

## IS1

### **Neuronal control of bone mass: Leptin as a regulator, the sympathetic tone as a mediator**

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One long term goal of our laboratory is to decipher the molecular mechanisms leading to degenerative diseases of the skeleton. One means we have used to address this question is to uncover novel physiological paradigms through the combined use of classical physiology and mouse genetics. The observation that osteoporosis invariably follows gonadal failure while obesity protects from it led us to hypothesize that bone mass, body weight and reproduction are regulated by the same hormones. This hypothesis is fairly restrictive as body weight and reproduction are regulated, to a large extent, via mechanisms involving hypothalamic relays. Testing this hypothesis *in vivo* revealed to our surprise that leptin is a powerful inhibitor of bone formation. Absence of leptin signaling leads to an increase in bone formation of such amplitude that it overcomes the increase in bone resorption caused by hypogonadism. Like all its other action leptin exerts its antiosteogenic function centrally and not peripherally and leptin serum levels do control bone mass in mice and in humans. Chemical leisoning, genetic and pharmacologic manipulations all followed by leptin infusion concurred to distinguish anatomically hypothalamic neuronal networks that are targets of leptin antiosteogenic function from those that are targets of its anorexigenic function. Classical parabiosis experiments performed between *ob/ob* mice followed by leptin infusion suggested that the mediator of leptin antiosteogenic function was of neuronal nature. This result and the low sympathetic tone that characterizes mice lacking leptin signaling suggested that the sympathetic nervous system may be the mediator of leptin antiosteogenic function. This assumption was confirmed in three ways. First, mice unable of producing norepinephrine and epinephrine and that are not obese have a high bone mass phenotype; second,  $\beta_2$  adrenergic receptors are present on osteoblasts; third, restoration of sympathetic tone in leptin-deficient mice corrected their high bone mass without affecting their body weight. Consequently,  $\beta$  blockers did prevent gonadectomy induced bone loss in mice by increasing bone formation parameters. Further progress in the regulation of bone mass by the sympathetic tone will be presented at the meeting.

## IS2

### **Wnt/ $\beta$ -catenin signaling promotes osteoblast activity and bone formation**

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Wnt proteins are now recognized as one of the major families of signaling proteins in development, which mutations lead to remarkable phenotypes in mouse, *Drosophila* and *Caenorhabditis elegans*. Among functions provided by Wnt proteins are such processes as embryonic induction, the determination of cell fate, and the modulation of cell polarity. The Wnt/ $\beta$ -catenin pathway, also called the canonical Wnt pathway, is the best studied of the Wnt pathways and is highly conserved through evolution. In this pathway, Wnt signaling inhibits the degradation of  $\beta$ -catenin, which can regulate transcription of a number of genes. Three distinct classes of receptor molecules have been associated with Wnt pathway activity: the Frizzled class of G-protein coupled receptors, proteoglycans, and recently a subclass of LDL-receptor-related proteins, LRP 5 and 6. Until recently, our knowledge of the involvement of Wnt signaling in bone formation was very limited, but over the past 2 years, several lines of evidence have implicated the Wnt/ $\beta$ -catenin canonical signaling pathway in osteoblast differentiation and bone formation. First, LRP5 deficiency in humans and in mice results in deficient bone formation and a severe osteoporosis (Gong et al. 2001; Kato et al. 2002). Furthermore, a mutation in the extracellular domain of LRP5 is genetically associated with higher bone mass in humans (Little et al. 2002; Boyden et al. 2002). Interestingly, LRP5 is expressed in osteoblasts both during embryonic development and postnatally. *In vitro*, activation of the canonical Wnt/ $\beta$ -catenin signaling, directly or via BMPs induces the expression of alkaline phosphatase (ALP) in pluripotent mesenchymal cells (Rawadi et al., 2002). Several lines of evidence clearly demonstrate that the effects of BMP2 on extracellular matrix mineralization by osteoblasts are mediated, at least in part, by the induction of a Wnt

autocrine/paracrine loop. In osteoblastic cells (MC3T3-E1), antagonizing the Wnt/LRP5 pathway by addition of the natural inhibitor Dkk1 lead to a decrease in ALP activity and reduced extracellular matrix mineralization. Inhibition of GSK-3 $\beta$ , an enzyme that phosphorylates  $\beta$ -catenin, is a key event downstream of LRP5. We therefore examined whether treatment with GSK-3 $\beta$  inhibitors could improve bone mass in LRP5<sup>-/-</sup> mice. We found that this treatment markedly increased bone volume, trabecular number, osteoblast number and bone formation rate in LRP5<sup>-/-</sup> animals, demonstrating that disruption of the canonical Wnt signaling pathway is responsible for reduced bone mass in Lrp5<sup>-/-</sup> mice. Enhancing the canonical Wnt signaling pathway by inhibiting GSK-3 $\beta$  could therefore be useful for treating low bone mass disorders.

### IS3

#### GENETIC FACTORS AND OSTEOPOROSIS

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Genetic factors play a major role in osteoporosis. Twin and family studies have shown that bone mineral density is highly heritable (60-80%), as are other key risk factors for osteoporotic fractures such as bone ultrasound, femoral neck geometry, bone turnover, vit D levels, muscle strength, age at menopause. Susceptibility to osteoporosis is mediated by the effects of many genes, although some diseases related to osteoporosis can rarely occur as the result of mutations in a single gene such as osteogenesis imperfecta. Many approaches are being pursued to identify the genes responsible including linkage studies in man and experimental animals and candidate gene studies. Linkage studies have identified multiple quantitative trait loci (QTL) for regulation of BMD and have indicated that the effects of these loci are site dependent and sex-specific. For the most part, the genes responsible for BMD regulation in these QTL have not been identified but allelic variations in at least two positional candidate genes BMP2 and TNFRSF1B have been identified that explain some of the linkage signal in QTL on chromosomes 20p12 and 1p36.

Most studies on the genetics of osteoporosis have used the candidate gene approach. The vitamin D receptor gene (VDR), the collagen type I alpha I gene (COL1A1) and oestrogen receptor gene alpha (ER) have been most widely studied. There is evidence to suggest that allelic variation in all three genes plays a role in regulating BMD, but the effects are modest and together, probably account for less than 5% of the heritable contribution to BMD. Genes may influence only certain phenotypes and not all genes that influence BMD will be important in fracture. Understanding the genetic determinants of osteoporosis has major clinical significance leading to novel molecular targets for drug design and diagnostic tests.

### IS4

#### FGF signalling in skeletal development

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Fibroblast growth factors (FGF) and their receptors (FGFR) play essential roles in skeletal development and growth. Activating mutations in the genes encoding FGFR1, FGFR2 and FGFR3 cause skeletal abnormalities including dwarfism, which involves altered FGFR3 function in long bone growth, and craniosynostosis, in which the skull sutures undergo premature bony fusion. Craniosynostosis affecting the coronal suture results from specific mutations of all three receptors, of which the roles of FGFR1 and FGFR2 are best understood. Elucidation of the mutations underlying the human defects has stimulated research using mouse models to establish the developmental link between genotype and phenotype. In mouse skull vault development, *Fgfr2* is expressed in preosteoblasts and *Fgfr1* is expressed in differentiating osteoblasts. Increased signalling by insertion of an FGF-soaked bead onto the fetal coronal suture causes down-regulation of *Fgfr2* and loss of proliferation, and up-regulation of *Fgfr1* and bone differentiation genes. This response mimics the effect of activating mutations of *Fgfr2* that enable ligand-independent dimerisation of the receptors, resulting in premature fusion of the coronal suture. The mechanism involves secretion of FGF ligands from the differentiating osteoblasts; these cells are in a relatively high FGF-concentration environment, in contrast to the low FGF concentration at the preostoblast-rich bone margin. Growth proceeds at an orderly rate as the newly differentiating cells secrete

FGF, converting the nearest preosteoblasts to osteoblasts, while the more peripheral preosteoblasts maintain growth at the margins of the osteogenic domain. Activating mutations of either *Fgfr1* or *Fgfr2* have the same effect on sutural growth, since both favour differentiation over proliferation leading to synostosis. Similarly, both loss- and gain-of-function mutations of *Fgfr2* cause coronal synostosis. The transcription factors ALX4 and MSX2 also affect the rate of calvarial bone differentiation, and appear to interact with *Fgfr1* and *Fgfr2* downstream of *Runx2*. Skeletal patterning in the skull vault, in which the sites of future sutures and hence FGFR signalling are established, involves the formation of a distinct boundary between skeletogenic neural crest and mesoderm very early in embryogenesis.

## IS5

### THE IMMUNE SYSTEM AND BONE REMODELLING

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In determining the fundamental mechanisms of bone formation and resorption, most attention has been paid to the role of either the osteoblast or osteoclast. It is well accepted that communication networks exist between these cells that are pivotal to the formation and activation of the osteoclast, and that the osteoclast also modulates osteoblast behaviour. However, studies into the action of cells of the immune system, particularly T, B and NK cells, to modulate the activity of either bone formation or resorption are in their infancy. These cells powerfully influence the growth and development of bone cells through the action of lymphocyte-derived cytokines. Several T cell-derived cytokines including IFN- $\gamma$ , IL-4, IL-10, IL-13, OCIL, sFRPs and GM-CSF act upon the osteoblast or directly upon osteoclast precursors to inhibit osteoclast formation, whilst RANKL, IL-6, IL-7 and IL-17 act to stimulate this process.

The primary role of activated T lymphocytes in inflammatory diseases such as arthritis to influence bone destruction through their production of TNF $\alpha$  and soluble RANKL is now accepted. Such a mechanism is also likely in the pathology of periodontal disease.

The capacity of naive T cells to influence bone remodeling is now emerging. Using genetically altered mice, in which targeted cellular populations can be specifically addressed *ex vivo*, we have demonstrated that IL-12 and IL-18 can inhibit osteoclast formation as a result of their actions upon T cells. Histo-morphometric analyses of IL-12, IL-18, and IL-12+IL-18 null mice reveal that these interleukins do indeed perturb normal bone architecture. Further, the actions of IL-18 were not restricted to impacting upon osteoclast formation, but also modulate osteoblast function.

More recently, the repertoire of factors that act through T cells to modulate osteoclast formation and or activity has been extended and includes IL-4 and estrogen. Each has previously been attributed to have direct actions upon osteoblasts or osteoclast progenitors, but clearly have the capacity to modulate bone turnover through a lymphocyte-dependent process.

Future studies are aimed to decipher the complex and overlapping effects of the many immune and hemopoietic cytokines, as well as determining the role that immune cells play in normal bone remodelling and in pathological conditions.

## IS6

### MOUSE MODELS TO STUDY INTERACTIONS BETWEEN THE IMMUNE SYSTEM AND BONE *IN VIVO*

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Functional interdependence between the immune system and bone is reflected in a number of regulatory molecules acting on the cells of both systems and common precursors for bone and immune cells, so that the disturbances of the immune system may affect bone metabolism, and vice versa. Our group is particularly interested in the interaction between the two systems *in vivo* and uses models of new bone formation in adult mice, such as endochondral osteogenesis induced by subcutaneous implantation of rhBMP-2 and intramembranous ossification induced by bone marrow ablation. Our results so far indicate that the immune system is particularly important for the creation of a restrictive immunological milieu at the site of new bone induction. For example, mice without functional B lymphocytes

(microMT gene knockout) showed greater cellular recruitment and proliferation but normal differentiation during endochondral osteogenesis. Also, acute removal of T lymphocytes enhanced new cartilage and bone formation *in vivo*, and stimulated osteoclast differentiation *ex vivo*. We also showed that apoptosis mediated by the Fas-Fas ligand system played an important part in new bone formation *in vivo*. Gld mice, which have a mutated, non-functional Fas ligand and subsequent lymphoproliferative disease, had greater whole body bone mineral density and greater trabecular bone volume than their wild-type controls. They also lost 5-fold less trabecular bone and had less osteoclasts on bone surfaces after ovariectomy-induced bone resorption. Moreover, they formed more bone in a model of intramembranous osteogenesis after bone marrow ablation, had less osteoclasts on bone surfaces and less apoptotic osteoblasts. Bone diaphyseal shafts and bone marrow stromal fibroblasts produced more osteoprotegerin (OPG) mRNA and protein than wild-type mice. These findings provide evidence that the disturbance of the bone system is a part of generalized lymphoproliferative syndrome and indicates the possible role of OPG as common regulatory in the immune system and bone. Although it is difficult to discern the role of individual cells or cytokines in *in vivo* models, they seem the best way to study the full complexity of the interactions between bone and immune system.

## IS7

### **Osteoblastic bone metastases**

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Certain solid tumors, such as prostate cancer, metastasize preferentially to bone, where they locally disrupt normal bone remodeling. Such metastases are classified as either osteolytic or osteoblastic based on the radiographic appearance of the lesion, but it is clear that bone resorption and bone formation are dysregulated in both types of metastatic disease. Recent clinical evidence indicates that both processes contribute to the metastatic phenotype, even in the same patient. We recently demonstrated that the vasoactive peptide, ET-1, stimulates the new bone formation associated with osteoblastic metastases. The breast cancer lines ZR-75-1, T47D and MCF-7 cause osteoblastic metastases in mice, and all three secrete ET-1. We showed that: 1) tumor-produced ET-1 stimulated new bone formation *in vitro* and osteoblastic metastases *in vivo* via ETA receptors; 2) ETA receptor blockade significantly reduced osteoblastic bone metastases and tumor burden in bone. Gene microarray analysis on RNA from mouse calvariae treated with ET-1 for 24 h showed significantly increased expression of *wnt5a*, *twist2*, and TGF $\beta$ -regulated genes. The mRNAs for the secreted proteins TIMP-3, PAI-1 and *cyr61/CCN1* were also increased. Thus, tumor-produced ET-1 has a major role in osteoblastic bone metastases by stimulating osteoblast proliferation and new bone formation. These effects may be mediated by modulation of *wnt*, *twist*, and TGF $\beta$  signaling pathways in osteoblasts and by enhanced secretion of active factors into the bone microenvironment.

Adrenomedullin is another tumor-produced factor with properties similar to ET-1. This 52 amino acid vasoactive peptide stimulated osteoblast activity and new bone formation and enhanced both osteolytic and osteoblastic metastases when expressed in lung A549 and prostate PC3 cells.

These experiments show that human tumor cells expressing multiple factors with opposing actions on bone cause complex metastatic responses in an animal model. Tumors in patients often express multiple bone-active factors, so an understanding of the effects of simultaneous multi-factorial expression is relevant to the clinical goal of treating and preventing metastatic bone disease.

## IS8

### **Bisphosphonates and the treatment of tumour-induced bone disease**

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In recent years the treatment of bone metastases by radiotherapy and systemic endocrine and cytotoxic drugs has been supplemented by the co-administration of bisphosphonates. These drugs are potent inhibitors of tumour-induced bone resorption that can relieve metastatic bone pain and improve the structural integrity of bone. Zoledronic acid and ibandronate are the most potent agents available and prevent 40-50% of the expected skeletal morbidity from advanced breast cancer. Zoledronic acid also significantly reduces the

risk of a skeletal complication in endocrine resistant prostate cancer (hazard ratio [HR] 0.64) and in a broad range of solid tumours other than breast and prostate cancers (HR 0.69). Bisphosphonates should now be considered for any patient with symptomatic bone metastases, especially when bone is the dominant site of metastasis. For economic reasons selection or prioritisation of patients for bisphosphonates may be necessary. It is now clear that the risk of a skeletal complication is related to the rate of bone resorption. Attention is now turning to the development of more rational treatment schedules using biochemical markers of bone metabolism to guide treatment in individual patients.

Animal studies and two clinical trials of oral clodronate have indicated that the adjuvant use of bisphosphonates may sufficiently alter the bone micro-environment to prevent metastasis in bone. Large (n= >3,000) randomised adjuvant trials are ongoing to evaluate the role of either clodronate or zoledronic acid in the adjuvant treatment of operable breast cancer.

Finally, bisphosphonates provide a safe and simple treatment for the prevention and reversal of cancer-treatment induced bone loss. This is an increasingly important complication of cancer treatment given the long survival that many patients now experience. Efficacy in the setting of androgen deprivation therapy for prostate cancer and following a chemotherapy-induced menopause in early breast cancer has been demonstrated. Currently the efficacy of bisphosphonates in the context of aromatase inhibitor use for breast cancer is the subject of several ongoing trials.

## **IS9**

### **Monitoring treatment response in osteoporosis**

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The objective of therapeutic monitoring in osteoporosis is to provide clinicians and patients with information that allows advantageous decisions to be made in individuals. There is considerable interest in the relationship between change in surrogates such as bone mineral density (BMD) or bone turnover markers (BTM) and fracture risk reduction. Within-trial surrogacy for apparent BMD change is generally weak (explaining less than a quarter of fracture risk reduction), but change in some BTMs seem more predictive in several studies. This may imply that change in bone turnover in response to therapy is of greater biological relevance than change in BMD. However measurement 'noise' can complicate the ability to compare measurements. For BMD, weak individual-level surrogacy and stronger trial-level surrogacy, might also be due to the impact of measurement error. Statistical approach and study design are important. Freedman and others have suggested statistical methods to evaluate the potential use of surrogates to replace the primary endpoint in therapeutic trials. The 'percentage of trial effect' explained by a surrogate is not directly related to prediction of response in individuals prescribed therapy. 'Percent explained' generally has a large confidence interval and it is difficult to compare different surrogates. Patient-eligibility criteria for studies designed to investigate the efficacy of drug therapy also differ from those which are appropriate to investigate the usefulness of a monitoring test. An intention-to-treat comparison with placebo is not necessarily optimal to determine the clinical usefulness of a monitoring test.

Utility of a test depends not only on predictive performance in clinical trials, but also on the exact way the test is to be interpreted and used in individual patients. The best threshold or percentage change in BTM is uncertain. The quantitative relationship between treatment effect on the surrogate and true endpoints will differ for different treatments. The demonstration that change in BMD or BTM predicts risk reduction does not in itself imply clinical utility. This depends on 1) the gain in information due to the test after prior information is considered, 2) the frequency of results deemed to require action, 3) the frequency of incorrect decisions, 4) the cost of the test, 5) the feasibility of altering management and 6) the consequences of decisions. The number of patients needed to investigate (NNI) to prevent one event should be defined. It does seem likely that BTM will be more useful than BMD to monitor therapy in osteoporosis. Before widespread application of monitoring rules it is important to try to estimate the effect on patient care.

## **IS10**

No Abstract

## IS11

### INITIAL EXPERIENCE WITH TERIPARATIDE (FORTEO) IN THE UNITED STATES

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Forteo was approved by the US Food & Drug Administration in November 2002. It is indicated for the treatment of postmenopausal women with osteoporosis, and for men with hypogonadal or idiopathic osteoporosis, who are deemed by their physicians to be at high risk for fracture. This presentation describes the first year commercialization experience for Forteo in the US. Forteo was introduced in a staged fashion, initially emphasizing physicians traditionally associated with osteoporosis: endocrinologists, rheumatologists, and a select group of internal medicine specialists. Focus has recently expanded to include broader groups. As of December 2003, approximately 45,000 patients had initiated Forteo. Forteo patients are 88% female, averaging 72 years in age. 70% of patients have experienced one or more prior fractures and there is a high prevalence of chronic disease co-morbidity. Insurance coverage for Forteo has been gratifying. 81% of patients seeking access to the drug have received it. To assist physicians with patient education, Lilly has created several programs. 'Managing Your Osteoporosis' is a group activity conducted by nurse educators. Patients receive individualized attention to injection technique and the osteoporosis disease state, as well as specific information regarding Forteo. Forteo Customer Care is available to patients who call a toll-free number with questions related to Forteo, the pen delivery device, and reimbursement. The Lilly Answer Center is another venue through which patients and health care providers can reach Lilly for assistance or to report adverse events. To date the great majority of calls have been requests for assistance with the pen. Forteo has been well-tolerated, with an adverse experience profile similar to that observed in clinical trials and listed in the product label. Hypercalcemia has been infrequently reported; it generally has been mild and responsive to reducing calcium supplements. Serious hypercalcemia (>13 mg/dl) has been reported in a handful of patients, most of whom either had baseline conditions known to be associated with hypercalcemia or developed such conditions while on treatment.

## IS12

### STRONTIUM RANELATE IN THE TREATMENT OF POSTMENOPAUSAL OSTEOPOROSIS

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Strontium (Sr) is a natural bone-seeking trace element which is incorporated into bone. In the 50's preliminary open therapeutic studies of Sr salts have suggested that Sr may have potential benefits for osteoporotic patients. These results incited Servier chemists to synthesize a new divalent salt of Sr, strontium ranelate (SR), composed of an organic moiety (ranelic acid) and of two atoms of stable Sr. *In vitro* SR appeared capable to increase proteins synthesis in osteoblasts cultures, to enhance preosteoblastic cell replication, and also to inhibit osteoclastic resorption. *In vivo* SR was shown to induce an uncoupling of bone remodeling in several animal models, stimulating bone formation and reducing resorption.

Results from a 2 year controlled phase II dose-response study in osteoporotic women suggested that ingestion of 2g. a day of SR increased bone mineral density (BMD) and reduced the incidence of new vertebral fractures (VF). A large phase III program was designed in women with severe postmenopausal osteoporosis and included two trials: one assessing the effects of 2 g. a day of SR on the risk of new VF (SOTI trial;1649 women), and one evaluating the effects on the risk of non spinal fractures (TROPOS trial;5091 women). A main statistical analysis was performed after 3 years. In SOTI, new VF occurred in fewer patients in the SR group than in the placebo group, with a risk reduction of 49% in the first year of treatment and 41% during the 3 year study period. SR increased lumbar spine BMD at month 36 by 14.4%. TROPOS study showed a significant 16% reduction in the relative risk of a first non spinal fracture in the group treated for 3 years with SR compared to placebo group (intention-to-treat population). A 41% reduction in the relative risk of hip fracture was demonstrated in the compliers' population. In both studies there was no significant differences between the groups in the incidence of serious adverse effects.

Although further studies are needed to elucidate the mechanisms behind the cellular effects of SR on bone formation and bone resorption, this compound already represents an effective and safe novel therapy of postmenopausal osteoporosis.

## OC1

### **INFLUENCE OF LRP5 GENE POLYMORPHISMS ON THE NORMAL VARIATION OF BONE MINERAL DENSITY**

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**Background:** Osteoporosis is a prevalent, highly heritable condition wherein genetic factors account for 50-80% of the inter-individual variation of BMD. Studies based upon cohorts with rare bone phenotypes characterised by extremely low or high BMD show the LRP5 gene is an important genetic modulator of BMD. We examined the frequency and influence of LRP5 polymorphisms within the normal population and report a role of LRP5 in the determination of BMD and development of osteoporosis in a normal population.

**Methods:** Case-control and family-based approaches were used to examine the role of the LRP5 gene in determining normal population variation of BMD. To assess the the entire BMD spectrum, 152 osteoporotic probands, their families (597 individuals) and 160 post-menopausal women with elevated BMD (T > 2.5) were recruited and lumbar spine, femoral neck and hip BMD measured. Polymorphisms detected by dHPLC and PAGE were genotyped using mass spectrometry and RFLPs. Within-family association studies were performed by QTDT and linkage analysis by SOLAR. Case-control comparisons of genotype and haplotype frequencies between groups with divergent BMD were performed by contingency-table analysis.

**Results:** 8 single nucleotide polymorphisms (SNPs) with allele frequencies of >5% were found in exons 8, 9, 10, 15, 18 and in introns 6, 7 and 21. Family-based association studies revealed the C171346A SNP in intron 21 was associated with hip BMD (p < 1 x 10<sup>-5</sup> in men, p=0.0019 in men and women). This association was confirmed in comparisons of osteoporotic probands and women with elevated BMD (p=0.03). Polymorphisms in exons 8 (C135242T, p=0.007) and 9 (C141759T, p=0.02) were also associated with BMD. Haplotypes composed of the SNPs G121513A, C135242T, G138351A and C141759T were associated with BMD when comparing osteoporotic probands and high BMD cases (p<0.003). The C165215T SNP in exon 18 was linked to lumbar spine, femoral neck and total hip BMD (parametric LOD scores = 2.8, 2.5 and 2.2 and non-parametric LOD scores = 0.3, 1.1 and 2.2 respectively). Lastly, linkage disequilibrium was present between the polymorphisms in exons 10, 15 and 18.

**Conclusion:** These results show that LRP5 polymorphisms are common and contribute to the determination of BMD in the general adult population. In particular, male BMD is strongly influenced by LRP5 gene polymorphisms.

## OC2

### **RAPID INHIBITORY EFFECTS OF GLUCOCORTICOIDS ON ERK ACTIVITY AND OSTEOBLAST PROLIFERATION OCCUR VIA TRANSCRIPTIONAL UPREGULATION OF MKP-1**

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Glucocorticoid (GC)-induced osteoporosis is characterised by decreased numbers of functional osteoblasts. We have found ERK activity to be central to serum-induced osteoblast proliferation *in vitro*, since inhibition of MEK activity by U0126 completely abolished both normal activation of ERK and proliferation in response to either serum or TPA. Dexamethasone (Dex) rapidly (<2hr) inhibits ERK activation, primarily affecting the sustained

phase of ERK activation required for nuclear shift and mitogenesis. This inhibition is reversed by co-treatment with the protein synthesis inhibitor, cycloheximide, and also by the glucocorticoid receptor antagonist, RU486, suggesting a classical GR-dependent, transcriptional mechanism. Furthermore, the negative effects of Dex on ERK activation are also reversed by the protein tyrosine phosphatase inhibitor, vanadate. Coupled with the rapidity of Dex action, this indicates immediate-early gene phosphatase involvement, and we therefore used quantitative, real-time pcr to examine expression profiles of the rapidly inducible dual-specificity phosphatases, MKP-1 and MKP-3. MKP-1 expression was 10-fold upregulated in both mouse and human osteoblast cell lines as rapidly as 30 min after Dex treatment and remained elevated for up to 24hr. MKP-3 was strongly down-regulated. MKP-1 protein was also markedly upregulated following 2-8 hr of Dex treatment, and this correlated precisely with dephosphorylation of ERK. At the cellular level, osteoblast proliferation was negatively affected by Dex treatment and this was reversed by both RU486 and vanadate. Overexpression of MKP-1 in MG63 osteoblasts blocked ERK activation and proliferative response to mitogens. Gene silencing of GC-induced MKP-1 using siRNA reversed the negative effect of GC treatment on ERK activity. Furthermore, MKP-1 was upregulated in wbc's of COPD patients undergoing high-dose methylprednisolone treatment for 28 days. We therefore propose MKP-1 upregulation as a novel and rapid mechanism accounting for the direct negative effects of glucocorticoids on osteoblasts.

### OC3

#### **PRENATAL GLUCOCORTICOID EXPOSURE RETARDS BONE GROWTH IN MICE AND IS ASSOCIATED WITH RAISED SERUM IGF-1**

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Between 3-10% of all live neonates are born small-for-gestational (SGA). While most SGA neonates normalise their stature, up to 15% do not experience catch up growth by 2 years. The underlying pathophysiology of this process of intrauterine growth retardation (IUGR) and the failure of catch-up growth is unclear. Poor growth in utero is associated with a failure to thrive after birth with an increased predisposition towards developing several chronic diseases in adulthood. SGA infants have higher levels of endogenous glucocorticoids (GC) at birth and GC retard growth prenatally in many mammals. The human model of IUGR leading to SGA offspring can be mimicked by administering exogenous GC to pregnant mice. The aims of this study were to determine the weights, crown rump lengths (CRL) and serum IGF-I, Insulin and IGFBP-2 levels on 1-day-old mice exposed to GC prenatally. Pregnant mice were exposed to dexamethasone (dex) (100 micrograms/kg/d) for the last 6 days of pregnancy. Controls received vehicle injections. The CRL and body weight were determined in 1-day-old mice (n=90). A sub-set of these mice (control:dex, 1:1) were injected with bromodeoxyuridine 1 hour before sacrifice, when blood was sampled and tibial length determined. Histological sections of tibiae were assessed for growth plate width and the chondrocyte proliferation rate. Dex treatment significantly decreased both body weight (11%; p<0.001) and CRL (7%; p<0.001). Female mice were more severely affected; body weight 16% decrease: (males 7%) and CRL 9% decrease (males 6%). There was a strong relationship between body weight and tibial length (r = 0.7, p<0.001). Median (10, 90 centile) serum IGF-I and IGFBP-2 in the dex treated groups were higher at 291ng/ml (282, 297) and 3190ng/ml (3001, 3204) (p<0.05) than the control group; serum insulin was unaltered. A significant negative association was found between serum IGF-I and CRL (r = 0.5, p<0.03). There were no significant differences in measured growth plate parameters. In conclusion, prenatal GC exposure reduces birth weight and length; this effect is more marked in the female offspring and is associated with raised IGF-I and IGFBP-2 levels raising the possibility of a state of IGF-I insensitivity.

## OC4

### ACTIVATORS OF PPAR ALPHA AND DELTA INCREASE BONE DENSITY IN INTACT MALE RATS

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A number of substances, in particular prostaglandin E2 (PGE2), are known which stimulate bone formation when administered to adult animals. *In vivo*, PGE2 is rapidly degraded, to PGA2, and we have previously reported that some of the bone anabolic effects of PGE2 may be caused by PGA2 (Still and Scutt, Prostaglandins and other lipid mediators, 65:21-31, 2001). PGA2 is known to bind to the family of PPAR nuclear receptors. Therefore, we investigated the effects of a number of PPAR agonists on colony formation using the CFU-f assay.

In these studies, PPAR alpha and/or delta agonists (Bezafibrate, Fenofibrate and linoleic acid) caused a dose-dependent increase in osteoblastic colony number, achieving numbers similar to, or greater than that of PGE2. PPAR gamma agonists (ciglitazone, 15-d PGJ2) had no effect on osteoblastic colony number.

The effect of these drugs was examined *in vivo*. Briefly, Wistar rats were injected daily with linoleic acid, Bezafibrate or Fenofibrate for 12 weeks. Metaphyseal bone mineral density was increased in all groups compared to the vehicle. Linoleic acid (0.3 mg/kg/d) and Bezafibrate (1 mg/kg/d) increased BMD by approximately 8%. Fenofibrate (1 mg/kg/d) increased BMD by approximately 11%.

Histomorphometric analysis at the proximal metaphysis shows an increase in bone volume, trabecular number and trabecular thickness. Further, macroscopic examination of the bones, showed a persistence of trabecular elements along the diaphysis. In a separate study, serum osteocalcin increased by 19%, 29% and 9% after 6 weeks of treatment with linoleic acid, Bezafibrate and Fenofibrate respectively.

In conclusion, PPAR alpha/delta agonists increase osteogenesis *in vitro* and increase bone mineral density *in vivo* suggesting that PPARs play a role in bone formation.

## OC5

### OSTEOBLAST FUNCTION IS INHIBITED COOPERATIVELY BY ACIDOSIS AND HYPOXIA

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Several lines of evidence indicate that osteoblast (OB) function is sensitive to extracellular pH. Bone formation by OB *in vivo* normally occurs adjacent to blood vessels, suggesting an important role for oxygen. We investigated the effects of pH and PO<sub>2</sub> on OB using a number of quantitative and qualitative methods. OB were harvested from neonatal rat calvariae by trypsin/collagenase digestion and cultured up to 35d in DMEM /10% FCS with 0.05 mg/ml ascorbate, 2 mM beta-glycerophosphate and 10 nM dexamethasone. pH was manipulated by addition of 5-25 mmol/l HCl, and PO<sub>2</sub> was varied between 20% - 0.2% O<sub>2</sub> (balance N<sub>2</sub>); 5% CO<sub>2</sub> was present in all experiments. We found that cell proliferation rate, assessed by <sup>3</sup>H-thymidine incorporation, decreased up to ~12 fold from d1 to d15 but was unaffected by pH reduction from 7.4 to 6.9 between days 3-15. The same pH reduction caused little or no decrease in collagen production, as assessed by <sup>3</sup>H-proline incorporation. Abundant matrix-containing nodules were formed in OB cultures at pH 6.9 but mineralisation of nodules was almost completely abolished, compared with pH 7.4. These results suggest that low pH causes an 'osteomalacic' condition due to increased Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> solubility and/or decreased alkaline phosphatase activity. In contrast, reducing PO<sub>2</sub> from 20% to 2% caused a 90% reduction in the area of bone nodules formed by OB after 35d but mineralisation itself did not appear to be affected. The inhibition of nodule formation in 2% O<sub>2</sub> was partly accounted for by reduction in cell proliferation (eg 70% after 5d). TEM studies suggested that collagen fibril organisation was impaired in 2% O<sub>2</sub>. In 0.2% O<sub>2</sub>, nodule formation was completely abolished. Tissue hypoxia is usually accompanied by acidosis. Thus, our results suggest that hypoxia and acidosis exert a powerful, cooperative inhibitory action on bone formation by reducing OB proliferation and the production plus subsequent mineralisation of organised collagenous matrix. These effects will be compounded *in vivo* by the strong stimulatory action

of hypoxia and acidosis on osteoclast formation and activity. Our findings emphasise the key role of the vasculature in bone.

## OC6

### CHARACTERISATION OF OSTEOCRIN, A NOVEL BONE-SPECIFIC PROTEIN, IN HUMAN TISSUE

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A recently identified novel bone-active molecule, osteocrin, which shares no homology with any known proteins, has been shown to be specifically expressed in cells of the osteoblast lineage. To further characterise this protein in human tissue we have used immunohistochemistry and image analysis in both *in vitro* and *in vivo* studies to correlate expression with osteoblast development and skeletal physiology.

Intense osteocrin expression was seen in primary human osteoblasts cultured for 2, 3 and 6 days in proliferating medium. Similar cultures in differentiating medium containing 200 nM hydrocortisone showed a 1.5-, 2.3- and 3.1-fold down-regulation ( $p < 0.05$ ) of osteocrin expression respectively at the same time points. In contrast, alkaline phosphatase expression in the same cultures increased with osteoblast differentiation and was elevated by hydrocortisone treatment at all time points. Control cultured dermal fibroblasts were negative.

High osteocrin expression was seen in sections from developing human neonatal rib bone, with intense immunoreactivity in osteoblasts on bone forming surfaces, in newly incorporated osteocytes and in some late hypertrophic chondrocytes. Interestingly, the primary spongiosa was generally less immunoreactive than the more mature bone distal to the growth plate. Osteocrin co-localised with osteocalcin in some osteoblasts but with less prolonged expression. In sections from adult human iliac crest bone biopsies, osteocrin expression was specifically localised to osteoblasts and young osteocytes at bone forming sites. The most pronounced immunoreactivity was seen in osteoblasts and osteocytes on the periosteal edge of the cortex and in Haversian systems within the cortex. Quantitative comparison between women treated with long-term, high-dose estradiol with known increased bone formation and untreated age matched post menopausal women demonstrated a significant increase in extent and intensity of osteocrin staining in the oestrogen-treated women at both cancellous (1.8-fold) and cortical (3.6-fold), ( $p < 0.05$ ) bone remodelling sites. Both pre-immune serum and sections of colon showed absence of staining.

These results demonstrate that osteocrin is specifically expressed by young active osteoblasts, particularly in developing bone and at sites of bone remodelling suggesting a role in bone formation. The down-regulation of osteocrin by hydrocortisone suggests that synthesis is linked to differentiation and suggests a role for osteocrin in the modulation of the mature osteoblast phenotype.

## OC7

### A NOVEL DUAL FLUORESCENCE ASSAY SHOWS THE PREFERENTIAL ADHERENCE OF OSTEOBLASTS TO RESORPTION SURFACES

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The mechanisms that control the targeting of osteoblasts to freshly degraded regions of bone are still mostly unknown. However, differences in the topography and molecular chemistry of resorbed versus unresorbed bone probably play a role in osteoblast adhesion and chemotaxis. We have developed an *in vitro* model that allows the simultaneous visualisation of osteoblasts and resorption pits and used this system to test whether osteoblasts preferentially adhere to resorbed bone surfaces.

Osteoclasts were isolated from the long bones of rabbits and osteoblasts from neonatal mouse calvaria. The osteoblasts were labelled with the viable fluorescent dye Cell Tracker Red. Dentin slices were labelled with the green fluorescent dye FluorX, and rabbit osteoclasts were cultured on these slices for 5-6 days. Next the osteoclasts were removed by sonication and Cell Tracker Red labelled osteoblasts seeded onto the slices. The osteoblasts were allowed to adhere for one hour, and after this any non-adherent cells were removed by vigorous washing in PBS. Both dentin slices and osteoblasts were visualized using

fluorescence microscopy, and images captured using a Progress C14 cooled CCD camera coupled to an image analysis workstation. Resorbed regions of dentin appeared as dark pits surrounded by green fluorescence and osteoblasts as small, red round objects. We developed an image analysis program that identifies resorption areas, and determines the position of osteoblasts relative to these resorption areas. We found that the cell density on resorbed areas was significantly higher (280% ?51%) than that on non-resorbed dentin.

In conclusion, osteoblasts preferentially adhere to resorbed dentin. This result is consistent with studies conducted on biomaterials that show osteoblast adherence depends on the chemistry and topography of a surface. Our newly developed dual fluorescence osteoblast adhesion assay could be used in future for determining the factors that control osteoblast adhesion to bone surfaces.

## OC8

### **MID FEMORAL NECK CORTICAL MORPHOLOGY CHANGES WITH AGE**

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In NHANES 3, Beck and Looker found 85% preservation of femoral neck (FN) bending resistance (calculated as section modulus) over 5 decades. The purpose of this study was to identify other age-related changes in the morphology of the mid cross-section of the FN, with implications for its capacity to resist fracture. Measurements were taken from peripheral quantitative computed tomogram (pQCT) images of a group of 81 Australian cadaveric femurs (35 F, aged 20 - 95, and 41 M, 19 to 84). All had died suddenly. The mid FN cross-section was segmented radially into eight regions and the cortical bone thickness (CT) and density (D) measured.

Gender differences were unimpressive:  $p = 0.14$ . In 6/8 regions there were considerable differences in CT between the young under fifty, (Un50,  $n=13$  F; 12 M) and the old, ( $p = 0.0001$  to  $0.03$ ). These effects were substantially attributable to CT increases with age inferiorly (+ 29%  $p = 0.000$ ) counterbalanced by anterior and posterior losses of 24% and 19% ( $p = 0.0001$ ). Furthermore the volumetric density of the bone tissue in these regions declined by 16% attributed to increased porosity. The effects of all these changes were to move the centroid medially away from the superoposterior cortex which in principle might have an adverse affect on stability.

According to standard concepts of mechanical stability CT uniformity, seen in the young, optimises fracture resistance to overloading from unusually loaded directions. Ageing was associated with a thickening of the inferior cortex and thinning of the cortex elsewhere. This local thinning effect was greatest where the loading forces experienced in stance are minimised, that is in the FN mid cross-section's neutral bending axis (anteriorly and posteriorly) and also superiorly. Increasing cross-section asymmetry will produce deterioration in the FN's capacity to take a load from a non-stance direction. We hypothesize that, without much changing its bending resistance in stance, ageing increases hip fragility and so fracture risk through the redistribution of bone tissue within the femoral neck cortex.

## OC9

### **IS DIVERSITY MORE IMPORTANT THAN QUANTITY OF PHYSICAL ACTIVITY IN MAINTAINING THE FRACTURE RESISTANCE OF THE PROXIMAL FEMUR?**

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Hip fractures occur as a direct consequence of cortical bone instability. Biomechanical principles suggest that larger bones would have greater bending resistance as measured by the section modulus (Z), which increases in direct proportion to the bone's diameter. However, structural stability, least for the lateral/superior cortex, and measured as critical buckling stress is compromised if the bone's cortices are either thinner or located further away (d-lat) from the section's centre of gravity. We hypothesised that aging, anthropometry and physical activity may conserve Z through distribution of bone tissue, to result in an optimal placement of the bone's centre of gravity for Z rather than cortical stability.

Hip structural analysis software was used to derive structural measurements from DXA scans on 1,359 men and women in the EPIC-Norfolk population-based prospective study. Up to 4

repeat DXA scans were done in 8 years of follow-up: 2 scans(n=954, 2.9y); 3 scans(n=722, 5.4y) and 4 scans(n=78, 7.5y). Weight, height and activities of daily living (ADL) were assessed on each occasion. A standard physical activity and lifestyle questionnaire was administered at baseline. The distance from the lateral bone surface to the centroid was measured on three narrow cross-sections; narrow neck(NN), intertrochanter(IT) and shaft(S). A linear mixed model was used to assess associations with predictors.

There were significant differences in the distance to the centroid in the 3 regions (greatest at IT and smallest at S,  $P<0.0001$ ). Men had greater d-lat than women (0.075cm difference,  $P<0.0001$ ). Aging was associated with medial shifting of the centroid that was greater in women NN (F&M=0.010cm/y), IT (F=0.011cm/y, M=0.005cm/y) and S (F=0.003cm/y, M=0.001cm/y)  $P<0.0001$ . Increasing weight and height were associated with greater d-lat ( $P<0.0001$ ). Among physical activity variables, lifetime activity ( $P=0.036$ ), walking/cycling for >1hr/day ( $P=0.001$ ), weekly time spent on weight bearing activity ( $P=0.009$ ), and FEV1 ( $P=0.016$ ) were associated with higher d-lat. Interestingly after adjusting for these variables, ADL scores as a measure of current activity and weekly time spent on low impact activities were associated with shorter d-lat ( $P<0.019$ ). Diversity of physical activities may be superior to stereotypic walking activity in maintaining proximal femur structural stability.

## OC10

### FRACTURES AND ALL-CAUSE MORTALITY IN A POPULATION SAMPLE OF ELDERLY WOMEN; OBSERVATIONS FROM THE MRC HIP STUDY

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Hip fracture is associated with increased mortality, a significant proportion of which is due to co-morbidity. The association between other fracture types, co-morbidity and mortality has not been widely reported. We studied the contribution of several clinical risk factors and incident fractures to all-cause mortality in a cohort of elderly women.

5212 women aged 75 years or over participated in the MRC HIPS study, a community based study of risk factors for hip fractures combined with a placebo controlled trial of clodronate (Bonafos). Self-reported medical history was recorded at study entry. Incident fractures were confirmed independently and definite high trauma fractures were excluded from analysis.

The incidence of fracture and mortality was lower than predicted for this population reflecting a 'healthy participant' bias. After a median follow-up of 4 years, 184 (3.5%) participants sustained hip fractures, 76 (1.5%) sustained clinical vertebral fractures, and 448 (8.6%) sustained appendicular (non-hip) fractures. Of the latter, 380 (7.3%) sustained exclusively limb fractures. 755 (14.5%) subjects died of various causes in the study period. At baseline, the mortality group were older, had lower body-mass index, and lower hip and forearm bone mineral density (BMD) (all  $P<0.01$ ). In univariate analysis rheumatoid arthritis (relative risk, 95% CI, 1.80, 1.13-2.85), current corticosteroid (CS) use (1.79, 1.24-2.57), stroke (2.70, 1.84-3.98), Parkinson's disease (2.04, 1.03-4.07), type 1 diabetes (2.26, 1.12-4.54), type 2 diabetes (1.82, 1.32-2.49), hip fracture (2.71, 1.96-3.75), and limb fracture (0.41, 0.28-0.62) were significantly associated with mortality. In multivariate logistic regression models, age (odds ratio, 95% CI, 1.09, 1.07-1.11), 1 standard deviation decrease in hip BMD (1.30, 1.20-1.42), CS use (1.74, 1.19-2.55), stroke (2.51, 1.68-3.75), type 1 diabetes (2.64, 1.26-5.52), type 2 diabetes (2.04, 1.46-2.83), hip fracture (2.20, 1.56-3.10), and limb fracture (0.42, 0.28-0.64) were independently associated with mortality. Limb fracture remained significant even when classified as upper limb (0.44, 0.27-0.70) and lower limb fracture (0.37, 0.16-0.87).

The study confirms the independent associations between hip fracture, co-morbidity and increased mortality. The mechanism of the association between incident limb (non-hip) fracture and decreased mortality requires further investigation and examination in other population samples.

## OC11

### **UMBILICAL CORD CALCIUM AND MATERNAL VITAMIN D STATUS PREDICT DIFFERENT LUMBAR SPINE BONE PARAMETERS IN THE OFFSPRING AT 9 YEARS**

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Environmental influences during intrauterine and early postnatal life modify the rate of childhood bone mineral accrual, with different effects on bone size and mineralization. In this study we explore the influence of umbilical venous calcium and maternal vitamin D status on the lumbar spine BMC and BMD of the offspring at nine years.

We studied 214 children (113 boys) included in a population-based study of maternal nutrition and fetal growth. The body build, nutrition and exercise of their mothers had been characterised during pregnancy; the children were followed up at age nine years to relate these maternal characteristics with whole body bone mass, estimates of volumetric bone density and body composition, measured using a Lunar DPX-L instrument.

At nine, the boys were significantly taller ( $p=0.01$ ) than the girls, and had higher age-adjusted lumbar spine (LS) BMC ( $p=0.006$ ) and bone area ( $p<0.001$ ). There was no difference in BMD ( $p=0.95$ ). Umbilical cord calcium, albumin, maternal mid upper arm circumference in late pregnancy, parental height, maternal vitamin D levels, paternal social class, and the child's birthweight were positively associated with LS BMC at 9 years ( $p<0.05$ ). Maternal smoking, and umbilical cord phosphate showed no association. When these univariate predictors were combined in a multivariate model, parental height ( $p<0.001$  mother,  $p=0.007$  father), cord calcium ( $p=0.017$ ) and male sex ( $p=0.03$ ) remained positive predictors of LS BMC. With the addition of the child's birthweight, cord calcium became insignificant ( $p=0.077$ ). The univariate predictors of BMD were similar, but low maternal vitamin D status was associated with lower BMD ( $p=0.021$  for lowest quintile of vitamin D versus remaining quintiles) in a multivariate model including maternal height ( $p=0.01$ ) and cord calcium ( $p=0.018$ ). The addition of child's birthweight ( $p=0.003$ ) removed the effect of cord calcium and maternal height, but low maternal vitamin D remained significant ( $p=0.002$ ).

Umbilical cord calcium predicts lumbar spine bone mineral content and density at 9 years, and low maternal vitamin D status is associated with lower BMD (a partially size-corrected measure), but not BMC at 9 years. This may imply a dichotomous mechanism underlying the effect of the early environment on bone size and mineralization.

## OC12

### **ANNUAL INTRAMUSCULAR VITAMIN D AND FRACTURE IN THE ELDERLY**

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The effectiveness of an annual intramuscular injection of vitamin D in preventing fractures among men and women aged 75 years and over in the general population, remains uncertain. We therefore performed a randomised double-blind placebo-controlled trial of 300,000 IU intramuscular vitamin D or matching placebo, administered every autumn over three years. The trial was performed in 4,354 men and 5,086 women aged 75 years and over, living in the general community and recruited from the patient registers of general practitioners in Wessex, England. The principal outcome measure was the incidence of all non-vertebral fractures.

After three years of follow up, 609 men and women had incident fractures (hip 110, wrist 107, ankle 24). Hazard ratios in the vitamin D group compared with the placebo group were 1.10 (95% CI 0.94-1.29,  $p=0.25$ ) for any first fracture and 1.48 (95% CI 1.01-2.17,  $p=0.04$ ) for first hip fracture; and 1.17 (95% CI 0.80-1.71,  $p=0.43$ ) for first wrist fracture, controlling for age and sex. Although the findings were similar among men and women, the difference between treatment groups for hip fracture appeared more pronounced among those aged 80 years and over, and among those without previous fractures. No apparent protective effect was observed when the cohort was stratified by age, previous fracture, or level of mobility. Analysis of serum PTH and 25-hydroxyvitamin D concentrations in a subset of subjects suggested that the intervention achieved a 20% suppression in peak winter PTH levels.

An annual intramuscular injection of 300,000 IU vitamin D is not effective in preventing hip and other non-spine fractures among elderly men and women resident in the general population.

### OC13

#### **NE-10790, A PHOSPHONOCARBOXYLATE ANALOGUE OF THE BISPHOSPHONATE RISEDRONATE, EXHIBITS DIRECT ANTITUMOR ACTIVITY *IN VIVO***

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In addition to being powerful inhibitors of bone resorption *in vivo*, bisphosphonates (BPs) also exhibit potent antitumor activity. *In vitro*, BPs inhibit tumor cell adhesion, invasion and proliferation, and they induce apoptosis of tumor cells. *In vivo*, BPs reduce skeletal tumor growth. However, because of their high affinity for bone mineral and rapid uptake in bone, tumor cells in the bone marrow may be exposed to BPs for too short a period to observe cytotoxicity. It is most likely that the antitumor activity of BPs in bone is mediated through inhibition of bone resorption which, in turn, deprives tumor cells of bone-derived growth factors. Conversely, a BP having a low bone affinity could act directly on tumor cells in the bone marrow because of its rapid release from bone mineral. To address this question, we compared the antitumor potency of the nitrogen-containing BP risedronate with that of its phosphonocarboxylate analogue (NE-10790) in which one of the phosphonate groups is substituted by a carboxyl group. NE-10790 had a 15-fold lower affinity for bone mineral compared to that observed with risedronate. *In vitro*, NE-10790 and risedronate inhibited proliferation of GFP-expressing B02 breast cancer cells (B02-GFP) (IC50s: 3.4 ± 1.0 and 0.5 ± 0.2 mM, respectively). Continuous treatment of mice with risedronate (0.15 mg/kg/day) almost completely inhibited bone destruction caused by B02-GFP cells (as judged by radiography) and substantially reduced skeletal tumor burden (as judged by fluorescence imaging and histomorphometry). NE-10790 (0.15 mg/kg/day), under similar experimental conditions, did not inhibit bone destruction whereas it drastically inhibited skeletal tumor burden (70% reduction). This lack of inhibitory effect of NE-10790 on bone destruction was consistent with the observation that NE-10790 was 8,000-fold less potent than risedronate (on a mg/kg basis) in inhibiting bone resorption in ovariectomized rats. Moreover, a continuous treatment of mice with NE-10790 (or risedronate), at a daily dose (0.15 mg/kg) that inhibited skeletal B02 tumor burden, did not inhibit the subcutaneous growth of B02-GFP cells. Overall, these findings strongly suggest that NE-10790 (because of its low bone affinity) transiently accumulates in bone and subsequently act on tumor cells to inhibit their growth.

### OC14

#### **BONE MARROW ENDOTHELIAL CELLS PRODUCE OSTEOPROTEGERIN: EVIDENCE FOR A ROLE IN THE DEVELOPMENT OF MYELOMA BONE DISEASE**

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Multiple myeloma (MM) is associated with the development of osteolytic bone disease, mediated by an increase in osteoclast activity. The cellular and molecular mechanisms responsible for promoting osteoclast formation are poorly understood. We have shown that the ligand for receptor activator of NF-κB (RANKL) is expressed by the myeloma cells. However, the role of osteoprotegerin (OPG), the decoy receptor for RANKL, in MM is less clear. The aim of this study was to determine whether murine bone marrow endothelial cells express OPG and whether this may play a role in the development of myeloma bone disease.

RT-PCR and flow cytometric analysis demonstrated that the murine bone marrow endothelial cells STR10 and STR12 express OPG. ELISA confirmed release of OPG. OPG was not detected in the supernatant from LE1SVO murine lung endothelial cells. Media conditioned by STR10 and STR12 cells, and containing OPG, was able to inhibit the formation of tartrate

resistant acid phosphatase positive, multinucleated cells and prevent bone resorption *in vitro*. OPG was expressed at a 1000-fold lower level in 5T33MMvt cells than in STR10 or STR12 cells and OPG protein could not be detected by ELISA in the myeloma cells. The addition of 5T33MMvt cells to either STR10 or STR12 cells resulted in a cell number-dependent decrease in OPG protein production. Quantitative RT-PCR confirmed that expression of OPG mRNA was decreased in STR10 and STR12 cells. Physical separation of 5T33MMvt and endothelial cells resulted in a down-regulation of OPG production. Furthermore, medium conditioned by 5T33MMvt cells also inhibited OPG production suggesting that a soluble factor is responsible. Neutralisation of TNFalpha, but not TGFbeta or bFGF, partially restored OPG levels suggesting that TNFalpha may be one of the factors involved in down-regulating OPG production by bone marrow endothelial cells.

In conclusion, these data demonstrate that bone marrow endothelial cells produce OPG and this is associated with a decrease in bone resorption *in vitro*. Myeloma cells decrease OPG production in bone marrow endothelial cells raising the possibility that this could alter the balance between OPG and RANKL in favour of RANKL and contribute to the development of myeloma bone disease.

## OC15

### **SQSTM1 MUTATIONS IN PAGET'S DISEASE: EVIDENCE FOR A FOUNDER EFFECT ON AN ANCESTRAL CHROMOSOME BEARING THE P392L MUTATION**

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Mutations in the SQSTM1 gene are a common cause of Paget's Disease of Bone (PDB) and the most common is a C/T substitution at position 1215, causing a proline to leucine amino acid substitution at codon 392 (P392L). Since marked geographical differences exist in the prevalence of PDB, we looked for evidence of a founder effect in chromosomes bearing the P392L mutation. We performed haplotype analysis in 311 cases of sporadic PDB and 357 controls from the UK by genotyping SNPs in exon 6 (C916T, G976A) and the 3'UTR (C2503T and T2687G). Two common haplotypes were found, accounting for 90% of alleles at the SQSTM1 locus; H1 (916T-976A-2503C-2687T) and H2 (916C-976G-2503T-2687G). There was a slight excess of H2 in cases compared to controls (46% vs. 41%), but the difference was not significant ( $p=0.07$ ). Analysis of haplotype background in 'sporadic' PDB cases, showed that the P392L mutation was carried almost exclusively on the H2 background (25/27, 92.6%) which is approximately 13.2 times (95% CI: 3.1, 56.4) more frequently than expected ( $p<0.0001$ ). Similarly, analysis of 13/70 PDB families who were previously found to carry the P392L mutation (Hocking et al, Hum Mol Gen 2002) showed that the mutation was carried almost exclusively on the H2 background (12/13, 92.3%), which is 16.8 times (95% CI: 2.1, 133.9) more often than expected ( $p=0.001$ ). In summary, our data show compelling evidence for a founder effect for the P392L mutation in this study population of predominantly UK descent where over 90% of mutations were on the same haplotype background (H2). These data differ from those reported by Laurin et al. (Am J Hum Genet 2002) who found that the P392L mutation in French-Canadian patients occurred with similar frequency on what they termed the PDB3H1 and PDB3H2 haplotypes (corresponding to H1 and H2 described here). Whilst our findings do not negate the suggestion that P392L is a recurrent mutation, the striking over-representation of P392L on H2 alleles indicates that the majority of UK subjects with SQSTM1 mediated PDB may be descended from a common ancestor.

## OC16

### **RANKL/OPG/RANK GENE EXPRESSION IN PERIPHERAL MONONUCLEAR CELLS FOLLOWING TREATMENT WITH ESTROGEN OR RALOXIFENE : POTENTIAL ROLE IN POST-MENOPAUSAL OSTEOPOROSIS**

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The RANKL/OPG/RANK pathway is the key downstream mediator of osteoclastogenesis. Mononuclear cells may be implicated in post-menopausal bone loss. The aim of the study was to assess the effects of estrogen or Raloxifene on RANKL, OPG and RANK gene expression in peripheral mononuclear cells (PBMCs). Twenty nine women with post-menopausal osteoporosis were treated with either estrogen (HRT) or Raloxifene for 12 months. BMD was measured at baseline and at 12 months. Serum CTX, OPG and RANKL were measured at baseline and at 1, 3, 6 and 12 months. PBMCs were isolated in 17 women (HRT : 6, Raloxifene : 11) and quantitative PCR analyses were carried out to determine any changes in RANKL, OPG and RANK expression. The effects of estrogen or Raloxifene in PBMCs *in vitro* were also assessed. BMD increased significantly following treatment with estrogen or Raloxifene (lumbar spine % change mean [SEM] : 4.3 [0.9],  $p < 0.001$ ). Serum CTX decreased following treatment (6 month: - 43.7 % [6.0],  $p < 0.0001$ ). Serum OPG declined gradually (12 month: - 26.4% [4.4],  $p < 0.001$ ). A smaller but significant reduction in serum RANKL was also seen at 6 month in a sub-group (n = 10) ( 6 month : - 19.6 % [9.2],  $p = 0.046$ ). We observed a significant decrease in RANKL, OPG and RANK gene expression in PBMCs (proportional change from baseline, 6 month : RANKL 50.0 [24.8] %  $p < 0.001$ , OPG : 21.7 [28] %  $p < 0.001$ , RANK : 76.6 [10.2] %  $p = 0.015$ ). The changes in OPG gene expression correlated significantly with changes in BMD ( $r = - 0.53$ ,  $p = 0.027$ ) and CTX ( $r = 0.7$ ,  $p = 0.0044$ ). Treatment of PBMCs *in vitro* with estrogen or Raloxifene also led to a down-regulation in RANKL, OPG, RANK gene expression and a reduction in osteoclast numbers and bone resorption. This study shows that both estrogen or Raloxifene led to a reduction in bone resorption associated with a decrease in the expression of RANKL/OPG/RANK in PBMCs. The data suggest that PBMCs may play an important role in the pathogenesis of post-menopausal bone loss and osteoporosis.

## OC17

### **NORMAL HUMAN OSTEOCLASTS ARE ACTIVATED BY ACIDOSIS**

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Rodent and avian osteoclasts (OC) are strongly activated to excavate resorption pits when extracellular pH is reduced, and acidification is an absolute requirement for resorption to occur. Systemic acidosis in humans causes bone loss and may play a role in the pathogenesis of osteoporosis. We have now investigated the effects of pH on the function of normal human OC. Human OC were generated by culturing peripheral blood mononuclear cells from healthy donors on 16mm<sup>2</sup> bovine bone chips ( $2 \times 10^5$  cells/chip; n=6-8) for 16d with RANKL (5ng/ml) and MCSF (20ng/ml) in pH 7.42 medium in 5% CO<sub>2</sub>. The resulting OC cultures were then acidified with 0-15 mmol/l HCl and maintained for a further 3d. Acid-base parameters were monitored by blood gas analyser; multinucleated OC and resorption pit areas were assessed 'blind'.

Reducing extracellular pH for the final 3d of culture caused striking, progressive increases in resorption pit formation by human OC, with peak stimulations of 5 to 6-fold at pH ~6.9-7.0. Numbers of OC (~100 / bone chip) were not significantly affected by acidification over 3d but were reduced in cultures kept continuously at low pH for 19d. The acid response curve of human OC differed from that of rat or mouse OC, in that it was shifted markedly in the alkaline direction, with half-maximal activation at pH~7.3 (as opposed to pH~7.1 for rodents). RT-PCR analysis indicated that mRNAs for cathepsin K and TRACP5 were upregulated at pH 7.0 in human OC cultures.

Acid-activation is a fundamental property of OC from all species studied to date, and extracellular H<sup>+</sup> now appears to be the long sought-after 'osteoclast activation factor'. The pH-activation profile of human OC corresponds with that of the H<sup>+</sup>-sensing human G-protein-coupled receptors reported to be present on bone cells (Nature 425:93-8, 2003). Such pH sensors may present an interesting new class of drug targets.

## C1

### **MOLECULAR DEFECT IN PSEUDOHYPOPARATHYROIDISM TYPE 1 B : 2 ILLUSTRATIVE CASES**

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We report 2 cases of pseudohypoparathyroidism type 1-b (PHP-1b) which is characterized by resistance to the renal actions of parathyroid hormone (PTH) and the absence of features of Albright's hereditary osteodystrophy (AHO) and discuss the molecular mechanisms underlying this disorder.

Case 1: a 12 year old girl presented with failure to gain weight and episodes of tremor consistent with carpopedal spasms. There was no relevant family history and clinical examination was unremarkable. Laboratory investigations showed a serum calcium of 1.54 mmol/l, phosphate 2.44 mmol/L, PTH 830 ng/l (10-65), 25 (OH) vitamin D 43 nmol/L. Thyroid profile was normal. Radiographs of her hands and wrists and renal ultrasound showed no abnormalities. Case 2: was diagnosed at the age of 9 when she presented with an unusual gait and learning difficulties. She was found to have a low serum calcium of 1.93 mmol/L. Phosphate was 2.45 mmol/L and PTH was >100 pmol/l (0.9-5.4). X-rays of her hands and wrists showed a slight coarse trabecular pattern and sub-periosteal erosion of the middle phalanges. Both patients were treated with 1-alpha-calcidol.

Molecular analysis in both patients showed a general methylation defect affecting exons NESP55, XL as well as exon A/B of the GNAS gene. In the renal proximal tubule, Gs-alpha expression occurs normally from the maternal allele. However, due to the broad methylation abnormalities, Gs-alpha expression is silenced in this part of the nephron from both parental allele, thus leading to PTH-resistance. Further characterization of the region of chromosome 20q13.3 containing the GNAS gene failed to show the 3-kb microdeletion located 220 kb centromeric of GNAS exon A/B identified in autosomal dominant PHP type 1b. Taken together, these data suggest that the genetic defect in our two sporadic cases of PHP type 1b remains to be identified, and it is conceivable that both cases are examples of a recessive form of the disease which could be caused by a homozygous (or compound heterozygous) mutation in a gene other than GNAS.

## C2

### **VANISHING BONE DISEASE: POTENTIAL FOR BISPHTHONATE TREATMENT**

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Skeletal angiomatosis (Gorham Stout's disease) is a rare condition of unknown aetiology characterised by local vascular proliferation and osteolysis. Although considered a benign condition with insidious onset, it can result in uncontrollable pain, pathological fracture, bony collapse and death dependent on the location of the lesions. To date radical excision surgery with associated morbidity and local recurrence has been the only treatment available for accessible, localised lesions. We present two cases of angiomatosis that demonstrate the spectrum of this disease and suggest a role for bisphosphonate treatment to inhibit the bone lysis associated with this condition.

Case 1 is a 10yr old girl who presented with pain in her right leg. X-ray showed her to have localised, massive osteolysis of the tibia and fibula. Skeletal angiomatosis was confirmed on biopsy and MRI. She was treated with high dose IV pamidronate, resulting in a reduction in her pain. Regional bone density measurement using DXA showed improvement at her tibia, with a 10% increase over 9 months. Follow up imaging has shown no further expansion of the lesions. Markers of bone turnover have reduced. She has been able to return to weight

bearing without problem. Bisphosphonate is being gradually withdrawn and follow-up continues.

Case 2 is a 20yr old man who presented with diffuse skeletal pain, a mass of his proximal right tibia and progressive systemic symptoms of fever, sweats and weight loss. X-ray showed an area of massive osteolysis and bony expansion of his proximal tibia, and cystic lesions of his pelvis and multiple vertebrae. Widespread skeletal angiomas was confirmed by biopsy and MRI. He was treated with IV pamidronate producing an improvement in his pain and systemic symptoms. However, due to a pathological fracture of his affected tibia and uncontrolled localised pain, he under-went an above knee amputation.

These two cases demonstrate that IV pamidronate therapy may be an effective way to control symptoms and limit progressive osteolysis in skeletal angiomas.

### C3

#### **LIMITED EVIDENCE OF RESCUE OF OSTEOCLAST-POOR OSTEOPETROSIS FOLLOWING 'SUCCESSFUL ENGRAFTMENT' BY CORD BLOOD FROM AN UNRELATED DONOR**

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Osteopetrosis (OP) is a genetic disorder characterised by severely reduced bone resorption resulting from a defect in either osteoclast (OC) development (OC-poor OP) or activation (OC-rich OP). The consequences are dense, fragile bones and a reduced bone marrow cavity associated with extramedullary haemopoiesis. Patients with OC-rich OP can be rescued by bone marrow or cord blood transplantation. However, little information exists concerning the success of transplantation as a treatment for OC-poor OP.

We report a child with OC-poor OP, diagnosed at age 7 by histopathology and radiology. At presentation, the patient was blind, anaemic, thrombocytopenic and leucopenic and had evidence of extramedullary haemopoiesis. At age 8, the child received a cord blood transplant from an unrelated donor. Engraftment was successful as circulating blood cells were of donor origin. However, 2 years post-transplant, there was virtually no rescue of the skeletal disease and there was persistent anaemia and extramedullary haemopoiesis. To investigate the phenotype further, peripheral blood mononuclear cells (PBMC) from the patient, pre and post transplant, were cultured on bone slices with M-CSF and RANKL for 2-3 weeks. Healthy individuals were used as controls. PBMC from the child prior to transplantation formed a few mono- and binucleate OCs (F-actin ring-positive cells, co-localising with vitronectin receptor and TRAP) *in vitro*. These cells expressed macrophage markers, CD11c and CD18, and were associated with small resorption lacunae (2% of the surface of the bone slices). Low levels of collagen fragments were released from these cultures, as assessed by CTx-ELISA (CrossLaps) (patient 1.86nM, control 93.6nM). In contrast, OC formed in PBMC cultures from the child following transplantation, showed results similar to control cultures: large numbers of OCs were present containing numerous nuclei; approximately 50% of the surface of bone slices was resorbed.

This is the first report of a successful haemopoietic engraftment failing to correct an osteopetrotic skeletal defect. The *in vitro* result supports previous studies showing that neither M-CSF nor RANKL rescue OC-poor OP. Finally, an explanation is required to explain why donor haemopoietic precursors that successfully engrafted this patient fail to 'home' to the bone marrow and correct the disease phenotype.

### P1

#### **TNF-ALPHA RECEPTOR 1 CONTROLS ENDOCHONDRAL BONE FORMATION IN ADULT MICE**

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Background and objectives: TNF-alpha, a major proinflammatory cytokine, exerts its role on bone cells through two receptors (TNFR1 and TNFR2). TNFR1, but not TNFR2, is expressed by osteoblasts, although its function *in vivo* is not fully understood.

**Methods:** We compared *in vivo* new bone formation in mice with TNFR1 gene knock-out (-/-) and wild-type mice, using two models of adult bone regeneration: intramembranous ossification following tibial marrow ablation and endochondral ossification induced by bone morphogenetic protein (BMP)-2. We analyzed histomorphometry of tibial marrow cavity and newly formed ossicles, as well as the expression pattern of genes for both bone-related markers and inflammatory cytokines by Northern blot, PCR, and quantitative PCR analysis.

**Results:** Intramembranous osteogenesis in animals without TNFR1 did not differ from the wild-type mice either in morphometric parameters or expression of bone-related markers (bone sialoprotein, osteocalcin, collagen alpha1(I), and osteopontin) and inflammatory cytokines (TNF-alpha, IL-1alpha). During endochondral osteogenesis, TNFR1 -/- mice formed more cartilage, and later on more bone and bone marrow. There was no difference in the expression of bone-related markers between the TNFR1 -/- and wild-type mice. Expression of BMP-7 was higher in TNFR1 -/- mice at all time points. The activity of gene for TNF-alpha in TNFR1 -/- mice was comparable to that in control mice, but the activity of IL-1alpha gene was higher in TNFR1 -/- mice. The TNFR1 -/- animals also had higher expression of RANKL and RANK, as assessed by quantitative PCR.

**Conclusion:** Signaling through the TNF receptor 1 seems to be involved in the restriction of new bone formation during endochondral but not intramembranous ossification in the adult mice. The site of TNFR1 effects seems to be recruitment and proliferation but not differentiation of cells during the osteogenic sequence. Higher expression of RANKL and inflammatory cytokines suggests the involvement of immune cells. This increases our knowledge of the role of TNF family members in bone biology and raises ideas for new therapeutic concepts.

## P2

### DENTIN MATRIX PROTEIN-1 AND BONE DEVELOPMENT

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Dentin matrix protein (DMP-1), a phosphoprotein highly linked to dentin formation, has recently been reported to have an important role in skeletal development. Previously we reported that adult mice lacking the gene for DMP-1 exhibit the characteristics of chondrodysplasia, osteoarthritis, and showed severe defects in mineralization. In this study, biomechanical assessment tests of bones from DMP-1 KO mice were performed.

Fifteen heterozygous, H, (DMP-1 +/-) and 15 KO (DMP-1 -/-) male mice were produced and used in this study. At 1, 3 and 7.5 months of age, the mice were sacrificed and 5 ulnae from each animal group were harvested and stored in 70% ethanol solution. Volumetric density (BMD) measurements of the intact ulnae were performed using peripheral quantitative computed tomography. Flexural tests were performed in 3-point bending, the ultimate load and stiffness were determined from the load-displacement curves. All data were analysed using Mann-Whitney U tests.

Density studies revealed that H mice had higher BMD than KO mice at all ages ( $p < 0.001$ ). In the H and KO mice, the cortical BMD peaked at 3 and 7.5 months, respectively. At 1 month, the mean cross-sectional areas of the ulnae were larger in H mice compared to KO mice (0.50 mm<sup>2</sup> Vs 0.33 mm<sup>2</sup>). However at 7.5 months of age, the reverse was observed (H = 0.75 mm<sup>2</sup> and KO = 0.98 mm<sup>2</sup>). Stiffness increased with age at a higher rate in H mice than KO mice (figure 2). Significant differences were observed at 3 months (\*\* $p < 0.01$ ) and 7.5 months (\* $P < 0.05$ ) between the two animal groups. There were no significant differences between stiffness values at 1 month.

This study has demonstrated that DMP-1 deficiency leads to: (1) severely compromised bone mineralization; (2) poor biomechanical properties of the long bone; and (3) delayed bone development and remodelling. In conjunction with previous findings that DMP-1 plays important roles in the early developmental stage of bone and the current study may suggest another important role for DMP-1 as a regulator for skeletal mechanostasis.

### P3

#### **COMBINED TREATMENTS OF BREAST CANCER CELLS USING BISPHOSPHONATES AND DOXORUBICIN**

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Bisphosphonates are commonly used to treat patients with metastatic bone disease, in order to prevent skeletal complications. Whether these anti-resorptive drugs also have direct anti-tumour effects remain to be established, but an increasing number of patients are receiving bisphosphonates in combination with cytotoxic drugs as part of their treatment. Previous work from our laboratory has shown that combining the potent bisphosphonate zoledronic acid (ZOL) with the commonly used cytotoxic drug paclitaxel *in vitro* results in a synergistic increase in apoptotic cell death. Here we compare the ability of three structurally different bisphosphonates to induce apoptotic cell death of breast cancer cells in combination with doxorubicin.

MCF7 breast cancer cells were treated with 0.05microM doxorubicin for 24 hours, and following removal of the drug, the cells were subsequently treated with either 25microM ZOL for 1 hour, 100microM alendronate for 1 hour or 500microM clodronate for 24 hours. The cells were further incubated in drug free media for up to 72 hours, and levels of apoptosis determined using Hoechst/PI staining and evaluation of nuclear morphology. In all cases, the level of apoptosis with a bisphosphonate alone was less than 1%, and approximately 2% with 0.05microM doxorubicin alone. This was not significantly different from levels in untreated controls.

We found a synergistic increase in apoptotic cell death of 13.65% when cells were treated with doxorubicin followed by ZOL. This was significantly greater than ZOL alone (0.75%,  $p=0.004$ ), or with doxorubicin alone (1.1%,  $p=0.004$ ). Sequential treatment using doxorubicin followed by alendronate also caused a synergistic increase in apoptosis, but the level of 3.23% was much lower compared with the ZOL group. In contrast, when cells were treated sequentially with doxorubicin then clodronate no synergistic increase in apoptosis was observed.

Our data shows that combined sequential treatment using doxorubicin followed by a bisphosphonate results in synergistic apoptosis, but only when nitrogen containing bisphosphonates are used. These results may have implications for patients who receive bisphosphonates while undergoing chemotherapy.

### P4

#### **ARE EPIGENETIC CHANGES IN DNA METHYLATIONS A SIGNIFICANT FACTOR IN THE ALTERED GENE EXPRESSION OF OSTEOARTHRITIC CHONDROCYTES?**

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Osteoarthritic chondrocytes express aberrant genes that appear to be stably propagated to daughter cells. Regulation of gene expression can be categorized as either short-term regulation by transcription factors or long-term phenotype-specific silencing of those genes that are never expressed. This silencing depends on DNA methylations, which represent 'epigenetic' changes, defined as altered regulation of gene expression without changes in genetic sequence. If cytosines of CpG dinucleotides in the promoter region are methylated, the gene may be silenced, even if the right transcription factors are present. We examined whether the aberrant gene expression in osteoarthritis may be due to clonally inherited epigenetic changes by determining the methylation status of mmp-9, one of the aberrantly expressed genes of osteoarthritis.

Femoral heads were obtained after hip replacement due to osteoarthritis or following femoral neck fracture, the latter showing no cartilage erosion. In osteoarthritic samples, cartilage was taken from eroded regions, which contained mostly clonal chondrocytes as confirmed by histology. Samples were ground in a freezer mill and DNA-extracted. To assess methylation status, treatment with methylation-sensitive restriction enzymes was followed by PCR amplification of a 350bp promoter region containing four potential methylation sites, with presence of the PCR band indicating methylations. HhaI cleaved one site very close to the transcription start site and AclI cleaved the other three. In all patients examined to date,

digestion with HhaI abolished the PCR band, indicating that this CpG was never methylated. However, a remarkable difference was found after digestion with AclI: in 8/9 osteoarthritic patients, the PCR band was no longer detectable, while in 4/5 non-osteoarthritic patients the PCR band was still present. These results indicate that all three AclI cleavage sites were methylated in the majority of chondrocytes from non-osteoarthritic patients, while at least one of the three AclI cleavage sites was unmethylated in osteoarthritic patients.

The present study provides the first 'proof-of-concept' data that epigenetic changes may be significant in the etiology of osteoarthritis. Further work needs to establish the generality of the present findings and whether de-methylations occur in the promoter regions of other genes that are aberrantly expressed in osteoarthritis.

## P5

### **NE10790, A PHOSPHONOCARBOXYLATE ANALOGUE OF RISEDRONATE, INDUCES HUMAN MYELOMA CELL APOPTOSIS *IN VITRO***

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Bisphosphonates (BPs) are widely used in the treatment of osteolytic bone disease associated with multiple myeloma (MM). In addition to their beneficial effects on bone disease in MM, BPs have recently been suggested to have anti-tumour effects. We and others have previously demonstrated that BPs can induce apoptosis of human MM cells both *in vitro* and *in vivo*. NE10790 is a phosphonocarboxylate analogue of the potent bisphosphonate risedronate, in which one of the phosphonate groups is replaced with a carboxyl group. This phosphonocarboxylate has a lower affinity for bone mineral and is a weak inhibitor of bone resorption, but has been reported to specifically inhibit Rab GGTase, and to inhibit tumour cell invasion *in vitro*. The aim of this study was to determine whether NE10790 has an anti-tumour effect in human MM cells *in vitro*, and to compare its effects with risedronate. Apoptotic cells were identified by characteristic changes in nuclear morphology and by a fluorescence in situ nick translation assay. Cell cycle analysis was performed by staining with propidium iodide followed by flow cytometric analysis. NE10790 significantly increased apoptosis in NCI H929, JJN-3, and RPMI 8226 MM cells in a dose-dependent manner ( $p < 0.0001$ ). JJN-3 cells were most sensitive to NE10790; a significant increase in apoptosis was detected following treatment with 1 mM NE10790 ( $p < 0.01$ ). Induction of apoptosis in MM cells was found not to be due to calcium chelation. Risedronate also dose-dependently increased apoptosis in JJN-3 cells, with a significant increase detected at 50  $\mu\text{M}$  ( $p < 0.05$ ). Treatment of JJN-3 cells with risedronate caused an accumulation of cells in the S-phase of the cell cycle at lower concentrations than that required to induce an increase in the sub-G0/G1 fraction (apoptotic). In contrast, NE10790 also induced an increase in the sub-G0/G1 fraction, but accumulation of cells in S-phase was not detected. These results demonstrate that the phosphonocarboxylate analogue of risedronate, NE10790, induces apoptosis in human MM cell lines *in vitro* in a dose-dependent manner. In addition, cell cycle analysis suggests that NE10790 may have a different mode of action than risedronate in induction of MM cell apoptosis.

## P6

### **GROUP III METABOTROPIC GLUTAMATE RECEPTORS ARE EXPRESSED IN BONE MARROW STROMAL CELLS AND ARE NEGATIVELY COUPLED TO NITRIC OXIDE SYNTHASE**

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Bone cells express a range of functional glutamate receptors. Since glutamatergic signalling is intimately linked with generation of nitric oxide (NO) in the central nervous system and NO plays an important signalling role in bone, we investigated the effects of glutamatergic stimulation on activity of nitric oxide synthase (NOS) in marrow stromal cell and osteoblast primary cultures. Femora were removed from male Wistar rats and the marrow cavity flushed with culture medium. Marrow stromal cells were cultured in alpha-minimal essential media, (15 percent fetal calf serum, penicillin, streptomycin, ascorbic acid and beta-glycerophosphate), in a humidified atmosphere of 5 percent carbon dioxide at 37 degrees for 16 to 30 days. NOS activity was assayed by measuring tritiated L-arginine-to-citrulline conversion. Expression of mGluRs was investigated using RT-PCR and Western blotting of

cell lysates. Single-cell calcium imaging was carried out with continuous saline perfusion, using cells labelled with Calcium Green-1. The properties of voltage-operated calcium channels were studied in marrow stromal cells using whole-cell patch clamp. Stimulation of osteoblasts with glutamate or N-methyl-D-aspartate had no detectable effect upon the NOS activity of osteoblasts. However, it reduced L-NAME-sensitive NOS activity in marrow stromal cells by approximately 40 percent. This effect was insensitive to inhibition of NMDA receptors by MK-801, or group I and group II metabotropic glutamate receptors (mGluRs) by alpha-methyl-carboxyphenyl-glycine, but was abolished by pre-treatment with (S)-2-amino-2-methyl-4-phosphonobutyric acid, an inhibitor of group III mGluRs. Using RT-PCR and Western blot analysis, we detected expression of the group III receptor, mGluR6, in marrow stromal cell lysates. Single cell calcium imaging showed a significant dose-dependent decrease in intracellular calcium in response to L-glutamate. This decrease was apparently sensitive to blockade of calcium influx by lanthanum, but not to disruption of calcium stores with thapsigargin. Whole-cell patch clamp demonstrated that L-glutamate had no effect on calcium influx via L-type voltage-operated calcium channels. Together, these findings indicate that group III mGluRs are expressed in bone marrow stromal cells and that they are negatively coupled to NOS. The coupling mechanism apparently involves inhibition of a calcium influx pathway that is independent of voltage-operated channels.

## P7

### **ROSUVASTATIN INHIBITS PROTEIN PRENYLATION AND BONE RESORPTION BY OSTEOCLASTS *IN VITRO***

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Statins are widely prescribed to inhibit HMG-CoA reductase activity in the liver, reducing the conversion of HMG-CoA to mevalonate and the subsequent production of cholesterol. Inhibition of HMG-CoA reductase also reduces protein prenylation by blocking the synthesis of farnesyl- and geranylgeranyl- diphosphate. We have shown that some statins are potent inhibitors of osteoclast function by causing loss of prenylated small GTPases, although they may also act as bone anabolic agents by promoting osteoblast activity.

We have investigated the effects of the hydrophilic statin rosuvastatin (RSV, Crestor) on protein prenylation in J774 macrophage-like cells and rabbit osteoclast-like cells, and on osteoclast-mediated bone resorption *in vitro*, in comparison to three other statins (cerivastatin/CER, pravastatin/PRA and simvastatin/SIM). All four statins prevented the prenylation of the small GTPase Rap1A in macrophages and osteoclasts within 24 hours in a concentration-dependent manner. The order of potency for inhibiting prenylation was CER > SIM > RSV > PRA. In J774 cells the lowest concentration that effected protein prenylation ranged from 0.01 microM for CER to 10 microM for PRA, or 0.1 microM CER to 100 microM PRA in osteoclast-like cells. Effects were observed on protein prenylation in both cell types within one hour of RSV treatment. Addition of mevalonic acid (100 microM) or geranylgeraniol (20 microM) at least partially restored protein prenylation, confirming RSV reduces protein prenylation in osteoclasts by inhibiting the mevalonate pathway.

The effect of RSV on osteoclast-mediated bone resorption was measured by culturing rabbit osteoclasts on dentine slices. All four statins decreased the number of active osteoclasts and inhibited bone resorption with the same order of potency and at similar concentrations to that observed for inhibition of protein prenylation. Furthermore, total osteoclast number was not significantly decreased at concentrations of statins that inhibited resorption, indicating that the anti-resorptive effect was due to changes in osteoclast function rather than to cytotoxicity.

In conclusion, RSV inhibits protein prenylation and inhibits osteoclast function *in vitro*. This suggests that, despite its hydrophilic nature, RSV has access into cells to a sufficient concentration to inhibit protein prenylation and affect osteoclast function *in vitro*.

## P8

### **OSTEOCLASTIC CORTICAL EROSION OF THE FEMORAL NECK IS POSITIVELY ASSOCIATED WITH SUB-PERIOSTEAL ALKALINE PHOSPHATASE EXPRESSION IN ELDERLY FEMALE CASES OF HIP FRACTURE AND CONTROLS**

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Hip fractures are associated with increased cortical and endocortical remodelling, reduced wall thickness of endocortical packets and increased cortical porosity at the femoral neck. Femoral neck width in the antero-posterior plane increases with time, being associated positively with lifetime physical activity. We hypothesised that exposure to mechanical loading may influence the sub-periosteal osteoblastic response to the weakening effect of post-menopausal intracortical bone resorption.

In 21 femoral neck biopsies from female subjects (13 with hip fracture) there was a positive association between the extent of osteoblastic periosteal alkaline phosphatase expression shown in frozen sections (as a marker of bone forming activity) and the percent of intracortical canals showing evidence of osteoclastic erosion (Goldner's stain;  $p=0.03$ ); this was stronger in the plane of locomotor loading and particularly strong in the inferior (compression) cortex ( $p=0.002$ ). The fracture group showed some differences from controls with significant elevation of % osteoid-bearing cortical canals in the fracture cases ( $n=35$ ) compared to the controls ( $n=23$ ) within the anterior ( $P=0.009$ ) and inferior regions ( $P=0.04$ ), while % eroded canals were also increased anteriorly ( $P=0.03$ ). In the fracture group (fracture;  $n=21$ , control;  $n=21$ ), lamellar thickness of endocortical bone packets were reduced both inferiorly ( $P=0.015$ ) and superiorly ( $P=0.045$ ); while % cortical and endocortical osteoid surface correlated positively ( $n=24$ ;  $P=0.041$ ).

Generally, these associations of intracortical with endocortical remodelling were consistent with both envelopes being regulated by common processes. Our results support the concept that there is slow growth of femoral neck width in post menopausal women by apposition of bone under the periosteum. Elevated sub-periosteal bone formation is associated quantitatively with increased osteoclastic erosion. In turn this is linked to post menopausal BMU imbalance in the intra- and endo-cortical envelopes. The maintenance of adequate femoral neck cortical width in the post menopause may depend in part on a sufficient sub-periosteal formative response to the mechanical loading stimulus of everyday physical activity.

## P9

### **MODIFICATIONS TO THE PHOSPHONATE GROUPS OF BISPHOSPHONATES AFFECTS THEIR POTENCY AND TARGET ENZYME SPECIFICITY**

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We and others have demonstrated that nitrogen-containing BPs inhibit bone resorption by inhibiting farnesyl diphosphate (FPP) synthase, thereby preventing the synthesis of isoprenoid lipids required for prenylation of small GTPases in osteoclasts. More recently, we showed that a weak anti-resorptive phosphonocarboxylate analogue of risedronate, NE10790, inhibits Rab geranylgeranyl transferase (Rab GGTase), thereby selectively preventing prenylation of Rab proteins in cells *in vitro*. We have now examined the effects of other modifications to the phosphonate groups of BPs i.e. removal of one of the phosphonate groups, and replacement of one of the hydroxyl groups on one or both of the phosphonate moieties with a alkyl group (to produce a phosphonoalkylphosphinate (PAP) or bisphosphinate (BPI), respectively).

NE10788, the monophosphonate analogue of risedronate, inhibited prenylation of Rabs in J774 macrophages and RAW 264 osteoclast-like cells at 4mM but had no effect on the prenylation of Rap1A. This compound, like NE10790, therefore inhibits Rab GGTase rather than FPP synthase. NE10788 also inhibited bone resorption *in vitro*, but unlike NE10790 also disrupted actin rings, suggesting that the anti-resorptive effect may not be a result of inhibition of Rab prenylation. By contrast with NE10788, the monophosphonate analogues of pamidronate or alendronate did not inhibit the prenylation of either Rab6 or Rap1A.

The BPs NE97220 and NE21656 inhibited prenylation of Rap1A and Rab6 with similar potency to risedronate (complete inhibition with 100µM). NE58029, the PAP analogue of NE97220, also inhibited prenylation of Rap1A and Rab6, but was approximately 4-fold less potent. By contrast, NE21608, the PAP analogue of NE21656 was completely inactive. Neither NE58052 nor NE21656 (the BPI analogues of these BPs) had any effect on protein prenylation.

In summary, the ability of bisphosphonates to inhibit FPP synthase requires the two phosphonate moieties in the P-C-P structure and is dramatically affected by the replacement of a hydroxyl group with an alkyl group on one or both of the phosphonate moieties, the conversion of one phosphonate group to a carboxylate, or removal of one phosphonate group. Furthermore, removal of one of risedronate's phosphonate groups, or replacing it with a carboxylate group, generates compounds that specifically inhibit Rab GGTase.

## P10

### **INNATE IMMUNE RESPONSES TO HUMAN MESENCHYMAL STEM CELL IMPLANTATION IN IMMUNOCOMPROMISED MICE**

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Human mesenchymal stem cells (hMSCs) have great potential for use in bone tissue engineering and cytotherapy. Experimental systems are required to assess their *in vivo* potentials. However, previous experiments showed that hMSCs are eliminated gradually and bone formation is limited after implantation in immunocompromised mice. The aim of current study was to investigate in more details in the innate immune system in response to xenogeneic implantation of hMSCs and the possible mechanisms of clearance.

Spleen and blood samples from normal and hMSC-implanted CB17 scid beige mice (CB10 SB) mice were collected for FACS analysis and compared with wild type Balb/c mice. Enhanced green fluorescent protein (GFP)-labelled hMSCs were implanted into the anterior tibialis muscle of CB17 SB mice. After killing the implanted tissues were collected for histochemical, immunohistochemical and histomorphometric analysis.

The results revealed that CB17 SB had lower proportion of T and B cells compared with wild-type mice, but the components of innate immune response, monocytes-macrophages, neutrophils and NK cells were over presented. Immunohistochemistry of myeloid cell markers in spleen were consistent with the FACS analysis. Transplants of hMSCs into CB17 SB mice showed osteogenesis. Histomorphometry demonstrated that the amounts of callus formation and ectopic bone formation in muscle were significantly higher than in sham animals at 1 week ( $p < 0.05$ ) but this was followed with elimination of bone tissue by remodelling at 2 and 4 weeks. Monocytes/macrophages, by mouse macrophage marker F4/80, were detected at the tissue sites of hMSC implantation and a complement receptor, CR3(5C6) which is expressed on both monocytes/macrophages and neutrophils showed a similar pattern. Both markers were significantly higher in hMSC-implanted samples compared with sham-operated controls ( $p < 0.01$ ). Macrophages and osteoclasts with tartrate-resistant acid phosphatase (TRAP) activities participated in the clearances of mineralised tissue formed by implanted hMSCs.

We conclude that the components of the innate immune system in immunocompromised mice are over-presented. Monocytes/macrophages, neutrophils and NK cells invade tissue sites containing implanted hMSCs and respond to xenogeneic osteogenesis. The resultant environment does not appear to be suitable for the long-term survive and differentiation of hMSCs. Further experimentations to develop experimental systems assessing the differentiation potentials of hMSCs are warranted.

## P11

### **IN VIVO BLOCKADE OF RETINOIC ACID RECEPTOR SIGNALLING INHIBITS CELL PROLIFERATION AND THE DIFFERENTIATION OF OSTEOCLASTS AND MATURATION OF CHONDROCYTES IN REGENERATING DEER ANTLER**

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Retinoic acid (RA) regulates both limb development and the regeneration of amputated appendages in amphibians. We have also shown that RA may play a role in the regenerating deer antler; it is synthesised at sites where retinoic acid receptors (RARs) and retinoid-X-receptors (RXRs) are expressed, and *in vitro* inhibits antler chondrocyte differentiation while promoting osteoclast differentiation. The aim of this study was to investigate the role of this pathway in the regenerating antler *in vivo* using a dominant negative RAR that blocks RA signalling.

Blockade was achieved by biolistic transfection of the surface of the antler blastema 24 hours after the previous antler had been shed. The blastema consists of a reepithelialising wound surface and subjacent mesenchymal cells that eventually give rise to antler tissues. One blastema was transfected with a dominant negative RAR construct (RAR-E), the contralateral side with a control beta-galactosidase expressing plasmid. Antlers were harvested after 7 (n=3) and 14 (n=6) days. Cryostat sections were stained for TRAP to identify osteoclast-like cells and for von Kossa to show mineralisation. PCNA staining was used to identify proliferating cells and type II collagen immunostaining to demonstrate areas of chondrogenesis.

The transfection efficiency in the central blastema was between 60 and 70 %. Five of the six antlers transfected with RAR-E and harvested at day 14 were smaller (by weight) than their contra-lateral control. However, the most distinct cellular effects were observed at day 7. Proliferation in the mesenchymal progenitor cell layer was decreased in RAR-E antlers (26.2±7.6 vs 47.9±9.2 % p<0.005) and the area of the central blastema containing TRAP+ve cells was also decreased (21,060 ±7,941 vs 39,174±11,2923 mm<sup>2</sup>). Conversely, in RAR-E antlers the area of the blastema showing staining for collagen type II was increased (13.0±11.3 vs 8.5±7.1 mm<sup>2</sup>), whereas the area of mineralised cartilage was decreased (36.1±21.7 vs 49.4±21.6 mm<sup>2</sup>).

In conclusion, this study has shown that in the antler blastema RAR signalling stimulates cell growth and osteoclast differentiation but appears to delay chondrocyte maturation. It thus provides functional *in vivo* data to show that RA may play an important role in regulating adult mammalian bone regeneration.

## P12

### **MYELOMA CELLS CAN PROMOTE OSTEOCLASTIC ACTIVITY RATHER THAN OSTEOCLAST RECRUITMENT *IN VITRO*; EVIDENCE FROM THE 5T2MM MURINE MODEL OF MYELOMA**

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A common clinical feature in multiple myeloma (MM) is skeletal destruction, which results in bone pain, pathological fracture and hypercalcaemia. Although increased osteoclastic bone resorption is responsible for the bone disease, the mechanisms responsible for promoting this activity are unclear. We have shown that CD138+ve primary human myeloma cells and 5T2MM murine myeloma cells express the ligand for receptor activator of NFκB (RANKL), raising the possibility that MM cells promote osteoclast formation and bone resorption in a stromal cell/osteoblast independent manner. The aim of the present study was to determine whether 5T2MM murine myeloma cells could induce osteoclast formation and bone resorption directly.

5T2MM murine myeloma cells were injected intravenously into C57BL/KaLwRij mice. All mice developed a serum paraprotein and the growth of 5T2MM cells in the bone marrow (BM). Radiographic and histological analysis of the tibia and femur demonstrated the presence of

lytic bone lesions and a reduction in cancellous bone area. Staining for tartrate resistant acid phosphatase (TRAP) activity demonstrated the presence of large numbers of TRAP+ve osteoclasts lining bone surfaces, adjacent to 5T2MM cells. 5T2MM cells were purified by density gradient centrifugation and incubated with peripheral blood mononuclear cells in the presence or absence of soluble RANKL (sRANKL). Addition of 5T2MM cells resulted in a significant reduction in the formation of TRAP+ve cells when compared to control. However, the presence of 5T2MM cells resulted in the formation of large resorption 'trails' on dentine slices, rather than the smaller pits seen in the absence of 5T2MM cells. The proportion of dentine surface undergoing resorption was identical to control. In all cases, sRANKL was required to induce osteoclastic bone resorption. In contrast, the addition of 5T33MM cells, which *in vivo* do not promote the development of lytic bone lesions or osteoclast formation, also inhibited TRAP+ve osteoclast formation and bone resorption *in vitro*. The demonstration that fewer TRAP+ve cells resorbed similar amounts of bone is consistent with 5T2MM cells promoting osteoclast activity and/or survival. These data suggest that myeloma cells isolated from the bone marrow promote osteoclastic activity directly rather than osteoclast recruitment.

### P13

#### **THE d2 SUBUNIT OF THE VACUOLAR H<sup>+</sup>ATPASE FORMS PART OF THE SPECIALIZED OSTEOCLAST AND RENAL PROTON PUMPS**

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Vacuolar-type H<sup>+</sup>ATPases are multi-subunit proton pumps essential for the acidification of intracellular compartments in eukaryotic cells. Specialized versions are also present at the plasma membrane of osteoclasts and renal intercalated cells (ICs), where they are critically involved in bone resorption and urinary acidification. H<sup>+</sup>ATPases comprise a V1 domain catalysing ATP hydrolysis and a V0 domain responsible for H<sup>+</sup> translocation. The precise contribution of many of the pump's subunits is not yet clear. The B, C, E, G, a, d and e subunits also have multiple isoforms encoded by different genes with differing expression patterns, and it is likely that these play an important role in the cellular localization and activity of H<sup>+</sup>ATPases. Using RT-PCR we have previously shown that in man, the d2 isoform is expressed predominantly in osteoclasts, kidney and lung, whereas d1 is ubiquitously expressed. We wished to determine whether the d2 protein forms part of the osteoclast ruffled border proton pump that contains the a3 subunit, defects of which cause recessive osteopetrosis, and/or the IC apical proton pump that contains the kidney-expressed a4 and B1 subunits, defects of which cause recessive distal renal tubular acidosis.

We have raised a novel polyclonal antibody (SK20) against human d2. SK20 recognizes a protein of appropriate size in membrane extracts from human, rat and mouse kidney, and does not cross-react with the d1 isoform. We found earliest human d2 expression at 12 weeks gestation. Immunolocalization in human neonatal rib bone sections revealed high intensity d2 staining in osteoclasts, which co-localized with a3 subunit staining. In human kidney sections, high intensity d2 staining was seen at the apical surface of ICs in the collecting duct and co-localized with a4 staining. No d2 staining was seen in proximal tubules.

These data demonstrate that d2 coexists with different a subunit isoforms in the specialized proton pumps of the osteoclast and the distal nephron, and further supports the hypothesis that subunit differences play a key role in H<sup>+</sup>ATPase localization and function. Exclusive co-expression of a3 and d2 in osteoclasts might represent a novel therapeutic target for the treatment of osteoporosis.

### P14

#### **SF-36 SCORE IS ASSOCIATED WITH FEMORAL BONE MASS IN MIDDLE AGED UK MEN**

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While several studies relate the impact of osteoporotic fracture on quality of life, no previous work has related bone mineral density (BMD) to SF-36, a validated questionnaire widely used to evaluate general health.

We therefore studied 498 men and 468 women born in Hertfordshire between 1931-39 who were still resident there in adult life. These individuals were recruited from a sample of 768 men and 714 women, who had completed a health questionnaire, detailing past medical history, drug history and SF-36, cigarette and alcohol consumption, physical activity and social class. Seven hundred and thirty seven men and 675 women attended a subsequent clinic; subjects were also invited to attend for bone densitometry (DXA), performed at the lumbar spine and femoral neck (Hologic QDR 4500). Full ethical approval and patient consent were obtained. The SF-36 questionnaire was mapped to 8 domains; physical function (PF), role emotional (RE), mental health (MH), bodily pain (BP), role physical (RP), social functioning (SF), vitality (VT) and general health perception (GH), with higher scores implying better status.

Both sexes were less likely to proceed from home questionnaire to bone densitometry if they had scored poorly for PF (men;  $p=0.007$  women;  $p=0.000$ ), RP (men;  $p=0.02$  women;  $p=0.000$ ), SF (men;  $p=0.03$ ,  $p=0.001$ ), VT (men;  $p=0.05$  women;  $p=0.000$ ), GH (men;  $p=0.009$  women;  $p=0.000$ ) or BP (men;  $p=0.02$  women;  $p=0.000$ ), or if they had sustained a fracture after the age of 45 years ( $p=0.04$ ). Among men, lower femoral neck BMD was associated with poor GH ( $p=0.07$ ) after adjustment for age, BMI, social class and lifestyle; higher total femoral BMD was associated with better scores for SF ( $p=0.03$ ), GH ( $p=0.02$ ), PF ( $p=0.03$ ) and VT ( $p=0.05$ ) in a multiple regression model but no relationships were apparent between SF-36 and lumbar spine BMD. No relationships were evident between SF-36 and BMD at any site in women. Adjustment for known co-morbidity (bronchitis, ischaemic heart disease, hypertension, diabetes, stroke) made little difference to our results.

We conclude that poorer functioning (assessed by SF-36) is associated with total femoral BMD in middle aged men (but not women), and may also significantly influence participation in research.

## P15

### TEMPORAL AND SPATIAL DELIVERY OF CELLS AND BIOMOLECULES USING BIOMINERALISED POLYSACCHARIDE CAPSULES FOR SKELETAL TISSUE ENGINEERING.

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The construction of biomimetic micro-environments with specific chemical and physical cues combined with temporal, spatial organization and modulation of distinct cell populations is of key importance in orderly tissue repair and regeneration. We have previously reported on the use of chitosan/alginate polysaccharide capsules to entrap and release viable human cell populations and functional quantities of growth factor *in vitro*. In this study, we detail a simple method for bead-in-bead generation wherein discrete 'guest' capsules within 'host' capsules can be generated containing different human cell populations and biomolecules and provide evidence for slow and programmed release of entrapped biological factors/cells and, ultimately *in vivo* bone formation.

Modulation of release profiles of different factors was confirmed using RNase and paranitrophenol phosphate release from bead-in-bead capsules with presence of both factors determined spectrophotometrically. Bead-in-bead capsules containing human bone marrow cells and rhBMP-2 were then generated. By controlling the extent of mineralization within the alginate/chitosan shell membrane, degradation of the shell wall and release of cells or rhBMP-2 into the surrounding medium could be finely regulated.

Human bone marrow stromal cells, hydroxyapatite particles (50-100microns) soaked in BMP-2 or collagen type I solution, were entrapped in polysaccharide capsules containing human bone marrow stromal cells and polysaccharide capsules were implanted subcutaneously for 5 weeks and for 8 weeks within diffusion chambers in athymic MF1-nu/nu mice. Mineralisation occurred in HBM capsules containing embedded BMP-2 'guest' capsules only with human bone marrow cells. Biomaterialised polysaccharide capsules provide secure microenvironments for the formation of tissue *in vivo* derived from entrapped human cell populations. After 5 weeks implantation in nude mice, capsules remained intact and were surrounded by extensive vascularisation, entrapped human bone marrow stromal cells generated extensive, dense and organised fibrous type I collagen. This study describes, for

the first time, the ability to generate bead-in-bead capsules consisting of spatially separated cell populations and temporally separated biomolecule release, entrapped within alginate/chitosan shells of variable thickness, mineralisation and stability. Such platform technologies and mesenchymal cell populations offer significant potential as multifunctional scaffolds and delivery vehicles in tissue regeneration of hard and soft tissues

## P16

### **STIMULATION OF HUMAN BONE MARROW STROMAL CELLS USING GROWTH FACTOR ENCAPSULATED CALCIUM CARBONATE POROUS MICROSPHERES**

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The fabrication of self-assembling biomineral-related complexes for cell growth and gene/biomolecule delivery offers important opportunities for skeletal repair. We have previously reported on the generation of microporous calcium carbonate spheres by simulation of microscopic shell biomineralisation with intricate architecture. In this study we have examined the potential of these microporous spheres for biomolecule/ DNA delivery, magnetic microbead entrapment for cell localisation and targeting as well as the use of the spheres as a passive matrix nucleators for bone formation, examined using an ex vivo bone defect model. In this work, 5-30 micron-sized calcium carbonate (vaterite) spheroids with elaborate sponge-like macroporous architecture were prepared by passive evaporation of water-in-oil microemulsions<sup>1</sup>. Incorporation of biomolecules within the vaterite microspheres during the synthesis procedure was achieved by mixing bioactive proteins, collagen type I, bovine haemoglobin or bacterial plasmid DNA with supersaturated calcium bicarbonate solutions prior to microemulsion preparation. The biological compatibility of microspheres on live chick bones and the potential of such a material to fill a bone defect was examined using the chorioallantoic membrane (CAM) assay. Fragments of plasmid DNA were incorporated into spheres as determined by staining with ethidium homodimer-1, viewed under confocal microscopy and positive immunostaining of IgG coated magnetic microbeads confirmed encapsulation within microspheres. Release of functional RNase A from microspheres was determined by degradation of RNA incubated with RNase A containing microspheres. FITC microscopy showed collagen type I could also be incorporated around these microstructures. The use of vaterite microspheres to adsorb and deliver active growth factors such as pleiotrophin and an admixture of bone morphogenetic proteins derived from an osteosarcoma was demonstrated using human bone marrow stromal cells. After 7 days ex vivo chorioallantoic membrane culture of pelleted vaterite spheres and human bone marrow cells placed into a chick femur defect resulted in the secretion of an organised collagen matrix. Vaterite spheres containing rhBMP-2 induced osteogenesis in promyoblast C2C12 cells. These studies demonstrate the development of facile techniques for the generation of inorganic scaffolds constructed from porous microspheres that are biocompatible, aid mineralization, and offer potential for growth factor / DNA delivery for skeletal tissue repair.

## P17

### **COL3A1: AN UNSUITABLE COMPARATOR FOR MEASURING COLLAGEN MRNA EXPRESSION LEVELS IN BONE OF PATIENTS WITH OSTEOGENESIS IMPERFECTA**

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Bone fragility in most patients with osteogenesis imperfecta (OI) results from mutations in type 1 collagen genes that lead to reduced collagen protein production (null phenotype) or to collagen with altered structural properties. COL3A1 protein is used as a comparator to determine changes in type 1 collagen protein expression in electrophoretic studies. We have previously described a real time PCR method to quantify the ratio of COL1A1:COL1A2 mRNA in skin fibroblast cultures from patients with OI. Truncating mutations and some amino acid substitutions in the triple helical domain of COL1A1 were associated with reduced COL1A1:COL1A2 mRNA ratio, with values lying within or beyond the range of a null genotype group. We have extended this study to evaluate the effect of mutations on COL1A1:COL1A2 mRNA ratio in bone.

Bone biopsies from 12 unrelated patients with varied OI presentations and known genotypes, 3 normal and 3 null genotype controls were collected into RNA Later[™]. Total RNA was extracted and converted to cDNA. Levels of COL1A1, COL1A2, COL3A1, beta 2-microglobulin (B2M) and albumin cDNAs were quantified using Taqman real-time PCR assays. Assays were optimized for probe/primer concentrations and amplification efficiency. Results were analyzed using the ABI Sequence Detection System software and the SPSS package.

The normalized ratio of COL1A1:COL1A2 mRNA expression in bone from OI null controls and patients was reduced by approximately 40% compared to normal controls (mean, 95% CI = Controls: 1, 0.79-1.25; Nulls: 0.59, 0.49-0.72; Patients: 0.65, 0.46-0.94). These findings concur with our previous data from skin fibroblast cultures. The pooled mean threshold cycle (Ct) for COL3A1 (35.4) and albumin (32.5) were considerably higher than those of B2M (21.6), COL1A1 (21.2) and COL1A2 (21.4). Additionally, COL3A1 showed significantly higher variance of Ct (21.0) compared to B2M (4.3;  $P < 0.001$ ) or albumin (1.5;  $P < 0.001$ ). COL3A1 is therefore not a suitable control to determine changes in type 1 collagen mRNA expression. If the high variance in type 3 collagen mRNA expression correlates with altered COL3A1 protein abundance, this may contribute to the small proportion of OI patients with null genotype who fail to have detectably abnormal protein levels using electrophoretic methods.

## P18

### A HISTOMORPHOMETRIC STUDY OF THE EFFECTS OF STEROID TREATMENT ON THE CORTICAL BONE OF THE ILIAC CREST

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Administration of glucocorticoids for the treatment of a variety of disorders is associated with increased bone loss and fracture risk. The effects of glucocorticoids on cancellous bone remodelling and structure are reasonably well documented but there are no reported histomorphometric studies in human cortical bone. The aim of this study was to investigate the effect of long-term glucocorticoids on iliac crest cortical bone in 14 patients, 9 females and 5 males, aged 18-48 years (mean 34 years), using Image analysis. Results were compared with those obtained in a group of premenopausal women with untreated endometriosis aged 23-40 years (mean 31.1 yrs).

Cortical width and area were similar in the two groups. However, cortical porosity was significantly higher in patients treated with glucocorticoids (10.5 + 11.8 %; mean + SD) compared with the untreated group (5.1 + 3.9 %;  $p = 0.006$ ). The Haversian canal number and density were also significantly higher in glucocorticoid treated patients (46.1 + 23.7 vs 27.8 + 18.6 and 16.6 + 13.0 vs 6.6 + 3.5 /mm<sup>2</sup>;  $p = 0.0003$  and  $p = 0.00002$  respectively), but Haversian canal area did not differ significantly between groups.

The mean wall width of the osteons was significantly lower in the treated patients compared to the untreated group (48.8 + 7.1 microns vs. 59.8 + 12.9 microns;  $p = 0.01$ ). Bone formation rate (microns squared/microns/day) and mineral apposition rate (microns/day) were also both significantly reduced in patients receiving long-term glucocorticoid therapy when compared to the untreated group (0.056 + 0.040 vs. 0.095 + 0.058 and 0.59 + 0.12 vs. 0.74 + 0.13;  $p = 0.05$  and  $p = 0.01$ , respectively). The proportion of canals with an eroded surface was lower in the treated group when compared with the untreated control group.

These results demonstrate that cortical porosity is increased in patients treated with long-term glucocorticoid therapy, due to an increase in the number rather than size of Haversian canals. This may be due to a long-term impairment of bone formation resulting in a significant decrease in both mean wall width of the osteons and bone formation rate at tissue level. Effects on cortical width were not demonstrated, possibly as a result of the relatively small sample size.

## P19

### **A LONGITUDINAL STUDY OF BONE-RELATED BIOCHEMICAL VARIABLES ACROSS THE MENOPAUSE**

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Cross-sectional studies have shown major changes in certain calcium-related variables at the menopause which needed to be validated by a longitudinal study which we have now completed.

We recruited by advertisement and initial screening, 104 normal premenopausal women over the age of 44 in whom we measured radiocalcium absorption, serum FSH and oestradiol, and collected venous blood and urine samples which were deep frozen. We then followed the subjects annually by questionnaire and hormone measurements until they had had amenorrhoea and a raised FSH level for at least one year, when radiocalcium absorption was remeasured and further blood and urine samples collected and deep frozen. We closed the study when 34 women had passed through the menopause, and then thawed the samples and measured 12 variables in paired blood samples and 6 in paired urine samples.

There were significant rises in total serum calcium and in the calculated ionised, ultrafiltrable and complexed calcium fractions and in serum bicarbonate, alkaline phosphatase, urine calcium and crosslinks excretion and significant falls in gastrointestinal absorption and renal tubular reabsorption of calcium (all  $P < 0.001$ ). There was no significant change in serum 1,25D, 25D, PTH, proteins, anion gap or creatinine. Despite these changes in many of the relevant biochemical variables, there were significant correlations between the first and second measured values with r-values ranging from 0.40 ( $P < 0.05$ ) for urine calcium up to 0.53 for ionised calcium, 0.56 for PTH, 0.57 for protein-bound calcium and 0.67 for radiocalcium absorption (all  $P < 0.001$ ).

We conclude that there are menopausal falls in calcium absorption without change in serum calcitriol and in tubular reabsorption of calcium without change in PTH which are probably due to the loss of direct effects of oestrogen on these end organs. There is also a probable rise in the PTH set-point. Despite these and other very significant changes in bone-related biochemical variables, the ranking of the subjects in respect of most of these variables remains relatively constant. These observations are of considerable potential significance for the understanding of calcium homeostasis in general and the changes at the menopause in particular.

## P20

### **HYPOVITAMINOSIS D AMONG HEALTHY ADOLESCENT GIRLS**

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Background: There has been a resurgence of vitamin D deficiency rickets among toddlers in the UK. We have recently observed an increase in the number of adolescents presenting with symptomatic vitamin D deficiency (Crocombe et al. Arch Dis Child 2004; 89: 197-199. This cross-sectional study was designed to determine the prevalence of hypovitaminosis D among healthy adolescent schoolgirls. Methods: Fifty one (28%) out of 182 girls [14 white (W) & 37 non-white (NW); median 15.3 yrs & range 14.7 to 16.6 yrs] attending year 10 of an inner city multiethnic girls' school took part in the study. We assessed their serum concentration of 25-hydroxyvitamin D (25OHD; a measure of vitamin D status) and related it to dietary intake of vitamin D, estimated duration of sunshine exposure (SE) and the percentage of body surface area exposed (%BSAE).

Results: Forty one (80%) girls were vitamin D deficient (25OHD < 15 ng/ml) and 9 (17%) were severely deficient (25OHD < 5 ng/ml). The median 25OHD concentration of W girls (14.9 ng/ml & range 7.3 to 29.3) was significantly higher ( $p < 0.001$ ) than that of NW girls (5.9 ng/ml & range 2.3 to 17ng/ml). The estimated intake of vitamin D in W and NW groups was 1.21 Åg/day & 1.42 Åg/day respectively; it was not related to 25OHD concentration in either group.

For the whole group, 25OHD concentration was related to the estimated SE ( $r=0.38$ ;  $p=0.007$ ) and %BSAE ( $r=0.41$ ;  $p=0.003$ ). In W girl the estimated SE and %SAE were significantly higher than that of NW girls,  $p=0.003$  and  $p=0.001$  respectively.

Conclusions: Hypovitaminosis D is common among healthy adolescent girls; NW girls were more severely deficient. Reduced sunshine exposure rather than diet explains the difference in vitamin D status of W and NW girls. Since vitamin D is essential for bone mass accrual during adolescence, vitamin D supplements should be given to girls with reduced sunshine exposure.

## P21

### REGULATION OF OSTEOCLASTOGENESIS BY RHBMP-2 AND RHVEGF

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BMPs, VEGF and other cytokines are released from bone during bone resorption. Recent study showed that VEGF caused a dose- and time-dependent increase in bone resorption *in vitro* and *in vivo*, and BMP-2 markedly enhanced osteoclast differentiation induced by sRANKL and M-CSF in mouse osteoclast culture system. The aim of this study was to further examine the effects of VEGF and BMP-2 on osteoclastogenesis using *in vitro* human osteoclast culture system.

Mononuclear cells were isolated by density gradient centrifugation from bone marrow washouts. Mononuclear cells were plated at  $1 \times 10^6/\text{cm}^2$  in MEM and 15% FCS. At day 7, the non-adherent cells were collected and cultured in 24-well plates or calcium phosphate (Ca-P) coated plates, with osteoclast-inducing media (OC media) containing sRANKL 30 ng/ml and M-CSF 30 ng/ml. rhBMP-2 (3, 30, 300 ng/ml) and VEGF (25 ng/ml) were added respectively or in combination to the cell culture, and the culture was kept for total 16 days. The number of TRAP positive multinuclear cells in each well and the resorptive pit areas on the Ca-P coated plates were compared.

By day 12-14, TRAP and VNR positive osteoclastic cells were found in all the experimental groups. The number of TRAP+ multinuclear cells were significantly reduced ( $p<0.05$ ) when rhBMP-2 (30 and 300 ng/ml) were present, and this was further reduced ( $p<0.01$ ) when rhVEGF was added together with rhBMP-2. Extensive lacunar resorption pits in the Ca-P coated plates were found in the culture treated with OC media and OC media with rhVEGF (25 ng/ml). The resorption pit areas were significantly reduced when rhBMP-2 was added at 30 and 300 ng/ml with or without rhVEGF (25 ng/ml,  $p<0.05$ ).

The present study had shown that the presence of rhBMP-2 at 30 and 300 ng/ml had strongly inhibited osteoclast differentiation and bone resorptive capability in the human osteoclast culture system, and the inhibition was further enhanced by the presence of rhVEGF. This study implies that VEGF and BMP-2 may be important, yet to be defined regulators, for osteoclastogenesis.

## P22

### EFFECT OF TRANSFORMING GROWTH FACTOR-BETA ON OSTEOCLAST PRECURSOR NFATC1 AND C-FOS EXPRESSION

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Understanding of the signalling pathway that commits mononuclear precursors to the osteoclastic lineage has advanced rapidly over the last few years. It is clear that osteoclast differentiation is initiated by osteoblast expressed RANKL, which activates a complex signalling cascade that results in the expression of osteoclast specific genes. In addition to RANKL we have shown that TGF-beta has an essential role in maintaining the responsiveness of non-committed precursors to RANKL. It is likely that TGF-beta facilitates osteoclastic differentiation through its ability to suppress anti-osteoclastic signals that commit precursors to inflammatory roles. Recently, we have shown that this is in part due to the ability of TGF-beta to induce a sustained elevation in the expression of SOCS3, which inhibits JAK/STAT activation by several anti-osteoclastic cytokines. However, SOCS3 expression does not account for all of the facilitative effects of TGF-beta. We therefore examined the

effect of TGF-beta on the expression of NFATc1, a transcription factor with an essential role in osteoclast differentiation.

In keeping with its ability to enhance osteoclast formation we found by Northern analysis that TGF-beta (0.4 ng/ml) augmented RANKL-induced (30 ng/ml) NFATc1 expression. Moreover, TGF-beta alone appeared to induce NFATc1 expression within 24 hours in cultures of non-committed osteoclast precursors. This increase was not due to an indirect augmentative action on osteoclast-inductive stimuli present in the culture environment as TGF-beta induced NFATc1 expression even in the presence of excess OPG (100 ng/ml) and anti-TNF-alpha antibodies (10 mg/ml).

The ability of TGF-beta to directly induce NFATc1 expression raises the question of why TGF-beta alone is unable to stimulate osteoclast formation. Evidence suggests that NFATc1 forms a complex with c-fos to control the transcription of osteoclastic genes. We therefore examined the effect of TGF-beta on c-fos expression in our system and found that, unlike RANKL, TGF-beta was unable to induce c-fos expression. Thus, our data suggests that a further mechanism by which TGF-beta could enhance osteoclast formation is through its ability to directly induce and augment RANKL-induced NFATc1 expression.

## **P23**

### **THE PRESENCE OF GLYCOSAMINOGLYCAN EPITOPES IN CHONDROGENIC CULTURES OF STRO-1+ IMMUNO-SELECTED HUMAN MARROW STROMAL CELLS**

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A potential candidate cell source for the biological repair of cartilage following trauma is the mesenchymal stem cell (MSC) population. The attraction in the use and manipulation of these cells lies in their proliferative capacity, multipotentiality and lack of immunogenicity. However, judicious selection of MSC populations for cartilage cell therapy over chondrogenic populations, or unselected marrow populations, will require demonstration of an appropriate glycosaminoglycan-enriched matrix. The aim of this study was to evaluate, using micromass pellet culture, the potential of STRO-1+ immuno-selected human MSC to form a cartilaginous phenotype in comparison to unselected human bone marrow populations and human articular chondrocytes. STRO-1+ marrow cells were isolated from human marrow aspirates (3 samples, 76±6 years of age) using magnetic activated cell sorting. Human articular chondrocyte populations were isolated by collagenase digest from femoral heads. STRO-1+ selected cells, unselected marrow stromal cells and human articular chondrocytes were cultured in pellet culture for 21 days in defined media containing 10 ng/ml TGFbeta3. Glycosaminoglycans (GAG) were extracted and measured by dye binding to di-methyl-methylene-blue (DMMB), with further examination by western blotting using a panel of unique antibodies for aggrecan (2-B-6, 3-B-3, 1-B-5, 7-D-1), Link (8-A-5) and type II collagen. In addition, pellet cultures were examined for histology and type II collagen immuno-histochemistry and data compared to articular cartilage.

No significant difference between pellet culture groups were observed (17.0 ± 3.1 mg GAG in unselected MSC; 15.8 ± 3.3 mg GAG in STRO-1+ selected MSC; 13.8 ± 2.7 mg GAG in chondrocytes) however, there was significantly (\*\* p<0.005) more GAG than in the monolayers of chondrocytes (1.3 ± 1.2 mg GAG) and significantly (\*\*\*)p< 0.001 less than in articular cartilage (51.4 ± 12.2 mg GAG). Pellets were positive for type II collagen by western blot. STRO-1+ samples expressed a variety of chondroitin sulphate epitopes, but with observably less material than in articular cartilage. These studies demonstrate the potential for chondrogenic differentiation of STRO-1+ selected MSC and the use of this culture system to delineate the pattern of GAG expression and extracellular matrix production critical in tissue engineering of cartilage for clinical application.

## P24

### **ADRENAL ANDROGENS REGULATE THE PRODUCTION OF AUTOCRINE AND PARACRINE FACTORS IMPLICATED IN BOTH BONE RESORPTION AND FORMATION IN A HUMAN OSTEOBLASTIC CELL LINE**

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The adrenal androgens ; dehydroepiandrosterone (DHEA) and androstenedione (ANDI) may have anti-resorptive as well as anabolic effects on bone. The aim was to assess the regulation of cytokines involved in bone remodelling by DHEA, ANDI and Dexamethasone (DEX) in a human osteoblastic cell line (HCC1). Expression of osteoprotegerin (OPG), RANKL, IL-6 was measured following treatment of the cells with DHEA, ANDI (1 pmol to 1 micromol) and DEX. The expression of a variety of cytokines and their receptors was assessed using a human cytokine/receptor gene array consisting of 268 cytokine-related cDNAs. An intensity ratio of the cDNA spots between the treated array and control array was set at >1.5 or < 0.66. DEX treatment (1nmol to 0.1 micromol) reduced OPG (0.1micromol : 33.7% [4.7] p < 0.001). OPG production also decreased with DHEA and ANDI (0.1 micromol) (DHEA 52.5% [7.8] p <0.001, ANDI 51.2% [4.5] p<0.001). This was not inhibited by co-treatment with the androgen receptor antagonist, flutamide (0.1 micromol). Co-treatment of ANDI (10 nmol, 1.0 micromol) with the aromatase inhibitor, ketoconazole ) reversed the inhibition in OPG production. DEX led to an increase in RANKL production (185% [87.7]) which was abolished by co-treatment with ANDI (77.3 % [12.1] p=0.08) and DHEA (47.4% [8.8] p=0.08 ). A significant reduction in IL-6 was observed with DEX (14.1[5.4] %, p < 0.01), ANDI (28% [9.7], p <0.02) and DHEA (0.1 micromol) (14% [9.8] p <0.05). OPG mRNA decreased with DEX (33.3% [1.3], p <0.001), ANDI (36% [1.3] p <0.001) and DHEA (48%<sup>1</sup>, p<0.02). DEX increased RANKL mRNA (391.1% [147], p=0.14). DHEA alone or with DEX resulted in a significant reduction in RANKL mRNA (76%<sup>3</sup> p=0.02, 46%<sup>3</sup> p<0.001). DHEA and ANDI reversed the DEX-induced increase in RANKL/OPG ratio. DEX had a mostly down-regulatory effect on many genes. In contrast, DHEA up-regulated over 30 genes including a number of growth factors which have been shown to promote osteoblastic proliferation. Our data suggest that the adrenal androgens may slow bone resorption through their conversion to oestrogens. DHEA may also stimulate osteoblasts and counteract some of the catabolic effects of glucocorticoids on the skeleton.

## P25

### **HUMAN PHOSPHO1 DISPLAYS HIGH SPECIFIC PHOSPHOETHANOLAMINE AND PHOSPHOCHOLINE PHOSPHATASE ACTIVITIES: A MEANS OF GENERATING INORGANIC PHOSPHATE IN MINERALISING CELLS**

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Skeletal mineralisation is dependent on the generation of inorganic phosphate (Pi) and traditionally this action has been attributed to the bone/liver/kidney isoform (TNAP) of alkaline phosphatase. However, some evidence exists to suggest the existence of other Pi generating phosphatases in bone. In newborn TNAP knockout mice, bone development and mineralisation appear to be normal, although hypomineralisation and other abnormalities of the skeleton and dentition have subsequently been observed. TNAP can also be removed from some preparations of matrix vesicles without reducing their potential to mineralise whilst specific inhibitory studies on TNAP provide additional evidence that other phosphatases are present within differentiating bone cells. We have previously reported a novel phosphatase, PHOSPHO1, whose expression is upregulated in mineralising osteoblast-like cells and is located to mineralising surfaces in both bone and cartilage. From these findings PHOSPHO1 has been implicated in the generation of Pi for mineralisation. PHOSPHO1 is a phosphatase, which belongs to the haloacid dehalogenase (HAD) superfamily of magnesium-dependent hydrolases. The natural substrate(s) of PHOSPHO1 is as yet unidentified and no information has been gathered with regard to its activity. In this study recombinant His-tagged PHOSPHO1 (rPHOSPHO1) was produced by inserting the coding region (from SaOS-2 cells) into a E. Coli expression vector. Following expression and subsequent purification

rPHOSPHO1 was used in phosphatase assays to identify two potential substrates, phosphoethanolamine (PEA) and phosphocholine (PCho). PHOSPHO1 catalyses the hydrolysis of PEA and PCho displaying  $K_m$  and  $V_{max}$  values of 3  $\mu\text{M}$  and 4.1  $\mu\text{mol}/\text{min}/\text{mg}$ , for PEA, and 11.4  $\mu\text{M}$  3.6  $\mu\text{mol}/\text{min}/\text{mg}$ , for PCho at optimal pH (6.7). Additionally, it was found that PHOSPHO1 is typical of most enzymes within the HAD superfamily, displaying high catalytic activity in the acid-to-neutral pH range and optimal activity in the presence of  $\text{Mg}(\text{II})$  compared to other divalent cations. These results suggest the involvement of PHOSPHO1 in the synthesis of ethanolamine and choline from phosphatidylethanolamine and phosphatidylcholine, respectively. Phosphatidylethanolamine and phosphatidylcholine are precursors in the synthesis of complex glycerolipids. These results therefore, provide a mechanism whereby  $\text{P}_i$  may be generated in mineralising cells from the degradation of phospholipids.

## P26

### CHONDROPTOSIS: A VARIANT OF APOPTOTIC CELL DEATH IN CHONDROCYTES?

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Evidence has accumulated in recent years that programmed cell death is not necessarily synonymous with the classical apoptosis, as defined by Kerr & Wyllie, but that cells use a variety of pathways to undergo cell death, which are reflected by different morphologies. Although chondrocytes with the hallmark features of classical apoptosis have been demonstrated in culture, such cells are extremely rare *in vivo*. We have examined the morphological differences between dying chondrocytes and classical apoptotic cells in growth-plate and osteoarthritic chondrocytes. Unlike classical apoptosis, chondrocyte death involves an increase in endoplasmic reticulum and Golgi apparatus. This is likely to reflect an increase in protein synthesis with retention of proteins in the ER leading to expansion of the lumen, whose membranes surround and compartmentalise organelles and parts of cytoplasm. The final removal of apoptotic remains does not involve phagocytosis, but a combination of three routes: 1) auto-digestion of cellular material within compartments formed by ER membranes; 2) autophagic vacuoles and 3) extrusion of cell remnants into the lacunae. Together these processes lead to complete self-destruction of the chondrocyte as evidenced by the presence of empty lacunae. The involvement of ER suggests that the endoplasmic reticulum pathway of apoptosis may play a greater role in chondroptosis than receptor-mediated and mitochondrial pathways. Lysosomal proteases, present in autophagic digestion, are likely to be as important as caspases in the programmed cell death of chondrocytes *in vivo*. We propose the term 'chondroptosis' to reflect the fact that such cells are undergoing apoptosis, albeit in a non-classical manner, but one that appears to be typical of programmed chondrocyte death *in vivo*. Chondroptosis may serve to eliminate cells that are not phagocytosed by neighbouring cells, which constitutes a crucial advantage for chondrocytes that are typically embedded in an extracellular matrix. Classical apoptosis in that situation is likely to lead to secondary necrosis with all its disadvantages. This may be the reason why most programmed cell death of chondrocytes *in vivo* appears to follow a chondroptotic and not the classical apoptotic pattern. At present the initiation factors or the molecular pathways involved in chondroptosis remain unclear.

## P27

### HYDROSTATIC PRESSURE AND UHMWPE PARTICLES MODULATE SYNTHESIS OF 1,25 DIHYDROXYVITAMIN D3 BY MACROPHAGES

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Hydrostatic pressure in the joint can be raised in aseptic loosening, but the mechanisms by which pressure could enhance loosening are unclear. We showed that hydrostatic pressures increased MP synthesis of various factors implicated in bone resorption<sup>1,2,3</sup>. These factors are known to be important in bone resorption (BR) and bone turnover, but the mechanisms by which these factors affect BR are unclear. 1,25-dihydroxyvitamin D3 (1,25D3) stimulates osteoclast activity. Macrophages (MP) can synthesise 1,25D3 and differentiate into osteoclast-like cells. We examined the effect of hydrostatic pressure on MP synthesis of 1,25D3. MP from normal human peripheral blood were exposed to physiological pressure

(34.5x10<sup>-3</sup>MPa) and/or UHMWPE particles and the effect on 1,25D3 synthesis examined. Synthesis of 1,25D3 was measured by HPLC and was increased in cells under pressure by a mean of 17% compared to controls. Image analysis of expression of 1 OHase (assayed by in situ hybridisation) showed a small increase in response to pressure (37%) and to particles (59%), and a larger increase to the two stimuli simultaneously (100%). These results show that exposure to either UHMWPE particles or pressurization (previously shown to increase cytokine synthesis) increases MP synthesis of 1,25D3. The effect of the two stimuli is additive, suggesting that 1,25D3 may be one of the factors which stimulates osteoclastic bone resorption in aseptic loosening. This synthesis could further exacerbate loosening, as both these stimuli are likely to be present *in vivo*.

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Role of cyclical pressure in release of M-CSF, chemokines and PGE2 and their role in implant loosening. JBJS 85-B(2) :288091; 2003

## P28

### MACROPHAGE ACTIVITY *IN VITRO* IS MODULATED BY TNF $\alpha$

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Hydrostatic pressure in the joint can be raised in aseptic loosening. We showed previously that hydrostatic pressure modulated synthesis by macrophages (MP) of several cytokines and other factors stimulating bone resorption, which could be important in the development of loosening. This study examined the relationship between synthesis of TNF $\alpha$  and other cytokines by MP. MP from human peripheral blood were exposed to pressure and/or UHMWPE particles. The effects of TNF $\alpha$  antibody was also examined. There was reduced synthesis of IL-1 $\beta$  and IL-6 by MP cultured with anti-TNF $\alpha$ , at the different pressures and cell densities tested. Incubation of MP at 1x10<sup>5</sup>/ml with anti-TNF $\alpha$  antibody reduced synthesis of all three cytokines at day 0 and day 7, when compared to cultures incubated without antibody. TNF $\alpha$  concentration increased with increasing cell density and exposure to pressure (34.5x10<sup>-3</sup>MPa). This increase was greatest (115%) in the cultures incubated for 7 days, at the highest cell density of 5x10<sup>5</sup>/ml and was less (33%) in cultures at 2.5x10<sup>5</sup>/ml. This density-dependent effect was not seen with cultures incubated overnight. Incubation of MP cultures at 5x10<sup>5</sup>/ml with antibody reduced TNF $\alpha$  synthesis to <1.0% of the control cultures. The antibody only reduced IL-1 $\beta$  to 81% and IL-6 to 69% of controls. Stimulation of cytokine synthesis by pressure did not overcome this reduction in TNF $\alpha$  and levels of IL-1 $\beta$  and IL-6 were similar to unpressurised cultures (88% and 73% of controls). A separate study looked at 1,25-dihydroxyvitamin D3 (1,25D3) and a positive correlation (r<sup>2</sup>=0.73) was demonstrated between TNF $\alpha$  and 1,25D3. These results suggest that TNF $\alpha$  may be an upstream initiator of IL-1 $\beta$  and IL-6 and that there is an interrelationship between pressure-related increases in TNF $\alpha$  and 1,25D3 production. Further research is necessary to elucidate this complex relationship, which has implications for the *in vivo* situation.

## P29

### EFFECTS OF VITAMIN K2 (MK-4) ON HUMAN OSTEOBLAST PROLIFERATION AND DIFFERENTIATION

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Vitamin K2 has been shown to affect the regulation of bone metabolism. MK-4 enhances gamma-carboxylation of bone glutamic acid residues and secretion of osteocalcin. In culture, MK-4 inhibits the formation and activity of osteoclasts, induces osteoclast apoptosis and increases the number of CFU-F/ALP<sup>+</sup> colonies, partly by inhibiting osteoblast apoptosis. Micromolar levels of MK-4 activate the nuclear receptor SXR and induce CYP3A4 and bone markers in human osteosarcoma cells.

We have investigated the effects of 0.1 nM to 10 microM MK-4 on the proliferation and differentiation of human bone-derived osteoblasts. Osteoblasts were cultured in medium containing increasing concentrations of MK-4. Cell proliferation after 8 days was measured using MTS tetrazolium compound. Conversion to formazan was measured at 490/600 nm in a plate reader. Cell proliferation progressively decreased at MK-4 concentrations less than 0.1 microM but then increased to give significantly greater proliferation of cells cultured with 10 microM MK-4 than of control cells with no MK-4.

Cells cultured for 2 and 12 days in small flasks were harvested into Trizol reagent for total RNA isolation. mRNAs for COL1A1, ALP, OC, RANKL, OPG, CYP3A4 and GAPDH were measured using real-time RT-PCR. Samples were quantified by the comparative cycle threshold (Ct) method for relative quantification of gene expression, normalized to GAPDH. Compared to control cells with no MK-4, there were significantly greater increases with time in ALP/COL1A1 mRNA in cells cultured with 0.1 nM to 10 nM MK-4 but less increase with time in this ratio in cells cultured with 10 microM MK-4 ( $p < 0.05$ ). OC mRNA levels did not change with time. In cells cultured with 0 nM, 0.1 nM and 1 nM MK-4, CYP3A4 mRNA remained unchanged with time while RANKL/OPG mRNA ratios decreased. Both of these mRNA measurements increased significantly at higher doses. Compared to controls, cultures with 10 microM MK-4 showed 25% less increase with time in ALP/COL1A1 mRNA but 7-fold greater increase in CYP3A4 mRNA and 16-fold greater increase in RANKL/OPG mRNA.

Thus, MK-4 concentrations less than 0.1 microM significantly reduce the proliferation and enhance the differentiation of cultured human osteoblasts compared to controls. However, at micromolar concentrations, MK-4 has the opposite effect.

### **P30**

#### **MATHEMATICAL MODEL SIMULATIONS SUPPORT THE REGULATORY ROLE OF THE MATRIX IN BONE FORMATION VIA EMBEDDED MITOGENIC FACTORS**

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The development of pharmaceutical treatments for bone disease can be enhanced by mathematical models that predict their effects on formation during cancellous bone remodelling.

The amount of bone formed and the duration of the formation phase at one micro-site on the surface of cancellous bone is simulated by changes in cellular activity (described by Michaelis-Menten equations) and the number of osteoblasts (calculated from a relationship that describes the proliferation of muscle stem cells in the presence of growth factors (Deasy et al., 2002)).

The model incorporates four feedback effects on the rate of bone formation:

1. the reduction in size of mature osteoblasts (and endoplasmic reticulum and Golgi apparatus) with differentiation, which limits the maximum capacity of cellular osteoid-forming activity ( $V_{Max,Obl}$ );
2. the reduction in cell numbers due to osteocyte formation;
3. the apoptosis of surplus osteoblasts that are not required for lining cell or osteocyte formation;
4. and, as mineralization progresses, the limited availability of osteoid substrate.

The model was parameterised by fitting simulations to data of changes in absolute osteoid seam thickness and its depth of formation and mineralization, together with relative changes in rates of osteoid formation and calcification (Eriksen et al., 1984).

Simulated depth of new bone formed was increased by 12.2% with a 2% increase in mitotic fraction, and increased by 6.4% with a 2% decrease in division time. The duration of formation was increased by 2.6% with a 2% increase in  $V_{Max,Obl}$ . A 2% change in other input variables produced changes in depth and duration of less than 2%.

Model simulations during sensitivity analysis provided insights into the different relationships between the amount of bone formed and the duration of bone formation, and the variables that determine either the number of mature osteoblasts, or their activity. The high sensitivity of bone formation to mitotic fraction supports the potential regulatory role played by the matrix via embedded mitogenic factors.

### P31

#### **PATHOPHYSIOLOGY OF BONE LOSS AFTER STROKE; A ROLE FOR VITAMIN D?**

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A 73 year old man was admitted with the sudden onset of left flaccid hemiparesis affecting his face, arm and leg. He had previously been well and independently mobile. A CT scan confirmed a right-sided thalamic haemorrhage and he was managed on the acute stroke unit. He consented to take part in an ongoing randomised double-blind placebo controlled study to prevent bone loss after stroke with a single 4mg zoledronate infusion. The baseline serum vitamin D (25OHD) was 13.7 ug/l (34.2 nmol/l) with calcium 2.2 mmol/l, alkaline phosphatase 99 U/l and PTH 25 ng/l (2.65 pmol/l). Although he was able to walk after 6 months, the majority of his day was spent self propelling in a wheelchair. Bone mineral density measurements at 0, 6 and 12 months revealed a 6.4% decrease at the hemiplegic total hip region, with a 1.2% increase at the unaffected total hip (method precision CV 1.03).

Vitamin D insufficiency is common in Japanese inpatients following stroke and in such patients vitamin D status has been correlated with BMD in the hemiplegic finger and post-stroke hip fracture. A sequential group of stroke inpatients from the aforementioned bisphosphonate study (n=33, 22M, 11F, mean age 71.1 +/- 10.7) had 25OHD levels measured within one month of stroke using a radioimmunoassay technique. A local cohort of healthy control patients (n= 96, 48M, 48F, mean age 69) had 25OHD levels measured at 2 monthly intervals for 14 months. From the healthy population, mean and SD values of 25OHD for any month were derived. Seasonally adjusted Z scores were subsequently calculated for the stroke patients enabling comparison with the healthy elderly group. In the stroke patients, the mean Z score of 25OHD was Z= -1.18 (95%CI: -1.00, -1.34).

Vitamin D insufficiency was common in this group of acute stroke inpatients and may have been present before admission. General immobility, unloading of the hip region and vitamin D osteopathy are likely to contribute to the bone loss observed following stroke. The case described illustrates the need for careful medical management of stroke patients at risk for bone loss, vitamin D insufficiency and fracture.

### P32

#### **BIOCHEMICAL BONE MARKERS IN COMBINATION WITH BONE MINERAL DENSITY ARE USEFUL IN IDENTIFYING PATIENTS WITH GREATEST FRACTURE RISK IN OSTEOPOROSIS**

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In Northern Ireland, bone markers have been measured over the past three years mainly to monitor response to anti-resorptive treatment in osteoporosis. Proposed national guidelines for the management of osteoporosis and related fractures threaten to overlook many women at high fracture risk. We studied bone mineral density (BMD) and bone markers in 79 local women to ascertain if markers of bone formation and resorption, when used alongside BMD, could improve identification of women at high fracture risk.

The women were divided into 3 groups i) postmenopausal osteoporotic, ii) postmenopausal non-osteoporotic and iii) premenopausal non-osteoporotic. This was done using a single BMD measurement by dual energy x-ray absorptiometry (DEXA). Serum bone formation markers (bone specific alkaline phosphatase (BSAP) and osteocalcin (OC)), and resorption marker (C-telopeptide of type 1 collagen (CTX)), were measured. A questionnaire detailing health including low trauma fracture history was completed by each patient.

Results showed serum bone formation markers increased with age and menopausal status even in those with normal BMD. There was a further increase seen in those with osteoporosis, but no previous fracture, when compared to postmenopausal non-osteoporotics (12% rise in mean BSAP, 10% rise in mean OC). A larger increase was seen between osteoporotics with previous fracture when compared to postmenopausal non-osteoporotic controls (70% rise in mean BSAP and 75% rise in mean OC). Mean serum CTX was relatively similar in non-osteoporotic pre- and post-menopausal women (2001 picomoles/litre and 2056 picomoles/litre respectively). In the osteoporotic women, when subdivided into those with

previous fracture and those without fracture, mean CTX was 59% higher in the fracture subgroup compared to the non-fracture subgroup (4327 picomoles/litre and 2721 picomoles/litre respectively). When compared to postmenopausal non-osteoporotics, the osteoporotic non-fracture subgroup had a mean CTX 32% higher; the fracture subgroup had a mean CTX more than twice that of postmenopausal non-osteoporotics (110% increase).

We conclude that the results support the combined use of bone markers and BMD measurement in assessment of fracture risk in osteoporotic patients. In particular, mean serum CTX remains similar in pre- and postmenopausal non-osteoporotics, but increases by over 100% in osteoporotics with previous fragility fractures.

### **P33**

#### **ULTRASOUND STIMULATES ATP RELEASE FROM HUMAN OSTEOBLASTS *IN VITRO***

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Low-intensity pulsed ultrasound (US) has been shown to cause a decrease in time to fracture healing, to reduce the incidence of delayed union, and to stimulate union in established non-united fractures in numerous studies. The mechanism by which US induces these responses, however, remains unclear although some growth factors and local mediators have been implicated in the fracture repair process following US stimulation. There is a growing volume of literature to support the role ATP in accelerating bone growth and remodelling. High concentrations of extracellular ATP are likely to be found at fracture sites due to cell lysis, and ATP is also released constitutively from intact osteoblasts; a process that is enhanced following mechanical stimulation. We therefore hypothesised that ATP release following US stimulation of human osteoblasts could be increased, and that this in turn might lead to changes in proliferation and gene expression in osteoblasts that would contribute to accelerated fracture healing. US was applied to SaOS-2 human osteoblast-like cells, and the concentration of ATP in the cell culture medium determined. The cells were subsequently harvested for investigation of gene expression using quantitative RT-PCR. Increased concentrations of ATP were detected in the cell culture medium of US-treated cells, and US also caused increased expression of c-fos, collagen I, and RANKL, and decreased expression of OPG by SaOS-2 cells. ATP stimulation of these cells caused similar changes in expression of these genes except collagen I. In addition, both US and ATP stimulated SaOS-2 cell proliferation. These findings indicate that US does cause ATP release by osteoblasts *in vitro*, and that this may contribute to the decrease in time to fracture healing that is commonly clinically observed by increasing expression of RANKL and decreasing expression of OPG by osteoblasts to promote osteoclastogenesis, and enhancing the proliferation of osteoblasts.

### **P34**

#### **TYPES OF ACTIVITY AND BARRIERS TO PARTICIPATION IN PHYSICAL ACTIVITY IN AN OLDER POPULATION WITH AND WITHOUT MINIMAL TRAUMA FRACTURES**

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**BACKGROUND AND OBJECTIVES:** Physical activity is essential for preserving bone health and preventing falls and fractures. In the older population participation in physical pursuits tends to diminish with age and perceived barriers to participation, and reasons for diminution should be ascertained. In this survey type and level of physical pursuits, and perceived barriers to participation were assessed in people aged 60 to 79 years, with and without recent low trauma fractures.

**METHODS:** A questionnaire was administered during interview to 207 patients (26; 12.6% male; 181; 87.4% female), to determine lifestyle behaviours in the three months preceding the fracture or interview. Excluded were those with diseases related to inactivity or osteoporosis. For the fracture group (Fg) (n = 120; 12.5% male) interviews were conducted in the fracture clinic or ward. For the non-fracture group (NFg n=87) obtained from local GP lists and matched for race, age and gender telephone interviews were conducted. Descriptive and qualitative analyses were used.

**RESULTS:** While the Fg were significantly less active than the NFg (p<0.01), the range of activities was similar for both groups. Gardening, walking and 'walking the dog' emerged as

most popular with participation ranging from 20% to 63%. Less than 10% participated in swimming, dancing or exercise classes and less than 5% in cycling, bowls, rambling, darts, fishing or golf.

More than half reported a decrease in activity since retirement (Fg 57% 68/120; NFg 55% 48/87) against an increase by only 7% and 18% respectively.

Prime barriers to participation to emerge were:

musculo-skeletal problems

- The arthritis in my (hands / knee) makes it difficult to continue with .... (handcrafts / walking)'.  
bereavement

- 'I used to (walk / play tennis) with my friend / partner but since s/he moved away / died I don't do that any more'.

and fear of falling

- 'I am afraid of falling again ....and so I don't go walking any more.'

In conclusion, the picture that emerged reveals a limited participation in a small range of activities with specific factors contributing to an inactive lifestyle. These factors should be addressed in strategies designed to encourage activity in older people.

### **P35**

#### **MORPHOLOGICAL CHECKING THE JAW DEFECT AFTER ITS REPLACING BY THE MATERIAL 'LITAR'**

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For the purpose of regenerating the bone tissue and preserving the viable tooth follicle we have used the hydroxyapatite-collagen material 'LitAr'. 'LitAr' is a highly structured material in which the inorganic component (hydroxyapatite) is formed as the result of a directed diffusion of calcium, phosphate and hydroxyl ions. The material porosity is equal to 70 , 75%. This ensures a high rate of vascularization.

We investigated the 'LitAr': biotransformation in the rudiment (permanent) zone on test animals (mongrel dogs' puppies) at the age of 3 months and determined the bone defect modeling place in the region of the 25th viable tooth follicle. It was investigated morphologically.

The material samples for conducting a histological investigation were removed in 2, 9, 15, 20 and 28 days. The collagen-apatite material microstructure is represented as thick separately lying collagen fibers covered with a thin apatite layer.

In the course of the microscopical investigation in two days after performing the operation we could observe the material collagen fibers, its salt component (hydroxyapatite), blood cellular elements (red blood corpuscles) and individual cellular elements of the connective tissue. 9 days later there were in large quantities cell and structural elements of the connective tissue (histiocytes, fibroblasts, collagen fibrils), there were formed vascular channels, we could observe a macrophage reaction of the cells, and the process of forming the unshaped connective tissue.

In 15 days we traced cellular elements of the hard formed connective tissue: fibrocyte, directed bundles of collagen fibers as well as we could reveal separate fragment of the implantation material surrounded by giant cells of foreign bodies.

In 20 days against the background of the solid connective formed tissue we could observe structures of the fresh bone tissue: osteocytes, osteoblasts, osteoclasts, ostial plates. In 28 days the bone tissue has taken the place of the connective tissue. The viable tooth follicle was cutting in the correct position.

Thus, the data obtained morphologically confirm the complete substitution of the collagenapatite material for bone structures for a relatively short time. The material 'LitAr' is no bar to forming and cutting the viable of the permanent tooth rudiment.

### P36

#### THE ADAPTATION OF THE PRE-PUBERTAL SKELETON TO HIGH INTENSITY EXERCISE: A STUDY OF BONE AND MUSCLE GEOMETRY

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A bone's strength is determined by its size, shape and amount of mass contained within the periosteal envelope. Therefore, to get an indication of a bone's adaptation to high intensity exercise, measurements other than bone mineral density should be made; baseline peripheral quantitative computed tomography (pQCT) data from an ongoing trial have been analysed to investigate these adaptations. We hypothesise that compared to sedentary controls (n=42) gymnasts (n=44) will have larger bones with thicker cortices and therefore greater stress-strain indices (SSI, related to bone bending strength), in the radius (R) and tibia (T). Muscle cross sectional area (CSA), an indicator of muscle force, will also be greater in the gymnasts.

Bone and muscle measurements were measured at 50% R and 65% T using pQCT (XCT-2000, Stratec, Germany); loop analysis was used to measure bone mineral content (BMC), cortical thickness, SSI, periosteal and endosteal circumferences.

Natural logs of bone and muscle variables were taken. Results are given as percent mean difference (ratio controls: gymnasts), p=. After adjustment for sex and height gymnasts had higher cortical bone area (R:13, 0.04; T:7, 0.01), mineral content (R:14, 0.04; T: 7, 0.03) and thicker cortices (R:17, 0.02, T:8, 0.02) in both the R and T than controls. Consequently their SSI was higher in both bones (R:14, 0.004, T:7, 0.04). Volumetric BMD was not different in either the R or T. Compared to controls the gymnasts also had greater muscle CSA (R:17, <0.001, T:6, 0.03) and grip strength (11, 0.03).

The bones of gymnasts have higher SSI than sedentary controls. This is likely to be achieved by an increase in cortical BMC and area; the increase in area itself reflects deposition of bone on the periosteal surface (R; p=0.004, T p=NS). These extremely small adaptations have a highly beneficial effect on the strength of the bone thus allowing the appendicular skeleton of pre-pubertal gymnasts to withstand the forces it is subjected to by muscles during activity.

### P37

#### BISPHOSPHONATES INDUCE APOPTOSIS AND INHIBIT ADHESION TO MINERALISED MATRIX IN LUNG CARCINOMA CELL LINES

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Lung carcinoma is one of the most commonly diagnosed cancers worldwide and systemic spread of the primary tumour is common: 80% of lung cancers metastasise to bone. In clinical studies, bisphosphonates (BPs) limit the progression of bone metastases in several carcinomas including lung cancer although the mechanisms involved are incompletely understood. BPs have several direct effects in cancer cells *in vitro* including induction of apoptosis and alteration of invasive properties, however, there is little data addressing the *in vitro* effects of BPs in lung cancer cells. This study therefore investigated the consequences of BP treatment for lung cancer cell survival and adhesion.

Two nitrogen containing BPs zoledronic acid (ZOL) and pamidronate (APD) and the non-nitrogen containing compound clodronate (CLOD) were tested against non-small cell and small cell lung carcinoma cell lines (n=3). Cell viability and number were determined using MTS dye reduction and Sulforhodamine B assay. Apoptosis was assessed by Cell Death ELISA. Cleavage of the substrate Ac-DEVD-pNA was used to determine caspase activation. Inhibition experiments were performed with the broad-spectrum caspase inhibitor Z-VAD-FMK. The effects of ZOL on adhesion were assessed by seeding equal concentrations of viable cells onto dentine matrix for 24 hours. Adherent cells were washed, fixed, stained and counted.

BPs reduced cell viability and number in a concentration and time dependent manner. ZOL and APD were consistently more potent than CLOD, with LD50s between 10 and >100 microM for ZOL and APD but from 500 to >2000 microM for CLOD. All BPs induced apoptosis by day three of treatment and this was associated with caspase activation to varying degrees. BP-induced loss of cell viability was partially abrogated by co-incubation Z-VAD-FMK.

Exposure to 1 or 100 microM ZOL for 24 hours inhibited adhesion to mineralised dentine, whereas 100 microM EDTA had minimal effect, indicating that the inhibition of adhesion was not due to calcium chelation.

BPs directly induce apoptosis in human lung carcinoma cell lines by both caspase dependent and independent means in addition to inhibiting their adhesion to mineralised dentine. This study provides a scientific basis for the clinical effects of BPs in lung cancer.

### **P38**

#### **INTERLEUKIN-1 BETA BLOCKS GLUCOCORTICOID INHIBITION OF OSTEOPROTEGERIN PRODUCTION IN OSTEOBLASTIC CELLS**

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The long-term treatment of patients with inflammatory disease using glucocorticoid drugs often results in bone loss and fractures. The mechanism of this effect is not understood but is thought to be partly due to inhibition of osteogenesis and partly to increased bone resorption. Bone resorption is largely controlled by the balance between the pro-resorptive cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) and the decoy receptor for RANKL, osteoprotegerin (OPG). OPG production by osteoblastic cells has been shown to be inhibited by glucocorticoids and therefore this may be an explanation for an increase in bone resorption.

Our aim was to see if the decrease in OPG protein production by glucocorticoids could be reversed by other agents such as inflammatory cytokines. We show that OPG production is stimulated by the pro-inflammatory cytokine interleukin-1 beta (IL-1) in MG63 cells and in primary osteoblastic cells and that this treatment overcomes glucocorticoid inhibition. We hypothesised that transrepression of the transcription factor Activator protein-1 (AP-1) by the glucocorticoid receptor was part of this mechanism. In order to investigate this we treated cells with phorbol myristate, a potent stimulator of protein kinase C which stimulates the activity and production of the transcription factor AP-1. We found that phorbol myristate stimulates OPG production and also overcomes glucocorticoid inhibition of OPG. This supports the hypothesis that AP-1 is important in the effect of glucocorticoids on OPG regulation.

### **P39**

#### **THE INHIBITION OF OSTEOPROTEGERIN PRODUCTION IN HUMAN OSTEOBLAST-LIKE CELLS BY DISSOCIATED GLUCOCORTICOID ANALOGUES**

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The long term treatment of patients with inflammatory disease using glucocorticoid drugs often results in unwanted side effects such as bone loss and fractures. The effects of glucocorticoids are mediated via the glucocorticoid receptor (GR), which becomes activated once bound to its glucocorticoid ligand. The anti-inflammatory effects of the GR are thought to be caused by transrepression of transcription factors, which would normally stimulate the expression of pro-inflammatory genes. Unlike the anti-inflammatory effects, the unwanted side effects of glucocorticoid treatment are thought to be caused by direct transactivation of various genes by the GR. Dissociation of the two properties of the GR can be achieved by mutation of the receptor or changing the structure of the ligand. Various glucocorticoid-like and non steroidal anti inflammatory drugs have been characterised that are capable of inducing transrepression with very little transactivation.

Bone resorption is largely controlled by the balance between the pro-resorptive cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) and the decoy receptor for RANKL, osteoprotegerin (OPG). OPG production by osteoblastic cells has been shown to be inhibited by glucocorticoids and therefore this may lead to an increase in bone resorption.

Our aim was to determine the effects of various 'dissociated' glucocorticoids on the production of OPG in osteoblast-like cells. This will hopefully shed light on the mechanism of glucocorticoid inhibition of OPG. Of the six compounds tested four were found to dose-dependently inhibit OPG production. ED50s of this effect are compared with so called

'symmetrical' glucocorticoids such as prednisolone. Preliminary data suggests that OPG inhibition is related to the transactivation rather than the transrepression potential of the ligands.

#### **P40**

##### **MEAT CONSUMPTION REDUCES THE RISK OF NUTRITIONAL RICKETS AND OSTEOMALACIA**

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Privational vitamin D deficiency results from limited skin exposure to ultraviolet light. The contributions of dietary vitamin D and other dietary factors to the risk of nutritional rickets and osteomalacia are uncertain; in particular, the possible role of meat in reducing rachitic and osteomalacic risk is controversial.

In two case-control studies of Asian late rickets and osteomalacia, seven-day weighed dietary intakes and measurements of daylight exposure were recorded in 43 cases of late rickets and 41 normal children and in 19 cases of osteomalacia and 72 normal women. The contributions of the food classes, nutrients, energy, age and daylight outdoor exposure to rachitic and osteomalacic risk were estimated by multiple logistic analysis.

Meat intake showed a significant negative relationship to rachitic and osteomalacic risk, maximal at zero intake [Relative risk (RR) 29.8 (95% C.I. 4.9-181);  $p < 0.001$ ]. Dietary fibre [RR 1.06 (95% C.I. 1.0-1.1);  $p = 0.043$ ] and daylight outdoor exposure [RR 0.98 (95% C.I. 0.97-0.99);  $p < 0.001$ ] showed significant positive and negative relationships to rachitic and osteomalacic risk. Calcium intake showed a significant negative relationship to the risk of severe rickets [RR 0.99 (95% C.I. 0.99-1.00);  $p < 0.01$ ]. Dietary vitamin D (including the recent upward revision of meat vitamin D content) did not contribute to rachitic or osteomalacic risk.

In the presence of endogenous vitamin D deficiency, the prevalence and severity of nutritional rickets and osteomalacia appear determined by low/absent meat intakes, high fibre and low calcium intakes acting singly or in combination. An established mechanism links low calcium and/or high fibre intakes with endogenous vitamin D deficiency. The mechanism of the protective effect of meat in reducing rachitic or osteomalacic risk associated with high calcium lacto-vegetarian diets is uncertain.

#### **P41**

##### **RESPONSES TO A SINGLE ORAL DOSE (50,000 IU) OF VITAMIN D2 IN OMNIVORE AND LACTOVEGETARIAN ASIAN AND WHITE WOMEN**

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Meat-eating omnivores have a higher drug oxidation rate than vegetarians, suggesting that meat protein may stimulate the oxidation of vitamin D to 25-hydroxyvitamin D (25OHD) via the hepatic mixed-function oxidase system. Since lactovegetarian white and Asian women have lower serum 25OHD concentrations than their omnivore counterparts, this hypothesis was examined by measuring the conversion of a single 1.25mg (50,000 IU.) oral dose of vitamin D2 to its hepatic (25OHD) and renal (1,25-dihydroxyvitamin D; 1,25D) metabolites in four groups of white and Asian lactovegetarian and omnivore women.

Following withdrawal of a basal blood sample, the oral dose of vitamin D2 was administered under supervision to 39 healthy white omnivore, 31 white vegetarian, 28 Asian omnivore and 10 Asian lactovegetarian women. Six further blood samples were withdrawn 2, 7, 14, 28, 56 and 84 days later for the estimation of serum 25OHD2, serum 25OHD3, 1,25D2, 1,25D3 and PTH concentrations. The significance of between-group differences in vitamin D metabolite and PTH concentrations was assessed by analysis of variance of areas under post-vitamin D2 dosing-time curves (AUC).

There were no significant between-group differences in serum 25OHD2 AUC values following vitamin D2 dosing. Serum 1,25D2 AUC values were significantly higher in Asian omnivore and lactovegetarian groups than in their white counterparts ( $p < 0.001$ ); there were no significant differences between omnivore and lactovegetarian groups of similar ethnicity. There were significant inverse correlations between pooled basal serum 25OHD3 concentrations in all four groups and serum 1,25D2 and PTH AUC responses to vitamin D2 dosing (1,25D2:  $r = -0.600$ ;  $p < 0.001$ ; PTH:  $r = -0.269$ ;  $p = 0.007$ ). The significantly greater serum 1,25D2 and PTH responses to vitamin D2 dosing in the Asian omnivore and lactovegetarian groups represent a response to their lower basal serum 25OHD3 concentrations.

The results of the present study do not support the hypothesis that meat consumption stimulates the hepatic conversion of orally administered vitamin D to 25OHD in omnivore women; the inferior vitamin D status of lactovegetarian women compared with omnivore women is not attributable to this mechanism and other causes need to be sought.

## **P42**

### **BREAST CANCER CELL ADHESION IS INHIBITED BY ZOLEDRONIC ACID BUT RESTORED BY CASPASE INHIBITION**

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Breast cancers preferentially metastasise to bone. The mechanisms associated with breast cancer bone metastasis development are incompletely understood but the molecular events that facilitate adhesion of breast cancer cells to bone matrix are thought to be critical. Nitrogen-containing bisphosphonates (NBPs), such as zoledronic acid (ZOL), have been shown to impair breast cancer cell adhesion and invasion *in vitro* and are known to limit skeletal morbidity in patients with osteolytic breast cancer metastases. NBPs induce caspase-dependent apoptosis in osteoclasts and malignant cells by mevalonate pathway inhibition resulting in reduced G protein prenylation and are also known to chelate calcium ions. This study aimed to investigate characteristics of ZOL-induced inhibition of breast cancer cell adhesion to protein matrices.

Protein matrices or MCF-7 and MDA-MB-231 breast cancer cells were exposed to investigational agents for 24 hours. Equivalent numbers of treated cells were seeded onto untreated mineralised dentine and unmineralised protein matrices for 1-24 hours, or untreated cells to treated matrices. Adherent cells were washed, fixed, stained and the dye eluted or cells counted.

Exposure of cells to ZOL for 24 hours at concentrations from 100nanoM - 100microM was associated with 40 - 80% impairment of adhesion to all matrices as previously, whilst seeding of untreated cells to matrices which had been exposed to 100nanoM - 100microM ZOL for 2 - 24 hours led to 20 - 40% inhibition of adhesion. Exposure of either cells or matrices to 100microM EDTA for 24 hours had no effect on adhesion indicating that ZOL-induced inhibition of adhesion was not solely a consequence of calcium ion chelation. Inhibition of adhesion was largely overcome by co-treatment of each cell line for 24 hours with ZOL together with the broad spectrum caspase inhibitor Z-vad-FMK.

In conclusion, these findings demonstrate that exposure of either breast cancer cells or protein matrices is inhibited by ZOL and suggest that this occurs at biologically relevant concentrations and durations of ZOL exposure. Furthermore, the results indicate that ZOL-induced inhibition of breast cancer cell adhesion is not a result of chelation of calcium ions and occurs by a caspase-dependent mechanism.

## **P43**

### **THE LOSS OF BONE CAUSED BY OESTROGEN-DEFICIENCY IS MEDIATED BY HYDROGEN PEROXIDE AND TNF-ALPHA EXPRESSION**

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Recently, we found that glutathione and thioredoxin, the major thiol antioxidants, and glutathione and thioredoxin reductases, the enzymes responsible for maintaining them in a reduced state, fell substantially in rodent bone marrow after ovariectomy, and were rapidly normalised by exogenous 17-beta oestradiol. Moreover, administration of N-acetyl cysteine or

ascorbate, antioxidants that increase tissue glutathione levels, abolished ovariectomy-induced bone loss, while L-buthionine-(S,R)-sulphoximine (BSO), a specific inhibitor of glutathione synthesis, caused substantial bone loss. These results suggest that oestrogen-deficiency causes bone loss by lowering thiol antioxidants in bone marrow.

How do lower antioxidant defences cause bone loss? All cells generate reactive oxygen species (ROS), and osteoclasts in particular produce them in large amounts. If thiol antioxidant defences are lowered, this will increase the half-life of ROS. Amongst ROS, hydrogen peroxide is sufficiently stable and membrane-permeant to transmit both intra- and intercellular ROS signals. Hydrogen peroxide also increases expression of TNF-alpha in many cell types, including osteoclasts. Blockade of TNF-alpha signalling has been shown to prevent oestrogen-deficiency bone loss in mice.

We therefore tested the role of hydrogen peroxide in oestrogen-deficiency bone loss; and tested whether the bone loss caused by BSO, which lowers antioxidant defences, was, like that of oestrogen-deficiency, caused by TNF-alpha signalling. We found that catalase prevented osteopenia in mice after ovariectomy. This suggests that oestrogen-deficiency causes bone loss through hydrogen peroxide, and that the lowering of antioxidant defences by oestrogen-deficiency is sufficient to lead to an increase in hydrogen peroxide production *in vivo*. We also found that BSO administration induced bone loss that is dependent, like that of oestrogen-deficiency bone loss, on TNF-alpha: bone loss was reduced by administration of soluble receptors for TNF-alpha, and abrogated in mice deleted for TNF-alpha expression. This suggests that lowering antioxidant defences induces TNF-alpha expression *in vivo*.

These experiments are consistent with a model for bone loss in which oestrogen deficiency lowers antioxidant defences in bone marrow and thereby increases hydrogen peroxide levels, which in turn induces expression of TNF-alpha, which causes bone loss.

#### **P44**

##### **THE GENERATION OF ANTIBODIES AGAINST ADULT HUMAN MESENCHYMAL STEM CELLS BY PHAGE DISPLAY**

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Adult bone marrow contains rare mesenchymal stem cells (MSC) that form colonies *in vitro* and differentiate into multiple stromal cell types *in vivo*. MSC and their immediate progeny are not identifiable morphologically and there are no definitive markers. We have used antibody phage display to generate monoclonal antibodies against MSC-enriched fractions of bone marrow mononuclear cells (BMMNC), to facilitate the identification and isolation of MSC within adult marrow.

In order to obtain our MSC-enriched target population, we have previously compared colony formation following immunoselection of different, antibody-defined BMMNC sub-populations. Of the antibodies tested, CD49a was shown to give the best enrichment of colony forming cells.

We have used a diverse synthetic phagemid library to generate phage antibodies and a simultaneous positive/negative selection strategy whereby library phage were incubated with unseparated BMMNC, and bound phage subsequently recovered from magnetically separated CD49a positive sub-populations. Selected library phage were amplified in *E. coli*, rescued with helper phage, concentrated from bacterial supernatants by polyethylene glycol precipitation and used for subsequent rounds of selection. After each round of selection, enrichment and diversity of library phage clones were assessed by titration and PCR/BstN-1 digestion respectively. When the diversity reduced, a dominant clone was analysed for binding to BMMNC, to CD49a BMMNC sub-populations and to cultured bone marrow stromal cells (BMSC) by FACS.

There was a substantial enrichment of library phage after 2 and 3 rounds of selection, and after 3 rounds of selection, sequence analysis confirmed that 1 clone had dominated the population (c15). Binding of c15 to unseparated BMMNC and CD49a positive sub-populations was minimal (2-3% above naive library). Binding was increased to 65% in BMSC cultured in the presence of dexamethasone, suggesting that this antibody may recognise stromal cells of the osteogenic phenotype.

In summary, we have generated phage antibodies against MSC-enriched BMMNC. Further tissue specificity and biochemical characterisation of 1 clone is now in progress and other

unique clones (from selection 2) will shortly be assayed for binding to target cells. It is hoped that phage antibodies generated in this way will provide a much needed resource for the study of mesenchymal stem cell differentiation.

#### **P45**

##### **ROLE OF THE IIIC ISOFORM OF FGFR2 IN MURINE PALATOGENESIS**

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Introduction: Crouzon syndrome is one of a group of human craniofacial syndromes caused by a mutation in the gene encoding fibroblast growth factor receptor type 2 (FGFR2 in human, Fgfr2 in mouse). It is characterized by premature fusion of cranial sutures (craniosynostosis), maxillary hypoplasia, shallow orbits with ocular proptosis, overcrowding of teeth and frequent high arched or cleft palate. The purpose of this project is to investigate the developmental mechanisms through which the Crouzon mutation causes mid-face hypoplasia and palatal abnormality. The project exploits a mouse model that has one of the most common Crouzon-type mutations, Fgfr2Cys342Tyr (Fgfr2C342Y). Methods: Three approaches were used: (1) comparative morphology; (2) cell proliferation analysis; (3) in situ hybridisation to detect altered gene expression. Histological techniques were used for evaluating sections of the palate, using haematoxylin and eosin staining for embryonic day (E)9.5 to E12.5 stages, and Mallory's trichrome for E13.5 to E15.5, to show bone and cartilage. Cell proliferation analysis was performed by intraperitoneal injection of 5'Bromodeoxyuridine (BrdU), at the required stage of pregnancy. The mice were killed 2 hours later, and the embryos rapidly dissected from the uterus, fixed, embedded in paraffin wax and sectioned. Nuclei that have taken up BrdU (indicating that they were synthesising DNA during the two-hour time period after injection) are detected using an anti-BrdU antibody technique and Zeiss KS400v3 image analyser system. Changes in gene expression secondary to the mutation will be analysed by in situ hybridization, using molecular probes for early markers of bone differentiation and FGFR signalling. Results: Results from E13.5-15.5 show significant morphologic differences in palatal development in wild-type mice as compared with heterozygote and homozygote mutants. There appears to be a pattern of development delay by one embryonic day in the heterozygotes and two days in the homozygotes as evidenced by orientation of palatal shelves and degree of shelf fusion. Result of cell proliferation studies for the corresponding embryonic days will also be shown. Conclusion: The Fgfr2Cys342Tyr mutation leads to developmental delay of palatal shelf elevation and fusion. Cell proliferation and gene expression studies are now in progress to investigate the cause of this delay.

#### **P46**

##### **BONE LOSS IN CHRONIC SPINAL CORD INJURY**

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Bone loss occurs rapidly following spinal cord injury (SCI), and osteoporosis and fragility fracture are recognised long-term complications. We audited the data of SCI patients attending a specialist centre to determine the degree of reduced bone mineral density (BMD), and to assess whether there are differences in the bone loss related to gender, site of lesion, or time since injury.

A retrospective analysis was made of medical records from patients with chronic SCI (>1 year post injury). All patients had BMD measurements by dual energy xray absorbitometry (Hologic QDR-Delphi) at the lumbar spine and hip. Reproducibility (as assessed by CV%) ranged from 0.8% to 1.6% across the skeletal sites. No patients were undergoing standing programmes or actively weight-bearing. In addition, no patients were on bisphosphonate treatment.

Data was available on 64 patients (48 men and 16 women). The mean age at time of scan was 40.8 years (range 15-68), with a mean time from injury of 11 years (range 1-56). There were 23 cervical, 38 thoracic and 3 lumbar lesions. Neurologically, 42 were complete injuries and 22 incomplete injuries.

The mean (+/- SD) Z-score at the spine was -0.01 (+/- 1.87), and this was not significantly different when compared to age- and sex-matched controls. At the total hip and femoral neck

the mean Z-scores were -2.00 (+/- 1.91) and -2.43 (+/- 1.43) respectively. These results were significantly reduced when compared to controls ( $p < 0.001$ ).

There was no correlation between time since injury and BMD, or between level of injury and BMD. There was also no significant difference in the results between the sexes. Following injury, 3 patients a fragility fracture (2 neck of femur and 1 tibia and fibula).

The results of this study confirm that hip BMD is significantly reduced in patients with SCI, although spinal BMD appears preserved even in patients with cervical injuries. We have also documented a risk of lower limb fragility fractures in our SCI patients, and this has been confirmed in larger epidemiological studies. Further therapeutic studies are required arrest the bone loss following SCI and to reduce subsequent fracture risk.

#### **P47**

##### **USE OF ALENDRONATE IN MANAGEMENT OF OSTEOPOROSIS FOLLOWING SPINAL CORD INJURY: A REPORT OF 4 CASES**

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Osteoporosis is a long-term complication of spinal cord injury (SCI), predominantly affecting the pelvis and lower extremities. The most important complication of this is fracture, and lower extremity fractures are more prevalent in the SCI population. Bisphosphonates are effective in increasing BMD and reducing fracture risk in postmenopausal and male osteoporosis, although to date there is limited data regarding their use in SCI patients.

In this retrospective analysis, we report 4 SCI patients who have been treated with oral alendronate at standard doses (10 mg/day or equivalent). There were 2 male and 2 female patients, with a mean age of 44 years (range 31-57). Two had cervical spine injuries (C4 and C6), and two thoracic spine injuries (T4 and T8). Neurologically, 3 had complete and 1 incomplete injuries. None were undertaking a weight-bearing or standing programme. BMD was measured at spine (L1-4) and hip using dual energy xray absorptiometry (Hologic QDR-Delphi) using standard techniques. Measurements were taken pre-treatment and after 12-24 months of alendronate therapy.

Treatment with alendronate was initiated an average of 12.75 years post-SCI (range 2-30). All patients tolerated therapy with no significant drug-related adverse effects. Significant reductions ( $p < 0.001$ ) in hip BMD were documented at baseline when compared to age- and sex-matched controls: mean (SD) Z-score at the total hip was -3.7 (0.12); and femoral neck was -3.66 (0.51). Although the mean (SD) lumbar spine BMD was -1.72 (1.85), this was not a significant reduction ( $p=0.103$ ). Following treatment with alendronate, BMD measurements at 12-24 months demonstrated significant increases at all skeletal sites. This was most marked at the lumbar spine (mean increase 6.59%) compared with the total hip (3.80%), and femoral neck (1.36%).

These 4 cases confirm that osteoporosis is a chronic complication of SCI, with deficits in BMD being more marked in the hip compared to spine. They also demonstrate that alendronate is effective in increasing BMD in SCI patients, with the % increases similar to those seen in idiopathic osteoporosis. Unfortunately, absolute hip BMD still remains low and bisphosphonate treatment may therefore be required earlier following SCI in an attempt to preserve bone mass.

#### **P48**

##### **CIRCULATING CORTISONE, BIOCHEMICAL BONE MARKERS AND BONE DENSITY**

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Cortisone is an inactive circulating corticosteroid that can be converted to the active corticosteroid cortisol by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1). 11 $\beta$ -HSD1 is expressed in osteoblasts and may play a role in age-related and glucocorticoid-induced osteoporosis. The kinetics of cortisone to cortisol conversion are such that enzyme activity is proportional to cortisone concentration. It would therefore be expected that in tissues expressing 11 $\beta$ -HSD1, cortisol generation will be greater when cortisone concentrations are higher. We have used these enzyme properties to measure the impact of

11b-HSD1 activity on bone *in vivo* by examining circulating cortisone, bone markers and bone mineral density (BMD) in women (n=135) and men (n=171) aged 61-73 years at baseline and followed up 4 years later. Serum cortisone, cortisol and osteocalcin were measured on morning fasting serum samples and type I collagen crosslinked N-telopeptide (NTx) on a timed morning urine collection. BMD was measured by DEXA at spine and hip.

Serum cortisone levels were negatively correlated with osteocalcin ( $r=-0.16$ ,  $p=0.06$  women;  $r=-0.20$ ,  $p=0.01$  men) but not urinary NTx ( $r=0.03$  women;  $r=-0.03$  men, both NS). Negative correlations between serum cortisone and spine BMD were apparent in unadjusted analyses ( $r=-0.18$ ,  $p=0.04$  women;  $r=-0.14$ ,  $p=0.07$  men) but cortisone did not predict femoral neck or total hip BMD or changes in BMD over time. In analyses adjusted for adiposity, osteoarthritis grade and life style variables the significance of these correlations did not change substantially ( $p=0.08$  for both men and women). All relationships were independent of cortisol concentrations. 11b-HSD1 expression appeared to result in a mean 20-27% inhibition of osteocalcin levels and a 12-15% reduction of spine BMD.

In this elderly cohort increased cortisol generation occurs within osteoblasts and negatively influences osteocalcin and spinal BMD. Serum cortisone, even though inactive, has a substantial effect on bone via local conversion to cortisol.

#### **P49**

##### **CALCIUM SUPPLEMENTATION DOES NOT MODIFY VITAMIN D STATUS IN 16-18 YEAR OLD BOYS**

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An adequate vitamin D status, as measured by 25-hydroxyvitamin D (25OHD), is thought to be a key factor governing peak bone mass, which may, potentially, reduce the risk of developing osteoporosis. An association between low calcium intake and low 25OHD has been demonstrated in animal studies (1, 2), and limited evidence has also been shown in humans (3). The aim of this study was to determine if dietary calcium intake was associated with 25OHD, and whether calcium supplementation reduced the winter decrease in 25OHD in 16-18 year old boys. One hundred and forty seven boys were randomised to receive either a calcium carbonate supplement (1000 mg Ca/day) or matching placebo for 14.7 months. Of these, 110 subjects provided non-fasting blood samples at baseline (between September and January), interim (between May and September) and final (between January and April) timepoints. Plasma was analysed for 25OHD by radioimmunoassay (DiaSorin, US). There was a non-significant increase in 25OHD between baseline and interim and a significant decrease between baseline and final (49.6 (16.6) vs 33.3 (12.8) nmol/l,  $p = 0.0004$ ). No significant difference in 25OHD was found between placebo and calcium supplemented subjects at either interim or final and no effect of tablet compliance was found. Dietary calcium intake was not significantly associated with 25OHD status at any timepoint and no interaction between calcium intake and supplementation was evident. In summary, in this group of older adolescent boys, there was no evidence for an association between usual calcium intake or calcium supplementation on 25OHD. It is possible that usual dietary calcium intake in these subjects was too high to significantly affect 25OHD, and that pronounced effects may only be evident at lower calcium intakes. Further studies are needed to examine whether lower calcium intakes affect 25OHD utilisation and whether this has functional consequences for bone accretion in adolescence.

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## P50

### OSTEOBLAST CHARACTERISATION AND GENE EXPRESSION DURING FAST GROWTH IN THE IMMATURE SKELETON

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In response to skeletal loading, bones increase their diameter by incorporation and the infilling of primary osteons. Comparing chickens with fast (F) and slow (S) growth potential we have previously reported that the tibia of fast growing birds had lowered mechanical properties, likely to be explained by increased cortical porosity. Indirect measurement of osteonal infilling indicated that this process was impaired in the fast strain. The aims of this study were to directly determine the infilling rate of the primary osteons and to compare osteoblastic characteristics of both strains. To quantify the rate of osteon infilling, tibiae were removed from 21-day-old chicks, which had been double labelled with calcein (80 and 8 h before death). The mineral apposition rate was  $F=11.51$  micrometers/day;  $S=28.16$  micrometers/day,  $P < 0.001$  and this data confirms previous histomorphometry results. Osteoblasts were grown and expanded in culture from explants of tibia of 21-day-old birds of both strains ( $n=4$ /strain). Osteoblast proliferation was determined by tritiated-thymidine uptake and differentiation by alkaline phosphatase (ALP) activity. At pre-confluency, cell proliferation was higher in the slow growing birds ( $F=7439$ dpm;  $S=11732$ dpm,  $P < 0.001$ ), but this pattern was reversed at confluency ( $F=10491$ dpm;  $S=1979$ dpm,  $P < 0.001$ ) and post confluency ( $F=4564$ dpm;  $S=1702$ dpm,  $P < 0.001$ ) and is a likely consequence of the earlier impairment of proliferation by contact inhibition in the slow growing strain. ALP activity (pNPP hydrol/30 min/mg protein) was only detected at post-confluency and was higher in the fast growing strain ( $F=1188$ ;  $S=216$ ,  $P < 0.001$ ). Gene expression of matrix proteins was determined by RT-PCR and quantified by densitometry. Collagen type 1 and osteonectin showed no difference in expression between fast and slow strains, but a higher level of expression of osteopontin was observed in the slow growing birds ( $F=382$ cnt/mm<sup>2</sup>;  $S=1205$ cnt/mm<sup>2</sup>,  $P < 0.01$ ). In conclusion, porosity was confirmed to be due to a lack of infilling within the primary osteons. Osteoblast proliferation rate was faster in the slow growing birds whereas differentiation was slower. This is in accord with our previous hypothesis that the fast growing birds are characterised by an increase in transit time through the osteoblast lineage. Osteopontin expression has been associated with mechanical loading but the significance of the lower osteopontin expression in the fast growing birds requires further study.

## P51

### TRANSIENT EXPOSURE TO HYPOXIA STIMULATES ATP RELEASE FROM OSTEOBLASTS

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Both extracellular nucleotides and hypoxia exert inhibitory effects on osteoblast (OB) function and stimulatory actions on osteoclast function. OB have been shown to release ATP constitutively. The inhibitory action of ATP on OB may be mediated via the P2Y<sub>2</sub> receptor subtype. Activation of G protein-coupled P2Y receptors leads to prompt rises in intracellular Ca<sup>2+</sup> and IP<sub>3</sub>. Earlier work from our group has shown that hypoxia stimulates ATP release from endothelial cells. Here we investigated whether hypoxia might exert a similar action on primary OB obtained from neonatal rats by trypsin/collagenase digestion. Cells were seeded at  $1 \times 10^5$  / well in 6-well plates and cultured for 15d in DMEM / 10%FCS. At d15 the medium was replaced with 1 ml serum-free DMEM, and cells were allowed to rest for 1 hr before transient exposure (for 0.5, 1.0 2.5 & 6.5 min) to 20% or 2% O<sub>2</sub> (both with 5% CO<sub>2</sub>, balance N<sub>2</sub>). Medium ATP concentrations were then measured using a standard luciferin-luciferase method. Basal ATP concentration was 0.5 plus/minus 0.1 nM; this was not significantly altered in plates gassed with 20% oxygen. Exposure to 2% O<sub>2</sub> increased ATP release, reaching a peak of 1.9 plus/minus 0.3 nM after 2.5 min. *In vivo*, however, it is reasonable to expect that equivalent numbers of OB would be bathed directly by a much smaller volume of fluid, perhaps of the order of a few microlitres; thus, ATP concentrations could be 2 orders of magnitude higher. To determine whether such concentrations of ATP might be biologically significant we used a fluorescence imaging plate reader (FLIPR) to study intracellular Ca<sup>2+</sup> responses of cultured OB to ATP. Concentrations of ATP equal or more than 200nM elicited

transient increases in intracellular  $Ca^{2+}$ , suggesting that hypoxia stimulated ATP release could be sufficient to activate P2Y receptors on OB. Earlier work has also demonstrated that 200nM ATP stimulates osteoclast formation and activity strongly (J Physiol 511:495-500; 1988). Our data suggest that ATP release in hypoxia might play a role in modulating osteoblast and osteoclast function.

## **P52**

### **EFFECTS OF ZOLEDRONIC ACID ON THE ADHESION AND INVASION OF PROSTATE CANCER CELLS AND THE INFLUENCE OF THE SDF-1/CXCR4 CHEMOKINE PATHWAY**

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Prostate cancer (CaP) preferentially metastasises to bone. Zoledronic acid (ZOL), like all bisphosphonates, strongly localises to bone. It inhibits the mevalonate pathway, which normally allows prenylation of G-proteins (by farnesylation or geranylgeranylation) - a crucial post-translational modification required for these proteins to localise to the membrane and hence function normally. In this study, we examined effects of ZOL on adhesion and invasion of DU145, LNCaP and PC-3 CaP cells. We investigated how these effects were influenced by altering prenylation, either directly using inhibitors of farnesyltransferase or geranylgeranyltransferase, or by adding farnesol (FOH) or geranylgeraniol (GGOH), cell-permeable analogues of intermediates in the mevalonate pathway that allow prenylation to occur in spite of upstream inhibition. We also investigated the role of the SDF-1/CXCR4 chemokine pathway on cell migration. SDF-1 is abundant in bone, and may influence the localisation of CaP cells.

Cells were exposed for 24 hours to various reagents. For adhesion, treated cells were placed onto dentine slices for 24 hours. For invasion, cells were added to the upper part of Matrigel Invasion Chambers, and left to invade across extracellular matrix-like material through to the lower half, for 48 hours. Cells were then fixed, stained and counted.

ZOL inhibited adhesion and invasion in a concentration-dependent manner. Co-treating with ZOL and GGOH had a greater rescuing effect than co-treating with FOH. Direct inhibition of geranylgeranylation had a similar or greater inhibitory effect than inhibiting farnesylation. Invasion was significantly increased by adding SDF-1 to the lower part of the invasion chambers. This effect was negated by adding SDF-1 or anti-CXCR4 antibody to the upper part, and significantly reduced by ZOL. The degree of inhibition of invasion caused by ZOL when SDF-1 was used as a chemoattractant was greater than with the original invasion assay, in which FCS alone is used as the chemoattractant.

ZOL inhibits CaP cell adhesion and invasion *in vitro*. The crucial mechanism appears to involve inhibiting geranylgeranylation of proteins - such as Rho, a G-protein known to be crucial in cytoskeletal re-organisation. The SDF-1/CXCR pathway has a clear influence on the migration of CaP cells across extracellular matrix material.

## **P53**

### **RELATIONSHIP BETWEEN STAGE OF OESTRUS AND BONE CELL ACTIVITY IN THOROUGHBRED HORSES**

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Musculoskeletal injuries are a major cause of wastage in racehorses, with evidence that risk of injury may be influenced by gender and season. We have shown that bone marker concentrations are higher in young male thoroughbreds than in females (mares), which probably reflects differences in bone size. However, during the summer breeding season concentrations of the carboxy-terminal propeptide of type I collagen (PICP) are higher in mares, and we therefore hypothesise that changes in sex hormone concentrations during the breeding season lead to changes in bone turnover in mares. This study aimed to establish the relationship between biochemical markers of bone cell activity and stage of oestrus cycle in mares.

Blood samples were collected monthly from 47 two year old thoroughbred mares in race training between April and September. Animals were categorised into oestrus and dioestrus

groups on the basis of serum progesterone concentrations at each sampling point. A progesterone concentration above (dioestrus) or below (oestrus) 1ng/ml being taken as the cut-off. Osteoclastin (OC) and P1CP were measured as markers of bone formation, and the carboxy-terminal telopeptide of type I collagen (ICTP) measured as a marker of bone resorption. Data were analysed using a mixed model analysis with repeated measures (PROC MIXED in SAS version 8e).

The proportion of animals identified as being in dioestrus increased from 47% in April to 77% in August. Stage of oestrus cycle had a significant effect on OC ( $P = 0.003$ ) and ICTP ( $P = 0.006$ ), with higher concentrations measured in dioestrus. For OC this difference was significant in April ( $P = 0.04$ ) and July ( $P = 0.03$ ). For ICTP the difference between oestrus and dioestrus was greatest during April ( $P = 0.07$ ) and May ( $P = 0.09$ ).

In conclusion this study shows that in horses, as in humans, bone cell activity changes during the oestrus cycle. Consistent with the role of oestrogen in other species, we found that lower circulating concentrations of oestrogen during dioestrus are associated with increased bone cell activity. It remains to be determined whether these changes place mares at increased risk of orthopaedic injury at specific times of year.

#### **P54**

Abstract withdrawn

#### **P55**

##### **CALCIUM PHOSPHATE COATED CONSTRUCTS FOR BONE TISSUE ENGINEERING**

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Biodegradable polymers such as polycaprolactone, in general, lack cell-recognition signals, which lead to poor cell adherence. This study focuses on optimisation of the polymeric scaffold for bone cellular activity using a calcium phosphate coating.

The matrix-type scaffolds were produced by fused deposition modelling whereas the foam-type scaffolds were produced by salt leaching. Coated scaffolds were produced by a simple biomimetic method using sodium silicate as a catalyst for the apatite nucleation. Bone marrow cells were harvested from trabecular bone marrow samples of patients undergoing routine total hip replacement surgery. After the fourth passage, cells were trypsinised and cell viability was examined by trypan blue exclusion. 300 000 cells were seeded on of each of the scaffolds. The scaffolds were placed into the incubator to allow the cells to adhere and culture medium was added after 4h. 1wk after the start of culture, the complete medium was replaced by osteogenic medium and changed every 2 days. The scaffolds were analysed for cellular activity using alamarBlue™ and alkaline phosphatase (ALP) assays using a spectrofluorometer. Scanning electron microscopy (SEM) was used to observe cell attachment. Material analyses were carried out using wavelength dispersive x-ray analysis (WDX) and x-ray diffraction (XRD) analysis. In addition, to study biocompatibility, coated and uncoated scaffolds were implanted into subcutaneously in BALB/c mice.

The results from alamarBlue™ and ALP assays showed better cell viability in the coated matrix-type scaffolds, whereas the coated foam-type scaffolds showed poor cell viability, possibly because the calcium phosphate coating had blocked the internal spaces of the non-interconnected porous foams. SEM results showed that more cells attached to the coated scaffolds, forming multi-layers, compared to the uncoated scaffolds. Foam-type scaffolds were better at trapping cells within their porous structure. WDX analysis showed that the coating layer had a Ca/P ratio of 1.68, similar to that of hydroxyapatite. This was confirmed by the XRD results. Biocompatibility studies showed no sign of adverse tissue inflammatory responses when the scaffolds were implanted *in vivo*. Therefore, current results indicate that the calcium phosphate-coated matrix-type scaffolds showed improved cell attachment, proliferation and differentiation.

## **P56**

### **INCREASED OSTEOBLASTIC DIFFERENTIATION IN CULTURED MARROW CELLS AFTER BLOOD LOSS OR SURGERY**

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Ex vivo studies of bone marrow cell differentiation following drug treatments or surgical procedures correctly use sham treated/operated control animals. In ovariectomy studies we have found a consistent significant increase in fibroblastic colony forming units (CFU-Fs) when bone marrow is cultured from sham-operated animals than cells from ovariectomised animals. The following experiments test the hypothesis that surgical trauma and/or blood loss increases the pool of CFU-Fs or their precursors in bone marrow.

Bone marrow was extracted post-mortem from the femora and tibiae of female Wistar rats weighing approximately 200g. The experimental groups were 1) intact non-anaesthetised control; 2) controls anaesthetised 2 weeks before euthanasia; 3) ovariectomised 2 weeks before euthanasia; 4) anaesthetised 2 weeks before euthanasia and 5ml.kg<sup>-1</sup> blood removed by direct cardiac aspiration, and 5) sham ovariectomy control 2 weeks before euthanasia (both ovaries exposed via a flank incision but not removed). Anaesthesia was by intraperitoneal injection of ketamine (90mg.kg<sup>-1</sup>) and xylazine (10mg.kg<sup>-1</sup>). Marrow cells were removed from the bones by gentle centrifugation and were cultured for 18 days in osteogenic conditions, after which they were stained sequentially for alkaline phosphatase activity, type I collagen and mineral. The total colony area and number of colonies were measured by image analysis.

All animals that had sham surgery or blood removal had greater numbers of larger colonies than intact controls, anaesthetised controls or cells from ovariectomised animals ( $p < 0.05$ ). (Total area/cm<sup>2</sup> +/- s.e.m, total colony number +/- s.e.m. Group 4: 0.261 +/- 0.0061, 20.1 +/- 1.39; Group 5: 0.272 +/- 0.0098, 22.7 +/- 1.39; Group 1: 0.236 +/- 0.0106, 17.7 +/- 0.76; Group 2: 0.096 +/- 0.0095, 10.1 +/- 0.085; Group 3: 0.125 +/- 0.0130, 14.3 +/- 0.19). The effects of the ketamine/xylazine anaesthetic was consistent with our other studies showing that these agents produce an inhibitory effect on CFU-Fs.

It is possible to speculate that blood loss or surgery upregulates haematopoietic cell lineage differentiation. This concomitantly upregulates the precursors of osteoblastic lineage cells. Alternatively the precursor pool affected by trauma/blood loss may remain sufficiently plastic that ex vivo, under osteogenic conditions, they can be induced to transdifferentiate towards an osteoblastic phenotype.

## **P57**

### **BOVINE BONE SUBSTITUTE IN REVISION KNEE ARTHROPLASTY**

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**INTRODUCTION:** Bovine bone substitute was used in seven patients undergoing revision knee arthroplasty for reconstruction of bone defects. Early clinical results are presented in this paper.

**MATERIALS AND METHODS:** This is a retrospective review. Between April 2000 and March 2003, bovine bone (Tutobone [trademark], Wescott-Medical, UK) was used in 7 revision arthroplasty cases (4 right knees & 3 left). There were 5 males and 2 females. The average age was 70.4 years. All revisions were carried out for aseptic loosening of the prostheses associated with massive osteolysis and bone loss.

The bone defects on the tibia and femur were as follows: (Obtained from operative records. Classified according to Anderson Orthopaedic Research Institute classification)

On the tibia, 3 cases had type I defect, 2 had type IIb defect and the remaining two cases had type IIA and type III defect each. On the femur there were 2 cases with type I defect, 3 cases with type IIA, and 2 cases with type IIB defect. There was no type III defect on the femur.

The defects on the tibia were corrected by impaction grafting and those on the femoral condyles were corrected by using bovine bone as bulk grafts. Semi-constrained constrained stemmed cemented modular knee prostheses (TC3, Depuy) were used in all. Clinical outcomes were recorded by the oxford knee Score. Serial radiographs were evaluated for graft density, integration, implant loosening, alignment and subsidence.

RESULTS: At recent follow-up, radiographs showed good graft integration, no loosening, and no subsidence of the implant and good prostheses alignment. The average Oxford Knee Score was 20.4.

CONCLUSION & DISCUSSION: The bone defects in these patients were successfully reconstructed with bovine bone. Bovine bone substitute is an alternative to autografts and allografts. Therefore there is no donor-site morbidity and it is cheaper and safer than allografts. It is an osteo-conductive matrix with intact type-I collagen that provides mechanical stability. Early bone integration with host bone was observed in our patients.

Early results are encouraging but long-term follow-up is needed.

## P58

### **ABNORMAL SEPTOCLASTS AT THE CHONDROOSSEOUS JUNCTION IN THE TL RAT ARE NORMALISED FOLLOWING CSF-1 TREATMENT**

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The septoclast is a unique cell specifically differentiated to facilitate angiogenesis at the chondroosseous junction. This cell is long and slender, lies just outside the invasive capillary sprouts, and stains intensely for the cysteine proteinase cathepsin B. The apex of the septoclast ends on the transverse septum in a structure that resembles the ruffled border of osteoclasts. The toothless (tl) rat, a naturally occurring autosomal recessive mutation in the *Csf1* gene resulting in profound deficiencies of bone-resorbing osteoclast, has severe, unrelenting osteopetrosis with a highly sclerotic skeleton, lack of marrow spaces, the failure of tooth eruption and a progressive, severe growth plate chondrodystrophy. Given that the tl rats have retarded bone growth at the COJ accompanied by suppressed angiogenesis and the absence of osteoclasts we decided to determine if septoclasts were similarly missing in the tl rat. We compared the COJ in the proximal tibia of tl rats and normal littermates with respect to number, distribution and cathepsin B activity of septoclasts. Injections of CSF-1 restores the osteoclasts population, bone resorption, growth and tooth eruption in tl rats but does not improve the growth plate phenotype, therefore we also examined the COJ in CSF-1 treated rats. In the normal COJ septoclasts were present as thin cells regularly aligned along the axis of the bone and exhibited strong staining for cathepsin B. tl rats had a reduced number of cathepsin B positive cells with morphology similar to septoclasts could be found somewhat further below the COJ than the normal littermates. The number and distribution of septoclasts at the COJ of the tl rat were restored with CSF-1 treatment. Ultrastructural evaluations of the COJ from the normal and tl rats confirmed these cells to be septoclasts. The presence of septoclasts in the tl skeleton confirms that these two catabolic cells not under identical developmental control. However, the reduced number and alignment of septoclasts in tl rats, and their restoration with CSF-1 treatment implies a role for CSF-1 in the normal differentiation of septoclasts. These data confirm that septoclasts play a significant role in bone growth at the COJ.

## P59

### **A ROLE FOR THE CANONICAL WNT SIGNALLING PATHWAY IN THE REGENERATING DEER ANTLER**

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Wnts are a family of paracrine and autocrine factors that regulate the growth and fate determination of many cell types, including osteoblasts. There is also evidence that Wnts may regulate regeneration in lower organisms. The aim of this study was to explore the hypothesis that the canonical Wnt signalling pathway regulates the function of progenitor cells in the distal tip of the regenerating deer antler, the only mammalian organ capable of complete regeneration. The objectives of the study were: (1) To localise activated beta-catenin (BCAT) in antler tissues (by immunocytochemistry) and to relate its localisation to cell proliferation (by PCNA staining), apoptosis (by Tunel staining) and to sites of PTHrP synthesis (by immunocytochemistry). (2) To determine the effect of activation or inhibition of Wnt signalling

on alkaline phosphatase (ALP) activity (by histochemical stain and biochemical assay) and on apoptosis in cultured antler progenitor cells (PER cells).

BCAT staining has a restricted localisation in the antler tip; there is positive staining associated with hair follicles and sebaceous glands, where there are also a number of PCNA +ve cells. There is a low level of staining in perivascular tissue, a site of osteoblast and osteoclast differentiation. However, the strongest staining is in mesenchymal progenitor cells in the distal antler tip below the perichondrium and PTHrP was co-localised in serial sections from this region. These cells have low levels of ALP activity and a high rate of proliferation (21.7±5.5% of cells) and apoptosis (63.9±9.2% cells). In cultures of progenitor cells from this region of the antler, LiCl, which activates BCAT via GSK3 $\beta$  inhibition, decreased ALP activity ( $p < 0.001$ ). In contrast, EGCG, which inhibits canonical wnt signalling via inhibition of Tcf transcription, stimulated ALP activity ( $p < 0.05$ ). Treatment of PER cells with PTHrP (10<sup>-7</sup>M) for 24 hours significantly increased the number of apoptotic PER cells (control: 2.8 ± 1.7%, PTHrP: 8.0 ± 2.4%  $p < 0.05$ ) whereas LiCl has no effect on apoptosis.

In conclusion, this study has provided evidence that activation of the canonical Wnt signalling pathway appears to maintain progenitor cells in the 'growth zone' of the antler in an undifferentiated state, which is necessary for continued regeneration.

## P60

### **CANONICAL WNT SIGNALLING REGULATES THE DIFFERENTIATION OF OSTEOCLASTS FROM REGENERATING DEER ANTLERS**

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Deer antlers are the only mammalian bones capable of complete regeneration and their extremely rapid growth involves extensive resorption of mineralised cartilage by osteoclasts. Recently we have found that active beta-catenin, a component of the Wnt signalling pathway, is expressed in antlers and a recent study has shown that activation of beta-catenin in osteoblasts led to a defect in bone resorption in a mouse model. The aim of the present study was to investigate the potential role of the Wnt pathway in regulating antler osteoclast differentiation.

Osteoclast formation was induced in micromass cultures (Faucheux et al. 2001) using PTHrP or RANKL. Cultures were treated for 9 days with LiCl, an activator of Wnt signalling, or Epigallocatechin-3-gallate (EGCG) which antagonises the transcriptional activation of Tcfs. End points studied were number of TRAP +ve multinucleated cells formed per dentine disc, percent area of dentine resorbed (determined by lectin staining and image analysis) and osteoclast activity (area resorbed per individual osteoclast). RANKL mRNA expression in LiCl treated cultures was studied by RT-PCR.

5mM LiCl significantly decreased the numbers of TRAP+ve osteoclasts in cultures treated with both PTHrP (by 35.3% ±19.9  $p < 0.05$ ) and RANKL (by 32.3%,  $p < 0.001$ ). LiCl also decreased the areas of dentine resorbed in PTHrP (by 26.0% ±0.5  $p < 0.01$ ) and RANKL (by 41.2%  $p < 0.001$ ) treated cultures. However, osteoclast activity was not affected by LiCl. RANKL mRNA expression was upregulated by PTHrP and this could be partly inhibited with LiCl. EGCG (1-25 micromolar) treatment did not change the numbers of osteoclasts formed but at a dose of 6 micromolar increased the activity of the osteoclasts and thus increased the area of dentine resorbed (twofold).

In conclusion, this study provides evidence that the Wnt pathway can regulate the differentiation and activity of osteoclasts in regenerating mammalian bone. Activation of beta-catenin inhibited antler osteoclast formation while inhibition of transcription by Tcfs increased osteoclast activity. The inhibition of osteoclast formation is, in part, indirect via changes in RANKL mRNA expression, however, other mechanisms are likely to be involved as LiCl also inhibited osteoclast formation in cultures stimulated directly with RANKL.

## **P61**

### **A MODULATORY ROLE FOR ADENOSINE IN BONE CELLS?**

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Nucleotide receptors can be classified into P1 receptors where adenosine is the primary ligand, and P2 receptors where the main ligands are ATP, ADP, UTP and UDP. Adenosine has a wide range of actions in many tissues, but there is no information on its role in bone physiology.

We have recently demonstrated the presence of the four P1 adenosine receptor subtypes (A1, A2A, A2B and A3) in human osteoprogenitor (HCC1) and bone marrow stromal (BMS) cells. In addition, NECA (1 microM), a non-specific adenosine receptor agonist, stimulated a 10-fold increase in IL-6 secretion by HCC1 cells, whilst a 10-fold higher concentration of adenosine, CCPA (A1 agonist) and IB-MECA (A3 agonist) stimulated secretion by 2.5-, 2-, and 3.5-fold respectively. CGS21860 (A2A agonist) had no effect. The effects of 100 microM NECA, adenosine and CGS21860 (13-, 5-, and 2-fold increase respectively) on cAMP stimulation paralleled that for IL-6 secretion and suggest that the functionally dominant receptor is the A2B subtype. Similar work with BMS cells also demonstrated an increase in IL-6 secretion with NECA, but only when cells were maintained in alpha-MEM not containing nucleotides. Alpha-MEM with nucleotides is used for routine culture of BMS cells in many laboratories, but we have shown that basally secreted IL-6 concentrations under these conditions are very high - further evidence that adenosine and other similar nucleotides might play a role in bone cell function.

Other work with HCC1 cells showed that adenosine, NECA, CGS21860 and CCPA, but not IB-MECA inhibited the production of osteoprotegerin, and that incubation with NECA for 24 hours increased cell numbers. In addition, (RT-PCR and immunocytochemistry) demonstrated that HCC1, BMS and MG63 cells express ecto 5'-nucleotidase, and that BMS and MG63, but not HCC1 cells, express adenosine deaminase.

Our data, for the first time describes a possible role for adenosine and the P1 receptors in the function of osteoprogenitor cells and osteoblasts. This might be especially important in conditions such as rheumatoid arthritis where high level of adenosine due to inflammation may influence the cross talk between osteoblasts and osteoclasts, as well as have direct effects on osteoblast and osteoclast differentiation and activity.

## **P62**

### **LYMPHOBLASTOID CELLS IN FIBRODYSPLASIA OSSIFICANS PROGRESSIVA - DO THEY PLAY A ROLE?**

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Fibrodysplasia Ossificans Progressiva (FOP) is a rare genetic disease in which progressive debilitating ossification of skeletal muscle connective tissue, tendons and ligaments occurs. Patients characteristically have shortened great toes and thumbs but no other obvious skeletal defects at birth. The pathology of FOP is relatively uncharacterised as patient samples are scarce, and the causative genetic mutation is unknown.

Ossification is often triggered by trauma to skeletal muscle, and is usually preceded by inflammatory lesions, which recede to leave areas of new bone. One current hypothesis regarding bone formation is that infiltrating inflammatory cells to the trauma site overexpress Bone Morphogenetic Protein 4 (BMP4), a protein that can initiate de novo bone formation. The released BMP4 is considered to act on cells present in the surrounding connective tissue causing endochondral bone formation. The present study aims to test this hypothesis.

To this end, lymphoblastoid cells isolated from FOP patients were cocultured with cells isolated from normal human skeletal muscle tissue. It was proposed that FOP cells would affect the skeletal muscle cells in a different manner to normal lymphoblastoid cells. After coculture with either normal or FOP inflammatory cells skeletal muscle cells were examined for proliferation (DNA increase) and differentiation (alkaline phosphatase activity) Under these conditions there was an insignificant difference between the effects of the two cell groups. However, there was a general effect of lymphoblastoid cells on all cocultures compared with

culture of skeletal cells alone. Lymphoblastoid cells caused a significant increase in proliferation ( $p < 0.01$ ), and concomitant decrease in alkaline phosphatase activity of the skeletal muscle cells.

Addition of the inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$ , which may be present in inflammatory lesions, to cocultures did not provoke FOP cells to affect muscle cells significantly differently to normal lymphocytes. In addition, BMP4 mRNA expression by FOP and normal lymphocytic cells was examined by real-time PCR and there was found to be no significant difference. These results suggest that BMP4 overexpression by infiltrating lymphocytes is not likely to be the cause of new bone formation in the cases of FOP investigated here and again raises the possibilities of genetic heterogeneity.

## P63

### STATINS: POTENTIAL CARTILAGE ANABOLICS?

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Statins that inhibit HMG-CoA reductase and so reduce the synthesis of cholesterol and isoprenoids have been shown recently to induce BMP2 production in bone *in vitro* and *in vivo*. This effect is mediated by the inhibition of the mevalonate pathway and more specifically by the inhibition of the prenylation of the small G proteins. An already large and growing number of growth factors which play significant roles in bone remodeling and repair have also been shown to have an anabolic action on cartilage. In addition the BMP family is well known to play an important role in cartilage matrix synthesis. However, the action of statins on cartilage matrix production by chondrocytes is unknown. We therefore investigated the effect of the statin, lovastatin, on markers of matrix formation *in vitro*.

We evaluated the regulation of cartilage matrix synthesis by lovastatin using articular bovine chondrocyte pellet culture. Glycoaminoglycan (GAG), and type II collagen (COL2) production were measured with an ELISA assay, cell proliferation was evaluated with a fluorescent method measuring the DNA quantities.

Production of COL2 and GAG proteins was increased in the presence of increasing amounts of Lovastatin after two and three weeks of stimulation. This time- and dose-dependent stimulatory effect was abolished by the addition of mevanolate or farnesol, two downstream metabolites of HMG-CoA reductase, indicating that matrix synthesis was a result of the inhibition of this enzyme. While cell proliferation continued throughout statin treatment, it was slower at higher concentrations.

These results suggest that Lovastatin increases COL2 as well as GAG synthesis by primary articular bovine cells through inhibition of the classical mevalonate pathway.

A commonly encountered problem in orthopaedics is cartilage tissue injury which heals incompletely or without full structural integrity. It is well established that bone morphogenic proteins (BMP) induce the production of new bone and cartilage so they may be useful therapeutically to stimulate healing of damaged articular cartilage. Statins may therefore be of use in cartilage repair and healing.

## P64

### THE VALUE OF TOTAL BODY BONE MINERAL DENSITY FOR THE ASSESSMENT OF FRACTURE RISK IN TRANSPLANT RECIPIENTS

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Introduction:

It is recognised that kidney transplant patients exhibit a high rate of bone loss in the post-transplantation period. This is principally because of long-term glucocorticoid therapy. This compounds their renal bone disease and increases their risk of fracture above that of age-matched controls. Although DEXA and lateral vertebral morphometry are well recognised tools to stratify the risk of osteoporosis, the value of TBBMD in the assessment of these patients fracture risk is established in this study which also investigates whether differences in regional BMC are associated with truncal or peripheral fractures.

Method:

64 patients were consecutively recruited from the nephrology service for a TBBMD measurement and Lateral Vertebral Assessment using a Lunar Prodigy GE scanner. A comprehensive fracture history was taken, TBBMD was measured excluding the skull. Peripheral bone mineral content (mean of the arms and legs) and truncal BMC were measured.

Results:

41 males and 21 females with a mean age of 58.2 (+/-12.5 SD) participated. 15 had osteoporosis, 24 had osteopaenia (as defined by WHO guidelines). 11, (18%) had vertebral fractures (Vfx), and 14 (23%) had a peripheral fracture (Pfx). The mean TBBMD for males was 1.165 (+/- 0.095), and for females was 1.023 (+/- 0.159). For every increase of 0.1gm/cm in TBBMD, there was a 0.47 reduction in the risk of any fracture. Patients with Vfx had lower truncal BMC ( $p < 0.01$ ) than those without Vfx. Patients with Pfx had lower peripheral BMC than those without Pfx ( $p < 0.02$ ).

Conclusions:

Modest decreases in TBBMD markedly increase the risk of fracture in kidney transplant patients. Regional BMC is significantly lower in those patients with fractures of either the trunk or periphery.

**P65**

**HUMAN OSTEOBLAST-LIKE CELLS HAVE HIGH DENSITY MAXI-K CHANNELS**

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A number of membrane potassium channels are increasingly implicated in apoptosis or survival of excitable cells, although the mechanisms involved are unclear. Inexcitable cells also possess many subtypes of potassium channels, their presumptive role being in volume control and secretion. It is likely that they also have a role in maintaining membrane potential and other cellular functions. Whole-cell recordings have shown that MG63 cells and human osteogenic precursor cells have outward currents carried by potassium ions, however few single channel data exist to aid further characterisation of the ion channels involved.

We have carried out single channel patch-clamp experiments in both human MG63 and SaOS osteoblast-like cells. We have also carried out RT-PCR using primer pairs for the maxi-K (HSLO) channel. Recordings were made from cells in non-confluent cultures. The bathing solution was mM: 150 NaCl; 3 KCl; 2 MgCl<sub>2</sub>; 2 CaCl<sub>2</sub>; 10 HEPES; 10 Glucose, pH 7.4. The recording pipette contained in mM: 140 KCl; 5 NaCl; 1 MgCl<sub>2</sub>; 1 CaCl<sub>2</sub>; 11 EGTA; 10 HEPES, pH 7.2. Giga-ohm seals (2-12 giga-ohms resistance) were achieved in about 50% of cells patched (n = 170).

Cell-attached recordings showed that both these cell types have a number of different single channel currents. The most prevalent channel was a large conductance channel. This channel was typically activated by strong depolarisation (> 80 mV), there being up to seven active channels in a patch at +80 mV. The open probability-voltage relationship showed marked hysteresis, the curve shifting to the left with successive depolarisations. The I-V relationship was linear up to 140 mV depolarised, the mean conductance being 227 ± 41 pS (n=15). Interestingly, this channel was activated at resting membrane potential by bath application of 10<sup>-4</sup> M glutamate (Mg<sup>2+</sup>-free Locke). In excised inside-out and outside-out patches (asymmetric K, n = 14), the channel was shown to be sensitive to both internal (2 mM) and external TEA (500 µM). RT-PCR confirmed the existence of this channel in MG63 cells.

This large conductance channel is a presumed target for hormones, local mediators and mechanical influences and such possibilities are now being tested.

**P66**

**SARCOMA-ASSOCIATED MACROPHAGES DIFFERENTIATE INTO OSTEOCLASTIC BONE-RESORBING CELLS**

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The cellular mechanisms which account for the formation of osteoclasts and bone resorption associated with an enlarging malignant mesenchymal tumour of bone are uncertain. Osteoclasts are marrow-derived multinucleated bone resorbing cells which express a macrophage phenotype. We isolated tumour-associated macrophages (TAMs) from a number of malignant mesenchymal tumour of bone and soft tissue (e.g. Ewing's sarcoma, haemangioendothelioma, leiomyosarcoma, osteosarcoma) and cultured them for 21 days in the presence and absence of M-CSF, RANKL and TNF-alpha. TAMs from all tumours differentiated into osteoclasts in the presence of M-CSF and RANKL, as shown by the formation of TRAP and VNR-positive multinucleated cells which were capable of carrying out lacunar resorption. Osteoclast formation was also seen when TAMs from Ewing's sarcoma were incubated with M-CSF and TNF-alpha. Bisphosphonates abolished bone resorption in these cultures. Our findings suggest that the growth of mesenchymal tumours in bone is likely to be due in part to osteoclast differentiation of mononuclear phagocyte osteoclast precursors present in the TAM population of sarcomas and that this process occurs by both RANKL-dependent and independent mechanisms.

**P67**

**SYNOVIAL FLUID PROMOTES MACROPHAGE AND OSTEOCLAST FORMATION IN INFLAMMATORY ARTHRITIS**

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Periarticular bone resorption is a feature of inflammatory arthritis due to rheumatoid disease, crystal arthritis and some form of osteoarthritis (OA). In these conditions the synovial fluid contains numerous inflammatory cells (mainly granulocytes and macrophages) that produce cytokines and growth factors (e.g. RANKL, OPG, M-CSF, TNF-alpha, IL-1) which influence osteoclast formation.

Synovial fluid from rheumatoid arthritis (RA), pyrophosphate arthropathy (PPA) and non-inflammatory OA patients was added to cultures of human peripheral blood mononuclear cells (PBMCs) in the presence and absence of RANKL and M-CSF. The addition of 10% synovial fluid to PBMC cultures resulted in the formation of numerous TRAP+ and VNR+ mononuclear cells which were capable of lacunar resorption. Marked stimulation of osteoclast formation and resorption was seen in PBMC cultures incubated with 10% RA and PPA but not non-inflammatory OA synovial fluid. We also noted that macrophages isolated from the synovial fluid of patients with inflammatory arthritis were capable of osteoclast formation when incubated with RANKL and M-CSF. In both monocyte and macrophage cultures, numerous small (macrophage-like) TRAP+ mononuclear cells that were capable of the formation of numerous shallow resorption pits and extensive areas of lacunar resorption. Very few multinucleated cells were found in synovial fluid-macrophage cultures.

Our studies indicate that there are likely to be soluble factors in inflammatory arthritis synovial fluid which are likely to promote bone loss (principally by mononuclear osteoclasts). Identification of the factors that promote osteoclast formation and resorption in synovial fluid should provide therapeutic targets for the treatment of inflammatory arthritis.

**P68**

**LIGHT, A TNF-SUPERFAMILY MEMBER, INDUCES OSTEOCLAST FORMATION IN VITRO; A NOVEL MEDIATOR OF BONE RESORPTION?**

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It is now recognised that members of the TNF ligand/receptor superfamily complex are important in osteoclast formation. RANKL and OPG play a central role in promoting and inhibiting osteoclastogenesis respectively. TNF-alpha regulates RANKL and OPG gene

expression in osteoblasts and can induce RANKL-independent osteoclast formation by a mechanism which operates at a lower level of efficiency than RANKL-induced osteoclastogenesis.

LIGHT is a member of the TNF-superfamily derived from an activated T cell cDNA library. LIGHT signals through the binding of two cell surface receptors: lymphotoxin-beta (LT-betaR) and herpes virus entry mediator (HVEM); a soluble decoy receptor (DcR-3) also binds LIGHT and, in a manner similar to OPG, competitively blocks the binding of LIGHT to its receptors.

We found that in long-term cultures of human monocytes incubated with M-CSF, or RAW 264.7 mouse macrophages, LIGHT markedly stimulated RANKL-dependent osteoclast formation. We also found that LIGHT promoted osteoclast formation and bone resorption in these cultures by a RANKL-independent mechanism. In contrast to TNFalpha, LIGHT resulted in the formation of numerous TRAP+ and VNR+ cells which were capable of extensive lacunar resorption. The addition of OPG and RANK:Fc had no significant effect on LIGHT induced osteoclast formation.

Flow cytometric analysis identified the presence of LT-betaR and HVEM on the surface of human peripheral blood mononuclear cells whereas LT-betaR was only expressed on CD14-positive osteoclast precursors.

Our results suggest that LIGHT, which is highly expressed by lymphocytes, granulocytes and monocytes, is involved in a pathway of osteoclast formation independent of RANKL, and is also capable of interacting and enhancing the effects of RANKL-mediated osteoclastogenesis.

## **P69**

### **RELATIONSHIP BETWEEN OSTEOPENIA AND LUMBAR INTERVERTEBRAL DISC DEGENERATION IN OVARIECTOMIZED RATS**

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Ovariectomy can cause bone loss in rats, but little is known about how it also induces lumbar intervertebral disc degeneration. This study investigated how estrogen deficiency affected intervertebral discs in ovariectomized rats. Thirty 3-month-old female Sprague-Dawley rats were divided randomly into three equal groups. The baseline control group was killed at the beginning of the experiment. An ovariectomy was performed in 10 rats and another group of 10 rats was subjected to a sham surgery. The ovariectomized rats were untreated after the surgery to allow for the development of moderate osteopenia. Bone mineral density measurement and bone histomorphometric analysis were applied to the segments of lumbar spines in all rats killed 6 months after postsurgery. The pathological changes of intervertebral discs were observed and the degree of lumbar intervertebral disc degeneration was scored by a histological scoring system. The bone mineral density of the spines in the ovariectomized group decreased significantly compared with the sham group. The bone volume indices in the ovariectomized group were significantly lower, but the bone turnover rate parameters were significantly higher than those in the sham group. The histological scores for lumbar intervertebral disc degeneration in the ovariectomized group were significantly higher than those in the sham group. There existed a significant negative correlation between the bone mineral density and Grade 2 discs in the ovariectomized rats. In conclusion, lumbar intervertebral disc degeneration occurs in the ovariectomized rats and the degeneration of cartilage end plates may be a pathogenic factor in disc degeneration.

## **P70**

### **THE EFFECT OF MECHANICAL STIMULATION ON OSTEOCYTE VIABILITY IN BONE**

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Physical activity is a key determinant of bone mass and health. It has been proposed that it contributes to the viability of bone cells through enhanced supply of nutrients and oxygen via the osteocyte lacuna/canicular system. In addition to healthy blood flow physical activity is

required for the full perfusion of cortical bone and during sustained unloading in vivo osteocytes become hypoxic and may die. We have studied the effect of chronic mechanical stimulation on osteocyte viability in bone maintained in a bioreactor.

The Zetos™ bioreactor is capable of maintaining bone biopsies ex vivo for up to 45 days. Trabecular bone samples from the proximal head of bovine femurs were prepared for use in bioreactor chambers. Samples were loaded for 5 minutes a day using either a walking (3000 Åstrain), jumping (3000 Åstrain) or control (no load) waveform repeated at 1 Hz for 28 days. Hydrogen peroxide treated negative control groups were also loaded in the same mode. After the experimental period, total cell numbers and cell viability were determined in unfixed cryostat sections by nuclear staining (DAPI) and lactate dehydrogenase activity (LDH) respectively. Cells were categorized as residing up to 1.5mm (outer zone) or >1.5mm (inner zone) from the biopsy surface perimeter. Osteocyte numbers were not significantly different between treatments and controls in any zone indicating a lack of clearance of dead cells. Osteocyte viability in control samples was lower in the outer zone than the inner zone. In the inner zone a higher percentage of live cells was observed in the walking (46.49 ± 8.9 SE n=3, p=0.05) and jumping (54 ± 4.2 SE, p= 0.006) samples compared to control (28.14 ± 8.3 SE). In the outer (less viable) zone jumping but not walking stimulation had a positive effect on osteocyte viability such that it reached similar levels to those in the more healthy inner zone (15.5 % control vs 50% jumping).

These data indicate that in this system, mechanical stimuli are capable of maintaining osteocyte viability. In addition they would suggest that vigorous mechanical stimulation is capable of cell rescue from the death associated with drilling trauma at the biopsy surface.

## P71

### **A SELECTIVE ESTROGEN RECEPTOR MODULATOR (SERM) INHIBITS OSTEOCYTE APOPTOSIS DURING ESTROGEN LOSS. IMPLICATIONS FOR BONE QUALITY MAINTENANCE**

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Ideally, Selective Estrogen Receptor Modulators should demonstrate all the positive bone associated effects of estrogens without their soft tissue side effects. While SERMs are known to alter bone formation and resorption in favour of bone mass, their full range of activities in bone are unknown. Estrogens are associated with positive effects not only on the quantity of bone, but also its quality including the maintenance of osteocyte populations through the inhibition of their apoptosis. Here we have used a rat model of ovariectomy (OVX) to determine whether the osteocyte sparing effect of estradiol can be mimicked by the SERM LY117018. Sixteen 12-week-old rats (Weight, 263) were divided into 4 treatment groups: sham operated (SHAM) (n=4), OVX (n=4), and OVX + 17 $\beta$ -estradiol (E2) (0.125mg/kg/day) (n=4), OVX + SERM (3mg/kg/day) (n=4). After 7 days treatment, radius and ulna were removed and the percentage of apoptotic osteocytes determined using an in situ nick-translation method. The success of ovariectomy was assessed by measurement of uterine weight. The proportion of apoptotic osteocytes present was five times higher in the OVX compared with the SHAM groups in both radius (1.09% vs 0.21%, respectively; p<0.01) and ulnae (1.40% vs 0.36%, respectively; p=0.001). Addition of estradiol to the OVX animals completely abrogated the increase in osteocyte apoptosis in both the radius (0.38%) and ulna (0.26%). Addition of the SERM to the OVX animals abrogated the increase in apoptosis to the same extent as estradiol in both the radius (0.35%) and ulna (0.38%). Nuclear morphology and electrophoresis of DNA confirmed the presence of apoptotic cells in the samples. In conclusion, estradiol and SERM are equally good at preventing the increase in osteocyte apoptosis engendered by OVX. These data point to the potential benefits of SERMs in the maintenance of both the quantity and the quality of bone in the post-menopausal individual. We will discuss the relevance of these data to our understanding of SERM function and the design of future disease intervention strategies.

**P72**

**THE ANTI-OXIDANT EFFECTS OF ESTROGEN AND ESTROGENIC DERIVATIVES IN THE PROTECTION OF OSTEOCYTES FROM OXIDATIVE STRESS INDUCED CELL DEATH *IN VITRO***

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Withdrawal of estrogen is the major factor determining post menopausal bone loss and has been associated with the apoptotic death of osteocytes. While estrogen replacement demonstrates clear estrogen receptor mediated benefits to bone cells little is known regarding estrogens' potential anti-oxidant effects in bone. Selective Estrogen Receptor Modulators (SERMs) are a class of non-steroidal compounds which exert estrogen agonist and antagonist effects on a number of target tissues. They demonstrate classic estrogen-like bone sparing effects and in addition have been shown to prevent ovariectomy induced osteocyte apoptosis *in vivo*. Our recent *in vitro* studies have suggested that estrogens ability to save osteocytes from oxidant induced death occurs independently of the oestrogen receptor and may be related to oestrogens known activity as an anti-oxidant. In this study we have sought to determine whether SERMs are capable of saving osteocytes from oxidant induced death through a non-receptor mediated route. Treatment of osteocyte cell line MLO-Y4 with H<sub>2</sub>O<sub>2</sub> caused a dose dependent induction of apoptotic cell death, which was significantly inhibited ( $p = 0.0019$ ) on pre-treatment with 17- $\beta$ -estradiol at near physiological concentration (10 nM). The saving effects of estradiol were shown to be receptor independent since pre-treatment of cells with estrogen receptor antagonist ICI 182,780 did not block the estradiol inhibition of apoptosis and the inactive stereoisomer 17- $\alpha$ -estradiol also significantly inhibited ( $p=0.0017$ ) oxidant induced apoptosis. The use of Selective Estrogen-Receptor Modulators (SERMS) Raloxifene and 117018 also significantly blocked oxidant induced apoptosis ( $p<0.0002$ ) however estrogen derivative Mestranol which lacks putative antioxidant moiety had no saving effect. 17- $\beta$ -estradiol, 17- $\alpha$ -estradiol and the SERMS all significantly reduced ( $p<0.002$ ) the number of reactive oxygen species positive cells in the presence of the free radical indicator 2'7'- dichlorodihydrofluorescein diacetate to levels comparable to potent anti-oxidant Vitamin E. It is possible that loss of osteocytes during estrogen insufficiency may occur through a failure to suppress the activity of naturally occurring or disease associated production of oxidant molecules. In addition these data suggest that estrogen and SERMs ability to save osteocytes from oxidant induced death might occur independently of estrogen receptor activation pointing to novel design criteria for pharmacological interventions.