



**Abstracts from the  
Bone and Tooth Society  
Annual Meeting 2005**

4-5 July 2005

**Birmingham, UK**

**Organiser**

**Dr Mark Cooper  
University of Birmingham**

# **Bone and Tooth Society**

The Society is the oldest and largest scientific society in Europe that is dedicated to further research into clinical and basic science problems related to mineralised tissues. The meeting attracts a wide audience from throughout the UK and, increasingly, from continental Europe and further afield. The presentations are traditionally a balance between clinical and laboratory-based studies. The participation of young scientists and clinicians is actively encouraged.

## **Committee 2005**

President: Richard Eastell (Sheffield)

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Sharyn Bord (Cambridge)

Peter Croucher (Sheffield)

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Richard Keen (London)

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## **Annual Meeting 2005**

### **Venue**

The meeting will take place at the University of Birmingham, Edgbaston, UK

The Society gratefully acknowledges the support of the following  
(list as at March 2005):

### **Major Sponsors**

Eli Lilly  
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### **CPD**

The meeting is approved for 13 CPD credits for full attendance.

### **Date of Next Meeting:**

4-6 July 2006

**[www.batsoc.org.uk](http://www.batsoc.org.uk)**

## **Awards**

The Bone and Tooth Society is delighted to make awards to the following, based on the review panel's assessment of their abstract. The Awards will be presented during the Awards Ceremony at 12.30 on Tuesday 5 July.

## **Scholarships**

Georgios Belibasakis (London) (OC2)

MAPK SIGNALLING AND RHO GTPASE ACTIVATION ARE PERTURBED BY PASTEURELLA MULTOCIDA TOXIN (PMT) DURING DIFFERENTIATION OF MURINE AND HUMAN OSTEOCLASTS

Roberto Desouza (London) (OC7)

THE OSTEOGENIC RESPONSE TO MECHANICAL LOADING IS SUPRESSED IN OLD FEMALE MICE BUT IS RECUELED AFTER PERIODS OF DISUSE

Isabel Orriss (London) (OC9)

P2X<sub>2</sub> BUT NOT P2X<sub>3</sub> RECEPTOR KNOCKOUT MICE DEMONSTRATE INCREASED BONE MASS AND WEIGHT

Helen Owen (Roslin) (OC8)

GROWTH PLATE CHONDROGENESIS AND LONGITUDINAL BONE GROWTH FOLLOWING EXPOSURE TO THE NOVEL GLUCOCORTICOID RECEPTOR LIGAND, AL-438

Scott Roberts (Roslin) (OC14)

OSTEOBLAST AND CHONDROCYTE MATRIX VESICLES CONTAIN PHOSPHO1: EVIDENCE FOR ITS ROLE IN MINERAL FORMATION

Carles Vilarino-Guell (Oxford) (OC10)

ESTROGEN RECEPTOR-ALPHA POLYMORPHISMS AFFECT CORTICAL BONE GROWTH IN CHILDHOOD IN A SITE-DEPENDENT MANNER

## **Bursaries**

Sweyn Garrioch (Aberdeen) (OC16)

BISPHOSPHONATES PREVENT ADHESION OF BREAST CANCER CELLS TO BONE IN VITRO BY INHIBITING PROTEIN PRENYLATION

Rowan Hardy (Birmingham) (P10)

AUTOCRINE REGULATION OF GLUCOCORTICOIDS BY 11 BETA-HYDROXYSTEROID DEHYDROGENASE TYPE 1: A MECHANISM FOR TISSUE-SPECIFIC REGULATION OF INFLAMMATION

Frank Joseph (Liverpool) (OC15)

THE CIRCADIAN RHYTHM OF OSTEOPROTEGERIN AND ITS ASSOCIATION WITH PARATHYROID HORMONE SECRETION IN ELDERLY MEN AND WOMEN

Stephen Kaptoge (Cambridge) (OC12)

VITAMIN K AND FRACTURE RISK: AN EFFECT ON BONE WIDTH NOT BMD?

Benit Maru (Bristol) (P5)

DIFFERENCES IN OESTROGEN RECEPTOR TRAFFICKING BETWEEN BONE AND MAMMARY CELLS

Anke Roelofs (Oxford) (OC17)

APOMINE, AN INHIBITOR OF HMG-COA-REDUCTASE, DOES NOT ACT BY INHIBITING PROTEIN PRENYLATION IN HUMAN MYELOMA CELLS IN VITRO

Danielle Scheven (Aberdeen) (P6)

THE ROLE OF CALCIUM IONS IN THE CELLULAR UPTAKE OF BISPHOSPHONATE DRUGS

Adam Taylor (Aberdeen) (OC5)

OSTEOCLASTS DERIVED FROM GUNMETAL MICE ARE FUNCTIONAL DESPITE HAVING DEFECTS IN RAB PRENYLATION

**Bone and Tooth Society Annual Meeting**  
**Birmingham, UK**  
**4-5 July 2005**  
**Outline programme**

	Monday 4 July	Tuesday 5 July
Morning	<p>09.30 Registration</p> <p>10.30 Symposium: Osteoclast biology Lorenz Hofbauer Steven Teitelbaum</p> <p>11.30 Oral communications</p> <p>12.30 Lunch</p>	<p>08.30 Symposium: Local regulators of bone metabolism Vincent Everts Clemens Löwik</p> <p>09.30 Oral communications</p> <p>10.30 Posters and coffee</p> <p>11.30 Poster discussion</p> <p>12.30 Awards/AGM Lunch</p>
Afternoon	<p>13.30 Symposium: Bioengineering and the skeleton David Hukins Richard Oreffo</p> <p>14.30 Posters and tea</p> <p>15.30 Symposium: The importance of skeletal shape and size Nicola Crabtree Thomas Einhorn</p> <p>16.30 Oral communications</p> <p>18.00 Close</p>	<p>13.15 Symposium: Nutrition and bone Ann Prentice Helen MacDonald</p> <p>14.15 Clinical Case Presentations</p> <p>15.00 Tea</p> <p>15.20 Clinical symposium John Bilezikian Fred Singer Michael McClung</p> <p>16.50 Close</p>
Evening	<p>20.00 Annual Dinner Birmingham Botanical Gardens</p>	

## Monday 4th July

- 09:30-10:20 Registration / coffee
- 10:20-10:30 **Opening ceremony**  
Chairman: Mark Cooper (Birmingham, UK)
- William Doe**, Dean of Medicine, University of Birmingham
- 10:30-11:30 **Symposium: Osteoclast biology**  
Chairmen: Tim Arnett (London, UK)/Jon Tobias (Bristol, UK)
- 10:30 IS1 ESTROGENS AND THE OPG/RANKL SYSTEM  
**Lorenz Hofbauer** (Marburg, Germany)
- 11:00 IS2 INTEGRIN FUNCTION IN OSTEOCLASTS  
**Steven Teitelbaum** (St Louis MO, USA)
- 11:30-12:30 **Oral communications**  
Chairmen: Peter Croucher (Sheffield, UK)/Steven Teitelbaum (St Louis MO, USA)
- 11:30 OC1 THE EFFECT OF AZD0530, A HIGHLY SELECTIVE SRC INHIBITOR, ON BONE TURNOVER IN HEALTHY MALES  
R A Hannon (Sheffield, UK)
- 11:42 OC2 MAPK SIGNALLING AND RHO GTPASE ACTIVATION ARE PERTURBED BY PASTEURELLA MULTOCIDA TOXIN (PMT) DURING DIFFERENTIATION OF MURINE AND HUMAN OSTEOCLASTS  
G Belibasakis (London, UK)
- 11:54 OC3 OSTEOCLAST FORMATION AND BONE RESORPTION ARE INHIBITED BY MEGAKARYOCYTES  
S Bord (Cambridge, UK)
- 12:06 OC4 CANINE DISTEMPER VIRUS INDUCES A DOSE-DEPENDENT INCREASE IN HUMAN OSTEOCLAST FORMATION AND FUNCTION IN VITRO  
A P Mee (Manchester, UK)
- 12:18 OC5 OSTEOCLASTS DERIVED FROM GUNMETAL MICE ARE FUNCTIONAL DESPITE HAVING DEFECTS IN RAB PRENYLATION  
A Taylor (Aberdeen, UK)
- 12:30-13:30 Lunch
- 13:30-14:30 **Symposium: Bioengineering and the skeleton**  
Chairmen: Thomas Einhorn (Boston MA, USA)/James Triffitt (Oxford, UK)
- 13:30 IS3 COLLAGEN FUNCTION IN BONE AND CARTILAGE  
**David Hukins** (Birmingham, UK)

- 14:00 IS4 BONE TISSUE ENGINEERING: HARNESSING BIOMIMETIC SCAFFOLDS AND MESENCHYMAL STEM CELLS  
**Richard Oreffo** (Southampton, UK)
- 14:30-15:30 Poster viewing / tea
- 15:30-16:30 **Symposium: The importance of skeletal shape and size**  
Chairmen: Andrew Pitsillides (London, UK)/Jonathan Reeve (Cambridge, UK)
- 15:30 IS5 SKELETAL SHAPE AND FRACTURE RISK IN PAEDIATRICS  
**Nicola Crabtree** (Birmingham, UK)
- 16:00 IS6 SKELETAL ADAPTATION TO MECHANICAL FORCES  
**Thomas Einhorn** (Boston MA, USA)
- 16:30-18:00 **Oral communications**  
Chairmen: Colin Farquharson (Roslin, UK)/Nick Shaw (Birmingham, UK)
- 16:30 OC6 CHARACTERISATION OF HUMAN FETAL POPULATIONS: A COMPARATIVE MODEL FOR SKELETAL AND STEM CELL DIFFERENTIATION  
S H Mirmalek-Sani (Southampton, UK)
- 16:42 OC7 THE OSTEOGENIC RESPONSE TO MECHANICAL LOADING IS SUPRESSED IN OLD FEMALE MICE BUT IS RECUED AFTER PERIODS OF DISUSE  
R L de Souza (London, UK)
- 16:54 OC8 GROWTH PLATE CHONDROGENESIS AND LONGITUDINAL BONE GROWTH FOLLOWING EXPOSURE TO THE NOVEL GLUCOCORTICOID RECEPTOR LIGAND, AL-438  
H Owen (Roslin, UK)
- 17:06 OC9 P2X<sub>2</sub> BUT NOT P2X<sub>3</sub> RECEPTOR KNOCKOUT MICE DEMONSTRATE INCREASED BONE MASS AND WEIGHT  
I R Orriss (London, UK)
- 17:18 OC10 ESTROGEN RECEPTOR-ALPHA POLYMORPHISMS AFFECT CORTICAL BONE GROWTH IN CHILDHOOD IN A SITE-DEPENDENT MANNER  
C Vilarino-Guell (Oxford, UK)
- 17:30 OC11 LATE EFFECTS OF VERY LOW BIRTH WEIGHT ON BONE MASS AT THE LUMBAR SPINE  
C M Smith (Sheffield, UK)
- 17:42 OC12 VITAMIN K AND FRACTURE RISK: AN EFFECT ON BONE WIDTH NOT BMD?  
S Kaptoge (Cambridge, UK)
- 18:00 Buses to Hall of Residence



19:30	Buses from Chamberlain Hall to Botanical Gardens
20:00	Dinner (Botanical Gardens)
23:00	Buses return to Chamberlain Hall

## Tuesday 5th July

- 08:00 Buses from Chamberlain Hall to meeting
- 08:30-09:30 **Symposium: Local regulators of bone metabolism**  
Chairmen: Michael Parfitt (Boston MA, USA)/Richard Oreffo (Southampton, UK)
- 08:30 IS7 WHAT DO BONE LINING CELLS REALLY DO?  
**Vincent Everts** (Amsterdam, The Netherlands)
- 09:00 IS8 OSTEOCYTE CONTROL OF OSTEOBLAST FUNCTION  
**Clemens Löwik** (Leiden, The Netherlands)
- 09:30-10:30 **Oral communications**  
Chairmen: Bronwen Evans (Cardiff, UK)/Agi Grigoriadis (London, UK)
- 09:30 OC13 SCLEROSTIN EXPRESSION BY OSTEOCYTES IS A DELAYED EVENT;  
IMPLICATIONS FOR THE CONTROL OF BONE FORMATION  
K E S Poole (Cambridge, UK)
- 09:42 OC14 OSTEOBLAST AND CHONDROCYTE MATRIX VESICLES CONTAIN  
PHOSPHO1: EVIDENCE FOR ITS ROLE IN MINERAL FORMATION  
S J Roberts (Roslin, UK)
- 09:54 OC15 THE CIRCADIAN RHYTHM OF OSTEOPROTEGERIN AND ITS  
ASSOCIATION WITH PARATHYROID HORMONE SECRETION IN ELDERLY  
MEN AND WOMEN  
F Joseph (Liverpool, UK)
- 10:06 OC16 BISPHOSPHONATES PREVENT ADHESION OF BREAST CANCER CELLS TO  
BONE IN VITRO BY INHIBITING PROTEIN PRENYLATION  
S Garrioch (Aberdeen, UK)
- 10:18 OC17 APOMINE, AN INHIBITOR OF HMG-COA-REDUCTASE, DOES NOT ACT BY  
INHIBITING PROTEIN PRENYLATION IN HUMAN MYELOMA CELLS IN  
VITRO  
A J Roelofs (Oxford, UK)
- 10:30-11:30 Poster viewing / coffee
- 11:30-12:30 **Poster discussion**  
Chairmen: Juliet Compston (Cambridge, UK)/Clemens Löwik (Leiden, The Netherlands)
- 11:30 P1 LOCALISATION OF HIF-2ALPHA TO SPECIFIC MATURATIONAL ZONES OF  
BOTH GROWTH PLATE AND ARTICULAR CARTILAGE  
A J Stewart (Roslin, UK)

- 11:35 P2 THE RESTRICTED POTENTIAL FOR RECOVERY OF GROWTH PLATE CHONDROGENESIS AND LONGITUDINAL BONE GROWTH FOLLOWING EXPOSURE TO PROINFLAMMATORY CYTOKINES  
V E MacRae (Glasgow and Roslin, UK)
- 11:40 P3 OSTEOBLAST CELL CYCLE REGULATION DURING DIFFERENTIATION DIFFERS FROM THAT OF EXPONENTIAL GROWTH: THE ROLE OF P27 AND CYCLIN D3  
A Sunters (London, UK)
- 11:45 P4 INTRAUTERINE PROGRAMMING OF MESENCHYMAL STEM CELL ACTIVITY  
S A Lanham (Southampton, UK)
- 11:50 P5 DIFFERENCES IN OESTROGEN RECEPTOR TRAFFICKING BETWEEN BONE AND MAMMARY CELLS  
B S Maru (Bristol, UK)
- 11:55 P6 THE ROLE OF CALCIUM IONS IN THE CELLULAR UPTAKE OF BISPHOSPHONATE DRUGS  
D M Scheven (Aberdeen, UK)
- 12:00 P7 STUDIES OF THE EXPRESSION IN HUMAN DUODENUM OF THE CALCIUM TRANSPORTER TRPV6 AND THE RELATIONSHIP TO VITAMIN D, AGE, SEX AND BONE MINERAL DENSITY  
J R F Walters (London, UK)
- 12:05 P8 OSTEOCLAST FORMATION POTENTIAL IS INCREASED AT TIMES OF PULMONARY INFECTION IN ADULTS WITH CYSTIC FIBROSIS  
E Shead (Cambridge, UK)
- 12:10 P9 LARGE POPULATION-BASED GEOGRAPHIC VARIATIONS IN DXA BMD IN MEN AND WOMEN ACROSS EUROPE. RESULTS FROM THE NETWORK FOR MALE OSTEOPOROSIS (NEMO) STUDY  
S Kaptoge (Cambridge, UK)
- 12:15 P10 AUTOCRINE REGULATION OF GLUCOCORTICOID BY 11 BETA-HYDROXYSTEROID DEHYDROGENASE TYPE 1: A MECHANISM FOR TISSUE-SPECIFIC REGULATION OF INFLAMMATION  
R S Hardy (Birmingham, UK)
- 12:30-13:15 **Awards**  
**Bone and Tooth Society AGM** (members)  
Lunch
- 13:15-14:15 **Symposium: Nutrition and bone**  
Supported by Alpro UK  
Chairmen: Roger Francis (Newcastle upon Tyne, UK)/David Reid (Aberdeen, UK)
- 13:15 IS9 CALCIUM AND BONE: HOW MUCH AND WHEN?  
**Ann Prentice** (Cambridge, UK)

- 13:45 IS10 FRUIT AND VEGETABLES IN BONE HEALTH  
**Helen MacDonald** (Aberdeen, UK)
- 14:15-15:00 **Oral communications – clinical**  
Chairmen: Richard Keen (London, UK)/Larry Raisz (Farmington, USA)
- 14:15 OC18 FAMILIAL HYPOMAGNESAEMIA-HYPERCALCIURIA AND  
NEPHROCALCINOSIS IN 2 SIBLINGS : 2 NOVEL HETEROZYGOUS  
MUTATIONS IN THE GENE ENCODING CLAUDIN-16  
G Hampson (London, UK)
- 14:30 OC19 A RANDOMISED CONTROLLED TRIAL (RCT) OF OSTEOPOROSIS  
SCREENING: LONG TERM EFFECTS ON USE OF MEDICATION AND  
FRACTURE RISK  
R J Barr (Aberdeen, UK)
- 14:45 OC20 THE RECORD TRIAL OF CALCIUM AND/OR VITAMIN D IN THE  
SECONDARY PREVENTION OF OSTEOPOROTIC FRACTURES  
A M Grant (Aberdeen, UK)
- 15:00-15:20 Tea
- 15:20-16:50 **Clinical Symposium**  
Chairmen: Richard Eastell (Sheffield, UK)/Neil Gittoes (Birmingham, UK)
- 15:20 IS11 MEDICAL APPROACHES TO HYPERPARATHYROIDISM  
**John Bilezikian** (New York, USA)
- 15:50 IS12 AETIOLOGY OF PAGET'S DISEASE OF BONE AND SUMMARY OF  
CURRENT TREATMENT GUIDELINES  
**Fred Singer** (Los Angeles, USA)
- 16:20 IS13 UPDATE ON TREATMENTS FOR OSTEOPOROSIS  
**Michael McClung** (Portland, USA)
- 16:50 End of Meeting

### IS1

#### Estrogens and the osteoprotegerin/RANK-Ligand system

LC Hofbauer<sup>[1]</sup>, V Viereck<sup>[2]</sup>. Division of Gastroenterology and Endocrinology<sup>[1]</sup> Philipps-University, Baldingerstr. D-35033 Marburg, Department of Gynecology and Obstetrics<sup>[2]</sup> University of Goettingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany

Bone remodeling is a life-long coordinated process of osteoclastic bone resorption and osteoblastic bone formation that rejuvenates the skeleton while maintaining its structure. Recently, receptor activator of NF- $\kappa$ B ligand (RANKL) has been identified as essential cytokine for the formation and activation of osteoclasts. The effects of RANKL are physiologically counter-balanced by the decoy receptor osteoprotegerin (OPG). The balance between RANKL and OPG is regulated by cytokines, hormones, and drugs, and crucially determines osteoclast functions and bone resorption. Alterations of the RANKL-to-OPG ratio are essential in the pathogenesis of bone diseases that result from increased bone resorption, including postmenopausal osteoporosis. Several lines of evidence implicate the OPG/RANKL system in the pathogenesis of osteoporosis following estrogen deficiency: (I) Estrogen receptor agonists are able to enhance OPG production in human osteoblasts through stimulating of osteoblastic differentiation. Our groups have demonstrated these effects for 17 $\beta$ -estradiol, the phytoestrogen genistein, and the selective estrogen receptor modulator raloxifene (II) Marrow stromal cells as well as B and T lymphocytes from postmenopausal women display higher levels of RANKL expression than premenopausal women or postmenopausal women on estrogen replacement therapy. In these women, RANKL expression is inversely correlated with serum levels of 17 $\beta$ -estradiol and positively with bone resorption markers. (III) The activity of c-jun N-terminal kinase (JNK), an intracellular signal following binding of RANKL to its specific receptor can be repressed by 17 $\beta$ -estradiol. (IV) Several studies have documented that polymorphisms in the OPG gene may confer an increased risk of developing postmenopausal osteoporosis. (V) In preclinical models of osteoporosis due to estrogen deficiency, bone loss was prevented by RANKL blockade using a modified OPG protein. A similar approach is currently being employed in clinical studies on postmenopausal women using monoclonal antibodies against RANKL. The role of the OPG/RANKL system in bone cell biology, its various interactions with estrogen receptor agonists, and progress of studies involving RANKL blockade against postmenopausal osteoporosis will be presented at this meeting.

### IS2

#### INTEGRIN FUNCTION IN OSTEOCLASTS

S L Teitelbaum. Washington University School of Medicine, St. Louis MO, USA

The osteoclast is the exclusive resorptive cell of the skeleton whose differentiation from members of the monocyte/macrophage family requires exposure to the cytokines, RANK ligand and M-CSF. Having assumed its mature phenotype, the cell's resorptive capacity depends upon its physical contact with bone. Integrins, which are the principal cell/matrix attachment molecules, are  $\alpha$ v/ $\beta$ 3 transmembrane heterodimers. Because our in vitro experiments indicated the  $\alpha$ v/ $\beta$ 3 integrin mediates osteoclastic bone resorption we deleted the  $\beta$ 3 integrin gene in mice and established they have increase bone mass due to dysfunctional osteoclasts. Using a retroviral strategy to transduce various  $\beta$ 3 constructs into osteoclasts lacking the integrin, we find the integrin subunit's cytoplasmic domain, and particularly  $\beta$ 3S752, is essential for  $\alpha$ v/ $\beta$ 3 function. The failure of  $\beta$ 3<sup>-/-</sup> mice to resorb bone reflects their inability to organize their cytoskeleton due to suppressed activation of the small GTPase, Rac. In keeping with this observation, mice conditionally deleted of both the Rac1 and Rac2 isoforms cannot normally degrade bone and as such have a trabecular mass more than three fold greater than wild type. Because the Vav family of guanine nucleotide exchange factors transmits Rac from its GDP to GTP bound form, we turned to mice deleted of these regulatory molecules and find those lacking the Vav3 isoform also have dysfunctional osteoclasts due to absence of  $\alpha$ v/ $\beta$ 3 integrin-mediated Vav phosphorylation. Finally, Rac is activated, in osteoclasts, by a signaling cascade in which Syk recruits c-Src to the  $\beta$ 3 integrin cytoplasmic domain. c-Src, in turn activates Syk which phosphorylates Vav3 leading to Rac-mediated organization of the osteoclast cytoskeleton. Thus,  $\alpha$ v/ $\beta$ 3 is not only the means by which osteoclasts recognize bone, but the integrin also transmits extracellular cues to intracellular signaling molecules to organize the resorptive cell's cytoskeleton. Consequently,  $\alpha$ v/ $\beta$ 3 is a current and attractive anti-osteoporosis therapeutic target.

### IS3

#### COLLAGEN FUNCTION IN BONE AND CARTILAGE

D W L Hukins. University of Birmingham, Birmingham, UK

This lecture summarises how collagen is able to reinforce the extracellular matrix (ECM) of three different types of tissue: meniscus (as an example of fibrocartilage), articular cartilage (as an example of hyaline cartilage) and cortical bone. In the first two examples, fibrils of interstitial collagens reinforce a proteoglycan gel; in the third example the ECM consists mainly of collagen and a mineral that resembles hydroxyapatite.

Collagen fibrils are stiff and strong in tension; the restoring forces in stretched fibrils oppose forces applied to tissues and so provide tensile reinforcement. However, like all fibres, they are expected to have low flexural and torsional stiffness; they will buckle under axial compression. Therefore, collagen fibrils can only provide appreciable reinforcement to ECM if they are oriented in the directions in which the tissue is stretched by applied forces. This expectation applies equally to types I, II and III collagen since they all form fibrils.

In fibrocartilage, collagen fibrils form fibres. In the menisci, inner fibres are oriented circumferentially so that they are stretched when the femoral condyles apply a compressive load to the surface of the tissue. An outer layer of fibres forms a wrapping that prevents the inner fibres from being pushed apart. When this system fails, tears develop.

In hyaline cartilage, individual collagen fibrils are dispersed in proteoglycan gel. In articular cartilage, fibrils in the surface zone are oriented so that they are stretched by the swelling pressure of the proteoglycan gel; this internal pressure combined with the tensile reinforcement, enables the tissue to withstand compression. Fibrils in the deep zone are oriented to anchor the tissue to the underlying calcified tissue. Tapered collagen fibrils have the advantage for reinforcing such systems that stress is distributed reasonably uniformly along their lengths.

In cortical bone collagen fibrils provide tensile reinforcement but they will also be subjected to compression. However, unlike the collagen fibrils in cartilage, they will not buckle because they are surrounded by very stiff mineral.

#### **IS4**

##### **BONE TISSUE ENGINEERING: HARNESSING BIOMIMETIC SCAFFOLDS AND MESENCHYMAL STEM CELLS.**

R O C Oreffo. Bone and Joint Research Group, Developmental Origins of Health and Disease, University of Southampton, SO16 6YD, UK

Bone is unique with a vast potential for regeneration from cells with stem cell characteristics with the capability, by definition, to self-renew as well as to give rise to daughter cells. With an increasing aging population, clinical imperatives to augment and facilitate tissue lost as a consequence of trauma or degeneration, have led to increased interest in these progenitor cells. Mesenchymal stem cells or human bone marrow stromal stem cells are defined as multipotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. These primitive progenitors exist postnatally and exhibit stem cell characteristics, namely low incidence and extensive renewal potential. These properties in combination with their developmental plasticity has generated tremendous interest in the potential use of mesenchymal stem cells to replace damaged skeletal tissues as a result of integration of stem cells, gene therapy and tissue engineering. To date, relatively little is known concerning the phenotypic characteristics, whether from a morphological or biochemical standpoint whilst direct in vivo confirmation of the lineage potential and plasticity or interconversion potential that exists of mesenchymal stem cells and osteogenic progenitor cells remains unclear. In essence mesenchymal stem cells could be cultured to expand their numbers then transplanted to the injured site or after seeding in/on shaped biomimetic scaffolds together with judicious selection of osteotropic growth factors to generate appropriate skeletal tissue constructs for, ultimately, bone repair.

Current concepts, approaches and challenges from work in the Southampton group are centered on: i) isolation, expansion and characterisation of the plasticity of fetal and adult osteoprogenitor populations tissue, ii) combination of osteoprogenitor cells with innovative self assembling biomimetic matrices and, iii) use of selected osteotropic agents and biomimetic scaffolds in an attempt to modulate the phenotype of the mesenchymal stem cell to generate mineralised bone tissue. Although clinical efficacy has yet to be achieved, development of protocols, tools and above all multidisciplinary approaches for de novo bone formation that utilise mesenchymal stem may improve the quality of life for many as a result of strategies to augment skeletal regeneration.

#### **IS5**

##### **SKELETAL SIZE AND FRACTURE RISK IN PAEDIATRICS**

N J Crabtree<sup>[1]</sup>, N J Shaw<sup>[1]</sup>. <sup>[1]</sup>Dept. Nuclear Medicine, Queen Elizabeth Hospital Birmingham & <sup>[2]</sup>Dept. of Endocrinology, Birmingham Children's Hospital, UK

Osteoporosis in adulthood is a heterogeneous disorder which may in part represent a failure of normal bone accretion in childhood. However osteoporosis and associated fragility fractures can also present earlier in life when a child either fails to gain bone or alternately loses bone due to chronic disease or pharmaceutical therapy. As such, both childhood and senile osteoporosis can be seen as a paediatric disease. To a large extent our bone shape, size and mass are genetically determined. However, environmental factors such as nutrition, physical activity and disease have an important and modifiable role on skeletal development.

Chronic diseases in children may affect their nutrition, mobility, and most frequently their growth such that when compared to healthy controls they may appear short. Being short is a consequence, not a diagnosis, of disease. It is also the most common cause of misinterpretation when assessing bone density by DXA. If skeletal size is reduced but bone mass optimally adapted to a reduced stature, a child may be at no greater risk of osteoporotic fracture. However, in contrast, if a child has reduced or appropriate height for their age but disproportionately reduced bone mass and/or reduced bone size, they will be at considerable risk of both incident and possible future fracture.

To add further confusion to the confounding effects of disease and growth on bone; gender and ethnic origin have also been shown to have a significant impact on fracture risk. However, known differences in body and bone sizes between these groups makes interpretation of results obtained from DXA analysis hazardous, and potentially misleading.

To date there have been several methodologies used to adjust for this size artifact; some based around attempts to estimate bone thickness by assuming particular geometry, whilst others have used allometric scaling in conjunction with multiple regression analysis, with indices of bone and body size. More recent techniques have concentrated on the functional aspects of the muscle-bone relationship in accordance to the Frost mechanostat bone model.

Evaluating the impact of bone and body size on the gender and ethnic differences may give an insight into the reported differences in adulthood fracture rates. However, no single DXA normalisation technique has shown an overwhelming ability to predict childhood fracture. As such a multifactorial approach to paediatric bone evaluation is recommended, evaluating both bone mass and bone size in relation to current and future fracture risk.

#### **IS6**

##### **SKELETAL ADAPTATION TO MECHANICAL FORCES**

T A Einhorn. Boston University Medical Center, Boston, USA

The skeleton is a physiologically dynamic organ system whose primary functions are to provide mechanical support for locomotor activities, physical protection of essential organs and tissues, and to act as a storage facility for systemic mineral homeostasis. Skeletal architecture displays a form-function relationship and this relationship guides the evolution, embryogenesis and ongoing adaptation of the skeleton to its mechanical strain environment.

Most studies of skeletal adaptation to mechanical forces have focused on the response of intact bone to changes in loading history or experimental loading conditions. Depending on a number of factors including the anatomy of the bone, its complement of cortical and trabecular tissue, and the rate at which the loads are applied, different outcomes have been obtained. Disuse is clearly related to a negative adaptive response while increases in loading demands are associated with bone formation. Interestingly, the most osteogenic signal is intermittent, short bursts of loading with ample periods of time between loading doses in order to permit an optimum osteogenic response.

One of the more interesting aspects of skeletal adaptation is the way by which bone responds to mechanical cues when it is undergoing a repair process. To study this, we have developed an experimental model in the rat in which a critical-sized

defect is stabilized with an external fixator. The mechanical strain environment is then altered either by distraction or an applied bending motion. Our results show that a defined rate and rhythm of distraction leads to the regeneration of substantial amounts of new bone, and the translation of the mechanical signal into molecular events is manifest as an increase in angiogenesis. On the contrary, when bending is applied, the process of endochondral ossification leads to the formation of a hyaline-like cartilage tissue with an abundance of proteoglycans, type 2 collagen, and the formation of gradients in collagen fiber angles. These gradients are similar to those found in normal articular cartilage. Taken together, these findings support the intriguing possibility that adaptive responses of the skeleton may be used to translate mechanical signals into therapies that promote bone formation, bone repair, bone regeneration and the engineering of skeletal tissues to form joints or joint-like tissues.

### **IS7**

#### **WHAT DO BONE LINING CELLS REALLY DO?**

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Bone modelling depends on the activity of osteoblasts for bone deposition and on osteoclasts to resorb this tissue. In addition to these cells, a large fraction of the bone surface is covered by the alleged bone lining cells. Since bone lining cells are generally considered to be relatively inactive, they received little attention thus far. However, these cells are present not only on surfaces characterized by a very low modelling activity (resting surface) but also at sites where osteoclasts are active. At the latter sites we noted their presence in Howship's lacunae that were vacated by osteoclasts. Here bone lining cells engulf and subsequently digest demineralised bone collagen fibrils protruding from the bottom of the resorption lacunae. In addition they endocytose and inactivate osteoclast secreted TRACP. They thus clean the bottom of the pit of non-digested bone matrix and inactivate enzymes left by the osteoclast. The cleaning is followed by the deposition of a thin protein layer that covers the bottom of the pit. After this, new bone is deposited in the pit either by bone lining cells that differentiate into osteoblasts or by osteoblasts entering the site. Cleaning of the bottom of the pit proved to be crucial for subsequent bone formation; without the cleaning activity bone deposition did not occur. Recently, we found that bone lining cells also play a role in the recruitment of osteoclasts. Pre-osteoclasts proved to strongly attach to bone lining cells, which was followed by withdrawal of the bone lining cells. Subsequently the pre-osteoclasts entered the cell-free areas and fused to form osteoclasts. We propose a dual role played by bone lining cells in the sequence of events leading to bone resorption and subsequent bone apposition. First, these cells attract and direct pre-osteoclasts to the site of bone where resorption has to occur, and second, following the resorption of bone by osteoclasts, the bone lining cells clean the bottom of the pit and prepare this site for bone apposition by osteoblasts. Thus it appears that bone lining cells fulfil several hitherto unrecognised important roles in coupling bone resorption to bone formation.

### **IS8**

#### **OSTEOCYTE CONTROL OF OSTEOBLAST FUNCTION**

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Abstract not received

### **IS9**

#### **CALCIUM AND BONE: HOW MUCH AND WHEN?**

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Calcium is an essential bone-forming mineral that must be supplied by the diet throughout life. The amount of calcium consumed may affect bone growth and development, reproductive performance, including the skeletal health of the mother and offspring, and the risk of osteoporosis in later life. A low calcium intake (less than about 400 mg/d) is a risk factor for osteoporosis in countries where fracture incidence is high. In addition, calcium is a powerful anti-resorptive agent and can be useful in reducing bone loss in patients with osteoporosis. However, at the public health level, there is no consensus about the amount of calcium that must be consumed at each stage of life to ensure optimal bone health and reproductive performance. Results from intervention studies suggest that, in most age groups, increased intakes of calcium are associated with increased bone mineral content and, in the case of adolescent boys, with increased bone growth, but not with changes in breast-milk calcium or maternal skeletal responses to lactation. In general, these studies have found no evidence of a relationship between effect of the intervention and customary calcium intake, making it difficult to identify a calcium intake that can be regarded as optimal. Dietary advice about calcium varies from one country to another but the trend in recent years towards higher reference values means that many populations, especially those in Africa and Asia, have calcium intakes that are considerably below recommendations. There is, however, little evidence that this impacts adversely on skeletal growth, reproductive performance or fracture risk, and suggests that these populations are well adapted to their low calcium intake. However, the mechanisms and limitations of this adaptation are unknown. It is possible that there would be health benefits associated with an increase in calcium intake in these populations but recent results suggest there may also be disadvantages. Evidence of benefit and an understanding of the underlying biological mechanisms are required before advocating an increase in population mean calcium intakes, although older individuals in countries with high fracture risk should be encouraged to ensure their calcium intake exceeds 400 mg/d.

## **IS10**

### **FRUIT AND VEGETABLES IN BONE HEALTH**

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Until recently, research on nutrition and bone health has focussed primarily on calcium and vitamin D. In the late 1990s two population-based studies in the UK (Aberdeen) and USA (Framingham) found that nutrients associated with fruit and vegetable intake were associated with higher bone mineral density measurements. At the same time a beneficial effect of vegetables on bone metabolism was observed in rats. These findings are compatible with the hypothesis of Wachman & Bernstein. They proposed that osteoporosis may be caused by chronic exposure to an acidic environment which is a result of consuming acid-generating foods. Fruit and vegetables may be protective because they produce alkaline metabolites. At the cellular level osteoclasts are unusual in that they prefer an acidic environment, whereas the opposite is true for most other cell types including osteoblasts. By making the environmental pH of the osteoclast more alkaline bone resorption can be reduced or even eliminated. Short-term human intervention studies, with organic salts of potassium, lasting several months suggest that alkaline metabolites reduce bone turnover but whether this is important in the long-term has not been proved. Also, although the 'acid metabolite' theory implies that dietary protein is detrimental to bone health, there is evidence to show that protein is beneficial. The relationship between the ratio of protein to potassium intakes and fracture risk in the Women's Health Initiative exhibited a U-shape curve, with greater number of fractures if either protein or fruit and vegetable intakes are low. Fruit and vegetables contain a number of other components that could plausibly influence bone metabolism: vitamin K (as a cofactor for carboxylation of osteocalcin), vitamin C (required for collagen cross-linking), folate (for clearing circulating homocysteine), phytoestrogens and flavonoids. A longer-term study comparing fruit and vegetable intake with alkaline metabolites is required to determine the mechanism behind the benefits of eating fruit and vegetables for bone health. The findings of a 2-year intervention study currently running in Aberdeen will be available next year. Whatever the outcome, it is clear that dairy products are not the only food group important for healthy bones but that fruit and vegetables have a supplementary role to play.

## **IS11**

### **MEDICAL APPROACHES TO PRIMARY HYPERPARATHYROIDISM**

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Well-established guidelines for parathyroidectomy (PTX) exist for patients with primary hyperparathyroidism (PHPT). Many patients, however, do not meet these criteria for PTX. For other reasons, some patients are not surgical candidates. Effective approaches to the medical management of PHPT are therefore desirable. Non-pharmacologic approaches include good hydration, ambulation, and a normal calcium intake. Estrogen therapy was used with some evidence for reduced serum calcium levels, but this approach has become less attractive with the results of the Women's Health Initiative study. In a short term pilot study, raloxifene reduced the serum calcium (Rubin et al., J Clin Endocrinol Metab, 2003). The bisphosphonates (BisP) are attractive because, as antiresorptives, they have the potential to control the active bone turnover characteristic of PHPT. Two randomized, blinded, placebo-controlled studies have shown that the bisphosphonate, alendronate (ALN), is associated with significant increases in lumbar spine and femoral neck bone density along with reductions in bone turnover markers. In one study (Chow et al. J Clin Endocrinol Metab, 2003), the serum calcium also fell. In another study (Khan et al. J Clin Endocrinol Metab, 2004), similar results were obtained for a longer 2-year period, but there was no reduction in the serum calcium. Parathyroid hormone (PTH) levels do not change. It would be desirable for a medical therapy to consistently reduce serum calcium and PTH levels. The calcimimetic, cinacalcet, has the potential to accomplish this aim because by binding to the calcium receptor, it increases the binding affinity of extracellular calcium for the calcium receptor with concomitant regulatory effects on PTH synthetic machinery of the parathyroid cell. In several studies, it has been shown the cinacalcet effectively normalizes the serum calcium in PHPT. (Shoback et al. J Clin Endocrinol Metab, 2003; Peacock et al. J Clin Endocrinol Metab, 2005). PTH levels fall significantly but not to the normal range. In parathyroid carcinoma, a more extreme form of PHPT, cinacalcet effectively reduces serum calcium levels, even when they are extremely high (Rubin et al. J Bone Min Res, 2004, abs). These results suggest that for patients in whom a medical approach to PHPT is desirable, a number of alternatives are showing promise.

## **IS12**

### **AETIOLOGY OF PAGET'S DISEASE OF BONE AND SUMMARY OF CURRENT TREATMENT GUIDELINES.**

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In 1883 the first report of Paget's disease in multiple family members appeared. Subsequently numerous reports of familial Paget's disease indicated that the disorder might be transmitted as an autosomal dominant trait. In 2002 the first description of a gene mutation associated with the disease was reported, P392L in the sequestosome 1 gene. In 1974 the presence of nuclear and cytoplasmic inclusions resembling the nucleocapsids of viruses of the Paramyxoviridae family was first reported in the osteoclasts of patients with Paget's disease. These were subsequently found to be a characteristic of the patients with familial and sporadic Paget's disease from many countries. Attempts to develop an animal model of Paget's disease have been made by targeting osteoclast progenitors in transgenic mice with the P392L sequestosome 1 gene mutation or the measles virus nucleocapsid (MVNP) gene. The P392L mutation increased osteoclast formation in the mice but did not produce a pagetic phenotype, while the MVNP gene did produce the pagetic phenotype. Based on the animal data and the fact that even elderly subjects with sequestosome 1 mutations may have no evidence of Paget's



disease, it is a reasonable hypothesis that sequestosome 1 mutations enhance susceptibility to Paget's disease while measles virus or other Paramyxoviridae viruses are the environmental factors which produce the pagetic phenotype. It is difficult to define the indications for treatment for Paget's disease due to the absence of large, long-term, randomized, placebo-controlled trials of drug therapy. There is general agreement that bisphosphonates are the drugs of choice and that the following are appropriate indications for treatment: bone pain, prevention of hearing loss, spinal cord dysfunction and hypercalcaemia. Some experienced practitioners would use drugs to attempt to prevent bone deformity, to prevent osteoarthritis in joints adjacent to pagetic bone and to reduce haemorrhage during orthopaedic surgery. Patients who have severe osteoarthritis of the hip or knee respond well to joint replacement. Osteotomy of the bowed tibia is generally effective in restoring mobility.

### **IS13**

#### **UPDATE ON TREATMENTS FOR OSTEOPOROSIS**

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The objective of treating men and women with osteoporosis is to reduce the probability of experiencing fractures, especially those of the spine and hip that are responsible for the morbidity associated with the disease. Since there are multiple factors that influence fracture risk, including both impaired bone strength and increased fall risk, there are several complementary strategies of management available. Targeted exercise programs and hip protectors are appropriate and effective for some patients. Correcting deficiencies of dietary calcium and especially vitamin D slows bone loss, improves muscle strength and reduces fracture risk in elderly adults. Pharmacological treatments to reduce bone turnover or to activate bone formation are known to preserve or increase bone strength. In patients with osteoporosis, these therapies offer substantially greater fracture protection than accomplished with calcium and vitamin D.

Multiple therapies have been documented to substantially reduce the risk of vertebral fractures. Importantly, treatment with alendronate, risedronate, raloxifene and teriparatide dramatically (by 75-95%) has reduced the likelihood of experiencing multiple vertebral fractures, effectively halting the devastating clinical consequences of spinal osteoporotic fractures. Several drugs including strontium ranelate, teriparatide, risedronate and alendronate have reduced the risk of non-vertebral fractures in postmenopausal women with osteoporosis. Bisphosphonates effectively reduce vertebral fracture risk in patients receiving glucocorticoids.

Each of our available agents is well-tolerated and some, like raloxifene, may have important extra-skeletal benefits. The choice of treatment for an individual patient depends upon the nature of their bone disease, their risks of vertebral or hip fracture and their other medical and clinical problems.

On the horizon are different versions or dosing regimens of familiar classes of drugs, and new drugs with unique mechanisms of action. While these new agents may not prove more effective than our current drugs in reducing fracture risk, they will provide important treatment choices with different dosing schedules and different tolerability profiles.

Important clinical challenges persist including the development of more efficient identification of patients at increased risk who would experience the greatest benefit from pharmacological intervention. Additionally, better strategies are required to increase both acceptance of and long-term persistence with therapy.

## OC1

### THE EFFECT OF AZD0530, A HIGHLY SELECTIVE SRC INHIBITOR, ON BONE TURNOVER IN HEALTHY MALES

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AZD0530 is a highly selective, dual-specific, orally available small molecule inhibitor of Src kinase and Bcr-Abl. Src kinase plays an essential role in RANK-mediated osteoclast activation, and may also inhibit osteoblast activity. To examine the effect of AZD0530 on osteoclast and osteoblast activity we have investigated the changes in markers of bone turnover in response to AZD0530 treatment in a multiple ascending dose study in healthy male volunteers (ages 18-55y). The study comprised placebo and 4 dose levels given to cohorts each of 12 volunteers. Volunteers in each cohort received a single dose of AZD0530 (60mg to 250mg) (n=9) or placebo (n=3) repeated, 7 or 10 days later, as multiple daily doses for 10 or 14 days. Serum and second morning urine were collected after overnight fast prior to and 24 and 48h after the single dose and the final dose. Further samples were collected 10-14 days after the last dose in the cohorts receiving the two highest doses. Resorption markers measured were serum cross-linked C telopeptide of type I collagen (sCTX), urinary cross-linked N telopeptide, corrected for creatinine (uNTX/Cr), and serum tartrate resistant acid phosphatase 5b (TRAP 5b). Formation markers measured were procollagen serum type I N terminal propeptide (PINP) and bone specific alkaline phosphatase (Bone ALP). Mean percentage change (95%CI) from baseline 24 hours after final dose at doses of 125mg, 185mg and 250mg respectively were -55 (-65,-43), -71(-77,-63), and -88(-91,-84) for sCTX -39(-56,-15), -69 (-78,-56) and -67 (-77,-53) for uNTX/Cr, -13 (-19,-5), -14 (-21,-6) and -11(-18,-3) for TRAP 5b, +23 (+3,+46), +33(+11,+58), and +13(-6,+35) for PINP. There were no significant changes in any markers at 60mg dose or placebo cohort or in Bone ALP at any dose. Provisional PK-PD modelling suggests the relationship between AZD0530 plasma concentrations and CTX suppression is well described by an inhibitory sigmoid E-max model. Levels of sCTX and uNTX/Cr but not TRAP 5b appear to rise back rapidly towards baseline following cessation of dosing with the two highest doses whereas PINP tends to decrease. We conclude that suppression of Src kinase activity inhibits osteoclast-mediated bone resorption. The potential effect of Src inhibition on bone formation warrants further investigation. AZD0530 may have therapeutic benefit in treating osteoclast-driven metastatic bone disease and osteoporosis.

## OC2

### MAPK SIGNALLING AND RHO GTPASE ACTIVATION ARE PERTURBED BY PASTEURELLA MULTOCIDA TOXIN (PMT) DURING DIFFERENTIATION OF MURINE AND HUMAN OSTEOCLASTS

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We have previously shown that the unique bacterial toxin, Pasteurella Multocida toxin (PMT), which activates the heterotrimeric G-protein, Gq, stimulates the small GTPase Rho and its downstream effector Rho kinase (ROK) in osteoblasts, leading to inhibition of osteoblast differentiation and bone nodule formation. PMT can also activate phospholipase C, protein kinase C and the Ras/MAP kinase pathway. However, PMT also targets osteoclasts and causes the bone resorbing disease, atrophic rhinitis in pigs, resulting in pathological bone resorption. Therefore, in this study, we have investigated the effects of PMT and Rho-ROK activation on osteoclast differentiation and activity, as well as on RANKL-stimulated MAPK activation.

In MCSF- and RANKL-stimulated cultures of murine bone marrow cells and human PBMCs, PMT inhibited osteoclast differentiation and resorption in a dose-dependent manner. This correlated with an inhibition of expression of the osteoclast markers, calcitonin receptor and cathepsin K, but with no effect on RANK or c-fms receptor expression. Biochemical studies demonstrated that PMT markedly induced the levels of active GTP-bound Rho in both mouse and human osteoclast precursors and mature osteoclasts, with no effects on Rac and only a slight increase in active Cdc42. To investigate the mechanisms of PMT action, we analysed the pathways downstream of RANKL signalling and Rho GTPase. PMT treatment of murine osteoclast precursors markedly blocked RANKL-stimulated p-p38, p-JNK and p-ERK levels. Interestingly, however, treatment of human or murine osteoclast precursors with the ROK inhibitor, Y-27632, rescued the inhibition of human osteoclast differentiation, with little effect on murine osteoclasts. Interestingly, RANKL-stimulated expression of c-Fos and NFATc1 proteins, two essential genes for osteoclast differentiation, were not affected by PMT, suggesting that the PMT effects are either downstream of c-Fos-NFATc1, or involve an alternative pathway. In contrast to the inhibitory effects on differentiation, PMT appeared to promote resorption of mature osteoclasts as addition of PMT to mature human osteoclasts increased the proportion of F-actin ring-containing, vitronectin receptor-positive osteoclasts, with a concomitant increase in resorption.

Overall, the signalling cascades induced by PMT in osteoclast precursors and mature osteoclasts yield important insights into the roles of the Rho-ROK and MAPK pathways during osteoclast differentiation and activation.

## OC3

### OSTEOCLAST FORMATION AND BONE RESORPTION ARE INHIBITED BY MEGAKARYOCYTES

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It has been previously reported that addition of megakaryocytes (MKs) to osteoblasts in vitro results in increased osteoblastic collagen and OPG production demonstrating the potential role of MKs in bone remodelling. To further investigate this role, we have investigated the effects of MKs on osteoclast formation and activity.

Human osteoclasts were generated from CD14 monocytes isolated from peripheral blood and cultured in the presence of M-CSF and sRANKL on dentine and calcium phosphate substrates. MKs were generated from CD34+ cells isolated from either human peripheral blood or cord blood and cultured in liquid medium for 6 days, after which time maturing MKs (CD61 positive cells) were isolated and added to monocyte cultures at either time 0 or at 3 days. After 9 days culture the number of osteoclasts identified morphologically and by TRAP staining were counted. Cells were removed and the area of resorption identified by von Kossa staining and quantitatively assessed by image analysis.

The addition of MKs to osteoclast cultures at day 0 inhibited the number of osteoclasts formed 1.9 fold (p > 0.003), whilst addition at 3 days had no effect on osteoclast number. The presence of MKs inhibited resorption 8.7-fold when co-cultured with osteoclasts from day 0 (p > 0.004), but only by 3.1-fold when co-cultured from day 3 (p > 0.01). In dose response experiments it was found that 1-10% of MKs added to monocyte cultures elicited the greatest inhibition of resorption.

Similar osteoclast cultures were treated with CD61 negative cells (non MKs) to confirm that the inhibition of osteoclast formation and activity was specifically due to MKs. Interestingly, these cultures showed an increase in the number of osteoclast-like cells formed, particularly when MKs were added at 3 days; however, there was no significant change in the % area resorption from osteoclasts cultured alone.

These results show that MKs directly inhibit both osteoclast formation and activity. The most pronounced effects were seen when MKs and osteoclasts were co-cultured from day 0 suggesting that MKs act primarily on osteoclast precursors.

#### **OC4**

##### **CANINE DISTEMPER VIRUS INDUCES A DOSE-DEPENDENT INCREASE IN HUMAN OSTEOCLAST FORMATION AND FUNCTION IN VITRO**

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Paget's disease is characterised by a dramatic increase in size and number of osteoclasts, leading to uncontrolled bone resorption. The aetiology of the disorder is still unclear, however evidence points to either a viral infection or a genetic susceptibility, or a combination of both. Previously we have shown that canine distemper virus (CDV) RNA is present in Pagetic bone samples. However, the effects of CDV on human osteoclast formation in vitro have not been previously studied.

Replicate cultures (n=3) of purified human osteoclast precursors were infected with increasing doses of CDV and cultured on dentine slices for 14 days. Osteoclasts were stained for tartrate-resistant acid phosphatase and the dentine slices were examined for evidence of resorption. Control cells were incubated in the absence of virus. In each case, 10 high-power microscopy fields were analysed.

CDV dose-dependently increased osteoclast number, numbers of nuclei per osteoclast, and size ( $p < 0.0001$ , ANOVA), and there was a concomitant increase in resorption on the dentine slices ( $p < 0.0001$ , ANOVA).

These results provide the first conclusive proof that CDV can infect human osteoclast precursors. The resulting increase in size and number of osteoclasts, and increased resorption, provide further evidence for the possible role of Paramyxoviruses in the pathogenesis of Paget's disease.

## OC5

### OSTEOCLASTS DERIVED FROM GUNMETAL MICE ARE FUNCTIONAL DESPITE HAVING DEFECTS IN RAB PRENYLATION

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Gunmetal (gm/gm) mice have an autosomal recessive mutation in the gene encoding Rab geranylgeranyl transferase (Rab GGTase), which causes a four-fold reduction in the level of this enzyme. Consequently, the geranylgeranylation of a subset of Rab proteins is reduced in some cell types in these mice, particularly melanocytes, megakaryocytes and platelets. As a result, gm/gm mice suffer from prolonged bleeding, thrombocytopenia and reduced platelet granule contents. We recently found that a weak anti-resorptive phosphonocarboxylate analogue of risedronate, NE10790, specifically inhibits Rab geranylgeranyl transferase (Rab GGTase). This compound prevents prenylation of Rab proteins in osteoclasts in vitro at concentrations that inhibit bone resorption, demonstrating that reduced Rab GGTase activity disrupts the bone-resorbing capacity of osteoclasts. We therefore investigated whether osteoclasts from gm/gm mice have defective prenylation of Rab proteins and whether this is associated with abnormal osteoclast function or a skeletal phenotype. Bone marrow cells were isolated from gm/gm and heterozygous (+/gm) mice (which have no defect in Rab prenylation) and stimulated to form osteoclasts in vitro using RANKL and M-CSF. As expected, Rab GGTase activity in gm/gm osteoclasts was markedly reduced compared to +/gm osteoclasts. The unprenylated form of numerous Rab proteins accumulated in the gm/gm, but not the +/gm osteoclasts, and the gm/gm osteoclasts were much more sensitive to further inhibition of Rab prenylation with NE10790. However, there was little difference in either the formation or resorptive activity of gm/gm and +/gm osteoclasts, indicating that gm/gm osteoclasts can function normally. Accordingly, pQCT analysis demonstrated that tibiae from gm/gm mice showed no signs of osteopetrosis compared to +/gm mice. Rather, cortical bone content was reduced by 11% in the gm/gm mice, due to an increased endosteal circumference (and therefore decreased cortical thickness), whereas there was little difference in trabecular bone parameters between gm/gm and +/gm mice. These results show that osteoclasts from gm/gm mice retain sufficient Rab GGTase activity to maintain osteoclast function, suggesting that more complete inhibition of Rab prenylation is required to disrupt osteoclast activity. The subtle bone phenotype of gm/gm mice may be caused by functional defects in other cells in the bone microenvironment such as megakaryocytes.

## OC6

### CHARACTERISATION OF HUMAN FETAL POPULATIONS: A COMPARATIVE MODEL FOR SKELETAL AND STEM CELL DIFFERENTIATION

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Multipotential mesenchymal stem cells (MSCs) or marrow stromal cells derived from adult human tissues offer tremendous potential for cell based tissue regeneration. However, to date, the plasticity, multipotentiality and characteristics of potential stem cells from fetal skeletal tissue remain poorly defined. We have examined, in preliminary studies, the multipotentiality and phenotypic properties of cell populations derived from human fetal femurs collected at 8-12 weeks post-conception in comparison to adult derived mesenchymal stem cell populations including those isolated using STRO-1 immunoselection. Fetal cells were culture expanded from explants in basal media then maintained for periods of up to 28 days in monolayer cultures in adipogenic and osteogenic conditions. Adipocyte formation was confirmed by morphology: large amounts of lipid accumulation were observed by Oil Red O and aP2 (FABP-3) staining. Osteogenic differentiation was also confirmed by Type I Collagen immunocytochemistry. Plasticity of fetal cells was demonstrated by the formation of large numbers of adipocytes within osteogenic populations. The growth of fetal cells on biomimetic scaffolds and their osteogenic activity was confirmed by confocal microscopy and Alkaline Phosphatase staining respectively. Cells were also maintained in chondrogenic conditions via the pellet culture method with established media conditions including TGF-beta3 with cultures taken to 7, 14, 21 and 28 days. Under these conditions, chondrocytes were embedded within lacunae and extensive matrix deposition was observed using Alcian blue/Sirius red staining. The chondrogenic phenotype was confirmed by expression of SOX-9 by immunocytochemistry. Differentiation and proliferation were accelerated in fetal populations compared to adult-derived immunoselected MSCs. In summary we demonstrate the proliferative and multipotential properties of fetal-derived chondrocytic cells, establishing their stem cell nature in direct comparison to STRO-1 immunoselected MSCs. Given the complexity, technical challenges and ethical issues surrounding current embryonic and embryonic germ cell research, human fetal cell populations may provide a unique half-way model and system to address stem cell differentiation in comparison to adult stem cell differentiation. Elucidation of immunogenicity and selective differentiation will confirm the potential of these fetal cells as a unique alternate model for skeletal regeneration.

## OC7

### THE OSTEOGENIC RESPONSE TO MECHANICAL LOADING IS SUPRESSED IN OLD FEMALE MICE BUT IS RECUED AFTER PERIODS OF DISUSE

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It has been suggested that ageing blunts the osteogenic response to load-bearing, and that this decline underpins ageing-associated increases in fracture incidence. Indeed, it is known that the mechanical thresholds for load-induced bone formation increase with ageing. We have previously shown that sciatic denervation increases mechanically-induced cortical new bone formation in young growing mice, and hypothesise that this influence will also therefore diminish with ageing.

Accordingly, female C57Bl/J6 mice were assigned into 2 groups. Right limbs of 18 month old mice in one group (n=5) were loaded (13N) to engender peak strains of 2000 microstrain on the tibial midshaft; contralateral tibiae used as controls. The second group (n=5) were submitted to right limb sciatic-neurectomy 100 days before they were 18-months old, kept for

100 days, and then loaded after appropriate calibration (7N) to generate identical peak strains; sham-operated contralateral tibiae used as controls. All animals were loaded on alternate days for 3 weeks, received a fluorochrome label on the third and last days of loading and killed 3 days later. Confocal images of transverse sections from 3 defined tibial diaphyseal sites were analysed. Periosteal enclosed bone (PEB) area, endosteal area, and new bone formation were measured.

As expected, aged tibiae failed to produce any increases in new bone formation in response to peak strains of 2000 microstrain; endosteal and PEB areas in these loaded tibiae were also similar to contralateral controls. We also confirmed that disuse produced marked decreases in PEB ( $p=0.008$ ) and increases in endosteal area. In contrast, however, we found that loading to identical peak strain levels in disused tibiae, surprisingly, produced significant increases in new bone formation ( $p=0.001$ ) and reversed the disuse-induced loss in PEB area. Current studies are examining whether this effect is also evident in trabecular bone.

Our findings support the notion that aged bones in a normal functional situation exhibit a reduced response to loading. This capacity to respond, however, is rescued by the imposition of periods of disuse. Establishing the basis of this disuse-related re-sensitisation to lower loads, but similar peak strain magnitudes, would allow a reduction in age-related fracture incidence.

### OC8

#### GROWTH PLATE CHONDROGENESIS AND LONGITUDINAL BONE GROWTH FOLLOWING EXPOSURE TO THE NOVEL GLUCOCORTICOID RECEPTOR LIGAND, AL-438

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Glucocorticoids (GC) are commonly used anti-inflammatory and immunosuppressive drugs. Long-term use can, however, result in a number of debilitating side effects such as growth retardation in children due to their actions on chondrocytes in the epiphyseal growth plate. Intense efforts have been made to maximise the beneficial effects but minimize the side effects of GCs. Recently, a non-steroidal anti-inflammatory agent (AL-438) that acts through the glucocorticoid receptor (GR) has been described. Studies with AL-438 have shown it retains full anti-inflammatory efficacy but has reduced negative effects on osteoblasts compared to those elicited by prednisolone (Pred) or dexamethasone (Dex). We have used the chondrocyte ATDC5 cell line to compare the effects of AL-438 with those of Dex and Pred on chondrocyte proliferation and differentiation in the epiphyseal growth plate. During chondrogenesis, Dex and Pred (10-6M) exposure for 24h resulted in a significant reduction in cell proliferation (30.3% and 18.8% respectively ( $p<0.05$ )), whereas exposure to AL-438 (10-6M) had no effect. Proteoglycan synthesis was also significantly reduced after exposure to Dex or Pred for 96h (56% and 53.9% respectively ( $p<0.05$ )) but not after AL-438 exposure. Alkaline-phosphatase (ALP) activity was significantly increased after exposure to Dex, Pred and AL-438. During terminal differentiation, cell proliferation, proteoglycan synthesis and ALP activity were unaffected by Dex, Pred or AL-438. At both time periods, apoptotic activity was also unaffected, as were gene expression levels of collagen II, collagen X and aggrecan. Fetal mouse metatarsals grown in the presence of Dex and AL-438 (10-6M) paralleled control bone growth until day 10 when Dex and AL-438 treated bones were 18 and 6% shorter (NS), respectively than control bones. The growth inhibitory effects of Dex were more apparent in the presence of IGF-I (100ng/ml) suggesting that Dex inhibits the growth stimulatory effects of IGF-I. These results indicate that the adverse effects Dex or Pred on chondrocyte proliferation and bone growth were attenuated following AL-438 exposure. This suggests that AL-438 has a reduced side effect profile on growth plate chondrocytes compared to other GCs, a property that could prove important in the search for new anti-inflammatory treatments for children.

### OC9

#### P2X<sub>2</sub> BUT NOT P2X<sub>3</sub> RECEPTOR KNOCKOUT MICE DEMONSTRATE INCREASED BONE MASS AND WEIGHT

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Accumulating evidence suggests extracellular nucleotides, signalling through P2 receptors, play a significant role in bone biology modulating both osteoblast and osteoclast function. These receptors have been characterised into the P2X ligand-gated ion channels and the P2Y G-protein linked receptors. Of the seven P2X receptor subtypes, P2X<sub>2</sub>, P2X<sub>4</sub>, P2X<sub>5</sub> and P2X<sub>7</sub> expression has been detected on bone cells. Using transgenic mice we have investigated the effects of P2X<sub>2</sub> and P2X<sub>3</sub> receptor deletion on bone phenotype. A PIXImus DEXA scanner was employed to measure bone mineral density (BMD) and bone mineral content (BMC) of these animals and their wildtypes at age 2, 4, 6 and 9 months. An intraperitoneal injection of 2.5% tribromoethanol in 2-methylbutan-2-ol at 0.16ml/10g was administered to induce anaesthesia. Each animal was scanned in triplicate with repositioning between each scan. Both P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>3</sub><sup>-/-</sup> mice had no overt phenotype and exhibited no gross abnormalities. At all ages P2X<sub>2</sub><sup>-/-</sup> mice exhibited significantly increased BMD, BMC and weight but the differences decreased in magnitude as the animals aged. For example, the BMD of P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>2</sub><sup>+/+</sup> at 2 months were  $0.0526 \pm 0.0006$  and  $0.0484 \pm 0.0005$  ( $p<0.001$ ), respectively but at 9 months were  $0.0540 \pm 0.0005$  and  $0.0515 \pm 0.0007$  ( $p<0.05$ ). Compared to P2X<sub>3</sub><sup>+/+</sup> controls, the P2X<sub>3</sub><sup>-/-</sup> mice demonstrated no significant differences in BMD, BMC or weight. In vitro, nucleotides stimulate osteoclast activity and inhibit bone nodule formation by osteoblasts, so would be expected to have a negative effect on bone remodelling balance in vivo. Deletion of the P2X<sub>2</sub> receptor, which is expressed on both osteoclasts and osteoblasts, could potentially limit the negative actions of endogenous nucleotides on bone, leading to increased BMD and BMC. In contrast, P2X<sub>3</sub> receptor expression has not been detected on bone cells and consequently is less likely to play a direct role in local regulation of skeletal remodelling. The age-related decreases in the BMD and BMC differences between P2X<sub>2</sub><sup>-/-</sup> and P2X<sub>2</sub><sup>+/+</sup> mice suggest the existence of compensatory mechanisms to overcome receptor deletion. These data provide further evidence for an in vivo role for purinergic receptors in modulating bone remodelling.

### OC10

#### ESTROGEN RECEPTOR-ALPHA POLYMORPHISMS AFFECT CORTICAL BONE GROWTH IN CHILDHOOD IN A SITE-DEPENDENT MANNER

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Recent studies in estrogen receptor-alpha (ESR1) knockout mice suggest that ESR1 acts to stimulate cortical bone formation at the periosteal envelope, particularly in the presence of dynamic mechanical strain. Although several studies in humans have analysed the relationship between ESR1 gene polymorphisms and bone mineral density, to what extent these influence cortical bone growth is currently unclear. We evaluated the hypothesis that ESR1 gene polymorphisms influence cortical bone growth in children, and that this effect differs at distinct skeletal sites according to the local strain environment. We related ESR1 gene polymorphisms to bone area (BA) at the upper limbs, lower limbs, trunk and spine, obtained from total body DXA scans performed at age 9 years in unrelated Caucasian children. DXA-gene associations were analysed by ANCOVA assuming a dose-responsive genetic model, following adjustment for height, weight, age, gender, Tanner stage (girls only), and measures of social position found to influence DXA variables in parallel studies. Results were available in 569 children (n = 324 for spine results). An exonic synonymous rs746432 polymorphism was significantly associated with arm BA (p = 0.002), such that rare allele copy number was negatively associated with BA. Similar, albeit weaker, associations were observed with leg BA and total body BA (p = 0.03 and 0.04 respectively), while no association was observed with spine or trunk BA (p = 0.9 and 0.5 respectively). Significant associations with BA were also observed for the rs2234693 (PvuII) intronic polymorphism (arm BA, p = 0.008; leg BA, p = 0.1; total body BA, p = 0.04; spine BA, p = 0.2; trunk BA, p = 0.2), while no association was observed between BA and the rs9340799 (XbaI) intronic polymorphism, D6S440 intronic microsatellite or rs2228480 exonic polymorphism. We conclude that although ESR1 gene polymorphisms affect cortical bone growth in childhood, this influence appears to be strongest at the appendicular skeleton, particularly the upper limbs. Further studies are required to explore the mechanisms involved in this genetic effect, and in particular whether these findings reflect a specific interaction between ESR1 function and the local strain environment.

### OC11

#### LATE EFFECTS OF VERY LOW BIRTH WEIGHT ON BONE MASS AT THE LUMBAR SPINE

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A low peak bone mass (PBM) is an established risk factor for fracture in later life. Children who were born with very low birth weight (<1500g, VLBW) have reduced bone mass compared to children born at full term. However, this reduced mass appears to be appropriate for their reduced body size, suggesting no reduction in bone strength during childhood. The effect of VLBW on PBM is unknown. A low bone mass in this population may become increasingly important as more VLBW infants survive into adulthood.

PA lumbar spine bone mass was measured, using dual energy x-ray absorptiometry (Hologic Discovery), in 62 young men and women (age 21 to 25 years); 36 born VLBW, 26 normal birth weight controls (C). There were 20 and 18 women, and 16 and 8 men in the VLBW and C groups respectively. Bone volume (BV) and volumetric bone density (bone mineral apparent density, BMAD) were estimated, using Carter's method (1997). Standard deviations scores (SDS), adjusted for age, height and weight, were calculated. The VLBW and C groups were compared using unpaired t-tests.

Height and weight were not significantly different between the VLBW and C groups. As expected, women were significantly shorter and lighter than men (p<0.002). Lumbar spine bone mineral density (BMD) was significantly reduced in the VLBW group. Mean (SD) BMD ZSc (Hologic output, adjusted for age only) was -0.68 (0.95), p = 0.02, in the VLBW group. There were no differences in BV SDS. BMAD SDS (mean, SD) was reduced in the VLBW group (-0.49 (0.93), p = 0.04.

PBM is reduced in VLBW young adults. This deficit is the result of reduced volumetric density, whereas bone size is normal. The deficit is still apparent after adjusting for differences in body size. Previous studies have suggested that VLBW children have reduced bone mass that is appropriate for bone and body size. We speculate that our results may suggest a deviation from normal bone mass accrual during puberty in VLBW individuals, which results in bones of normal size, but of reduced density. This reduced PBM may have implications for future fracture risk in VLBW individuals.

### OC12

#### VITAMIN K AND FRACTURE RISK: AN EFFECT ON BONE WIDTH NOT BMD?

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Most studies on nutrition and bone health have studied bone mineral density(BMD) as a surrogate endpoint for fractures, but bone geometry is also an important BMD independent determinant of fracture risk. For the same bone mass, wider bones have greater bending resistance as measured by the section modulus(Z).

We analysed data from 670 women aged 67-79y at recruitment into the longitudinal population based EPIC-Norfolk study to assess effects of dietary nutrients on subperiosteal diameter (PD) and Z. Up to 4 hip DXA scans were performed within 8 years of follow up: 2 scans(n=472;2.9y), 3 scans(350;5.5y) and 4 scans(79;7.4y). Hip structural analysis software was used to derive structural parameters from the DXA scans at the narrow neck(NN), intertrochanter(IT) and shaft(S) regions. Nutrient intakes were estimated as the mean intake from up to three 7-day diet diaries completed at baseline(n=670), 1.5 years(383) and 3 years(296).

Both PD and Z increased with age: PD NN[0.020 cm/yr 95% CI (0.018, 0.023)]; IT[0.015 (0.012, 0.017)]; S[0.006 (0.004, 0.008)]; Z all regions [0.015 cm<sup>3</sup>/yr (0.013, 0.018)]; and were positively associated with weight, height and lifetime physical activity (all P<0.059). Higher intake of vitamin K was independently associated with having larger PD [ $\beta$ =0.033 (0.011, 0.054) cm per 100ug/d difference P=0.003], an effect that did not significantly differ by region(P=0.141). Higher potassium and carotene intakes modified the effect of aging on Z, increasing the rates of Z increase by 0.003 cm<sup>3</sup>/yr per 1 SD increase(P<0.029). Alcohol intake was positively associated with Z(P=0.028). There was no significant effect of vitamin K on BMD. In a subset of 277 women who had biochemical measurements, the vitamin K effect on PD was independent of effects of sex hormone binding globulin and creatinine which increased rates of PD expansion with aging(P<0.025). The results indicated significant effects of dietary nutrients on bone width and section modulus. Their statistical significance was in part attributable to improved precision resulting from repeated measurements of these rather imprecise indices. The effect of vitamin K on bone width was consistent with evidence from previous studies associating higher vitamin K intake with lower risk of hip fracture.

### OC13

#### SCLEROSTIN EXPRESSION BY OSTEOCYTES IS A DELAYED EVENT; IMPLICATIONS FOR THE CONTROL OF BONE FORMATION

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Sclerostin, the SOST gene product is an important regulator of bone formation. We have investigated the precise location of sclerostin in fresh frozen undecalcified sections of adult human iliac crest bone using histochemistry. 14 biopsies from patients participating in a study of bone loss in hemiplegia were taken within 3 months of an acute stroke. The spatial relationships between sclerostin positive osteocytes and forming/mineralising surfaces were evaluated using serial sections stained for alkaline phosphatase (ALP) and unstained sections with demeclocycline labelling. Overall, 86% of 6231 cortical osteocytes were positive for sclerostin, whereas 74% of 1018 cancellous osteocytes were positive. Osteoblasts and lining cells were consistently negative for staining in all biopsies. Sclerostin negative osteocytes were located significantly closer to haversian canals and endosteal/periosteal surfaces (Median Distance from Surface, MDS 53.5 microns, IQR 31.5-84.9) than sclerostin positive cells (MDS 88.6 microns, IQR 60.8-116.8; p<0.0001, paired t-test). A detailed analysis of individual osteons/cortical BMU's (280 osteons from 5 patients) indicated that the distribution of sclerostin positive and negative osteocytes was strongly related to the forming status of the osteon. ALP positive (forming) osteons were significantly more likely to contain osteocytes negative for sclerostin (chi square 33.5, p<0.0001). 96.4% of newly formed osteocytes (embedded within the previous 16 days) were negative for sclerostin. Osteocytes in mineralised bone were positive for sclerostin with diffuse staining in canaliculi. Sclerostin secretion by new osteocytes is therefore a delayed event. These findings are consistent with the concept that newly embedded osteocytes secrete sclerostin after the onset of mineralisation to inhibit cortical bone formation and BMU infilling by cells of the osteoblast lineage

### OC14

#### OSTEOBLAST AND CHONDROCYTE MATRIX VESICLES CONTAIN PHOSPHO1: EVIDENCE FOR ITS ROLE IN MINERAL FORMATION

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We have previously reported a novel phosphatase, PHOSPHO1, whose expression is upregulated in mineralising osteoblast-like cells and is located to mineralising surfaces in bone and cartilage. PHOSPHO1 belongs to the haloacid dehalogenase superfamily of hydrolases and is capable of cleaving phosphoethanolamine (PEA) and phosphocholine (PCho) to generate inorganic phosphate (Pi). As hydroxyapatite crystallisation occurs within matrix vesicles (MVs) of tissue non-specific alkaline phosphatase (TNAP)-deficient mice and individuals with hypophosphatasia, it is likely that another enzyme is responsible for elevating the intravesicular concentration of Pi. Thus, we hypothesise that PHOSPHO1 scavenges Pi from PEA and PCho in order to generate the concentration required for initial hydroxyapatite crystal formation inside the MV lumen. For this to occur, functional PHOSPHO1 must be present within MVs. Therefore the aims of this study were to confirm bone-specific expression of phospho1 and show that PHOSPHO1 is both present and active within MVs. Real-time PCR of murine tissues revealed that phospho1 expression in bone (tibial diaphysis) was approximately 150-fold higher than the gut (lowest expression). MVs were isolated from chicken growth plate and Western blotting, using an avian specific PHOSPHO1 antiserum, allowed identification of two forms of PHOSPHO1, 30.4 and 28.6 kDa, within the MVs relating to alternative start sites. To allow detection of active PHOSPHO1 within MVs we isolated MVs from murine calvarial osteoblast cultures, obtained from TNAP null (-/-) and TNAP heterozygous (+/-) mice. The cells were grown in the presence of ascorbic acid and beta-glycerophosphate for 15 days post confluence. Although we show here that human TNAP has a lower specific activity towards PEA compared to human PHOSPHO1, at physiological pH, we adopted this strategy to eliminate the possibility that TNAP hydrolysis of PEA may mask PHOSPHO1 activity. Using PEA as a substrate we found that the TNAP+/- and TNAP-/- MVs had a PEA hydrolase activity of 4100 (450) and 990 (86) pmol Pi released/min/mg lysate, respectively. These results show that PHOSPHO1 is highly expressed within bone and the

protein present within MVs is in an active state. These data further support our hypothesis that PHOSPHO1 plays a central role in matrix mineralisation.

#### OC15

##### THE CIRCADIAN RHYTHM OF OSTEOPROTEGERIN AND ITS ASSOCIATION WITH PARATHYROID HORMONE SECRETION IN ELDERLY MEN AND WOMEN

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**BACKGROUND:** Parathyroid hormone (PTH) is normally secreted in a circadian rhythm and modulates bone turnover through the differential stimulation of receptor activator for nuclear factor kappa B ligand (RANKL) and suppression of osteoprotegerin (OPG), both of which are fundamental factors in regulating bone turnover. We have studied the relationship between PTH and OPG over a 24-hour period.

**METHODS:** Hourly peripheral venous blood samples were obtained from 6 healthy elderly women (mean age 68.2 plus/minus 2.1 years) and 6 healthy elderly men (mean age 68.2 plus/minus 1.9 years) with normal bone mineral density. Plasma PTH (1-84) and OPG were measured on all samples. Cosinor analysis was performed to analyze circadian rhythm parameters. Cross-correlation and Pearson's analysis was used to determine the relationship between variables and whether one time series led another. Cross-correlation analysis determines the correlation between two time series of equal length that have been paired, data point by data point, and then one of the time series is shifted by one or more time points (lag time) and the correlation process is repeated.

**RESULTS:** Significant circadian rhythms were observed for PTH and OPG in the women and men ( $p < 0.05$ ). Secretory patterns of PTH and OPG were out-of-phase during a 24 h period and maximal negative correlation between PTH and OPG ( $r = -0.5$ ) was observed when PTH changes preceded OPG changes by one hour in women and two hours in men. Pearson's correlation analysis confirmed that the diurnal rhythm of PTH correlated significantly and negatively with that of OPG ( $r = -0.4$ ;  $p < 0.05$ ).

**CONCLUSION:** We have demonstrated that peripheral blood concentrations of OPG demonstrate a concerted circadian rhythm in elderly non-osteoporotic postmenopausal women and elderly men, which may, in part, be regulated by the circadian changes in PTH concentration that control bone turnover on a daily basis. A significant decrease in OPG in response to increasing PTH may result in increased bone resorption by osteoclasts.

#### OC16

##### BISPHOSPHONATES PREVENT ADHESION OF BREAST CANCER CELLS TO BONE IN VITRO BY INHIBITING PROTEIN PRENYLATION

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Bisphosphonates are an important class of drugs used to treat cancer-associated bone diseases. Recent studies with animal models have suggested that, as well as preventing bone destruction, some bisphosphonates may also prevent the spread of tumour cells in bone, perhaps by interfering with cell adhesion and invasion. However, the exact molecular mechanisms involved remain unclear. Nitrogen-containing bisphosphonates (N-BPs) are potent inhibitors of FPP synthase and prevent the prenylation of small GTPases, some of which are known to play key roles in cell adhesion. We therefore examined whether the inhibitory effect of zoledronic acid (ZOL) and other bisphosphonates on tumour cell adhesion in vitro is due to inhibition of protein prenylation.

MCF-7 breast cancer cells were treated with 0.1-100µM ZOL for 24h. The adherent cells were removed from culture flasks then seeded onto discs of dentine and cultured for 3h. Discs were rinsed, fixed and stained with toluidine blue, then the cells were counted. 1µM or more ZOL caused a concentration-dependent decrease in the adhesion of MCF-7 cells; 1µM inhibited adhesion by approximately 50%. Treatment of MCF-7 cells with 5µM or more ZOL was also associated with the accumulation of the unprenylated form of Rap1A (indicative of inhibition of FPP synthase), which could be detected in cell lysates by western blotting. In addition, the inhibitory effect of ZOL on cell adhesion and protein prenylation could be overcome by the addition of geranylgeraniol, a substrate that restores the geranylgeranylation of small GTPases in the presence of N-BPs.

10µM risedronate (RIS) and the bisphosphonate NE11808 (potent inhibitors of FPP synthase) also prevented protein prenylation and inhibited tumour cell adhesion, whereas the structurally-related analogues NE58051 and NE11809 (which are poor inhibitors of FPP synthase) had little effect on cell adhesion or protein prenylation. Furthermore, NE10790 (the phosphonocarboxylate analogue of RIS that only prevents the prenylation of Rab proteins) did not affect tumour cell adhesion, even at a concentration of 1mM.

These observations strongly suggest that N-BPs inhibit the adhesion of tumour cells to bone in vitro by preventing the prenylation of certain small GTPases. Whether this mechanism occurs in vivo remains to be determined.

#### OC17

##### APOMINE, AN INHIBITOR OF HMG-COA-REDUCTASE, DOES NOT ACT BY INHIBITING PROTEIN PRENYLATION IN HUMAN MYELOMA CELLS IN VITRO

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Apomine (tetraisopropyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethyl-1,1-diphosphonate) is a 1,1 bisphosphonate ester, which originally attracted attention for its cholesterol-lowering properties. In recent years a possible role for apomine as a novel anticancer agent has emerged. We have previously demonstrated that apomine induces apoptosis in human myeloma cells in vitro, and exerts antitumour effects in the 5T2MM murine myeloma model, as determined by decreases in serum paraprotein levels and decreases in the proportion of 5T2MM cells in the bone marrow. Apomine is thought to



act by rapidly upregulating proteasomal degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate limiting enzyme in the mevalonate pathway. All well-known drugs that interfere with the mevalonate pathway, including statins and the nitrogen-containing bisphosphonates, inhibit downstream protein prenylation. This is believed to be their major mechanism of action. The aim of this study was to investigate whether apomine acts by inhibiting protein prenylation. The prenylation status of Rap1a and Rab6 in JJJN-3, RPMI 8226 and NCI H929 human myeloma cells treated with apomine was assessed by SDS-PAGE and western blotting. Whereas treatment with 500 microM alendronate or 5 microM lovastatin clearly led to an accumulation of unprenylated Rap1a and Rab6 in the cells, treatment with 10 microM apomine had no detectable effect on protein prenylation after 24, 48 or 72 h of treatment. This was not due to a lack of effect of apomine in general, as apoptosis was strongly increased (up to 60 percent apoptosis) as compared to the control in the same samples that were analyzed for protein prenylation. In support of these findings, both mevalonate (MVA) and geranylgeraniol (GGOH) were unable to prevent induction of apoptosis by apomine, whereas both MVA and GGOH completely prevented the induction of apoptosis by lovastatin, as measured by an in situ nick translation assay. In conclusion, no evidence was found for an inhibition of protein prenylation by apomine in myeloma cells, and consistent with these findings, apoptosis induced by apomine could not be prevented by either MVA or GGOH. These findings are unexpected and strongly suggest another mode of action for apomine in the induction of apoptosis in these cells.

### OC18

#### FAMILIAL HYPOMAGNEAEMIA-HYPERCALCIURIA AND NEPHRO-CALCINOSIS IN 2 SIBLINGS : 2 NOVEL HETEROZYGOUS MUTATIONS IN THE GENE ENCODING CLAUDIN-16

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A 7 year old boy was investigated following the findings of marked renal insufficiency and nephrocalcinosis in his 18-month old sister. He too was found to have nephrocalcinosis with increased urinary fractional excretion of magnesium : 12.4% (normal <4%) and urinary calcium : 5.7 mmol (< 2.5/24 hours). His serum magnesium was low : 0.63 mmol/l but serum calcium was normal : 2.38 mmol/l. He had renal impairment with a GFR of 51 ml/min, partial distal renal tubular acidosis, defective urinary concentrating ability and elevated serum PTH : 600 ng/l. Renal ultrasound revealed severe bilateral nephrocalcinosis. X-rays of his wrists, hands and knees did not show any abnormalities. Therapy with thiazide diuretics and magnesium supplements failed to halt the progression of the disorder. Both children subsequently underwent renal transplantation. Both children's parents are unaffected and there is also one unaffected sibling. There was no history of consanguinity. Mutation analysis revealed 2 novel heterozygous mutations in the Claudin-16 (CLDN 16) gene, also known as Paracellin-1, in both affected siblings; one mis-sense mutation in exon 4 : C646T which results in an amino acid change Arg 216Cys in the second extracellular loop of Claudin-16 and a donor splice-site mutation which changes intron 4 consensus splice site from 'GT' to 'TT'. Both parents are heterozygous for one of the 2 mutations and the unaffected brother is heterozygous for the paternal mutation (splice-site mutation). Claudin-16 or Paracellin-1 is a member of a family of membrane-bound proteins that constitute the inter-cellular tight-junction. The protein is exclusively expressed in the thick ascending part of Henle's loop where it is involved in the paracellular reabsorption of calcium and magnesium. Consequently patients with inactivating mutations in this gene experience severe renal calcium and magnesium wasting and progressive renal failure.

### OC19

#### A RANDOMISED CONTROLLED TRIAL (RCT) OF OSTEOPOROSIS SCREENING: LONG TERM EFFECTS ON USE OF MEDICATION AND FRACTURE RISK

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Osteoporosis is a common condition occurring in 1 in 2 women but current management is unsatisfactory because it is generally not introduced until a major fracture has occurred. Population screening programmes are one means to identify menopausal women with low bone mineral density (BMD) and an elevated risk of future fracture but require to be proven effective in a RCT.

In 1993 a random sample of 4800 women aged 45-54 years living within 32km of Aberdeen, Scotland, was selected from the community health index. Subjects were randomised in equal numbers to screening (active) or no screening (inactive controls). Only the active women were assessed and those found to be in the lowest quartile of BMD were suggested to consider HRT treatment and informed about other lifestyle factors to reduce fracture risk. Nine years after randomisation a follow-up questionnaire was mailed to both groups to assess the effect of screening on the uptake of treatment and on the incidence of fractures.

Response rates after 9 years were 56.8% in the control group and 59.7% in the active group. No significant differences were observed in age, weight, height or self-reported general health, nor in self-reported disease states with the exception of hyperparathyroidism (controls 0.5%, actives 1.1%, p=0.05). 52.4% of the active group reported taking HRT compared with 44.5% of the control group (p<0.001). Similarly the active subjects were significantly more likely to report current or past use of vitamin D, calcium, alendronate, etidronate or raloxifene than the control subjects (36.6% of the active group and 21.6% of the control group reporting to have taken some form of osteoporosis medication (p<0.001)).

In a per protocol analysis of self-reported incident fractures a 23.7% reduction in fractures (of any site) in the active group was observed (RR=0.763 95%CI=0.579-1.006) which increased to 25.2% following adjustment for age, weight and height (RR =0.748, 95%CI=0.567-0.986).

No significant difference was observed in the number of fallers (controls=13.9% vs actives=15.6%,  $p=0.202$ ) or the rate of falls (controls=0.28falls/year vs actives=0.30falls/year,  $p=0.503$ ).

Screening for low bone density significantly increases the use of HRT and other treatment for osteoporosis and reduces fracture incidence.

## **OC20**

### **THE RECORD TRIAL OF CALCIUM AND/OR VITAMIN D IN THE SECONDARY PREVENTION OF OSTEOPOROTIC FRACTURES**

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It is unclear if calcium and/or vitamin D are effective in the secondary prevention of fractures. We investigated the effect of calcium and/or vitamin D on the incidence of further fractures in people aged 70 years and over with a low-trauma fracture.

We undertook a placebo-controlled, 21 centre UK-based trial. 5292 participants from fracture clinics or wards were randomised to daily 1g calcium, 800IU (20mcg) vitamin D3, both, or placebo. The principal outcome was new, low-trauma fracture. The study had 80% power ( $2P < 0.05$ ) to detect an absolute reduction in fractures from 15% to 12%. Other outcomes were health status (as assessed by EQ-5D and SF-12), mortality, hospital admissions, change of residence, falls and adverse events. Planned sub-group analyses examined the effect of age, gender, type of fracture at recruitment, time since fracture, latitude of recruitment centre, calcium intake, vitamin D status, body weight, and compliance. Recruitment started in 1999 and follow-up ended in 2004.

Recruitment fractures included proximal femur (17%) and distal forearm (35%). At recruitment most participants were within 3 months of a fracture and were able to walk out of doors unaccompanied, although some required walking aids. The main reasons for trial ineligibility were low mental test score (43%) and current treatment for osteoporosis (34%). 698 (13.2%) had a further low-trauma fracture, of which 183 were hip fractures. There were no statistically significant differences between those allocated calcium and those not (331 (12.6%) vs. 367 (13.7%); HR 0.94, 95% CI 0.81 to 1.09); those allocated vitamin D and those not (353 (13.3%) vs. 345 (13.1%); HR 1.02, 95% CI 0.88 to 1.19); and those allocated both calcium and vitamin D versus placebo [165 (12.6%) vs. 179 (13.4%)]. Also, no differences were detected in all reported fractures, X-ray confirmed fractures, hip fractures, death, falls, quality of life, or in sub-group analyses. Compliance with calcium was significantly poorer, in part reflecting gastrointestinal symptoms.

The findings do not support the use of routine supplementation with calcium and/or vitamin D for the prevention of further fractures in people with a recent low-trauma fracture.

## **P1**

### **LOCALISATION OF HIF-2ALPHA TO SPECIFIC MATURATIONAL ZONES OF BOTH GROWTH PLATE AND ARTICULAR CARTILAGE**

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Vascular endothelial growth factor (VEGF) is involved as an angiogenic stimulus during normal skeletal development, growth and repair. However, the molecular basis of the control of VEGF expression in relation to growth plate differentiation and vascularisation is poorly understood. Using Percoll density gradient centrifugation, chick chondrocytes were separated into populations of different maturational phenotype. Differential display analysis of the chondrocyte populations showed highly upregulated expression of hypoxia-inducible factor-2alpha (HIF-2alpha) mRNA during chondrocyte differentiation. HIF-2alpha is a homologue of the HIF-1alpha transcription factor, both of which play a role in the activation of a number of genes, including VEGF. The elevated expression of HIF-2alpha during chondrocyte differentiation was accompanied by increased VEGF expression. HIF-1alpha mRNA was also found to be expressed but at similar levels in all of the chondrocyte fractions. Analysis of the murine chondrocyte cell line, ATDC5, which undergoes ordered maturation indicated that HIF-2alpha gene and protein expression also increased in parallel with chondrocyte differentiation. This observation was supported by immunohistochemistry on sections of mouse bone which showed staining corresponding to the presence of HIF-2alpha in hypertrophic growth plate chondrocytes. HIF-2alpha is therefore likely to be involved in the initiation of blood vessel formation in the growth plate, a process crucial for endochondral ossification and bone growth. The presence of HIF-2alpha was also observed in articular chondrocytes but was restricted to the superficial tangential zone, which suggests the source of oxygen for these cells comes from the underlying subchondral bone.

## **P2**

## THE RESTRICTED POTENTIAL FOR RECOVERY OF GROWTH PLATE CHONDROGENESIS AND LONGITUDINAL BONE GROWTH FOLLOWING EXPOSURE TO PROINFLAMMATORY CYTOKINES

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Abnormal growth patterns are commonly observed in children with chronic inflammatory diseases. These disorders are associated with increased pro-inflammatory cytokine production, which may influence growth through a local effect in the epiphysis. We determined the effects of IL-1-beta, IL-6 and TNF-alpha on the growth of murine ATDC5 chondrogenic cells and neonatal metatarsals. At 100-0.1ng/ml, IL-6 had no effect on ATDC5 proliferation or proteoglycan (PG) synthesis during terminal differentiation. At 10 and 100ng/ml, TNF-alpha reduced proliferation (>70%) and PG synthesis (>40%) (P<0.05). At all concentrations, IL-1-beta reduced proliferation (>80%) and PG synthesis (>30%) (P<0.05). Cytokines in combinations (all 10ng/ml) produced no additive or synergistic effects. TNF-alpha and IL-1-beta (100 and 10ng/ml) markedly reduced the mRNA expression of aggrecan, collagen II and X, whereas IL-6 had no effect. A reduction in metatarsal growth was induced by IL-1-beta (71%) and TNF-alpha (45%) (10ng/ml; P<0.05). An additive effect of IL-1-beta + TNF-alpha was observed (110% decrease; P<0.05). IL-6 did not alter metatarsal growth. The ability of ATDC5 cells to spontaneously recover from cytokine exposure was studied. The cytokines were removed after a 7-day treatment during chondrogenesis, and PG synthesis was assessed at 14 days. TNF-alpha induced cell death and detachment from the culture wells. PG synthesis was decreased (41%) by a 7-day IL-1-beta treatment (P<0.001 vs control). This difference was maintained (38%) following recovery (P<0.001 vs control). However PG synthesis was greater (16%) in the 'recovery cells' compared to those treated with IL-1-beta for 14-days (P<0.05). Metatarsals were exposed to cytokines for a 4-day period, and spontaneous recovery was assessed following a further 4-day recovery period in the absence of cytokines. At day 8, metatarsal growth was reduced 52% and 87% following exposure to IL-1-beta and IL-1-beta + TNF-alpha respectively (P<0.05 vs control). At day 8, metatarsals that were exposed to TNF-alpha for the first 4-days grew a similar amount to controls. These studies show that both IL-1-beta and TNF-alpha induced inhibitory effects on chondrocyte dynamics in ATDC5 cells and metatarsals. Both cytokines may be associated with growth retardation in children with inflammatory diseases, and that catch-up growth potential following exposure may be restricted.

### P3

#### OSTEOBLAST CELL CYCLE REGULATION DURING DIFFERENTIATION DIFFERS FROM THAT OF EXPONENTIAL GROWTH: THE ROLE OF P27 AND CYCLIN D3

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The mechanisms controlling osteoblast proliferation are of fundamental importance for understanding the molecular basis of metabolic bone disease and hyperproliferative disorders of bone. Mesenchymal stem cells and committed pre-osteoblasts which are induced to differentiate into osteoblasts must proliferate in a phase of clonal expansion before differentiating into mature osteoblasts. However, the regulation of cell cycle genes during this clonal expansion is poorly understood. Here, we examine the changes in key cell cycle regulators during osteoblast differentiation in vitro.

In differentiating primary rodent calvarial osteoblasts, BrdU labelling experiments revealed that proliferation levels were highest in developing bone nodules during the early post-confluent phase of differentiation. Western blotting showed that upon stimulation of differentiation, the expression of both cyclin D3 and the cell cycle inhibitor p27 increased, whilst that of cyclin D1 and p21 decreased. Immunostaining experiments confirmed that cyclin D3 and p27 were expressed at very high levels largely in the developing bone nodules, suggesting that p27 and cyclin D3 were specifically regulating osteoblast proliferation. Functional assays using immunoprecipitation and immunodepletion experiments revealed that p27 formed complexes with cyclin D1 and CDK4, whereas cyclin D3 associated with CDK4, and all complexes supported kinase activity. However, there was little association between p27 and cyclin D3 proteins during differentiation. The expression of the cell cycle inhibitor p16 was also shown to be significantly elevated in differentiated osteoblast cultures during the mineralisation phase. Immunocytochemistry revealed that the expression of cell cycle inhibitor p16 was confined to mineralised osteoblast nodules, and was associated with increased expression of the senescence marker beta-galactosidase. Evidence that osteoblast cell cycle is targeted during transformation comes from c-Fos overexpressing mice, which develop osteosarcoma. Overexpression of c-Fos in wild-type osteoblasts inhibits osteoblast differentiation, and increases expression of cyclin D1 whilst cyclin D3 expression is decreased. Taken together, these data suggest that p27 and cyclin D3, as well as p16 play important roles in osteoblast proliferation and differentiation, and that cell division is regulated by different mechanisms during exponential growth and differentiation. This differentiation-specific cell cycle regulation may be the target of transforming oncogenes during osteosarcoma formation.

### P4

#### INTRAUTERINE PROGRAMMING OF MESENCHYMAL STEM CELL ACTIVITY

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Epidemiological studies suggest skeletal growth is programmed during intrauterine and early postnatal life. We hypothesize that age-related decrease in bone mass has, in part, a fetal origin and are investigating this using a rat model of maternal protein insufficiency. Dams received either 18% (control) or 9% (low protein) diet during pregnancy, and the offspring (n=194) studied at selected time points (4, 8, 12, 16, 20, 47 weeks).

Maternal weight was unaffected by diet during pregnancy. Control diet mothers had significantly larger litters. At delivery, the control mean pup mass was significantly less compared to restricted diet pups. In contrast, the reverse was observed at 4 weeks indicating modulation of growth trajectory. In males, this lag disappeared by 8 weeks, but has remained in females.

Total number of colony forming units (indicative of colony forming efficiency and proliferation potential of mesenchymal stem cells) increased with age and was similar with both diets. The total number of alkaline phosphatase (AP) positive CFU (indicative of osteogenic potential and differentiation) increased with age with both diets, but to a lesser extent in the control diet. The percentage of AP positive CFU dropped from 45% to 15% over 20 weeks with the control diet, but remained constant at 30% with the restricted diet. These results were observed in both sexes and were unaffected by the addition of Vitamin D3, IGF-1, or Growth Hormone. However, specific AP activity increased over 20 weeks in the control

group, but decreased in females with the restricted diet. Total protein levels followed the same trend. Specific AP activity was significantly higher with the restricted diet in both males and females at 4 weeks, and females at 8 week. However, levels were significantly higher with the control diet in females at 16 and 20 weeks, and males at 12 weeks. These data indicate that a low protein diet in utero affects both mesenchymal stem cell activity and osteogenic potential in the offspring. These results further support the need to understand the key role of the nutritional environment in early development on programming of skeletal development with implicit consequences in later life.

## **P5**

### **DIFFERENCES IN OESTROGEN RECEPTOR TRAFFICKING BETWEEN BONE AND MAMMARY CELLS**

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It is well established that some compounds acting as oestrogen receptor antagonists in breast cancer cells can mimic effects of oestrogen in bone cells although the mechanisms remain unclear. Recent studies have demonstrated the importance of receptor trafficking (both nuclear-cytoplasmic and intra-nuclear) for oestrogen receptor (ER)-alpha function. Thus, DNA binding and transcriptional activation are thought to be followed by ER-alpha ubiquitination, which enables receptors to bind nuclear matrix proteins (freeing binding sites on DNA for subsequent rounds of ER-alpha binding and transcription), an effect revealed by a reduction in ER-alpha mobility. We hypothesise that cell context-dependent signalling of oestrogen antagonists may reflect differences in ER-alpha trafficking and have tested this by monitoring distribution and mobility of ER-alpha-enhanced green fluorescent protein (EGFP) after transient transfection into breast and bone-derived cell lines (MCF-7 and ROS) and cultured for 24hr in the presence of 10<sup>-8</sup> M 17beta-oestradiol (E2), 4-hydroxytamoxifen (4-HT), ICI 182,780 (ICI) or control serum-free medium. In control MCF-7 cells the vast majority (>99%) of ER-alpha-EGFP was within the nucleus and this was unaltered by any of the treatments, whereas in control ROS cells approximately 10% of the ER-alpha-EGFP was cytoplasmic and this was increased to 45% by ICI. The mobility of intra-nuclear ER-alpha-EGFP was also determined by fluorescence recovery after photo-bleaching (FRAP). Maximal recovery from photo-bleaching, an indication of the proportion of ER-alpha-EGFP that remains mobile, was similar for both cell types in the presence of control medium and E2. However, whereas 4-HT significantly reduced the maximal recovery (from 35.5+/-2.7% to 23.2+/-0.9%, P<0.05) in MCF-7 cells, recovery in ROS cells was unaffected. Moreover, while ICI completely blocked recovery from photo-bleaching in MCF-7 cells (reduction to 0.1+/-0.1%), partial recovery was observed (20.1+/-1.4% (ICI) compared to 38.2+/-2.8% (controls)) in ROS cells. Thus oestrogen antagonists differentially influence the proportion of nuclear ER-alpha-EGFP that is mobile in MCF-7 and ROS cells, as well as the amount of ER-alpha-EGFP that is cytoplasmic. We conclude that ER-alpha trafficking differs significantly between MCF-7 and ROS cells in the presence of ICI and 4-HT, which may relate to the cell context-dependence of transcriptional activity of oestrogen antagonists.

## **P6**

### **THE ROLE OF CALCIUM IONS IN THE CELLULAR UPTAKE OF BIPHOSPHONATE DRUGS**

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Nitrogen-containing bisphosphonates inhibit bone resorption by inhibiting farnesyl diphosphate synthase, preventing the prenylation of small GTPases such as Rap1A. However, the factors that influence the internalisation of bisphosphonates into osteoclasts and other cells, such as J774 macrophages, remain unclear. To address this, we synthesised a fluorescently-labelled analogue of alendronate (AF-ALN). We have recently demonstrated that AF-ALN is internalised into vesicles within J774 cells by a process involving fluid-phase endocytosis, and that calcium chloride stimulates the uptake of AF-ALN into J774 cells, quantified by flow cytometry. In this study, we also examined the effect of 10-fold molar excess of magnesium chloride and strontium chloride. Whilst magnesium chloride had no effect on AF-ALN internalisation, strontium chloride, like calcium chloride, increased the amount of AF-ALN uptake. EGTA or the bisphosphonate clodronate (CLO) reduced AF-ALN internalisation and prevented ALN-induced accumulation of unprenylated Rap1A in a concentration-dependent manner. By contrast, the monophosphonate analogue of ALN (which, unlike bisphosphonates, does not chelate calcium ions) had no effect on AF-ALN uptake or ALN-induced accumulation of unprenylated Rap1A. These results suggest that endocytosis of bisphosphonates is enhanced by calcium ions and can be prevented by the removal of extracellular calcium.

To confirm that the inhibitory effect of CLO and EGTA on AF-ALN uptake was the result of chelation of calcium ions from the medium rather than a direct cellular effect, J774 cells were pre-treated with 1mM CLO or 1mM EGTA for 4 hours, then cultured for a further 4 hours in fresh medium containing 0.1mM AF-ALN with either CLO or EGTA, or in fresh medium containing 0.1mM AF-ALN alone. Pretreatment with CLO or EGTA did not affect the uptake of AF-ALN, and CLO or EGTA only reduced the uptake of AF-ALN when present in the medium simultaneously with AF-ALN.

Taken together, these results demonstrate that calcium ions, or similar divalent cations such as strontium, but not magnesium, enhance the endocytic uptake of bisphosphonates, perhaps by forming a macromolecular complex or microprecipitate that is more efficiently internalised by endocytic cells. It is therefore likely that bisphosphonates are also internalised by resorbing osteoclasts as a calcium complex in the resorption lacuna.

## **P7**

### **STUDIES OF THE EXPRESSION IN HUMAN DUODENUM OF THE CALCIUM TRANSPORTER TRPV6 AND THE RELATIONSHIP TO VITAMIN D, AGE, SEX AND BONE MINERAL DENSITY**

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**Background:** Dietary calcium is absorbed by intestinal cells and plays an essential role in bone mineralization and the prevention of osteoporotic fractures. The rate-limiting step at the apical brush-border membrane is now thought to involve the TRPV6 calcium channel/transporter. The aim of the present studies was to determine the extent that factors including vitamin D metabolites may influence the expression of TRPV6 in human duodenum.

**Methods:** Endoscopic duodenal biopsies and blood samples were obtained from 33 fasting volunteers. Subjects with significant diseases including duodenal ulceration, malignancy and coeliac disease were excluded. RNA was prepared and gene expression determined by quantitative real time RT-PCR (Taqman). Results were normalized to GAPDH transcript values. Blood measurements included 1,25-dihydroxyvitamin D (1,25(OH)2D) and 25-OH vitamin D. 16 subjects had DXA bone mineral density (BMD) measurements at hip and lumbar spine.

**Results:** Duodenal TRPV6 transcript expression levels correlated positively with 1,25(OH)2D ( $r=0.47$ ,  $p<0.01$ ) but this relationship was much stronger in males than in females. In men ( $n=10$ ), the association of 1,25(OH)2D and TRPV6 ( $r=0.87$ ,  $p<0.01$ ) was similar at all ages. In women ( $n=23$ ), there was no significant overall relationship of TRPV6 with 1,25(OH)2D ( $r=0.20$ ) but there was a significant negative correlation with age ( $r=-0.69$ ,  $p<0.001$ ). In women aged over 50 ( $n=13$ ), TRPV6 expression was significantly lower than those under 50 ( $n=10$ , median 0.32 vs. 0.58 arbitrary units,  $p<0.001$ ), and was not positively related to 1,25(OH)2D. There was no significant association of TRPV6 with 25-OH vitamin D levels. Expression of the vitamin D receptor was positively related to TRPV6 in all groups except the older women, where median levels were also significantly lower than in the younger women (0.60 vs. 0.86,  $p<0.02$ ). The relationship of TRPV6 expression with BMD was less strong and did not reach significance.

**Conclusions:** TRPV6 expression in human duodenum is vitamin D-dependent but this relationship is lost in older women, who have lower levels of transcripts for both TRPV6 and the vitamin D receptor. These findings can explain the vitamin D resistance and lower fractional calcium absorption seen in older women.

## P8

### OSTEOCLAST FORMATION POTENTIAL IS INCREASED AT TIMES OF PULMONARY INFECTION IN ADULTS WITH CYSTIC FIBROSIS

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Osteoporosis is characterised by low bone mass, bone fragility and an increased susceptibility to fracture. Approximately 25% of adults with cystic fibrosis (CF) have low bone mass and disease severity is the most consistent correlate. Aris and co-workers have shown that biochemical indices of bone resorption are increased [<sup>1</sup>] and that there is a temporal relationship between these and levels of inflammatory markers during infective pulmonary exacerbations in CF [<sup>2</sup>].

The aim of this study was to investigate the relationship between pulmonary infection and the osteoclast precursor population in peripheral blood in adults with cystic fibrosis by measuring CFU-GM growth before (baseline), during (day 1 and 14) and after (day 42) an infective exacerbation. Ten patients (4 male, mean [SD] age 22.9 years [4.0], BMI 20.5 kg/m<sup>2</sup> [3.0]) were recruited. Patients were in a stable condition (defined criteria) at the time of the baseline blood test. Patients were primarily colonised with *Pseudomonas aeruginosa* and none had been prescribed oral glucocorticoids within the previous 3 months. CFU-GM colonies were grown using peripheral blood mononuclear cells obtained by ficcol density separation. Cells were plated at  $3 \times 10^5$ /ml in Methocult medium (Stem Cell Technologies Inc.).

Data at each timepoint are expressed as percentage variation from baseline (mean [SD]). At day 1, the increase in number of CFU-GMs was +38% [4.7], at day 14 +77% [29] and at day 42 +39% [29]. Changes in mean CFU-GM numbers at day 1 were not significant (one-way ANOVA,  $p>0.05$ ). However, most patients did show significant increases ( $p<0.05$ ) between baseline, day 1 and day 42.

Our results suggest that pulmonary infective exacerbations in CF are associated with increased generation of osteoclast precursors in peripheral blood. Possible candidates are pro-resorptive cytokines such as IL-1, IL-6, TNF-alpha and RANKL, the production of which is known to be increased during infection. Stimulation of bone resorption by such factors during infective episodes is consistent with the observed relationship between bone mass and infection and may thus be important in the pathogenesis of bone disease in CF.

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## P9

### LARGE POPULATION-BASED GEOGRAPHIC VARIATIONS IN DXA BMD IN MEN AND WOMEN ACROSS EUROPE. RESULTS FROM THE NETWORK FOR MALE OSTEOPOROSIS (NEMO) STUDY

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Patterns of BMD change with age and between-population variations have been mostly studied in women but little is known about men. As part of the Network for Male Osteoporosis (NEMO) Study, we analysed BMD data from population-based sample of 5,139 men and 6,601 women aged 19-95 years (mean=60y SD=14) from 39 centres across Europe to assess between centre variations in mean BMD levels; effects of age, weight, height; and between-gender contrasts. BMD was measured at the femoral neck, trochanter, and/or L2-L4 spine using DXA densitometers manufactured by Hologic ( $n=16$ ), Lunar ( $n=19$ ), Norland ( $n=4$ ) and Sopha ( $n=1$ ). Densitometers were cross-calibrated with the European Spine Phantom. Analysis was by linear regression including polynomial terms in age, weight, and height to assess curvature of associations.

There were highly significant between-centre differences in mean BMD levels in both genders for the 3 regions ( $P<0.0001$ ), except female spine. The difference between the highest and lowest BMD centre in men ranged from 1.3 population SD's at the femoral neck to 1.6 SD's at the spine. The differences were larger in women ranging from 1.9 to 2.8 SD's respectively. In men, femoral neck and trochanter BMD ( $n=4724$ ) were best described by a 3-degree (femoral neck) and 4-degree (trochanter) polynomial in age ( $P=0.001$ ), 2-degree polynomial in weight ( $P<0.0001$ ), and a negative linear effect of

height( $P < 0.037$ ). None of these effects differed significantly by centre( $P > 0.05$ ) and explained 28% and 21% of the total variance in femoral neck and trochanter BMD. Spine BMD( $n = 3773$ ) was best described by a 2-degree polynomial in age and weight( $P < 0.001$ ), and 19% of the total variance was explained. In women, femoral neck and trochanter BMD( $n = 6195$ ) were best described by 4-degree polynomial in age, 2-degree polynomial in weight and a negative linear effect for height (trochanter). The effect of age and weight significantly differed by centre( $P < 0.009$ ) and explained 42% and 36% of the variance in femoral neck and trochanter BMD respectively. Spine BMD in women( $n = 4936$ ) was described by 3-degree polynomial in age and linear effects for weight and height, explaining 17% of the variance. In conclusion, there is considerable geographic variation across Europe in BMD and effects of age and weight varied by centre in women.

#### **P10**

##### **AUTOCRINE REGULATION OF GLUCOCORTICOIDS BY 11 BETA-HYDROXYSTEROID DEHYDROGENASE TYPE 1: A MECHANISM FOR TISSUE-SPECIFIC REGULATION OF INFLAMMATION**

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Acute inflammation plays an important role in the normal immune system by helping to coordinate host responses to danger signals such as infection. In most cases the inflammation is rapidly resolved but in chronic inflammatory diseases such as rheumatoid arthritis (RA) the inflammation persists leading to localized accumulation of potentially damaging immune cells. Although it remains unclear why inflammation persists in some tissues and not in others recent studies have shown that stromal cells such as fibroblasts play a pivotal role in directing local immune responses and disease persistence. We have hypothesized that activation of glucocorticoids (GCs) via the enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) is an important facet of stromal cell function by providing an autocrine anti-inflammatory pathway. To test this hypothesis we have assessed changes in 11beta-HSD1 expression and activity in fibroblasts isolated from different tissue sites (epidermis, bone marrow, synovium) in  $n = 6$  subjects. Cells were cultured in the presence or absence of various inflammatory stimuli (TNFalpha, IL-1, IL-4, IFNgamma) for 24 hrs and expression of target genes assessed by quantitative RT-PCR. The glucocorticoid receptor isoforms alpha and beta were expressed to a similar degree in all tissues and showed no significant change in expression following treatment with cytokines. However, baseline expression of 11beta-HSD1 mRNA varied considerably for the different fibroblast types: synovial >> bone marrow > dermal. These variations were matched by differences in cortisone metabolism with synovial fibroblasts showing the highest capacity for endogenous cortisol generation. Expression of 11beta-HSD1 increased in dermal (56-fold), bone marrow (9-fold) and synovial (7-fold) following treatment with TNFalpha, with similar results for IL-1. However, IL-4 and IFNgamma had no effect. We postulate that tissue-specific variations in the autocrine activation of GCs via 11beta-HSD1 may play an important role in defining the impact of stromal cells on the persistence of inflammatory disease.

#### **P11**

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

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Ovariectomy (OVX) can cause bone loss in rats, but little is known about how it also induces lumbar intervertebral disc degeneration (LVD). This study investigated how estrogen deficiency affected intervertebral discs in OVX rats. Thirty 3-month-old female SD rats were divided randomly into three equal groups. The baseline control group (BL) was killed at the beginning of the experiment. An ovariectomy was performed in 10 rats (OVX group) and another group of 10 rats was subjected to a sham surgery (Sham group). The OVX rats were untreated after the surgery to allow for the development of moderate osteopenia. Bone mineral density (BMD) measurement and bone histomorphometric analysis were applied to the segments of lumbar spines in all rats killed 6 months after surgery. The pathological changes of intervertebral discs were observed and the degree of LVD was scored by a histological scoring system. The BMD of the spines in the OVX group decreased significantly compared with the Sham group. The bone volume indices in the OVX group were significantly lower, but the bone turnover rate parameters were significantly higher than those in the Sham group ( $P < 0.01$ ). The histological scores for LVD in the OVX group were significantly higher than those in the Sham group ( $P < 0.01$ ). There was a significant negative correlation between the BMD and Grade II discs in the OVX rats ( $P = 0.042$ ). In conclusion, LVD occurs in the OVX rats and the degeneration of cartilage end plates may be a pathogenic factor in disc degeneration.

**P12****EVALUATION OF AUTOLOGOUS BONE MARROW MESENCHYMAL STEM CELL-CALCIUM PHOSPHATE CERAMIC COMPOSITE FOR LUMBAR FUSION IN RHESUS MONKEY INTERBODY FUSION MODEL**

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Autologous bone marrow mesenchymal stem cell (BMSC)-calcium phosphate ceramic composites were constructed in vitro and implanted as a bone graft substitute for lumbar anterior interbody fusion in rhesus monkeys to determine the osteogenic capacity of the composites. Nine adult rhesus monkeys underwent lumbar L3-L4 and L5-L6 discectomy and interbody fusion via an anterior retroperitoneal approach. Two fusion sites in each animal were randomly assigned to two of three treatments: autogenous tricortical iliac crest bone graft (autograft group) or cell-free ceramic graft (ceramic group) or BMSC-ceramic composite graft (BMSC group). Autologous BMSCs were culture-expanded and stimulated with osteogenic supplement. The spinal fusion segments were evaluated by radiography, biomechanical testing, histologic analysis and histomorphometric analysis at 3 months post-surgery. The BMSC group could achieve lumbar interbody fusion superior to the ceramic group both biomechanically and histologically. The BMSC group and the autograft group showed equivalent biomechanical stiffness. Ceramic residues were significantly greater in the ceramic group versus the BMSC group. The results indicate that BMSC-ceramic composites can enhance bone regeneration and achieve osseous spinal fusion 3 months after the implantation in rhesus monkey interbody fusion model.

**P13****ANALYSIS OF NON-ADHERENCE WITH BISPSPHONATE TREATMENT FOR OSTEOPOROSIS IN UK PATIENTS**

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Adherence to bisphosphonate therapy for osteoporosis is important to improve bone mineral density and reduce fractures (1,2,3). Adherence to bisphosphonates in the UK is poor, even with weekly regimens (4). This research aims to review a breakdown of non-adherence with available bisphosphonates, and compare to adherence with therapies for other asymptomatic chronic conditions, using a UK database (DIN-LINK).

DIN-LINK collates sampled information from approximately 100 UK general practices. Information was extracted from this database, to analyse non-adherence patterns in patients starting bisphosphonate therapy between December 2002 and November 2003 (5) These patients had not received osteoporosis therapy (excluding vitamins/minerals) in the previous 12 months. Patients were classified as non-compliant (did not return for a prescription within 1.5 times the prescription length at least once in the follow-up period before restarting), non-persistent (stopped treatment, without restarting), adherent (compliant and persistent), or switched therapy (to another drug for the same condition). All patients remained 'available' for treatment (ie; did not change residence or die), and were followed for twelve months.

Data for diuretics/beta-blockers for hypertension and statins for hyperlipidaemia was extracted for comparison (5).

Adherence 'breakdown' results (after one year) were: Adherent/Non-persistent/Non-compliant/Switched therapy

Weekly bisphosphonate: 33%/24%/38%/6%

Daily bisphosphonate: 22%/52%/24%/3%

Diuretic/beta-blocker: 21%/35%/-/-

Statin: 39%/13%/-/-

Adherence is lowest in the daily bisphosphonate and hypertensive patients, followed by weekly bisphosphonates, and then statin therapy. These differences may be due to various factors including perceived efficacy/value, tolerability, co-morbidity and convenience of administration.

The current pattern of adherence to bisphosphonates is poor. Even in patients initiated on weekly treatment, only a third adhere fully over one year, and a quarter of patients stop taking any osteoporosis treatment altogether. This is likely to be adversely affecting health outcomes. Less frequent (weekly) bisphosphonate dosing may have influenced adherence, but this remains sub-optimal - further improvements (eg: convenient administration or patient education) are warranted to optimise therapeutic outcomes in osteoporosis (1).

1 Reginster J-Y & Lecart MP. 2004

2 Yood RA et al. 2003

3 Caro JJ et al. 2003

4 DIN-LINK data (Osteoporosis Report 4, All Diagnoses, MAT October 2004), CompuFile Limited

5 DIN-LINK data (Osteoporosis, hypertension, hyperlipidaemia compliance reports, MAT November 2004), CompuFile Limited

**P14****THE EFFECTS OF ZOLEDRONIC ACID AND DOXORUBICIN IN COMBINATION ON BREAST CANCER CELL INVASION IN VITRO**

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The bisphosphonate zoledronic acid (ZOL) and the cytotoxic drug doxorubicin (DOX) induce synergistic levels of apoptosis in breast cancer cells (BCC) when DOX is given before ZOL. As ZOL and DOX have individually been shown to reduce BCC invasion and migration, respectively we have investigated if these drugs also act synergistically on BCC invasion in vitro.

Methods: For each assay, MCF7 cells were treated with 0.05micM DOX for 4 hours followed by 1 or 10micM ZOL for 24 hours. Separate samples were exposed to the drugs in the reverse sequence. To study invasion, MCF7 cells were either grown on Transwell membranes coated with Matrigel or in a 24-well plate. Cells were treated sequentially using the above drug combinations, prior to starting the invasion assays for 48 hours. Cell growth and apoptosis was also assessed under the same conditions.

Results: Invasion of MCF7 cells treated with ZOL and DOX was significantly reduced when compared with control, but the effect was dependent on drug sequence. When cells were treated with 1micM ZOL, invasion was only significantly reduced if cells were pre-treated with DOX (p less than 0.001, versus control (fresh medium alone)). For 10micM ZOL, invasion was reduced, when administered before or after the DOX (for both sequences, p less than 0.001, versus control). However when growth was taken into account, treatment with 10micM ZOL alone caused a significant reduction in MCF7 growth (p less than 0.05), whereas 1micM ZOL, either alone or when combined with DOX, did not significantly reduce cell growth. Apoptosis was unaffected by any of the drug combinations.

Conclusions: Pre-treatment of BCC with 0.05micM DOX followed by 1micM ZOL reduces invasion when cells were grown on Matrigel. 10micM ZOL, pre- or post-DOX also reduces invasion, but for this combination inhibition of cell growth may contribute to the reduction in invasion observed.

## P15

### INTRAUTERINE PROGRAMMING OF SKELETAL DEVELOPMENT

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Epidemiological studies suggest skeletal growth is influenced by the intrauterine and early postnatal environments. We hypothesize that age-related decrease in bone mass has, in part, a fetal origin and are investigating this using an ovine model. Rams were exposed to a global nutrient restricted maternal diet in utero (50% total nutrient requirements, 0-30 days gestation), UC, n=6), postnatal nutrient challenge to reduce body weight to 85% target weight from weaning (12 weeks) to 25 weeks postnatal age (CU, n=5), or both (UU, n=7). Control diet (CC, n=11). All measurements on offspring were taken at 2.5 years.

Anthropometric measurements of femurs showed no significant differences between groups, however all measurements showed a trend, compared to the control group, for the mean to be lower in the CU group, higher in the UU group, and similar in the UC group. When analysed by pQCT, femurs showed no significant differences between the groups, although the same trends were again observed, this time with cortical and trabecular Bone Mineral Density (BMD), Bone Mineral Content (BMC), cortical thickness and trabecular area. These remained after normalising to body mass. Examination of vertebrae (CC n=2, UC n=2, and UU n=2, analysed in triplicate) using DXA showed BMD in the UC group was significantly lower than CC, whereas the UU group was similar to CC. No differences were found in BMC. Significance was lost when results were normalised to mass. In a further study to examine if maternal nutrient restriction before conception had any effect, vertebrae from control (n=3) and male offspring from sheep exposed to a 50% nutritional restriction for 30 days before conception (n=8) were tested in duplicate. No significant differences were found with BMD or BMC.

These results suggest that reduced nutrition in early gestation or early postnatal life negatively impacts on bone structure while bone formation is unaltered, or enhanced, with combined pre and postnatal nutrient restriction indicating that minimal mismatch between the pre- and postnatal nutrient environment is beneficial to skeletal development. These results indicate the key role of the nutritional environment on programming of skeletal development with implicit consequences in later life.

## P16

### SENSITIVITY OF FINITE ELEMENT ANALYSIS OF X-RAY IMAGES (FEXI) TO ANATOMICAL VARIATIONS OF THE PROXIMAL FEMUR - A SIMULATION APPROACH

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FEXI (Finite Element analysis of X-ray Images) is an osteoporotic fracture prediction technique. Routine two-dimensional clinical radiographic bone images, such as DXA scans, are used to create a 2D plane stress model for finite element analysis. Compressive mechanical loading is simulated to determine the stiffness of the bone.

We have investigated the sensitivity of FEXI to variations in trabecular bone density, hip-axis length, hip angle, and head anteversion. A virtual anatomy proximal femur was created using the 'vxt' volume graphics library. The resultant bone model was passed through an x-ray simulator to create a 2D image suitable for analysis by FEXI. We found that FEXI-derived stiffness increased linearly with trabecular bone density, decreased non-linearly with hip-axis length, decreased gradually with neck angle, but was insensitive to head anteversion. Multi-parameter sensitivity analysis (MPSA) was performed to determine the relative sensitivity of each parameter on the overall stiffness of the bone. FEXI was most sensitive to hip-axis length (17.4), followed by trabecular bone density (7.9), neck angle (3.4) and head anteversion (0.9).

In conclusion, a simulation approach helps us to understand and analyze the various anatomical parameters that affect the stiffness and hence strength of a bone susceptible to osteoporotic fracture. Clinical validation of the FEXI approach will require extensive analysis of large datasets.



## P17

### DNA TRANSFECTION OF BONE CELLS AT HIGH EFFICIENCIES WITHIN BIOMINERALISED POLYSACCHARIDE CAPSULES

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Polysaccharide (alginate and chitosan) capsules coated with a unique self-assembled semi-crystalline shell of calcium phosphate were developed as enclosed biological reaction vessels for the spatial and temporal delivery of primary human cells and bioactive factors. The aim of this study was to demonstrate plasmid DNA encapsulation, delivery and transfection of adjacent cells inside capsules, embedded capsules and plated cells covered by exogenous solution containing DNA capsules.

Bacterial plasmid DNA and/or bone cells (SaOS) was added to solution of sodium alginate solution supplemented with phosphate ions and mixed thoroughly. Alginate droplets were fed through a syringe into a solution of chitosan supplemented with calcium ions. Guest capsules were inserted into soft, pliable host capsules soon after immersion in chitosan solution. Capsules were then immersed in basic media in plastic well-plates for up to 7 days to enable transfection to occur. Encapsulated bone cells were stained with standard X-Gal to show transfected cells expressing beta-galactosidase.

Here we demonstrate delivery of plasmid DNA from embedded capsules into a surrounding host capsule followed by transfection of encapsulated bone (SaOS) cells therein. The measured transfection efficiency was 65 percent at 7 days. Furthermore, DNA delivery and transfection was demonstrated within capsules containing an admixture of bone cells and plasmid (+51 percent) and from capsules containing DNA alone suspended in media over plated bone cells (+5 percent). We also demonstrate capsule transfection of cells *in vivo*; a small proportion of which were released. The ability to regulate shell decomposition and release of capsule contents provides a mechanism for programmed release of gene modulated cells into the biological environment. The mechanism of plasmid transfer from the alginate gel into the cell is still under investigation however, plasmid was found to be strongly associated with the chitosan/ calcium phosphate shell as shown by ethidium-homodimer-1 staining of encapsulated DNA and this may assist the transfer from gel to cell.

Programmed non-viral delivery of genes using biomaterial constructs is an important approach to gene therapy and orchestrated tissue regeneration. These unique biomaterialised polysaccharide capsules provide a facile technique, and enclosed biomimetic micro-environments with specifiable degradation characteristics, for the safe encapsulation and delivery of functional quantities of plasmid DNA.

## P18

### DISCRIMINATION OF BMD-MATCHED RECENT HIP FRACTURE SUBJECTS BY FEXI (FINITE ELEMENT ANALYSIS OF X-RAY IMAGES)

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The preferred method of assessing the risk of a fragility fracture is currently a measure of bone quantity, as bone mineral density (BMD) by dual energy X-ray absorptiometry (DXA). However, other bone quality factors contribute to the overall risk of fracture, including anatomical geometry and the spatial distribution of bone.

FEXI analysis of routine two-dimensional clinical radiographic images, such as DXA scans, involves the simulation of a mechanical compression test by 2D plane-stress finite element analysis, and provides a measure of whole bone stiffness (N/mm). A novel feature is that the grey level of each image pixel is converted into a corresponding value of Young's modulus in the finite element model.

We have investigated the ability of FEXI of the proximal femur to discriminate between 15 female subjects who had recently suffered a non-traumatic hip fracture (F), their DXA scan performed within 1 week of the subject being chair-mobile, and 15 age, sex, and total hip BMD-matched control (C) subjects with no previous fracture or steroid use. The subject descriptive statistics were age (years): C = 78.5 +/- 2.0, F = 79.1 +/- 5.0; weight (Kg): C = 55.2 +/- 7.8, F = 60.4 +/- 8.8; total hip BMD (g cm<sup>-2</sup>): C = 0.701 +/- 0.118, F = 0.700 +/- 0.119; and FEXI (N/mm): C = 24.7 +/- 1.6, F = 25.7 +/- 2.0. Paired-sample T-test analysis (C - F) gave: age = -0.6 +/- 5.7, p = 0.691; weight = -5.2 +/- 9.1, p = 0.045; total hip BMD = 0.0004 +/- 0.006, p = 0.801; and FEXI = -0.96 +/- 1.74, p = 0.051. The fracture cohort was therefore significantly heavier, contrary to expectation, and exhibited a higher stiffness of the proximal femur.

This pilot study suggests that FEXI has the potential to provide a measure of bone quality that may predict fracture risk independent of BMD. Facilitated by semi-automated segmentation of the proximal femur from a hip DXA image and a batch processor that enables multiple scans to be analysed concurrently, FEXI may be readily implemented into clinical trials.

**P19**

## APPLICATION OF A NOVEL BONE SIMULATOR TO THE ASSESSMENT OF BONE QUALITY

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We have developed a 3D bone remodelling simulation environment that considers the net effect of osteoblast and osteoclast activity on biopsy and computer-generated cancellous bone samples. The finite element solver predicts the stiffness of the resultant structures, where each voxel is converted directly into a finite element. An integrated histomorphometry tool allows the relationship between structure and mechanical properties to be explored. A graphical interface enables 3D stereoscopic visualization of the bone structures at every stage of an analysis, together with 'fly-throughs' and automatic movie generation.

3D natural tissue bone structures are obtained from microCT scans, with typical resolutions of 15-25 microns. This data is used directly in the stochastic simulator which is based upon the concept of the basic multi-cellular unit (BMU), where either net resorption or net formation is considered at bone/marrow surfaces. The simulation considers the probability that any surface voxel will be activated into a BMU and if activated, the length of the resorption cavity or bone deposition. Simulation of anabolic treatment following varying degrees of initial resorption yielded a hysteresis relationship between the stiffness and density of bone, due to the sensitivity to trabecular perforation.

The simulator also allows the user to specify complex multi-functional non-linear modelling and remodelling rules, based on, for example, local bone age, stress/strain profile and location within a structure. The age of each bone voxel is recorded during simulation so that activity can be biased away from recent remodelling sites. Similarly, values of strain energy density in each voxel can be used to influence activity, facilitating strain-weighted adaptation.

**P20**

## DEVELOPMENT OF TISSUE ENGINEERING STRATEGIES FOR CARTILAGE GENERATION: MICROMASS AND THREE DIMENSIONAL CULTURES USING HUMAN CHONDROCYTES AND AN IMMORTALIZED CELL LINE

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Monolayer cultures of the ATDC5 murine chondrocytic cell line are widely used to study chondrocyte differentiation. However, the 3-dimensional (3D) microenvironment is known to play an important role in promoting cell-matrix interactions during chondrogenesis. This study was therefore aimed at engineering facile, robust 3D models for cartilage generation, which would aid development of clinically useful constructs. The potential of ATDC5 cells to generate cartilage was compared to human articular chondrocytes in 3D micromass pellet cultures and by tissue engineering strategies involving biodegradable scaffolds and bioreactor technology. Proliferating ATDC5 cells and confluent human articular chondrocytes, expanded prior in monolayer cultures, were harvested for pellet culture and dynamic seeding onto polyglycolic acid (PGA) fleece within a 'high aspect ratio vessel' rotating bioreactor. After a 21-day culture period, explants were analysed for chondrogenic differentiation by histology (Alcian blue/ Sirius red, alkaline phosphatase {ALP}) and for expression of typical chondrocytic markers, sox-9, aggrecan and type II collagen, by immunohistochemistry and RT-PCR. Chondrogenic differentiation in ATDC5 and articular chondrocyte pellets was evidenced by distinct chondrocytes, expressing typical chondrocytic proteins, in lacunae embedded in a cartilaginous matrix of type II collagen and proteoglycans. In pellets of both cell types, gene expression for aggrecan and type II collagen matched protein expression. Although sox-9 gene expression matched protein expression in articular chondrocyte pellets, sox-9 protein expression in ATDC5 pellets was not complemented by gene expression, which remained restricted to the proliferating ATDC5 cells of the monolayer. Staining for ALP was not detected in pellets of both cell types. Explants of ATDC5 cells cultured on PGA fleece in the bioreactor were reminiscent of cartilaginous structures composed of numerous chondrocytes, staining for typical chondrocytic proteins, in lacunae embedded in a matrix of type II collagen and proteoglycans. In comparison, articular chondrocyte explants exhibited areas of sox-9, aggrecan and type II collagen- expressing cells growing on fleece, and discrete islands of chondrocytic cells embedded in aggrecan and type II collagen-rich matrix. This study highlighted the crucial role played by the 3D microenvironment in modulating cell-matrix interactions in cartilaginous explants exhibiting gene and protein expression profiles reflective of the in vivo scenario.

**P21**

## ATP RELEASE AND P2 RECEPTOR EXPRESSION BY HUMAN CHONDROCYTES.

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The role of P2 receptor ligands (ATP and other nucleotides) as extracellular messengers is well established, mediating a wide variety of cellular processes. P2 receptors are divided into two families, the ligand-gated ion channel P2X receptors (P2X1-7) and the G-protein-coupled P2Y receptors (P2Y1,2,4,6,11,12,13,14). Previous data from our group and others have been fundamental in establishing that purinergic signalling has a highly influential role in the bone microenvironment, modulating multiple functions including osteoblast proliferation, osteoclast formation, osteoclast resorptive capacity, and apoptosis of both osteoblasts and osteoclasts. Whilst purinergic signalling in bone has been elucidated and is being targeted for drug intervention relatively little is known as to the role of purinergic signalling in

cartilage. Early work demonstrated that chondrocytes express enzymes to breakdown extracellular ATP, and that extracellular ATP has contrasting effects on cartilage proteoglycan turnover depending on the source of cartilage. In addition, extracellular ATP was also shown to synergise with factors known to be involved in rheumatoid arthritis (RA) and osteoarthritis (OA). These earlier studies provide tantalizing evidence for purinergic regulation of chondrocyte behaviour; however, no consensus study clearly profiles P2 receptor expression by chondrocytes.

Using RTPCR we investigated the expression of P2 receptors by primary human chondrocytes and the immortalized human chondrocyte cell line C-20/A4. These cells express P2Y<sub>1,2,4,6</sub> receptors when cultured in monolayer, and shift towards predominant expression of P2Y<sub>6</sub> when cultured in alginate beads. In addition, we investigated the release of ATP from human chondrocytes using a luciferin/luciferase bioluminescence assay. We found that chondrocytes constitutively release ATP, and that this release is modulated by mechanical stimulation induced by fluid flow.

Mechanical loading is known to be an important regulator of chondrocyte metabolism; we believe that ATP released from chondrocytes following mechanical load and acting via P2 receptors represents an additional pathway in the transduction of the mechanical signal. Aberrant expression of P2 receptors or increased release of ATP that may occur in diseased states and/or following trauma will result in deregulated purinergic signalling and altered cartilage turnover, leading to a progressive destruction of the cartilage tissue and loss of function as seen in RA and OA.

## P22

### THE EFFECT OF SIMVASTATIN ON THE HUMAN OSTEOBLAST-LIKE CELL, HTB-96

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Statins are competitive inhibitors of the enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme in cholesterol biosynthesis. Hence, their wide use for the treatment of hypercholesterolemia. Mundy et al. (1999, *Science*, 286: 1946-1949), identified statins as bone stimulatory agents. Simvastatin, have been found to increase the expression of BMP-2 mRNA that triggers a cascade of events leading to increased bone formation. However, the exact mechanism for this stimulatory action is not fully elucidated. The aim of this study was to explore the possible mechanisms responsible for the enhanced bone formation. The hypothesis is that the anabolic effect of statins on bone may be partly due to stimulation of osteoblast proliferation and differentiation and/or inhibition of osteoblast apoptosis.

**Methods:** The effect of different concentrations of simvastatin (5 micromolar to 1 nanomolar) on the human osteoblast-like cells HTB-96, at different incubation times was examined. Osteoprotegerin (OPG) concentration of the culture medium was measured using a modified ELISA assay. Alkaline phosphatase (ALP) activity of the cell lysate was measured using a colorimetric method. Cell apoptosis was determined using a DNA fragmentation method.

**Results:** Simvastatin (10<sup>-7</sup> Molar, 1 and 3 micromolar) caused a significant increase in OPG concentration of the culture medium at most incubation times. The most significant (p<0.001) increase was found at 18 hours by 10<sup>-7</sup> Molar (2.48 ± 0.09 versus 1.83 ± 0.09 [ng/ml]). Simvastatin at only 5 micromolar concentration, at all incubation periods caused a significant increase in ALP activity. This was most significant (p<0.001) at 18 hours (3.4 ± 0.5 versus 0.75 ± 0.15 U/100,000 cells/ml). Apoptosis of HTB-96 cells was significantly inhibited by simvastatin (10<sup>-7</sup> Molar and 1 micromolar) at all incubation times. The most significant anti-apoptotic effect was observed (p<0.05) as early as 6 hours by 10<sup>-7</sup> Molar (0.08 ± 0.01 versus 0.15 ± 0.02 OD at 405/495 nm). Surprisingly, higher doses of simvastatin (3 and 5 micromolar) resulted in significantly increased apoptosis.

**In conclusion,** these results suggest that the anabolic effect of simvastatin may be arbitrated in part through an inhibition of osteoblast apoptosis and stimulation of osteoblast activity.

## P23

### OSTEOCYTE DENSITY IN BONE: THE EFFECT OF HORMONAL OR MECHANICAL DISRUPTION

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Osteocytes are considered fundamental to regulating load-induced bone mass and architecture. How this is achieved has not been elucidated, but communication between other osteocytes and surface osteoblasts is assumed obligatory. Although osteocyte density (OD) has been shown to differ in skeletal sites and reduce with age, the effect of hormonal status or a change in local mechanical loading environments on OD has hitherto been overlooked. This study was designed to ascertain whether OD is affected by ovariectomy, or altered by a lower mechanical demand.

Female rats were ovariectomised at 50 days, or had maxilla molars removed at 64 days. Control animals remained untreated. After 207 days, all animals were sacrificed and mandible, ulnae, and parietal bones were removed and fixed in formaldehyde. Following this, bones were decalcified in 10% EDTA, and prepared for cryosectioning.

Nuclei in 10micron thick sections were stained with haematoxylin and visualised using an Olympus BHS microscope linked to a Kontron image analysis system. Known field areas comprising matrix only were captured and osteocyte numbers counted.

In the control group, there was a significant difference in OD in alveolar (1592 ± 238/mm<sup>2</sup>) compared with basal (807 ± 175/mm<sup>2</sup>) mandibular bone. Similarly, there was a significant difference in OD between the ulnae (830 ± 45/mm<sup>2</sup>) and calvariae (589 ± 57/mm<sup>2</sup>).

In the ovariectomised group, alveolar bone OD was reduced (1228 ± 160/mm<sup>2</sup>, p=0.01) as was that in the ulna (660 ± 73/mm<sup>2</sup>, p=0.03) compared with controls, consequently there was no difference in OD between the ulnae and calvariae (673 ± 56/mm<sup>2</sup>). Calvarial OD did not differ from the control group.

There was no difference in OD in the basal mandibular bone of animals that had had maxilla molars extracted (846 ± 166/mm<sup>2</sup>) compared with controls. However, this was not so in alveolar bone, where OD increased (2007 ± 131/mm<sup>2</sup>, p<0.001).

These data indicate that during normal bone growth, the number of osteoblasts incorporated into bone matrix depends on the skeletal site. Moreover, that this osteoblast to osteocyte incorporation ratio is sensitive to hormonal status and the local mechanical loading environment suggests that the osteoblast: osteocyte ratio is significant.

**P24**

SULPHATED GLYCOSAMINOGLYCAN STAINING INTENSITY IN BONE: EFFECT OF A CHANGE IN MECHANICAL LOADS  
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Sulphated glycosaminoglycans (GAGs) of bone matrix have been proposed to have a number of various roles in bone, including regulation of the mineralization process. Alterations in the magnitude or principle direction of habitual mechanical loads through bone might influence the local concentration of these GAGs and, thus bone mineral content (BMC), which is not equal throughout the skeleton.

Groups of female rats were fed a soft diet (powdered chow as a paste) for 8 weeks, whilst a control group was fed the standard chow. After the experimental period animals were sacrificed; mandible, ulna, and calvaria were collected and fixed in formaldehyde. Following this, bones were decalcified in 10% EDTA and prepared for cryosectioning. 10micron sections were immersed in 0.05% Alcian Blue GX in 0.025M acetate buffer solution containing 0.025M MgCl<sub>2</sub> at a final pH of 5.8 for 18 hours to stain sulphated GAGs. The staining intensity was quantified using a Vicker's M85A scanning and integrating microdensitometer. 10 fields were measured in the calvariae and ulnae. 10 fields were measured from 4 regions of the mandible; upper and lower regions of both the buccal and lingual alveolar bone.

GAG staining intensity in calvaria was  $54.3 \pm 1.4$  M.I.E. x 100 which was lower than that measured in ulna;  $74.3 \pm 3.1$  M.I.E x100,  $p < 0.001$ .

In the mandible of animals fed a hard diet, staining on the buccal aspect of alveolar bone was consistently lower than the staining on the lingual aspect in both the upper and lower regions (upper and lower buccal:  $55.7 \pm 4.7$  M.I.E.x100, upper and lower lingual:  $65.2 \pm 5.5$  M.I.E.x100,  $p=0.02$ ). In animals fed a soft diet, this pattern was reversed with GAG staining intensity in buccal aspect being higher than the lingual aspect (upper and lower buccal:  $74.8 \pm 4.0$  M.I.E.x100, upper and lower lingual:  $60.9 \pm 4.9$  M.I.E.x100,  $p < 0.005$ ).

These data indicate that alterations in the mechanical loading environment can regionally influence either the absolute GAG content, or the availability of GAGs for staining. Either way, this represents a local, loading-related matrix remodelling event which could influence matrix integrity.

**P25**

VARIATIONS IN HUMAN OSTEOPROGENITOR ADHESION PATTERNS REVEAL A METHOD FOR IN SITU INVESTIGATION OF SUB-POPULATION BEHAVIOUR IN HETEROGENEOUS CELL POPULATIONS: IMPLICATIONS FOR TISSUE REGENERATION

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In many cases an understanding of tissue heterogeneity is necessary to link advances in molecular- and cell-biology to tissue-level parameters in a meaningful and insightful way, for example to inform tissue regeneration strategies. However, many experimental techniques disturb the natural tissue balance making it difficult to draw realistic conclusions concerning in situ sub-population behavior. In this study we present a widely applicable and non-invasive means to quantify population heterogeneity by combining experiment and mathematical analysis, using adhesion patterns within human osteoprogenitor populations as an illustration.

We examined patterns of adhesion in STRO-1 immunoselected and unselected osteoprogenitor samples with reference to patterns of adhesion in the homogeneous osteoblast-like MG63 continuous cell line. Adhesion was examined on tissue culture plastic (TCP) and Fibronectin (FN) surfaces, using cell area as a readily obtainable and cell-specific measure of spreading. The underlying distribution of cell areas for each cell type and surface were investigated and correlations between data sets examined using a combination of graphical and non-parametric statistical methods. Mappings between area distributions were used to quantitatively explore heterogeneity of tissue composition and selective regulation of sub-populations by surface modification. In particular, correlations between STRO-1+/- profiles and MG63 profiles allowed quantification of the degree of heterogeneity of composition of these populations, while mappings between populations on different surfaces allowed quantification of tissue response to environmental modification. This study demonstrates that STRO-1+/- populations are profoundly different in composition and response to surface modification by FN, and outlines these differences. This work is of immediate interest to the musculoskeletal research field since it provides a sensitive means to quantify bone progenitor/marrow population properties and thus explore in situ regulation of function. Furthermore, the current results suggest that this method has considerable potential to understand generic in situ sub-population behavior and tissue organisation and thus will find wide applicability beyond the immediate musculoskeletal field.

**P26**

ZOLEDRONIC ACID INHIBITS CELL TO BONE MATRIX INTERACTIONS IN HUMAN BREAST AND LUNG CARCINOMA CELL LINES

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Carcinomas of the lung and breast frequently metastasise to bone causing substantial clinical complications and reducing life expectancy. Adhesion of malignant cells to bone matrix and associated proteins is thought to be a key step in this process. Bisphosphonates (BPs), particularly the nitrogen containing bisphosphonate zoledronic acid (Zol), have been shown to significantly limit the progression of bone metastasis in several cancers including lung and breast. We investigated the effect of clinically relevant concentrations of BPs on the adhesion of lung and breast carcinoma cell lines to mineralised bone matrices and basement membrane proteins to elucidate a scientific basis for this action.

Lung (n=3) and breast (n=2) carcinoma cell lines were treated with 1nanoM, 1 or 100 microM Zol or 100microM EDTA for 24 hours. The cells were harvested and equal numbers of cells seeded onto dentine matrices or Matrigel (basement membrane protein) coated plates for a further 24 hours. Adherent cells were washed, fixed, stained and counted. Parallel experiments on cell viability, number and the induction of apoptosis were performed using standard laboratory methods. To elucidate the specific basement membrane proteins involved in adhesion cells were screened using a CytoMatrix TM kit consisting of fibronectin, vitronectin, laminin, collagen I and IV strips as per manufacturer's instructions. Zoledronic acid was found to significantly reduce adhesion to mineralised bone matrix and basement membrane proteins in both breast and lung cancer cell lines, for example, 100microM Zol inhibited cell adhesion by 50% in MCF-7 breast cells. EDTA did not affect adhesion indicating that the effects seen were not attributable to calcium chelation. Moreover inhibition of adhesion was observed at lower concentrations and earlier time points than those required to induce a reduction in cell viability or number or to induce apoptosis indicating that cell death was also not a significant factor. Our findings demonstrate that BPs significantly and directly reduce adhesion to mineralised matrices and suggest that impaired cell-matrix interactions may play a role in BP-induced cancer cell death.

## **P27**

### **A METHOD FOR DETERMINING SKELETAL RATIOS FROM DXA SCANS**

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Dual-energy X-ray absorptiometry (DXA) is the gold-standard technique for bone densitometry when measuring osteoporosis and fracture risk. Nevertheless, DXA scans clearly contain unused information on bone size (i.e. length and width) that could potentially be used to improve the prediction of fracture risk. Moreover, the use of skeletal ratios is ideal for gene specificity in genetic studies. The Twins UK Registry at St Thomas' Hospital holds enough bone densitometry data to allow thorough exploration of two bone measurement techniques which will essentially provide genetic analysis of skeletal ratios.

The aim of the study was to investigate the optimal method for measuring bone size from DXA.

Exploring the extensive database, 50 twin files were selected according to whether their DXA scans had been repeated on the same Hologic QDR-4500W DXA machine. The two methods of measurement performed were:

\* Reticule - manual measurement of images on paper using a magnifying reticule and ruler (+/- 0.1cm)

\* LPC (Linear Pixel Count) - counting the number of horizontal lines created on the DXA computer screen using the special analysis software

Data was entered into a table of total height, length of spine and the lengths of principal bones (femur, tibia and radius). Microsoft Excel and STATA 8 were used for the analysis.

Method: R=Reticule, L=LPC

Time Per Scan (mins): R[10], L[7]

Sample Ratios (spine): spine: height R[1:3.3], L[1:3.1]; spine: femur R[1:1.22], L[1:1.21]; spine: tibia R[1:1.38], L[1:1.42]; spine: radius R[1:2.06], L[1:2.05]

Mean CV and range for all measures (%):

Inter-operator error (1999 & 2003) R[2.19 (0.71-4.42)], L[1.77 (0.47-2.76)]

Intra-operator error (One week) R[2.89 (0.83-6.08)], L[1.65 (0.43-2.66)]

The sample ratios confirm that both methods measure the same length. A variance ratio test showed LPC to have less standard deviation than reticule for measures showing inequality between methods.

In conclusion, LPC is a faster and significantly more accurate method than reticule. Methods to adapt LPC for wider scale use will be explored.

## **P28**

### **TRANSFORMING GROWTH FACTOR-BETA-INDUCED CHANGES IN GENE EXPRESSION IN MURINE BONE MARROW MACROPHAGE PRECURSORS**

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Osteoclasts are multi-nucleated cells that resorb bone. They are derived from cells of haemopoietic origin and differentiate into mature osteoclasts in the presence of M-CSF and RANKL. Transforming growth factor-beta (TGF-beta) is an essential co-factor for this process; however the mechanism by which it primes precursors for osteoclast formation is not completely understood.

A close relationship between osteoclasts and the immune system is emerging. This concept is underlined by transgenic studies showing that several immune factors affect skeletal phenotype and bone metabolism. Genome-wide microarray technology has become an increasingly common tool for investigators looking at differential gene expression in response to a particular treatment. Therefore, murine M-CSF-dependent monocyte/ macrophage precursors were cultured on plastic in the presence of TGF-beta for 5 days. Using the Affymetrix mouse genome 430 2.0 array, we aimed to identify immune-based molecules affected by TGF-beta, with a potential role in osteoclast biology.

TGF-beta enhanced gene expression of two Immunoglobulin (Ig) superfamily receptors, F11 (JAM-1, JAM-A, PAM-1) and poliovirus receptor-related-3 (PRR3) compared to the M-CSF control, by 9.4 fold (n=2) and 10.5 fold (n=4), respectively. TGF-beta also induced gene expression of the haemopoietic cell antigen, sialophorin (CD43, leukosialin), by 3.2 times that of the control (n=2).

We have identified several immune molecules whose mRNA expression is enhanced by TGF-beta in murine myeloid precursors after 5 days. F11 and PRR3 are both members of the Ig receptor superfamily and assist cell adhesion in other cell types. Interestingly, F11 receptor activation increases intracellular free calcium in epithelial cells. Engagement of sialophorin in human T-lymphocytes induces calcium mobilisation by tyrosine phosphorylation of phospholipase C-

gamma2, thereby promoting nuclear localisation of NFAT transcription factors, in addition to enhancing its' DNA binding activity. Recently it has been demonstrated in osteoclasts that calcium fluxes are important to activate the critical transcription factor NFATc1. Therefore, these effects of F11 and sialophorin in other cell types make them interesting candidates for further analysis in osteoclasts. Further characterisation of their roles in osteoclasts may provide further insight into the augmentative role of TGF-beta in osteoclast formation.

### **P29**

#### **SILICON DISTRIBUTION IN BONE**

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Previous studies have shown that silicon is required for normal bone formation. In addition there is evidence that many silicon containing bioactive materials show osteogenic activity resulting from the release of silicon from the material. We have shown that supplementation of osteoblastic cultures with silicon and calcium can mimic the effect of bioactive materials like pseudowollastonite. The aim of the current study was to examine the distribution of silicon in bone tissue. Previous studies have reported precise localisation of silicon to regions of osteoid matrix about to undergo mineralisation. Methods: Rat tibia were embedded in methacrylate resin and cut longitudinally using a diamond band-saw. Specimens were polished using diamond suspension and polishing cloths to a finish of 1 micron. Using Scanning Electron Microscopy (SEM), back-scattered images of the bone tissue were collected and elemental line scans were performed with EDAX.

Results: Analysis on 3 month old rat tibia has shown consistent results irrespective of the region of tissue examined. In all cases there were low, but significant levels of silicon detected above background in all mineralised bone tissue. Levels appear similar between regions of cortical and trabecula bone. No significant increase of silicon concentration was seen in regions undergoing mineralisation. Controls using pure metal standards and silicon substituted apatites suggest that the silicon detected is not an artefact. Levels of silicon appear low but in the region of 0.2% w/v as determined by reference to silicon substituted standards.

Conclusions: Previous work has demonstrated that addition of silicon to differentiating bone cells results in accelerated nodule formation. The current studies confirm the presence of silicon in bone at much higher levels than the surrounding connective tissue. However, the pattern of localisation is radically different from that previously reported with silicon being uniformly found in mineralised tissue. The presence of silicon in normal bone tissue suggests that the effects of adding silicon to cultured cells may be relevant to normal bone differentiation.

### **P30**

#### **ABERDEEN VERTEBRAL MORPHOMETRY SYSTEM TO DETECT PREVALENT VERTEBRAL FRACTURES IN THE OPUS STUDY.**

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### **P31**

#### **DIFFERENTIAL EFFECT OF PPAR AGONISTS ON HUMAN RECOMBINANT RANKL GENERATED OSTEOCLAST CULTURE IN VITRO**

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The nuclear receptor and transcription factor, peroxisome proliferator-activated receptor (PPAR), regulates the activity of transcription factors in the adipogenic differentiation and inflammatory response pathways. Three isoforms of PPAR have been identified: PPAR alpha, beta and gamma, each with specific functions. We investigated the expression of PPAR receptors and the effects of PPAR specific agonists, on osteoclast formation and osteoclast function. Human peripheral blood mononuclear cells (PBMCs) were stimulated with human recombinant RANKL and M-CSF to generate osteoclasts. RNA was extracted at days 0, 7, 14 and 21 and RT-PCR for all three PPAR receptor isoforms demonstrated their expression throughout the culture period. In parallel cultures, PPAR agonists (1nM to 10uM) were added from the beginning of the culture till day 14, when mature osteoclasts were formed and the number of osteoclasts assessed by counting TRAP positive cells with 3 or more nuclei. PBMCs were also grown on dentine wafers without the addition of any compounds until day 14. Once mature osteoclasts were formed, the PPAR agonists (1nM to 1uM) were added for 7 days and the extent of resorption was measured. Activation of all PPAR isoforms with specific agonists (ciglitzone, L165041 and GW9578) resulted in significant dose dependent inhibition of osteoclastogenesis ( $p < 0.05$ ). Dose dependent inhibition of osteoclast resorption was observed with, ciglitzone, a PPAR-gamma specific agonist ( $p < 0.05$ ), whereas L165041, a PPAR-beta specific agonist, resulted in significant dose dependent stimulation of osteoclast resorption ( $p < 0.05$ ). GW9578, a PPAR-alpha specific agonist, suppressed osteoclast resorption when 0.1uM was added to the culture but higher and lower concentrations of GW9578 were not as effective. These data establish a link between PPARs and osteoclastogenesis in PBMC cultures containing RANKL and M-CSF, and support a role for differential PPAR signaling pathways in modulation of osteoclast formation and function.

### P32

#### DIFFERENCES IN THE POTENTIAL BINDING OF PHOSPHATE CONTAINING COMPOUNDS TO BONE MINERAL ARE REVEALED BY THE NOVEL USE OF HYDROXYAPATITE COLUMN CHROMATOGRAPHY

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Bisphosphonates (BPs) are the most widely used class of antiresorptive agents for the treatment of metabolic bone diseases, and are known to bind strongly to bone mineral. There is a growing appreciation that small but potentially important differences exist among the BPs, not only in their relative potencies but also in their duration of action. Our recent studies suggest that unexpected differences in mineral binding affinities contribute both to potency and to reversibility of action. In order to study the differences in mineral binding that may relate more directly to the retention and diffusion of BPs, and other non-BP phosphate containing compounds through bone, we have developed a novel method based on FPLC, using hydroxyapatite columns (3 x 25 mm) to which phosphate containing compounds can adsorb and be eluted by using increasing phosphate buffer gradients (1-1000mM) at pH 6.8. The individual compounds emerge as discrete peaks detectable by UV absorbance and/or mass spectrometry. Under the conditions used the retention times (mins; mean +/- SEM) for the BPs were 22.0 +/- 0.1 for zoledronate, compared with 16.16 +/- 1.17 for risedronate, and 7.33 +/- 0.08 for NE10790, a risedronate analogue in which one of the phosphate groups is replaced by a carboxyl group. These elution patterns were consistent and the elution times statistically different ( $p < 0.05$ ). Some biological phosphates eg ADP and ATP bind less strongly while others, including AMP, phospho-tyrosine and p nitrophenyl phosphate were not significantly retained on the hydroxyapatite columns.

These results confirm that differences in hydroxyapatite binding affinities are an important feature of BPs, and suggest that the side chains may contribute to mineral binding affinities in addition to the P-C-P backbone. These differences may help to explain the variations in retention and persistence of effects of BPs observed in animal studies as well as in clinical studies.

### P33

#### OSTEOBLAST DIFFERENTIATION IN COCULTURE: DIRECT CONTACT WITH ENDOTHELIAL CELLS INCREASES ALKALINE PHOSPHATASE ACTIVITY BUT ABROGATES VEGF RESPONSIVENESS

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Vascular endothelial growth factor (VEGF) is implicated in the coupling of osteogenesis to endothelial cell (EC) behaviour. We have previously found: i) that osteoblasts (OB) unlike EC increase their VEGF secretion in response to osteogenic prostanoids, like PGE<sub>2</sub>, ii) that EC exhibit greater intracellular signalling responses to VEGF than OB, including prostanoid production, suggesting a paracrine action of OB-derived VEGF in bone. We have hypothesised that ECs facilitate osteogenesis in response to OB-derived VEGF and that this cross talk is regulated by PGs. We examined whether coupling requires soluble mediators or direct cell contact. To distinguish between these, we used: i) a non-contact system in which human umbilical vein EC (HUVEC) and human OB (HOB) are separated by a 0.4micronM filter, or ii) a direct-contact co-culture system. Long-term co-culture in both systems promoted increased HOB alkaline phosphatase (ALP). Treatment of HUVEC, but not HOB, with VEGF165 in non-contact co-culture conditions promoted further significant increases in HOB differentiation. In contrast, exogenous VEGF165 failed to promote HOB differentiation when applied in direct contact co-culture. Thus, VEGF exerts an indirect EC-mediated effect on OB behaviour, unless direct cell contact between OB and EC is possible. To determine whether autocoids contribute to this crosstalk we used a pharmacological approach. This showed: i) that L-NIO, a nitric oxide synthase inhibitor, reduced but did not abolish HUVEC-related increases in HOB ALP, ii) that a VEGF-receptor blocker had no effect, but iii) that NS398, a selective COX-2 inhibitor,

significantly enhanced EC-induced increases in HOB ALP activity and, importantly that this enhancement was reversed by exogenous PGH<sub>2</sub> (COX-2 product). Our studies indicate that VEGF enhances EC-induced OB differentiation. A lack of direct VEGF effect on OB demonstrates the requirement for EC, suggesting that OB-derived VEGF promotes osteogenesis via EC crosstalk. Blunting of this effect by direct OB:EC contact, suggests that VEGF is used by OB to influence only remote EC. In contrast, the effects of PGs and COX-2 inhibition in co-culture, where direct contact is possible, suggest that PGs are vital negative regulators of OB: EC cross talk when these cells are in close proximity.

### **P34**

#### **HYPOXIA INHIBITS CARTILAGE FORMATION IN MICROMASS CULTURES**

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It is increasingly apparent that oxygen tension plays an important role in regulating the activities of bone cells. Previous work in our laboratory has shown that lowering pO<sub>2</sub> to 2% strongly increases osteoclastogenesis and inhibits the formation of bone by osteoblasts. Chondrocytes and osteoblasts differentiate from pluripotent mesenchymal precursors. Cartilage is an avascular tissue, and chondrocytes are thought to experience a hypoxic environment. However the effect of hypoxia on cartilage formation in vitro is unclear. We used micromass cultures of mesenchymal cells from chick limb buds to investigate the effects of pO<sub>2</sub> on cartilage production. Limb buds were isolated from embryos at Hamburger Hamilton stage 22-24, and mesenchymal cells were released from the ectoderm by treatment with 2.5% trypsin. Released cells were pelleted and resuspended in 50:50 Hams F12 / DMEM containing 10% FCS, and 2 x 10<sup>5</sup> cells in 20 microlitre droplets were placed onto 24-multiwell trays and incubated at 37 degrees C/5% CO<sub>2</sub>. After 2 h cells were overlaid with medium and then incubated at 37 degrees C for 4 days. Oxygen tension was controlled by placing culture dishes into humidified, gas tight fuse boxes which were purged with gas mixtures containing 20% - 2% O<sub>2</sub>, 5% CO<sub>2</sub>, balance N<sub>2</sub>. Cartilage nodules were stained with alcian blue and analysed using image analysis software. The formation of cartilaginous nodules, measured by quantitative image analysis of alcian blue stained cultures, was significantly inhibited below 5% O<sub>2</sub>, with a maximum 6-fold decrease in nodule area in 2% O<sub>2</sub>. Addition of 10 micromolar ATP (an angiogenic factor released by osteoblasts upon exposure to hypoxia) to micromass cultures caused a significant, 4-fold increase in cartilage nodule area, indicating that ATP was unlikely to be mediating the inhibition of cartilage formation in hypoxia. This effect of ATP contrasts with its inhibitory action in bone nodule forming osteoblast cultures. The mesenchymal tissue in limb buds is avascular, but the present results suggest that it is reasonably well oxygenated, and that hypoxia does not act to promote the initial stages of cartilage formation. However, these findings do not preclude a role for hypoxia in chondrogenesis at later stages of development.

### **P35**

#### **MAINTENANCE OF BONE MINERAL DENSITY IN MEN : POTENTIAL ROLE OF THE GONADOTROPIC HORMONES**

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Considerable attention has focused on the effects of testosterone (T) and oestradiol on the male skeleton. However the role of the gonadotropic hormones; luteinising hormone (LH) and follicle-stimulating hormone (FSH) in the maintenance of bone mass in men is unclear but gonadotropin receptors are present in bone. This study aimed to investigate the relationship between bone mineral density (BMD) and gonadotrophin levels and whether LH/FSH/T levels were associated with polymorphic variants in the KAL-1 gene which encodes a protein implicated in the development of gonadotropin-releasing hormone neurones. We studied 155 men aged (mean [SD]) 57.6 [13.7] years, 63 with osteoporosis and 92 controls. Areal BMD (ABMD) and volumetric BMD (VBMD) were assessed at the femoral neck and lumbar spine and ABMD at the total hip. Gonadotrophins were assayed by standard immunological techniques. The following KAL-1 polymorphisms were genotyped: codon 611(exon 12) 'T' to 'C' and rs1918152 (promoter region) 'A' to 'G' changes. The groups were similar with regard to age. However controls had a higher BMI than the osteoporosis group ( 26.8 [4.2] vs. 24.0 [4.3], p <0.0002). Population backward multiple linear regression analyses showed significant positive associations between LH and femoral neck (ABMD, p=0.002; VBMD, p=0.008) and total hip (ABMD, p=0.003) BMD following correction for all variables including BMI, age, dairy intake, and exercise. In contrast, negative associations were seen between BMD and FSH at the femoral neck (ABMD, p=0.006; VBMD, p=0.001), total hip (ABMD, p=0.0006) and lumbar spine (VBMD, p=0.003; ABMD, p=0.03). There was no relation between BMD and total testosterone in any region. Increasing age was associated with FSH (p<0.0001) and LH levels (P<0.0001). A positive association was found between fracture and FSH (p=0.03) after correction for other risk factors. No correlation was found between gonadotrophin levels and KAL-1 polymorphisms. Testosterone levels were negatively associated with BMI (p=0.02) and positively with exercise (p=0.03) and the KAL-1 gene promoter polymorphism (p=0.01). This study suggests that LH and FSH may be implicated in the pathogenesis of osteoporosis in men.

### **P36**

#### **SEEING BONES AT THE NANOSCALE**



#### - AN INSIGHT TO BONE NANO-MECHANICS -

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For decades, the understanding of the mechanical properties of bones and tendons has been the source of major research interests. As a new era of instrumentation became available, the scale of the studies engendered by this interest became smaller and smaller. Thus, until recently, observing and measuring the physical properties of bones and mineralised tissues at a sub-micron scale was still a difficult task. This limitation, in terms of instrumentation, prevented the study of the most elementary constitutive protein, the collagen molecule.

We have used state-of-the-art 'atomic force microscopy' to image a variety of collagenous structures and perform mechanical measurements on hetero-trimeric type I tropo-collagen molecules. High-resolution imaging demonstrated a mean ( $\pm$  Std. Dev.) contour length of (287 $\pm$ 35) nm and height of (0.21 $\pm$ 0.03) nm. Sub-molecular features, namely the coil-pitch of the molecule, were also observed, appearing as a repeat pattern along the length of the molecule, with a repeat length of  $\sim$ 8 nm that is comparable to the theoretical value.

Single molecule force spectroscopy on collagen molecules allowed us to establish the stretching pattern of a single molecule, where both the mechanical response of the molecule and pull-off peak are convoluted in a single feature. By interpreting the mechanical response obtained, using a worm-like chain model of entropic elasticity, we extracted the value of the effective contour length of the molecule at (202 $\pm$ 5) nm. This value was smaller than that given by direct measurement, suggesting that the entire molecule was not being stretched during the force measurements; this is likely to be related to the absence of covalent binding between probe, sample and substrate in our experimental procedure. An observed irregularity in the stretching pattern (10% of events) was attributed to an interruption in the amino-acid sequence of the trimeric molecule or to molecular unwinding.

These results were aimed at understanding the non-linear region of the well-know stress-strain curve observed in the macro-mechanical testing of tendon, for example. As such they are a precursor to wider studies on alteration in the stability and mechanical properties of the collagen molecule in bone diseases.

#### P37

##### OSTEOPOROSIS FEATURES IN VITAMIN D DEFICIENCY AREA

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Background and objectives. Osteoporosis (OP) problem is especially important for areas located at high latitude leading to restricted ultraviolet light exposure (Scharia S.H., 1998). So, in West Siberia annual solar radiation mean is less, than in tropic zone in 4 times, and in 2 times than in the middle part of Russia. Besides for West Siberia territory there are typical severe climate conditions, following to low Calcium nutrition. Our investigation was aimed to determination OP features in vitamin D deficiency conditions.

Methods. We observed West Siberia inhabitants - 615 persons from 18 to 84 years old (average age 49,3 $\pm$ 12,4 years) by DEXA in ultra- (UD) and mediobasal (MD) forearm parts according to WHO criteria (1994). There were 13% men and 87% women, 55,1% of them were postmenopausal (PW) during 9,9 $\pm$ 7,52 years.

Results. Anamnestically the OP fractures frequency was 16,9%, including 10,6% in men, 20,2% in women with normal menstrual cycle (MW) and 69,2% in PW group. According to DEXA in UD bone mass loss was registered in 66,3% patients, including OP in 18,5%; in MD 59,8% and 18,0% correspondingly. In men older 50 years OP was found in 25,9%, osteopenia in 55,6%; in women older 50 years in 39,9% and 45,3% correspondingly. The most OP frequency was confirmed in PW: only 10,8% women in this age group had normal BMD; at the same time in MW group and in men these indexes were equal (32,5%). The average T-criteria mean was found -1,41 $\pm$ 1,277 SD in UD and -1,45 $\pm$ 1,257 SD in MD. Importantly, maximal average T-criteria means were found in age group of 30-39 years: -0,85 $\pm$ 1,172 SD in UD, -0,63 $\pm$ 0,626 SD in MD. T-criteria indexes in persons at the age of 18-20 years and 60-69 years were similar: -1,86 $\pm$ 0,850 SD and -2,19 $\pm$ 1,191 SD in UD ( $p > 0,05$ ); -2,35 $\pm$ 1,377 SD and -2,49 $\pm$ 1,242 SD in MD ( $p > 0,05$ ), but in 30% of young patients osteopenia had prime character.

Conclusion: The received data show high OP frequency in West Siberia, probably due to vitamin D deficiency and low Calcium supplementation, what needed to create state food fortification programme for vitamin D and calcium addition.

#### P38

##### THE ROLE OF ROS IN OSTEOCLASTS

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We have previously shown that reactive oxygen species (ROS) stimulate osteoclast formation. RANKL is able to stimulate small amounts of ROS used for signalling. The ROS induced by RL are essential to the osteoclast. By overexpressing glutathione peroxidase (GPX) and peroxiredoxin (PRX), enzymes that catalytically remove H<sub>2</sub>O<sub>2</sub>, we were able to abrogate osteoclastic differentiation. This inhibition of osteoclast formation was associated with suppression of NF $\kappa$ B activation. However, there is a body of evidence that osteoclasts make ROS when they are resorbing bone, more than is required for cell signalling. This suggests that ROS are involved not only in differentiation, but function in the mature osteoclast. The major source of ROS is the NADPH oxidase (NOX) of mononuclear phagocyte cells, from which the osteoclast is derived. NADPH oxidase is a multicomponent system that catalyses the formation of superoxide ions. We therefore went on to identify the NOX present in osteoclasts, and found that the catalytic subunit of the respiratory burst oxidase (gp91phox or NOX2) is substantially downregulated by RL. Further, the homologue NOX1, implicated in vascular cell signalling was also very low. We were unable to detect other known homologues, NOX3, 4 and 5 or DOUX 1 and 2. We also assessed the levels

of antioxidant defences. We found that resorbing osteoclasts expressed antioxidant defences at high levels. Compared to macrophages, osteoclasts expressed high levels of peroxidases, together with thiols, ROS scavengers such as GSH and proteins such as metallothionein. The high levels of defences against ROS are consistent with the ability of osteoclasts to make large amounts of ROS, beyond those needed for signalling. However, none of the known NOXs are expressed at high enough levels. This suggests that osteoclasts generate ROS through an as-yet unidentified member of the NOX family.

### **P39**

#### **BONE EXTRACTS CAN STIMULATE THE SECRETION OF OSTEOPROTEGERIN IN THE OSTEOSARCOMA CELL LINES MG-63 AND SAOS-2**

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Cells of the osteoblast lineage express the membrane-bound protein RANKL, along with its secreted decoy receptor osteoprotegerin (OPG). OPG is able to bind to RANKL and prevent it from binding to its receptor RANK on the osteoclast surface. RANK/RANKL binding is necessary for osteoclastogenesis and levels of bone resorption are regulated via relative levels of RANK/RANK-L/OPG expression/secretion. Osteoclast-mediated bone resorption has the potential to release matrix and growth factors from the bone matrix. Physiologically, the release of these factors may influence the activity of cells in the vicinity of bone resorption. Indeed, demineralised bone matrix has previously been shown to stimulate stromal and osteoblast cell proliferation and maturation. However, whether bone matrix-derived factors regulate osteoclast formation and activity is unknown. In this study, we have examined the effects of bone extracts on OPG secretion by osteoblast lineage cells.

Cortical bone powder was prepared from femurs of 6 subjects, the bone powder was extracted with 0.5M EDTA, and desalted using a Centricon Plus 20 centrifugal filter. Confluent MG63 and SAOS-2 cells were treated with 1-2 micrograms of extract protein for up to 3 days, and OPG secretion was determined by ELISA. Cell number was assessed using the MTS assay.

In both cell lines examined the bone extract stimulated OPG production above that seen in the control cells at all time points examined. MG-63 cells showed an increase of 210% in the average OPG secretion per 1000 cells on day 1 ( $p < 0.05$ ), rising to 257% on day 3 ( $p < 0.0005$ ). Similarly treatment of the SAOS-2 cells produced an average increase of 227% on day 1 ( $p < 0.0005$ ), which again increased with time reaching 387% on day 3 ( $p < 0.0005$ ). These data demonstrate that bone-derived factors stimulate OPG secretion by cells of the osteoblast lineage, suggesting that factors released through osteoclast activity may form part of a negative feedback mechanism to prevent excessive bone loss.

### **P40**

#### **STATINS INCREASE OSTEOPROTEGERIN PRODUCTION AND APOPTOSIS IN OSTEOBLASTIC CELLS**

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Osteoblastic cells secrete a soluble glycoprotein called osteoprotegerin (OPG) which acts as a decoy receptor by binding to the receptor activator of nuclear factor kappa-B ligand (RANKL) and also for tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). Thus, OPG acts to inhibit RANKL-mediated bone resorption but may also have a role in regulating TRAIL-mediated apoptosis.

Statins are specific inhibitors of HMG coenzyme A reductase which is an enzyme acting early on in the mevalonate pathway which leads to the production of cholesterol, hence, the use of these drugs as treatment for hyperlipidemia. Statins have also been shown to stimulate bone formation in rodents, to inhibit osteoclast differentiation and to have a tumour growth inhibitory potential by causing apoptosis. Our aim was to see if the influence of statins on OPG production was related to their initiation of apoptosis. These effects might contribute to the increase in bone formation previously seen with statins.

Mevastatin and lovastatin (1 microM) cause MC3T3-E1 and SAOS-2 cells to increase OPG secretion into the culture medium over a 48 hour incubation period without significant cell death. A higher concentration (10 microM) of these statins causes a more elevated secretion of OPG and results in up to 50% cell death in the 48 hour incubation period. There appears to be a direct relationship between the elevation of OPG and the extent of cell death. A significant increase in message for OPG occurred after 24 hours with mevastatin. Mevalonate, a metabolite produced by HMG coenzyme A reductase, prevented the stimulation of OPG secretion by mevastatin in both SAOS-2 and MC3T3-E1 cells. In conclusion, statins cause an increase in OPG by an indirect mechanism which appears to be associated with an increase in apoptosis.

**P41****BONE-METABOLISM MARKERS IN MEN WITH COELIAC DISEASE OR OSTEOPOROSIS**

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The effect of Coeliac Disease (CD) on bone is controversial. We have measured bone-specific alkaline phosphatase (BALP, IDS Ltd, UK), total alkaline phosphatase (TALP) and beta CTx (Serum CrossLaps, Nordic Bioscience, Denmark) in serum, and calcium/creatinine ratios (uCa/Cr) in 24h urine collections from 45 men with CD (some of whom had osteoporosis) and 45 age-matched healthy controls (HC). CD was treated by calcium, and gluten avoidance. We also studied 38 age-matched men with untreated Osteoporosis (OP), defined by vertebral fracture.

Between-run CV for the BALP assay was 8.4% at 8.6mcg/L, 2.5% at 12mcg/L and 1.2% at 49mcg/L. Within-run CV was 5.6% at 8.4mcg/L and 2.2% at 33mcg/L. Second samples, taken from 13 HC after 1 year, gave a pooled within-subject CV of 10.1%. For CTx, within-run CV was 9% for the normal range, which is at the very low end of the assay range, and between-run CV was 15%. Pooled within-subject CV was 26%. CTx data were available for only 20 of the age-matched HC.

Median BALP was 11.8mcg/L (5.0-33.5) in CD, 8.2mcg/L (5.0-14.0) in HC and 9.5mcg/L (5.7-27.6) in OP. (CD versus HC,  $P < 0.0001$ ; CD versus OP,  $P = 0.004$ ; OP versus HC,  $P = 0.05$  (Wilcoxon)). TALP was 80IU/L (40-153) in CD, 57 (35-91) in HC and 67 (43-274) in OP. (CD versus HC,  $P = 0.0002$ ; CD versus OP,  $P = 0.34$ ; OP versus HC,  $P = 0.01$ ). Median CTx was 0.25mcg/L (0.05-1.41) for CD, 0.20 (0.10-1.28) for HC and 0.32 (0.06-3.90) for OP (OP versus HC,  $P = 0.033$ ; OP versus CD,  $P = 0.08$ ).

BALP correlated closely with TALP in CD ( $r^2 = 0.80$ ) but much less in HC ( $r^2 = 0.24$ ) (Pearson). The correlation in OP was even weaker than in HC ( $r^2 = 0.11$ ). CTx correlated rather weakly with BALP only in CD ( $r^2 = 0.17$ ), and slightly more with PTH ( $r^2 = 0.25$ ).

Median uCa/Cr was 0.17 (0.006-0.589) in CD, 0.24 (0.029-0.653) in HC and 0.27 (0.11-0.83) in OP (CD versus OP,  $P = 0.05$ ).

Neither BALP nor TALP fell during treatment of CD.

BALP is raised in CD more than TALP or CTx, and, unlike either, is higher in this condition than in our sample of untreated osteoporosis patients. Its unresponsiveness to treatment should be investigated.

**P42****EXPRESSION OF TYPE VI COLLAGEN INVERSELY CORRELATES WITH THE MATURITY OF BONE MATRIX**

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We raised a monoclonal antibody against the alpha 3 chain of type VI collagen and used it to investigate the expression of collagen VI in bone and bone cell lines.

Initially we undertook an immunological survey of various sections of human bone at different stages of maturity. The immunohistochemistry observed in this study demonstrates for the first time, that expression of this antigen inversely correlates with the development and maturation of the bone, since antigen expression was detected in immature bone, but was absent from mature bone matrix. The data presented in this study shows collagen VI expression in osteoblasts but not bone lining cells, traces around the pericellular area of osteocytes, abundant expression throughout immature but not mature osteoid and its prevalence in the marrow stroma of both immature and mature bone. Antibody access to collagen VI appears not to be masked by the mineral component of bone, as immunoreactivity was associated with immature osteoid but was absent from more mature unmineralised osteoid. In support of this, masking of collagen VI in the mature bone was ruled out, as the pattern of staining remained the same even after decalcification did not result in antigen retrieval.

We also investigated the expression of type VI collagen in three different human osteosarcoma cell lines, MG-63, TE85 and SaOS-2. These cell lines represent osteoblast cells at different stages of maturation. The degree of differentiation of human cells of the osteoblast lineage is associated with progressive changes in the expression of collagen VI, with the least well differentiated being positive and the most well differentiated being negative for collagen VI expression. This inverse association between the expression of collagen VI and TNAP is very interesting as it mirrors the association of STRO-1 and TNAP expression in osteoprogenitor cells.

The overall results are consistent with type VI collagen having a role in stabilising the extracellular matrix during development by providing the initial scaffolding, with an array of cell and matrix molecule binding sites, upon which mineralisation takes place but is subsequently removed as mineralisation proceeds.

**P43****REPEATABILITY OF ACTIVE SHAPE MODELLING OF THE PROXIMAL FEMUR FOR RADIOGRAPHS AND DXA SCANS**

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The shape of the femur is an important factor in fracture risk. One method for assessing this is the active shape model (ASM). An ASM of the proximal femur has been built from radiographs which, when used in combination with bone mineral density, significantly improved the separation of fracture and control subjects in comparison to either method alone. When presented with an image, the ASM automatically searches the image and locates the outline of the femur. This study tests the accuracy of this search process in radiographs and DXA scans.

A 29-point ASM was used for both radiographs and DXA images. Reference points were marked by a combination of automatic search and manual adjustment. Two error measurements were obtained, the point-to-line error, which indicates how far points lie from the outline and the point-to-point distance, indicating the total displacement between points.

A fully automatic search using the ASM produces an identical result each time it is run and so, in one sense, is perfectly repeatable. This study examined the discrepancy between the reference outlines and the results of an automatic search.

Radiographs were digitised at 348 microns/pixel and a 20-image training and test set was used for the ASM. For an automatic search, the median point-to-line error was 1.32 mm (25-75 percentiles 1.09-1.71 mm) and point-to-point error 2.17 mm (1.60-2.75 mm) (median presented as data were not normally distributed).

DXA scans from the Lunar Expert densitometer were assessed using a 360-image training set and a 22-image test set. A fully automated search had a point-to-line error of 1.73 mm (1.55-2.07 mm) and point-to-point error of 2.69 mm (2.39-3.34 mm). When manual adjustment of the scans was permitted, the point-to-line improved to 1.33 mm (1.11-1.65 mm) and point-to-point to 2.08 mm (1.75-2.63 mm). The time taken for an experienced user to assess 10 scans was recorded, including the automatic search, and any manual adjustment required. The mean time per scan was 35 seconds (stdev 16 s).

This study found that a fully automatic active shape model could successfully identify the outline of the femur in both radiographs and DXA images.

#### **P44**

##### **BIM AS A POTENTIAL TARGET IN OSTEOBLAST SURVIVAL**

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Osteoblast lineage cells undergo apoptosis following withdrawal of growth factors, detachment from matrix and treatment with bone toxic drugs, such as glucocorticoids (GC). Signalling through kinases such as ERK and PI3-kinase plays a critical role in osteoblast survival and this is compromised during anoikis (which affects signalling downstream of integrins and FAK) and following withdrawal of mitogens. GC-induced apoptosis of osteoblasts shows features similar to both anoikis and growth factor withdrawal, and can be prevented by treatment with vanadate which activates signalling driven by tyrosine phosphorylation. This survival-promoting activity can be partially blocked by U0126 and Wortmannin, indicating involvement of both ERK and PI3-K respectively.

Since apoptosis signalling is very closely regulated by kinase-mediated proteasomal degradation and control of sub-cellular compartmentalisation, we examined the regulation of the pro-apoptotic protein, Bim (Bcl-2 Interacting Mediator of Cell Death). As shown in other cell types, Bim protein levels are regulated by the 26S proteasome and by ERK and PI3-K signalling in osteoblasts. Levels of Bim were four times higher than the control after treatment (24h) with the proteasome inhibitor MG132 even in the presence of serum. The three major isoforms of Bim, BimEL, BimL and BimS, are overexpressed in response to detachment in MBA15.4 (mouse), hPOB (human) osteoblasts and primary human bone marrow stromal cells (hBMSC), peaking within 24h of detachment and remaining high for at least 72h. During this time massive osteoblast apoptosis occurred. BimEL was upregulated following 24h serum starvation in MBA15.4, concurrent with a 50% loss of viability (trypan blue exclusion) and a 15 fold increase in apoptosis (DAPI stain and TUNEL). In MBA15.4 osteoblasts 10-6M dexamethasone upregulated BimEL protein in a time-dependent manner, with levels peaking at 48h and remaining elevated at 72h. This cell line undergoes GC-induced apoptosis, induced three-fold after 72h treatment with 10-6M Dex and rising to eight-fold after six days (TUNEL). Basal levels of Bim protein are hardly detectable in MBA15.4 and MG63 cell lines and in hBMSC. Establishment of its physiological roles and how damage signals relay through Bim will facilitate manipulation of pro-survival signalling in vulnerable skeletal cells in the clinical setting.

#### **P45**

##### **ENVIRONMENTAL AND GENETIC EFFECTS ON OSTEOCLAST RECRUITMENT IN OSTEOPOROTIC AVIAN BONE**

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Modern hybrids of domestic laying hen produce 1 egg/day for 50 weeks. For this entire laying period normal bone turnover ceases; only medullary bone (MB) is formed, a loosely woven bone type of limited structural value. MB is resorbed overnight for eggshell formation when there is no dietary calcium intake but structural bone may also be resorbed leading to increased fracture risk. Surveys indicate around 30% of the UK flock of 90 million hens suffer at least one lifetime fracture (a severe welfare issue). Avian osteoporosis can be mitigated by allowing increased activity and by genetic selection but the underlying mechanisms are not clear.

To investigate the hypothesis that avian osteoclast numbers are affected by environment and genetics, established lines of hens selected for osteoporosis resistance or susceptibility (OP-RES and OP-SUS) were reared communally to 16 weeks then divided equally into an AVIARY or individual CAGES. Birds were culled at 25 and 56 weeks. Left tibiotarsi mid-diaphyses were processed for histomorphometry. Sections were reacted for tartrate-resistant acid phosphatase (TRAP) to measure, using ScionImage4.02 software, osteoclast numbers (N.Oc.), areas (Tot.Oc.Ar.), and counterstained with Toluidine Blue for MB areas per field. Cortical bone cross-sectional area (Ct.B.Ar.) was calculated. Ct.B.Ar. was also used to derive breaking stress (BStr) from 3-point bending tests on the right tibiotarsi.

2-way ANOVA with Fisher's PLSD post-hoc tests revealed the following effects:

OP-RES hens had greater BStr, Ct.B.Ar. and MB areas, and lower Mean N.Oc and Tot.Oc Area than OP-SUS hens at both 25 and 56 weeks of age (25wks: P<0.001 for all, except Tot.Oc.Ar., P<0.01. 56wks: B.Str,Ct.B.Ar.,MB area, P<0.001, N.Oc., Tot.Oc.Ar.,P<0.05).

AVIARY hens had greater BStr and Ct.B.Ar. than CAGE hens at both 25 and 56 weeks of age (25wks:BStr, $P<0.05$ , Ct.B.Ar., $P<0.01$ , 56wks:BStr, $P=0.01$ , Ct.B.Ar., $P<0.001$ ). Mean N.Oc and Tot.Oc.Ar. were higher in the CAGE hens than the AVIARY hens at 25 weeks (N.Oc., $P<0.001$ , Tot.Oc.Ar., $P<0.05$ ) but were not significantly different at 56 weeks. MB areas were unaffected by environment at either age. There were no interactions of line and housing. We conclude that genetic selection for skeletal traits and appropriate environment both reduce osteoclast recruitment in avian bone, improving bone strength.

#### **P46**

##### **DIFFERENTIAL SENSITIVITY OF NORMAL OSTEOBLAST-LIKE CELLS AND OSTEOSARCOMA CELLS TO TRAIL AND AN AGONIST ANTIBODY TO TRAIL RECEPTOR-2**

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TRAIL (TNF-related apoptosis-inducing ligand) is produced by activated T-cells. It induces apoptosis of many cancer cell lines in vitro and has specific anti-tumour activity in vivo. At least 50% of human cancer cell lines tested, from various tissues, are TRAIL sensitive, while most normal cells, including osteoblasts, are resistant. However many osteosarcoma cells are also resistant. TRAIL binds to four membrane-bound receptors, inducing apoptosis through the death receptors DR4 and DR5, but also binding to the decoy receptors DcR1 and DcR2. In addition osteoprotegerin (OPG), another member of the TNF receptor superfamily produced by osteoblastic cells, binds TRAIL and can act as a soluble decoy receptor. The resistance of normal cells to TRAIL, and the differential sensitivity of cell lines and tumour types, is poorly understood. This study aimed to investigate the action of TRAIL and an agonist antibody to DR5 on normal and transformed osteoblast-like cells.

Using flow cytometry, we found that the human osteosarcoma cell line, MG63, expressed both death receptors DR4 and DR5 on the cell surface, but neither of the decoy receptors, while both the normal human osteoblast-like cell line hPOB-tert, and primary normal human osteoblastic cells, expressed DR5 and the decoy receptor DcR2. Cultures of MG63s and hPOB-tert were treated with TRAIL or an agonist antibody to DR5 and cell viability and apoptosis assayed by alamar blue and nick translation, respectively. When treated with rhTRAIL at a dose of 50ng/ml, neither MG63 nor hPOB-tert cells exhibited any reduction in viability. As MG63s do not express either of the decoy receptors, this could not explain their lack of response to TRAIL, while concomitant treatment of the cultures with excess of a neutralizing antibody to OPG demonstrated that their endogenous OPG production was not exerting a protective effect. In contrast, when treated with 1microgram/ml DR5 agonist antibody, apoptosis of MG63s increased by 600% at 72 hours, while hPOB-tert cells remained unaffected in spite of their high level of DR5 expression. These findings highlight differences in the response to TRAIL receptor agonists among osteoblast-like cells which may lead to the development of novel therapeutic targets.

#### **P47**

##### **SYNERGISTIC INDUCTION OF 11 BETA-HYDROXYSTEROID DEHYDROGENASE EXPRESSION IN OSTEOBLASTIC MG-63 CELLS BY CYTOKINES AND GLUCOCORTICOIDS**

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Glucocorticoids (GCs) are important therapeutic agents for a wide-variety of inflammatory diseases but are known to have potentially detrimental side-effects such as osteoporotic bone-loss. These effects of GCs are mediated via the intracellular GC receptor (GR) and by local GC metabolism catalyzed by 11 beta-hydroxysteroid dehydrogenase (11 beta-HSD). We have postulated that autocrine activation of GCs via 11 beta-HSD1 plays a key role in counteracting the effects of inflammatory cytokines but may also exacerbate the effects of therapeutic steroids on bone-forming osteoblasts. We have examined the effects of GCs and the inflammatory cytokine interleukin-1 (IL-1) on expression of 11 beta-HSD1 in osteoblast models. Treatment of osteoblastic MG-63 cells with IL-1 (0.1-10 ng/ml) or dexamethasone (100 nM) for 48 hrs upregulated 11 beta-HSD1 (90-fold and 60-fold respectively) with little change in GR alpha or GR beta expression. However, treatment with IL-1 in combination with dexamethasone or cortisol synergistically enhanced 11 beta-HSD1 expression (1400-fold and 1380-fold respectively). This was in complete contrast to effects on other gene products such as osteoprotegerin (OPG) and COX-2 which were also induced by IL-1 but suppressed by GCs alone or when combined with IL-1. Similar synergistic effects on 11 beta-HSD1 expression were also observed with TNF alpha and enzyme activity studies indicated that combined IL-1 and dexamethasone increased the capacity for cortisol generation in MG-63 cells. Transcriptional analyses using variable lengths of a 2.5 kb 11 beta-HSD1 promoter showed regions of IL-1 induction but did not reveal sites of synergistic induction with dexamethasone. These data suggest that the synergistic effect of GCs and IL-1 on 11 beta-HSD1 are due to a novel transcriptional regulation mechanism as opposed to a non-specific anti-inflammatory response. We postulate that exposure to GCs and inflammatory cytokines substantially increases the capacity for autocrine activation of endogenous GCs in osteoblasts. Further analysis of this mechanism may provide new insights on the pathogenesis of steroid-induced osteoporosis.

#### **P48**

##### **POTENTIAL FOR HUMAN MESENCHYMAL STEM CELL AUGMENTATION OF IMPACTED BONE GRAFT AND A SYNTHETIC GRAFT SUBSTITUTE**

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Demand for bone graft is outstripping supply. This is particularly pertinent in the field of revision hip surgery where impaction bone grafting of the femur and acetabulum has impressive results in the short and medium term but often requires up to 6 donated femoral heads. Spine and selected tumour and trauma cases are also eminently suitable for this mode of bone stock replacement. This study details the histological and biochemical findings of a parallel in-vitro and in-vivo study. Bone allograft and a Beta Tricalcium Phosphate graft substitute with a quoted porosity of 60% were seeded with unselected or STRO-1+ selected human mesenchymal stem cell fractions and evaluated in an impaction bone grafting model. Both studies demonstrated cellular viability, activity and osteogenesis after 4 weeks culture in-vitro and in-vivo. The success of autologous stem cell implantation on impacted graft substitute with or without the addition of morsellised allograft may be applicable to clinical use in the future.

#### **P49**

##### **A BIPHASIC PATTERN OF OSTEOBLAST GENE EXPRESSION BY DIFFERING CONCENTRATIONS OF PTH 1-34**

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Parathyroid hormone (PTH) has complex effects on bone remodelling, and its actions are poorly understood. Low intermittent doses increase bone formation, whereas high and continuous doses of PTH cause a decrease in bone mineral density (BMD). In this study we have investigated whether the opposing responses to PTH exposure may be induced solely by varying agonist concentration.

Quiescent osteoblast-like SaOS-2 cells were induced with concentrations of PTH 1-34 ranging from 0.01-100ng/mL for 8 hours. Gene expression changes were subsequently measured by quantitative PCR using a Biorad iCycler. At low concentrations of PTH (0.01-0.1ng/mL) a pattern of anabolic gene expression was apparent: increased collagen-1 (P<0.05) and OPG expression (P<0.05), and decreased RANKL expression (P<0.05). Conversely at higher concentrations of PTH 1-34 (10-100ng/mL) the opposite changes in gene expression were noted, in agreement with previous findings, and revealing a biphasic effect on gene expression by PTH at differing concentrations. No significant change in PTH receptor expression was observed at any concentration of PTH 1-34 induction.

Examination of levels of expression of OPG and N-terminal pro-peptide of collagen-1 (P1NP) protein mirrored the mRNA findings. In addition, this pattern of gene expression was maintained throughout a 16-day culture period where cells were continuously stimulated with concentrations of PTH 1-34 ranging from 0.01-100ng/mL. Reporter cell studies revealed that the half-maximal induction of the immediate early gene c-fos occurred at doses significantly higher than those inducing increased gene expression of OPG and collagen-1, and a similar pattern of cAMP induction following PTH 1-34 induction was observed. The signalling mechanism behind this induction of anabolic gene expression therefore remains to be fully elucidated. These findings indicate that exposure of bone cells to varying concentrations of PTH may play a role in the observed opposing effects of PTH on BMD.

#### **P50**

##### **ACCELERATED BONE TURNOVER IN VITAMIN D-DEFICIENT PATIENTS**

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Vitamin D deficiency is associated with hip fractures. This is principally due to the associated complications of osteomalacia and secondary hyperparathyroidism. A serum vitamin D (25(OH)D) level greater than 20ng/ml is usually considered replete (Lips, 2001). Increased bone turnover and bone loss has been reported in Vitamin-D deficient patients. The objective of this study was to compare the biochemical bone turnover in vitamin D-deficient patients to those who were vitamin D-replete.

One hundred consecutive patients attending a Bone Protection Clinic had serum vitamin D (25(OH)D) and serum Parathyroid Hormone (PTH) measured along with the bone markers serum C-telopeptide (CTx), Osteocalcin (OC), Procollagen type amino-terminal peptide (PINP). Creatinine Clearance (CrCl) was calculated using the Cockcroft and Gault formula. A serum level of 25(OH)D< 20ng/ml was taken as Vitamin D-Deficient.

The mean age of the subjects was 70 (range; 31-91). There were 9 males and 91 females. 26% of patients were vitamin D-deficient (11.7 +/-0.9 ng/ml v 33.6 +/- 1.0 ng/ml). Significant increases were noted in the bone markers in the deficient patient group compared to the replete group. P1NP increased by 76% (84.9 +/- 12.4 ng/ml v 48.0 +/- 6.7 ng/ml, p <0.007). CTx increased by 66% (0.486 +/- 0.05 ng/ml v 0.292 +/- 0.02 ng/ml, p <0.0002). OC increased by 28% (28.7 +/- 3.3 mg/ml v 22.3 +/- 1.6 mg/ml, p <0.05). PTH was also 33% higher in the deficient group (43.1 +/- 5.7ng/ml v 32.5 +/- 1.7 ng/ml, p <0.02). The compromised renal function in the vitamin D-deplete was not severe enough to alter bone marker interpretation (41.2 +/- 2.6 ml/min v 56.9 +/-2.8 ml/min, p<0.002). Of note, PINP, which is excreted via the hepatic route, recorded the greatest difference between the two groups. Vitamin D levels below 20ng/ml are associated with significantly higher concentrations of PTH and consequently, a mean increase of approximately 50% in biochemical bone turnover activity. Therefore maintenance of 25(OH)D above 20ng/ml is essential in the prevention of accelerated bone turnover and subsequent bone loss and fractures.

#### **P51**

##### **INCREASED COMMITTED OSTEOGENIC CELLS IN THE BONE MARROW OF TARTRATE-RESISTANT ACID PHOSPHATASE KNOCKOUT MICE**

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Tartrate-resistant acid phosphatase (TRAP) is an iron containing protein expressed by osteoclasts, macrophages and dendritic cells. TRAP is secreted by osteoclasts during bone resorption and its serum activity correlates with the level of resorptive activity in certain bone diseases. Although its precise role is unknown we have shown that mice lacking TRAP have developmental deformities of the limbs and axial skeleton. Femurs from knockout mice have increased tensile strength, increased MMP2 activity and contain more total collagen cross-links. Osteoclasts from knockout animals demonstrate defective bone resorption in vitro. These mice also have defective osteoblast function leading to enhanced mineralisation contributing to the observed increased mineral density. The development of osteoblasts from undifferentiated progenitor cells occurs through a series of cellular transitional stages, which can be based on morphological, biochemical, and molecular criteria. Alkaline phosphatase (ALP) expression is an early marker of osteogenic differentiation that identifies committed bone cells in a heterogeneous cell population. The aim of this study was to investigate whether TRAP knockout mice contain more ALP positive cells and enhanced ALP activity. Bone marrow was isolated from 8 week old TRAP deficient and normal (wild-type) mice and prepared for either single-colour (FITC) flow cytometry, using a monoclonal ALP antibody, or for determining ALP activity. Flow cytometry revealed greater numbers of ALP positive cells (p<0.05) with increased fluorescence in the TRAP knockout bone marrow, compared to the wild type. TRAP knockout mice also showed an increase in ALP activity (p<0.05). We conclude from these studies that bone marrow deficient in TRAP contain greater numbers of committed osteogenic cells, based on ALP expression and activity. Preliminary data also suggests that bone marrow from TRAP knockout mice has reduced numbers of stromal progenitor cells, on the basis of STRO-1 expression. Overall, this suggests that TRAP may play a role in regulating osteogenic differentiation.

#### **P52**

##### **GENERATING OSTEOCLASTS FROM CHILDREN**

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Historically osteoclasts have been difficult to study in vitro. Using newly established techniques it is now possible to generate osteoclasts from human peripheral blood mononuclear cells (PBMCs). This methodology has been used to study osteoclast differentiation and function in adulthood, but very little data exists on samples from children. Our aim has been to establish this method using adult volunteers, and to investigate osteoclastogenesis during childhood.

PBMCs have been seeded on to glass coverslips (CS) and dentine slices (DS), and allowed to adhere for 2 hours. Non-adherent cells were removed, and adherent cells cultured with M-CSF (25ng/ml) and RANKL (30ng/ml) for 14 days (CS) and 21 days (DS) respectively. Osteoclasts were defined as multinuclear (3 or more), TRAP positive cells, and functionality demonstrated by pit formation on dentine. We have tested 9 batches of fetal calf serum (FCS), compared the use of CS versus glass chamber slides (GS), and tested a range of cell seeding densities. Results are expressed as mean (+/-SD) number of TRAP positive multinuclear osteoclasts per x20 field.

Of the 9 FCS batches evaluated, 3 resulted in significant osteoclast formation, with 60-80% resorption of DS. Comparing CS and GS, and 2 different cell seeding densities, the number of osteoclasts observed were: 10.4+/-2.24 and 5.5+/-2.85 respectively (0.75 million cells), and 10.67+/-2.96 and 4.28+/-1.64 (0.5 million cells). For cell densities (0.75, 0.5 and 0.4 million respectively) seeded on to CS in the presence of 2 different batches of FCS, results were: serum 1, 8.44+/-4.52, 13.83+/-5.03, and 0.72+/-0.75; serum 2, 10.4+/-2.24, 10.67+/-2.96, and 1.02+/-1.25. Our established method now uses CS in serum 2 (from Invitrogen) with 0.5 million PBMCs seeded on each one, enabling adequate osteoclastogenesis and dentine pit formation.

Using these conditions, and collecting 8 mls of blood, we have cultured osteoclasts from healthy children, 6 and 9 year-old girls - 0.83+/-0.24 and 8.3+/-4.55 osteoclasts respectively. However, we did not observe osteoclast formation from 2

samples of PBMCs derived from a 16 year-old with acute lymphoblastic leukaemia. We are now developing this work further to investigate age and sex specific differences of osteoclast differentiation and function during childhood.

### **P53**

#### **CYTOKINE PRODUCTION BY MONONUCLEAR CELLS IN PATIENTS UNDERGOING THERAPY FOR THYROID DISEASES**

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Thyroid disorders have a significant impact on bone loss. The mechanisms of action of thyroid hormone on bone remodelling remain poorly defined. Tri-iodothyronine (T3) may regulate osteoclastic activity through osteotropic cytokines. Mononuclear cells are implicated in the regulation of bone turnover. This study investigated bone turnover and mononuclear cell cytokine production in patients with thyroid disorders. The study population consisted of 14 patients with newly-diagnosed hypothyroidism aged mean 53.1 [SEM 5.2] years (group 1), 11 subjects with hyperthyroidism 46.5 [5.1] years (group 2) and 11 euthyroid controls. Group 1 was seen at baseline, and 6 and 12 weeks after thyroxine initiation. Group 2 were seen prior to radio-iodine therapy and after 6-8 weeks. Thyroid function tests, serum CTX, bone-specific alkaline phosphatase (BSALP) were measured in both groups and 6 weeks apart in controls. Peripheral mononuclear cells (PBMCs) were isolated and cultured for 4 days. IL-6, TNF-alpha, vascular endothelial growth factor (VEGF) were assayed in the media by ELISA. CTX increased in Group 1 (baseline: 0.2175 [0.027] ng/ml; 6 weeks: 0.3342 [0.036] p=0.01; 12 weeks: 0.3737 [0.017] p=0.02) and decreased in Group 2 (baseline CTX: 0.5902 [0.018], 6 weeks: 0.4473 [0.147] p=0.07). No significant changes in BSALP were observed. IL-6 and TNF-alpha increased significantly at 6 weeks in Group 1 (IL-6/protein ratio; base-line: 1.5 [0.3], 6 weeks: 2.9 [0.8] p=0.05, 12 weeks: 1.3 [0.2], TNF-alpha; baseline: 0.45 [0.05], 6 weeks: 1.3 [0.45] p=0.05, 12 weeks: 0.43 [0.08]). In contrast, a reduction was seen in IL-6 and TNF-alpha production in Group 2 at 6 weeks (IL-6; baseline: 2.8 [0.85], 6 weeks: 1.1 [0.21] p=0.01, TNF alpha; baseline: 5.3 [2.5], 6 weeks: 0.7 [0.25] p=0.001). VEGF increased significantly in Group 2 only (baseline: 10.0 [4], 6 weeks: 16 [3.3] p=0.02). Cytokines levels were stable in the control group. These data suggest that cytokine or growth factor synthesis by PBMCs may be implicated in bone remodelling in thyroid disorders.

### **P54**

#### **EXPRESSION OF BRUTON'S TYROSINE KINASE IN OSTEOCLASTS**

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Evidence using tyrosine kinase inhibitors, such as genistein, herbimycin A, and PP2, has shown that tyrosine kinases are crucial for osteoclastogenesis. Mice deficient in Src tyrosine kinase have severe osteopetrosis resulting from defective osteoclasts as well as enhanced osteoblast differentiation and bone formation. Inhibition of Src kinases prevents activation of a related family of non-receptor tyrosine kinases - the Tec kinases. We have previously shown that the Tec family member, Bruton's tyrosine kinase (Btk), is required for LPS-induced TNF production in monocytes and macrophages. This study aims to elucidate the role of Btk in osteoclast differentiation and activation.

Adenoviral constructs encoding wild-type Btk (AdBtk) and kinase-deficient Btk (AdBtkKDel) were used to look at the effect of Btk overexpression on osteoclast formation and activation. Adenoviruses encoding GFP alone (AdGFP) or an empty adenovirus (Ad0) were used as additional controls. Monocytes were elutriated from peripheral blood mononuclear cells and treated with 100 ng/ml M-CSF for 72 h; these precursor cells were infected with adenovirus for 2 h, washed and then incubated for either 7 (TRAP) or 21 days (dentine) in the presence of RPMI (10% FBS) and M-CSF 25ng/ml and RANKL 20ng/ml.

Osteoclasts, like monocytes and macrophages, express endogenous Btk.

To examine the effect of Btk overexpression, cells were infected with increasing doses of viral particles from 10:1 to 100:1 multiplicity of infection (moi). In response to AdBtk there was a moi specific dose response and at 100:1 there were two times more TRAP+ osteoclasts compared to AdGFP and Ad0 and AdBtkKDel. Each of the adenoviruses, with the exception of Ad0, express GFP allowing the examination of osteoclast morphology by fluorescent microscopy. Osteoclasts infected with AdBtk were larger in surface area compared to control viruses or uninfected cells however the average number of nuclei was not significantly different. As there are no dominant negative versions of this kinase, we are currently constructing short hairpin RNA adenoviruses to examine the effects of ablating endogenous Btk expression as well as the other Tec family kinases; Tec and Bmx.

These data suggest that Btk is playing a role in actin reorganisation in osteoclasts, a role previously described in mast cells, and we are currently investigating this using confocal microscopy.

### **P55**

#### **K+ CHANNELS REGULATE THE FUNCTION OF HUMAN OSTEOBLAST-LIKE CELLS**

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We have previously shown in single channel patch-clamp recordings from both human MG63 and SaOS-2 osteoblast-like cells that a number of distinct conductances exist (Wann et al. 2004a, b). In cell-attached recordings the most prevalent



channel is a maxi-K<sup>+</sup> channel. However, smaller conductance K<sup>+</sup> channels and other less selective cation channels are also present. RT-PCR using primer pairs for the maxi-K<sup>+</sup> (HSLO) channel and for TRPM4B has confirmed that these gene products are expressed in MG63 cells. The key question we are now investigating is what precisely is the role of such channels in osteoblast-like cells.

To this end we have grown both MG63 and SaOS-2 cells in the presence of the K<sup>+</sup> channel blockers, TEA (1mM) and 4-AP (100 microM) to assess cell viability and examined the mineralisation (Alizarin red S staining) of SaOS-2 cells in the presence of TEA (5 mM). We have also determined the nanoscopic structure of the hydroxyl apatite crystallites from mineralised cell cultures using small angle X ray scattering (SAXS) in the absence and presence of TEA (1 mM).

These modest concentrations of blockers produce no significant change in cell viability after 24, 48 or 72 hours, as judged by trypan blue exclusion. Lower concentrations modify proliferation rate, and reduce significantly the mineralisation of SaOS-2 cells after 96 hours.

Nanocrystalline size and shape is also dependent on K<sup>+</sup> channel function. SAXS data indicate that the crystallite thickness obtained from osteoblasts derived from bone marrow stromal cells is 2.5nm, comparable to that seen in human bone. Other preliminary results show that the blocker TEA (1 mM) changes both the crystal size to above 3nm, and the shape of crystallites became more polydisperse as these cells mineralise in culture.

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## P56

### ADENOSINE AND GLUTAMATE MAY INTERACT TO REGULATE THE ACTIVITY OF BONE CELLS

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Understanding how bone is formed is required for the development of therapies for diseases such as osteoporosis and rheumatoid arthritis. Important roles for ATP and glutamate in bone formation have been described, but there is little information on the naturally occurring metabolite of ATP, adenosine, which acts through a different class of receptors (P1). In the CNS interactions between adenosine and glutamate receptors are implicated in Long Term Potentiation (LTP), where neuronal responses are altered by previous signalling episodes. Here we show that osteoblasts and osteocytes express adenosine receptors, spontaneously produce adenosine, and that glutamate may modulate adenosine responses in bone cells.

We have used HCC1 (pre-osteoblast), MG-63 and SaOS-2 (osteoblast-like) and MLO-Y4 (osteocyte-like) cell lines. Adenosine production, as measured by reverse-phase HPLC, was 4-7 (HCC1 and MG63) and less than 0.04 (SaOS-2 and MLO-Y4) micromoles per hour per million cells. The release of adenosine by MG63 cells was stimulated, by 10 and 15 fold respectively, in the presence of 0.01 and 0.1 millimolar glutamate.

Osteoblasts express ionotropic and metabotropic glutamate receptors. RT-PCR showed that all four adenosine receptors, A1, A2a, A2b and A3 and the enzymes involved in the catabolism and metabolism of adenosine (CD73 and adenosine deaminase) are also expressed in these cell lines.

Since IL-6 is an important regulator of bone cell activity, we determined whether adenosine and glutamate influence IL-6 release by osteoblasts. The universal adenosine receptor agonist, NECA (1 micromolar), increased IL-6 secretion by 10 fold in HCC1 cells whereas 10 micromolar adenosine, CCPA (A1 agonist) and IB-MECA (A3 agonist) stimulated secretion by 2.5, 2 and 3.5 fold respectively. CGS21680 (A2a agonist) had no effect on IL-6 secretion. Interestingly, whilst glutamate (0.1-1 millimolar) failed to stimulate IL-6 release, it appeared to inhibit (up to 25%) basal and NECA stimulated IL-6 secretion in HCC1 cells.

These findings demonstrate that osteoblasts release adenosine that may activate adenosine receptors to increase release of IL-6. Furthermore, activation of glutamate receptors in osteoblasts appears to stimulate adenosine release and inhibit the adenosine-induced increase in IL-6 release. Targeting interactions between adenosine and glutamate signalling pathways may be of therapeutic importance.

## P57

### WHAT IS THE FUNCTION OF GLUTAMATE TRANSPORTER VARIANTS IN BONE CELL SIGNALLING?

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It is established that glutamate receptor activation can influence the differentiation and activity of both osteoblasts and osteoclasts in vitro. L-glutamate signalling during excitatory neurotransmission is regulated by excitatory amino acid transporters (EAATs) that remove glutamate from the synapse. Identification of the glutamate transporter, GLAST-1 (EAAT1), as a mechanically-regulated gene in osteocytes, implicated glutamate as an osteogenic signal in bone (1).

GLAST-1 is a 'high affinity' sodium-dependant glutamate transporter that acts as a transporter and a glutamate gated ion channel. We have cloned a novel variant of the GLAST gene, called GLAST-1a, in which exon 3, encoding 46 amino acids, is spliced out. Hydrophobicity analysis of the GLAST-1a amino acid sequence predicts a reverse orientation in the plasma membrane (2). The five members of this transporter family (EAATs 1-5) show extensive sequence divergence, but the conservation of their topology allows equivalent domains to be aligned. Analysis of intron/exon structure and potential splice sites reveal that all but EAAT5 retain the potential for an equivalent, in frame, splicing event. This evolutionary conservation led us to investigate GLAST-1a function in *Xenopus* oocytes and GLAST variant regulation in MLO-Y4 osteocytes.

GLAST-1a expressed in *Xenopus* oocytes mediates radiolabelled-glutamate uptake at similar rates to GLAST-1. Whole cell clamping of GLAST-1a expressing oocytes revealed glutamate concentration-dependant current changes consistent with transport and ion channel activity. Intriguingly, co-injection of GLAST-1 and GLAST-1a cRNA into the same oocyte alter responses to glutamate indicating a regulatory role for the splicing event that may explain its evolutionary conservation.

GFP-tagged GLAST-1 and 1a expression constructs and immunohistochemistry with an antibody that recognises both GLAST variants revealed that localisation of GLAST variants within MLO-Y4 cells varied in response to mechanical and

glutamate stimulation. It is unclear how these responses are mediated since osteocytes do not exhibit the expected glutamate-dependent increases in intracellular calcium associated with glutamate receptor activation. Thus GLAST-1 variants may directly mediate mechanical signalling in osteocytes by acting as ion channels or receptors. Elucidation of this signalling mechanism may allow us to mimic the osteogenic potential of mechanical loading, thus offering a novel approach to increase bone mass.

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#### **P58**

##### **THE IMPACT OF REDUCED BONE MINERAL DENSITY AND OSTEOPOROTIC FRACTURES ON QUALITY OF LIFE IN POSTMENOPAUSAL WOMEN.**

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The increasing prevalence of osteoporosis mandates that healthcare professionals take a comprehensive view of the scientific investigation, treatment, and management of the disease. Despite recent therapeutic advances, the enhancement of quality of life for patients with osteoporosis remains an elusive goal. Health-related quality of life covers physical, mental, and social well-being. Quality of life may be measured for evaluation of treatment effects or for the assessment of the burden of the disease of osteoporosis. The main complications and complaints regarding to osteoporosis are caused by fragility fractures which are associated with suffering and decrease of physical function, social function, and well-being, but the impact of reduced bone mineral density BMD is less clear.

The aim of the study was to investigate the QOL perception in 458 postmenopausal women who visited Outpatient Osteoporosis Department of IAM for an osteoporosis screening program.

All subjects underwent BMD measurements by DXA in lumbar spine. Data regarding to fractures were collected using X-rays and medical documentation provided by study participants. QOL was measured using special questionnaire, partially based on QUALEFFO, EuroQol and EQ-5D instruments.

Study participants were subdivided into three groups: subjects with osteoporosis, osteopenia, and with normal BMD values (according to WHO criteria). A significant difference was found for the domains which described physical function ( $p < 0.001$ ) and general health perception ( $p < 0.05$ ) (osteoporotic vs normal BMD). After segregation subjects according to whether or not they had osteoporotic fractures (vertebral or femoral) a significant difference was found in osteoporotic subjects for domains which described physical function ( $p < 0.001$ ), mental function ( $p < 0.05$ ) and general health perception ( $p < 0.001$ ). Multiple regression analyses in whole study population showed that, independently of age, both femoral or vertebral fractures and low spine BMD had significant influence on QOL perception.

Our study on postmenopausal women demonstrated, that independently of former fractures, QOL impairment can be caused by reduced spinal BMD.

#### **P59**

##### **THE EARLY EFFECT OF RECOMBINANT PTH ON URINARY CALCIUM IS DETERMINED PRINCIPALLY BY THE EXTENT TO WHICH BONE TURNOVER HAS INCREASED**

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Recombinant PTH has recently become available for the treatment of severe osteoporosis. Occasionally it may have the side effect of hypercalcaemia or hypercalciuria. The mechanism by which urinary calcium changes with this treatment needs further clarification. The aim of this study is to identify whether changes in bone turnover during early PTH therapy affect the urinary calcium levels.

43 patients (42 female) with severe osteoporosis, mean age 75 (range 51-90 years), had serum levels of procollagen type-1 aminoterminal peptide (P1NP), PTH, C-telo-peptide (CTx) and 24-hour urinary calcium (UCa) measurements taken before starting rPTH treatment and at follow up, mean time 4.1 months after commencing therapy. For analysis, the group was divided into patients who had an increase in UCa (Group A: n=22) and those who's UCa had decreased (Group B: n=21), at follow up. Average age was 73 years in group A and 76 years in group B. All patients took supplemental calcium and vitamin-D.

There was no significant difference between baseline levels of P1NP, PTH and CTx between the groups. Baseline UCa levels in the groups were similar, 3.02mmol/L (Standard error of the mean = +/-0.4) in group A and 4.90mmol/L (+/-0.75) in group B. At follow up, average UCa levels in group A had increased to 4.90 (+/-0.6) while in group B, they had decreased to 3.30 (+/-0.5). Following 4 months rPTH therapy there was an increase in P1NP levels in group A from 35.5 (+/-5.5) to 126.0 (+/-21.0) ng/ml and in group B from 35.8 (+/-7.3) to 79.0 (+/-11) ng/ml. This represents a 60% difference in the increase in P1NP level between the groups ( $p < 0.05$ ). PTH levels at follow up did not differ between the two groups and in both groups were lower than baseline. There was a greater increase in CTx in group A: 0.332 (+/-0.06) to 0.724 (+/-0.11) compared to group B: 0.271 (+/-0.03) to 0.555 (+/-0.10) in group B, but this was not significant. Four patients at follow up (9.3%) had developed new hypercalciuria.

The principle factor determining 4-month UCa levels was the degree to which bone turnover was increased on rPTH therapy. Hypercalciuria is relatively common in patients treated with PTH so urinary calcium levels should be closely observed during therapy.

#### **P60**

##### **THE NEED TO INCREASE AWARENESS OF OSTEOPOROSIS**

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Osteoporosis is in part a preventable disease. To be actively involved in prevention however, the public need to be aware of the disease, its risk factors and recommended risk reducing measures. Therefore how much is actually known about osteoporosis and what are the most effective methods of increasing awareness?

To assess the level of osteoporosis knowledge in patients attending for DEXA scanning and to review whether receiving an osteoporosis information leaflet or having a discussion with a doctor about the disease improved knowledge.

A questionnaire was devised to assess knowledge of osteoporosis risk factors, risk-reducing measures and signs/symptoms. Patients also stated whether patients had ever had a conversation with a doctor about osteoporosis. Questionnaires were issued in two centres; Glasgow Royal Infirmary, UK (120 patients), and Christchurch, New Zealand (56 patients). 60/120 Glasgow patients were given an osteoporosis leaflet to read prior to attending.

57/176 (32%) patients knew no risk factors, 34/176 (19%) no risk reducing measures and 69/176 (39%) no signs/symptoms of osteoporosis.

87/176 (49.4%) had a previous conversation with a doctor about osteoporosis. This group had increased osteoporosis knowledge in all areas tested when compared to the 88 who hadn't.

The 60/176 (34%) patients given an osteoporosis leaflet to read prior to attending showed no increased knowledge.

Overall, knowledge of osteoporosis was poor. It was relatively increased in all areas tested in those who had a previous discussion with a doctor about the disease. Patient information leaflets alone appear to be an ineffective source of information.

Osteoporosis is a large and expanding health concern. In order to address this issue, the public need to be made more aware of the disease allowing them to be actively involved in preventative measures from an early age.

While implementing large-scale osteoporosis education programmes would be beneficial, we are however perhaps overlooking the simple but highly effective role that individuals can play. By making health care professionals more aware of the limitations that currently exist in knowledge and therefore the need to discuss osteoporosis with their patients, a key method of increasing awareness can be provided.

## **P61**

### **CRYOPRESERVATION OF HUMAN ARTICULAR CARTILAGE**

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Autologous chondrocytes implantation (ACI) provides a method for treating disabling cartilage defects, which are normally confirmed after initial surgical arthroscopy. Cartilage is harvested at a second arthroscopy, with the isolated chondrocytes immediately expanded and then re-implanted six weeks later. If intact human cartilage can be viably cryopreserved, material from patients could be stored at initial arthroscopy until required. This would provide a more flexible ACI method, with the prospect of reducing the number of operations required. However there is no clear evidence to suggest intact human cartilage can be viably cryopreserved. Therefore the aim of this study was to investigate whether viable chondrocytes can be isolated from cryopreserved intact human articular cartilage.

Human articular cartilage samples, retrieved from patient undergoing total knee replacement, were cored as 5 mm diameter discs then minced to approximately 0.1 mm<sup>3</sup> size pieces. Chondrocytes were isolated by digesting minced cartilage tissue in 400 unit/ml collagen type II (Gibco) in DMEM at 37 degree C for 16 hours in a tissue culture incubator. Samples were freezing at the following stages; intact cartilage discs, minced cartilage and chondrocytes immediately after enzymatic isolation. The freezing process were controlled by a step cooling system at -1 degree C/minute from 4 to -30 degree C and -2 degree C/minute from -30 degree C to -80 degree C and liquid nitrogen for 24-48 hours. After completing of isolation, cell viability was examined using LIVE/DEAD fluorescent staining. Isolated chondrocytes were then cultured in 20% FCS DMEM for 2 weeks. Alamar blue assay for cell proliferation was performed at day 4, 7 and 14. The numbers of cell colonies were counted at day 7.

The results showed that the viability of isolated chondrocytes from control, cryopreserved intact AC discs, minced AC and isolated then frozen samples were 68.49%, 26.34%, 30.62% and 35.85% respectively. Alamar Blue assay and colonies counting showed that these cells formed colonies in culture and proliferated over the period in culture of two weeks.

We conclude that viable chondrocytes can be isolated from cryopreserved intact human AC. Further study is warranted before this method could be employed to patients benefit in ACI.

**P62****INDICATORS OF POOR HEALTH AND PHYSICAL FRAILTY PREDICT BONE LOSS AT THE HIP**

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We have previously observed that indicators of frailty such as low serum albumin, low serum thyroid hormone T3 and poor physical strength predicted vertebral fracture but not non-vertebral fracture.

We carried out a 10-year prospective study of a population-based group of 375 women, ages 50 to 85 years, to identify predictors of rates of bone loss. At baseline we collected fasting blood samples for biochemical measurements and obtained medical and lifestyle data by questionnaire. Incident vertebral fractures were determined by a single radiologist from spinal radiographs at 0, 2, 5, 7 and 10 years and BMD of the lumbar spine and femoral neck (FN) was measured at these times. The annual percentage rate of bone loss was calculated from a regression line. Only those subjects who attended for 3 or more visits were included (n=255). Percentage rather than absolute FN rate of change was used in the analysis, as absolute rate of change was inversely related to bone mineral content (p=0.05).

Neither height, weight nor body mass index predicted the rate of change, but older age was associated with greater annual bone loss (rho=-0.17, p=0.007). Indicators of poor health that were associated with bone loss included low serum albumin levels (rho=0.25, p<0.001) and a poor self-assessment of health on a scale of 1 to 5 (rho=-0.15, p=0.016). These predicted bone loss independently of age and of each other. Physical weakness and lack of stamina were indicated by poor grip strength, also associated with bone loss (p<0.001), and by self-assessed difficulty in standing (p=0.001) or sitting (p=0.037) for continuous periods. These were also independent of age.

By logistic regression, FN bone loss was also associated with the risk of incident vertebral fracture (p<0.001, odds ratio=2.03 for each SD of percentage rate of bone loss). The rate of change at the lumbar spine was not associated with incident fractures.

We conclude that factors relating to poor health and physical frailty predict not only vertebral fractures as previously shown, but also the rate of bone loss at the femoral neck. This rate of bone loss is also significantly associated with incident vertebral fractures.