DENOSUMAB DECREASES CORTICAL POROSITY IN POSTMENOPAUSAL WOMEN WITH LOW BONE MINERAL DENSITY


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Cortical bone constitutes 80% of skeletal mass, and 70% of bone loss during ageing is the result of intracortical remodelling which results in porosity that compromises bone strength.1,2 Denosumab increased volumetric BMD (vBMD) of the cortical compartment of the distal radius, as assessed by HRpQCT, and increased polar moment of inertia, a surrogate of strength, as estimated by QCT.3 We now report the changes in cortical porosity that occurred during the 1-yr study.

Postmenopausal women with a mean (SD) age of 60.6 (5.4) yrs and mean BMD T-scores at the spine, total hip, and radius of −2.44, -1.30 and -1.85, respectively, were enrolled and randomly assigned in a double-blind, double-dummy fashion to denosumab 60 mg Q6M (N=83), alendronate 70 mg QW (N=82), or placebo (N=82). Porosity was evaluated in the compact appearing cortex of the distal radius at baseline and month 12 from HRpQCT scans using an enhanced method that identifies the cortex with automatic threshold segmentation.4 Pores above ~82 μm are identifiable and was expressed as percent of the total cortical volume. The accuracy and reproducibility of this method have been reported.5,6 Baseline cortical porosity was 2.6%. During 12 months, cortical porosity increased in placebo subjects, remained unchanged in alendronate treated subjects, and tended to decrease in denosumab treated subjects. Denosumab reduced cortical porosity by 8.18% (P<0.01) compared to placebo.

Porosity may be underestimated in these provisional analyses because thresholding discards low density and trabecularized cortex and porosity below ~82 μm is not quantified. Ongoing work exploring nonthreshold methods to assess porosity within the compact-appearing and fragmented cortex may identify additional differences between therapies. Within these constraints we infer that denosumab prevented the progression of porosity seen with placebo, an effect that is likely to improve bone strength and reduce fracture risk.


LONG-TERM DENOSUMAB TREATMENT IN POSTMENOPAUSAL WOMEN WITH OSTEOPOROSIS: RESULTS FROM THE FIRST TWO YEARS OF THE FREEDOM TRIAL EXTENSION


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Aim: We report the 2 yr interim results of an open-label extension study designed to evaluate up to 10 years of long-term efficacy and safety of denosumab in the treatment of postmenopausal osteoporosis.

Methods: Postmenopausal women who completed the FREEDOM study were invited to participate in the extension study. All women receive denosumab (60 mg) every 6 months and daily calcium and vitamin D. For women who received placebo during FREEDOM, the data presented here reflects 2 years of denosumab treatment (cross-over group). For women who received denosumab during FREEDOM, the data presented here reflects 5 years of continuous denosumab treatment (long-term group).

Results: Of the women who completed FREEDOM, 70% (4550) enrolled in the extension (2207 cross-over; 2343 long-term). Similar to those in the denosumab group in the extension study. All women receive denosumab (60 mg) (cross-over group). For women who received denosumab during FREEDOM, the data presented here reflects 5 years of continuous denosumab treatment (long-term group).

Results: Of the women who completed FREEDOM, 70% (4550) enrolled in the extension (2207 cross-over; 2343 long-term). Similar to those in the denosumab group in FREEDOM, the cross-over group in the extension study had significant gains ($P<0.0001$) in the lumbar spine (7.9%) and total hip (4.1%) BMD in the first two years. In the long-term group, there were further significant increases ($P<0.0001$) in BMD to a total of 13.7% (lumbar spine) and 7% (total hip) from FREEDOM baseline. Serum C-telopeptide (CTX) was rapidly reduced following denosumab dosing in both groups, with the characteristic attenuation of CTX reduction at the end of the dosing period. New vertebral and nonvertebral fracture incidence remained low in both groups. Incidences of adverse events (AEs) and serious AEs (SAEs) were similar to or lower than in the FREEDOM study. In particular, incidence rates of SAEs of infection in the long-term group were similar to or lower than in the FREEDOM study.

Conclusions: The interim safety and efficacy results from this extension study are consistent with the original FREEDOM study results and provide long-term exposure data for up to 5 years.

(1) Cummings NEJM 2009; 361:756

### TABLE 1. Correlations between porosity, osteocalcin and undercarboxylated osteocalcin in middle-aged men

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tibia</th>
<th>Radius</th>
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</thead>
<tbody>
<tr>
<td>UcOC</td>
<td></td>
<td>uOC</td>
</tr>
<tr>
<td>Porosity within compact-appearing cortex</td>
<td>$r = 0.57^{***}$</td>
<td>$r = 0.60^{***}$</td>
</tr>
<tr>
<td>Porosity of the transitional zone</td>
<td>$r = 0.59^{***}$</td>
<td>$r = 0.63^{***}$</td>
</tr>
<tr>
<td>Fraction of the trabecular (medullary) compartment that is void</td>
<td>$r = 0.59^{***}$</td>
<td>$r = 0.46^{*}$</td>
</tr>
</tbody>
</table>

*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. OC uOC (undercarboxylated osteocalcin). (Data were adjusted for age and BMI).
THE CONTRIBUTION OF SOFT TISSUE OSTEOPROGENITORS TO SPINAL FUSION
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Background: Spinal fusion surgery has been performed for almost a century to prevent the deterioration of spinal anomalies. This highly invasive surgical procedure uses bone graft and/or bone morphogenetic proteins (BMPs) to stimulate fusion (union) of multiple vertebrae. The identity of osteogenic progenitors that these osteogenic stimuli act upon is poorly understood. We have hypothesized that myogenic and/or vascular progenitors from the adjacent musculature may contribute to fusion.

Methods: We created a newly refined surgical methodology for induction spinal fusion in mice. This procedure features a midline approach and delivery of BMP-2 via collagen sponges inserted between the spinal processes and the adjacent muscles. BMP-induced bone was visualized in 3D by microCT. Experiments were performed in MyoDHcre+;Z/AP+ and Tie2hcre+;Z/AP+ transgenic conditional reporter mice. In these mice, staining for the heat-stable human alkaline phosphatase reporter permitted lineage tracking of myogenic and vascular progenitors in vivo.

Results: Dose response experiments with rhBMP-2 have illustrated that robust bone formation can be achieved with 10 μg rhBMP-2, however fusion can still be obtained with doses as low as 1 μg rhBMP-2. Histological staining with Picosirisius red/Alician blue show the early presence of cartilage, which is later replace with bone. Lineage stains have shown that MyoD-lineage cells contribute significantly to bone formation in this model. Data from tracking of Tie2-lineage cells is expected to also be extremely informative.

Conclusion: These data suggest a contribution from cells from the soft tissue adjacent to the spine to the fusion process. These cells may represent a novel target cell population for therapeutic intervention to maximise bone formation.

EFFECT OF CO-MORBIDITIES ON FRACTURE RISK: FINDINGS FROM THE GLOW STUDY
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Introduction: Greater awareness of the relationship between co-morbidities and fracture risk can improve fracture prediction in algorithms such as FRAX®. We utilised a large, multinational cohort study to investigate the effect of comorbidities on fracture risk.

Methods: 52,960 women were recruited from The Global Longitudinal Study of Osteoporosis in Women (GLOW). GLOW is an observational prospective study of women aged 55 years and older recruited through 723 primary physician practices in 17 sites in 10 countries. At baseline, women completed a questionnaire that recorded comorbidity history and prevalent fragility fracture. Incident clinical fracture history was recorded annually. A comorbidity index, defined as number of baseline comorbidities from the following was derived: high blood pressure, high cholesterol, heart disease, stroke, asthma, chronic obstructive pulmonary disease (COPD), arthritis (reported osteoarthritis & rheumatoid arthritis), inflammatory bowel disease, coeliac disease, cancer, diabetes, multiple sclerosis and Parkinson’s disease.

Results: 3224 (6.1%) women sustained an incident fracture over 2 years of follow-up. Comorbidities were common; 26,215 women (49.5%) reported hypertension, and 26,084 women (49.3%) high cholesterol levels. All recorded comorbidities were significantly associated with fracture.
except high cholesterol, and coeliac disease; the strongest association was seen with Parkinson’s disease [HR 2.13 (95% CI 1.51, 3.01), p<0.0001]. The HR of fracture increased with increasing comorbidity index [HR 1 vs. 0 comorbidities: 1.08 (0.93, 1.25); (HR 4+ vs. 0 comorbidities: 1.49 (1.27, 1.74), after adjustment for age, BMI, prior fracture, current steroid use, alcohol more than 3 drinks/day and current smoking]. The comorbidities that contributed most to fracture prediction, in order of importance, were: arthritis, Parkinson’s disease, COPD, diabetes and multiple sclerosis. Conclusion: Comorbidities as captured in a comorbidity index, contribute significantly to fracture risk. Arthritis and Parkinson’s disease carry a particularly high risk of fracture. Increasing comorbidity index was associated with increasing fracture risk.

30 ABSOLUTE FRACTURE RISK-BASED CLINICAL THRESHOLD FOR INTERVENTION: A DECISION CURVE ANALYSIS


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Aim: This study was sought to determine the absolute fracture risk threshold for intervention by using the decision curve analysis (DCA) method.

Methods: The present study was part of the ongoing Dubbo Osteoporosis Epidemiology Study which involved 2216 (1358 women) participants, aged 60+ as of 1989, whose bone health has continuously been monitored since 1989. Baseline measurements included age, femoral neck BMD, prior fracture, and falls. During the follow-up period, 426 women and 149 men had sustained a low-trauma fracture. We considered 3 Cox’s proportional hazards models for predicting fracture risk: model I included prior fracture; model II, age and BMD; and model III included all four factors. From each model, we estimated 10-year predicted probability of any and hip fractures for each individual. We then calculated the “net benefit” for each intervention threshold (T). The net benefit (NB) is a function of total sample size (n), true positives (TP) and false negatives (FN) as follows: NB=TP/n-(FN/n)*T/(1-T).

Results: The DCA suggested that for any fracture, optimal NB reached when the 10-year fracture risk is 10% regardless of models used; however, at this risk level, almost all individuals are subject to intervention. For risk threshold between 10-20%, optimal NB was achieved with model III, following was model II. For risk threshold between 20-40%, the NB was slightly declined in women and more steeply in men. For hip fracture, models II and III yielded similar optimal NB at threshold at 6% in women and between 6%-10% in men. Clinical decision based on prior fracture alone yielded suboptimal NB for hip fracture. For all models and for a given threshold, the NB was greater in women than in men.

Conclusion: These results suggest that a 10-year risk of any fracture between 10-20% or hip fracture risk between 6-10% provides optimal net benefit.

31 TRANSCRIPTIONAL INDUCTION OF ADAMTS5 BY AN NF-KB FAMILY MEMBER RELA/P65 IN CHONDOCYTES DURING OSTEOARTHRITIS DEVELOPMENT

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ADAMTS5 (aggrecanase-2) is known to be a crucial proteinase that degrades joint cartilage during osteoarthritis (OA) development. To elucidate the molecular network as a therapeutic target of OA, the present study attempted to identify transcription factors to induce ADAMTS5 expression and examined the underlying mechanism. Exhaustive comparison of the genomic sequences of about 2 kb of 5′-end flanking regions of human, macaca, and mouse ADAMTS5 genes revealed that the 1.4 kb region upstream of the transcriptional start site was highly conserved among species. The sequence search in this region predicted the consensus binding motifs of NF-κB, C/EBP, GATA, AP-1, OCT, SOX, STAI; and HIF. We therefore created expression vectors of 12 representative transcription factors for these sites, and transfected them in chondrogenic ATDC5 and non-chondrogenic HeLa cells with a luciferase reporter construct containing the 1.4 kb ADAMTS5 gene fragment. Among the transcription factors, an NF-κB family member, RELA/p65, most strongly stimulated the luciferase activity in both cells. In the ADAMTS5 genes, there were three NF-κB binding motifs: -1,196/-1,187, -896/-887, and -424/-415 bp, in which deletion, mutagenesis, and tandem-repeat analyses of the luciferase assay identified the core responsive regions of RELA/p65 to be the two upstream motifs. Electrophoretic mobility shift assay revealed the binding of nuclear extracts of RELA/p65-overexpressed COS-7 cells with the two NF-κB motif oligonucleotide probes. The specificity of the binding was verified by the cold competition with excess amount of the unlabelled wildtype probe and by the supershift with an
antibody to RELA/p65. Retroviral overexpression of RELA/p65 markedly increased the *Adamts5* expression in ATDC5 cells. Furthermore, IL-1β, a putative inducer of the NF-κB signal as well as OA development, enhanced *Adamts5* and *Rela/p65* expressions in ATDC5 cells. The *Adamts5* induction by IL-1β was suppressed by the knockdown of *Rela/p65* with its specific siRNA transfection. Finally, in the experimental OA model by surgical induction of instability in the knee joints of 8-week-old mice, *Adamts5* and *Rela/p65* were co-expressed in chondrocytes of the degraded joint cartilage. In conclusion, we identified *Rela/p65* as a potent transcriptional activator of ADAMTS5 in chondrocytes during OA development. The molecular network related to the RELA/p65-ADAMTS5 axis may thus represent a therapeutic target for OA.

32 EFFECTS OF MONOSODIUM URATE (MSU) CRYSTALS ON CHONDROCYTE VIABILITY AND FUNCTION: IMPLICATIONS FOR DEVELOPMENT OF JOINT DAMAGE IN CHRONIC GOUT

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Aim: Chondrocytes, the stromal cells of cartilage, are important mediators of cartilage degradation in arthropathies such as osteoarthritis and rheumatoid arthritis. In gout, focal cartilage damage occurs within the joint at sites of urate crystal deposition. We hypothesised that interactions between chondrocytes and monosodium urate monohydrate (MSU) crystals contribute to cartilage damage in chronic gout.

Methods: MSU crystals were prepared by recrystallisation of uric acid. Cultures of primary human chondrocytes were prepared from cartilage obtained from patients undergoing knee or hip arthroplasty. These cells were cultured under non-adherent conditions using tissue culture plates coated with poly-(2-hydroxyethyl methacrylate). PicoGreen and alamarBlue assays were used to assess chondrocyte viability following culture with MSU crystals. Quantitative real-time PCR was used to determine changes in gene expression in chondrocytes cultured with MSU crystals. Joint samples from patients with gout were stained with toluidine blue and analysed for cartilage morphology.

Results: MSU crystals rapidly reduced chondrocyte viability in a dose-dependent manner. The reduction in chondrocyte viability was specific to MSU crystals, as soluble uric acid did not alter cell viability. Culture with MSU crystals reduced mRNA expression of matrix proteins and increased mRNA expression of degradative enzymes such as MMPs (matrix metalloproteases) and ADAMTS (A disintegrin and metalloproteinase with thrombospondin motifs) peptidases. In cartilage samples from patients with gout, cartilage adjacent to tophus was highly disordered with loss of normal architecture and reduced proteoglycan staining.

Conclusions: These data indicate that MSU crystals may contribute to cartilage damage in gout through reduction of chondrocyte viability and promotion of a catabolic state.

33 THE CROSS TALKS BETWEEN WNT/Β-CATELIN AND RAC-1 SIGNALING IN REGULATION OF MAINTENANCE AND FUNCTION OF SUPERFICIAL CELL LAYER IN ARTICULAR CARTILAGE

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Aim: Articular cartilage has poor ability to regenerate and repair, and thereby proceed toward osteoarthritis. The superficial layer (SFL) in articular cartilage has received much attention as an initial site of cartilage degeneration, however, the exact regulation of SFL function remain largely unclear. We have found that Wnt/β-catenin signalling is critical for maintenance of SFL in articular cartilage using the loss- and gain-of-function approaches of β-catenin (Col2CreER/βcatenin and Col11ΔβcateninER, respectively). Rac-1, one of the small GTPase has been shown to regulate β-catenin signalling pathway. In this study, we tested whether β-catenin and Rac-1 signalling pathways have a crosstalk in regulation of SFL function in articular cartilage.

Methods: We isolated the superficial cells (SFCs) that show strong and rapid attachment on fibronectin substrate from mouse articular cartilage and determine the effect of β-catenin and Rac-1 signalling on proliferation and differentiation in SFCs. We also examined the regulation of SFL function by analyzing the articular cartilage in cartilage-specific Rac-1 deficient mice (Col2Cre-Rac-1<sup>fl/fl</sup>).

Results: SFCs expressed higher levels of Wnts and receptors for Wnt ligands as well as articular surface markers such as *Lubricin* and *Asporin* as compared with
chondrocytes. Treatment of Wnt3a, an inducer of Wnt/β-catenin signalling, stimulated proliferation and maintained high expression of Lubricin in SFCs over passages. In contrast, Rac-1-deficient-SFCs expressed lower levels of Lubricin and Asporin than the control cells, and failed to activate β-catenin signalling even after treatment of Wnt3a. At 3- and 6-month-old of ages, Rac-1 deficient articular cartilage was deformed and contained significantly thinner SFL with less number of cells as compared with the control articular cartilage. Interestingly, the similar phenotypes have been also observed in the articular cartilage in b-catenin deficient-mice. Interestingly, the similar phenotypes have been also observed in the articular cartilage in b-catenin deficient-mice. Conclusions: These results suggest that Rac-1 and Wnt/β-catenin signalling could cooperatively regulate SFL proliferation and function.

34 INHIBITION OF PROTEIN KINASE-D PROMOTES CARTILAGE REPAIR AT INJURED GROWTH PLATE IN RATS

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Injured growth plate cartilage is often repaired by bony tissue, impairing bone growth and causing growth defects in children. Currently, molecular events leading up to the undesirable bony repair remain unclear.

Aim: This study utilised a rat growth plate injury model to investigate the potential role during growth plate bony repair of protein kinase-D (PKD) which is known to regulate osteoblast differentiation transcription factor osterix.

Methods: Following surgical injury at the proximal tibial growth plate, young rats received four once-daily injections during days 5–9 of vehicle or 2.35 mg/kg gö6976 (a PKD inhibitor known for its inhibitory effects on osterix), and injured growth plate samples were collected at day 10.

Results: MicroCT analysis revealed that bone volume at the injury site was significantly lower following gö6976 treatment compared to the vehicle control (P<0.05). Histological analysis showed that PKD inhibition resulted in an increase in% of mesenchymal repair tissue (P<0.001), a decrease in bone trabeculae and bone marrow tissues, and more cartilaginous tissue within the injury site. Consistently, gö6976 treatment decreased mRNA expression at the injury site of bone related genes (osterix and osteocalcin) and increased levels of cartilage related genes (collagen-2a and Sox9). In support, in vitro experiments with rat primary bone marrow stromal progenitor cells showed that addition of gö6976 promoted chondrogenic differentiation resulting in a significant increase in collagen-2a expression (P<0.05).

Conclusions: These results suggest that PKD is an important factor for growth plate bony repair and blocking PKD activity after growth plate injury may result in less bone formation and potentially more desirable cartilage repair.

35 NOTCH/RBPJ/HES1 SIGNAL IN CHONDROCYTES MODULATES THE TERMINAL STAGE OF ENDOCHONDRAL OSSIFICATION DURING SKELETAL GROWTH AND OSTEOARTHRITIS DEVELOPMENT

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Here we have examined the role of the Notch signalling pathway in chondrocytes during the endochondral ossification process that is essential for skeletal growth and osteoarthritis (OA) development. In cultures of mouse primary costal chondrocytes and chondrogenic ATDC5 cells, Notch1, 2, the transcriptional effector Rbpj, and the target transcription factor Hes1 were strongly expressed in their terminal differentiation stages during Mmp13 and Vegfa expression, while Notch3, 4 and other target Hes/Hey members were little expressed throughout the differentiation stages. In the limb cartilage of mouse embryos and in the knee joint cartilage of a mouse experimental OA model with surgical induction of instability, intracellular domains (ICD) of Notch1, 2 were localized in the nucleus of highly differentiated chondrocytes in the hypertrophic zone and in the degraded cartilage, respectively, while they remained in the cytoplasm of less differentiated chondrocytes of the proliferative zone and undegraded cartilage. Rbpj and Hes1 were also coexpressed in the nucleus of highly differentiated chondrocytes, while other Notch ICDs and Hes/Hey members were not detected in either cartilage. We then created conditional knockout mice of Rbpj in chondroprogenitor cells (Sox9-Cre;Rbpjfl/fl) and chondrocytes (Col2a1-Cre; Rbpjfl/fl). Although the Sox9-Cre;Rbpjfl/fl mice died shortly after birth, the embryos exhibited dwarfism with impaired matrix degradation and vascular invasion into the cartilage primordia due to decreases of Mmp13 and Vegfa expression. When we created the experimental OA model in a
Col2a1-Cre;Rbpj\textsuperscript{fl/fl} mouse line with partial Rbpj inactivation causing normal skeletal growth, the knee OA development was suppressed as compared to the Rbpj\textsuperscript{fl/fl} littermates, with prevention of the terminal stage of endochondral ossification, similar to the Sox9-Cre;Rbpj\textsuperscript{fl/fl} limb cartilage. Retroviral overexpression of Notch1-ICD or Notch2-ICD in ATDC5 cells caused enhancement of Alizarin red and ALP stainings, as well as Mmp13, Vegfa, and Hes1 expression. On the contrary, a Notch inhibitor DAPT suppressed these markers in immature murine articular chondrocytes. Luciferase analyses revealed that the Hes1 transfection enhanced the MMP13 and VEGF\textsubscript{A} promoter activity most potently among the Hes/Hey members. In conclusion, the Notch/Rbpj/Hes1 signal in chondrocytes modulates the terminal stage of endochondral ossification during skeletal growth and OA development, indicating it to be a possible therapeutic target of OA.

38 PREVALENCE OF SARCOPENIA IN OLDER WOMEN: THE GEELONG OSTEOPOROSIS STUDY

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Aim: While sarcopenia develops in all individuals with advancing age, the extent varies progressively. We aimed to determine the prevalence of sarcopenia in older women. Using the European Working Group on Sarcopenia in Older People recommendations, we defined sarcopenia in terms of both low muscle mass and function.

Methods: Subjects were randomly-selected women aged 20–94 yr enrolled in the Geelong Osteoporosis Study. Muscle mass was measured as total lean mass by DXA (Lunar) and expressed as a percentage of body mass. Low lean mass was defined as more than 1 SD below the mean \((Lunar) and expressed as a percentage of body mass. Low muscle mass was measured as total lean mass by DXA (Lunar) and expressed as a percentage of body mass. Low lean mass was defined as more than 1 SD below the mean (Lunar) and expressed as a percentage of body mass. Low lean mass was defined as more than 1 SD below the mean. Therapeutic target of OA.

Results: Among 436 women aged 60–94 yr, 143 had low lean mass, 179 had TUG >10 s and 70 had both, meeting criteria for sarcopenia. Age-specific prevalence for sarcopenia was 60–64 yr 9.5%, 65–69 yr 10.1%, 70–74 yr 16.0%, 75–79 yr 25.8%, 80–84 yr 15.2% and 85+ 19.1%. Sarcopenia was associated with lower physical activity scores (by 24.5%, \(p<0.001\)), increased likelihood of a fall (OR=1.87, 95% CI 1.11–3.14, \(p=0.02\)) and high falls-risk (OR=2.36, 95% CI 1.30–4.29, \(p=0.005\)); no association was detected with functional reach or BMD.

Conclusions: The prevalence of sarcopenia was greatest for age 70–79. Prevalence data age-standardised to national levels (2001) suggest that sarcopenia affects 15.2% of Australian women aged 60+. Cross-sectional analyses reveal that women with sarcopenia are habitually less active and more likely to be at risk of falling.

39 ROLE OF TMEM119 IN THE MUSCLE OSSIFICATION SIGNALING

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Fibrodysplasia ossificans progressive (FOP) is a genetic disease in which heterotopic ossification occurs in muscle. However, the details of the heterotopic ossification in FOP remain unclear. Our previous studies suggested that Smad3 independently of TGF-β is related to the bone anabolic action of PTH. We identified the PTH-responsive Smad3-related molecule, Tmem119, as an osteoblast differentiation factor. The constitutively activating mutation (R206H) of the bone morphogenetic protein (BMP) receptor, ALK2, underlies the molecular pathogenesis of FOP. In the present study, we performed a DNA microarray analysis between empty vector and ALK2 (R206H)-transfected mouse myoblastic C2C12 cells, and Tmem119 was one of the genes identified, whose expression was increased >3.5 times in the experimental vs. control group. Stable Tmem119 overexpression induced the commitment of
C2C12 cells into osteoblasts and their mineralization. On the other hand, differentiation of myoblastic cells into myotubes was suppressed, and differentiation into chondrocytes was little affected. Moreover, transcriptional activity of the BMP-2 signalling molecules, Smad1/5, was increased in the absence of exogenous BMP-2 in C2C12 cells. We then analyzed the mechanism whereby Tmem119 induced the commitment of myoblasts into osteoblasts using C2C12 cells. Stable Tmem119 overexpression enhanced endogenous BMP-2 levels, and a reduction in endogenous Tmem119 by specific siRNA suppressed BMP-2 levels. BMP-2/4 neutralizing antibody and dorsomorphin, an ALK2 inhibitor, antagonized the enhancement by Tmem119 of alkaline phosphatase (ALP) and osteocalcin (OCN). Although Tmem119 interacts with Runx2, Smad1 and Smad5, Tmem119 siRNA antagonized the BMP-2-induced ALP and OCN, but not Runx2 and Osterix, mRNAs, in C2C12 cells. In conclusion, we showed that Tmem119 promotes the differentiation of myoblasts into osteoblasts and the interaction with the BMP signalling pathway occurs downstream of Runx2 and Osterix in myoblasts. Tmem119 may play a critical role in the commitment of myoprogenitor cells to the osteoblast lineage.

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THE ROLE OF OSTEOCALCIN IN GLUCOCORTICOID-INDUCED METABOLIC DYSFUNCTION
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We recently demonstrated that osteoblast-targeted disruption of glucocorticoid (GC) signalling attenuates GC-induced bone loss, metabolic dysfunction and obesity, while preventing the marked decrease in circulating osteocalcin usually seen with GC treatment. In the present study, we investigated the role of osteocalcin in GC-induced insulin resistance and glucose intolerance. Seven-week-old CD1 outbred mice were treated with placebo or corticosterone (1.5 mg/week) for 28 days. On day 7, osteocalcin was replaced via hepatic transfection of a non-viral DNA plasmid containing the osteocalcin gene driven by the albumin promoter (pLIVE, Mirus). We transfected either a wildtype osteocalcin construct (wt-OCN) able to undergo gamma-carboxylation, or a mutant osteocalcin construct (mOCN) which cannot be carboxylated. Empty vectors were used as controls. Insulin tolerance tests (ITT) and oral glucose tolerance tests (oGTT) were performed 0, 7, 14, 21 and 28 days into GC treatment. Weight, body composition and food intake were monitored throughout. Successful transfection was confirmed via detection of the GFP-containing pLIVE-vector in mouse hepatocytes 7-28 days post-transfection. GC-treatment resulted in complete suppression of serum osteocalcin levels at d7 and increasing insulin resistance over the 4-week observation period. GC-treated mice receiving the mOCN construct on day 8 regained their insulin tolerance in a time-dependent manner, with glucose levels falling to 60% of baseline in response to insulin on day 28. Glucose tolerance followed the same pattern. In contrast, GC-treated mice transfected with either the empty vector or the wt-OCN construct remained insulin resistant and glucose intolerant.

In conclusion, the adverse effects of exogenous GC on insulin sensitivity and glucose tolerance in mice can be overcome by replacing noncarboxylated (but not carboxylated) osteocalcin in the circulation. These data provide evidence that the osteoblast-specific peptide, osteocalcin, plays a central role mediating the effects of exogenous GC on systemic energy metabolism.

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TREATMENT WITH INTERLEUKIN 6 RECEPTOR ANTIBODIES INHIBITS PROSTATE CANCER GROWTH IN A MURINE MODEL OF BONE METASTASIS
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Aim: In patients with metastatic prostate cancer, high circulating interleukin-6 (IL-6) levels have been associated with poor clinical outcomes. IL-6 has also been linked to a more aggressive phenotype and progression of hormone
refractory prostate cancer. In this study, we investigated the effect of the IL-6 on human prostate cancer growth in vitro and in vivo.

Methods & Results: Treatment of the human prostate cancer cell line, PC3, with RANKL up-regulated IL-6 mRNA expression 2-fold within 4 hrs. Interestingly, treatment of PC3 cells with IL-6, in turn, increased RANK expression 2-fold, and PTHrP expression 4-fold. The effects of IL-6 on RANK expression were blocked by treatment of PC3 cells with the anti-human IL-6 receptor antibody, tocilizumab. These data suggest that RANKL, IL-6 and RANK (or RANKL, IL-6 and RANKL) may form a ‘feed-forward’ loop that promotes cancer growth in bone.

In vivo studies: PC3 cells were implanted intratibially into 5-week-old nude male mice. Mice were then randomized into 2 groups (n=8 each), receiving either tocilizumab (50 mg/kg/3 d) or vehicle. Zoledronic acid (ZA) (100 μg/kg/3 d) was co-administered in a subset of mice (n=8) to determine the contribution of the bone microenvironment to tumour growth. Mice were monitored by x-ray imaging on d17, d24 and d30 (sacrifice). Compared to controls, treatment with tocilizumab significantly inhibited radiographic osteolysis from d17 onwards. Cotreatment with ZA completely prevented osteolysis.

Conclusion: As tumour-derived IL-6 increased tumour cell RANK expression, and bone-derived RANKL increased tumour cell IL-6 expression, the inhibitory effects of tocilizumab on tumour growth may be due to the interruption of a ‘feed-forward’ loop between tumour and bone cells which involves RANKL, IL-6 and RANK (or PTHrP). Our data indicate that IL-6 plays an important role in the metastatic growth of prostate cancer cells in bone and may be a potential therapeutic target in prostate cancer bone metastasis.

51 TIMING OF ADVERSE OUTCOMES FOLLOWING OSTEOPOROTIC FRACTURES IN ELDERLY WOMEN AND MEN: IMPLICATION FOR LONG TERM MANAGEMENT

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Background: The long term risks of fracture and mortality following osteoporotic fracture in the elderly are not clear, partly due to their interdependency (i.e., risk of fracture depends upon survival). In this situation, Kaplan-Meier analysis can be unreliable. Thus cumulative incidences of re-fraction, mortality and sustaining both outcomes in elderly men and women using competing risk analyses were examined.

Methods: Subjects from the Dubbo Osteoporosis Epidemiology Study were followed (1989-2007). Initial and subsequent fractures and mortality status obtained. Competing risk models with 4 possible outcomes: death without re-fracture, death following re-fracture, re-fracture but alive, and event-free were considered.

Results: Of the 2245 women and 1760 men (29,660 and 20,171 p-yrs, respectively), 952 women and 342 men had an initial osteoporotic fracture. Of these 23% women and 21% men re-fractured and 28% women and 39% men died within 5 yrs. After 5 yrs both mortality and re-fracture rates decreased significantly. Long term (>5-10 yr) cumulative re-fracture incidence was reduced in both sexes by the competing risk of death, particularly in older age groups. Following re-fracture 50% of women and 75% of men died within 5 yrs so total 5-yr mortality was 41% in women and 58% in men. However, total mortality (post initial and re-fracture) was elevated above population mortality for ≥10 yrs following initial fracture with most of the 5–10 yr excess mortality due to that following re-fracture. Re-fracture within 5 years was associated with a higher mortality than re-fracture after 5 yrs [adjusted HR 2.44 (95% CI, 1.73-3.45)].

Conclusion: Re-fracture and mortality were highest immediately post fracture, however, excess mortality exists up to 10 yrs post fracture, primarily due to that following re-fracture. However, those who survived remaining re-fracture-free 5–10 yrs post initial fracture had a very low risk of further adverse outcomes, suggesting a less aggressive approach may be appropriate for this population.

52 ALTERED OSTEOCYTE FUNCTION IN OSTEOARTHRITIS: A POSSIBLE PATHOLOGICAL ROLE IN SUBCHONDRAL BONE SCLEROSIS

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Background & Aim: Subchondral bone sclerosis is a recognised manifestation of osteoarthritis (OA) joint. The osteocyte cell network is now considered to be central to the regulation of bone homeostasis but it is still unknown whether the integrity of the osteocyte cell network is altered in OA patients.

Method: The pathophysiology of the osteocyte network in OA tissues were studied in tibial knee specimens obtained.
from patients undergoing knee replacement surgery. Type 1 control (n=5) vs. type 4 OA (n=5) subchondral bone volumes was assessed using microCT. Osteocyte cell number and lacunae per unit area were counted. Scanning electron microscopy (SEM) was performed to detect any morphological variations. Immunoassaying techniques were applied to observe the relative expression strength of osteocyte specific markers and matrix metalloproteinases (MMPs) in samples graded according to disease severity.

Results: Compared with type 1 controls, type 4 OA subjects showed significant increase in the average number of osteocyte lacunae. There was an increase in the number of average osteocyte nucleus in the type 4 OA patient group. Morphological scanning electronic microscopy images showed defective organization of osteocyte-canaliculus system in type 4 OA patients compared to controls. Type 4 OA patients had a lower proportion of osteocytes expressing sclerostin compared to type 1 controls. Conversely, the expression of dentin matrix protein-1, matrix metalloproteinases-1 (MMP-1), MMP-9, and ADAMTS4 were all significantly higher in type 4 OA osteocytes. MicroCT results showed a 20% increase in bone volume in the type 4 OA patient group compared to type 1 controls (p =0.049).

Conclusion: Dysregulation of osteocytic proteins occur in the course of OA development and appears to be central to altered bone and mineral metabolism in this patient population and is likely to be a critical determinant contributing to pathological changes in OA subchondral bone.

A GAIN-OF-FUNCTION TYPE MUTATION OF THE Natriuretic Peptide Receptor B CAUSES ACCELERATION OF SKELETAL GROWTH AND OSTEOPOROTIC CHANGE IN HUMANS AND MICE


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Background: C-type natriuretic peptide (CNP)/Natriuretic peptide receptor type B (NPR-B) signalling pathway is known to play an essential role in endochondral ossification. Clinical report: The proband is a 12-year-old boy. He had fractures at ages 11 and 12 years and was referred to the pediatric department for evaluation of suspected skeletal dysplasia with advanced growth, fragile bones, and arachnodactyly of hands and feet. Height was 177.0 cm (+2.7 SD). Weight and arm span were in the normal range. Blood pressure was normal. Physical examination showed Marfanoid habitus and markedly longer and wider halluces. Laboratory findings were normal except for increased bone formation and resorption markers. BMD Z-score corrected for his height was –3.9. His mother and maternal grandmother showed the same phenotype as well as severe scoliosis. Although the phenotype resembled overproduction of CNP due to a chromosomal translocation, his karyotype was normal.

Objective: To determine whether the patients’ phenotype results from excessive CNP/NPR-B signalling.

Design and Method: (1) Direct sequencing of the coding regions of the Natriuretic peptide precursor C (NPPC) and NPR-B genes was performed. (2) To compare cGMP production between wildtype and mutant NPR-B (WT and Mut, respectively), HEK293A cells were transfected with vectors containing WT and Mut and cGMP concentrations were measured after CNP incubation. (3) Furthermore, transgenic mice in which Mut was expressed in chondrocytes under the control of ColIIa2 promoter/enhancer were generated.

Results: (1) We identified a novel heterozygous missense mutation resulting in a p.Val883Met substitution within the catalytic domain of NPR-B. (2) Treatment with CNP increased HEK293A cGMP levels in a dose-dependent manner. Mut always showed concentrations significantly higher than WT even without CNP (p <0.05). Moreover, circulating levels of cGMP were also increased in the patients. (3) Mut transgenic mice exhibited a phenotype similar to that of the patients. Soft x-rays showed that the mineralized cancellous bone mass was significantly decreased. Stronger osteoporotic change/bone deformity was observed in mice with the highest Mut mRNA expression and advanced with aging.

Conclusions: We present the first report of a 3-generation family with tall stature due to a gain-of-function NPR-B mutation. Although NPR-B mRNA expression is not restricted to bone, the patients’ phenotype seems to be confined only to the bone. Similarly, Mut transgenic mice demonstrated acceleration of skeletal growth and osteoporotic change.

DUAL EFFECTS OF PIM INHIBITION ON MYELOMA: INDUCTION OF BONE FORMATION AND TUMOR SUPPRESSION

Multiple Myeloma (MM) closely interacts with bone marrow microenvironment to enhance tumor growth along with progression of devastating bone destruction.

We recently reported that MM-bone marrow stromal cell (BMSC) interaction potently upregulate in MM cells the serine/threonine kinase Pim-2 which acts as a critical anti-apoptotic regulator in MM cells (Leukemia, 2011). We also found that the MM cells suppressed osteoblast differentiation from BMSCs along with up-regulation of Pim-2 in BMSCs. In the present study, we therefore explored the role of Pim-2 in osteoblastogenesis and the effects of Pim inhibition on tumor growth and bone destruction in MM. Treatment with Pim-2 siRNA or the Pim inhibitor SMI-16a facilitated mineralized nodule formation by BMP-2 in MC3T3-E1 cells. However, enforced expression of Pim-2 in MC3T3-E1 cells abrogated the mineralized nodule formation, suggesting antagonism of bone formation by Pim-2. The Pim inhibition further up-regulated smad1/5 and p38MAPK phosphorylation as well as osterix expression induced by BMP-2 in MC3T3-E1 cells. Importantly, the Pim inhibition restored mineralized nodule formation in MC3T3-E1 cells suppressed by MM cell conditioned media. Furthermore, treatment with SMI-16a 20 mg/kg i.p. every other day markedly decreased MM tumor size without apparent loss of bone both in MM mouse models with intratibial injection of murine STGMI MM cells and in human INA6 MM cell-bearing SCID-rab MM models, while control mice exhibited extensive bone destruction along with tumor expansion in the bone marrow and outside the bone in microCT images and in bone sections. These results suggest that Pim-2 induced in BMSCs by MM cells plays as a negative regulator for bone formation in MM, and that Pim inhibition is able to resume bone formation while reducing tumor burden in MM. Therefore, Pim inhibitors may become a candidate of novel therapeutic agents targeting the MM-BMSC interaction.

Aim: To investigate the effects of a pan-retinoic acid receptor (RAR) agonist (all-trans retinoic acid; ATRA) and a pan-RAR antagonist (NRX194310) on osteoblast differentiation.

Methods: We investigated the effects of ATRA and NRX194310 on osteoblast differentiation of Kusa4b10 stromal cells. We also determined the effects of ATRA and NRX194310 on the differentiation of primary osteoblast lineage populations obtained from collagenase-digested bone. These cells are as follows: mesenchymal stem cells (Sca-1+, CD51-), osteoprogenitors (Sca-1+, CD51+) and osteoblast (Sca-1-, CD51+) cell populations. The effects on osteoblastic differentiation were assessed by Alizarin Red staining for mineralisation and qRT-PCR. To further assess the effects of the RAR pan-antagonist on bone, we gavage fed C57BL/6 mice daily for 10 days with NRX194310 (0.5 mg/kg/day). Results: RARa and RARg subtypes were highly expressed in all osteoblast lineage populations. ATRA (1 μM) significantly inhibited both mineralisation and the expression of osteoblast markers downstream of Runx2 (Osterix, Alkaline Phosphatase, PTH Receptor 1 and Osteocalcin) in all cell populations compared to DMSO-treated controls. In contrast, NRX194310 (1 μM) accelerated mineralisation and expression of genes associated with mature osteoblasts, with a more pronounced effect observed in immature progenitor cells. Supportive of this, ATRA inhibited and NRX194310 significantly potentiated PTH-stimulated cAMP production in Kusa4b10 cells (P<0.01, n=4). Histomorphometry indicated a significant increase in mineralised bone surface in mice treated with NRX194310 compared to DMSO controls (P<0.05, n=4). However, micro computed tomography (μCT) data showed no significant change in bone volume with 10 days of NRX194310 treatment. Conclusions: These studies demonstrate that ATRA inhibits differentiation of osteoblast lineage cells whereas NRX194310 promotes osteoblast differentiation in vitro and bone formation in vivo. Our data also help to explain why high doses of vitamin A are associated with risk of osteoporosis.

Acknowledgments: Dr R.A.S. Chandraratna kindly provided the NRX194310 for these studies.

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Aim: Fracture healing in mice is accelerated in the absence of B and T cells, but dissection of the independent role of each of these lymphoid populations has not been undertaken. Osteoblasts support B cell development therefore we were interested to determine if B cells reciprocate any contribution to osteoblast maintenance and function.

Methods: Immunohistology was used to examine distribution of cell populations of interest in either wildtype or µMT deficient mice (lack mature B cells). Adapted histomorphometry was used to quantify the extent of physiological endocortical osteoblast bone surface and osteal macrophage (osteomac) canopy. Bone repair was assessed in a stabilized tibial injury model that heals predominantly via intramembranous ossification resulting in complete intramedullar and intercortical bridging of the defect site by 7 days post injury.

Results: Immunohistochemistry for the B220 antigen indicated that mature B cells were randomly distributed throughout bone marrow of wildtype mice with no propensity or aversion for endosteal regions or sites of bone modelling and/or remodelling. In the endocortical diaphyseal region, adapted histomorphometry demonstrated that wildtype and µMT deficient mice had a similar extent of osteocalcin+osteoblast bone surface (63±3.4% vs. 74±7%, respectively, p=0.13). The extent of the osteoblast-associated osteomac canopy was also comparable in these mice (77±1.6% vs. 71±4.3%, respectively, p=0.13). In a tibial injury model, B220+ B cells were occasionally scattered within areas of high anabolic activity in wildtype mice. Boney bridging (collagen type 1+ matrix area within the injury site), area of F4/80 (macrophage marker) staining and area of TRAP (osteoclast marker) staining within the injury site were similar in µMT deficient and wildtype mice.

Conclusions: Osteoblast bone forming surface and intramembranous ossification during bone healing are unimpeded in the absence of mature B cells suggesting that these lymphoid cells do not influence anabolic bone modelling in vivo.

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SELECTIVE SEROTONIN RE-UPTAKE INHIBITORS (SSRIS) INHIBIT HUMAN OSTEOCLAST AND OSTEOBLAST FORMATION AND FUNCTION

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SSRIs are widely used antidepressants and one of the most commonly used medications. Our group was amongst the first to document a link between SSRI use and reduced BMD. Limited studies in animal and human models indicate that SSRIs may directly regulate serotonin signalling in bone cells. However the mechanism of action of SSRIs on human osteoclast (OC) and osteoblast (OB) formation and function remains unclear. Gene expression levels of serotonin (5-HT) receptors, serotonin transporter (5HTT) and tryptophan hydroxylase-I (Tph1) were assessed in OC precursors, mature OC, non-mineralising and mineralising primary human OB by real time PCR. OC formation and resorption in the presence of a number of SSRIs was assessed in CFU-GM derived cells treated with RANKL and M-CSF for 14 d. OB were cultured with SSRIs for 28 d and assessed for alkaline phosphatase (ALP) activity and bone mineralisation. Cell viability and apoptosis was determined by flow-cytometric annexin V assessment. OC (precursor and mature) and OB (nonmineralising and mineralising) expressed Tph1, 5HTT and 5-HTR1B. 5HTR2A was expressed only in OB, whereas 5HTR2B expression increased dramatically from precursor to mature OC. Except for citalopram (C), the SSRIs all dose dependently inhibited OC formation and resorption over the range of 1–10 μM in the order of potency: sertraline(S)>fluoxetine(Fx)>paroxetine(P)>fluvoxamine(Fvm). SSRIs (except C) also inhibited ALP and bone mineralisation by OB in a similar order, but only at 30 μM. SSRIs induced apoptosis in both OC precursors, and OB in an identical pattern to inhibitory effects observed in OC and OB. Treatment with serotonin alone had no effect on either OC or OB parameters. These data demonstrate that SSRIs inhibit bone cell function via apoptosis, but with differing potencies. Given the capacity of SSRIs to sequester in the bone marrow at high concentrations over several months, these data may explain the loss of BMD with chronic use.

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C-FOS PLAYS AN ESSENTIAL ROLE IN UP-REGULATION OF RANK EXPRESSION IN OSTEOCLAST PRECURSORS

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Aim: We previously reported that osteoclast precursors were detected as RANK-positive cells in bone tissues (J Cell Biol 184:541, 2009). RANK-positive cells were observed in bone tissues in RANKL^{−/−} mice, but not in c-Fos^{−/−} mice. On the other hand, F4/80-positive macrophages were similarly observed in bone tissues in both osteopetrotic mice. These results suggest that c-Fos, but not RANKL, is required for the up-regulation of RANK in osteoclast precursors. Then, we analyzed the mechanism of c-Fos-mediated up-regulation of RANK in osteoclast precursors.

Methods: Frozen tibial sections were prepared from wildtype mice, RANKL^{−/−} mice, and c-Fos^{−/−} mice and subjected to immunostaining for c-Fms (a receptor of M-CSF). Spleen macrophages (SPMs) were prepared by the treatment with M-CSF of spleen cells obtained from wildtype mice and c-Fos^{−/−} mice. Those SPMs were used for experiments on RANK expression, osteoclast differentiation, and c-Fos and RANK overexpression.

Results: (1) c-Fms-positive cells were detected in bone tissues of c-Fos^{−/−} mice and RANKL^{−/−} mice as well as wildtype mice. (2) The expression levels of RANK and c-Fos in wildtype SPMs were increased by the treatment with M-CSF In contrast, the upregulation of RANK was not observed in c-Fos^{−/−} SPMs. (3) The RANK expression in c-Fos^{−/−} SPMs was increased by the over-expression of c-Fos. (4) Osteoclastic differentiation of c-Fos^{−/−} SPMs could not be rescued by the overexpression of RANK.

Conclusions: We showed for the first time that c-Fos induced by M-CSF plays an essential role in the upregulation of RANK in osteoclast precursors. Researchers have believed that c-Fos plays an essential role under the RANK-mediated signals in osteoclast precursors to differentiate into osteoclasts. Our results suggest that c-Fos plays essential roles not only in RANKL-induced formation of osteoclasts but also in M-CSF-induced formation of osteoclast precursors.

59 ONCOSTATIN M POTENTLY INDUCES IL-6 AND RANKL EXPRESSION IN MOUSE SYNOVIAL FIBROBLASTS AND SYNERGISES WITH IL-1
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Aim: Oncostatin M (OSM) is a multipotent cytokine expressed in rheumatoid arthritic and osteoarthritic synovial tissues. OSM alone, or together with proinflammatory cytokines like IL-1, can stimulate synovial fibroblasts (SFs) to promote inflammation and joint destruction. We examined acute effects of OSM, IL-1 and their combination on SF expression of IL-6 and RANKL.

Methods: SFs were isolated from nonarthritic mice and stimulated with mouse OSM (2 ng/mL), mouse IL-1 (10 ng/mL) and their combination for 1, 6 and 24 h. Gene expression was assessed by quantitative RT-PCR; protein by flow cytometry and ELISA.

Results: In SFs, OSM and IL-1 increased IL-6 mRNA expression 80-fold at 6 h; OSM further increased expression 135-fold at 24 h. Profound synergistic upregulation of IL-6 mRNA and protein occurred when submaximal doses of OSM and IL-1 were combined (>1000-fold, mRNA; ~150-fold, protein). OSM and IL-1 both increased RANKL mRNA at 6 h (OSM, 9-Fold; IL-1, 4-Fold), with OSM increasing RANKL expression 20-fold at 24 h. Combining OSM and IL-1 enhanced RANKL mRNA expression at 24 h (100-fold), but without synergism. OSM also stimulated mRNA expression of its coreceptors (OSMR, 6-fold; gp130, 3-fold). Furthermore, OSM increased IL-1 receptor mRNA and protein expression. While IL-1 did not regulate its own receptor, it induced OSMR expression 3-fold. Importantly, the effects of OSM were dependent on OSMR expression.

Conclusions: OSM, acting alone or in synergy with IL-1, potently stimulates IL-6 and RANKL expression in SFs, with its actions dependent on OSMR expression. The synergism between OSM and IL-1 may be due to the crossregulation of their respective receptors. This study suggests a significant role for OSM, acting through OSMR, in contributing to inflammation and bone destruction in arthritic joints.

60 EPIGENETIC REGULATION OF OSTEOCLAST DIFFERENTIATION: POSSIBLE INVOLVEMENT OF JMJD3 IN THE HISTONE DEMETHYLATION OF NFATC1
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Recent studies have revealed that gene expression is controlled by epigenetic mechanisms such as chromatin histone modifications and DNA methylation, and that the expression of key developmental genes tend to be regulated by the trimethylation and demethylation of histone H3 lysine 4 (H3K4me3) and lysine 27 (H3K27me3). Osteoclast differentiation is tightly controlled by two essential cytokines, macrophage colony-stimulating factor and receptor activator of nuclear factor κ B ligand (RANKL). However, the role of epigenetic regulation in osteoclast differentiation is poorly understood. We applied massively
parallel sequencing of the chromatin immunoprecipitation products to investigate the H3K4me3 and H3K27me3 modification patterns around the transcription start site (TSS) of several transcription factors known to be important for osteoclastogenesis, i.e., Mitf, Nfkb1, Nfkb2, Mitf, Fos, and Nfatc1. H3K4me3 was present in both osteoclast precursors and osteoclasts in TSS of all of these transcription factors, except for Mitf. The H3K27me3 marks were present in a relatively broad peak centered on the TSS of Nfatc1, but not of the other transcription factors. Following the treatment with RANKL and subsequent osteoclast differentiation, a marked reduction in the level of H3K27me3 at the Nfatc1 locus was observed. Since the most likely explanation of H3K27me3 demethylation at the Nfatc1 locus is the involvement of H3K27me3 demethylases, we examined the expression of H3K27me3 demethylases during osteoclast differentiation. The expression of Jumonji domain containing-3 (Jmdj3), but not Utx, was time-dependently increased in osteoclast precursors and recruited in the vicinity of the TSS of Nfatc1 after stimulation with RANKL. In addition, gene silencing of the Jmdj3 gene by short hairpin RNA reduced demethylation of H3K27me3 around the TSS of Nfatc1 and markedly suppressed RANKL-induced osteoclastogenesis. These results suggest that demethylation of H3K27me3 in the vicinity of the TSS of the Nfatc1, regulated by Jmdj3, plays a key role in RANKL-induced osteoclast differentiation.

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TARGETED DISRUPTION OF THE GLUCOCORTICOID RECEPTOR IN ADIPOCYTES RESULTS IN, AN OSTEOSCLEROTIC PHENOTYPE, INCREASED FAT MASS AND GROWTH RETARDATION IN MICE

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Aim: The mechanisms by which glucocorticoids exert their receptor-mediated effects on bone and fat cells are poorly understood. In the present study we aimed to elucidate the role of the glucocorticoid receptor (GR) in adipocytes, and its interaction with bone, through characterisation of an adipocyte GR-deficient mouse line.

Methods: GRflox/flox mice were crossed with Fabp4Cre mice to generate GR-Fabp4Cre mice, in which the cre recombinase is under the control of the mouse fatty acid binding protein 4 (Fabp4) promoter. Cre-negative-GRflox/flox mice served as denote wildtype (WT) controls. Mice were analysed for postnatal skeletal changes (by whole body bone and cartilage staining), body composition (by DAX) and bone volume (by microCT).

Results: GR-Fabp4Cre and their WT littermates had a similar phenotype at birth, with normal skeletal size and regular bone and cartilage staining. Both groups developed normally until day 6, when GR-Fabp4Cre mice started to display a pleiotropic phenotype with significant growth retardation, pronounced alopecia followed by premature death within 2 weeks after birth. On day 10, skeletal size and body weight were significantly reduced in GR-Fabp4Cre mice when compared to WT littermates (p<0.05). Analysis of body composition revealed a significant increase in total body fat mass and a significant decrease in total body lean mass in GR-Fabp4Cre mice compared to WT littermates (p<0.05 for both). In contrast, trabecular bone volume was significantly increased in Fabp4-GRko mice (p<0.05). Despite delayed secondary calcification (Figure, arrows), GR knockout in adipocytes significantly increased tibial BV/TV compared to WT mice (p<0.05 compared to WT littermates). In addition, calvaria bone density was increased in GR-Fabp4Cre mice, indicating that both endochondral and intramembranous bone formation are altered by adipocyte specific GR knockout mice.

Conclusion: Adipocytic glucocorticoid signalling through the GR may play an important role in the postnatal development and growth of mice with profound skeletal effect.

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ANALYSIS OF THE ROLES OF FGF23 IN FETUS - SPECIFIC MINERAL METABOLISM USING HYP MOUSE

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Aim: Serum levels of phosphate (Pi) in fetus are maintained higher than maternal levels during late gestation, although the underlying mechanism remains unclear. We have previously reported that placenta expressed Klotho and might be a target of FGF23 signalling. In the current study, we have investigated whether FGF23 plays a role in fetus-specific mineral metabolism, using Hyp mice with high levels of serum FGF23.

Methods: Blood samples from E18.5 pregnant Hyp (Phex–/–) and wildtype (WT) mice, and their male fetuses were subjected to the measurement of calcium (Ca), Pi, and
FGF23. Genotyping was performed by genomic PCR to distinguish male *Hyp* (*Phex*<sup>Hyp/-</sup>) fetuses from WT fetuses delivered from *Phex*<sup>WT</sup> mothers. Gene expression in placenta was analyzed by real-time PCR.

Results: Although the Pi level in *Phex*<sup>Hyp/-</sup> mothers was lower than that in WT mothers, Pi levels in fetuses were comparable among the 3 groups; those from WT mothers and *Phex*<sup>Hyp/-</sup> and WT fetuses from *Phex*<sup>Hyp/-</sup> mothers. The Ca level in *Phex*<sup>Hyp/-</sup> fetuses was significantly lower than that in WT littersmates. The FGF23 level in *Phex*<sup>Hyp/-</sup> mothers was higher than that in WT mothers. WT fetuses from both *Phex*<sup>Hyp/-</sup> and WT mothers had equivalently low levels of FGF23. On the other hand, FGF23 level in *Phex*<sup>Hyp/-</sup> fetuses was about 20-fold higher than that in *Phex*<sup>Hyp/-</sup> mothers. The expression of vitamin D receptor (Vdr) was decreased in placenta from *Phex*<sup>Hyp/-</sup> mothers. In isolated trophoblasts, FGF23 induced the phosphorylation of ERK1/2 and the expression of Egr-1, and decreased the expression of Vdr.

Conclusions: Materno-fetal Pi transport is accelerated in *Phex*<sup>Hyp/-</sup> mothers with high FGF23 levels, independently of the genotype of fetuses, suggesting that maternal FGF23 might play a role in Pi transport. On the other hand, FGF23 in fetuses may be involved in vitamin D metabolism rather than Pi transport.

63 A COMPUTATIONAL APPROACH TO UNDERSTANDING FUNCTIONAL BEHAVIOUR OF BONE MULTICELLULAR UNITS

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Bone remodelling maintains the functionality of skeletal tissue by locally coordinating bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts). This coordination is operated within so-called bone multicellular units (BMUs). While several properties of bone cell-cell communication have been assessed experimentally, a comprehensive understanding of the functional behaviour of a BMU from its cells and regulatory factors in a spatio-temporal framework remains to be elucidated. In this contribution, we will present two computational models of cortical BMUs that address this question. In the first model, we show how some of the most important cell communication pathways currently known to exist between osteoblasts and osteoclasts (such as RANK-RANKL-OPG, TGFβ) are able to organise the cells into a travelling structure corresponding to the progression of a cortical BMU. This model allows to understand the spatio-temporal mechanisms of action of the regulatory factors, leading to segregated but functionally-coordinated cells (1). In the second model, we study microscopic bone resorption mechanisms in cortical BMUs and show how the life history of the osteoclasts (generation, apoptosis, nuclei renewal) influences their movement pattern and collective behaviour. These properties strongly influence the shape and extent of the developing resorption cavity, and so functional resorption by BMUs.

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64 VISCOELASTIC RESPONSE OF PTH, IBANDRONATE AND COMBINATION TREATMENT IN Ovariectomized Rat Femur CORRELATING WITH BMD

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Viscoelastic response upon loading is one of the properties exhibited by bone. Little has been reported on viscosity changes existing in osteoporotic bone or with treatments. Since bone viscoelasticity correlates to load-bearing capacity, the aim of this study is to investigate the viscoelastic properties of ovariectomized and drug-treated rat femurs.

15 SD rats were divided into 5 groups: (1) SHM: sham surgery; (2) OVX: ovariectomy surgery and treated with vehicle saline; (3) PTH: 10 μg/kg PTH treated ovariectomized rats; (4) IBN: 7 μg/kg ibandronate treated ovariectomized rats; (5) COM: ibandronate and PTH concurrent treated ovariectomized rats. Rats were euthanized at week 12. After metaphyseal region scanning by μCT and pQCT, femurs were embedded in epoxy and polished. After rehydration, nanoindentation was conducted using CSM mode to determine elastic modulus (E) and hardness (H).

Basic creep test was conducted using the 8bitg model. Viscosity (η) is computed based on the curve fitting of displacement by nonlinear regression. μCT analysis suggested that IBN and COM group had a better effect than PTH in preserving trabecular bone in terms of BV/TV, Tb.Th, SMI, BS/BV, Tb.Sp and Tb.N. In BMD, viscosity and SSly, COM group were significantly higher than the other two monotherapy groups. In 12 wks, BMD-η, SSly-η are both positively correlated (R=0.844 and 0.863 respectively, p<0.01 for both), whilst the E or H showed weak correlation with BMD or SSly.

Our results suggest that:
1. The osteoporotic deterioration does exist in bone viscoelasticity while treatments can dramatically restored decreased $\eta$ and $E$.
2. $\eta$, which strongly correlated with BMD and SSly, presented its potential to be another bone quality surrogate.
3. PTH has the highest $E$ among treatments. However, $\eta$ of PTH group was significantly lower than the other two. We hypotheses that different drugs have various specialties in improving nanolevel bone quality.

(1) Kim DG, Sarandeep SH et al. J Biomechan Engin 2010;132:1

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WHOLE VERTEBRAL BODY STRENGTH PREDICTED BY BONE MINERAL DENSITY FROM DXA AND BY BONE MICROARCHITECTURE FROM MICRO-CT

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The positive relationship between areal BMD (aBMD) derived from DXA and bone strength underpins aBMD as a good predictor of fracture risk. However, the predictive validity of aBMD for osteoporotic vertebral fractures remains suboptimal. The diagnostic sensitivity of DXA may be improved by assessing vertebral aBMD from lateral projections, compared to the commonly used posterior-anterior (PA) projections. X-ray microCT allows nondestructive three-dimensional structural characterisation of entire bone segments at high resolution. The aim of this study was to assess vertebral aBMD by both PA- and lateral-projection DXA and bone volume (BV) by microCT, and to compare their ability to predict whole vertebral body strength determined experimentally.

Eight human cadaver spines (mean age at death 78±10 years) were immersed in a water bath and scanned by DXA in PA and lateral projections; aBMD for L2 and L3 vertebrae was calculated. The L2 and L3 vertebrae were then dissected from each spine and entirely scanned by microCT (18 $\mu$m pixel size). BV was calculated over the microCT trabecular bone volume of the entire vertebrae. The vertebral bodies were then tested to failure in uniaxial compression to determine ultimate load.

aBMD by lateral-projection DXA and BV by microCT were both highly predictive of ultimate load ($r^2=0.70$, and $r^2=0.81$, both $p<0.01$). aBMD by lateral-projection DXA was highly predictive of BV assessed by microCT ($r^2=0.68$, $p<0.01$). Conversely, aBMD by PA-projection DXA had a lower coefficient of determination with ultimate load ($r^2=0.37$, $p<0.05$) and with BV ($r^2=0.29$, $p<0.05$). The standard-error-of-the-estimate in predicting ultimate load decreased by 31% when using aBMD from lateral-projection DXA, compared to PA-projection DXA.

These findings highlight the capability of aBMD assessed using lateral-projection DXA to predict vertebral strength, and provide a basis for further exploring the clinical application of lateral-projection DXA analysis.

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BIOMATERIAL SCAFFOLDS FOR MUSCULOSKELETAL REGENERATIVE MEDICINE: AN IN VITRO ANALYSIS

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Background: Injuries to bone and tendons can cause major morbidity in healthy, active people. The ability to provide a scaffold that encourages appropriate cell attachment, growth, and ultimately tissue regeneration, could improve the clinical outcomes from injuries such as rotator cuff tears and nonunion fractures.

Aim: Several scaffold materials of both natural and synthetic origin have been tested in this study to evaluate their potential utility in musculoskeletal regenerative medicine.

Methods: Four different scaffolds were evaluated as biomaterials: Spidrex 543 (Oxford Biomaterials Ltd, UK), a spider-like silk fabric; Endoform® (Mesynthes, NZ), a decellularised ovine forestomach matrix; 3D collagen gels and FiberWire® (Athrex, Inc, US), a polyethylene and polyester composite, commercially available suture currently utilised in orthopaedic surgery. Attachment and growth of primary osteoblasts and tenocytes were analysed using live-dead staining and alamar blue fluorescence. Morphological phenotype was assessed using confocal microscopy and cell differentiation was evaluated by differential gene expression.

Results: Osteoblasts and tenocytes both successfully adhered to and grew on the Endoform®, the silk and within the 3D collagen gels, whereas the orthopaedic suture material proved unsuitable for cell attachment/growth.
Gene analysis and morphology in the three permissible scaffolds suggest cells retain their phenotype when cultured in them. The 3D culture systems support increases in proliferation and differentiation, notably, gene expression of key osteoblastic markers alkaline phosphatase, osteocalcin and bone sialoprotein were increased 33-, 240- and 34-fold, respectively, in osteoblasts cultured within 3D collagen gels for 72 h (P≤0.05) compared to osteoblasts in 2D cultures. Conclusions: We have identified a number of biomaterial scaffolds that have potential for use in bone and tendon regeneration. Further testing is required to determine if they support tissue formation.

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NOVEL LOCI ASSOCIATED WITH BONE MINERAL DENSITY: RESULTS OF A MULTIPOINT LINKAGE ANALYSIS OF EXTENDED PEDIGREES

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Genomewide association studies have identified several genes that explain less than 5% of BMD variance. We hypothesise that there exists genes with major effect size, and that these genes can be identified by linkage studies within families. The present study was designed to discover novel genes that are associated with BMD using an extended pedigree design. The Dubbo Osteoporosis Genetics Study (DOGS) was designed as a multigenerational familial investigation, which includes 509 individuals across 84 pedigrees, aged between 18 and 95 years. The individuals and pedigrees were selected based on a proband who had relatively high BMD (Z-score >1.28). BMD was measured at the femoral neck (FN), lumbar spine (LS) and total body (TB) by DXA (GE-Lunar, Madison, USA). Genotypes for 503 markers across the genome were determined using a deCODE microsatellite panel. Variance components analysis and multipoint linkage analysis were performed using SOLAR. Data from 194 men and 315 women (average age 52.3), among whom 54% were aged 50 years and over, were analysed. The average BMD Z-score was +0.31 for FNBMD and +0.57 for LSBMD. Heritability analysis indicated that 49% and 65% of the variance of FNBMD and LSBMD, respectively, was due to genetic factors. Multipoint linkage analysis identified multiple loci that were linked to FNBMD, with the most significant result being at chromosome 3q25 (LOD score 3.11), which explains up to 50% of the total additive genetic variance. For LS the highest LOD score was observed at chromosome 1q23 (LOD score 1.80), explaining up to 35% of the total additive genetic variance. These loci have not been previously identified. These results suggest that there exists novel loci that could play an important role in the regulation of BMD.

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GLUCOCORTICOID THERAPY DETERIORATES BONE STRENGTH BUT INCREASES POST-YIELD ENERGY TO FRACTURE BY THE REDUCTION OF DEGREE OF MINERALIZATION OF BONE IN A MICE MODEL OF RHEUMATOID ARTHRITIS


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Aim: Patients with rheumatoid arthritis (RA) have a greater risk of fracture including insufficiency fracture, relative to general population. Chronic inflammation and impaired mobility as well as the use of glucocorticoids (GC) are thought to be major causes of bone fragility in RA. However, the mechanisms for high incidence of insufficiency fractures in GC-treated RA patients are not fully understood. To address this, we investigated the effects of GC and RA on bone quality, quantity, and biomechanical properties, using a mice model of RA.

Methods: Five-month old male human tumor necrosis factor transgenic (hTNFtg) mice and wildtype (WT) littermates were used. Treatment was performed using slow release pellet of prednisolone (5 μg/kg/day) or placebo. Mice were killed at 0, 14, 28 and 42 post-treatment and compositional change of bone was assessed by Raman spectroscopy. Bone quantity, microstructure, and biomechanical properties of Tibia and 2nd lumbar vertebral bodies were then assessed by microfocal computed tomography and biomechanical testing.

Results: GC and hTNF transgene additively decreased mechanical strength, rigidity/stiffness, and energy to yield in both tibiae and vertebral bodies. In tibial torsion test, GC reduced energy to failure without changing the ratio of post-yield energy to total energy in WT mice, while GC increased energy to failure and the ratio of post-yield energy to total energy in hTNFtg mice. In compressive test of vertebral body, the ratio of post-yield energy to total energy was decreased by GC in hTNFtg mice but not in WT mice. Microstructures of bone were deteriorated mainly by hTNF transgene, while degree of mineralization was decreased by both hTNF and GC.

Conclusions: The results of this study suggest that GC additively decreases bone strength in RA and that hypomineralization of bone in GC-treated RA, which increases...
ductility of bone, are associated with increased risk of insufficiency fracture.

69 PREVENTION OF WEAR PARTICLE-INDUCED OSTEOLYSIS BY A NOVEL V-ATPASE INHIBITOR SALIPHENYLHALAMIDE (SALIPHE) THROUGH INHIBITION OF OSTEOCLAST MATURATION AND BONE RESORPTION


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Wear particle-induced aseptic prosthetic loosening is a major complication after total joint arthroplasty and is well established that the bone resorbing osteoclasts is responsible for the extensive bone destruction (osteolysis) associated with wear particle-induced peri-implant loosening. Thus, inhibition of osteoclastic bone resorption may serve as a potential therapeutic avenue for prosthetic loosening. Here, we demonstrate that two selective V-ATPase inhibitors, saliphenylhalamide and bafilomycin, attenuate wear particle-induced osteolysis in a mouse calvarial model. In vitro biochemical and morphological assays revealed that the inhibition of osteolysis is partially attributed to a disruption in osteoclast acidification and polarization, both a prerequisite for osteoclast bone resorption. Interestingly, V-ATPase inhibitors also impaired osteoclast differentiation via the inhibition of RANKL-induced NF-κB signalling pathway. In conclusion, we showed that V-ATPase inhibitors affected multiple physiological processes including osteoclast differentiation, acidification and polarization, leading to inhibition of osteoclast bone resorption in vitro and wear particle-induced osteolysis in vivo. The results of the study provide proof that V-ATPase inhibitors, such as saliphenylhalamide, are potential antiresorptive agents for treatment of wear particle-induced osteolysis to prevent aseptic prosthetic loosening.

72 PAGET’S DISEASE OF BONE-ASSOCIATED SEQUESTOSOME 1/P62 MUTANT PROTEINS INCREASE RANKL-INDUCED AP-1 SIGNALLING AND AFFECT CELLULAR CO-LOCALISATION WITH KEY SIGNALLING INTERMEDIATES AJUBA AND TRAF6

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Aim: Paget’s disease of bone patients commonly harbour a mutation in the Sequestosome 1/p62 (p62) gene. Most known mutations manifest within or cause deletion of the Ubiquitin-associated (UBA) domain of p62, a scaffold protein that forms complexes with both TRAF6 and the LIM-protein Ajuba in RANKL-induced signalling to NF-κB. We have previously shown that p62 mutant proteins increase NF-κB signalling compared with wildtype p62. The aim of this study was to investigate the effect of p62 mutations on AP-1 activity, and the interaction of p62 with key signalling intermediates in the AP-1 and NF-κB pathways, important for osteoclastogenesis.

Methods: HEK293 cells stably expressing RANK were transfected with an AP-1 luciferase reporter with empty vector or p62 (wildtype or mutant). Cells were treated with RANKL or left untreated. Lysates were prepared and tested for AP-1 activity. For co-immunoprecipitations, p62 (wildtype or mutant) was coexpressed with either TRAF6 or Ajuba. p62 protein was immunoprecipitated, bound to beads and, following extensive washes, p62 and bound interacting proteins were eluted and processed for Western blot analysis.

Results: We observed that p62 mutant proteins are associated with increased AP-1 activity compared with wildtype p62 and the empty vector control. Additionally, we observed that Ajuba induces AP-1 activity under basal and RANKL-induced conditions, an effect that is abrogated by p62 co-expression. We also found that wildtype p62 appears to aggregate with key signalling intermediates Ajuba and TRAF6 in the nucleus, whereas UBA-deficient p62 interacts with these proteins primarily in the cytosol.

Conclusions: We conclude that aggregation of signalling intermediates by p62 is the potential mechanism underlying signalling repression that is observed with p62 over-expression. Furthermore, the increased signalling observed with p62 mutant proteins may be due to decreased capacity for client-protein aggregation and/or altered cellular localisation.

76 ANALYSIS OF OXIDATIVE STRESS AND ANTIOXIDANT ENZYMES IN MECHANICAL STRESS RESPONSE


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FKBP10 MUTATION IN SAMOA

Phenotype resulting from a founder variable osteogenesis imperfecta

Aim: Mechanical loading plays an important role in maintaining homeostasis both in skeletal muscle and bone. Reduced mechanical stimulation leads to the enhancement of oxidative stress in skeletal muscle, and the alteration of the expression pattern of antioxidant enzymes, resulting in muscle atrophy. However, the relevance of mechanical stimulation and oxidative stress in bone remains to be fully elucidated.

Method: This study investigated the oxidative stress level and the gene expression pattern of antioxidant enzymes in bone using tail suspension to clarify whether skeletal unloading regulates oxidative stress and antioxidant capacity in bone.

Result: Hindlimb unloading significantly increased the level of reactive oxygen species in bone marrow cells as well as the serum level of an oxidative stress marker. Hindlimb unloading also upregulated the expression of CuZn-SOD (Sod1), one of the major antioxidant enzymes in the cytoplasm, but not any other antioxidant enzymes (Sod2, Cat and Gpx1). The tails of Sod1-deficient (Sod1−/−) and wildtype mice (WT) were suspended for 2 weeks to investigate the physiological role of Sod1 on mechanical unloading. DXA revealed that Sod1−/− mice showed a significant decrease by 1.7-fold in the femur BMD in comparison to the WT mice. Similarly, a microCT analysis showed that Sod1 deficiency significantly reduced BV/TV by unloading (Sod1−/−: H48%, WT: H24%, p<0.01). The dynamic bone formation parameters revealed that the Sod1 deficiency exacerbated the decline of the bone formation rate and mineralizing surface by unloading, while no difference was observed in the bone resorption parameters between Sod1−/− and WT, thus indicating that Sod1 insufficiency exacerbated bone loss under mechanical unloading conditions due to the suppression of osteoblastic bone formation activity.

Conclusion: These results indicate that reduced mechanical stimulation modified the antioxidant capacity and Sod1 plays a protective role in oxidative stress due to unloading in bone.

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VARIABLE OSTEOGENESIS IMPERFECTA PHENOTYPE RESULTING FROM A FOUNDERS FKBP10 MUTATION IN SAMOA

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Mutations in FKBP10, which encodes the collagen prolyl cis-trans isomerase chaperone protein FKBP65, have recently been discovered to cause a recessively-inherited variant of osteogenesis imperfecta. We have identified 17 individuals in 10 independent families originating from the Samoan islands who share one FKBP10 mutation. One group presents at birth with Bruck syndrome-like features of talipes and flexion contractures; and/or neonatal fractures—these patients do not attain independent mobility. Patients in the second group present later in childhood, typically with pain on walking or long bone fractures aged 4–18 years. The difficulty with ambulation is the result of progressive acetabular protrusion which leads in some to severe impairment of mobility. The diversity of phenotype was present even within families. Short stature, macrocephaly, platybasia, Wormian bones, white sclerae with normal hearing and teeth were common to all patients. The short stature is exacerbated by progressive scoliosis, a major cause of reduced life expectancy.

The Samoan mutation [c.948_949insT] in FKBP10 has not been previously described. It creates a frameshift with a premature stop codon in exon 7 and results in mRNA instability, so that no protein is produced. In all but one of these families, affected individuals were homozygous for this mutation. Two affected siblings from a family with one Samoan parent were compound heterozygous for the Samoan mutation and the previously described c.831_832insC mutation.

We estimate this mutation has a frequency of ~1 in 50–100 in the Samoan population; it is probably a founder mutation carried by early settlers to Samoa ~1000 BCE.

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ADVERSE CARDIOVASCULAR EFFECTS OF CALCIUM SUPPLEMENTS MAY NOT PERSIST AFTER DISCONTINUATION OF SUPPLEMENTS: 5-YEAR FOLLOW UP OF THE AUCKLAND CALCIUM STUDY.


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Aims: In a 5 y randomized placebo-controlled trial of 1 g/day calcium citrate in 1471 postmenopausal women, relative risks for myocardial infarction (MI) and stroke with calcium were 1.49 and 1.37, respectively1,2. These findings are further supported by the results from meta-analyses of placebo-controlled trials of calcium supplements3,4.
We wished to determine whether the adverse cardiovascular effects of calcium supplements persist after discontinuation of supplements.

Methods: Approximately 5 y after completion of the trial, we collected information on MI, stroke, and post-trial calcium supplement use in the 1408 surviving participants. 1174 participants were contacted by telephone. Information on all 1408 participants was obtained from national databases of hospital admissions and deaths.

Results: During an average of 9.1 y of follow-up, 138 women (52 during, 86 post-trial) had an MI, 158 had a stroke (59 during, 99 post-trial) and 257 women died (63 during and 194 post-trial). Post-trial calcium supplement use was similar in both treatment groups (35-37%). When analysed on an intention to treat basis (n=1471), there was no difference in the risk of myocardial infarction (HR 1.04, CI 0.74-1.45), stroke (HR 1.04, CI 0.76-1.42), or death (HR 1.16, CI 0.91-1.48) between women originally allocated to calcium and those allocated to placebo. There were no differences in the risk of MI (HR 0.82, CI 0.55-1.22) or stroke (HR 0.84, CI 0.58-1.22) in the post-trial period between women originally allocated to calcium and those allocated to placebo. There were also no differences in the risk of MI or stroke between women who took calcium post-trial and those who did not, in either treatment group.

Conclusion: The adverse cardiovascular outcomes seen in the original clinical trial did not persist in the post-trial period. Confounding by post trial calcium supplementation did not influence this conclusion.

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(2) Bolland et al. BMJ 2008;336:262
(3) Bolland et al. BMJ 2010;341:c3691
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85 DOES CALCIUM SUPPLEMENTATION WITH AND WITHOUT VITAMIN D INCREASE CARDIOVASCULAR RISK? A CLINICO-BAYESIAN INTERPRETATION

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Background: Recent analyses suggest that calcium supplementation±vitamin D (Ca±D) is associated with increased risk of myocardial infarction (MI). However, the effect size was modest, and could be due to bias. In this study, we re-examined the likelihood that such a clinically relevant association exists using a Bayesian meta-analysis approach.

90 PREVENTION OF SKELETAL DAMAGES WITH FOLINIC ACID SUPPLEMENTATION IN YOUNG RATS RECEIVING LONG-TERM METHOTREXATE CHEMOTHERAPY

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With the development of chemotherapy and increasing childhood cancer survivor rates, skeletal complications such as bone growth arrest and fractures during and after chemotherapy have become significant problems for paediatric cancer survivors. It is therefore important to develop preventative strategies for these skeletal defects. Methotrexate (MTX), a commonly used chemotherapeutic agent in paediatric cancers, has been shown to cause bone growth defects both clinically and in experimental animals.

Aim: The current study examined the effects of chronic high-dose methotrexate (MTX) chemotherapy on bone
growth of young rats, and potential protective effects of antidote folic acid (FA) in protecting bone growth during long-term MTX chemotherapy.

Methods: During the induction phase, rats received injections of saline, MTX or MTX+FA (MTX at 0.65 mg/kg, FA at 0.87 mg/kg, 5 days on/9 days off), followed by the maintenance phase of twice weekly injections for 4 weeks (MTX at 1.3 mg/kg and FA at 1.3 mg/kg).

Results: Histological analysis revealed that at the growth plate, chronic MTX treatment caused reduction in columnar chondrocyte numbers, induction of chondrocyte apoptosis and chondroclast recruitment. In the metaphysis, ex vivo x-ray microtomography (μCT) revealed MTX caused overall reduction of trabecular bone volume, which was due to increased osteoclast density, induction of osteoblast apoptosis and increased adipocytes. Furthermore, plasma from MTX-treated rats was able to induce ex vivo osteoclast formation from normal bone marrow cells, suggesting systemic contribution to bone resorption. FA supplementary treatment was able to alleviate MTX-induced histological and cellular damages in both the growth plate and metaphysis.

Conclusion: These findings indicate that FA supplementation can prevent growth plate and metaphyseal damages from chronic MTX administration, and may potentially be useful in paediatric patients who are at risk of skeletal growth suppression as a result of chronic MTX chemotherapy.

91 METHOTREXATE CHEMOTHERAPY-INDUCED BONE LOSS AND MARROW ADIPOSY IS ASSOCIATED WITH DeregULATION OF THE WNT/B-CATENIN SIGNALLLING PATHWAY

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The chemotherapeutic agent methotrexate (MTX), commonly used to treat a variety of cancers, has been shown to cause bone defects including osteoporosis. The current study sought to characterise the effects of MTX treatment on bone/fat volume and differentiation potential of bone marrow stromal progenitor cells in a rat model and to identify potential regulatory mechanisms. The Wnt/β-catenin signalling pathway is an integral regulator of bone formation and adipogenic differentiation, therefore this study aimed to investigate the impact of its modulation for the prevention of MTX-induced bone loss and marrow adiposity. MTX treatment (5 consecutive daily doses at 0.75 mg/kg) caused a significant reduction in trabecular bone volume parallel to an increase in marrow adiposity. Cell culture studies illustrated that while osteogenic differentiation capacity of isolated marrow cells was reduced, adipogenic potential was markedly increased on day 9. Consistently, RT-PCR gene expression analyses revealed osteogenic transcription factors Runx2 and Osterix to be decreased but adipogenic genes PPARγ and FABP4 upregulated on days 6 and 9 in the marrow stromal population. Furthermore, Wnt-10b illustrated to be important for appropriate osteoblast/adipocyte commitment, had reduced mRNA expression in the bone marrow stromal population following MTX treatment, yet regulation returned to normal by day 14. Concurrent administration of 6-bromoindirubin-3'-oxime (BIO) (at 0.2 mg/kg), an inhibitor of glycogen synthase kinase 3β (GSK-3β) involved in destabilising β-catenin and thus Wnt signalling, alleviated MTX-induced transient changes in bone/fat volume, osteogenic/adipogenic commitment and gene expression profiles including Wnt target gene cyclin D1. These findings illustrate that short-term MTX chemotherapy induces a transient switch in differentiation potential towards adipogenesis at the expense of osteogenesis and that Wnt/β-catenin signalling plays an important role in these defects, illustrating a potential future therapeutic target for preventing bone loss and marrow adiposity resultant of chemotherapy.

92 THE VITAMIN D RECEPTOR PROMOTES HUMAN PROSTATE CANCER CELL GROWTH VIA A LIGAND INDEPENDENT PATHWAY

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Aim: Bone is a frequent site for prostate cancer metastasis. We have previously reported that vitamin D deficiency promotes human prostate cancer cell growth in bone. However, little is known about the role of the vitamin D receptor (VDR) in this context. The present study aimed to define the role of the VDR in human prostate cancer growth in vitro and in vivo.
Methods & Results: VDR expression was knocked down by stable expression of shRNA in PC3 cells (PC3-VDR-KD), with nontarget cells (PC3-NT) generated as controls. VDR mRNA knock down was 85% and induction of CYP24 mRNA expression by 1,25(OH)2D3, normally seen in VDR expressing cells, was abrogated in PC3-VDR-KD cells, indicating effective disruption of VDR signalling. Treatment of PC3-NT cells with 1,25(OH)2D3 significantly reduced cell growth by up to 51% as compared to untreated PC3-NT cells. Surprisingly, growth of PC3-VDR-KD cells in ligand-free cultures was also reduced by 49% (compared to NT cells). Moreover, cell migration was increased by 10% in PC3-VDR-KD cells. Of note, PC3-VDR-KD cells did not respond to treatment with 1,25(OH)2D3.

To further investigate the effects of VDR knockdown in vivo, PC3-NT and PC3-VDR-KD cells were implanted subcutaneously in nude mice, and tumor growth was monitored for 69 days. Compared to NT cells, VDR knockdown resulted in significantly smaller tumors from day 12 onwards. Similarly, when PC3-NT or PC3-VDR-KD cells were implanted into the tibiae of vitamin D sufficient mice, disruption of VDR signalling resulted in significantly smaller osteolytic lesions from day 17 onwards (x-ray analysis).

Conclusion: These results suggest a novel ligand-independent role of the VDR in promoting prostate cancer cell growth and suppressing invasive cell potential (migration). This novel function of the unliganded VDR contrasts with the known anti-proliferative actions of the liganded VDR and may offer new therapeutic approaches in cancer treatment.

93 HOW WELL DO THE FRAX (AUS) AND GARVAN CALCULATORS PREDICT FRACTURES FROM THE GEELONG OSTEOPOROSIS STUDY (GOS)

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The FRAX calculator (AUS)1 estimates the 10-year absolute risk of fractures at the hip, spine, humerus and wrist (“major osteoporotic fractures”) whereas the Garvan calculator2 predicts the 10-year absolute risk of fracture at the hip, spine, wrist, metacarpal, humerus, scapula, clavicle, lower limb, pelvis and sternum (“osteoporotic fractures”). The calculators use 11 and 5 risk factors, respectively. This study aims to assess the ability of both calculators to predict fracture in a cohort of women followed prospectively for 10 yr. An age-stratified random population-based sample of women was recruited by GOS during 1993–7 (n=587; age 60–90 yr). Risk factors measured at baseline visit included: femoral neck BMD, falls, prior fracture, weight, height, parental fracture, smoking, glucocorticoid usage, secondary osteoporosis, and alcohol consumption. Subjects were followed biennially for 10 yr (median 9.17 yr, IQR: 4.79-10). Fractures documented and verified radiologically were only those sustained after a low trauma event. Absolute risk of fracture was calculated using both calculators. Number of predicted fractures was calculated by the sum of the absolute risks adjusted by time in the study. A one-sample chi-squared test assessed the difference between the observed and predicted number of fractures. The areas under the receiver operating characteristic curves (AUC) were calculated.

There were 38 hip, 15 major osteoporotic, and 127 osteoporotic fractures observed. The FRAX calculator (with BMD) predicted 20.1 hip fractures (p<0.01) and 49.5 major osteoporotic fractures (p<0.0001) whereas the Garvan calculator predicted 54.8 hip (p<0.02) and 127.5 osteoporotic fractures (p=0.96). There was no significant difference in AUC using FRAX (hip: AUC=0.75, 95% CI=0.68-0.82, major osteoporotic: AUC=0.63, 95% CI=0.64-0.75, osteoporotic: AUC=0.69, 95% CI=0.64-0.74) or Garvan calculators (hip: AUC=0.77, 95% CI=0.70-0.84, major osteoporotic: AUC=0.70, 95% CI=0.65-0.70, osteoporotic: AUC=0.70, 95% CI=0.65-0.75).

The Garvan calculator predicted fragility fractures extremely well although modestly overestimated hip fractures. The FRAX calculator substantially underestimated both hip and osteoporotic fractures.

(2) Nguyen, N.D., et al., Osteoporos Int, 2007;18:1109

94 FRAGILITY FRACTURE AND OSTEOARTHRITIS: INTERACTIONS BETWEEN BONE MINERAL AND BONE MASS INDEX

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Increased BMD and BMI are associate with increased risk of osteoarthritis (OA) and reduced risk of fragility fracture. However, little is known about the relationship between fragility fracture and osteoarthritis. This study
sought to examine the interactions between BMD and BMI in the determination of the OA-fracture relationship. The study was part of the on-going Dubbo Osteoporosis Epidemiology Study, which involved 2,412 women and 1,452 men aged between 46–99 years. Baseline BMD was measured at femoral neck (FNBMMD) and lumbar spine (LSBMD) by DXA. Osteoarthritis was ascertained by self-report. The incidence of fragility fracture was ascertained by X-ray report. A total 1,077 participants (691 women and 386 men) had reported a diagnosis of OA. Overall, the risk of OA was associated significantly with increased LSBMD in men (odds ratio [OR] 1.34, 95% CI, 1.19-1.52) and women (1.20; 1.09-1.32). Elevation in FNBMMD was significantly associated with increased risk of OA in men (OR 1.16; 1.02-1.32), but not in women. When stratified by BMI, significant association remained between OA risk and high LSBMD amongst women with BMI <25 kg/m² (OR 1.26; 1.07-1.47) and BMI >30 kg/m² (1.26; 1.05-1.51); and in men with BMI <25 kg/m² (OR 1.75; 1.37-2.27) or BMI 25–30 kg/m² (1.21; 1.02-1.43). OA was associated with an increased risk of fracture. After adjusting for LSBMD, women with OA had significant increased fracture risk (OR 1.41; 1.16-1.72), particularly in those with BMI 25–30 kg/m² (OR 1.45; 1.05-1.99). However, the association was not significant in men. These data suggest that high BMD is associated with a greater risk of OA in both men and women, especially those with low BMI; and hence, BMD could be a useful measure for identifying individuals at high risk of OA, particularly among those at lower BMI spectrum. Despite having higher bone density, women with self-reported OA, especially those overweight, have an increased risk of fragility fracture, suggesting that the OA-fracture association is mediated via non-BMD factors.

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**ENDOGENOUS PARATHYROID HORMONE IS ASSOCIATED WITH REDUCED CARTILAGE VOLUME IN VIVO IN A POPULATION-BASED SAMPLE OF ADULT WOMEN**


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**Aim:** PTH has complex actions on bone, and recent animal and in vitro studies also suggest that PTH may affect articular cartilage. However, little is known of the relationship between PTH and joint structure in vivo, thus, the aim of this study was to examine the association between endogenous PTH and cartilage volume in vivo in a healthy adult population with no symptoms of knee OA.

**Methods:** Magnetic resonance imaging of the dominant knee was performed on 101 asymptomatic females aged 35–49 years (2007–9), from which knee cartilage volume was determined. Blood samples were obtained 10 years prior (1994–7), and stored at −80°C for random batch analyses. Serum intact PTH was quantified by chemiluminescent enzyme assay. Serum 25-hydroxyvitamin D (25(OH)D) was assayed using an equilibrium radioimmunoassay after extraction with acetonitrile.

**Results:** A 1-unit (pmol/L) increase in PTH was associated with reduced medial cartilage volume [regression coefficient± standard deviation, p-value] (−0.7±0.3, p=0.03), after adjustment for age, BMI and bone area. The association was sustained after further adjustment for seasonal variation (−0.8±0.3, p=0.02), and for 25(OH)D with a sinusoidal adjustment (−0.06±0.4, p=0.04), and calcium supplementation (−0.9±0.3, p=0.01). No associations were observed with lateral cartilage volume (0.2±p≤0.3). After excluding subjects with osteophytes, to account for the possibility of subjects having signs of pre-clinical OA, results remained similar (−0.9±0.4, p=0.01), including after further adjustment for season (−1.2±0.4, p=0.007), and 25(OH)D (−1.0±0.4, p=0.01).

**Conclusions:** This study suggests increased levels of PTH might be detrimental to cartilage in humans in vivo. Animal studies suggest that increased PTH may reduce the ability of cartilage to heal following minor injury. This may explain our results, particularly given the effect we observed in the medial compartment which is exposed to higher loads during weight bearing compared to the lateral compartment.

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**FAT MASS AND FRACTURE RISK IN ELDERLY MEN AND WOMEN: A PROSPECTIVE STUDY**

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**Background & Aim:** The relationship between body fat mass and fracture risk is controversial, due primarily to lack of prospective data. The present study sought to examine the association between whole body and abdominal fat mass and fracture risk in postmenopausal women and elderly men.
Materials & Methods: The present study was part of the ongoing Dubbo Osteoporosis Epidemiology Study (DOES), in which a random sample of more than 2000 men and women aged 60+ years has been continuously followed up for 21 years. The present study was based on a cohort of 1129 participants (361 men and 768 women), whose total body BMD scans were available. BMD at the femoral neck and lumbar spine, total body fat mass and abdominal fat mass were measured by DXA (GE-LUNAR Corp, Madison, WI). Baseline characteristics of participants including age, height, physical activity, history of falls, smoking and prior fracture were ascertained at the initial visit. The incidence of low-trauma and non-pathological fractures was ascertained from x-ray reports. The Cox’s proportional hazards regression was used to evaluate the association between fat mass and fracture risk, with adjustment for baseline covariates.

Results: During the median 5 years of follow-up, 19 (5%) men and 107 (14%) women had sustained a fragility fracture. Women with fracture had lower BMD, lower body fat mass and body weight than those without a fracture. In women, increased risk of fracture was associated with lower abdominal fat mass (hazard ratio/standard deviation[HR/SD]: 1.33; 95% CI: 1.07-1.65), after adjusting for age (HR/SD: 1.32; 1.08-1.63), femoral neck BMD (HR/SD:1.26; 1.03-1.56), and prior fracture (HR/SD:1.41; 1.15-1.73). Compared with women in the highest tertile of abdominal fat, those in the lowest tertile had a 2.1-fold (95% CI: 1.25-3.55) increase in fracture risk. Further analyses revealed that lower body fat mass was also associated with increased fracture risk, but the association was not independent of BMD. The magnitude of association between fat mass and fracture risk (HR/SD: 1.32; 1.08-1.63) was greater than that of body weight and fracture risk (HR/SD: 1.15; 0.94-1.41). Approximately 27% of fracture liability was attributable to abdominal fat mass.

Conclusion: Lower total body fat mass, particular lower abdominal fat, was significantly associated with increased fracture risk in women, not in men. These results suggest that the incorporation of fat mass into existing fracture prognostic models may enhance their predictive accuracy.

Sclerostin induces osteocyte support of osteoclast formation and osteoclast activity

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Sclerostin is a product of mature osteocytes and a potent negative regulator of bone formation (1). Our recent study showed that sclerostin affects osteoblasts in an anti-anabolic manner (2). However, studies employing a neutralising antibody against sclerostin have reported decreased bone resorption markers (3), indicating that sclerostin may also have a catabolic action. The aim of this study was to investigate potential catabolic actions of sclerostin via the RANK-RANKL pathway.

The effect of recombinant human sclerostin (rhSCL) on pro-oSTEOCLASTIC gene expression was tested in cultures of human primary immature osteoblasts and differentiated late-osteoblast/pre-osteocyte cultures, as well as the mouse osteocyte-like cell line, MLO-Y4. To examine the functional effects of rhSCL on resulting pro-oSTEOCLASTIC activity, MLO-Y4 cells plated onto a bone-like substrate were primed with rhSCL for three days and then either mouse splenocytes or human peripheral blood-derived mononuclear cells (PBMC) were added. Resorptive activity was analysed after 14 days of culture. As apoptosing osteocytes have been shown to support osteoclast formation, the effect of rhSCL on MLO-Y4 apoptosis was assessed by caspase assays and nuclear morphology.

Sclerostin dose-dependently up-regulated the expression of RANKL mRNA and down-regulated that of OPG, increasing the RANKL:OPG mRNA ratio in late osteoblast/pre-osteocyte-like cultures and in MLO-Y4 cells. In cocultures of rhSCL treated MLO-Y4 cells and osteoclast precursors, osteoclast resorptive activity increased approximately 7-fold. The increased resorption was abolished by co-addition of recombinant OPG. rhSCL treatment also increased TRAP-positive multinucleated cell formation, and significantly increased the size of the formed cells. No detectable increase of caspase activity was observed in rhSCL-treated MLO-Y4 cells and the nuclear morphology did not change, indicating that the pro-oSTEOCLASTIC effect was not as a result of MLO-Y4 cell death. Our findings show for the first time that sclerostin, in addition to its anti-anabolic activity, acts on viable osteocytes to promote osteoclast formation and activity, and does so in a RANKL-dependent manner.

(1) Li X et al., JBMR (2009)
(2) Atkins GJ et al. JBMR Epub ahead of print (2011)
(3) Eddleston A et al. JBMR (2009)
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LOWER SERUM 25-HYDROXYVITAMIN D LEVELS ARE ASSOCIATED WITH GREATER ALL-CAUSE AND CANCER-RELATED MORTALITY AMONG AUSTRALIAN ADULTS: FINDINGS FROM THE AUSTRALIAN DIABETES, OBESITY AND LIFESTYLE STUDY (AUSDIAB)

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Aim: Low serum 25OHD levels have been associated with morbidity and mortality, but the relationship with mortality in Australia has not been investigated. Thus, we examined the association between 25OHD and overall and cause-specific mortality in Australian adults.

Methods: Multivariate-adjusted Cox proportional hazards regression models were used to estimate the relative mortality risk of adults (n=10,542) aged ≥25 years, using data from the 1999–2000 AusDiab study linked to mortality records [National Death Index] for deaths until 16/7/2007 for all-cause, CVD and cancer-related mortality. The fully adjusted model included: age, sex, season, latitude, ethnicity, education, smoking, waist circumference, exercise, diabetes status, hypertension, use of lipid-lowering medication, serum cholesterol, triglycerides, HDL-C, history of diabetes status, hypertension, use of lipid-lowering medication, subjects finishing a five year RCT of calcium supplementation (CAIFOS) (1), they were then recruited into a five-year epidemiology study. Baseline serum 25(OH)D concentration was determined using the LC-MS/MS method. The total hip DXA BMD was measured at year one. Clinical incident osteoporotic fractures were ascertained by adverse events diary returned to the study centre every four months and confirmed by radiographic report. Mortality data were obtained from the WA mortality registry.

Results: During follow-up (median 7-years), 530 (5.0%) participants died (173 CVD- and 213 cancer-related deaths) and these participants had lower 25OHD levels compared to those who survived (57 vs. 64 nmol/L, P<0.001). All-cause mortality risk was increased by 60% in the lowest compared to highest 25OHD quartile, and cancer-related mortality was increased by 79-88% across the lowest three quartiles (Table). There was a trend for CVD and non-CVD/cancer related deaths to be greater in those in lowest quartile, but the HRs were not significant. Similar results were observed after excluding the 101 participants who died within 2-years of follow-up.

Conclusion: Lower serum 25OHD levels were independently associated with an increased risk for all-cause and cancer-related mortality in Australian adults.

Table: Hazard ratios (95% CI) for mortality by quartiles of 25OHD

<table>
<thead>
<tr>
<th>Mortality</th>
<th>Serum 25OHD Quartiles, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;47</td>
</tr>
<tr>
<td>All-cause</td>
<td>1.60 (1.20, 2.14)</td>
</tr>
<tr>
<td>CVD</td>
<td>1.51 (0.92, 2.48)</td>
</tr>
<tr>
<td>Cancer causes</td>
<td>1.86 (1.13, 3.04)</td>
</tr>
<tr>
<td>Non-CVD/cancer</td>
<td>1.49 (0.86, 2.60)</td>
</tr>
</tbody>
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VITAMIN D INSUFFICIENCY IN OLDER WOMEN: PREVALENCE AND IMPACT ON BONE DENSITY, FRACTURE RISK AND MORTALITY

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Aim: Low vitamin D status may have negative effects on health outcomes in older people. However, there are few longitudinal data in older Australian women. This study aimed to examine the prevalence of vitamin D insufficiency and its association with bone density, 10-year fracture risk and mortality in older community-dwelling Western Australian women.

Methods: The study subjects were 1383 women aged 70–85 years when recruited in 1998 from the population. After finishing a five year RCT of calcium supplementation (CAIFOS) (1), they were then recruited into a five-year epidemiology study. Baseline serum 25(OH)D concentration was determined using the LC-MS/MS method. The total hip DXA BMD was measured at year one. Clinical incident osteoporotic fractures were ascertained by adverse events diary returned to the study centre every four months and confirmed by radiographic report. Mortality data were obtained from the WA mortality registry.

Results: 400 (28.9%), 504 (36.4%) and 479 (34.6%) subjects had insufficient (serum 25(OH)D <75 nmol/L), insufficient (serum 25(OH)D 50–75 nmol/L) and ideal vitamin D status (serum 25(OH)D ≥75 nmol/L), respectively. Adjusting for baseline age, weight, calcium intake, physical activity, season and calcium treatment, subjects with vitamin D insufficiency had 3.6% lower total hip BMD compared to those with ideal vitamin D status (794±6 vs. 823±6 mg/cm², P=0.001). Vitamin D insuf-
Vitamin D is important for bone, cartilage and muscle function. However, there is little data on its association with pain. The aim of this study was to describe the association between serum 25OHD and change in knee pain over five years.

Methods: Longitudinal population-based study of randomly selected older adults \( (n=766) \). Serum 25OHD was assessed by radioimmunoassay and knee pain using the WOMAC questionnaire at baseline and again after five years. We used linear regression with adjustment for season, age, sex and BMI. We also examined potential structural mechanisms for any effect by additionally adjusting for radiographic osteoarthritis, bone marrow lesions, chondral defects and muscle strength.

Results: Participants were aged 50–80 years (mean 62 years), 50% were male with a mean WOMAC score of 3.2 (range 0–39). Mean serum vitamin D was 53.8 nmol/l (range 13–166 nmol/l), with 4.2% of participants having moderate deficiency (≤25 nmol/l). Knee pain (total WOMAC score) was stable in participants with vitamin D 25–50 and ≥50 nmol/l but worsened over five years in persons with vitamin D <25 nmol/l (b = −1.02, \( p=0.002 \)), with consistent results within each of the pain subscales. This association persisted after adjustment for covariates.

When vitamin D was analysed as a continuous measure, there were no associations between vitamin D and change in WOMAC score (b = −0.12, \( p=0.2 \)). This effect was largely independent of structural factors.

Conclusions: Serum vitamin D level in the osteomalacic range (<25 nmol/l) is an independent predictor of worsening or incident knee pain over five years suggesting a lag time between the development of low levels and pain. This suggests supplementing levels below this will prevent worsening knee pain.

101 ANNUAL HIGH-DOSE ORAL VITAMIN D3: IS THE INCREASED RISK OF FALLS ATTRIBUTABLE TO CHANGES IN MUSCLE STRENGTH?

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We have previously reported increased falls and fractures in a RCT using a single annual dose of 500,000 IU cholecalciferol (D3) administered orally for 3–5 years to 2,256 older women\(^1\). The increased rate of falling in the D3 group was higher in the first 3 months post-dosing \((p=0.017)\). Aim: To investigate if the increased falls are associated with muscle function.

Methods: Serial biochemistry and physical assessments were done on a study of 97 randomly selected participants. Serum 25-hydroxyvitamin D (25D) and muscle marker alpha antichymotrysin (ACT) were measured using immunoassay (DiaSorin) and ELISA (G Biosciences), respectively. Hip flexion muscle strength was measured using Nicholas™ Manual Muscle Tester and is reported as change at 3-month post dose, ≥2 years after baseline. The peak force (kg) required to break an isometric muscle contraction was measured as the examiner applied force against the participant (average of 3 trials/participant). Our post hoc analysis used a regression model at 3-months post-dose and included age, baseline strength and change in 25D as covariates. The analysis was stratified by whether or not the change in 25D was more than 150% of the baseline 25D.

Results: Baseline 25D was 49 nmol/L and increased to 124, 93 and 62 nmol/L in the D3 group at 1-, 3- and 12-month postdose. Increases in 25D up to 150% were associated
with progressively increased strength whereas larger increases in 25D (>150%) were associated with decreasing strength (mean strength change associated with 10 nmol/L unit increase in 25D = 0.38 kg (95% CI: 0.1, 0.7) vs. –0.26 kg (95% CI: -0.6, 0.06); <150% vs. >150% increase in 25D, respectively; heterogeneity p = 0.003). Change in ACT also suggests a threshold (p = 0.029).

Conclusion: These findings suggest a threshold effect of vitamin D status following annual high-dose D3 and are consistent with a U-shaped association reported between frailty status and 25D levels.

(1) Sanders KM, Stuart AL, Williamson EJ, et al., JAMA 2010
(2) Ensrud KE et al., JCEM 2010

102 MATERNAL VITAMIN D SUPPLEMENTATION DURING PREGNANCY PREVENTS VITAMIN D DEFICIENCY IN THE NEWBORN: A RANDOMISED CONTROLLED TRIAL

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Vitamin D (Vit D) deficiency is increasing due to lifestyle factors affecting sun exposure.

Aim: To determine if maternal Vit D supplementation during pregnancy prevents neonatal Vit D deficiency, in Vit D deficient mothers.

Methods: A randomised controlled trial was conducted over 12 months from 2008–2009 in a metropolitan Melbourne (latitude 38°S) tertiary maternity hospital antenatal clinic. 48 of 70 mothers with singleton pregnancies diagnosed with Vit D deficiency (serum 25OHD Vit D <75 nmol/l) at 12–16 weeks gestation, consented to be randomised to Vit D supplementation with 2000 IU cholecalciferol orally daily until delivery (n=23), or no supplementation (n=25). At 28 weeks, those remaining Vit D deficient on Vit D retesting in the treatment group received doubled cholecalciferol (4,000 IU) until delivery.

Results: Mean maternal 25-OH Vit D concentration at delivery was significantly higher (p<0.0001) in neonates of supplemented mothers (81 nmol/L, 95% CI 70–91 nmol/L) compared with neonates of control mothers (42 nmol/L, 95% CI 34–50 nmol/L). There was a significant positive correlation between maternal 25-OH Vit D and umbilical cord 25-OH Vit D concentrations at delivery (Spearman Rank correlation coefficient 0.88; p<0.0001). Mean supplemented maternal 25-OH Vit D concentration at delivery was significantly higher (p<0.0001) (71 nmol/L, 95% CI 62–81 nmol/L) compared with control mothers (36 nmol/L, 95% CI 29–42 nmol/L). There were no significant differences in baseline maternal 25-OH Vit D at enrolment (p=0.9) between supplemented (32 nmol/L, 95% CI 26–39 nmol/L) and control mothers (33 nmol/L, 95% CI 26–39 nmol/L).

Conclusion: Vit D supplementation of Vit D deficient pregnant women prevents neonatal Vit D deficiency.

103 TREATMENT WITH ORAL CHOLECALCIFEROL 2000 IU AND 5000 IU ON SERUM VITAMIN D, PTH, BONE TURNOVER AND MUSCLE STRENGTH IN PATIENTS WITH VITAMIN D DEFICIENCY

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Aim: To determine the optimal dose of cholecalciferol required to achieve a target serum 25(OH)D level >75 nmol/L and its relationship to both bone turnover and muscle strength.

Methods: 30 vitamin D deficient patients (serum 25(OH)D <50 nmol/L) randomly assigned to two groups – i.e., 2000 IU/day and 5000 IU/day. Collected at baseline, at 2 months and 3 months post therapy: (a) clinical demographics, (b) dietary calcium recall, (c) physical tests of muscle function, and (d) biochemistry. Statistical analysis using paired student T-test and analysis of variance (ANOVA). Regression analysis was used to determine relationship between serum 25(OH)D, PTH and bone turnover.

Results: 26 (87%) patients completed 3 months of therapy. Percentage increase in serum 25(OH)D (compared to baseline) was 82.7% in 2000 IU group and 219.5% in 5000 IU group. While all participants (100%) achieved a serum 25(OH)D concentration >75 nmol/L, only 5 subjects (45.4%) in 2000 IU group compared to 14 subjects (93.3%) in 5000 IU group achieved final 25(OH)D concentration >75 nmol/L (p<0.01). Mean serum calcium increased from 2.35+0.09 mmol/L to 2.39+0.08 mmol/L after cholecalciferol 2000 IU daily (p=0.55) and from 2.35+0.10 mmol/L to 2.38+0.08 mmol/L after cholecalciferol 5000 IU daily (p=0.55). Serum PTH levels normalised in most patients (n=19; 73.0%). In the
regression analysis, the serum PTH levels began to rise as the serum 25OHD concentrations decreased below 70–75 nmolar range. All parameters of muscle strength showed trends in improvements following the administration of both the 2000 IU and 5000 IU doses. No patient reported untoward side effects and no patient developed hypercalcaemia.

Conclusion: Treatment for 3 months with oral cholecalciferol 5000 IU daily may be more effective than 2000 IU daily in achieving optimal serum 25OHD concentrations in vitamin D deficient patients.

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GROWTH FROM BIRTH TO ADULTHOOD AND BONE MINERAL DENSITY DATA FROM THE NEW DELHI BIRTH COHORT
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We studied the relationship of height and BMI during childhood with adult bone mineral content (BMC) and areal and volumetric bone density (aBMD, vBMD) in the New Delhi Birth Cohort, India. Participants were 565 men and women aged 33–39 years, whose weight and height was recorded at birth and annually during infancy (0–2 years), childhood (2–11 years) and adolescence (11 years–adult). Lumbar spine, femoral neck and forearm BMC and aBMD were measured using DXA; lumbar spine and femoral neck vBMD were calculated. Birth length, and height and height gain during infancy, childhood and adolescence were positively correlated with adult BMC (p<0.01 all sites except birth length with femoral neck). Correlations increased with height from birth–6 years, then remained constant for later height measurements. There were no associations with vBMD. BMI at birth, and during childhood and adolescence was also positively correlated with BMC (p<0.01 for all sites). BMI at 11 years, and BMI gain in childhood and adolescence, were correlated with aBMD and vBMD (p<0.001 for all); these correlations strengthened with increasing age of BMI measurement. All associations with height and BMI in early life were attenuated after adjustment for adult height and BMI respectively. We conclude that greater skeletal growth in utero and during infancy are associated with higher peak BMC, sharing a causal pathway with attainment of adult height. Greater BMI gain in childhood and adolescence is associated with higher peak aBMD and vBMD, sharing a causal pathway with attainment of adult BMI.

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LONGITUDINAL CHANGE OF BONE GEOMETRY IN THE MID FEMUR OF GROWING CHILDREN
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Introduction & Aim: Long bones of the appendicular skeleton are often assumed to be symmetrical particularly in the mid-shaft section which is predominantly cortical bone. This region of bone may however respond asymmetrically to pubertal maturation and gender influences. In this study we tested the hypothesis that the femoral mid-shaft is growing in a symmetrical pattern along its length.

Method: Prepubertal healthy children (26 boys and 20 girls) aged 8–11 years were studied at baseline and after 26–48 months (pubertal stage Tanner 2 to 4). MRI was used to acquire serial contiguous slices (6 mm) of the entire femur. Bone geometry of the proximal two thirds, midsection (50%) and distal third were compared longitudinally across genders.

Results: Changes in total area (TA), cortical area (CA) and midshaft area (MA) were all significant (p<0.001) from baseline at the three sites (Table 1) without gender differences. When the three sites were compared and analysed separately for gender, there was no difference between enlargement of CA at proximal and distal slices for boys or girls. At the distal slice MA expansion was highly significant (p<0.0001) in both genders compared to the mid and proximal slices. In girls, there was no significant difference between proximal and mid slices for MA. The changes in TA were significantly different (p<0.001) between all sites for both genders.
Conclusion: Growth induces long bone geometrical adaptation in response to stimuli in a site specific pattern. These results confirm that the mid femoral shaft is not a symmetrical hollow cylinder. Caution should be used in interpreting results of single slice examinations (e.g., pQCT) within this section of bone.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Bone Area Change from Baseline: Mean (CI 95% lower bound, upper bound)</th>
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<tbody>
<tr>
<td></td>
<td>Total Bone area (mm$^2$)</td>
</tr>
<tr>
<td>Male</td>
<td>Females</td>
</tr>
<tr>
<td>Proximal Slice</td>
<td>127.8 (90.5,155.6)</td>
</tr>
<tr>
<td>Mid Slice</td>
<td>144.9 (116.8,173.0)</td>
</tr>
<tr>
<td>Distal Slice</td>
<td>166.6 (135.6,197.5)</td>
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that muscle function is also affected in these individuals based on several small clinical studies and reduced muscle differentiation in a limb-specific Nf1 knockout mouse. The importance of muscle contribution to the skeletal phenotype of scoliosis progression and deficient bone healing are also poorly understood. In this study we aimed to examine types of scoliosis progression and deficient bone healing are also poorly understood. In this study we aimed to examine muscle function in Nf1+/− heterozygous and Nf1MyoD−/− homozygous mice.

Methods: Grip strength testing studies and botox-induced atrophy/regeneration experiments were performed in the Nf1−/− mouse strain. Nf1MyoD−/− mice were generated by crossing the MyoD−/− mouse strain with the Nf1floxflox strain to induce a muscle-specific knockout. Phenotyping experiments were performed in cultured Nf1MyoD−/− myoblasts and examining Nf1MyoD−/− embryos and neonates.

Results: No significant decrease in muscle strength was seen in the Nf1−/− mouse. However, compared to wildtype control mice, Nf1+/− mice showed an inferior recovery following botox treatment and injected muscles exhibited evidence of extensive fibrosis. Nf1MyoD−/− mice showed severe running and were typically destroyed by mothers before postnatal d6. Weight and muscle histology were performed on early neonates and late stage embryos showing significant decreases in total muscle mass but no overt muscular dystrophy or fibrosis. Primary Nf1MyoD−/− myoblasts were examined for their ability for myogenic as well as osteogenic differentiation. Notably, Nf1MyoD−/− myoblasts showed decreased alkaline phosphatase and matrix mineralization under pro-osteogenic conditions.

Conclusions: These data show further evidence for a key role for NF1 in muscle development and/or maintenance. Further rescue experiments and studies examining the importance of NF1 muscle deficiency in the NF1 skeletal phenotype are underway.

III

RAP-011 AUGMENTS CALLUS FORMATION IN CLOSED RAT FRACTURES

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Aim: RAP-011, a fusion of the extracellular domain of the activin type IIA receptor to a murine IgG-Fc fragment, antagonizes Activin signalling. We hypothesized RAP-011 could be used to augment fracture healing.

Methods: 52 male Wistar rats underwent closed femoral fractures by three-point bending. Rats received twice-weekly subcutaneous injections of RAP-011 (10 mg/kg) or Vehicle. Endpoints were 2, 4, and 6 weeks for radiography and histology outcomes.

Results: Earlier bony union with RAP-011 was indicated by x-ray at 2 and 4 weeks. Histomorphometry indicated hastened cartilage removal, with a 49% reduction in callus percent cartilage at 2 weeks with RAP-011 ($p<0.05$). At 6 weeks, RAP-011 resulted in a superior bony callus. QCT of the fractured femora revealed increases in total bone mineral content (BMC) (31%, $p<0.01$), total bone volume (BV) (36%, $p<0.05$), and periosteal bone circumference (16%, $p<0.05$). These resulted in a 93% increase in calculated polar moment of inertia ($p<0.01$). Comparable results were seen with microCT. Callus length, by x-ray, increased by 32% ($p<0.01$), 18% ($p<0.05$), and 16% ($p<0.01$) at 2, 4, and 6 weeks respectively. RAP-011 treatment produced mild systemic effects by 6 weeks, measured in the contralateral femora by QCT. Small but statistically significant increases in total BV (8%, $p<0.05$), periosteal perimeter (4%, $p<0.05$), and predicted moment of inertia (15%, $p<0.05$) were observed.

Conclusions: These data suggest significant early and late stage affects of RAP-011 in the promotion of fracture repair. Early union, more rapid cartilage removal, increased callus length and size, and a calculated stronger callus were all promoted by RAP-011. These data suggest that Activin signalling may be a valuable pathway for targeting for orthopaedic intervention. Future studies will confirm mechanical strength, optimize dosing regimens, and test alternative surgical models.

Acknowledgements: Funding and reagents from Acceleron Pharma. ACEH-011 (the human analogue to RAP-011) currently under clinical development by Celgene Corporation.

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A GENOMEWIDE ASSOCIATION STUDY OF BONE MINERAL DENSITY: RESULTS FROM THE ODENSE ANDROGEN STUDY

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Aim: To identify genetic variants affecting peak bone mass in a cohort of young men by using genomewide genotyping
and to test association between the associated SNPs and BMD in elderly men.

Methods: We used a two-step study design comprised of a discovery cohort of 783 young men aged 20–30 and a replication cohort of 600 elderly men aged 60–76. In the discovery cohort, participants were selected on the basis of BMD of the hip; genomewide genotyping using Affymetrix 5.0 Array was performed in the 100 participants with the highest and lowest BMD, respectively. We retested the ten SNPs with the lowest p-values in the elderly cohort for association with BMD of the hip and lumbar spine as well as occurrence of potentially osteoporotic fractures.

Results: Of the ten SNPs selected from the microarray analysis, three SNPs reached significance in the replication cohort. We found rs1335858 (p = 0.001) and rs8021947 (p = 0.026) significantly associated to BMD of the hip and rs8112088 (p = 0.028) associated to BMD of the lumbar spine. The effect size of rs1335858 was in order of a change in BMD of 5.5% across genotypes explaining 1.2% of the total variance of BMD. None of the SNPs were found significantly associated with fracture occurrence although fracture frequency changed by a factor of two across genotypes for rs1335858. All three SNPs are in areas not previously connected to BMD by genomewide association studies.

Conclusions: We found three SNPs associated with BMD of the hip/lumbar spine. Rs1335858 was significantly associated with BMD of the hip after correction for multiple testing in the replication cohort and had a substantial effect size. The impact on fractures, however, did not reach significance.

113 FRACTURES AND FALLS WITH CHRONIC ANTI EPILEPTIC DRUG USE AND PATIENT AWARENESS OF THE ISSUE

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Aim: To evaluate the prevalence of fractures and falls in epilepsy patients taking antiepileptic drugs (AED) and assess their level of awareness about AED-related bone health and fracture risk.

Methods: A cross-sectional survey was conducted in epilepsy clinic outpatients and a nonepileptic comparison sample. Detailed information on their fall and fracture history was collected. Data of nonepileptic nonAED-user subjects from other studies approved by the Melbourne Health Human Research Ethic Committee who met the selection criteria were included for comparison.

Results: 150 AED-users (72 males, 78 females, median age = 39.3 years, IQR: 28.1) and 506 healthy comparison subjects (314 female, 192 male non-AED-users, median age = 41.8 years, IQR: 27.7) were studied. The prevalence of previous fractures was increased in users at vertebrae (p = 0.009), clavicle (p = 0.013) and ankle (p = 0.039). Users had significantly greater history of multiple fractures (p = 0.001) than nonusers. Within users, fracture risk increased with age (p = 0.032), longer therapy duration (p = 0.001) and polytherapy (p = 0.007). Nonseizure-related fractures (69% of cumulative fractures during therapy) occurred more than seizure-related fractures. In female users the prevalence of falls (p = 0.027) and multiple falls (p = 0.028) in the preceding year was significantly higher than in female nonusers. In all users, nonseizure-related falls were more frequent than seizure-related falls. Less than 30% of epilepsy patients were aware of the association of AED use with increased risk for fractures, decreased BMD or falls.

Conclusion: A clinical sample of patients with epilepsy taking AEDs has increased fracture risk. Those who are older, receiving longer term AED treatment and on polytherapy are at particular risk of fractures. Female AED-users have an increased prevalence of falling and of multiple falls. Patients on chronic AED therapy need information about their increased risk of falling and fractures, and strategies to minimize these major adverse effects.

114 SERUM URIC ACID IS ASSOCIATED WITH BONE LOSS AND BODY COMPOSITION IN WOMEN: A LONGITUDINAL STUDY

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Oxidative stress has been linked to osteoporosis. Serum uric acid (UA), a strong endogenous antioxidant, has been associated with higher BMD, lower bone turnover and lower prevalence of fractures in a large cross-sectional study of men. Whether this relationship is present in...
women and how UA relates to changes in BMD longitudinally has not been examined. A sample of 356 peri- and postmenopausal women, mean age 60.5 years was studied. Each individual had baseline BMD and body composition measurements by DXA and at least one repeat measure, on average 9.7 years later. Rate of change in BMD was expressed as percent gain or loss per year. UA, calcitropic hormones and bone turnover markers were measured at the final visit. Cross-sectional data analyses revealed that women with higher UA levels had significantly higher absolute BMD measures at all skeletal sites. Multiple regression analyses showed a strong association between UA and BMD at all skeletal sites at baseline and follow-up visits. Body weight and its components such as lean mass (LM) and fat mass (FM) were also significantly related to serum UA. The association between serum UA and BMD remained significant in multiple regression analyses after accounting for possible confounders including LM and FM. Regression analyses of the longitudinal BMD data demonstrated significant associations between serum UA levels and rates of change in BMD at all skeletal sites. Rates of change in body weight and LM, but not FM, were also significantly associated with serum UA levels. However after adjustment for changes in LM, associations remained significant for lumbar spine, forearm and whole body BMD but not for hip BMD. Higher serum UA levels appear to be protective for bone loss in peri- and postmenopausal women and this relationship does not appear to be explained by changes in body composition measures. This commonly measured biochemical parameter may be a useful marker of risk of osteoporosis in peri and postmenopausal women.

II5
SAFETY AND EFFICACY OF DENOSUMAB IN GIANT CELL TUMOUR OF BONE (GCTB)
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Aim: GCTB is an osteolytic tumour that contains osteoclast-like giant cells and mononuclear cells expressing RANKL. Denosumab is a fully human monoclonal antibody that binds RANKL, inhibiting osteoclast activity. We report the safety and efficacy results of a 12-month interim analysis in an open-label, phase 2 study of denosumab in GCTB.

Methods: Eligible subjects with surgically unsalvageable GCTB (cohort 1) or salvageable GCTB with planned surgery (cohort 2) received subcutaneous denosumab 120 mg 4-weekly (120 mg loading dose on days 8 and 15). The primary objective was to evaluate the safety of denosumab. Analyses also included physicians’ subjective assessments of disease progression and the proportion of cohort-2 subjects for whom surgery was delayed, reduced in scope, or not required. Safety analyses included all subjects who received denosumab; efficacy analyses included subjects who received denosumab for ≥6 months. Results: Most enrolled subjects (87%) had a Karnofsky status ≥80% at baseline and 52% had recurrent unresectable disease. AEs were reported in 126/158 subjects (80%) who received denosumab; most frequent were fatigue (15%) and headache, back pain, and extremity pain (13% each). Osteonecrosis of the jaw was reported in 3/158 subjects (1.9%). No other serious AEs were attributed to denosumab. Two subjects died on study; neither death was attributed to denosumab. Hypocalcaemia was reported in 7 subjects (4%). Based on physicians’ subjective assessment of disease status at 12 months, there was no disease progression in 72/73 evaluable cohort-1 subjects (99%). Among 23 cohort-2 subjects who had planned surgery at baseline, 15 (65%) did not undergo surgery within the first 12 months of the study; 5/8 subjects (62%) underwent less morbid surgical procedures than planned.

Conclusion: Denosumab was well tolerated in subjects with GCTB and was associated with inhibited disease progression and reduced requirements for surgery.

II6
PARATHYROID HORMONE EXCESS AND DEFICIENCY: IS PETER REALLY ROBBED OR PAUL PAID?