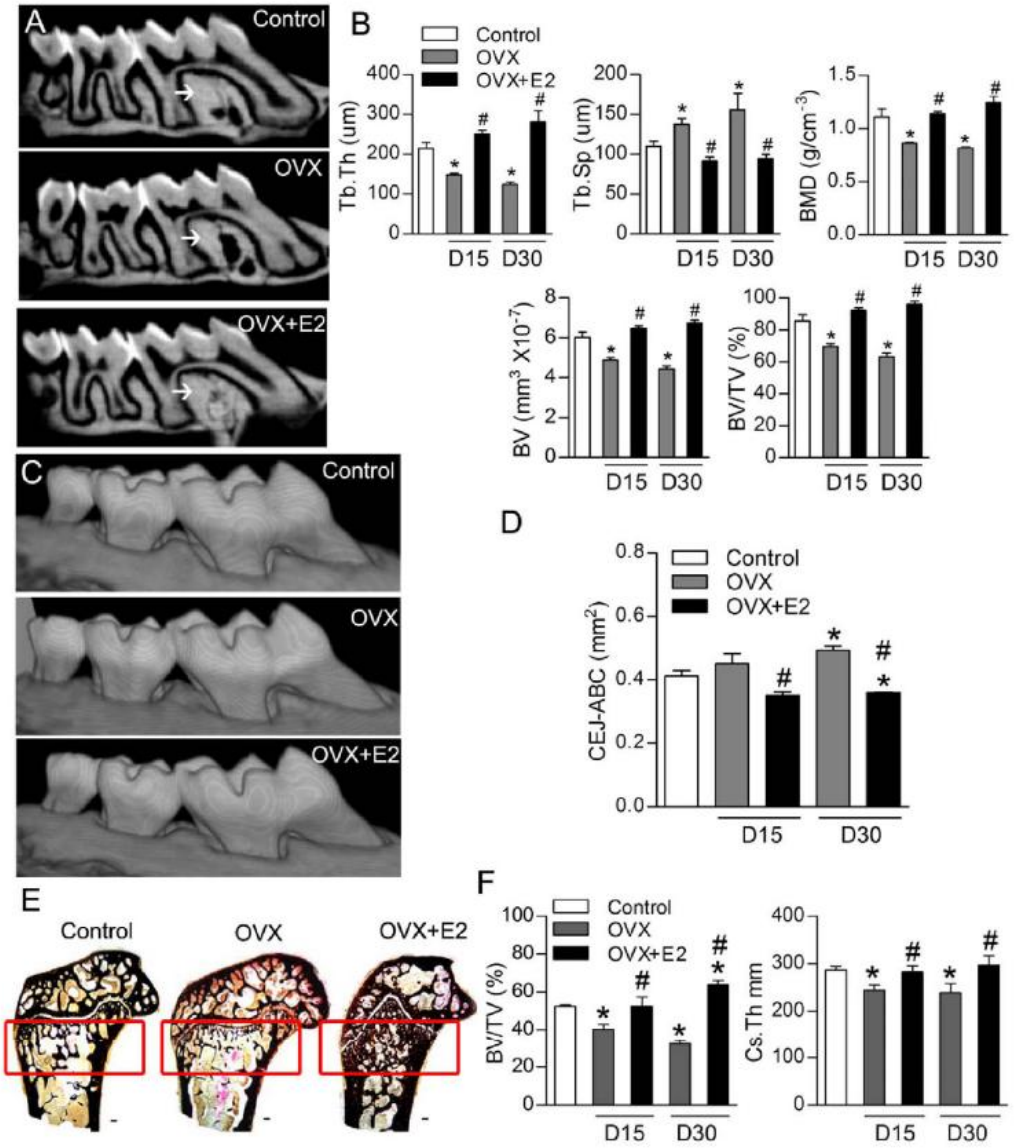

Fig. 3 – Effect of ovariectomy and 17β-Estradiol (E2) on the alveolar bone, area between the cemento-enamel junction and the alveolar bone crest (CEJ-ABC) and femur in Balb/c mice. The mice were bilaterally ovariectomized and immediately implanted with subcutaneous capsules containing corn oil (OVX) or E2 (17β-Estradiol, OVX + E2) and euthanized by decapitation on days 15 (D15) and 30 (D30) after ovariectomy A – MicroCT representative images of Control, D30 OVX and D30 OVX + E2 mice (white arrows indicate the analyzed area). B – Balb/c mice maxillary microCT results in experimental period of D15 and D30: trabecular thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), bone mineral density (g/cm^{-3}), bone volume (BV, mm^3) and percent bone volume (BV/TV %). C – CEJ-ABC high quality 3D representative image of Control, D30 OVX and D30 OVX + E2 mice. D – Alveolar bone crest (ABC) loss quantification performed through the measurements of CEJ-ABC area (mm^2) in the palatal face of the first, second and third molars on days D15 and D30. E – Von Kossa staining representative images of Control, D30 OVX and D30 OVX + E2 mice (Scale bar = 100 μm), rectangle shows the proximal epiphysis and metaphysis investigated area. F – Balb/c mice Von Kossa staining histomorphometric analysis of the femur trabecular bone on days D15 and D30: trabecular bone (BV/TV%) and cortical thickness (Cs.Th – μm). Scale bar = 100 μm. Five mice were used for each time-point. Data were expressed as mean ± SEM. $^*p < 0.05$ – statistical difference from Control group. $^\#p < 0.05$ – statistical difference from OVX group. One-way ANOVA with Newman–Keuls multiple comparison test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

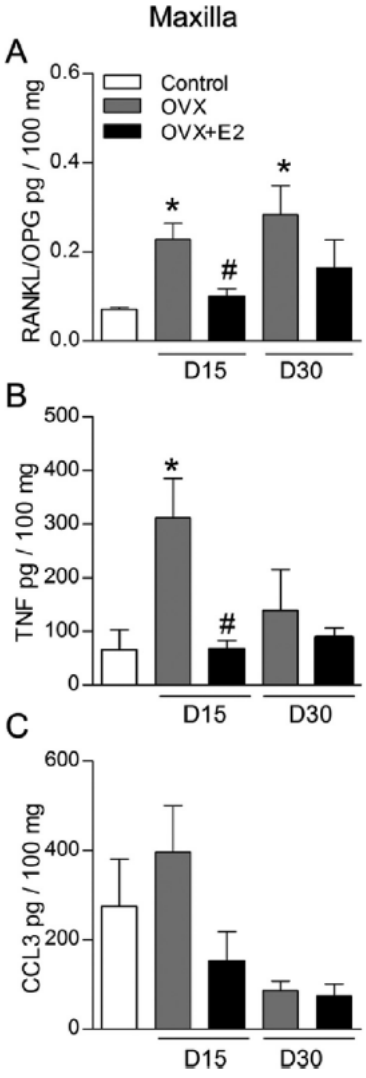

Fig. 4 – Ovariectomy (OVX) increases the expression of TNF-α and CCL3 and RANKL/OPG ratio 15 days (D15) after ovariectomy in the C57BL6/J maxilla but only RANKL/OPG ratio remains augmented in the period of 30 days (D30). These responses were reversed in OVX + E2 mice. Results of maxillary bone RANKL/OPG ratio (A), TNF-α (B) and CCL3 (C). Five mice were used for each time-point. Data were expressed as mean ± SEM. $^{*}p < 0.05$ – statistical difference from control group. $^{\#}p < 0.05$ – statistical difference from OVX group. One-way ANOVA with Newman–Keuls multiple comparison test.

rapid impairment in the bone remodelling given that a reduction of bone microarchitecture in both maxilla and femur were found on D15 and the alveolar bone loss was increased on D30 after OVX in both mice strains – C57BL6/J and Balb/c.

Despite the fact that C56BL6/J and Balb/c mice have distinct prototypical innate immune response Th1- and Th2-type, respectively,[24] there were quite similar effects of ovariectomy in both strains. Therefore, similar to what was observed in long bones, alveolar bones also suffer significantly from lack of oestrogen on D30 in both strains.

A possible relationship between oestrogen deficiency and alveolar bone loss is seen in the experimental periodontitis.[28] Although not the main cause of periodontal disease, oestrogen deficiency alters alveolar bone microarchitecture compromising their mechanical stability[31,32] and consequently affecting disease progression.[29,30] Furthermore, oestrogen lack, osteoporosis/osteopenia and periodontitis have common mechanisms that contribute to bone loss by increasing the production of inflammatory cytokines,[33–35] which may exacerbate bone effects. Even difficult to be proved in clinical studies, experimental results from us and others[28–30] may account for oestrogen deficiency as a risk factor of periodontal disease.

Many molecular mechanisms are involved in bone loss, in particular there have been several studies demonstrating the role of OPG, RANKL, and TNF-α in mediating bone loss in long bones after ovariectomy.[12,36] Consistently with the latter findings, we observed changes in levels of cytokines. Moreover, changes in cytokine levels were normalized by replacement with oestrogen. Therefore, these cytokines increased expression appear to be the underlying mechanism explaining the bone phenotype of ovariectomized mice.

We have found that CCL3 is important for mechanical-loading-induced alveolar bone loss.[37] Here we found, after OVX, a significant increase of CCL3 production in serum, but not in maxilla. It is possible that CCL3 contributes to the observed phenotype, a tenet that deserves further investigation in the future.

Emerging evidence indicates that osteoporosis raises an individual's susceptibility to periodontal breakdown.[4–8] Herein, significantly increased progression of alveolar bone loss is observed in patients with osteopenia.[6,38] Our results indicate that decreased maxillary alveolar bone parameters should be carefully analyzed, particularly in advanced aged patients, and the hypothesis of osteoporosis could be considered.

In conclusion, oestrogen deficiency, as observed in ovariectomized mice, is accompanied by bone loss, a feature also observed during menopause in humans. Here, we show using microCT that oestrogen-deficiency also causes significant alveolar bone loss. Mechanistically, we show that oestrogen controls negatively the expression of cytokines, including RANKL/OPG and TNF-α, which favour bone loss. Our results suggest that further studies should evaluate whether cytokines may contribute to bone loss during menopause and whether these cytokines are effective targets to prevent bone loss.

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Competing interests

The authors declare that there is no conflict of interest concerning the manuscript or to any of coauthors.

Ethical approval

Study was approved by the Ethics Committee on the Use of Experimental Animals of the Federal University of Minas Gerais (protocol 39/2011).

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PARTE II

Nossos primeiros resultados demonstraram que a deficiência estrogênica causa perda óssea alveolar, a qual está relacionada ao aumento de TNF-α e da proporção RANKL/OPG. O estradiol atua via os receptores ERα e ERβ (Krum, 2011; Syed *et al.*, 2005; Weitzmann and Pacifici, 2006) e as células ósseas apresentam ambos receptores estrogênicos (Chokalingam *et al.*, 2012; Krum, 2011). Desta forma, nosso segundo objetivo foi analisar se o receptor ERα participa na perda óssea alveolar associada a deficiência de estradiol e os mecanismos envolvidos. Os resultados e discussão referentes ao **objetivo 2** serão apresentados no formato do artigo científico 2.

ARTIGO CIENTÍFICO 2

Estrogen receptor alpha deficiency is associated with reduced maxillary alveolar bone quality.

Abstract

Introduction: Estrogen deficiency results in systemic bone loss. However, the contribution of estrogen receptor alpha (ERα) in maintenance of alveolar bone microarchitecture and its correlation to interleukins is not yet well defined. **Methods:** Bone remodeling was induced by orthodontic tooth movement (OTM) in 8-10 weeks old females and males homozygote $ER\alpha^{+/+}$ (wild type - WT) and $ER\alpha^{-/-}$ (ERKOα) mice. The maxillary bone samples were submitted to microCT, molecular analysis and energy dispersive spectroscopy (EDS). Bone marrow cells from WT and ERKOα mice were isolated to evaluate osteoblast and osteoclast diferentiation. **Results:** Both female and male ERKOα demonstrated an osteoporotic phenotype in the femur and vertebrae. Maxillay alveolar bone loss and OTM were augmented in ERKOα mice and associated with decreased calcium percentage levels and increased expression of IL-33 in the periodontium. *In vitro* osteoclasts and osteoblasts differentiation from BMC were significantly higher in ERKOα than WT mice. **Conclusion:** Data provide herein evidence that estrogen receptor alpha (ERα) contributes in the osteoprotective effect on alveolar maxillary bone.

Key words: estrogens, maxilla, alveolar bone loss, IL-33, osteoporosis, estrogen receptor alpha

Introduction

Estrogens are important regulators of bone metabolism (Manolagas *et al.*, 2013). Estrogen deficiency, as found in post-menopause women, may lead to osteopenia and osteoporosis, caused by a disruption of bone remodeling mainly due to osteoclast-induced bone resorption (Cummings *et al.*, 1985; Ginaldi *et al.*, 2005). Long bones, wrists and vertebrae are the most affected sites of osteoporosis (Lufkin *et al.*, 1998; Riggs *et al.*, 1998). Furthermore, post-menopause women also experience decreased mandibular bone density and a higher incidence of periodontal disease and tooth loss (Deguchi *et al.*, 2008; LaMonte *et al.*, 2013; Lerner, 2006; Nicopoulou-Karayianni *et al.*, 2009; Tezal *et al.*, 2005; Yoshihara *et al.*, 2004).

Ovariectomized (OVX) rodents have been established as reliable animal models for estrogen deficiency-induced osteoporosis (Thompson *et al.*, 1995). Previous studies from our group and others had demonstrated that ovariectomy in rats and mice results in loss of maxillary bone and increase in osteoclastic activity (Bezerra *et al.*, 2013; Ejiri *et al.*, 2008; Tanaka *et al.*, 2002; Tanaka *et al.*, 2003).

Furthermore, estrogen deficiency may cause TNF-α, RANKL and IL-6 unbalanced production in the periodontal tissue (Cenci *et al.*, 2000; Roggia *et al.*, 2001; Shu *et al.*, 2008; Streckfus *et al.*, 1997), which might be prevented by 17ß-estradiol replacement (Macari *et al.*, 2015). Besides estrogen, IL-33 which is a member of IL-1 family, acts as a bone protector (Keller *et al.*, 2012; Schulze *et al.*, 2011) and inhibits TNF-α bone resorption (Zaiss *et al.*, 2011) through decrease in osteoclast number and is associated with increased production of anti-osteoclastogenic cytokines as IL-10 (Saleh *et al.*, 2011). However, the correlation between ovariectomy with estrogen decrease and IL-33 has not been reported yet.

Most of the estrogen actions at cellular level are mediated by estrogen receptors alpha (ERα) and beta (ERβ) by regulating directly (classical) or indirectly (indirect) DNA binding (Krum, 2011; Syed *et al.*, 2005; Weitzmann and Pacifici, 2006). The effects of estrogen in

bone occur in part via ERα found in both, osteoblasts and osteoclasts (Chokalingam *et al.*, 2012; Vidal *et al.*, 2000). The constitutive activation of ERα in osteoblasts stimulates production of osteoprotegerin (OPG) and interleukin-6 (IL-6) and increases bone mineral density in the femur (Ikeda *et al.*, 2011). The deletion of ERα gene from osteoclasts, in turn, results in trabecular bone loss, increased number of osteoclasts (Martin-Millan *et al.*, 2010), and decreased apoptosis of mature osteoclasts (Nakamura *et al.*, 2007; Novack, 2007). The deleterious disruption of ERα gene from osteoblasts compromises bone strength (Melville *et al.*, 2014) and reduces trabecular and cortical bone volume in the femur and tibia (Almeida *et al.*, 2013; Maatta *et al.*, 2013). Accordingly, the global ERα deficient (ERKOα) females mice exhibit decreased bone diameter while males decreased bone density (Walker and Korach, 2004). However, controversy remains with respect to the phenotype of the trabecular bone in ERKOα mice, considering that increase (Syed *et al.*, 2011) and decrease (Lindberg *et al.*, 2001b) of tibia trabecular thickness have been reported. Therefore, the impact of ERα deletion on the maxillary alveolar bone has not been determined yet and the importance of ERα in the function and metabolism of the alveolar bone remains elusive.

Mechanical loading stimulates bone formation (Ehrlich and Lanyon, 2002), however estrogen receptor α deficient mice display a reduced response on cortical bone to mechanical loading (Callewaert *et al.*, 2010; Lee *et al.*, 2003; Lee *et al.*, 2004; Lee and Lanyon, 2004; Windahl *et al.*, 2013). There are no reports concerning the role of ERα in mechanical loading-induced maxillary bone remodeling.

This study aimed to characterize ERKOα mice maxillary bone phenotype and the role of the inflammatory molecules in this process. Additionally, we intend to verify the differences between genders and if ERα has a role in bone remodeling induced by mechanical loading.

Material and methods

Experimental protocol

Heterozygote ERα$^{+/-}$ male mice were backcrossed with heterozygote ERα$^{+/-}$ female mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) in a C57BL/6 background. The littermates were genotyped at 4–5 weeks of age by PCR (Syed *et al.*, 2011) to separate the females and males homozygote ERα$^{+/+}$ (wild type - WT) and ERα$^{-/-}$ (ERKOα) mice (n=5 per group). The experiment was performed at Otago University (Dunedin, New Zealand) when the littermates were 8-10 weeks old. The animals were treated under the ethical regulations for animal experiments, defined by the Otago University Animal Ethics Comittee (Application number 102/13). Uterus, maxilla, femur and vertebrae (lombar L1) were collected for analysis.

Induced alveolar bone remodeling

The alveolar bone remodeling was induced by orthodontic tooth movement (OTM) as previously described (Taddei *et al.*, 2012). A force of 0.35 N was exerted in the mesial direction of the upper right first molar with a Ni-Ti 0.25×0.76 mm coil spring (Lancer Orthodontics, San Marcos, CA, USA) which was bonded between the first molar and the incisors. The left side (without appliance) of the alveolar bone and peridontium was used as control (C) and the right side was used as experimental side (OTM). The maxillary alveolar bone was collected 12 days after OTM and used for microtomography (microCT), energy dispersive spectroscopy (EDS) and molecular analysis (RT-PCR).

MicroCT

The maxillary alveolar bone, femur and vertebrae (L1) were fixed in 10% formalin for 48 hours and scanned using a microCT system (Skyscan 1172 X-Ray microtomograph,

Aartselaar, Belgium). The images were reconstructed by NRecon software (Skyscan, Aartselaar, Belgium) and analyzed by CT-Analyzer software (Ctan, Skyscan, Belgium). The calibration was carried out with known density calcium hydroxyapatite phantoms. High-resolution scans with an isotropic voxel size of 8.62 were acquired (50 kV, 0.5 mm aluminum filter, 0.5° rotation angle). The analysis for the percentage of bone volume/total volume (BV/TV%), bone volume (BV), bone mineral density (BMD), structure model index (SMI), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) was performed in the furcation area of the first molar root.

Energy Dispersive Spectroscopy (*EDS)*

Maxillary samples were dehydrated in ethanol serial solutions and left overnight at room temperature. The samples were coated with gold and scanned with a field emission scanning electron microscope (JEOL Ltd, Tokyo, Japan) fitted with a JEOL 2300F EDS system (JEOL Ltd, Tokyo, Japan) for calcium (Ca^{2+}) mineral content percentage analysis as previously described (Marchini *et al.*, 2012).

mRNA extraction and real time PCR

For RT-PCR analysis, we extracted total RNA from the periodontal ligament and surrounding alveolar bone samples from the left upper first molars by using the Trizol mRNA extraction using columns and on-column DNAse treatment (RNeasy Mini Kit, Qiagen Inc, Valencia, CA, USA). The integrity of RNA samples was checked by analyzing 1 µg of total RNA on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturers' instructions. After RNA extraction, complementary DNA was synthesized by using 2 µl of RNA through a reverse transcription reaction using Quanti TectRT kit (Qiagen Inc, Valencia, CA, USA). The targets analyzed were: interleukin-33 (IL-33), tumor necrosis

factor alpha (TNF-α), receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG) and RANKL/OPG ratio. The mRNA levels were measured by means of Real Time PCR using SYBR Green PCR Master Mix. Each plate was run in the machine using a thermo cycling protocol consisting of an initial pre-incubation step at 95°C for 10 min to increase detection sensitivity, then 45 amplification cycles starting at 95°C for 10 sec, 60°C for 30 sec, and 72°C for 1 sec, followed by a single cooling step at 40°C for 10 sec. The resulting data from each plate were run through an absolute quantization/second derivative maximum analysis using the LightCycler® 480 software 1.5.0.39. The sequences of the primers were designed based on nucleotide sequences in the Ensembl and NCBI database were used (Table 1). Expression of each gene was normalized using the mean expression of two housekeeping genes PGK1 (Phosphoglycerate kinase 1) and TBP (TATAA-box binding protein) expression in the sample using the Ct method and $2^{-\Delta Ct}$ calculation.

Osteoclast generation

Bone marrow cells (BMC) were obtained from the femurs of WT and ERKOα mice. The BMC were incubated in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and soluble macrophage colony-stimulating factor (M-CSF) (100 ng/ml; Peprotech, London, UK) for 6 days to generate pre-osteoclasts. Soluble receptor activator of nuclear factor-$_K$B ligand (RANKL) (100 ng/ml; Peprotech) were then added until day 8. Cells were fixed with acetone, citrate and formaldehyde 37% and stained by tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich). The percentage of TRAP-positive cells was determined counting the TRAP-positive cells in ten fields (magnification 40x) per well.

Osteoblast generation

Osteoblastic differentiation in adherent bone marrow mesenchymal stromal cells was induced by culture in medium containing 10% FBS, 100 μM L-ascorbic acid 2 –phosphate, 10 nM Dexamethosone, 5 mM β-glycerophosphate (β-GP). The culture media was replaced once every 3 days. At day 14 and 21 post induction, the levels of alkaline phosphatase activity was assessed using SensoLyte pNPP Alkaline Phosphatase Assay kit (AnaSpec, CA, USA) as per the manufacturer's instructions.

The calcium deposits formed at the end of 21 days were quantified using alizarin–red staining. The cell monolayers grown on 24 well plates were washed three times with PBS and then fixed with ice-cold 70% ethanol for 1 hour. Following fixation, the calcium deposits were washed thoroughly with distilled water and stained with 40 mM alizarin-red (pH 4.1) . The extracted stain was then dried at room temperature and the percentage of calcium deposits were measured using the Image J software (National Institutes of Health, USA).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The differences between groups were performed by t-test and two-way ANOVA with Bonferroni multiple comparison test. $p < 0.05$ was considered statistically significant.

Results

Uterus weight

To confirm ERKOα mice phenotype the uterus weight was measured. ERKOα mice demonstrated significant decrease in uterus horn compared to WT mice (WT 4.43 ± 0.71, ERKOα 1.67 ± 0.16 mg/g).

ERα receptor deletion causes bone loss in femur and vertebrae of female and male mice

Femur microCT analysis demonstrated that deletion of ERα receptor resulted in significantly decreased BV/TV%, BV, BMD and Tb.Th in both female and male ERKOα mice (Fig. 1A and 1B). No differences were seen in Tb.Sp and Tb.N. Male WT mice exhibited increased BV/TV%, BV and diminished Tb.Sp compared to female WT mice (Fig. 1A and 1B). This difference between male and female was not seen in ERKOα mice.

The vertebrae of female ERKOα mice exhibited decreased BV/TV%, BV, Tb.Th, Tb.N and increase in SMI values, whereas only BV/TV%, BV and Tb.Th were reduced in the male ERKOα mice vertebrae (Fig. 1C and 1D). Similarly to femur, the vertebrae of male WT mice demonstrated enhanced BV/TV%, BV, Tb.Th and decreased Tb.Sp when compared to female WT mice (Fig. 1C and 1D). Additionally, male ERKOα mice had increased Tb.N and decreased Tb.Sp compared to female ERKOα mice (Fig. 1C and 1D).

ERα receptor deletion is detrimental for alveolar maxillary bone

Female ERKOα mice demonstrated alveolar bone loss with decrease in BV/TV%, BV, Tb.Th, Tb.N and increased Tb.Sp and SMI values (Fig. 2A and 2B). Meanwhile, male ERKOα mice alveolar bone had decrease in BV/TV%, BV, Tb.Th and increased Tb.Sp (Fig. 2A and 2B). Male WT mice showed increased SMI and diminished BV/TV%, BV when compared to female WT mice (Fig. 2A and 2B), which was not seen in ERKOα mice.

ERKOα mice demonstrates increased induced bone remodeling with decreased calcium percentage

Mechanically induced bone remodeling, measured by amount of orthodontic tooth movement (OTM), was higher in female ERKOα and male ERKOα mice compared to respective WT (Fig. 3A and 3B). Male WT mice showed significant decreased OTM when compared to female WT mice (Fig. 3A and 3B). The control side, without orthodontic appliance, of both ERKOα mice genders had lesser calcium (Ca) percentage than WT.

IL-33 expression is augmented in periodontium of ERKOα mice

Molecular analysis was carried out to better understand maxillary alveolar bone in ERKOα mice. RT-PCR analysis revealed enhanced expression of IL-33 in both ERKOα mice genders (Fig. 4A). TNF-α level was diminished in male ERKOα mice (Fig. 4B) but not in female ERKOα (Fig. 4A) when compared to WT mice. Similar fold change levels of RANK (Fig. 4C), RANKL (Fig. 4D), OPG (Fig. 4E) and RANKL/OPG ratio (Fig. 4F) was seen in WT and ERKOα mice in both genders. These results might indicate that maxillary alveolar bone loss in ERKOα mice acts in a RANK and RANKL independent manner. Moreover, male ERKOα mice had decreased TNF-α (Fig. 4B) and RANK (Fig. 4C) levels compared to female ERKOα mice.

Osteoclasts and osteoblasts formation is accentuated in the absence of ERα receptor

Bone marrow cells from WT and ERKOα were cultivated under specific supplements to differentiated in osteoclasts and osteoblasts. Our results demonstrated increased osteoclasts numbers in ERKOα mice compared to WT (Fig. 5A and 5B).

Osteoblasts calcium deposits were significantly increased in ERKOα mice in comparison to WT mice (Fig. 5C and 5D). Alkaline phosphatase (ALP) levels were enhanced in osteoblasts from ERKOα mice after 14 and 21 days (Fig. 5E and 5F).

Discussion

The antiresorptive effects of estrogen are important to preserve alveolar bone microarchitecture (Bezerra *et al.*, 2013; Bonnet *et al.*, 2013; Macari *et al.*, 2015). In the current study, the results demonstrated for the first time that ERα receptor deletion is detrimental for alveolar maxillary bone. ERKOα mice also showed increased bone remodeling induced by mechanical force. Moreover, ERKOα mice present augmented osteoclasts and osteoblasts differentiation from bone marrow cells.

Estrogen has a profound effect in various organs and tissues, including reproductive tract and skeletal system (Lubahn *et al.*, 1993; Riggs *et al.*, 2002). ERKOα mice have confounding systemic effects triggered by general estrogen lack like increased estrogen serum levels, shorter long bones in both sexes, infertility in female mice with decreased uterus weight (Walker and Korach, 2004). Our result demonstrated decreased uterus weight in female mice which is in accordance with the literature (Lindberg *et al.*, 2002) and similar to findings in ovariectomized mice (Macari *et al.*, 2015). Although ERKOα mice, just like any other transgenic mouse, present some systemic side effects it has been a valuable animal to study the consequences of the complete lack of ERα in bone metabolism.

ERKOα mice long bones and vertebrae phenotype is controversial in literature. We demonstrated that female and male ERα receptor deficient mice femur and vertebrae have disruption of bone architecture. Accordingly, Lingberg *et al.* (2001b) have found decreased Tb.Th in tibia of male ERKOα mice. Conversely Syed *et al.* (2011) reported increased Tb.Th in the tibia of 3 month old female ERKOα mice and no difference was seen in the lumbar spine. Similarly, Parrika *et al.* (2005) demonstrated increase in trabecular bone formation in one year old male and female ERKOα mice. The reasons for this discrepancy might be explained by the animal's age and the methods used for analysis. In our study we have used 8-10 weeks old mice, while Syed *et al.* (2011) and Parrika *et al.* (2005) had used 3 month and

1 year aged mice, respectively. We have analyzed femur and vertebrae by microCT while others have used the tibia and histomorphometric parameters (Lindberg *et al.*, 2001b; Syed *et al.*, 2011).

It is already known that estrogen lack yield increased maxillary bone remodeling in rats, as measured by the amount of tooth movement (Salazar *et al.*, 2011). However, our study firstly demonstrates the participation of ERα receptor in the maintenance of maxillary bone and its importance in bone remodeling. Our results are not in agreement with literature which demonstrated decreased response of the ulna and tibia to mechanical loading in ERKOα mice (Callewaert *et al.*, 2010; Lee *et al.*, 2003; Lee *et al.*, 2004; Lee and Lanyon, 2004; Saxon *et al.*, 2012; Windahl *et al.*, 2013). However, most of these studies were focused in cortical bone. Our findings demonstrated increased alveolar bone remodeling in ERKOα mice which might be explained by the increased rate of bone remodeling in the maxillary bones compared to long bones (Huja and Beck, 2008). We are in accordance with Saxon *et al.* (2012) that demonstrated enhanced cortical bone formation rate in the femur of female ERKOα mice.

Estrogen receptor β (ERβ) also is responsible for regulation of the skeleton (Couse and Korach, 1999; Saxon *et al.*, 2012). Female and male ERβ deficient mice are fertile and ERβ is indispensable for normal ovarian morphology and function (Lindberg *et al.*, 2001a; Walker and Korach, 2004). ERα and ERβ exert opposing effects in the regulation of bone longitudinal growth (Krege *et al.*, 1998; Vidal *et al.*, 2000) and ERβ participates in aged related bone loss (Windahl *et al.*, 2001).

Previous studies described the presence of ERβ in human gingival and periodontal tissue (Jonsson *et al.*, 2004; Liang *et al.*, 2008) and that ERα regulates osteogenic differentiation of human periodontal ligament tissue-derived mesenchymal stem cells which may be involved in the pathogenesis of estrogen related periodontal disease (Cai *et al.*, 2013). However, it seems therefore, that ERα receptor is of great importance to maxillary alveolar bone

maintenance once ERα receptor deletion is detrimental for alveolar maxillary bone in female and male mice causing bone microarchiteture deterioration.

We have verified gender-dependent parameters in WT mice, with male mice exhibiting increased bone volume of femur and vertebrae and diminished maxillary alveolar bone compared to the female. However, these differences between genders are not seen in ERKOα mice which might suggest the importance of ERα receptor in mice to maintain physiological bone levels (Vidal *et al.*, 2000).

The main mineral component of bone is hydroxyapatite, a mineral form of calcium (Ca^{2+}) and phosphorus (P) (Boskey, 1981). Analysis of the changes of the Ca^{2+} and P ratio may contribute for the assessment of bone health (Bonjour, 2011). We found that either female or male ERKOα mice exhibited reduced maxillary bone Ca^{2+} percentage, reinforcing the importance of ERα receptor in bone quality. Marchini *et al.* (2012) found no difference in the Ca^{2+} concentration of the alveolar bone between sham and ovariectomized female rats; on the contrary, the OVX mice showed the highest average in Ca/P ratio.

It is already known that maxillary bone remodeling is influenced by inflammatory mediators (Andrade *et al.*, 2007; Taddei *et al.*, 2013). Our results showed that IL-33 expression is augmented in periodontium of ERKOα mice. IL-33 is expressed in bone tissue and acts as an osteoprotective molecule (Keller *et al.*, 2012; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011). The alveolar bone loss in ERKOα mice seems to be related with the increase of IL-33 (Mun *et al.*, 2010). However, the relation between estrogen and IL-33 in bone remains unclear and further studies should be conducted.

Interestingly, despite the augmented maxillary alveolar bone loss, both female and male ERKOα mice did not showed increase in the expression of osteoclastic markers, TNF-α, RANK, RANKL and OPG levels. Literature has demonstrated the contribution of RANKL, OPG and TNF-α in mediating bone loss after estrogen lack (Aoki *et al.*, 2006; Roggia *et al.*,

2004). In our study, male ERKOα mice exhibited decreased TNF-α besides the fact of the osteoporotic alveolar bone phenotype, which may be associated with the cofounding system effects present in ERKOα mice. Conversely to our study, Ikeda *et al.* (2011) reported that OPG and IL-6 production are modulated by ERα receptor in osteoblasts transgenic mice. However, our findings showed no significant difference for RANK, RANKL and OPG in ERKOα mice which might be explained by Liang *et al.* (2008) which demonstrated that estrogen regulates OPG and RANKL only via ERβ in the periodontium.

In vitro results showed enhanced $TRAP^+$ cells formation, increased osteoblasts calcium deposition and alkaline phosphatase activity in BMC from ERKOα mice, which is in agreement with previous reports (Nakamura *et al.*, 2007; Parikka *et al.*, 2005; Syed *et al.*, 2011). It may suggest that maxillary alveolar bone, femur and vertebrae osteoporotic phenotype are associated with increased osteoclast differentiation prevailing from osteoblast activity.

In conclusion, we provide herein evidence that estrogen receptor alpha (ERα) participates in maxillary alveolar bone protection possibly via down-regulation of osteoclastogenesis.

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Table 1 - Oligonucleotide sequences for different bone markers genes.

Gene	Primer sequence forward (F) and reverse (R)
Il33 (IL-33)	F: CAAAGTTCAGCAGCACCGCAG R: TTATGGTGAGGCCAGAACGGAG
Tnf (TNF-α)	F: GCGACGTGGAACTGGCAGAA R: TTTGGGAACTTCTCATCCCTTTGGG
Tfnrsf11a (RANK)	F: AGCATCCCTTGCAGCTCAACA R: TTCCGTTGTCCCCTGGTGTG
Tnfsf11 (RANKL)	F: CGTGCAGAAGGAACTGCAACAC R: TGGTGAGGTGTGCAAATGGCT
Tnfrsf11b (OPG)	F: TCATCCAAGACATTGACCTCTGTGA R: GCTGCTCGCTCGATTTGCAG

Figure 1 - Bone microarquitecture of femur and vertebrae in female and male Wild-Type (WT - C57BL6/J) mice and estrogen receptor alpha deficient (ERKOα) mice. A - Femur representative images of female and male WT and ERKOα mice. B - Femur microCT results. C - Vertebrae representative images of female and male WT and ERKOα mice. D - Vertebrae microCT results. MicroCT parameters: percent bone volume (BV/TV %), bone volume (BV, mm^3), structure model index (SMI), bone mineral density (BMD, g/cm^{-3}), trabecular thickness (Tb.Th, µm), trabecular separation (Tb.Sp, µm) and trabecular number (Tb.N, 1/mm). Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from WT. $^{+}$ $p < 0.05$ - statistical difference from female mice. Statistical analysis was performed Two-way ANOVA with Newman-Keuls multiple comparison test.

Figure 1

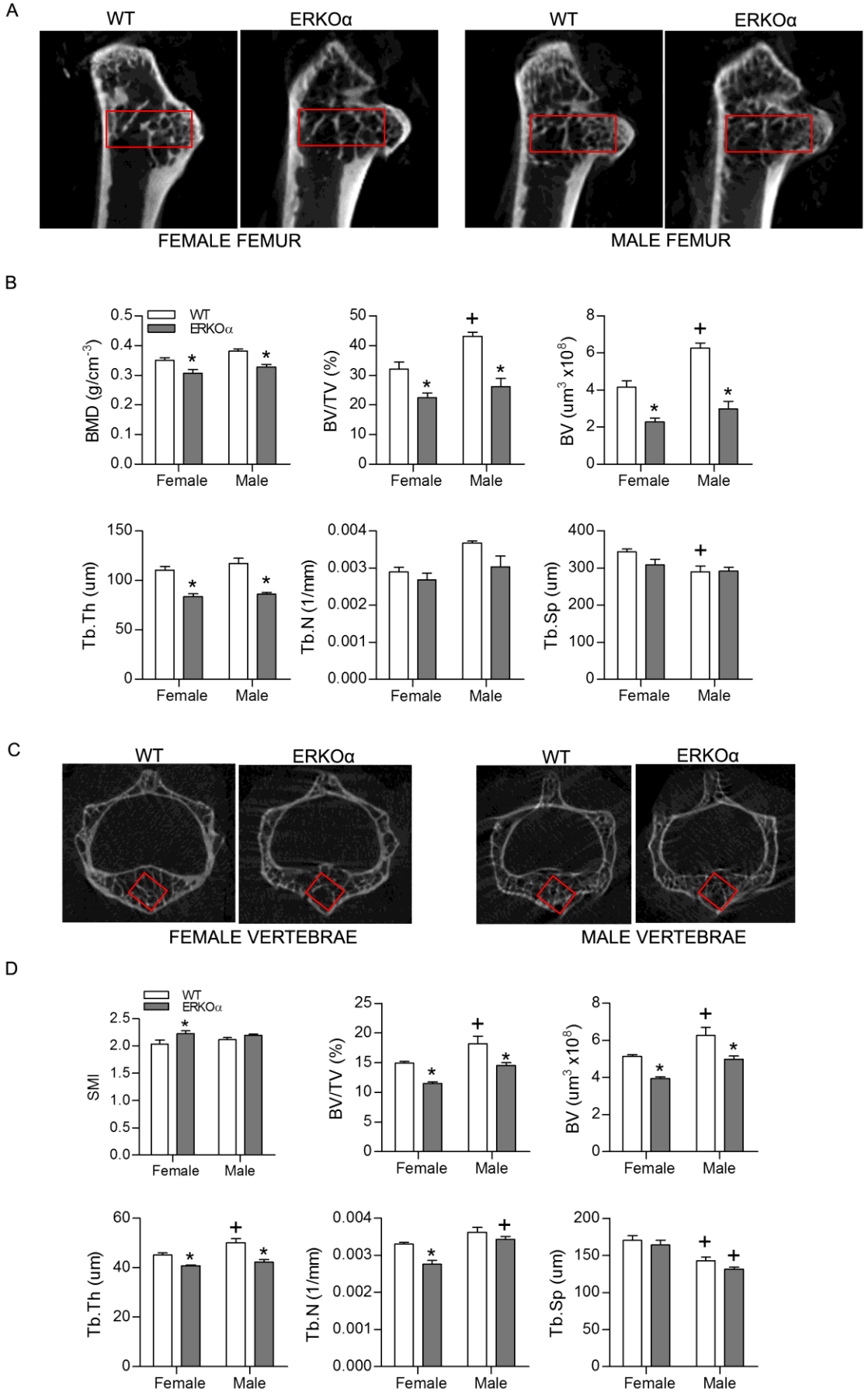

A
WT
ERKOα
WT
ERKOα
FEMALE FEMUR
MALE FEMUR
B
WT
ERKOα
BMD (g/cm-3)
BV/TV (%)
BV (um3 x10^8)
Tb.Th (um)
Tb.N (1/mm)
Tb.Sp (um)
Female
Male
C
WT
ERKOα
WT
ERKOα
FEMALE VERTEBRAE
MALE VERTEBRAE
D
WT
ERKOα
SMI
BV/TV (%)
BV (um3 x10^8)
Tb.Th (um)
Tb.N (1/mm)
Tb.Sp (um)
Female
Male

Figure 2 - Microarquitecture of maxillary bone of the female and male Wild-Type (WT - C57BL6/J mice) and estrogen receptor alpha deficient mice (ERKOα). A - Maxillae representative images of female and male WT and ERKOα mice. B - MicroCT results. MicroCT parameters: percent bone volume (BV/TV %), bone volume (BV, mm^3), structure model index (SMI), trabecular thickness (Tb.Th, µm), trabecular separation (Tb.Sp, µm) and trabecular number (Tb.N, 1/mm). Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from WT. $^+$ $p < 0.05$ - statistical difference from female mice. Statistical analysis was performed Two-way ANOVA with Newman-Keuls multiple comparison test.

Figure 2

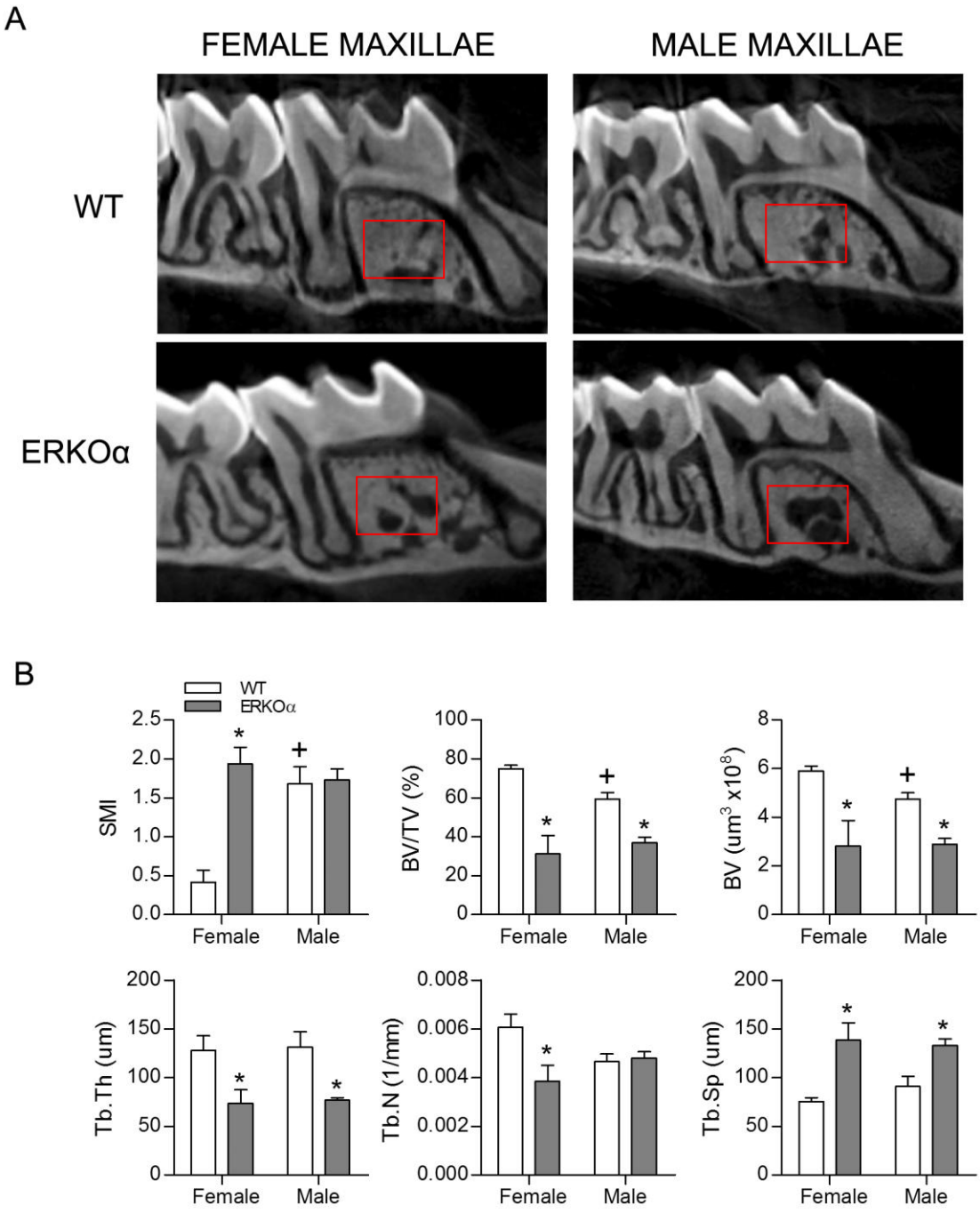

A
FEMALE MAXILLAE
MALE MAXILLAE
WT
ERKOα
B
WT
ERKOα
SMI
BV/TV (%)
BV (um3 x10^8)
Tb.Th (um)
Tb.N (1/mm)
Tb.Sp (um)
Female
Male

Figure 3 - A - Representative images of female and male WT and ERKOα mice maxillary bone submitted to orthodontic tooth movement. B - Deletion of estrogen receptor alpha (ERα) increases orthodontic tooth movement (OTM) in ERKOα female and male mice. C - Calcium (Ca) percentage in the furcation area of the first molars in WT and ERKOα mice. Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from WT. Statistical analysis was performed by t-test. $^{+}$ $p < 0.05$ - statistical difference from female mice. $^{\#}$ $p < 0.05$ - statistical difference from control side. Statistical analysis was performed Two-way ANOVA with Newman-Keuls multiple comparison test.

Figure 3

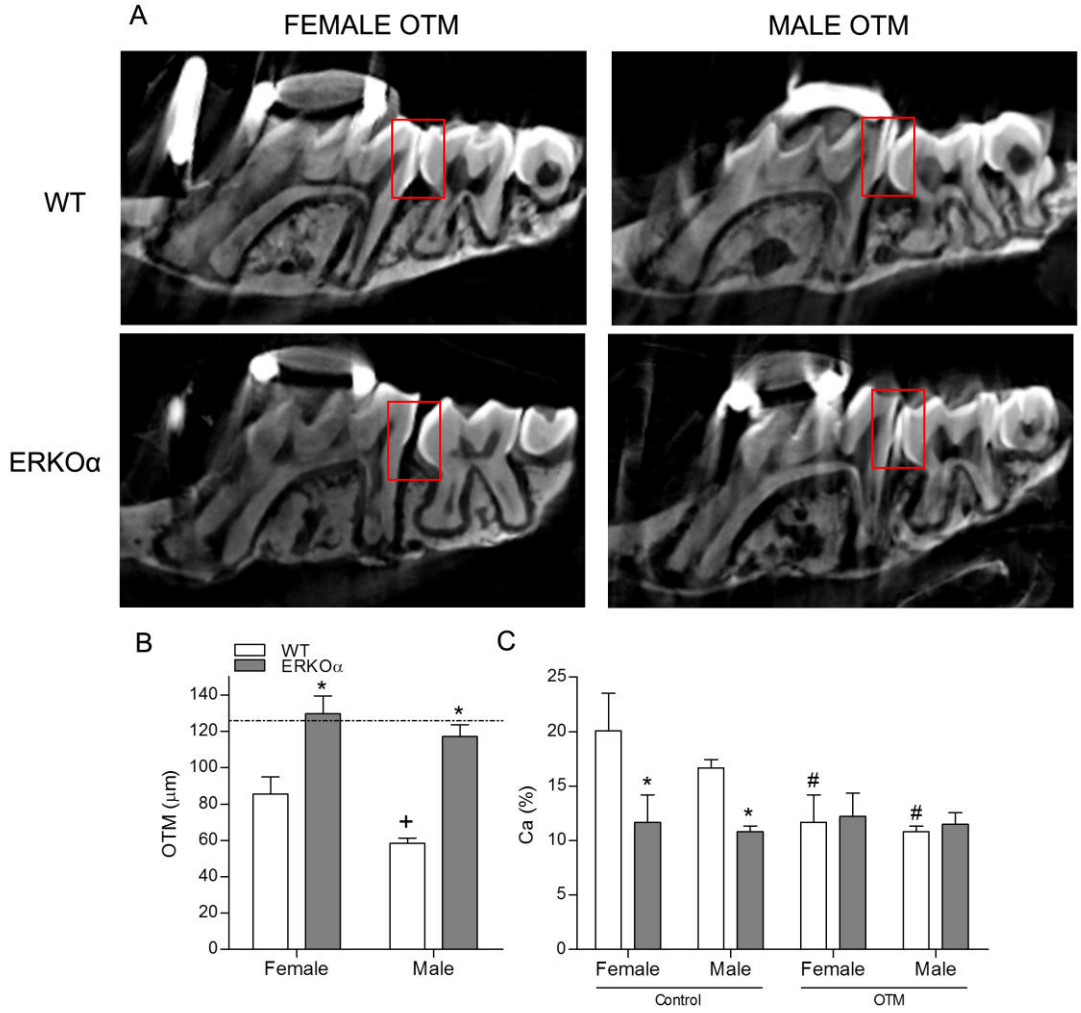

A
FEMALE OTM
MALE OTM
WT
ERKOα
B
WT
ERKOα
OTM (μm)
140
120
100
80
60
40
20
0
Female
Male
C
Ca (%)
25
20
15
10
5
0
Female
Male
Female
Male
Control
OTM

Figure 4 - (A) mRNA fold change of interleukin-33 (IL-33), (B) tumor necrosis factor alpha (TNF-α), (C) receptor activator of nuclear factor kappa-B (RANK), (D) ligand of the receptor activator of nuclear factor kappa-B (RANKL), (E) osteoprotegerin (OPG) and (F) RANKL/OPG ratio in the alveolar bone and periodontium samples of female and male WT and ERKOα mice in control side. Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from WT. $^{+}$ $p < 0.05$ - statistical difference from female mice. Statistical analysis was performed Two-way ANOVA with Newman-Keuls multiple comparison test.

Figure 4

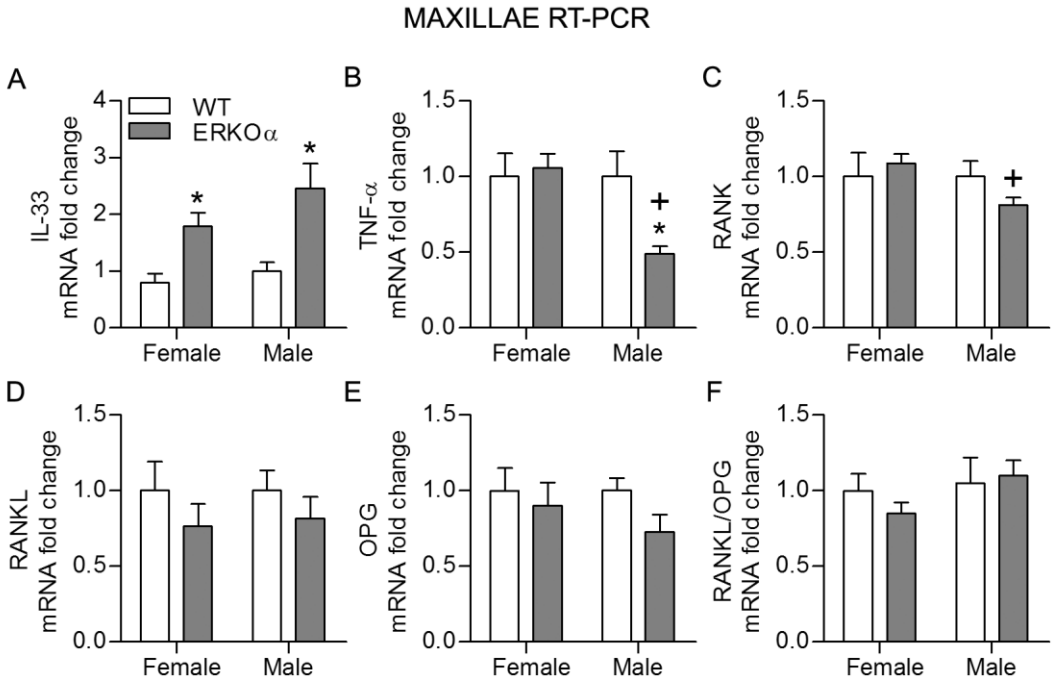
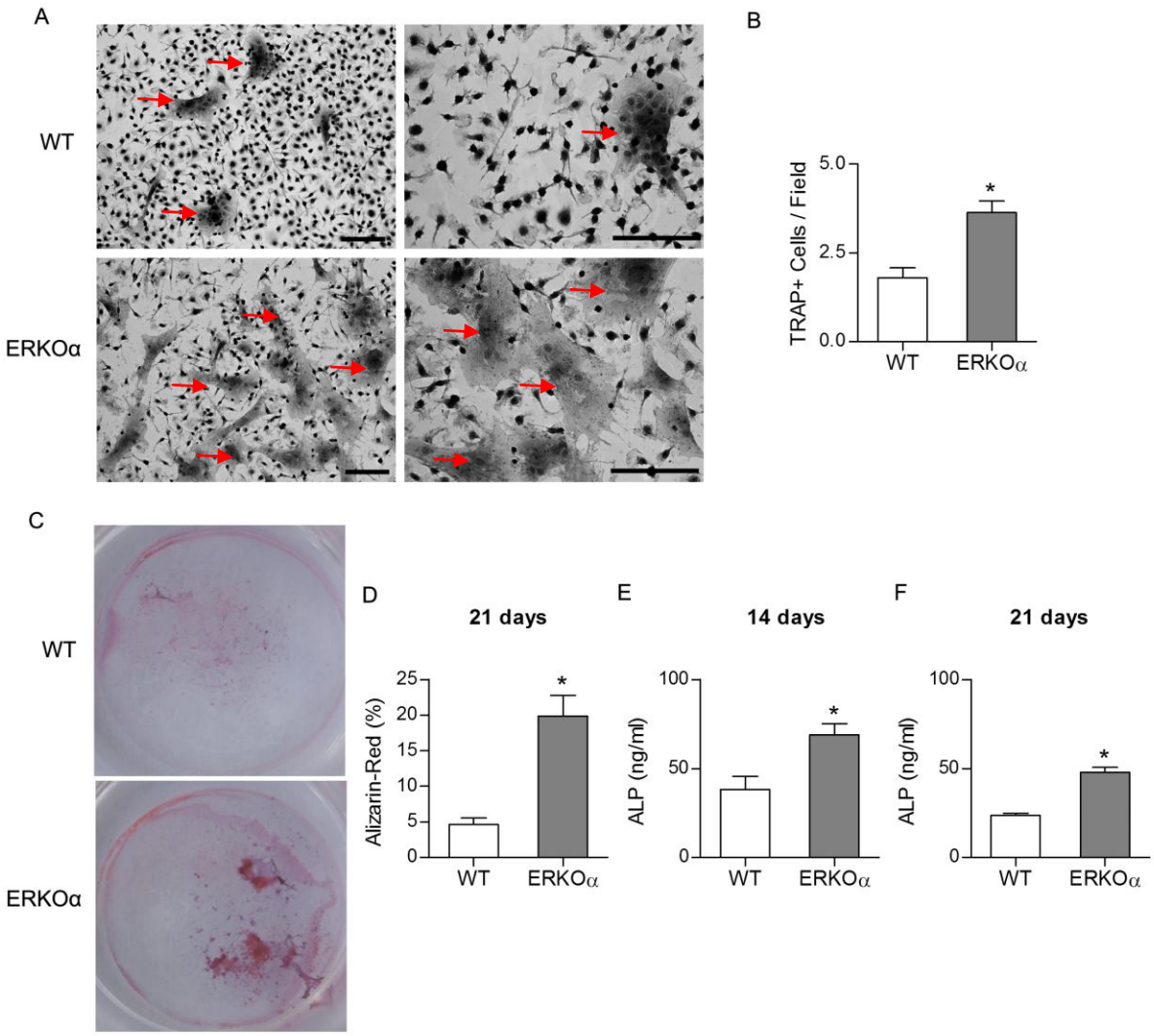

MAXILLAE RT-PCR
A
IL-33
mRNA fold change
WT
ERKOα
B
TNF-α
mRNA fold change
C
RANK
mRNA fold change
D
RANKL
mRNA fold change
E
OPG
mRNA fold change
F
RANKL/OPG
mRNA fold change
Female
Male

Figure 5 - A and B – Osteoclast differentiation from bone marrow cells of Wild-Type (WT - C57BL6/J) mice and estrogen receptor alpha deficient (ERKOα) mice. C and D - Bone marrow cells of WT and ERKOα were induced to osteoblast differentiation. Osteoblasts calcium deposits were stained with alizarin red. E and F - ALP levels after 14 and 21 days of osteoblast differentiation. Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from WT. Statistical analysis was performed by t-test. Scale bar 100 um.

Figure 5


A
WT
ERKOα
B
TRAP+ Cells / Field
5.0
2.5
0.0
*
WT
ERKOα
C
WT
ERKOα
D
21 days
Alizarin-Red (%)
25
20
15
10
5
0
*
WT
ERKOα
E
14 days
ALP (ng/ml)
100
50
0
*
WT
ERKOα
F
21 days
ALP (ng/ml)
100
50
0
*
WT
ERKOα

PARTE III

Nossos resultados nos permitem inferir que o receptor de estradiol ERα participa na perda óssea alveolar. Os animais ERKO apresentam aumento da perda óssea alveolar e da síntese da interleucina-33 (IL-33) nos ossos maxilares. A IL-33 é uma molécula inflamatória da família da IL-1 que, assim como o estrogênio, participa na proteção óssea por meio da diminuição da reabsorção óssea e estímulo da produção da matriz óssea (Keller *et al.*, 2012; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011). Entretanto, não há estudos na literatura que indiquem a relação dos estrogênios e IL-33 nos ossos maxilares, sendo este nosso terceiro objetivo. Os resultados e discussão referentes ao **objetivo 3** serão apresentados no formato do artigo científico 3.

ARTIGO CIENTÍFICO 3

The IL-33/ST2 paradigm: lack of anti-resorptive effect in osteoporosis model.

ABSTRACT

Introduction: Regulation of bone homeostasis by 17β-estradiol (E2) is mediated by a network of inflammatory molecules that directly affects the bone cells. In same way, interleukin-33 (IL-33), a member of the interleukin-1 (IL-1) family, acts through ST2 receptor and has inhibitory effects in bone resorption. **Purpose:** To investigated the effect of ST2/IL-33 in alveolar bone loss using a model of osteoporosis. **Methods:** Balb/c (Wilde-type-WT) and $ST2^{-/-}$ mice were ovariectomized and implanted with oil- (OVX) or E2-containing capsules (OVX+E2). Ovary-intact mice were used as controls. Mechanical maxillary bone remodeling was induced by orthodontic tooth movement (OTM). Histomorphometric analyses were done to evaluate, bone volume, osteoclasts and osteoblasts counts, range of OTM and molecular analysis to determine expression of bone markers. Isolated bone marrow cells (BMC) from Balb/c ovary-intact, OVX and OVX+E2 mice were used to evaluate effect of E2 and IL-33 in osteoclasts differentiation. **Results:** Similar effects of OVX (loss of bone mass) were observed in long bones of WT and ST2 deficient mice. However, OVX of $ST2^{-/-}$ mice did not induce bone loss in maxillae. Ovariectomy also resulted in increase of bone resorption in WT mice submitted to mechanical loading. In contrast, OVX $ST2^{-/-}$ mice demonstrated reduced OTM and bone resorption and increased number of osteoblasts, when compared to WT OVX group. Molecular analysis of periodontium after bone remodeling induction (OTM) demonstrated that OVX yielded increased expression of TNF-α, RANK and RANKL/OPG ratio and diminished expression of RUNX2, IL-10 and semaphorin3A in WT mice. In ST2 deficient mice, OVX produce similar

effects when analyzing RANK, RANKL/OPG ratio and RUNX2. In contrast, production of TNF-α and IL-10 was not affected by OVX and expression of semaphorin3A was slightly increased in OVX ST2 deficient mice. *In vitro* results confirmed that E2 and IL-33 were able to suppress osteoclasts formation. Nevertheless, when BMC were taken from OVX mice, IL-33 treatment did not affect osteoclastogenesis. **Conclusion:** These results indicate that estrogen deficiency abrogates osteoprotective effect of IL-33/ST2 in alveolar bone. It would be linked to apparent osteoclasts unresponsiveness to IL-33 in conditions of estrogen lack.

KEY WORDS: interleukin-33, estrogens, maxilla, alveolar bone loss, osteoporosis

Introduction

The decline in estrogen levels associated with menopause causes trabecular and cortical bone loss in women and is associated with a high bone remodeling rate, which can lead to an osteoporosis process (Kanis *et al.*, 2013) being the proximal femur and vertebral bodies the most common affected sites. Estrogen plays a central role in bone homeostasis/remodeling which is a dynamic process and is orchestrated by bone-producing, osteoblasts, and bone-resorbing cells, osteoclasts (Nakamura *et al.*, 2007; Novack, 2007; Okazaki *et al.*, 2002; Saika *et al.*, 2001; Weitzmann and Pacifici, 2006).

Estrogen has direct effects in both osteoblasts and osteoclasts by affecting the secretion of several cytokines. Estrogen deficiency results in bone loss mainly via up-regulation of osteoclastogenesis through the increased production of tumor-necrosis factor alpha (TNF-α) (Roggia *et al.*, 2001), interleukin-1 (IL-1) (Kimble *et al.*, 1995), and interleukin-6 (IL-6) (Jilka *et al.*, 1992). Estrogen inhibits receptor activator of nuclear factor kappa B ligand (RANKL)-induced osteoclastic differentiation (Chen *et al.*, 2014). Another mechanism by which estrogen contributes to protect bone loss is the up-regulation of osteoprotegerin (OPG) secretion by osteoblastic (Hofbauer *et al.*, 1999) and marrow stromal cells (Saika *et al.*, 2001). Moreover, estrogen inhibits bone resorption via up-regulation of IL-10 (Tural *et al.*, 2013) and semaphorin3A (Sema3A) administration can exert an osteoprotective function in estrogen lack conditions after ovariectomy (Hayashi *et al.*, 2012; Tse, 2012). Therefore, estrogen effects on bone homeostasis are dependent on inflammatory cytokines regulation. A disruption of this inflammatory network balance of the pro and anti-resorptive factors may result in bone loss.

Interleukin-33 (IL-33) is one cytokine that may influence bone scenario (Keller *et al.*, 2012; Mun *et al.*, 2010; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011) and is

involved in T cell-mediated immune responses (Schmitz *et al.*, 2005). IL-33 can be released upon cell injury as an alarmin (Haraldsen *et al.*, 2009). IL-33 is a member of IL-1 family which acts as a pro-inflammatory cytokine via a cell-surface receptor complex of ST2 and IL-1 receptor accessory protein (Schmitz *et al.*, 2005) leading to the activation of intracellular signaling pathways (Ali *et al.*, 2011). IL‑33 can act as a pro- or anti-inflammatory cytokine and is associated with the first line of host defense against pathogens in parasitic infection and atherosclerosis, but it is also associated to severe pathological changes in the lung, digestive tract (Palmer and Gabay, 2011) and exacerbation of rheumatoid arthritis (Verri *et al.*, 2010).

Recent reports support the evidence of a direct effect of IL-33 in bone cells *in vitro* and *in vivo* (Keller *et al.*, 2012; Mun *et al.*, 2010; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011). Some studies indicate that IL-33 is an important bone-protecting cytokine which inhibits osteoclast differentiation from bone marrow precursor cells even in the presence of RANKL and macrophage colony-stimulating factor (M-CSF) (Keller *et al.*, 2012; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011) and is associated with an increased production of anti-osteoclastogenic cytokines like IL-4, IL-13, IL-10 and IFN-γ (Saleh *et al.*, 2011; Zaiss *et al.*, 2011). Mice lacking IL-33 receptor (ST2) display increased bone resorption and low trabecular bone mass phenotype caused by increased osteoclastogenesis (Keller *et al.*, 2012; Schulze *et al.*, 2011). In contrast, in human $CD14^{+}$ monocytes, IL-33 has also been documented to stimulate formation of functional osteoclasts and induce resorption mediated by TNF-α receptor-associated factor 6 (TRAF6) (Mun *et al.*, 2010). Similarly, IL-33 is expressed in osteoblasts and its expression is increased during osteoblast differentiation (Schulze *et al.*, 2011) which promotes osteoblastic matrix mineral deposition (Saleh *et al.*, 2011). IL-33 and estrogen seems to have a clear relationship with bone metabolism and both apparently prevent bone resorption. However, there are no studies demonstrating ST2/IL-33

effects in bone loss associated with estrogen deficiency. Herein, we investigated the effect of ST2/IL-33 in alveolar bone loss by using two different experimental models: osteoporosis and mechanically-induced bone remodeling.

Material and methods

Experimental protocol

Female Balb/c wild-type (WT) and mice deficient for the ST2 receptor ($ST2^{-/-}$), 8-10 weeks old, were obtained from the University of São Paulo. The animals were treated under the ethical regulations for animal experiments, defined by the Ethics Committee on the Use of Experimental Animals of the Federal University of Minas Gerais (protocol 39/2011 and 130/2012). Ovary-intact regularly cycling WT and $ST2^{-/-}$ mice (n=5 per group) were used to determine the phenotype of the uterus, maxillary bone and femur of the ST2 receptor deficient mice. The animals were decapitated on the day of proestrus of the estrous cycle, characterized by elevated serum estrogen levels (Nelson *et al.*, 1981).

Next we investigated the effect of ST2/IL-33 in alveolar bone loss by using a model of osteoporosis WT and $ST2^{-/-}$ mice (n=5 per group). The animals were bilaterally ovariectomized and immediately implanted with subcutaneous capsules containing 60 µg/implant/mouse of 17β-estradiol (E2) (Sigma-Aldrich, St. Louis, MO; OVX+E2) or corn oil (OVX) (Macari *et al.*, 2015). The animals were euthanized by decapitation 30 days after ovariectomy and the uterus, maxilla and femur were collected for analysis. The ovariectomy success was determined by the uterus weight.

Alveolar bone remodeling induced by mechanical loading

The alveolar bone remodeling was induced by orthodontic tooth movement (OTM) using a Ni-Ti 0.25×0.76 mm coil spring (Lancer Orthodontics, San Marcos, CA, USA). The

appliance was bonded between upper right first molar and the incisors and exerted a force of 0.35 N in the tooth mesial direction as previously described (Taddei *et al.*, 2012). For histomorphometric and molecular analysis, the left side (without appliance) of the alveolar bone and periodontium was used as control (C) and the right side with induced bone remodeling by 12 days of mechanical loading with orthodontic tooth movement was used as experimental side (OTM).

Histomorphometric analysis

The right and the left maxillary halves were fixed in 10% formaldehyde, decalcified in 14% EDTA and embedded in paraffin. Sagittal sections of 5 µm were stained with hematoxylin and eosin; tartrate resistant acid phosphatase (TRAP; Sigma-Aldrich, Saint Louis, MO, USA) and Masson's Thrichrome. Five sections per animal were evaluated under light microscope (Olympus AX70 Light, Tokyo, Japan). The tooth movement was quantified as previously described (Taddei *et al.*, 2012).

The furcation area of the first molar root was selected for analyses of the alveolar bone volume/total volume percentage (BV/TV%) by using Image J software (National Institutes of Health, USA). The mesial side of the first upper molar distal-buccal root was used for the TRAP positive osteoclast counts and the distal side of the same tooth was used for histomorphometric quantification of Masson's Thrichrome stained osteoblasts to determine the osteoblasts number per bone perimeter (ObN/BPm). All osteoblasts were identified and counted according to their morphology (Takano-Yamamoto and Rodan, 1990).

The femurs were fixed in 10% formaldehyde, dehydrated and embedded in resin (Technovit 7200, Exakt, Kulzer GmbH, Wehrheim, Germany). The blocks were cut in a sagital plane using a diamond band saw fitted into a precision slicing machine (Exakt, Apparatebau, Norderstedt, Germany), reduced to a thickness of about 50 µm using a cutting –

grinding device (Exakt) and stained by Von Kossa staining. The proximal metaphysis and the diaphysis were analyzed under a light microscope connected to a digital camera (PowerShot A620, Canon, Tokyo, Honshu, Japan) to obtain BV/TV% measured using the Image J software.

mRNA extraction and real time PCR

For RT-PCR analysis, we extracted total RNA from the periodontal ligament and surrounding alveolar bone samples from the upper first molars by using the RNeasy kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturers' instructions. Gingival tissue, oral mucosa and tooth were discarded. The integrity of RNA samples was checked by analyzing 1 µg of total RNA on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturers' instructions. After RNA extraction, complementary DNA was synthesized by using 3 µg of RNA through a reverse transcription reaction using Quanti TectRT kit (Qiagen Inc, Valencia, CA, USA).The targets analyzed were: semaphorin 3A (Sema3A), interleukin-10 (IL-10), receptor activator of nuclear factor kappa-B (RANK), receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), tumor necrosis factor alpha (TNF-α), runt-related transcription factor 2 (RUNX2). The mRNA levels were measured by means of Real Time PCR using TaqMan chemistry (Invitrogen, Carlsbad, CA, USA) in a Viia7 instrument (Life Technologies, Carlsbad, CA) using inventoried optimized primers/probes sets (Invitrogen, Carlsbad, CA, USA), with basic reaction conditions (40 cycles) of conditions were 95°C (10 minutes), 94°C (1 minute), annealing at 56°C (1 minute) and 72°C (2 minutes). The results were depicted as the relative level of gene expression and were calculated in reference to internal controls GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and β-actin expression in the samples.

Osteoclast generation

Bone marrow cells (BMC) were obtained from the femurs of ovary-intact Balb/c mice (regularly cycling animals with no surgical interventions), OVX and OVX+E2 mice. The BMC were incubated in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and soluble macrophage colony-stimulating factor (M-CSF) (100 ng/ml; Peprotech, London, UK) for 6 days to generate pre-osteoclasts. Soluble receptor activator of nuclear factor-$_{K}$B ligand (RANKL) (100 ng/ml; Peprotech) were then added until day 8. In some experiments, we added soluble 17β-Estradiol 10^{-8} M (Sigma-Aldrich) and IL-33 (20 ng/ml) (Sigma-Aldrich). Cells were fixed with acetone, citrate and formaldehyde 37% and stained by tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich). The percentage of TRAP-positive cells was determined by the proportion of TRAP-negative and TRAP-positive cells in ten fields (magnification 40x) per well.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). The differences among groups were analyzed by two-way ANOVA followed by the Bonferroni post-hoc test. $p < 0.05$ was considered statistically significant.

Results

Efficacy of ovariectomy and E2 replacement

To confirm the ovariectomy success the uterus weight was measured. The uterine horns of ovary-intact $ST2^{-/-}$ mice showed atrophy compared with ovary-intact WT mice ($p < 0.05$) (WT 7.95 ± 0.54, $ST2^{-/-}$ 5.42 ± 0.69 mg/g). Similar uterus atrophy was verified in both WT and ST2 deficient mice after OVX. Accordingly, in all groups it was reversed by E2 replacement (WT OVX 1.37 ± 0.09, WT OVX+E2 10.32 ± 0.45, $ST2^{-/-}$ OVX 1.19 ± 0.05, $ST2^{-/-}$ OVX+E2 9.39 ± 0.32 mg/g) ($p < 0.05$).

IL-33/ST2 protects femur and maxilla from bone loss

Deletion of ST2 receptor resulted in decreased BV/TV% ($p < 0.05$) in femur (Fig. 1A and 1B) and maxillary alveolar bone ($p < 0.05$) (Fig. 2A and 2B).

IL-33/ST2 is important for alveolar bone resorption in an estrogen lack condition

The histomorphometric analysis of the femur (Von Kossa staining) demonstrated similar reduction of BV/TV% in WT and $ST2^{-/-}$ mice after 30 day of OVX ($p < 0.05$) (Fig. 1C and 1D). Differently from the femur, $ST2^{-/-}$ OVX mice presented increased BV/TV% in the alveolar bone compared to $ST2^{-/-}$ OVX+E2 and WT OVX mice ($p < 0.05$) (Fig. 2C and 2D).

IL-33/ST2 lack of protection in the mechanically induced bone remodeling in an osteoporotic situation

During mechanically induced bone remodeling, ovariectomy resulted in augmented bone resorption (measured by amount of orthodontic tooth movement) ($p < 0.05$) (Fig. 3A), number of osteoclasts ($p < 0.05$) (Fig. 3B and 3C) and decreased number of osteoblasts ($p < 0.05$) (Fig. 3D and 3E) in WT mice. ST2 deletion also resulted in higher OTM ($p < 0.05$)

(Fig. 3A), osteoclasts counts ($p < 0.05$) (Fig. 3B and 3C) and decreased osteoblast numbers ($p < 0.05$) (Fig. 3D and 3E) compared with WT. Intriguingly, ovariectomized ST2$^{-/-}$ mice exhibited augmented numbers of osteoclasts ($p < 0.05$) (Fig. 3B and 3C) and osteoblasts counts ($p < 0.05$) (Fig. 3D and 3E) but significantly diminished OTM ($p < 0.05$) (Fig. 3A) compared to WT OVX and ST2-/- OVX+E2.

IL-33 induces maxillary bone resorption in WT ovariectomized mice via down-regulation of IL-10 and Sema3A in a RANK, RANKL/OPG independent manner

Molecular analyses were done using alveolar bone and periodontium in order to understand mechanisms involving ST2/IL-33 and estrogen deficiency. The Control side, with no induced bone remodeling, WT and ST2$^{-/-}$ mice exhibited no changes in TNF-α expression ($p > 0.05$) (Fig. 4.1. A). Ovariectomy increased the expression of RANK (Fig. 4.1. B) ($p < 0.05$) and RANKL/OPG ratio (Fig. 4.1. C) ($p < 0.05$) and decreased the levels of RUNX2 (Fig. 4.1. D) ($p < 0.05$) in WT and ST2$^{-/-}$ mice. The expression of IL-10 (Fig. 4.1. E) was diminished in WT OVX and ST2$^{-/-}$ mice ($p < 0.05$) while ST2$^{-/-}$ OVX mice demonstrated increased IL-10 levels. Ovariectomy yielded decreased levels of Sema3A (Fig. 4.1. F) ($p < 0.05$) in WT OVX and ST2$^{-/-}$ mice however no difference in Sema3A expression ($p > 0.05$) was seen in ST2$^{-/-}$ OVX mice.

OTM induced significant augmented expression of all markers analyzed ($p < 0.05$), the only exception was RANKL/OPG ratio ($p > 0.05$) (Fig. 4.2).

Moreover, the OTM side displayed TNF-α augmented levels in WT OVX mice, however ovariectomy did not change TNF-α expression in ST2$^{-/-}$ OVX mice. All experimental ST2$^{-/-}$ mice groups demonstrated diminished levels of TNF-α ($p < 0.05$) when compared to WT (Fig. 4.2. A). Ovariectomy caused decreased expression of IL-10 in WT OVX mice ($p < 0.05$) but no effect was seen in ST2$^{-/-}$ OVX mice ($p > 0.05$) (Fig. 4.2. E) although IL-10

mRNA expression was increased in $ST2^{-/-}$ OVX mice in comparison to WT OVX mice ($p < 0.05$).

WT OVX and $ST2^{-/-}$ OVX mice showed similar patterns of mRNA expression (OTM side) with increased levels ($p < 0.05$) of RANK (Fig. 4.2. B), RANKL/ OPG ratio (Fig. 4.2. C) and decreased expression of RUNX2 ($p < 0.05$) (Fig. 4.2. D). In same way these cytokines did not exhibited statistical difference between WT and ST2 deficient mice ($p > 0.05$).

Ovariectomy yielded decreased expression of Sema3A in WT OVX mice (OTM side), while $ST2^{-/-}$ OVX mice exhibited increased expression of the same cytokine ($p < 0.05$) (Fig. 4.2. F). Even though Sema3A demonstrated reduced expression in all $ST2^{-/-}$ mice groups ($p < 0.05$), down-regulation of Sema3A induced maxillary bone resorption in ST2 receptor deficient mice ($p < 0.05$) (Fig. 4.2. F).

Absence of IL-33 anti-osteoclastogenic effect in cells derived from ovariectomized mice

To better elucidate the association between estrogen and IL-33 in osteoclast differentiation, bone marrow cells from ovary-intact, OVX+E2 and OVX WT mice were cultivated in the presence of M-CSF and RANKL and treated with soluble 17β-Estradiol 10^{-8} M (E2) and/or IL-33 (20 ng/ml). E2, IL-33 and E2+IL-33 treatments suppressed osteoclasts formation in cultures of BMC from ovary-intact mice ($p < 0.05$) (Fig. 5A and 5B) and OVX+E2 mice ($p < 0.05$) (Fig. 5C and 5D).

The BMC from OVX mice exhibited enhanced TRAP+ cells formation when compared to cells from OVX+E2 group ($p < 0.05$). E2 and IL-33 treatment also suppressed the osteoclast differentiation in BMC from OVX group ($p < 0.05$). However, IL-33 did not result in modification of osteoclasts generation when BMC were taken from OVX mice (Fig. 5C and 5D).

Discussion

Estrogen and IL-33 play a key role in bone regulation through the control of the activity of bone-forming osteoblasts and bone-resorbing osteoclasts (Cenci *et al.*, 2003; Imai *et al.*, 2009; Keller *et al.*, 2012; Mun *et al.*, 2010; Nakamura *et al.*, 2007; Okazaki *et al.*, 2002; Saika *et al.*, 2001; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Srivastava *et al.*, 2001; Weitzmann and Pacifici, 2006; Zaiss *et al.*, 2011). Our results demonstrated that ST2 deficiency results in significant bone loss in femur and maxilla. While ovariectomy similarly reduced femur bone volume of wild-type and ST2 deficient mice, the alveolar bone of $ST2^{-/-}$ was not affected by estrogen lack. Moreover, under mechanical stimuli, ST2 and estrogen individually confers osteo-protection, but in absence of both (ovariectomized ST2 deficient mice) this phenotype was reversed. *In vitro* experiments confirmed anti-osteoclastogenic effects of IL-33 and estrogen; however IL-33 effect is lost in the absence of estrogen. These results indicate that under no disease condition and mechanical stimuli, estrogen lack abrogates osteoprotective effect of IL-33/ST2 in alveolar bone. It would be linked to apparent osteoclasts unresponsiveness to IL-33 in conditions of estrogen lack.

Estrogen is important for bone health and its lack increases bone loss which occurs mainly via increased number of osteoclasts (Imai *et al.*, 2009; Imai *et al.*, 2010; Nakamura *et al.*, 2007), up-regulation of TNF-α, RANKL (Cenci *et al.*, 2000; Roggia *et al.*, 2001) and down-regulation of IL-10 (Luo *et al.*, 2011; Tural *et al.*, 2013). We demonstrated that ovariectomy have yielded increased bone loss in femur and maxillary bone, augmented the mechanical loading OTM, increased number of osteoclasts TRAP+ cells associated with increased TNF-α (OTM side), RANK and RANKL/OPG ratio production (Control and OTM sides) and decreased expression of RUNX2, IL-10 and Sema3A in the induced bone remodeling side (Control and OTM sides). Our results are in agreement with Bonnet *et al.* (2013) which verified femur and alveolar bone loss after ovariectomy, although he analyzed

the mandibular bone and our study is focused in the maxillae. Accordingly, estrogen lack bone resorption is mediated by TNF-α (Roggia *et al.*, 2001) and OPG (Shu *et al.*, 2008) up-regulation and Sema3A administration would decrease OVX bone loss (Hayashi *et al.*, 2012).

Similarly to estrogen, IL-33 also protects bone via osteoclasts regulation by directly acting on osteoclast precursor (Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011) and inducing expression of IL-10 (Saleh *et al.*, 2011). In the absence disease condition IL-33 is expressed in the bone tissue and act as a bone-protective cytokine (Keller *et al.*, 2012; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011). In accordance with Keller *et al.* (2012), which verified that IL-33 has a bone protective function, our findings revealed that IL-33 protects maxilla and femur from bone loss during bone remodeling in the absence disease condition. Our results showed that $ST2^{-/-}$ animals displayed decreased BV/TV% in the femur and maxillary bone which corroborate with the literature (Schulze *et al.*, 2011; Zaiss *et al.*, 2011). There was also increased mechanical loading-induced bone remodeling indicating that ST2 deficient mice present increase bone loss. These findings are in accordance with previous observations demonstrating that IL-33 is a potent suppressor of osteoclast activation (Keller *et al.*, 2012; Schulze *et al.*, 2011) and its absence culminate in increased bone resorption.

Our main goal was to determine the association between estrogen and IL-33 in bone remodeling. IL-33 is a member of IL-1 family and has been associated with inflammatory bone diseases, as arthritis (Palmer *et al.*, 2009; Talabot-Ayer *et al.*, 2014; Verri *et al.*, 2010) and periodontal disease (da Luz *et al.*, 2014; Koseoglu *et al.*, 2014). The literature demonstrated that in a pathological condition as rheumatoid arthritis, IL-33 was seen as an enhancer regulator of TNF-α-induced pro-inflammatory function (Kunisch *et al.*, 2012). Our findings suggested that in the course of osteoporosis, ST2 deficient mice exhibited reduced maxillary bone loss and amount of tooth movement in comparison with WT OVX mice.

Conversely, the number of osteoclasts remained the same in mice lacking ST2 receptor with or without the ovariectomy. These results might indicate that osteoclasts function, but not osteoclasts recruitment, is affected by IL-33 during estrogen lack.

It is already known that estrogen promotes early osteoblasts differentiation (Okazaki *et al.*, 2002) and that IL-33 expression is increased during these period (Schulze *et al.*, 2011). In our study we counted the osteoblasts in distal side of the first upper molar distal-buccal root of WT and $ST2^{-/-}$ mice. Our results demonstrated that ovariectomy and IL-33/ST2 receptor absence caused osteoblasts number impairment. However, in presence of both estrogen and IL-33 (WT OVX+E2 mice) or their complete absence ($ST2^{-/-}$ OVX mice) the maxillary bone recovered the osteoblast counts.

Sema3A which is expressed by osteoclasts and osteoblasts can exert an osteoprotective function (Hayashi *et al.*, 2012) and regulate bone remodeling (Fukuda *et al.*, 2013). Our result exhibited that ovariectomy yielded decrease in Sema3A levels in WT mice, corroborating with previous data which demonstrated that Sema3A administration had decreased bone loss after ovariectomy both by inhibiting osteoclastic bone resorption and promoting osteoblastic bone formation (Hayashi *et al.*, 2012; Tse, 2012). In same way, $ST2^{-/-}$ mice showed diminished levels of Sema3A although ovariectomy did not demonstrated significant difference, indicating that the maxillary bone resorption might be associated to Sema3A down-regulation in IL-33/ST2 absence and that Sema3A and IL-33 are related to alveolar bone loss in an estrogen deficiency situation.

One of the molecular mechanisms by which estrogen exerts its inhibitory effects on osteoclasts bone resorption is by TNF-α down-regulation (Roggia *et al.*, 2001; Roggia *et al.*, 2004), however it is know that IL-33 could induce the production of TNF-α (Moulin *et al.*, 2007). We investigated some molecular markers in the periodontium, including TNF-α, in order to better understand the correlation between estrogen and IL-33 in the bone phenotype.

Accordingly to the literature, our results demonstrated that in estrogen lack with IL-33 presence (WT OVX mice) there was increased TNF-α expression (OTM side). Similarly, there was reduced expression of TNF-α in IL-33 absence ($ST2^{-/-}$ mice).

Estrogen protects bone via up-regulation of IL-10 (Luo *et al.*, 2011; Tural *et al.*, 2013), which is known to inhibit bone resorption (Zhang *et al.*, 2014). However the effect of IL-33 on IL-10 levels is controversial because no effect on IL-10 levels (Schmitz *et al.*, 2005) and increased IL-10 expression (Saleh *et al.*, 2011) was seen in literature after IL-33 stimuli. We verified that estrogen lack in the presence of IL-33 (WT OVX mice) caused decrease in IL-10 expression and no difference in IL-10 levels was seen after ovariectomy in ST2 receptor deficient mice. Our findings suggest that IL-33 contributes to increase bone resorption in osteoporotic process via down-regulation of IL-10.

In addition, we analyzed RANK and RANKL/OPG ratio and osteoblast marker RUNX2. Estrogen is able to inhibit the expression of inflammatory cytokines such as RANK, RANKL/OPG ratio (Onal *et al.*, 2012; Shu *et al.*, 2008). Conversely, IL-33 bone protection acts in an RANK and RANKL/OPG independent manner (Schulze *et al.*, 2011). In agreement with the literature, our results demonstrated similar expression pattern of RANK and RANKL/OPG and RUNX2 (OTM side) between WT and $ST2^{-/-}$ mice groups, which indicate that bone resorption is not correlated to those cytokines and there is no interaction between estrogen and IL-33 in these process. The Control side, with no induced bone remodeling, $ST2^{-/-}$ mice exhibited increased levels of RANKL/OPG ratio and decreased mRNA expression of RUNX2 which might explain the augmented osteoclasts and diminished osteoblasts numbers in these mice group. Ovariectomy in the Control side of $ST2^{-/-}$ OVX mice increased the RANKL/OPG and RUNX2 patterns described above although the osteoclasts number remain the same and there was an increase in the osteoblasts counts. The

results indicated that estrogen lack in ST2 deficient mice might affect these molecular inflammatory mediators however they are not correlated to the cellular changes.

It is well known that estrogen (Imai *et al.*, 2009; Schiller *et al.*, 1997) and IL-33 (Keller *et al.*, 2012; Mun *et al.*, 2010; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011) inhibits osteoclasts differentiation *in vitro*, however their correlation is still unclear. We confirmed that estrogen and IL-33 were able to suppress osteoclasts formation. Nevertheless, when BMC were taken from OVX mice, IL-33 treatment did not affect osteoclasts differentiation.

IL-33 acts through ST2 receptor, can act as a pro- or anti-inflammatory cytokine (Palmer and Gabay, 2011; Zaiss *et al.*, 2011). IL-33 would protect bone (Saleh *et al.*, 2011), however our results revealed that in a pathologic condition, as osteoporosis, estrogen lack abrogates osteoprotective effect of IL-33/ST2 in alveolar bone. It would be linked to apparent osteoclasts unresponsiveness to IL-33 in conditions of estrogen lack. Further studies are warranted to investigate the interaction between estrogen and IL-33/ST2 in bone.

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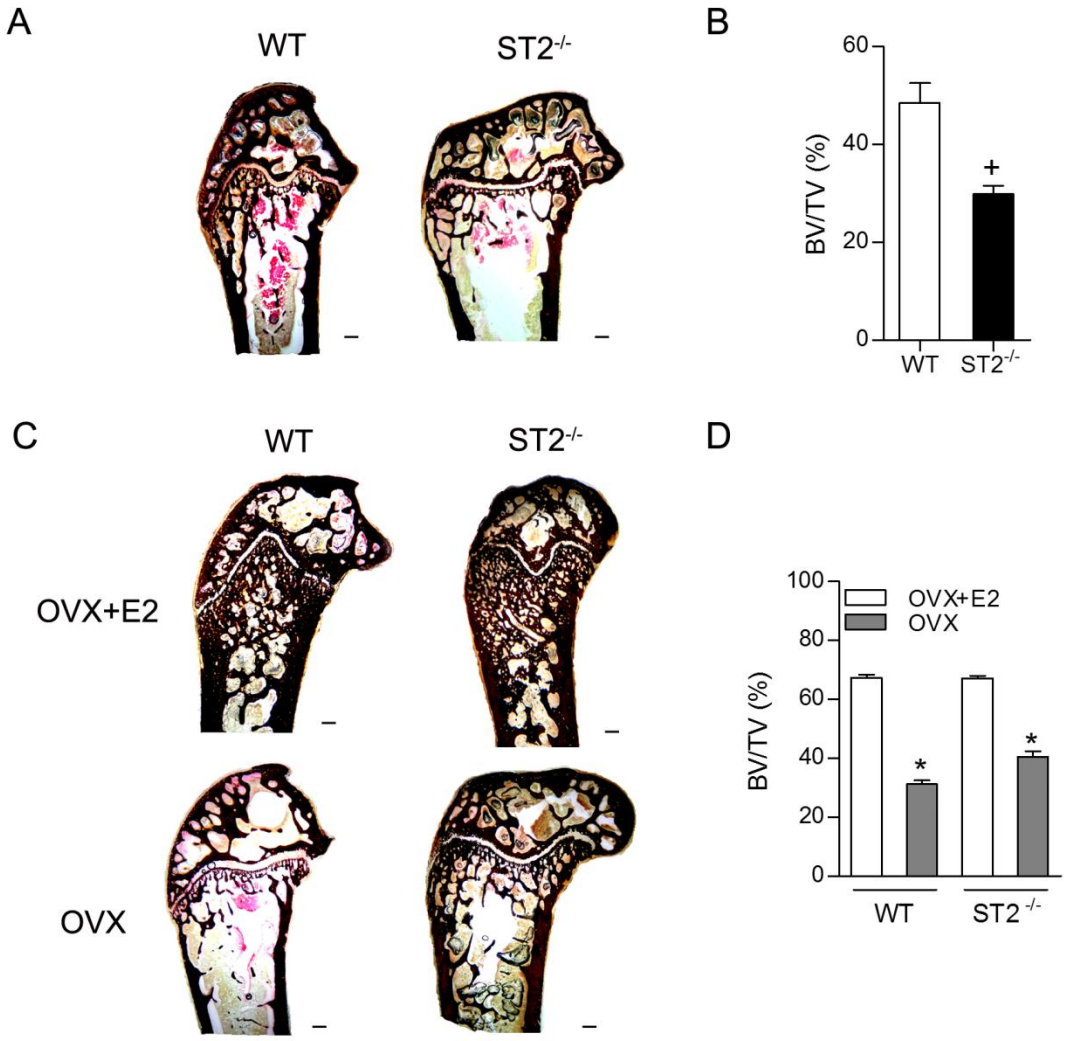
FIGURES AND LEGENDS

Figure 1 - IL-33/ST2 protects femur from bone loss in absence disease condition and does not participate in femur bone resorption in an estrogen lack condition. A - Von Kossa staining representative images of WT and $ST2^{-/-}$ mice femurs (Scale bar = 100 µm). B - ST2 receptor deficient mice showed decreased BV/TV% in femur. C - Von Kossa staining images from OVX+E2 and OVX treatment WT and $ST2^{-/-}$ mice femurs. D - Histomorphometric analysis of the femur demonstrated similar BV/TV% bone loss in WT and $ST2^{-/-}$ mice after 30 day of OVX. Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from OVX+E2 group. $^{+}$ $p < 0.05$ - statistical difference from WT mice within same treatment. Two-way ANOVA with Bonferroni multiple comparison test.

Figure 1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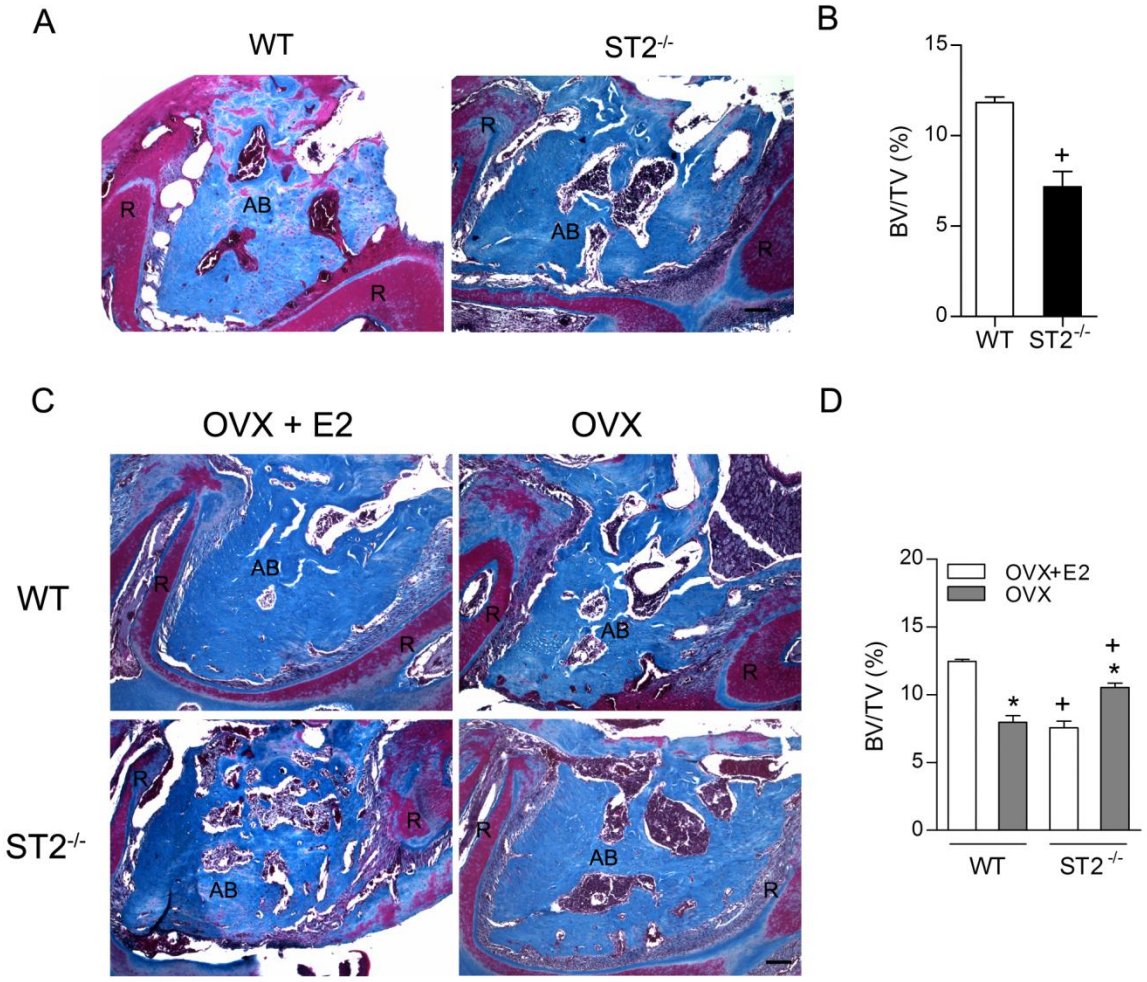
ST2 -/-

Figure 2 - IL-33/ST2 protects maxilla from bone loss in absence disease condition and is important for alveolar bone resorption in an estrogen lack condition. A - Von Kossa staining representative images of WT and $ST2^{-/-}$ mice maxillae (Scale bar = 100 µm). B - ST2 receptor deficient mice showed decreased BV/TV% on maxillary bone. C - Von Kossa staining images from OVX+E2 and OVX treatment WT and $ST2^{-/-}$ mice maxillae. D - Histomorphometric analysis of the maxillae demonstrated increased BV/TV% bone loss in WT OVX and $ST2^{-/-}$ OVX+E2. Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from OVX+E2 group. $^{+}$ $p < 0.05$ - statistical difference from WT mice within same treatment. Two-way ANOVA with Bonferroni multiple comparison test.

Figure 2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WT
ST2 -/-

Figure 3 - Deletion of IL-33/ST2 receptor reduces orthodontic tooth movement (OTM) induced bone remodeling in an osteoporotic situation. We evaluated the effect of the estrogen lack and absence of ST2 receptor in the number of tartrate-resistant acid phosphatase positive cells (TRAP+) osteoclasts cells and osteoblasts per bone perimeter (ObN/BPm) in the control (C) and mechanically induced bone remodeling (OTM) sides. Ovariectomy resulted in augmented bone resorption (measured by amount of OTM) (A), increased number of osteoclasts (B and C) and decreased number of osteoblasts (D and E) in WT mice. ST2 deletion also resulted in higher OTM (A), increased osteoclasts counts (B and C) and diminished number of osteoblasts (D and E) compared with WT. Intriguingly, ovariectomized $ST2^{-/-}$ mice exhibited augmented numbers of osteoclasts (B and C), increased osteoblasts counts (D and E) and significantly diminished OTM (A) compared to WT OVX and ST2-/- OVX+E2. A - Changes in the amount of tooth movement (OTM) in WT and $ST2^{-/-}$ mice. B - The number of TRAP-positive osteoclasts was evaluated in the mesial side of the disto-buccal root of the first molar (black arrows indicate TRAP-positive osteoclasts). C - Number of osteoclasts in the control (C) and orthodontic tooth movement (OTM) sides. D - The distal side of the disto-buccal root of the first molar was used for osteoblasts evaluation by Masson's Thricrome staining (yellow arrows indicate osteoblasts and white arrows indicate osteoclasts). E - Osteoblasts counts in distal side of the disto-buccal root of the first molar. AB, alveolar bone; R, root. Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from OVX+E2 group. $^{\#}$ $p < 0.05$ - statistical difference from control (C) group. $^{+}$ $p < 0.05$ - statistical difference from WT mice within same treatment. Two-way ANOVA with Bonferroni multiple comparison test.

Figure 3

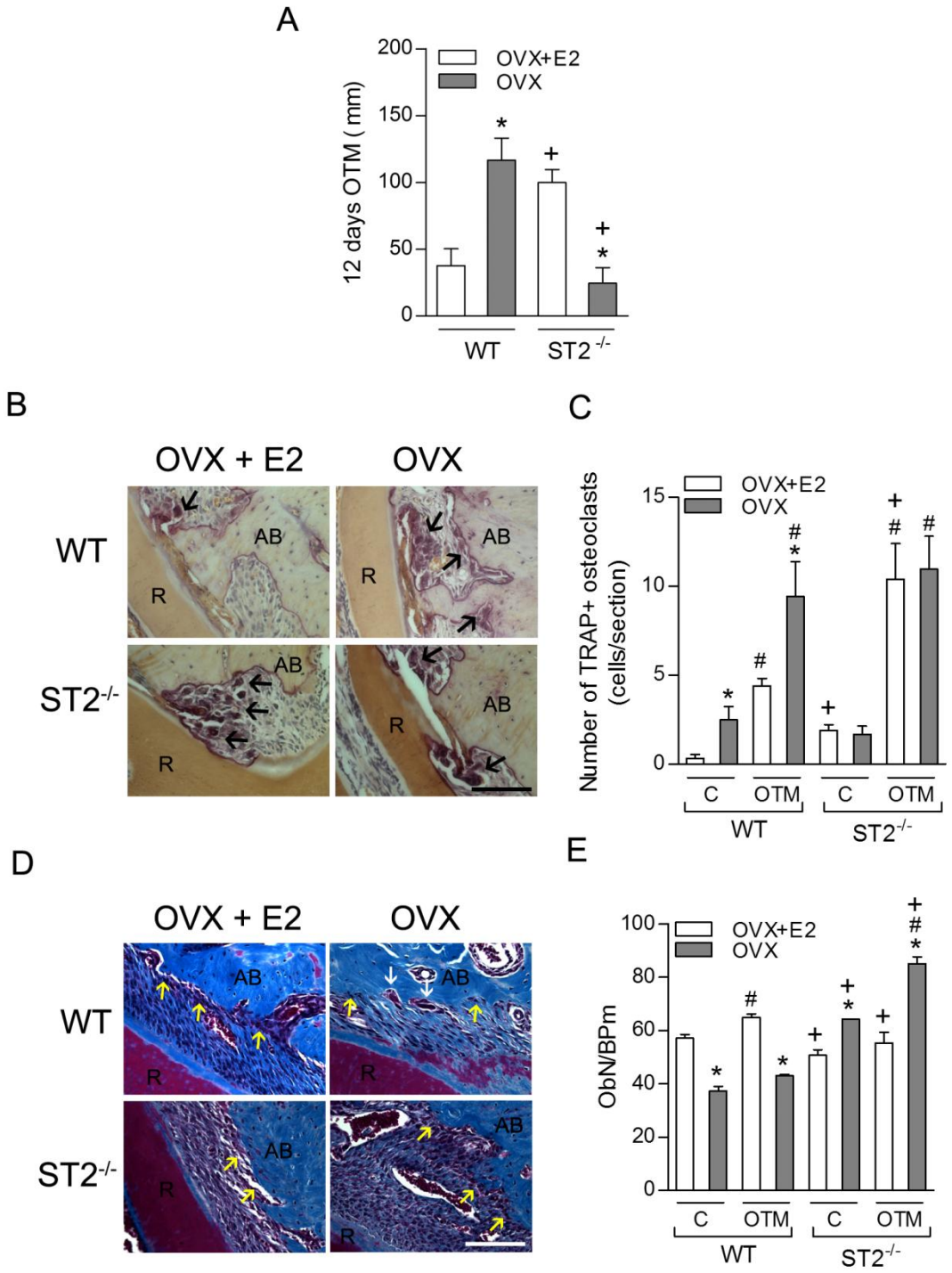

A
12 days OTM (mm)
OVX+E2
OVX
WT
ST2 -/-
B
OVX + E2
OVX
WT
ST2-/-
AB
R
C
Number of TRAP+ osteoclasts (cells/section)
OVX+E2
OVX
C
OTM
WT
ST2-/-
D
OVX + E2
OVX
WT
ST2-/-
AB
R
E
ObN/BPm
OVX+E2
OVX
C
OTM
WT
ST2-/-

Figure 4 - mRNA expression of tumor necrosis factor alpha (TNF-α) (A), receptor activator of nuclear factor kappa-B (RANK) (B) and receptor activator of nuclear factor kappa-B (RANK) (D)and its ligand RANKL (RANKL)/osteoprotegerin (OPG) ratio (C), runt-related transcription factor 2 (RUNX2) (D), interleukin-10 (IL-10) (E) and semaphorin 3A (Sema3A) (F) in WT and $ST2^{-/-}$ mice alveolar bone and periodontium samples after 30 of ovariectomy in the maxillary left side (without appliance - control) (Fig. 4.1.) and the right side with induced bone remodeling by 12 days of mechanical loading with orthodontic tooth movement (OTM) (Fig. 4.2.). Five mice were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from OVX+E2 group. $^{+}$ $p < 0.05$ - statistical difference from WT mice within same treatment. Two-way ANOVA with Bonferroni multiple comparison test.

Fig. 4.1. - Control

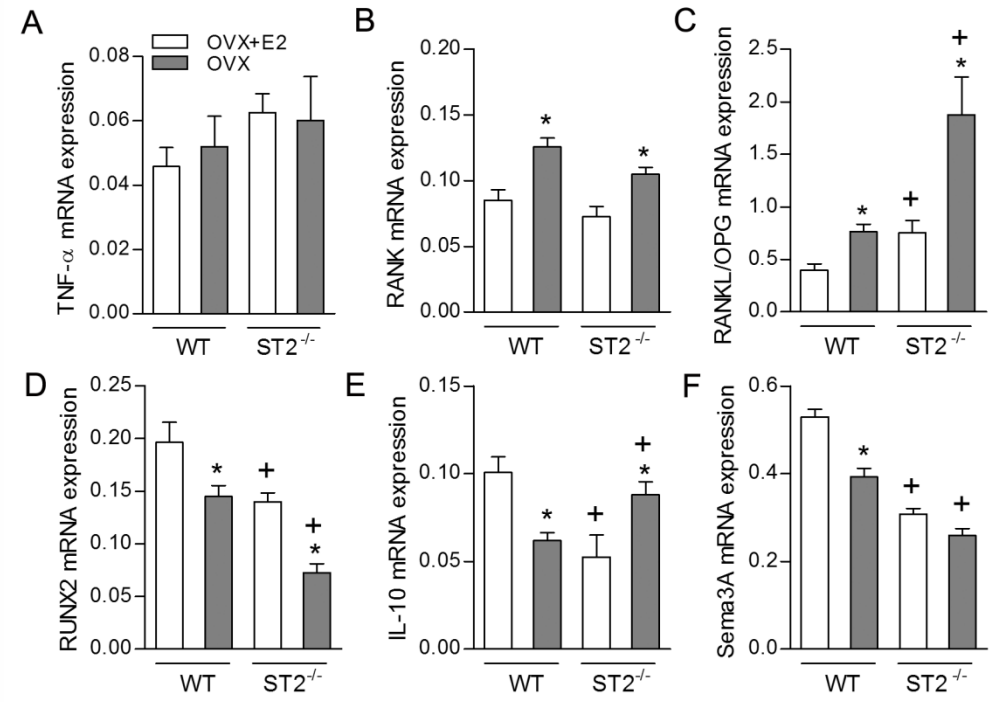


Fig. 4.2. - OTM

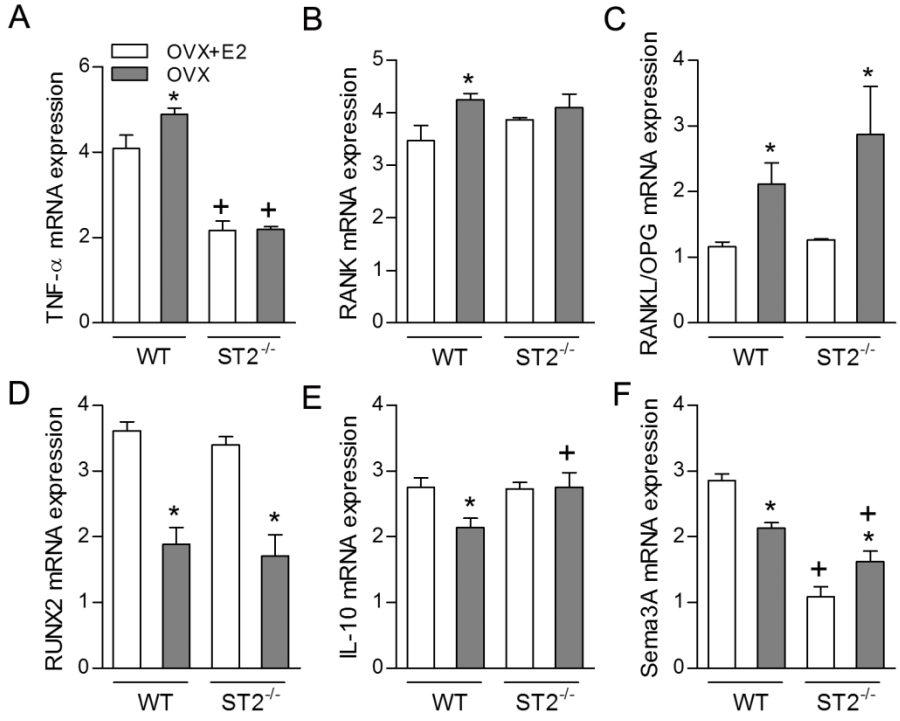
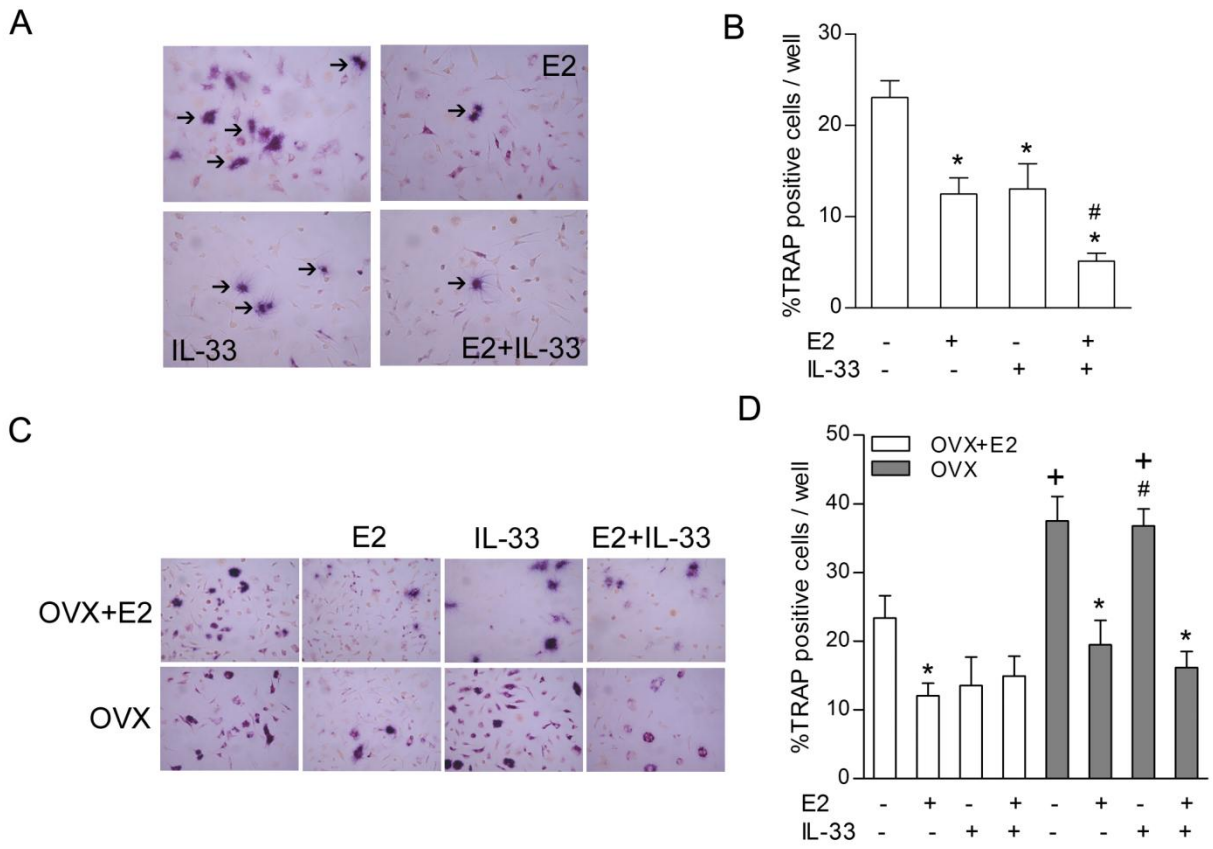


Figure 5 - Bone marrow cells from Balb/c mice were cultivated in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-$_K$B ligand (RANKL). A - Cell culture of osteoclasts tartrate-resistant acid phosphatase positive cells (TRAP+) in ovary-intact mice treated with soluble 17β-Estradiol 10^{-8} M (E2) and/or IL-33 (20ng/ml) (osteoclasts indicated by black arrows). B - Osteoclasts TRAP+ cells of ovary-intact mice suppression by estrogen, IL-33 and E2+IL-33 treatment. C - Representative images of OVX+E2 and OVX mice osteoclasts TRAP+ cell culture. D - Number of TRAP+ cells of OVX+E2 and OVX mice treated with soluble E2 10^{-8} M and/or IL-33 (20ng/ml). Osteoclast differentiation was suppressed in the bone marrow cells of OVX+E2 mice by estrogen and IL-33 treatment. However IL-33 did not suppressed osteoclast TRAP+ cells in OVX mice cells. Three femurs were used for each time-point. Data were expressed as mean ± SEM. * $p < 0.05$ - statistical difference from OVX+E2 group. $^{\#}$ $p < 0.05$ - statistical difference from control (C) group. $^{+}$ $p < 0.05$ - statistical difference from mice within same treatment. Two-way ANOVA with Bonferroni multiple comparison test.

Figure 5


A
E2
IL-33
E2+IL-33
B
%TRAP positive cells / well
30
20
10
0
E2 - + - +
IL-33 - - + +
C
E2
IL-33
E2+IL-33
OVX+E2
OVX
D
OVX+E2
OVX
%TRAP positive cells / well
50
40
30
20
10
0
E2 - + - + - + - +
IL-33 - - + + - - + +

4. CONSIDERAÇÕES FINAIS

Um estudo realizado na América Latina estima que a prevalência da osteoporose na coluna vertebral seja em torno dos 12% a 18%, e na região proximal do fêmur entre 8% a 22%, em mulheres a partir 50 anos de idade (Morales-Torres and Gutierrez-Urena, 2004). Acredita-se que cerca de 25% das mulheres no período pós-menopausa nos EUA apresentem algum tipo de fratura como consequência da osteoporose (Melton, 1995). As fraturas na região proximal do fêmur estão associadas com um grande número de mortes e invalidez além dos altos custos com medicamentos comparados com outros tipos de fraturas geradas pela osteoporose (Cummings *et al.*, 1985). A incidência destas fraturas dobraram de número nos últimos 25 anos e a estimativa é que em torno de 6 milhões de pessoas no mundo sofrerão de fratura na região proximal do fêmur até 2050 (Porter *et al.*, 1990). À medida que a população idosa mundial aumenta, este tipo de fratura pode se tornar uma "epidemia ortopédica" (Cummings *et al.*, 1985). Assim, estima-se um aumento dos custos em vários países gerando um grande problema econômico e social. Desta forma, há a necessidade de mais estudos para compreendermos melhor os mecanismos envolvidos na osteoporose o que poderá contribuir para identificação de novos alvos para intervenções terapêuticas.

O perfil dos pacientes que procuram tratamento ortodôntico mudou nas últimas décadas ocorrendo aumento do número de pacientes adultos e idosos (Rinchuse *et al.*, 2007). Esta mudança na demanda trouxe desafios biológicos para o atendimento ortodôntico, muitas vezes por tratarem-se de pacientes com alterações sistêmicas ou sob uso de medicamentos controlados (Zahrowski, 2009a; b). Dentre estes, pode-se citar a deficiência de estrogênios e a osteoporose comuns nesta faixa etária, principalmente em mulheres no período pós-menopausa, e que podem interferir na movimentação dentária ortodôntica (Ghoneima *et al.*, 2010; Salazar *et al.*, 2011; Zahrowski, 2009a). A deficiência de estrogênio aumenta a

reabsorção óssea pelos osteoclastos sendo o principal fator etiológico, em mulheres, para a osteoporose (Cooper *et al.*, 1992). A administração de diversas drogas anti-resorptivas são recomendadas para o tratamento e controle da osteoporose, tais como bifosfonatos, raloxifeno, terapias de reposição hormonal, administração de calcitonina, hormônio da paratireóide e ranelato de estrôncio (Henriksen *et al.*, 2011). Porém, a utilização dos bisfosfonatos pode gerar atrasos na movimentação dentária ortodôntica e até causar osteonecrose dos ossos maxilares (Ghoneima *et al.*, 2010). Além disso, o uso de medicamentos de forma contínua, no intuito de tratar ou aliviar a sintomatologia de tal doença, resulta também em um desequilíbrio na remodelação óssea (Ghoneima *et al.*, 2010; Shoji *et al.*, 2011; Zahrowski, 2009a). Portanto, é importante investigar como a idade e possíveis interações farmacológicas afetam o tratamento ortodôntico (Rinchuse *et al.*, 2007). A negligência do profissional no diagnóstico e na condução do caso em pacientes com alterações sistêmicas ou sob tratamento medicamentoso de rotina pode ocasionar efeitos indesejáveis significativos como recidiva e baixa resposta ao tratamento (Lewiecki *et al.*, 2011).

A hidroxiapatita é o principal componente mineral do osso, sendo a principal fonte de cálcio e fósforo de nosso organismo (Boskey, 1981). A manutenção da homeostase de cálcio e massa óssea são mantidos por meio da atividade equilibrada entre a reabsorção óssea pelos osteoclastos e a formação óssea realizada pelos osteoblastos (Boyce *et al.*, 2012). O osso é um tecido altamente dinâmico e sua remodelação fisiológica depende de fatores sistêmicos e locais onde qualquer desequilíbrio pode acarretar perdas ósseas excessivas, como observado na osteoporose (Teitelbaum, 2000). Além disso, o osso pode sofrer modificações frente a estímulos de força mecânica e crescimento (Boyce *et al.*, 2012; Eriksen, 2010; Feng and McDonald, 2011; Robling *et al.*, 2006).

Os estrogênios são importantes reguladores da remodelação óssea (Lorenzo *et al.*, 2008; Rauner *et al.*, 2013), atuando no tecido ósseo via receptor de estrogênio ERα e ERβ (Lindberg *et al.*, 2001; Lindberg *et al.*, 2002; Nakamura *et al.*, 2007; Vidal *et al.*, 2000). A diminuição dos níveis de estrogênio ocorre principalmente em mulheres no período pós-menopausa, aumentando assim a susceptibilidade de desenvolvimentos processos patológicos como a osteoporose. O papel protetor dos estrogênios no tecido ósseo está associado ao aumento da sobrevida dos osteoblastos e osteócitos, mas principalmente pela indução da apoptose dos osteoclastos (Lea *et al.*, 1999; Lorenzo *et al.*, 1998; Nakamura *et al.*, 2007). A perda óssea induzida pela deficiência de estradiol é causada por uma complexa interrelação entre hormônios e citocinas que leva à alteração da remodelação óssea (Weitzmann and Pacifici, 2006).

Desta forma, considerando-se a importante relação entre o osso, estrogênio e moléculas inflamatórias, fica clara a importância de aprofundar o conhecimento desta inter-relação, principalmente em patologias ósseas como a osteoporose. Atualmente, a literatura é bem clara com relação à perda óssea em ossos longos causada pela falta de estrogênio (Imai *et al.*, 2010; Khosla, 2010; Riggs *et al.*, 2002). Entretanto, o efeito da falta de estrogênio nos ossos alveolares ainda não está bem esclarecido.

Nossos resultados permitiram observar a importância do estrogênio na manutenção da integridade do osso alveolar da maxila. A ovariectomia (OVX) tem demonstrado ser um modelo confiável de indução de osteoporose por falta de estrogênio (Thompson *et al.*, 1995). Em nosso estudo, camundongos ovariectomizados de diferentes linhagens apresentaram perda da microarquiterura e crista óssea alveolar sendo estes acompanhados pela perda de osso trabeculado no fêmur. A reposição hormonal com estradiol (E2) reverteu completamente este fenótipo. Este pocesso de perda óssea alveolar por OVX foi acompanhado pelo aumento

de moléculas inflamatórias como TNF-α e a proporção RANKL/OPG na maxila, que também foram revertidas após a reposição hormonal.

Adicionalmente, verificamos que a proteção do osso alveolar depende do receptor de estrogênio ERα e que sua ausência promove aumento do processo de remodelação óssea na maxila, diminuição da porcentagem de cálcio na região da furca dos primeiros molares e aumento da IL-33 no periodonto. Além dos efeitos nos ossos maxilares, os animais deficientes para o receptor de estrogênio ERα apresentaram efeito sistêmico demonstrado pela perda óssea no fêmur e vértebras. Nossos resultados *in vitro* demonstraram aumento do número de osteoclastos assim como aumento da atividade dos osteoblastos na ausência de ERα. Porém, mais estudos são necessários para melhor compreensão das vias de ativação do ERα nos ossos alveolares.

Além do estrogênio, outras moléculas inflamatórias estão envolvidas na proteção do osso como a IL-33. Seu mecanismo de ação envolve a estimulação da formação de matriz óssea pelos osteoblastos porém seu principal mecanismo de ação seria a inibição da osteclastogênese (Keller *et al.*, 2012; Saleh *et al.*, 2011; Schulze *et al.*, 2011; Zaiss *et al.*, 2011). Dados da literatura sugerem que a IL-33 apresenta papel protetor no tecido ósseo, entretanto, no caso da artrite reumatóide a IL-33 participa no recrutamento de neutrófilos agravando assim a doença (Verri *et al.*, 2010). Sendo assim, a IL-33 dependendo da situação em que se encontra, pode participar de forma pró- ou anti-inflamatória (Miller, 2011). Em nosso estudo, os animais deficientes de receptor ERα apresentaram fenótipo ósseo maxilar osteoporótico associado ao aumento de IL-33, com isto decidimos analisar melhor a correlação entre a IL-33 e o estrogênio.

Realizamos o trabalho utilizando animais selvagens (WT) e deficientes para o receptor de IL-33 ($ST2^{-/-}$) submetidos ou não a OVX. Os animais $ST2^{-/-}$ demonstraram perda óssea alveolar, aumento da remodelação óssea, aumento do número de osteoclastos e

diminuição de osteoblastos semelhantes aos WT OVX, sendo estes dados acompanhados pela diminuição de IL-10 e Sema3A. Adicionalmente, animais $ST2^{-/-}$ OVX não apresentaram perda óssea maxilar, simultaneamente aos animais WT na presença de estrogênio. Nossos resultados *in vitro* demonstraram que a osteoclastogênese foi suprimida pelo estrogênio e IL-33. No entanto, a IL-33 não foi capaz de suprimir a diferenciação dos osteoclastos na ausência de estrogênio. Estes dados sugerem que o papel "osteoprotetor" da IL-33 depende da presença de estrogênio.

O aprofundamento do conhecimento do efeito da osteoporose nos ossos maxilares apresenta grande relevância clínica uma vez que a falta de estrogênio pode acarretar em aumento da incidência de doenças inflamatórias tais como doenças periodontais e complicações em situações clínicas que exigem remodelamento ósseo como reparo de fraturas, implantes osseointegrados e movimentação dentária. Este fato ganha maior proporção, principalmente nos dias atuais, com o aumento da população idosa mundial.

5. CONCLUSÕES

Os resultados deste trabalho permitem-nos concluir que:

1. A falta de estrogênio acarreta perda óssea alveolar com aumento da expressão de TNF-α e da razão RANKL/OPG.

2. Os efeitos do estrogênio no osso alveolar ocorrem pelo menos em parte via ERα.

3. Os efeitos "osteoprotetores" de IL-33/ST2 no osso alveolar, por sua vez, não são observados em condições de redução de estrogênio.

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ANEXO A

Parecer do Comitê de Ética em Experimentação Animal

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 39/2011,** relativo ao projeto intitulado "***O papel dos receptores de quimiocinas CCR1, CCR2 e CCR5 na reabsorção óssea induzida por ovariectomia***", que tem como responsável(is) **Tarcília Aparecida Silva** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **27/ 04/2011.**

Este certificado expira-se em **27/ 04/ 2016.**

CERTIFICATE

We hereby certify that the **Protocol nº 39/2011,** related to the project entitled "***The role of CCR1, CCR2 and CCR5 chemokine receptors in bone resorption in ovarectomized mice***", under the supervisiors of **Tarcília Aparecida Silva,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **April 27, 2011.**

This certificate expires in **April 27, 2016.**

Belo Horizonte, 2 de Maio de 2011.

Profª. Jacqueline Isaura Alvarez-Leite
Coordenadora do CETEA/UFMG

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(Mod.Cert. v1.0)

Otago University Animal Ethics Committee

Hunter Centre
C/o Faculty Office
Medical School

14 November 2013

Prof D Grattan
Department of Anatomy
Otago School of Medical Sciences

Dear Prof Grattan,

Application No. 102/13

I have pleasure in advising you that the Animal Ethics Committee at its meeting on 13 November has given approval for your application for use of animals in a project entitled *'Investigate the association between chemokines and hormones in bone of female mice'*. This is approved with the following provisos:

a) in # 2.5.1: i) please note genotyping by tail tip is categorized as an ET.

ii) if animals lose 15% or more of their body weight they should be graded a "C".

[Please revise the boxes in the Totals line in light of these comments.]

b) in # 2.5.1 B: please provide a specific reference from your previous work to justify the animal numbers, bearing in mind the recent review of the University's Code of Ethical Conduct, the Auditors were very critical of the current level of justification of animal numbers presented by our PIs, stating that it does not meet the NAEAC (National Animal Ethics Advisory Committee) guidelines. Consequently, the Committee asks that you revisit this section, providing a clearer justification of numbers requested, using **one** of the following options:

pilot data and a power calculation, OR a biostatistical review, OR cite your own work, i.e. a publication that is reviewable by the AEC, or relevant published literature which clearly shows that the group sizes are appropriate.

c) in # 3.1: there appears to be no reference or description of Exp. 4 here. Please clarify.

d) in # 4.2.2: the Committee recommends that if there is weight loss due to pain or difficulty eating the pain medication be adjusted accordingly. Please confirm.

e) in # 5.4.1 A + B: the information in these two sections is contradictory. Please revisit A and confirm the correct answer, in light of the details you have provided in B and C.

f) in # 6.3 + # 8.4.4: "CO_2 Chamber" has been ticked in one section but not in the other. Please revisit and correct accordingly.

g) the Committee recommends that Soraia Macari attend a Manipulation Technique Meeting run by the Animal Welfare Office to discuss her skills and experience to undertake the work as outlined.

h) the Animal Ethics Committee considers that a site visit is necessary for this protocol. Site visits are the main strategy for ensuring good practice, and are an important aspect of communication between the Committee and the investigator. Therefore, **before this project commences** you must contact the Animal Welfare Office who will arrange this visit to occur at an appropriate stage of the work, and at a mutually convenient time.

This visit is requested to observe the attachment of the orthodontic coil device. Please feel free to contact the Animal Welfare Office if you wish further clarification.

i) please confirm in your response to this letter that all personnel listed on the original application and your Head of Department have been made aware of any changes to the original protocol as a result of these provisos.

In any correspondence on this subject, including the ordering of animals for your research, would you please quote the application number given above.

Approval is given on the basis that the Code of Ethical Conduct for the Manipulation of Animals will be adhered to and accurate records of animal usage will be maintained.

Please be aware that there is now a requirement to submit a brief report on the outcome of this project. Information regarding the format of this report, plus a request for the standard animal use statistics will be mailed to you just prior to the expiry of this protocol.

Permit holders are asked to advise the Secretary of the Animal Ethics Committee when an approval will be activated, when a project has been completed or when an approval will not be activated due to lack of funding.

Responses should take the form of a letter providing commentary or answers to the questions asked. ***Please do not resubmit a new corrected protocol unless specifically requested. As the Committee members will not necessarily have access to this letter when evaluating your response to these provisos, please include a brief summary of the original questions you are responding to.***

Yours sincerely

Barbara Lee
Secretary
Otago University Animal Ethics Committee

Please Note: this protocol is only approved subject to further information being received which addresses the specified provisos. Failure to provide information as requested within six weeks of receipt of this letter will result in this approval being withdrawn. **Notwithstanding the above provisos, for the purposes of the release of research funds, this letter may be considered as formal approval.**

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Senhor(a) Professor(a) Tarcília Aparecida Silva,

Após análise de sua solicitação de avaliação do projeto O papel da IL-33 na remodelação óssea induzida por movimentação ortodôntica, submetido a esta comissão pelo protocolo 130 / 2012, a CEUA decidiu **aprovar** a sua solicitação.

Justificativa: Aprovado na reunião do dia 09/05/2012

Para acessar ao seu projeto clique no link:
http://www.ufmg.br/bioetica/cetea/ceua/
Belo Horizonte, 10/05/2012.

Atenciosamente.

Sistema CEUA-UFMG
http://www.ufmg.br/bioetica/cetea/ceua/

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