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MEETING REPORT

Progress in RANK ligand biology: bone and beyond

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Meeting Report from the 2012 International Bone Academy, 3–4 February 2012, Brussels, Belgium

Introduction

The RANK/RANKL/OPG signaling pathway is no longer a strictly bone phenomenon—this was the message of the 2012 meeting of the International Bone Academy. Indeed, true to its title, *Progress in RANK Ligand Biology: Bone and Beyond* delved not only into the role of the pathway in the skeleton, but also into its crucial involvement in non-skeletal tissue processes. A total of 168 physicians and scientists, from 29 different countries all over the world, convened in Brussels, Belgium, for the 3–4 February meeting, which was supported by Amgen in collaboration with GlaxoSmithKline. The 2012 International Bone Academy, accredited by the European Accreditation Council for Continuing Medical Education, featured state-of-the-art lectures delivered by leaders in the bone field and related areas, as well as stimulating roundtable discussion. Talks on the functions of the RANK/RANKL/OPG pathway in immunity and infection, mammary gland development and breast cancer, and vascular calcification confirmed the importance of the pathway beyond bone. This meeting report provides summaries of the 12 talks given at the meeting. Publication of the report, including author, publication and open access fees, was supported by an educational grant from Amgen and GlaxoSmithKline. The report was then guest-edited by Henry Kronenberg.

Plenary Talk 1: Role of RANK, RANKL and OPG in Bone Disease

In the *Role of RANK, RANKL and OPG in Bone Disease*, Professor Stuart Ralston (University of Edinburgh, Scotland) gave an overview of the crucial function of the RANK/RANKL/OPG signaling pathway in osteoporosis, Paget's disease of bone and osteopetrosis, with a particular focus on the genetic defects characteristic of each of these disorders. The molecular constituents of the pathway were first identified in four seminal papers published in the late 1990s.[1–4] Receptor activator of nuclear factor-κB (RANK), a receptor that sits on the surface of osteoclast precursors, binds its ligand (RANKL) that is made by stromal cells, osteoblasts, osteocytes, hypertrophic chondrocytes and T cells. Binding of the receptor to the ligand stimulates the differentiation of the osteoclast precursors into mature osteoclasts, and activates the latter as well, leading to an increase in bone resorption. Meanwhile, osteoprotegerin (OPG), a decoy receptor for RANKL, opposes this process by preventing RANKL from binding to RANK. By operating in this manner, OPG hinders osteoclast precursor differentiation, with an ensuing inhibition of bone resorption. Thus, bone health depends upon a proper ratio between OPG and RANKL, two of the skeleton's most crucial regulatory molecules.

Loss-of-function mutations in RANK, particularly in the cysteine-rich portion of the receptor, as well as mutations in RANKL can cause osteopetrosis, a rare familial disease that is characterized by anemia, bone marrow failure, fractures, bone pain, nerve compression syndromes, osteoarthritis and osteomyelitis. Osteopetrosis is a complex disease of osteoclast differentiation and function, characterized by osteoclast-rich and -poor forms, as well as by a host of mutations in non-RANK/RANKL genes. In support of a role for RANKL mutations in osteopetrosis, Professor Ralston pointed to research showing that the addition of recombinant RANKL and macrophage colony-stimulating factor to peripheral blood monocytes, which were isolated from patients with osteoclast-poor osteopetrosis resulting from RANKL mutation, corrected the inability of these cells to differentiate into mature osteoclasts *in vitro*.[5] Meanwhile, revealing the importance of RANK mutations in osteopetrosis, when osteoclast precursors were obtained from individuals suffering from osteoclast-poor osteoporosis resulting from RANK mutation, the addition of RANKL and macrophage colony-stimulating factor to these cells failed to correct the osteoclast differentiation defect seen in this instance.[6]

Similar to osteopetrosis, Paget's disease of bone, a high bone turnover disorder affecting ~2% of the elderly population and characterized by lytic lesions that cause pain, fracture, deformity, deafness and osteoarthritis, is also caused by a multiple of genetic defects, including mutations in components of the RANK/RANKL/OPG pathway. For instance, an activating mutation in RANK causes an early onset form of Paget's disease typified by arthritis in the hands, an excess of woven bone, and other abnormalities.[7] As another example, inactivating mutations of OPG cause juvenile Paget's disease, a form of Paget's first identified in 2002.[8] Paget's disease can also result from mutations in the sequestosome 1 gene (*SQSTM1*), which encodes a scaffolding protein necessary to transduce

RANK/RANKL signaling into the cell nucleus.[9,10] Although Professor Ralston also emphasized the importance of environmental factors in the pathogenesis of Paget's disease, the genetic influence in this condition is undeniable, as the higher number of risk alleles a person possesses, the greater his or her risk of developing Paget's. Unpublished data from Professor Ralston's research also indicate that the severity of Paget's is linked to the number of risk alleles that patients carry.

Mutations in RANK, RANKL and OPG, in the form of common single-nucleotide polymorphisms, have also been flagged in genome-wide association studies of osteoporosis,[11,12] as well as in meta-analysis[13] of those studies. Such mutations, along with other mutations linked to osteoporosis, have small effect sizes and account for just a small percentage of the genetic variance in bone mineral density (BMD) observed in osteoporosis. Although this 'missing heritability' may result from the incomplete capture of other common alleles, it may also reside in copy number variants, or in rare alleles with large effect sizes that studies have been unable to identify thus far.

Interestingly, as Professor Ralston described in the last part of his presentation, an acquired deficiency of OPG, resulting from the production of neutralizing autoantibodies against it, can also have a role in osteoporosis. Indeed, a 2009 case report published in the *New England Journal of Medicine* described the presence of such antibodies in a high-turnover osteoporosis patient also suffering from celiac disease and autoimmune hypothyroidism.[14] This 40-year-old man responded remarkably well to the bisphosphonate, zoledronic acid, which prevented further fractures and increased BMD. In unpublished work, Dr Ralston has used enzyme-linked immunosorbent assay to detect anti-OPG antibodies in idiopathic osteoporosis patients; those with the antibodies exhibit lower BMD and more fractures.

Ultimately, Professor Ralston concluded, understanding the genetic basis of osteopetrosis, Paget's disease and osteoporosis, including the genetic alterations in RANK/RANKL/OPG signaling that have been identified in these disorders, has provided the bone field with a better understanding of the pathogenesis of bone disease. In the future, the hope is that genetic markers can be used to identify individuals at risk of osteoporosis and other bone diseases and to identify those most likely to respond to particular treatments. The bone field also looks forward to the identification of as-yet-undiscovered genes that may suggest new drug targets.

Plenary Talk 2: Osteocytes and Bone Remodelling

When looking at the understanding of bone remodeling from a historical perspective, it is breathtaking to witness the path the osteocyte has taken. Completely overlooked 30 years ago in favor of osteoblasts and osteoclasts, osteocytes, the most plentiful cells in bone, have emerged as the key cellular regulators of the remodeling process, to say nothing of their crucial role in processes like mineral homeostasis and matrix mineralization. In *Osteocytes and Bone Remodelling*, Professor Joanna Price (University of Bristol, UK) focused on one particular aspect of the bone remodeling process, in which osteocytes have a proven role: the adaptive response to mechanical strain, with resultant effects on osteoblasts and osteoclasts.

That osteocytes might be major players in bone remodeling is evident from the anatomy of these cells and how they are situated within bone tissue. Although embedded in bone, osteocytes can connect to the bone surface, as well as to each other, through cytoplasmic processes that go through the bone in tunnels known as canaliculi. Further suggesting a key role for osteocytes is what happens to bone when they are absent. In transgenic mice lacking 70–80% of their osteocytes, for instance, bone exhibits the intracortical porosity, microfractures, osteoblast dysfunction and trabecular bone loss characteristic of fragile, aging bone.[15]

The earliest evidence linking osteocytes to mechanotransduction comes from experiments with turkeys whose osteocytes exhibited changes in enzyme activity in response to mechanical loading of the ulna.[16] Since the publication of these results in the late 1980s, strong evidence for the role of osteocytes in mediating the effects of mechanical loading, and for the importance of the estrogen receptor alpha (ERα) in this process, has emerged. For instance, using a more refined mouse tibia/fibula loading model and gene array technology, Professor Price's group has found that compared to wild-type animals, knockout mice missing ERα exhibit a large decrease in the number of genes regulated at various time points after mechanical loading. These mice are also unable to form new bone in response to mechanical loading.[17]

Of the molecular signaling pathways mediating the osteocyte response to mechanical loading, the Wnt pathway has become one of the most important. Synthesized by osteocytes, sclerostin, an endogenous inhibitor of the Wnt signaling pathway through its binding to the LRP5/6 receptor on the surface of osteoblasts, has emerged as a particularly important component of the load-induced response. Indeed, *in vivo* in mice, mechanical loading[18] has been found to inhibit sclerostin expression in trabecular bone's secondary spongiosa, a region that exhibits bone formation in response to loading; the more bone formation there is, the greater the suppression of sclerostin.

In addition to its important function during mechanical loading, osteocytes also have a key role in bone's response to unloading, and Professor Price presented several lines of evidence to make this case. For instance, while wild-type mice lose bone with unloading, and exhibit an increase in sclerostin expression upon unloading, mice engineered to lack most of their viable osteocytes neither lose bone nor exhibit elevated sclerostin levels after unloading.[15] Furthermore, as one might expect, knockout mice missing sclerostin do not lose bone in response to unloading.[19]

Along with sclerostin, RANKL produced by osteocytes also now appears as a key factor in the bone response to unloading. Research published last year in *Nature Medicine*[20,21] demonstrated that osteocytes are an essential source of RANKL, and that mice lacking RANKL expression in their osteocytes do not lose bone upon unloading, and in fact exhibit osteopetrosis. This paradigm-shifting work demonstrates that the key source of RANKL during bone remodeling is not the osteoblast or its progenitor cells, which has been the prevailing view, but rather the osteocyte. Furthermore, Professor Price described *in vitro* work showing that sclerostin upregulates osteocytic expression of RANKL.[22] With these new findings come new questions; which population of osteocytes produces RANKL, and whether sclerostin has autocrine effects, are two key ones.

The final question to which Professor Price turned was whether osteocytic stimulation of bone resorption is necessary to mediate the bone formation that occurs in response to loading.

Recent studies using a mouse axial tibia loading model have shown that the bisphosphonate risedronate did not have any meaningful impact upon the loading-induced bone formation response, which suggests that resorption is not necessary for mechanical loading to induce an anabolic response in bone, at least in this experimental setting.[23] In addition, consistent with clinical evidence that the anabolic effect of parathyroid hormone (PTH) is diminished in patients who have received bisphosphonates, data from a mouse ulnar loading model that is characterized by an absence of resorption indicates that PTH augments mechanical loading's bone-building effect.[24] This demonstrates once again that the mechanical loading response does not depend upon bone resorption.

In light of recent research revelations, Professor Price finished with an analogy to illustrate the osteocyte's role in bone remodeling. Consider osteoclasts as rank-and-file workers tasked with the job of resorbing bone. Osteoblasts too serve as rank-and-file laborers that do the job osteoclasts have created for them—forming new bone. Osteocytes, however, have a higher calling: they oversee their osteoblast and osteoclast employees. Thus, thought to be unimportant 30 years ago yet now, in this new light, viewed as the master regulator of bone remodeling, the osteocyte is neglected no more.

Plenary Talk 3: Biology of Fracture Healing

Fracture healing is a complicated, multi-stage process that depends upon a well-orchestrated, ordered sequence of events in which osteoblasts and osteoclasts have a critical role, just as they do in normal bone remodeling. In the *Biology of Fracture Healing*, Professor Larry Suva (University of Arkansas for Medical Sciences, Little Rock, USA) focused on the events underlying indirect (secondary) fracture healing. In contrast to direct (primary) fracture healing, indirect fracture healing, the most common type of such healing, is characterized by the formation of a fracture callus that stabilizes a healing fracture and that is ultimately remodeled by osteoclasts and osteoblasts into healthy new bone.

Professor Suva began his talk by describing three main phases of the indirect fracture healing process. During the first, an acute inflammatory phase, a hematoma that forms at the fracture site degenerates, ultimately to be replaced by well-vascularized fibrous tissue by 3–5 days post-fracture. During the second, a reparative phase, periosteal cells that have transformed into chondroblasts make cartilage, osteoblasts start to lay down woven bone, and by ~3 weeks post-fracture, a fracture callus of bone and cartilage that bridges the fracture gap has formed. Finally, in the third phase, the fracture is remodeled by osteoclasts—a step that depends upon RANKL produced by osteocytes—that resorb old bone, and by osteoblasts that form new bone.

A number of factors, such as anti-resorptive agents, affect fracture healing. In the case of alendronate and denosumab, the influence of these two agents is to delay cartilage removal and callus remodeling, and to increase bone strength and stiffness in healing fractures.[25] Smoking and nicotine also affect fracture healing. In this regard, Professor Suva described some of his own studies on rats exposed to tobacco smoke; these animals exhibit delays in callus formation. Similarly, rats exposed to nicotine display delays in bone formation. In each case, the delay occurs during the early stages of fracture healing, at 7 days post-fracture, but over time fracture healing indicators begin to resemble those seen in control animals. Nonetheless, an examination of the cells from animals exposed to smoke reveals increases in osteoblast recruitment and mineralization *ex vivo* in culture, which highlights the reality that findings from *in vivo* experiments may not mesh with those coming from studies of cells in culture.

Professor Suva also focused on the importance of vascularization during fracture healing. Experiments in adult mice have revealed that non-stabilized fractures exhibit increased vascularity, as seen in an increase in vessel length and surface density, compared with un-fractured limbs and stabilized fractures.[26] Meanwhile, studies using a mouse model of type 1 diabetes have shown that diabetic animals display decreased fracture healing compared with non-diabetic animals, a defect that can be rescued by insulin administration.[27] These studies also reveal that the insulin receptor is expressed only in regions of very active bone formation. Furthermore, in a rat model of type 2 diabetes, negative effects on fracture healing are also observed.[28]

Other factors with adverse consequences for proper fracture healing include alcohol, as well as cisplatin chemotherapy.[29] However, the news is not all bad: certain interventions can in fact improve fracture healing, including several pharmacological approaches. For instance, inhibin A, a member of the transforming growth factor-β superfamily of signaling molecules and an antagonist of the activin IIA receptor, enhances fracture healing by stimulating osteoblast proliferation, in a distraction osteogenesis fracture healing model in mice.[30,31] Similar to inhibin A, PTH(1-34) also enhances fracture healing.[32] Still, despite the knowledge generated from animal studies of fracture healing, the clinical utility of agents such as PTH(1-34) remains uncertain.

Plenary Talk 4: The Effects of Osteoporosis Therapy on Fracture Healing

Fractures that do not heal properly represent a significant problem for the orthopedics community, with approximately 5–10% of all fractures exhibiting delayed or non-union. The agents that clinicians use to treat osteoporosis may also have a role to play in these cases, as Professor Suva described on behalf of Per Aspenberg (Linköping University, Sweden) in *The Effects of Osteoporosis Therapy on Fracture Healing*.

One treatment option for fractures showing delayed or non-union is recombinant teriparatide. A study published in 2010 by Professor Aspenberg and colleagues examined the utility of this agent in a prospective, randomized, double-blind trial of approximately 100 postmenopausal women with fractures of the distal radius.[33] A 20 μg dose of teriparatide, the dose typically used for osteoporosis, administered daily for 8 weeks within 10 days of the fracture, decreased the median time from fracture to healing from the 9.1 weeks seen with placebo to 7.4 weeks, although no such statistically significant effects were found for a 40 μg dose. Professor Suva noted, although, that these types of fractures usually heal by 5 weeks, while in this study the effect of teriparatide versus placebo was not evident at that time. Published last year, another study of intact PTH (1-84) provided similar results in 65 elderly osteoporotic women with pelvic fractures. In this prospective, randomized controlled study, 100 μg of PTH(1-84) daily improved the time to fracture

healing from 12.6 weeks in the control group to 7.8 weeks in the treatment group, with the latter also showing improvements in pain scores.[34]

Statins are another class of drugs that investigators have examined for their potential to improve fracture healing. More than a decade ago, preclinical studies in a rodent fracture repair model found that high doses of these cholesterol-lowering agents resulted in larger calluses, although not more mature ones, compared with controls, as well as in increases in the load-to-failure.[35] Unfortunately, these results could not be replicated in humans, as a clinical study of simvastatin found no effect on fracture healing in a prospective, double-blind, randomized controlled trial in ~60 patients with fractures of the distal radius.[36]

Fracture healing experts have also been interested in anti-sclerostin antibodies, which remove sclerostin's inhibitory effect on the Wnt signaling pathway, as an approach to enhance fracture healing. Preclinical studies in cynomolgus monkeys show that administration of these agents results in larger, stronger and more mature calluses.[37] In another anti-sclerostin antibody study in rats that had screws placed in the proximal tibia, researchers documented increases in bone strength and density around the screw–bone interface. This finding, if it can be translated into the clinical realm, could offer a way to improve implant stability.[38] Meanwhile, similar results come from studies of anti-Dickkopf-1 antibodies that, similar to the anti-sclerostin antibodies, increase Wnt signaling.[39]

Finally, other agents are under consideration as potential enhancers of fracture healing, including bisphosphonates, which appear to increase the lifespan of implants. However, future studies are needed to illuminate the clinical value both of agents already in hand, as well as agents in development, in the fracture healing setting.

Plenary Talk 5: Cancer and Bone. Where Are We Today?

Oncology researchers must pay close attention to bone disease. Not only do some of the treatments used in the oncology setting induce bone loss in cancer patients, but many cancers, including breast cancer, prostate cancer and lung cancer, have a strong predilection to metastasize to bone, causing significant skeletal morbidity, including pathologic fracture, spinal cord compression and the need for radiotherapy and surgery. In *Cancer and Bone. Where Are We Today?* Robert Coleman (Weston Park Hospital, Sheffield, UK) described the utility not only of bisphosphonates in the clinic for the treatment of bone disease in the cancer setting, but also the value of denosumab, demonstrating the relevance of the RANK/RANKL/OPG pathway well beyond the osteoporosis arena.

With regard to cancer treatment-induced bone loss, Professor Coleman focused on breast cancer, where the mainstay treatments used for this disease, including chemotherapy-induced menopause, ovarian suppression, aromatase inhibitors and tamoxifen (in premenopausal women), all can cause bone loss and fractures. For instance, breast cancer patients on aromatase inhibitor therapy lose bone at a rate of approximately 2.5% per year, a number that balloons to more than 7.5% per year for women with chemotherapy-induced menopause, compared with the naturally occurring bone loss rate of 1% per year observed in postmenopausal women. Fortunately, a number of clinical trials of various bisphosphonates, particularly zoledronic acid, risedronate and ibandronate, have proven the value of these agents in increasing BMD in the cancer treatment-induced bone loss setting.[40] Likewise, in the only fracture intervention study in the oncology field, denosumab decreased the risk of new vertebral fractures at both 1-, 2- and 3-year time points, compared with placebo, in prostate cancer patients receiving androgen deprivation therapy.[41]

In addition to cancer treatment-induced bone loss, bone metastasis is an especially difficult problem in cancer because of the 'vicious cycle': tumor cells stimulate osteoblasts to express RANKL, which ultimately enhances osteoclastic bone resorption, which then releases factors from the bone matrix that further fuel tumor growth.[42] Early studies of adjuvant clodronate in breast cancer patients showed significant effects of this bisphosphonate on disease-free and overall survival, particularly in older, postmenopausal women. Nonetheless, it was the Austrian Breast and Colorectal Cancer Study Group trial-12 (ABCSG-12) investigation that finally changed the way physicians treat bone metastasis.[43] This landmark study randomized 1800 premenopausal patients with endocrine-responsive stage I/II breast cancer to receive the gonadotropin-releasing hormone agonist goserelin and the SERM tamoxifen, or goserelin and the aromatase inhibitor anastrozole, with or without zoledronic acid. Results at 84 months' follow-up show that zoledronic acid has a statistically significant advantage both in terms of disease-free survival (a 29% decrease in disease-free survival events) and in overall survival (a 39% decreased risk of dying), compared with patients not receiving this bisphosphonate. Professor Coleman highlighted that most of the benefit with regard to disease-free survival was observed outside of bone, as indicated, for example, by reductions in distant metastases and loco-regional recurrence. Interestingly, subgroup analysis shows that the disease-free survival benefit is observed primarily in patients over 40 years of age; these are individuals in whom ovarian function is completely eliminated by goserelin.

Unfortunately, unlike ABCSG-12, the much larger AZURE trial, in which stage I/II breast cancer patients were randomized to receive standard therapy with or without zoledronic acid, could find no significant differences in disease-free survival or invasive disease-free survival for the whole study population.[44] However, AZURE did indicate benefits on disease-free survival and invasive disease-free survival in a subgroup of patients who were postmenopausal for more than 5 years, compared with those who were premenopausal, perimenopausal or whose menopausal status was unknown. Furthermore, this effect based on menopausal status was not seen for first bone recurrence, but rather for first recurrence outside of bone (a 30% decrease in the >5 years postmenopausal group). However, the risk of first recurrence outside of bone was actually increased in the younger group of patients (by 32%). Professor Coleman suspects that a shift—from inhibins to activins—in the molecular drivers that control bone remodeling in the postmenopausal scenario may be the culprit behind this adverse outcome. Consistent with these findings are results from ZO-FAST, another zoledronic acid trial in postmenopausal women taking aromatase inhibitors, which also documented a disease-free survival benefit.[45]

Professor Coleman also presented results from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-34 phase 3 trial of adjuvant clodronate.[46] Here, investigators randomized more than 3000 stage I/II breast cancer patients receiving

adjuvant standard therapy to clodronate or placebo. As with the zoledronic acid trials, NSABP B-34 found beneficial effects on distant metastasis-free interval and other parameters only in a subgroup of patients older than 50 years of age. Likewise, the German Adjuvant Intergroup Node-positive Study of ibandronate documented a trend towards a benefit only in a subgroup of patients aged 60 years and older.[47] Finally, a trial of denosumab in more than 1400 castrate-resistant prostate cancer patients showed that this anti-resorptive increased the time to bone metastasis.[48]

Concerning the prevention of skeletal morbidity in the advanced cancer setting, numerous studies show that zoledronic acid reduces the risk of skeletal-related events in a variety of cancers, including breast cancer, prostate cancer, other solid tumors, lung cancer and renal cell cancer. Zoledronic acid also has a beneficial effect on pain.[49] Meanwhile, results from three large (~5500 patients in total), identical, randomized, double-blind, active-controlled trials comparing denosumab to zoledronic acid in patients with a variety of tumors[50–52] show that denosumab delays the time to first on-study skeletal-related event by about 8 months, compared with zoledronic acid, with a relative risk reduction of 17%. This benefit was observed for a variety of tumor types, as well as for a number of skeletal-related events including pathological fracture, spinal cord compression and the need for surgery or radiation to bone. While these studies found no difference in pain relief, patients receiving denosumab did experience an improvement in the time to worsening pain. These trials also found no differences in terms of progression-free and overall survival, with a similar incidence of osteonecrosis of the jaw. Denosumab-treated patients also exhibited improvements in quality of life.

Professor Coleman ended his presentation with discussion of two novel agents. Radium-223 is a radioisotope and calcium mimic that targets new bone growth in and around bone metastases. Data show that this agent improves overall survival by about 3 months, compared with placebo, in the setting of advanced prostate cancer.[53] Finally, early anecdotal data indicate that cabozantinib may also have a role to play in the cancer and bone setting, with bone scans revealing tumor response in patients receiving this inhibitor of multiple protein kinases.

Keynote Lecture with Dinner. The Future of Bone Imaging: *In Vivo* Evaluation of Bone Remodeling

During *The Future of Bone Imaging: In Vivo Evaluation of Bone Remodeling*, Professor Ralph Müller (ETH Zürich, Switzerland) discussed the impact that novel *in vivo* imaging techniques could have in the bone field. The imaging techniques available to investigators today provide micro- and ultrastructural data on bone remodeling that surpass what traditional bone densitometry can deliver. Yet, they do not illuminate bone remodeling processes *in vivo*.

Preclinical studies in mice using *in vivo* imaging to examine the response to ovariectomy indeed suggest the fruitfulness of this approach.[54] In young (15-week-old) mice, a μCT study examining the 6th caudal vertebra in ovariectomized and sham-operated mice found that postmenopausal osteoporosis could be modeled in these animals. With regard to static bone morphometric measures, the study found that bone microstructure worsened over time in ovariectomized mice, compared with the sham-operated animals. While both groups showed comparable increases in trabecular bone volume fraction during the first 2 weeks of the experiment, at 12 weeks the ovariectomized animals exhibited a statistically significant decrease in that measure. Accounting for this change, the ovariectomized group exhibited statistically significant decreases in trabecular thickness and trabecular number, as well as an increase in trabecular spacing.

One can also look at dynamic morphometric measures by analyzing serial *in vivo* μCT data. For instance, compared with sham-operated animals, ovariectomized mice exhibit increases in bone formation rates starting at 8 weeks, and an increase in bone resorption rates throughout the entire 12 weeks. Changes in other dynamic parameters, such as mineralizing surface, eroded surface, mineral apposition rate and mineral resorption rate, can also be monitored over time. Furthermore, superimposing μCT images taken before and after ovariectomy, and comparing them to superimposed images from sham animals, enables the visualization of how bone microstructure changes over time; this technique allows the investigator to recognize where bone is resorbed, where it is formed and where it remains quiescent. In fact, results using this technique show that, by 12 weeks, the ovariectomized animals no longer lose bone, but their remodeling rate is twice as high, compared with the sham-operated animals.

Meanwhile, finite element analysis of the different μCT images demonstrates that the bone loss observed in the ovariectomized animals is paralleled by a decrease in bone strength over time. This approach allows the investigator to visualize, in particular regions of the bone, including both cortical and trabecular areas, exactly where strength is diminished. Professor Müller also emphasized that the same technique of extracting dynamic bone morphometry parameters from *in vivo* μCT scans taken at various time points can be used to understand the bone formation and resorption responses to mechanical loading, the latter an intervention that has potential as an anabolic therapy.[55]

In the long run, with these types of studies, Professor Müller envisioned a future of 'physiome maps' to help overcome barriers to a better understanding of bone microstructure. In particular, one limitation the bone field now faces is the difficulty in making meaningful comparisons between the results of different studies, as study design differs from one investigation to the next, as do the techniques used to evaluate the various bone parameters and the baseline characteristics of study animals. A physiome map overcomes these problems by showing, on just a single time axis, and for any particular parameter such as bone volume/total volume, trabecular thickness, cortical area fraction or cortical thickness, how those parameters change over time, according to the treatment in individual subjects. With a physiome map approach, comparisons of the effects of anabolics like PTH, anti-resorptives like bisphosphonates and mechanical loading, for instance, can be made, with clear and quick visualization of the varying responses to each treatment.[56] Professor Müller concluded by noting that the simple addition of results from future longitudinal studies to the physiome maps is an easy way to extend them. *In vivo* techniques have great potential, yet the combination of anatomical imaging techniques such as *in vivo* μCT with molecular imaging modalities—so-called multimodality molecular imaging—may offer an even larger opportunity.[57]

Plenary Talk 6: Therapeutic Antibodies: Past, Present and Future

As a therapeutic antibody with proven benefit for a serious disease, denosumab is in good company. Indeed, clinicians now use these agents to treat a host of diseases, especially cancer and immune disorders, and of the 10 top-selling pharmaceuticals in 2010, 6 were in fact antibodies. Despite their success, both clinically and commercially, antibodies still have ample potential to become even more powerful and effective therapeutics. This was the message of Sir Gregory Winter's (Medical Research Council, and Trinity College, Cambridge, UK) lecture on *Therapeutic Antibodies: Past, Present and Future.*

To understand why antibodies have been so successful as a therapeutic strategy, Sir Gregory Winter looked to their intrinsic properties. Y-shaped, multi-pronged adaptor proteins, antibodies have variable (V) regions that bind to antigen and block its action; the binding of this region to cell surface antigen can also result in apoptosis. Meanwhile, antibodies have a long serum half-life of 14–30 days, because their large size helps them to elude filtration by the kidneys. Antibodies are also able to evade the endocytic activity that would normally lead to digestion of serum proteins. Furthermore, the Fc region of antibodies signals the immune system to mount a killing response when, for instance, it binds to receptors on neutrophils, macrophages and natural killer cells.

In comparison to small-molecule drugs, then, antibodies have many advantages. Because they have large binding sites, they exhibit high affinity and specificity for their targets, decreasing the likelihood of toxic off-target effects. Their ability to block protein–protein interactions, their long serum half-life and their use of effector mechanisms also distinguish them from small-molecule agents. However, antibodies do have some distinct disadvantages, as they do not have access to small sites, extravascular or intracellular targets, nor can they be given orally. They may also provoke immunogenic reactions.

Today's engineered therapeutic antibodies take advantage of the beneficial intrinsic properties of natural antibodies, thanks to important technological advances. In the 1970s, rodent monoclonal antibodies arrived on the scene, but they were immunogenic because of their non-human nature, and had poor effector function in humans.[58] The next stage of antibody evolution witnessed mouse-human chimeric antibodies that were less immunogenic and had better effector function in humans than the rodent versions that preceded them.[59,60] Antibody engineers then worked on developing humanized antibodies[61,62] until, finally, they were able to make completely human antibodies,[63,64] as in the case of denosumab.

Despite the therapeutic success of engineered antibodies, there is still great opportunity to improve them, particularly by building upon the inherent strengths of natural antibodies. For instance, efforts are underway to further increase target specificity by designing bi-specific antibodies capable of hitting two targets instead of one.[65,66] Antibodies can also become better killers, by increasing the binding of the Fc region to natural killer cells by altering Fc protein or sugars, for example. Drug conjugates that unite small, extremely toxic, chemical drugs to antibodies can also accomplish this feat. Investigators are also looking to build upon another strength of natural antibodies—their long half-life—by tinkering with the natural endocytic process, whereby a recycling receptor binds to an antibody within an endocytic vesicle and recycles that antibody to the cell surface, thus preventing it from degradation. Introducing mutations into the Fc portion of the antibody increases the binding of the recycling receptor to the antibody, further extending the antibody's half-life.[67]

Other avenues to engineer very long-lasting activity of antibodies include the design of slow-release formulations, and, as in the case of denosumab, the administration of very large doses, as Sir Gregory Winter pointed out. Denosumab is given only once every 6 months, but he foresees the arrival of an era in which therapeutic antibodies need be administered only once a year.

Plenary Talk 7: The RANKL Pathway in Immunity and Inflammation

In recent years, studies of the crosstalk between the skeleton and the immune system have brought the nascent field of osteoimmunology into the spotlight. In *The RANKL Pathway in Immunity and Inflammation*, Professor Georg Schett (University of Erlangen-Nuremberg, Germany) focused on rheumatoid arthritis, a chronic inflammatory autoimmune disease, and the role of RANKL in this disorder, to illustrate the important connection between bone and the immune system.

In rheumatoid arthritis, the production of autoantibodies leads to inflammation, which then leads to joint damage, but bone damage is also a key feature of the disease. Bone damage occurs very early in the course of rheumatoid arthritis, progresses rapidly and is not repaired as it would be under normal circumstances. Indeed, periarticular osteoporosis is common near sites of inflammation, and generalized osteoporosis is also observed.[68] Inflammation's detrimental effect on bone is also evident in the increase in fracture risk associated with it; the more inflammation that is present, the greater the risk of fracture. Meanwhile, decreases of 2.5 and 5% in vertebral and femoral neck BMD, respectively, are apparent just in the first year of rheumatoid arthritis, and these numbers double in the second year if disease activity remains uncontrolled.[69]

With this background, Professor Schett then turned to the mechanisms underlying the bone erosion characteristic of rheumatoid arthritis. In preclinical studies, mice engineered to lack osteoclasts do not exhibit bone erosion when rheumatoid arthritis is induced by the inflammatory cytokine tumor necrosis factor (TNF)-α.[70–72] Meanwhile, inhibiting RANKL, which blocks the formation of osteoclasts in inflamed joints, prevents the bone erosion normally observed in arthritic mice;[73,74] the administration of OPG has the same effect.[75] RANKL inhibition also prevents the BMD loss seen in arthritic rodents.[76,77] These positive effects of RANKL inhibition occur even though the inhibition does not have any impact on inflammation itself,[75,76] which suggests that interfering with the RANK/RANKL/OPG pathway may have few detrimental effects on the immune system. Professor Schett further noted that animals that overexpress OPG do not exhibit immune system defects.

Moving from animals to humans, a phase 2 study of denosumab in rheumatoid arthritis patients supports preclinical findings. Indeed, denosumab prevents bone erosion as assessed both by MRI and X-ray,[78] although it too had no effect on rheumatoid arthritis disease activity. However, this lack of a direct effect on rheumatoid arthritis itself limits denosumab's value

in this area, especially as rheumatologists can already rely on agents, such as anti-TNF-α drugs, that do both.

In the last part of his talk, Professor Schett focused on the factors that drive osteoclast formation in rheumatoid arthritis. A key 25-year-old paper from the osteoimmunology field's early days found that co-culturing activated T cells with macrophages led to the production of an osteoclast-activating factor, later identified as RANKL.[79] It is now understood that certain subsets of T cells, such as TH17 cells, stimulate osteoclastogenesis, while others, such as TH2 cells and T regulatory cells (Tregs), inhibit it. Along with RANKL, additional inflammatory cytokines such as TNF-α, interleukin (IL)-1, and IL-6 also have a key role in quickening the pace of osteoclastogenesis, which helps to explain why bone erosion occurs even in the first year of rheumatoid arthritis. This picture of osteoclast formation generated from preclinical studies is enhanced by clinical investigations[80] showing that the blockade of TNF-α with the monoclonal antibody certolizumab stops bone erosion. Similar results have been achieved by using the antibody tocilizumab, which blocks the binding of IL-6 to its receptor, and by using the IL-1 receptor antagonist anakinra. Other cytokines such as IL-33 actually inhibit osteoclast differentiation, illustrating the complexity of immune regulation of bone. All of these links between the skeleton and the immune system observed in rheumatoid arthritis highlight the relevance of the field of osteoimmunology and make it an area likely to continue receiving heightened interest in the coming years.

Plenary Talk 8: RANK Ligand in Mammary Gland Development and Breast Cancer

As the title of the 2012 International Bone Academy meeting suggests, bone does not have a monopoly on RANK/RANKL/OPG signaling. Indeed, an important role of this pathway is also observed in both healthy and cancerous mammary gland tissue, as Professor Eva González-Suárez (Bellvitge Institute for Biomedical Research, Barcelona, Spain) explained in *RANK Ligand in Mammary Gland Development and Breast Cancer*. Co-author of one of the three *Nature* papers that launched this area of research,[81–83] Professor González-Suárez focused on her results from a mouse mammary gland model of breast cancer.

During normal mammary gland development, most of which takes place after birth, this structure evolves specific properties, dictated by hormones such as estrogen, progesterone and prolactin, during the stages of prepuberty, puberty, pregnancy, lactation and involution. During pregnancy, mammary alveolar cells, which consist of secretory epithelial cells, differentiate upon parturition. A number of genetic pathways control this process, including the RANK/RANKL/OPG pathway. Indeed, both RANK and RANKL are expressed in mouse mammary epithelial cells during pregnancy, and are tightly regulated. In particular, during mid-gestation in mice, overexpression of RANK and RANKL is observed, as a consequence of the activity of progesterone, prolactin, PTH and PTHrP. The phenotype of RANK and RANKL knockout pregnant mice illustrates the importance of RANK/RANKL/OPG signaling in mammary gland development, as the mammary epithelial cells from such animals exhibit decreased proliferation and survival.[84]

Research has also demonstrated a key role for the RANK/RANKL/OPG pathway during mammary tumor formation and metastasis.[81] Evidence for this assertion comes from a RANK-overexpressing transgenic mouse model in which a synthetic progesterone derivative, which upregulates RANKL, and a carcinogen are used to induce mammary carcinogenesis. Compared to wild-type mice, transgenic animals exhibit increased susceptibility to mammary carcinogenesis in this model. Indeed, the mice develop tumors much earlier than control animals, and also exhibit an increased number of preneoplastic lesions, and an increased incidence of spontaneous and progesterone derivative/carcinogen-induced adenocarcinomas. The transgenic animals also develop spontaneous, palpable mammary gland tumors.

Such findings suggest that inhibiting the RANK/RANKL/OPG pathway may have anti-tumor effects, and indeed this was the case in the mouse experiments. In fact, pharmacologically inhibiting RANKL with a soluble receptor reduces the incidence of both palpable mammary tumors and adenocarcinomas, and decreases the number of preneoplastic lesions. Interestingly, because RANK and RANKL are also expressed in both malignant and premalignant lesions in wild-type animals, the study also examined what effect the RANKL inhibitor had in those cases. Here too, and again using a progesterone derivative and a carcinogen to induce tumors, the inhibitor reduces the incidence of palpable tumors in the wild-type mice, compared to mice receiving a placebo injection. In addition, both the RANK-overexpressing and the wild-type mice exhibit reductions in proliferation and cyclin D1 expression (the latter a measure of cell cycle progression) in mammary epithelial cells. Furthermore, compared to placebo-injected animals, wild-type mice that receive the RANKL inhibitor exhibit decreased cell proliferation and increased apoptosis in normal epithelial cells, in hyperplasias and neoplasias (*in situ*) cells, as well as in adenocarcinoma cells. Finally, when comparing the effects seen with the RANKL inhibitor to zoledronic acid, wild-type animals treated with various doses of the bisphosphonate do not exhibit any reductions in the time to palpable lesions, tumor incidence or tumor proliferation. In sum, these results argue for an important role of the RANK/RANKL/OPG pathway in tumor development.

Professor González-Suárezthen discussed whether RANKL has a role in other tumor models. Here she focused on a mouse model in which tumorigenesis is driven by overexpression of the rat homolog of human epidermal growth factor receptor 2. Expression of the latter is found in some breast cancers, and an anti-human epidermal growth factor receptor 2 antibody, trastuzumab, has been developed for the treatment of breast cancer. In this model, progesterone remains at physiological levels. Compared to normal epithelial cells, *in situ* neoplasias and adenocarcinomas exhibit decreased levels of RANKL and highly increased levels of RANK, in this human epidermal growth factor receptor 2 tumor-driven model, and research is underway to understand this finding. Meanwhile, pharmacological inhibition of RANKL decreases the incidence of spontaneous mammary gland tumors and preneoplastic lesions, as well as spontaneous lung metastases that occur in this model. Related research using mice with breast-specific knockout of RANK shows that ablation of RANK protects the mice from progesterone-mediated effects on cell proliferation,[82] which suggests that RANKL inhibition could work synergistically with chemotherapy. Professor González-Suárez also turned to recent mouse studies demonstrating that progesterone induces the expansion of adult mammary stem cells in the mammary stem cell niche during

pregnancy and the luteal cycle; these cells are thought to have a role in breast cancer.[85,86] Paracrine RANK/RANKL signaling may explain this finding.

Thus, RANK/RANKL/OPG signaling has an important role in tumor development in preclinical models. While there are few data in humans, RANKL is in fact expressed in human invasive breast cancer, both in the tumor compartment as well as in surrounding stromal cells, such as infiltrating lymphocytes and fibroblast-like cells. Expression of RANK is also observed in the tumor compartment, but the degree to which tumors express RANK remains unclear. Nonetheless, these findings, however preliminary, suggest that RANKL inhibition could potentially be a way to prevent breast cancer in the clinical setting.

Plenary Talk 9: Osteoporosis and Cardiovascular Diseases Sharing Common Mechanisms

The RANK/RANKL/OPG pathway has an important role in bone disease, inflammation and immunity, normal mammary gland development and breast cancer. Now cardiovascular disease can also be added to that list, an unsurprising fact considering the mechanistic links between cardiovascular disease, particularly arterial calcification, and osteoporosis. These links were the focus of Lorenz Hofbauer's (Technical University, Dresden, Germany) presentation, *Osteoporosis and Cardiovascular Diseases Sharing Common Mechanisms*.

Genetics is a good place to start to appreciate the links between osteoporosis and cardiovascular disease, as variations in several osteoporosis genes, including estrogen receptor-α and -β, OPG, TNF-α and matrix gla protein (MGP), are also associated with vascular diseases. Furthermore, several knockout mice phenotypes are characterized by a combined vascular-skeletal phenotype, including three to which Professor Hofbauer devoted most of his talk: mice missing MGP, fetuin-A or OPG.

MGP is a small matrix protein that has a high affinity for hydroxyapatite, modulates cartilage metabolism and acts as a scavenger for calcium phosphate. Knockout mice missing MGP exhibit a skeletal phenotype characterized by short stature and fractures.[87] In addition, they exhibit osteopenia, as well as defects in cartilage development including the presence of calcifications in the growth plate, a lack of the columnar organization seen in normal cartilage, as well as a dearth of hypertrophic chondrocytes. Meanwhile, these knockout animals also have an accompanying vascular phenotype, characterized by diffuse arterial calcification throughout the body. In fact, they even exhibit cartilage in the vascular wall microarchitecture.

Similar to MGP-deficient mice, those missing fetuin-A also exhibit both a bone and a vascular phenotype. Fetuin-A is found in the serum as a locally and systemically active protein that binds with high affinity to mineral. It functions to inhibit the formation/precipitation of apatite precursor mineral, binding calcium into a soluble 'calciprotein' peptide, thus acting in a manner similar to the way that high-density lipoprotein interacts with cholesterol. Fetuin-A also stimulates phagocytosis of insoluble calcium precipitates. These properties lead to a vascular phenotype *in vivo*; fetuin-A knockout mice exhibit ectopic calcification, with calcium precipitates deposited in organs such as the kidney, skin, lung and myocardium.[88] The knockouts also exhibit a skeletal phenotype typified by secondary hyperparathyroidism, osteopenia and an increased number of osteoclasts.

Similar to the MGP and fetuin-A knockouts, animals lacking OPG also exhibit vascular and bone phenotypes. Most organs produce OPG, including cells of the vascular system such as endothelial and vascular smooth muscle cells. That OPG might have an important role in the vasculature comes from an examination of OPG knockout mice.[89,90] One very striking finding is that death of these animals results more often from hemorrhage than from osteoporosis. Bleeding is caused by aortic aneurysms in association with calcification in the aorta and renal arteries. Administration of OPG after birth improves this phenotype by decreasing (though not eliminating) arterial calcification, while increasing OPG levels through genetic means during gestation completely reverses the phenotype. This suggests that in addition to vascular calcification, other defects, such as impairments in vascular microarchitecture, are important. Furthermore, in rat models, vascular calcification induced by warfarin or vitamin D intoxication can be prevented by administering OPG, likely not because of a direct effect on the vasculature but through an effect on bone, so that mineral remains at normal levels rather than being deposited in the vasculature.[91] Furthermore, in experiments using a human RANKL knock-in mouse that has glucocorticoid-induced osteoporosis, denosumab not only decreases bone loss, but also decreases vascular calcification, with an inverse correlation between aortic calcium content and BMD, and a positive correlation with bone resorption, observed in these studies.[92,93] In short, RANK/RANKL/OPG signaling has an important part to play in both bone and the vascular system.

In the final part of his presentation, Professor Hofbauer turned to the question of whether vascular calcification is an active process driven by cellular differentiation events, or whether instead it is a passive, degenerative process. In favor of the former, there is evidence that vascular smooth muscle cells can trans-differentiate into osteoblast-like cells. In addition, recent work has documented the presence of circulating osteoblast-lineage cells in humans;[94] such cells could form mineralized nodules *in vitro*, and bone in an *in vivo* transplantation assay. However, he said that the data now more strongly support vascular calcification as a passive, degenerative process driven by the release, from stressed vascular smooth muscle cells, of matrix vesicles and apoptotic bodies that serve as the nidus for calcification. This process proceeds in the absence of calcification inhibitors such as fetuin-A or MGP. Clinical evidence supports this view. For instance, in children with renal insufficiency—patients who exhibit accelerated vascular calcification—OPG and fetuin-A levels increase as they get older, likely to counteract the increased vascular calcification they experience. Fetuin-A levels decrease the longer these children are on dialysis, and levels of MGP also decrease.[95] Taken together, then, passive mechanisms, rather than cell differentiation events, appear as the major players in vascular calcification.

Plenary Talk 10: Cardiovascular Diseases and Osteoporosis: Clinical Perspectives

Evidence in support of a relationship between cardiovascular disease and osteoporosis derives not only from basic science research, but also from epidemiological and clinical studies as well. In *Cardiovascular Diseases and Osteoporosis: Clinical Perspectives*, Douglas Kiel (Harvard Medical School, Boston, USA) discussed the insight gleaned from such studies, with a

particular focus on data from the Framingham Heart Study. This epidemiological study, which began more than 50 years ago, has followed more than 5000 men and women from the town of Framingham, Massachusetts. Inclusion of both the offspring, as well as the third generation, of the original Framingham cohort has added another 9000 subjects to the investigation.

Aware of the progression of abdominal aortic calcification, as determined by X-ray, as the Framingham study progressed over the years,[96] Professor Kiel and his co-investigators found associations between calcification and cardiovascular disease, with both men and women in the highest tertile of calcification exhibiting the highest incidence of coronary heart disease over a 22-year period, with similar results found with regard to congestive heart failure.[97,98] Associations between calcification and various bone parameters have also been documented in over 1300 men and women taking part in the Framingham Offspring Study. For instance, in women, though for unexplained reasons not in men, an inverse association between the degree of coronary artery and abdominal aortic calcification, as measured by CT, and volumetric BMD was found; women with the highest BMD exhibited the lowest calcification scores.[99] As another example, in a longitudinal study that followed more than 550 subjects belonging to the Framingham cohort over a 25-year period, women (although again, not men) with the most bone loss, as measured by changes in metacarpal cortical area, exhibited the highest increases in aortic calcification scores.[96] Similarly, in another cohort of more than 2000 subjects, those in the highest quartile of metacarpal cortical area had the lowest incidence of coronary heart disease, although again such statistically significant differences were not found in men.[100]

Moving from the Framingham subjects to other cohorts, Professor Kiel pointed to findings consistent with data in the Framingham cohort. For instance, a prospective, epidemiological study in Denmark that followed more than 2500 healthy postmenopausal women for 7.5 years found that those with the greatest bone loss at the hip had the highest increases in aortic calcification scores. In addition, the severity of aortic calcification predicted hip fracture, as the greater the severity of calcification, the higher the fracture risk.[101] Meanwhile, a prospective study of nearly 800 subjects from the MINOS study of male osteoporosis found that men with the most aortic calcification and the lowest BMD exhibited the highest risk of non-vertebral fracture.[102] A recent systematic review of studies that have found associations between cardiovascular disease and osteoporosis concluded that individuals with prevalent subclinical cardiovascular disease have an increased risk of bone loss and fractures, although it is uncertain whether, conversely, low BMD increases cardiovascular risk.[103]

Do the bone field's clinical trials of anti-resorptives illuminate the relationship between osteoporosis and cardiovascular disease? The HORIZON Recurrent Fracture Trial of zoledronic acid suggests that these drugs may indeed have a beneficial effect on cardiovascular disease.[104] This study documented a 28% decrease in all-cause mortality in individuals taking zoledronic acid, compared with placebo, at 36 months of follow-up. This decrease could not be explained by baseline risk factors for mortality in the study population, or by new clinical fractures that occurred during follow-up, but rather by acute events such as arrhythmias. In fact, there was a trend for zoledronic acid–treated patients to be less likely to die from arrhythmias than placebo-treated subjects who died.[105] However, the factors accounting for the mortality reduction seen in HORIZON remain unclear.

Several studies, though, have found no effect of bisphosphonate treatment on cardiovascular measures. For instance, an investigation of oral and intravenous ibandronate in 474 postmenopausal women taking part in two randomized, placebo-controlled trials testing the effects of this bisphosphonate on bone mass and fracture risk found no effect on the progression of aortic calcification over a 3-year period.[106] Meanwhile, in a substudy of the FREEDOM trial of denosumab, in more than 2000 subjects at high risk for cardiovascular events, results revealed that denosumab had no effect on cardiovascular events or on vascular calcification over 3 years.[107] Although these findings may have disappointed the bone field, the links between osteoporosis and cardiovascular disease, as indicated by epidemiological and clinical studies, make this area an intriguing one for future research.

Plenary Talk 11: Targeting the RANKL Pathway–Clinical Trial Update

In *Targeting the RANKL Pathway—Clinical Trial Update*, the final session of the 2012 International Bone Academy, Serge Ferrari (Geneva University Hospital, Switzerland) presented the latest results from clinical trials of denosumab. To provide context to understand recent data, Professor Ferrari first described results from FREEDOM, the clinical trial that established denosumab's value in treating osteoporosis. In the FREEDOM study, nearly 8000 women, aged between 60 and 90 years and who had T-scores less than -2.5, though not less than -4 at the lumbar spine, were randomized to receive denosumab or placebo.[108] For the study's primary end point of new vertebral fracture, denosumab decreased the risk of fracture by 68% compared with placebo. For the secondary end points of non-vertebral and hip fractures, denosumab reduced the risk of fracture by 20 and 40%, respectively, compared with placebo.

With this context in mind, Professor Ferrari then moved to more recent data. Recent subanalysis of patients at higher risk for new vertebral and hip fractures substantiates statistically significant reductions in both outcomes, but particularly for hip fracture.[109,110] Indeed, at 3 years, patients aged 75 years and older receiving denosumab exhibited a 62% reduction in hip fracture compared with high-risk patients who received a placebo, whereas such a statistically significant reduction could not be found in a lower-risk group. Furthermore, at 3 years, denosumab decreased the risk of wrist fracture by 40% in a high-risk group of patients with a T-score $\leqslant -2.5$.[111]

Going beyond the 3-year time frame of the FREEDOM trial, the FREEDOM extension study has now provided data on the effects of denosumab after 6 years of treatment.[112] In this open-label, active treatment study, subjects who had received denosumab in the FREEDOM trial took the drug for another 3 years, while a crossover group of FREEDOM placebo subjects received 3 years of treatment. Much of the suppression of serum CTX levels seen during the FREEDOM trial was maintained in the long-term denosumab group of the extension trial, in which levels of this marker were still reduced by about 60%. However, an increased variability of this measurement was seen, perhaps due to differences in the timing of sampling of subjects, and to the small number of subjects in the analysis. A continued

increase in BMD at both the lumbar spine and total hip was also observed in the extension trial's long-term denosumab group. Here, extension group subjects exhibited a 15% increase in lumbar spine BMD at 6 years compared with baseline, and a 7.5% increase in total hip BMD. This finding is particularly interesting as extension studies with bisphosphonates do not show this pattern. Furthermore, at 6 years, the incidence of new vertebral fractures remained stable and low. Non-vertebral fracture incidence was also low and had in fact decreased further at 6 years. Importantly, adverse events remained stable over the course of the extension study. Indeed, the incidence of osteonecrosis of the jaw was low (two cases in the 6th year of denosumab, two cases in the crossover group), and there were no cases of atypical femoral fractures; only the incidence of eczema was modestly increased. The study, however, was not powered to determine whether the incidence of these rare events was affected by therapy.

To understand the further decrease in non-vertebral fractures seen with denosumab, Professor Ferrari then explored the bone densitometric and structural factors that could explain denosumab's effect in this regard. In the case of bisphosphonates, increases in BMD correlate poorly with the decreases in fractures seen with these drugs. However, in the case of denosumab, at 36 months, the increase in hip BMD explains 87% of the reduction in non-vertebral fractures, and the decrease in vertebral fractures is also, to a large extent, explained by increases in BMD.[113] This contrast to oral bisphosphonates may result from differences in compliance with treatment, or because most denosumab-treated patients gain hip BMD over time, while a substantial number of patients taking oral bisphosphonates may actually lose BMD over time (perhaps because of compliance issues), although bisphosphonates effectively prevent hip fractures. Another potential explanation is that, in clinical trials with bisphosphonates, changes in hip BMD levels tend to plateau around 2 or 3 years at about a gain of 4%, compared with the continuous gain in BMD seen after 6 years of denosumab treatment. Ultimately, though, the clinical significance of the finding is unclear.

To ascertain whether alternative explanations might be at play, Professor Ferrari then described results from a QCT substudy of a small number of patients from the FREEDOM trial. In this study of 62 subjects, denosumab increased volumetric hip BMD as well as bone mineral content at all hip bone compartments, including at cortical and subcortical sites.[114] Effects on cortical/subcortical sites could mean that denosumab works by decreasing cortical porosity. Finite element analysis of the QCT scans further indicates that denosumab increases hip strength at both trabecular and cortical compartments, an improvement that is strongly associated with an increase in spine strength.[115]

A 2010 phase 2, double-blind, HR-pQCTpilot study that randomized nearly 250 postmenopausal patients to receive denosumab, alendronate or placebo for 12 months further supports an impact of denosumab on cortical porosity.[116] In this study, greater changes in volumetric BMD, at both the trabecular and cortical compartments of the distal radius, were observed with denosumab, compared with the alendronate and placebo groups. Other recent results have shown that, at 12 months, cortical porosity at the distal radius decreased in the denosumab group, compared with the placebo group, which exhibited increased cortical porosity.[117]

One obvious explanation for the decrease in cortical porosity witnessed with denosumab is a reduction of bone turnover that is greater with denosumab than with alendronate. A new and intriguing possibility, however, is that a transient increase in PTH production resulting from denosumab treatment could be at work. Indeed, results indicate that while cortical porosity increases with increasing levels of PTH in the alendronate group, cortical porosity decreases with increasing levels of PTH in the denosumab group. The specific hypothesis is that, as bone resorption inhibition is underway, the spike in PTH levels seen with denosumab could potentially explain the decrease in cortical porosity, leading to the gain in volumetric BMD and potentially explaining why a continuous gain in BMD as well as a reduction in fractures is seen at the hip with denosumab. To illustrate that plausible biology bolsters this speculative idea, Professor Ferrari pointed to PTH's ability to target the osteocyte and suppress the production of the osteocyte-secreted, natural bone formation inhibitor, sclerostin.[118] Thus, the 2012 International Bone Academy ended near where it began, with bone's most common cell type.

Conflict of Interest

Professor Aspenberg is a consultant for Amgen and Eli Lilly & Co., and is a stockholder for AddBIO AB. Professor Coleman has received speaker fees from Amgen, is a consultant for Amgen and Novartis and has provided expert testimony for Novartis. Professor Ferrari has received research grants from Amgen, Novartis and MSD, and is a consultant for Amgen/GSK, MSD, Eli Lilly and Novartis. Professor Hofbauer has received research grants from Amgen, Novartis and Nycomed, and is a consultant for Amgen, Merck and Novartis. Professor Kiel has received research grants from Amgen, Novartis and Merck, and is a consultant for Amgen, Merck, Novartis and Eli Lilly. Professor Ralston is a consultant for Amgen, Merck, Novartis, Pfizer and Lilly. Sir Gregory Winter is a consultant for F-Star, Covagen and Biosceptre, and is the Director of Bicycle Therapeutics. The remaining speakers declare no conflict of interest. NAA received an author fee paid from an educational grant made to IBMS by Amgen and GlaxoSmithKline.

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