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## Pharmacological manipulation of fracture healing and bone remodeling

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2010

[Link to publication](#)

*Citation for published version (APA):*

Harding, A. K. (2010). *Pharmacological manipulation of fracture healing and bone remodeling*. [Doctoral Thesis (compilation), Orthopaedics (Lund)]. Department of Orthopaedics, Lund University.

*Total number of authors:*

1

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## List of papers

- I. Åstrand J, Harding AK, Aspenberg P, Tägil M. Systemic zoledronate treatment both prevents resorption of allograft bone and increases the retention of new formed bone during revascularization and remodelling. A bone chamber study in rats. *BMC Musculoskelet Disord* 2006 Aug 4; 7: 63.
- II. Harding AK, Aspenberg P, Kataoka M, Bylski D, Tägil M. Manipulating the anabolic and catabolic response in bone graft remodeling: Synergism by a combination of local BMP-7 and a single systemic dosis of zoledronate. *J Orthop Res* 2008 Sep; 26(9): 1245–9. Nominated to Atomium Culture.
- III. Harding AK, Toksvig-Larsen S, Tägil M, W-Dahl A. A single dose of zoledronic acid enhances pin fixation in high tibial osteotomy using the hemicallotasis technique. A double-blind placebo controlled randomized study in 46 patients. *Bone* 2010 Mar; 46(3): 649-54. Epub 2009 Nov 11.
- IV. Harding AK, W-Dahl A, Geijer M, Toksvig-Larsen S, Tägil M. No decrease in time to fracture healing in high tibial osteotomies after a single bisphosphonate infusion. A randomized study in 46 patients. Submitted.

# Abbreviations and definitions

<b>ADL</b>	activities of daily living		
<b>angiopoietins</b>	growth factors that promote angiogenesis		
<b>ATP</b>	adenosine triphosphate	<b>IL-1</b>	interleukin 1, a cytokine
<b><math>\beta</math>-catenin</b>	an intracellular signal protein used by Wnt	<b>IL-6</b>	interleukin 6, a cytokine
<b>BCC</b>	bone conduction chamber	<b>KOOS</b>	Knee injury and Osteoarthritis Outcome Score, patient-relevant outcome measure
<b>BMC</b>	bone mineral content (g)	<b>M-CSF</b>	macrophage colony-stimulating factor. Essential for osteoclast differentiation
<b>BMD</b>	bone mineral density (g/cm <sup>2</sup> )	<b>MSC</b>	mesenchymal stem cell, a multipotent stem cell that can differentiate into a variety of cell types
<b>BMP</b>	bone morphogenic protein. Local-acting signaling proteins.	<b>ONJ</b>	osteonecrosis of the jaw
<b>BMPR</b>	bone morphogenic protein receptor	<b>OP-1</b>	osteogenic protein-1, recombinant BMP-7
<b>BV/TV</b>	bone volume/total volume	<b>OPG</b>	osteoprotegerin, a protein secreted by osteoblasts that act as a decoy receptor for RANKL
<b>CFU-F</b>	colony-forming unit–fibroblast	<b>PDGF</b>	platelet-derived growth factor
<b>DBM</b>	demineralized bone matrix	<b>PLA</b>	polylactic acid, a biodegradable biopolymer made from natural sugar sources
<b>DEXA</b>	Dual Energy X-ray Absorptiometry, method to measure bone mineral content and bone mineral density	<b>Prenylation</b>	lipid modification of a protein, important for protein-protein interaction and signaling
<b>Dickkopf</b>	a secreted Wnt antagonist that binds to a part of its receptor complex	<b>PPI</b>	pin performance index
<b>FPFS</b>	farnesyl pyrophosphate syntase, enzyme in the mevalonate pathway, important for bisphosphonate function	<b>PTH</b>	parathyroid hormone
<b>GDF</b>	growth differentiation factors, members of the TGF- $\alpha$ superfamily	<b>PTHr1</b>	parathyroid hormone receptor 1
<b>HA</b>	hydroxyapatite	<b>QOL</b>	quality of life
<b>HCO</b>	hemicallotasis osteotomy	<b>R1 and R2</b>	side-chains in the chemical structure of bisphosphonates
<b>Histomorphometry</b>	histological quantification of volumes, surfaces, cell numbers, and tissue dimensions involved in bone formation and resorption	<b>RANK</b>	receptor activator of nuclear factor $\kappa$ B. Expressed on the cell surface of osteoclasts and osteoclast precursors
<b>HKA angle</b>	hip-knee-ankle angle. A radiographic method for determining	<b>RANKL</b>	receptor activator of nuclear factor $\kappa$ B ligand. Expressed on

	the surface of osteoblasts and marrow stromal cells	<b>TGF-<math>\beta</math></b>	transforming growth factor $\beta$ , three known subtypes in humans
<b>ROI</b>	region of interest, the area in the DEXA image selected for measuring	<b>TNF-<math>\alpha</math></b>	tumor necrosis factor $\alpha$
<b>Sclerostin</b>	a secreted Wnt antagonist that binds to a part of its receptor complex	<b>VEGFs</b>	vascular endothelial growth factor, stimulates growth of new vessels
<b>Smad</b>	intracellular signaling proteins used by the TGF- $\beta$ superfamily to signal	<b>Wnt</b>	the mammalian homologue of wingless (a gene in the drosophilia fruit fly). Wnt is a family of growth factors
<b>Small GTPases</b>	signaling proteins in osteoclasts	<b>WOMAC</b>	Knee and hip osteoarthritis index, developed at Western Ontario and McMaster Universities

# Introduction

## Clinical background

Drug treatment is an important cornerstone of modern medicine, successfully changing the course of many diseases. However, in orthopedic surgery, drugs are still surprisingly seldom used. Technical advances, such as new fracture fixation devices and joint prostheses, have revolutionized the lives of many orthopedic patients, but biological advances in the treatment are few. Yet, even today, some fractures fail to heal, despite numerous operations applying the most modern devices. Limb amputations are sometimes the only remaining solution in these severe cases of non-uniting fractures.

The healing of a fracture is influenced by many factors. Systemic factors often depend on the general health of the patient, such as nutritional status or generalized disease such as diabetes or anemia, but also on life-style factors like drugs and smoking. Local factors, such as the type of bone or rather the quality of blood supply and access to progenitor cells, also affect the prerequisites for the fracture to heal. About 5–10% of all fractures fail to heal, leading to delayed healing, fibrous healing or non-healing (Bostrom and Camacho 1998; Bouxsein et al. 2001). For the patient this means at least prolonged immobilization, pain or maybe even a reoperation, and for both the patient and the society an economic loss.

The use of bone graft in the treatment of non-unions is well documented and known to improve the outcome of surgery. Autografts are harvested from the patient him- or herself but give post-operative morbidity in 8–25% of cases (Summers and Eisenstein 1989). Instead of autografts, recombinant bone-inducing proteins such as the bone morphogenic proteins (BMPs) have been introduced into orthopedic surgery and are approved for use in fractures, non-unions and spinal fusion. However, the drugs are expensive, and no study has proven them to be better than autograft, as regards the healing rate of pseudarthroses (Friedlander et al. 2001).

Autografts and bone anabolic drugs are aimed at improving the anabolic bone-forming osteoblasts when insufficient callus is being formed. Delayed healing or non-union can however also be a result of a premature catabolic response by the osteoclasts, triggered either by instability or by a too rigidly fixated fracture. Sometimes non-union is due to a combination of an anabolic insufficiency and a premature catabolism of the sparse new-forming callus. The inability of the BMPs to perform better than autograft in pseudarthrosis healing in the clinical studies must be considered surprising and maybe a premature osteoclasts-mediated catabolism by the BMP (Wutzl et al. 2006, Jensen et al. 2009) is the cause. Bisphosphonates reduce the osteoclastic catabolic activity in experimental studies, but the use of bisphosphonates in orthopedic conditions other than osteoporosis and bone metastases is limited. If the delayed healing or non-union is a failure of both the anabolic and catabolic responses, theoretically the combination of BMP and a bisphosphonate would be effective. If anabolism is sufficient, a bisphosphonate given alone without the BMP might have the capacity to shorten the healing time of a more benign fracture such as the osteotomy of the proximal tibia in gonarthrosis.

Fracture treatment by external fixation has been developed and refined during the last century. It offers several advantages for severely comminuted fractures, where other methods would fail. It is minimally invasive and therefore reduces the risk of devitalization and infection in open fractures. Limb reconstruction with external fixation can be used in bone lengthening and correction of deformities. The fixation of the pins in all these situations is important and maybe the most critical for the treatment by external fixation. In several studies, both animal and clinical, improved fixation has been described with the use of hydroxyapatite-coated pins (Moroni et al. 1998, 2001, 2008; Magyar et al. 1997). Perhaps the anti-catabolic effect of the bisphosphonates could also be used in this context to influence the bone-pin interface

**Table 1. Experimental studies on local or systemic bisphosphonate effect on fracture healing**

Author (year)	Model	Bisphosphonate	Dose	Effect
Gerstenfeld et al. 2009	Closed femoral fracture, mouse	Alendronate s.c. compared to RANKL inhibitor and control	0.1 mg/kg, twice weekly	Delayed callus remodeling. Increased strength and stiffness
Greiner et al. 2008	Closed femoral fracture, rat	Zoledronate coated titanium K-wires	20 µg/implant	Increased callus area. Earlier mechanical stability
McDonald et al. 2008	Closed femoral fracture, rat	Zoledronate s.c.	0.1 mg/kg one dose 1 week postop. or 0.02 mg/kg weekly for 5 weeks	Increased hard callus BMC, volume and strength. Delayed remodelling with weekly dosing
Amanat et al. 2007	Closed femoral fracture, rat	Zoledronate i.v. and local	0.1 mg/kg , i.v., 0, 1 or 2 weeks postop. 0.01 mg/kg local	Increased callus BMC, volume and strength. 2 weeks postop. superior.
Amanat et al. 2005	Open femoral fracture, rat	Pamidronate s.c. and local	3 mg/kg, s.c., one dose at surgery. 0.1 mg/kg or 1.0 mg/kg local	Increased callus BMC, volume and strength in systemic treatment.
Li et al. 2001	Open femoral fracture, rat	Icandronate s.c.	10 µg/kg or 100 µg/kg three times/week for 2 weeks or continuous	Higher stiffness and ultimate load. Delayed remodelling in continuous, high dose
Adolphson et al. 2000	Colles fracture, human, plaster splint	Clodronate p.o.	400 mg twice daily	Increased BMD of fractured radius
Li et al. 1999	Open femoral fracture, rat	Icandronate s.c.	10 µg/kg or 100µg/kg 3 times/week for 2 weeks preop. or continuous	Larger callus in continuous treatment
Madsen et al. 1998	Tibial fracture, rat	Clodronate s.c.	10 mg/kg daily 28 days preop. and 28 days postop. or saline	Increased BMC and BMD of fractured tibia. No effect on callus size and mechanical strength
Nyman et al. 1996	Tibia double osteotomies, rat	Clodronate s.c.	10 mg/kg daily, 6 weeks	Delayed maturation of callus
Peter et al. 1996	Open radial fracture, dog	Alendronate p.o.	2 mg/kg/day. 9 weeks preop. or 16 weeks postop., or both	Larger callus in continuous treatment. No adverse effect on union, strength or mineralization
Goodship et al 1994	Osteotomy gap tibia, sheep	Pamidronate i.v.	0.5 mg/kg once a week. 4 preop. and 12 postop.	Increased external callus and ultimate torsional strength
Hyvönen et al. 1994	Closed femoral fracture, rat	Clodronate s.c.	0, 3, 10 or 30 mg/kg once a week. 1, 2, 3, 4, 8, 12 or 22 weeks	No altered histological appearance of callus, no delayed healing.

by delaying the bone resorption around the pins and maybe prevent early loosening. In previous experimental studies both local and systemic treatment with bisphosphonates has proven effective in enhancing the fixation of pins (Skoglund et al. 2004).

## Hypotheses

In the present study we address two separate tasks in fracture healing: (1) whether fracture healing can be faster or rather achieved earlier; and (2) if fracture healing can be better, i.e. decrease the

**Table 2. Clinical studies on the application of BMP for fractures or non-unions**

Author (year)	Model	Type of BMP	Effect
Kanakaris et al. 2008	Tibial non-unions	rhBMP-7	Healing rate 89.7%
Ristiniemi et al 2007	Distal tibial fracture	rhBMP-7 vs. collagen	Accelerated healing
Giannoudis et al. 2007	Pelvic ring instability and non-union	BMP-7 with or without ABG	Healing rate 89%
Swiontkowski et al. 2006	Open tibial fracture	BMP-2 0.75 or 1.5 mg/ml vs. no implant	Reduced frequency of secondary interventions
Dimitriou et al. 2005	Tibial-, femoral-, humeral-, forearm-, clavicle non-unions	rhBMP-7, 3.5 mg with or without ABG	Healing rate 92.3%
Govender et al. 2002	Open tibial fracture	BMP-2 0.75 mg/ml or 1.5 mg/ml vs. no implant	Reduced frequency of secondary interventions. Accelerated fracture and wound-healing
Friedlaender et al. 2001	Tibial non-unions	BMP-7 vs. ABG	Healing rate 85%, on par with autograft

ABG = autologous bone graft

incidence of pseudarthrosis/delayed healing and offer a solution to pseudarthrosis/delayed healing as they occur.

We suggested four hypotheses to address these questions:

1. Can we prolong the time between the doses with a bisphosphonate, also in fracture healing, as the trend has been in osteoporosis treatment?
2. Is it possible to improve the net biological effect of the BMPs by controlling the resorption, thereby increasing the remaining amount of callus?
3. Can we replace the expensive, hard-to-handle anabolic drugs with an anti-catabolic drug such as a bisphosphonate?
4. Is it possible to use the biologic effect of the bisphosphonate to improve pin fixation?



## Fracture healing and remodeling

The capacity of the bone for repair and regeneration in response to injury or surgical treatment is substantial. Both fracture healing and remodeling involve a complex set of regulated signaling pathways that control the formation of new bone matrix and the resorption of damaged bone at the disease or injury site. These pathways are responsible for recruiting and activating the different cells and growth factors that are involved in the process of fracture healing. The sources of cells and signals in fracture healing may come from the periosteum and the bone marrow space at the site of the damaged bone (Gerstenfeld et al. 2003), but also from the surrounding muscles which appear to be an important secondary source when the bone ends are necrotic, as in an open fracture (Tägil et al. 2009; Liu et al. 2009). The coordination of multiple cell types and cellular processes recapitulates the pathway of normal embryonic development (Gerstenfeld et al. 2003; Ferguson et al. 1999) and partly the growth in the epiphysis in the child (Gerstenfeld et al. 2003).

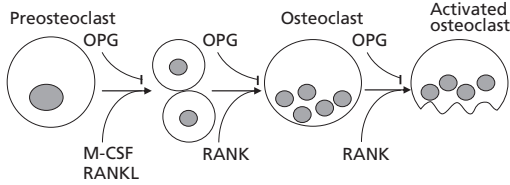
Fractures heal by endochondral and/or intramembranous ossification (Gerstenfeld et al. 2003). Endochondral bone formation usually occurs in regions that are mechanically less stable and immediately adjacent to the fracture site, whereas intramembranous ossification occurs at the proximal and distal edges of the callus (Einhorn 1998; Gerstenfeld et al. 2003; Dimitriou et al. 2005). Fracture trauma involves not only an interruption of skeletal integrity but also disruption of the normal vascular structures and disruption of the marrow architecture. This results in the infiltration of inflammatory cells, macrophages, and degranulating platelets during formation of a hematoma (Einhorn 1998, Brighton and Hunt 1991). This first inflammatory response is coordinated by and involves the secretion of a range of cytokines and growth factors including IL-1, IL-6, and TNF- $\alpha$  that play a role in initiating the repair cascade. TGF- $\beta$ , PDGF and BMP-2 initiate the callus formation. GDF-8 controls cellular proliferation and recruits mesenchymal stem cells (Ai-Aql et al. 2008; Cho et al. 2002;

Gerstenfeld et al. 2003; Dimitriou et al. 2005). Mesenchymal cells that have differentiated into chondrocytes proliferate and synthesize cartilaginous matrix. In the final stages of this soft callus production, the chondrocytes undergo hypertrophy and mineralize the cartilaginous matrix before undergoing apoptosis (Barnes et al. 1999). During this stage angiopoietins and VEGFs are induced, which stimulate vascular ingrowth from vessels in the periosteum (Gerstenfeld et al. 2003).

The next stage is remodeling of soft callus into hard callus. In this stage the soft callus cartilage/fibrocartilage is gradually removed and replaced with woven bone (Barnes et al. 1999; Einhorn 2005). Osteoclasts are recruited from the blood stream for the resorption and osteoblasts locally for the formation of woven bone. The cells involved in the resorption of the soft callus may not be osteoclasts, but instead another large multinucleated cell termed chondroclast (Cole and Walters 1987; Gerstenfeld et al. 2003). The early new bone equivalent to the hard callus is irregular and under-remodeled. Hard callus does form, also in the absence of a cartilaginous template as intramembranous bone formation. The vasculature is critical for formation of the hard callus and VEGFs are up-regulated to stimulate neo-angiogenesis.

The final stage comprises the remodeling of the hard callus into the original cortical and/or trabecular bone configuration to restore the anatomical structure essential to support the mechanical loads (Gerstenfeld et al. 2003). The remodeling is a coupled process of bone resorption by the osteoclast followed by the formation of lamellar bone by the osteoblast.

The stages described above overlap. The outcome of the fracture repair process can be described as a balance between the anabolic (bone forming) and catabolic (bone resorbing) responses instead of the temporarily ordered stages (Little et al. 2007). Many of the cell types recruited to the early stage fracture site as well as the associated regulatory growth factors and cytokines are parts of a general response to tissue injury. Some growth



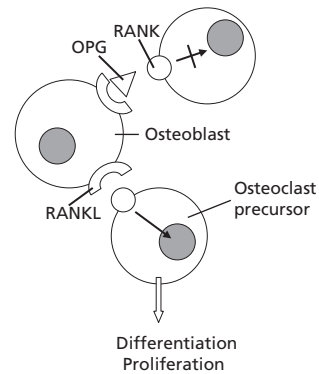
**Figure 1.** Differentiation and activation of osteoclasts. M-CSF and RANKL are essential for osteoclastogenesis. OPG can bind RANKL and thereby inhibit osteoclast differentiation.

factors are non-specific to bone and these processes are referred to as non-specific anabolism and non-specific catabolism (Schindeler et al. 2008), generic to all kinds of tissue injury and including proliferation and expansion of non-differentiated progenitor cells. Specific anabolism and catabolism implies differentiation into more specialized cells with unique function such as osteoblasts or osteoclasts. The speed of fracture healing may be determined by the process of non-specific anabolism and catabolism (recruiting cells, revascularization), while the strength of repair relates to the mechanically driven balance between bone-specific anabolism and catabolism (Schindeler et al. 2008).

## Cells

### Osteoclasts

Osteoclasts are multinucleated cells of hematopoietic lineage. They are members of the monocyte/macrophage lineage and are formed via multiple cellular fusions from their mononuclear precursors (Väänänen and Laitala-Leinonen 2008). Their proliferation and differentiation depend on the presence of two different cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor  $\kappa$  B ligand (RANKL) (Figure 1). M-CSF is expressed by osteoblasts and stromal cells in both soluble and membrane-bound forms (Felix et al. 1996; Teitelbaum 2000) and RANKL is expressed on the surface of osteoblasts and marrow stromal cells (Lacey et al. 1998; Takahashi et al. 1999; Boyle et al. 2003). M-CSF binds directly to its receptor on the surface of the early osteoclast progenitor and thereby provides signals required for proliferation (Teitelbaum



**Figure 2.** RANK is activated by the binding of RANKL. OPG act as a decoy receptor and block the interaction of RANK and RANKL.

2000). M-CSF is thought to be crucial for the survival and proliferation of osteoclast precursor cells as it induces the proliferation, supports their survival and up-regulates the expression of receptor activator of nuclear factor  $\kappa$  B (RANK), which is a prerequisite for osteoclast precursor cells (Arai et al. 1999; Ross and Teitelbaum 2005). RANK is expressed on the cell surface of osteoclasts and their precursors (Boyle et al. 2003). Binding of RANK to the extracellular protein RANKL promotes several of the steps leading to the development of mature, multinucleated, bone-resorbing osteoclasts. RANKL induces activation of intracellular pathways, which leads to the induction of an essential transcription factor that is translocated into the nucleus and starts transcription of its target genes that promote osteoclast differentiation (Takayanagi 2007).

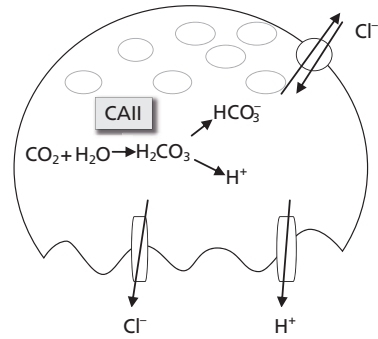
The protein osteoprotegerin (OPG) which is secreted by the osteoblast acts as a decoy receptor that competes with RANK for RANKL and thereby inhibits osteoclast differentiation (Simonet et al. 1997) (Figure 2). Skeletal mass may be determined by the relative concentrations of RANKL and OPG (Hofbauer et al. 2001). In OPG knockout mice the production and function of osteoclasts are unregulated and the result is osteoporosis (Bucay et al. 1998). Besides having an effect on osteoporosis (Bekker 2001), OPG treatment has been shown to decrease bone resorption and preserve the structure of the femoral head in an animal model of ischemic osteonecrosis (Kim et al. 2006). In an animal study of tibial fractures, OPG treatment impaired

the remodeling process and decreased the material properties of the callus tissue (Ulrich-Vinther and Andreassen 2005).

Osteopetrosis is a heterogeneous group of heritable conditions in which there is a defect in osteoclastic bone resorption (Tolar et al. 2004). The mutations affect processes that are essential for the resorption, such as acidification of the bone. In RANK or RANKL knockout animals, osteoclasts cannot be produced, and the animals develop osteopetrosis. In humans a mutation in the gene-encoding RANKL leads to an osteoclast-poor form of osteopetrosis (Sobacci et al. 2007). These individuals present with severe osteopetrosis, but progress more slowly than other types of autosomal recessive osteopetrosis associated with normal or elevated numbers of non-functional osteoclasts, where the mutations result in impaired function of osteoclasts (Tolar et al. 2004). BMPs that promote osteoblastogenesis have also been found to have an effect on osteoclast formation. In vitro studies show that BMPs increase the RANKL/OPG ratio (Wutzl et al. 2006) or act directly on cells of the osteoclastic lineage (Okamoto et al. 2006; Jensen et al. 2009) and thereby result in increased osteoclastogenesis. Osteoclast activity is also regulated by calcitropic hormones. Vitamin D promotes the differentiation of osteoclasts from the monocyte macrophage stem cell precursors by stimulation of RANKL production of osteoblasts. Parathyroid hormone (PTH) binds to its receptor, PTH receptor 1 (PTH1R) on osteoblasts and thereby induces the production of M-CSF and RANKL (Ma et al. 2001; O'Brien et al. 2008). Calcitonin inhibits bone resorption by acting directly on osteoclasts through its receptor (Yasuda et al. 1998).

### ***Osteoclast function***

To resorb bone, the differentiated osteoclast adheres tightly to the surface through a ring-like sealing zone that is rich in the cytoskeletal protein actin (Tolar et al. 2004). When the osteoclast is resorbing it is polarized and forms three distinct areas: the basolateral membrane, the sealing zone and the ruffled border (Väänänen and Laitala-Leinonen 2008). The ruffled border membrane is the actual resorbing organ. The basolateral membrane, which is not in contact with bone material, is important in exocytosis, a process in which a

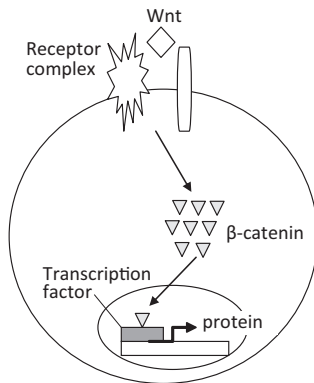


**Figure 3.** In the osteoclast cytoplasm, carbonic anhydrase II (CA II) forms carbonic acid ( $H_2CO_3$ ) from carbon dioxide ( $CO_2$ ) and water; the  $H_2CO_3$  dissociates to bicarbonate ( $HCO_3^-$ ) and a proton ( $H^+$ ). The protons are transported through the ruffled border into the resorption lacuna by a vacuolar proton pump ( $H^+$ -ATPase), generating a pH of 4 to 5 in the extracellular space adjacent to bone. To ensure electro neutrality, a chloride channel (CLC7) simultaneously transports the counter ion chloride. The organic matrix of the bone is removed through enzymatic activity, with cathepsin K, a cysteine protease, as a major actor. The degradation products and debris are transported within vesicles through the osteoclastic cytoplasm and released at the basolateral membrane.

vesicle releases its contents when it fuses with the cell membrane. Resorption occurs through acidification of the bony surface, by targeted secretion of hydrochloric acid into a closed compartment called the resorption lacuna that leads to dissolution of mineral matrix and bone mineral, such as hydroxyapatite (Figure 3). This is a unique feature of the osteoclast (Väänänen and Laitala-Leinonen 2008). The osteoclast half-life is approximately 10 days. They can likely move from one resorption site to another during this time. Bisphosphonates that are bound to bone mineral will be released during the dissolution of bone mineral and then taken up by endocytosis by the osteoclast. Inside the osteoclast, the bisphosphonates affect enzymatic systems and lead to apoptosis of the osteoclast (Russel et al. 2007).

### ***Osteoblasts and osteocytes***

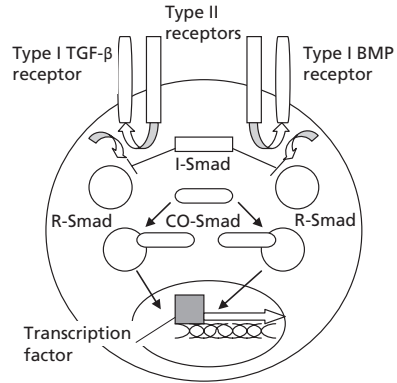
Osteoblasts originate from bone-marrow-derived pluripotent mesenchymal stem cells (MSCs) of the colony-forming unit-fibroblast (CFU-F) lineage that also gives rise to fibroblasts, chondrocytes, myocytes and adipocytes (Bodine and Komm 2006). Osteoblast differentiation is promoted by



**Figure 4.** Overview of the canonical Wnt pathway. Extracellular binding of Wnt to the receptor complex causes intracellular accumulation of  $\beta$ -catenin that can induce the expression of target genes after translocation to the nucleus.

lipid-modified glycoproteins of the wingless (Wnt) family, bone morphogenic proteins (BMPs), and several transcription factors. Two transcription factors essential in the signaling pathways that induce osteoblastogenesis are Runx2 and Osterix (Phimphilai et al. 2006; Nakashima et al. 2002).

Wnt molecules are a large family of growth factors that mediate fundamental biological processes such as embryogenesis, organogenesis and tumorigenesis (Bodine and Komm 2006). Although Wnt proteins signal through several pathways to regulate cell growth, differentiation, function and death, the Wnt/ $\beta$ -catenin or canonical pathway appears to play an important role in bone formation (Pieters et al. 2008). Activation of Wnt/ $\beta$ -catenin signaling occurs upon binding of Wnt to a receptor complex on the cell surface of the osteoblast (Figure 4) (Bhanot et al. 1996; Wehrli et al. 2000). In early stages the differentiation of mesenchymal stem cells to osteoblast precursors is stimulated while alternative differentiation of these stem cells toward adipocytes or chondrocytes is inhibited (Pieters et al. 2008). Wnt signaling has been shown to reduce osteoblast and osteocyte apoptosis *in vivo* and increase bone formation by stimulating differentiation and replication of osteoblasts (Krishnan et al. 2006). The stimulation of differentiation can be referred to as bone-specific anabolism and the replication as unspecific anabolism. Interruption of the Wnt signaling results in reduction in bone mass and a defect in osteoblast proliferation and



**Figure 5.** The BMP and TGF- $\beta$  family members use the Smad signaling pathways. When the ligand associates with the type I receptor, the type II receptor binds to and phosphorylates the type I receptor. Type I receptor phosphorylates R-Smad proteins, which in turn associates with CO-Smads in a complex. This complex moves across the nuclear membrane into the nucleus, where it associates with transcription factors and induces specific gene expression and protein production

maturation (Kato et al. 2002). Loss of function in one of the genes of the receptor complex is found in some individuals with idiopathic or early onset osteoporosis (Pieters et al. 2008).

BMP induces osteoblast differentiation through an intracellular Smad protein dependent signal pathway (Figure 5) (Kawabata et al. 1998; ten Dijke et al. 2003). BMPs bind to specific transmembrane BMP receptors (BMPR) type I and II (Miyazono et al. 2005). Upon binding of BMP, the receptors dimerize and the receptor autophosphorylates. This starts a signaling pathway inside the cell, consisting of Smad proteins, and leads to the translocation of a Smad protein-complex into the nucleus (Kawabata et al. 1998; ten Dijke et al. 2003). Inside the nucleus the Smad protein-complex interacts with transcription factors that are important for osteoblast differentiation (Phimphilai et al. 2006). The transcription factors control the expression of genes that are required for the cell to function as an osteoblast. Runx2 and Osterix are two important transcription factors for osteoblast differentiation. Their function is so critical that mice deficient in the production of either of these transcription factors have skeletons completely composed of cartilage (Phimphilai et al. 2006; Nakashima et al. 2002). The maturation of osteoblasts is blocked and the differentiation of osteoblasts is absent. Runx2

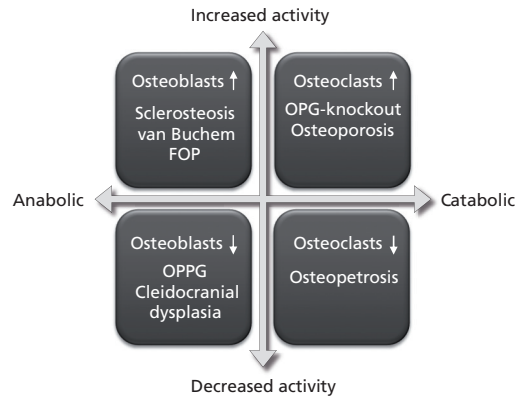


**Figure 6.** Extensive heterotopic bone formation typical for FOP (Shore et al. 2006).

seems to act upstream of Osterix and is required for Osterix expression (Nakashima et al. 2002). In humans, loss of a single copy of Runx2 leads to a defect of intramembranous bone formation resulting in the disease cleidocranial dysostosis (Zhang et al. 2000). A heterozygous mutation in one of the BMP receptors leads to fibrodysplasia ossificans progressiva (FOP) (Shore et al. 2006), an autosomal dominant human disorder of bone formation that causes developmental skeletal defects and extensive debilitating bone formation within soft connective tissues (Figures 6 and 7).

### ***Osteoblast function***

Osteoblasts have the ability to form bone tissue by the secretion of alkaline phosphatase, type I collagen, proteoglycan, bone sialoprotein and osteopontin. Osteoblasts do not function individually



**Figure 7.** Mutations or variations in signaling pathways of osteoblasts and osteoclasts result in changed activity and ratio of the cells. FOP = fibrodysplasia ossificans progressiva, OPG = osteoprotegerin, OPPG = osteoporosis pseudoglioma

but are found in clusters along the bone surface, lining on the layer of bone matrix that they are producing (Hadjidakis and Androulakis 2006). Bone matrix is produced by deposition of collagen I. The collagen matrix becomes mineralized, presumably by the activity of alkaline phosphatase (Wennberg et al. 2000). Osteoblasts are also involved in the osteoclast differentiation as they produce RANKL and osteoprotegerin to modulate osteoclast activity (Takahashi et al. 1999; Simonet et al. 1997). The half-life of an osteoblast is approximately 100 days.

### ***Osteocytes***

Osteoblasts that become embedded in the new bone matrix during bone formation are transformed to osteocytes, the most abundant cellular component of the bone. Once embedded into the bone matrix, the former osteoblasts, now osteocytes, cease their bone-forming activity. An important role of osteocytes, and their network of cell processes, is to function as strain and stress sensors, signals that are very important for maintaining bone structure (Burger and Klein-Nulend 1999). Osteocytes communicate with neighboring osteocytes and with cells on the bone surface via a meshwork of cell processes, which are located inside canaliculi within the bone matrix (Franz-Odenaal et al. 2006). The osteocytes produce sclerostin, a glycoprotein and an antagonist to the Wnt pathway. Sclerostin inhibits Wnt signaling by blocking a part

of its receptor-complex leading to reduced osteoblast activity (van Bezooijen et al. 2007). Sclerostin is regarded as a key inhibitor that regulates the normal extent of bone formation and consequently protects against the deleterious effects of uncontrolled bone formation (Poole et al. 2005). Loss of function of the sclerostin protein results in sclerostosis and van Buchem disease, two conditions of severe hyperostosis (Pieters et al. 2008). Sclerostin antibodies have been suggested as a treatment of osteoporosis (Hoeppner et al. 2009) as an anabolic drug. Treatment of osteoporotic rats with sclerostin function-blocking antibody increased the bone

mass and bone strength (Li et al. 2009). Another protein secreted by the osteoblasts and osteocytes is Dickkopf. Like sclerostin it binds to a part of the Wnt receptor complex and blocks the canonical Wnt pathway (Mao et al. 2001).

A special receptor for PTH, parathyroid hormone receptor 1 (PTHr1), is expressed in osteocytes and in osteoblasts. Activation of PTHr1 in transgenic mice decreases the expression of sclerostin and elevates the signaling from the receptor complex, which results in higher bone mass (O'Brien et al. 2008). Osteocytes can live for several decades.

# Proteins and drugs in bone healing and remodeling

## Anabolic proteins and drugs

Bone healing and remodeling can be affected by various proteins and drugs acting on cells and signaling pathways involved in these complex processes. Alone or in combination, they have been tested in animal experiments but also in clinical studies. PTH increases the rate of remodeling and induces both formation and resorption (O'Brien et al. 2008). Continuous treatment increases net bone resorption, but with intermittent administration the net effect is bone formation (Andreassen et al. 1999). PTH thereby acts as an anabolic drug in bone repair and remodeling (Skripitz et al 2000). The anabolic effect of PTH is caused by increased osteoblast number due to down-regulation of sclerostin and elevated Wnt signaling (O'Brien et al. 2008). The catabolic effect of PTH is caused by stimulating osteoclast formation by binding to osteoblastic cells, increasing the production of RANKL and M-CSF (Ma et al. 2001; O'Brien et al. 2008).

PTH has been used clinically in the treatment of osteoporosis (Lindsay et al. 1997; Misof et al. 2003) with a distinct effect. The drug has also been used in fracture healing, and in a randomized clinical study of distal radial fractures a shorter healing time was observed with daily injections of low-dose PTH (Aspenberg et al. 2009). Statins are another group of drugs found to have anabolic bone effects in animals (Mundy et al. 1999) and were also tested in a similar randomized study comparing simvastatin and saline, also in distal radial fractures. No difference was found between the active compound and the control (Patil et al. 2009). In an animal study by Morgan et al., the combination of BMP-7 in a bone defect and daily injections of PTH led to greater bone volume as well as better microstructural organization and integration of the new-formed bone (Morgan et al. 2008).

Strontium resembles calcium and in the same group in the periodic system and therefore easily incorporated in bone. It has been used in treatment of postmenopausal osteoporosis and bone-

biopsy specimens from patients treated with strontium ranelate show a reduction in bone resorption (Canalis et al. 2007). Apart from these anticatabolic prerequisites, the drug also has anabolic properties and appears to be an ideal drug for bone metabolism (Hamdy 2009; Marie 2006). No clinical studies of fracture healing exist for strontium.

## BMP

BMP molecules are soluble, local-acting signaling proteins, which bind to specific receptors on the surface of a mesenchymal progenitor cell and are capable of inducing these cells to differentiate into osteoblasts. Such a substance was prophesized by Hippocrates (460–370 BC), who observed the regenerative potential of bone and speculated that natural products and endogenous substances in the human body are superior therapeutic agents for clinical application (Reddi 1997). Over 2000 years later, Senn demonstrated in 1889 that decalcified bone implants could be used in the treatment of osteomyelitis and certain bone deformities (Reddi 1997; 2001). A Swedish surgeon, Gustav Levander, showed in 1938 that when cell-free alcoholic extracts of bone are injected into the muscles of rabbits, cartilage and bone form at the site of injection. Levander suggested that bone regeneration takes place as the result of some specific bone forming substance activating the non-specific mesenchymal tissue (Levander 1938). Levander's finding was later confirmed by Annersten (1940) and Bertelsen (1944) after which Lacroix (1945) named the hypothetical substance osteogenin. In 1965 Urist showed that segments of demineralized, lyophilized rabbit bone matrix, DBM, induced new bone when implanted intramuscularly in rabbits (Urist 1965). He called the substance bone morphogenic protein and the phenomenon of inducing bone at a non-skeletal site osteoinduction.

Bone induction induced by demineralized bone is a multistep cascade resembling the sequence of events observed during embryogenesis (Reddi and Huggins 1972). The first, at least partially, puri-

fied osteoinductive substance was named osteogenin (BMP-3) (Sampath et al. 1987). At almost the same time, BMPs were purified (Wang et al. 1988), sequenced and synthesized recombinantly (Wozney et al. 1988). By screening human genomic libraries the genes for BMP-2b and BMP-3 were isolated and a new gene termed OP-1 (osteogenic protein-1), later BMP-7 (Özkaynak et al. 1990).

To date, at least 20 BMP-related proteins have been identified (Miyazono et al. 2005; Nakase and Yoshikawa 2006; Alaoui-Ismaili and Falb 2009). Except for BMP-1, which is a pro-collagen C protease, they are all members of a family of secreted signaling molecules referred to as the TGF- $\beta$  superfamily (Wozney and Rosen 1998; Reddi 2001). The TGF- $\beta$  superfamily consists of structurally related proteins that are found in different kinds of organisms from fruit fly to human. The ability to manufacture proteins through recombinant technology led to the commercialization of two BMPs, BMP-7 (OP-1) and BMP-2, both approved for clinical use to repair bone. The BMPs have a short half-life and only a small amount is retained at the repair site due to rapid local clearance. BMPs are therefore combined with implanted carriers to avoid breakdown and increase their concentration at the bone repair site. The carriers must be biocompatible and can be made from (I) natural polymers, such as collagen, (II) inorganic materials such as hydroxyapatite, (III) synthetic polymers, such as polylactic acid (PLA), (IV) composite materials such as collagen plus inorganic materials (Seeherman and Wozney 2005). One of the first carriers was type I collagen, modified to limit breakdown and to decrease its immunogenic properties. In one of the commercially available proteins, Osigraft (rhBMP-7), the amount of active protein appears to be small. One vial contains 3.5 mg BMP-7 per 1000 mg collagen (Bishop and Einhorn 2007). A collagen sponge with BMP-2 retains only 5% of BMP-2 after being implanted for 14 days (Senta et al. 2009). Other ways of administration are therefore being tested, and the ideal would be to have local production of the gene product, and gene therapy has been another strategy for producing BMPs locally (Baltzer and Lieberman 2004). The results have been promising in small animals but not in larger animals (Senta et al. 2009).

### *BMP receptors*

BMP treatment has been shown to induce osteogenic differentiation in myoblasts (Katagiri et al. 1994; Yeh et al. 2002). Osteoblasts, myoblasts, chondrocytes and adipocytes arise from the same pluripotent mesenchymal stem cell of the colony-forming unit-fibroblast (CFU-F) lineage. BMPs bind to transmembrane receptors on a cell's surface, and two types of receptors, types I and II, are required for signal transduction (Miyazono et al. 2005). Three type I receptors and three type II receptors have been identified. Type II receptors have serine/threonine kinase domains that are constitutively active and phosphorylate glycine/serine domains in the type I receptors upon ligand binding, leading to activation of the type I receptors (Yamashita et al. 1996; Miyazono et al. 2005). The BMP receptors (BMPR) IA and II are reported to be ubiquitously expressed, whereas IB is tissue-specific. The sensitivity of the myoblast to BMP-2 treatment has been shown to be correlated to the expression of BMPR-IA and the expression of BMPR-IA is enhanced by BMP-2 treatment. Fibroblasts do not show the same response when treated with BMP-2 (Liu et al. 2009).

Fibrodysplasia ossificans progressiva has been linked to a heterozygous mutation in a BMPR type I (Shore et al. 2006). The mutation is believed to cause constitutive activation of the receptor. Mesenchymal cells expressing this receptor are more sensitive to undergo BMP-induced osteoblast differentiation and mineralization (van Dinther et al. 2009). Mutations in the BMPR-II gene have been found in some patients with familial pulmonary arterial hypertension (Machado et al. 2009).

### *TGF- $\beta$*

Another protein that participates in the fracture healing and could be described as anabolic is the transforming growth factor beta (TGF- $\beta$ ). TGF- $\beta$ s are ubiquitous in the body and they stimulate proliferation of cells of mesenchymal origin. The most concentrated natural source of TGF- $\beta$ s is the platelets, which release them in the early inflammatory phase. Like the BMPs the TGF- $\beta$ s signal through serine-threonine receptors (Yamashita et al. 1996) and the Smad signaling pathway inside the cell (Kawabata et al. 1998). The difference between the intracellular signaling of BMPs and



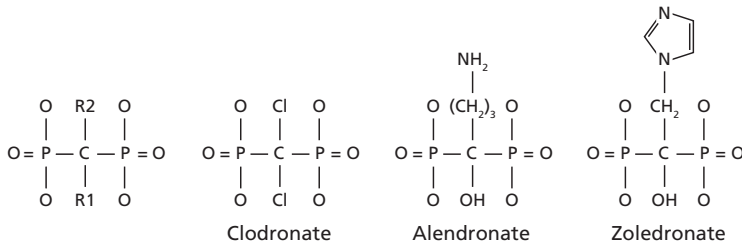


Figure 8. The generic structure of bisphosphonates and schematic drawing of the chemical structure of clodronate, alendronate and zoledronate. R2 side chain pointing upwards.

TGF- $\beta$ s appears to concern which of the different Smad proteins that the receptor associates with (Miyazono et al. 2005). The TGF- $\beta$  and BMP pathways interact with each other through multiple mechanisms. They compete in interaction with certain Smad proteins and regulate the expression of others. In this way there is a cross-talk between the BMP and the TGF- $\beta$  signaling pathways (Maeda et al. 2004; Miyazono et al. 2005). During fracture healing the TGF- $\beta$  seems to be present during all stages and it peaks during chondrogenesis. Because they are produced by degranulated platelets after initial injury they are suggested to be involved in the initiation of callus formation. While the BMPs induce differentiation of osteoprogenitor cells into osteoblasts (bone-specific anabolism), the TGF- $\beta$  induce proliferation of the osteoprogenitors (unspecific anabolism). Treatment of a stable fracture with TGF- $\beta$ 2 increases the callus volume but not the bone content in the callus. In a mechanically unstable fracture neither the callus volume nor its bone content is increased, but the fibrous component of the callus is increased (Barnes et al. 1999). The signaling cross-talk between BMP and TGF- $\beta$  pathways plays a crucial role in the regulation of osteoblastic differentiation (Maeda et al. 2004) and thereby affects bone healing and remodeling. Inhibition of the TGF- $\beta$  signaling accelerates the BMP-induced osteoblastic differentiation (Maeda et al. 2004) and may be a treatment option in conditions where the anabolic part of fracture healing and remodeling needs to be boosted.

### Anti-catabolic drugs and proteins

The protein osteoprotegerin (OPG), which is

secreted by the osteoblast and acts as a decoy receptor for RANKL on the surface of the osteoclast and thereby inhibits osteoclast differentiation (Simonet 1997), can be described as an anti-catabolic drug or protein. A single injection of OPG in postmenopausal women has demonstrated that bone turnover can be substantially reduced (Bekker 2001). OPG treatment has been shown to decrease bone resorption and preserve the structure of the femoral head in an animal model of ischemic osteonecrosis (Kim et al. 2006) far more effectively than by bisphosphonates (Kim et al. 2005). In an animal study of tibial fractures, OPG treatment impaired the remodeling process and decreased the material properties of the callus tissue (Ulrich-Vinther and Andreassen 2005). Denosumab, a RANKL antibody given as an infusion every 6 months, has been shown to reduce the risk of fracture in postmenopausal osteoporosis (Cummings et al. 2009).

### Bisphosphonates

Bisphosphonates were first synthesized in the 1800s, and have only been used in medicine since the 1960s. The early use of bisphosphonates was industrial, mainly as corrosion inhibitors but also as complexing agents in the textile, fertilizer and oil industries. Chemically, the bisphosphonates are stable analogues of naturally occurring inorganic pyrophosphate but have a P-C-P bond instead of a P-O-P bond. They have two side-chains (R1 and R2) that are attached to the carbon atom (Figure 8). The two phosphonate groups are essential for binding to bone mineral, such as hydroxyapatite (HA), and together with the R1 side chain they act as a “bone hook”. A hydroxyl group (OH) or an amino group at the R1 position increases the affini-

ity for calcium and thus bone mineral (van Beek et al. 1998; Russell et al. 2007). Bisphosphonates adhere to bone mineral to such an extent that the binding can practically be considered as permanent and lasts until bone is resorbed. The structure and three-dimensional conformation of the R2 side chain determine anti-resorptive potency and affect binding to HA (Russell et al. 2007; van Beek et al. 1998, Nancollas et al. 2006). Bisphosphonates containing a nitrogen atom in an alkyl chain as R2 side chain, as in alendronate, are 10–100-fold more potent than etidronate, which does not contain any nitrogen atom (Russell and Rogers 1999). If the bisphosphonates contain a nitrogen atom within a heterocyclic ring, as in risedronate and zoledronate, they are up to 10,000-fold more potent than etidronate in some experimental systems (Russell and Rogers 1999). Bisphosphonates that share a common P-C-P structure, and have OH at the R1, can have different binding affinities for hydroxyapatite (Nancollas et al. 2006), and zoledronate has the highest affinity to hydroxyapatite (Nancollas et al. 2006).

The gastrointestinal uptake of bisphosphonates is low and the bioavailability for alendronate is <1% (Gertz et al. 1995) and about 50% of the absorbed dose is taken up selectively by the skeleton (Cremers et al. 2005). After an intravenous infusion of zoledronate, the renal excretion is 40% of the total dose, with the majority of excretion occurring within 24 hours (Chen et al. 2002). The retention is greater in patients with disease states of high bone turnover and is influenced by other factors, such as renal function (Cremers et al. 2005). The bisphosphonates with higher binding affinity to bone have lower urinary excretion. Within bone, bisphosphonates initially localize to regions where new bone mineral is being deposited and also where bone is being resorbed by osteoclasts, preferentially in areas of high bone turnover (Sato et al. 1991). The bisphosphonates stay there until they are released during the bone resorption by the osteoclasts (Russell et al. 2007).

#### *Classification and function of bisphosphonates*

Bisphosphonates can be classified into at least two major groups due to different modes of influencing the osteoclast. The first generation of bisphosphonates comprises the non-nitrogen-containing

bisphosphonates, such as etidronate and clodronate. In the osteoclast these are metabolically incorporated into non-hydrolyzable analogs of adenosine triphosphate (ATP) (Frith et al. 1997), which inhibit the adenine nucleotide translocase in the mitochondrial membrane (Lehenkari et al. 2002). The mitochondrial permeability is thereby affected, which initiates a caspase activation leading to an irreversible step toward apoptotic cell death (Benford et al. 2001). The second group comprises the more potent, nitrogen-containing bisphosphonates, such as risedronate and zoledronate. These bisphosphonates, containing a nitrogen atom in one of the alkyl chains, are referred to as the second generation of bisphosphonates, and those containing a nitrogen atom within a heterocyclic ring as the third generation. Members of this group interfere with other metabolic reactions as an inhibitor in the mevalonate pathway (Luckman et al. 1998). The mevalonate pathway is found in all cells of virtually all known prokaryotic as well as eukaryotic organisms. The major target in the mevalonate pathway is the enzyme farnesyl pyrophosphate synthase (FPPS), which is inhibited by all of the clinically used nitrogen-containing bisphosphonates (van Beek et al. 1999). By inhibiting FPPS, the synthesis of farnesyl pyrophosphate and its downstream metabolite geranylgeranyl pyrophosphate is prevented. These metabolites are required for lipid modification (prenylation) of small GTPases, crucial signaling proteins regulating a variety of cell processes important for osteoclast function, including cytoskeletal arrangement, membrane ruffling, trafficking of intracellular vesicles and cell survival (Coxon and Rogers 2003). The proteins accumulate in their unprenylated form, and prenylated small GTPases cannot be replaced in the osteoclast following normal protein turnover. The inhibition of FPPS by nitrogen-containing bisphosphonates appears to be the cause of the acute-phase reaction to nitrogen-containing bisphosphonates, resulting in fever and “flu-like” symptoms.

#### *Safety*

Osteonecrosis of the jaw (ONJ) has been identified as a potential and serious complication, particular with long-term and high-dose intravenous bisphosphonate therapy in malignant disease,

especially with the potent nitrogen-containing bisphosphonates (Rizzoli et al. 2008). Most reported ONJ cases have been in cancer patients receiving intravenous bisphosphonates. The etiology of osteonecrosis is unknown, and any causal role for bisphosphonates remains unproven, but there seems to be a strong clinical correlation between ONJ and bisphosphonate therapy (Ruggiero and Drew 2007). Other reported risk factors associated with the development of ONJ include history of dento-alveolar trauma, inflammatory dental disease and use of chronic steroids in conjunction with bisphosphonates (Ruggiero and Drew 2007). The inhibition of osteoclasts and thereby bone resorption and remodeling is one hypothesis of the mechanism behind ONJ (Ruggiero and Drew 2007); anti-angiogenic properties of certain bisphosphonates is another (Wood et al. 2002). Few cases of ONJ have been reported in patients who have been given oral therapy with various bisphosphonates for benign disorders (Rizzoli et al. 2008).

Subtrochanteric insufficiency fracture in patients with long-term bisphosphonate therapy is another drug-related side effect that has been reported and discussed. The incidence of stress fractures was estimated at 1/1000, considered acceptable, since the bisphosphonate treatment reduces the incidence of any fracture by 15/1000 (Schlicher and Aspenberg 2009).

In our studies we use zoledronate, a third generation potent bisphosphonate developed for intravenous use. Zoledronate belongs to the group of heterocyclic nitrogen-containing bisphosphonates and is the most potent of the clinically used bisphosphonates. It has strong affinity for bone mineral and strong inhibitory activity in FPPS in osteoclasts. Both features appear to contribute to its high potency as well as its prolonged duration of action. In the clinical situation it is administered once yearly to patients with osteoporosis but has been used up to every fourth week in patients with malignancies, the latter maybe the reason for the appearance of ONJ in these patients.

# Mechanics

## Callus distraction

The first known successful lengthening of deformed limbs was reported by Codivilla in 1905 (Codivilla 1905), who described the use of an osteotomy of the cortex and immediate application of traction force to a calcaneal pin (Codivilla 1905). Ombredanne was first to use an external fixator for limb lengthening. In 1913 he reported having performed a lengthening of a bone, a femoral osteotomy, at a rather high rate of 5 mm per day (Aronson 1997). In 1923 Bier described how bony consolidation can be expected if the ends are left in contact a few days after osteotomy (Giebel 1992) and in 1927, Abbott introduced the idea of a latency period after the osteotomy to promote the formation of bone (Abbott 1932).

In Kurgan, Siberia, in the former USSR, Gavriil A Ilizarov started in 1950s to use a ring fixator for treating fractures and deformities. To optimize the process of callus distraction, he did extensive basic research and was the one who coined the term “distraction osteogenesis”. Initially, his patients were treated for fractures and non-unions, but later, he developed the techniques of bone transportation and limb lengthening (Aronson 1994). Ilizarov studied the effects of various distraction rates and distraction frequencies on the bone regenerate and the soft tissues around the bone and found a distraction rate of 1 mm per day to be the most appropriate (Ilizarov 1989). The rhythm of the distraction, as recommended by Ilizarov, is 0.25 mm four times a day (Ilizarov 1989). Other experimental work has shown that sporadic rhythms of distraction inhibit bone formation (Aronson 1994). Increases in the distraction rate can be detrimental to healing and lead to a distraction gap filled with mostly fibrous tissue (Ilizarov 1989; Choi et al. 2004).

The callus distraction is divided by some authors into four phases: resting phase, distraction, neutralization and dynamization phase (Giebel 1992) but by others into three phases: latency (resting phase), distraction, and consolidation (Cho 2007 et al.; Ai-Aql et al. 2008). The resting phase corre-

sponds to the first 6–10 days after the corticotomy. During this phase the regenerate or callus precursor forms and corresponds to the early stages of fracture healing (Ai-Aql et al. 2008). During the subsequent distraction phase, distraction is undertaken at a rate of 1 mm per day. The callus is elongated and a central fibrous zone forms, called the fibrous interzone. This is rich in chondrocyte-like fibroblasts and oval cells, morphologically an intermediate between fibroblasts and chondrocytes (Vauhkonen et al. 1990; Aronson 1994; Sato et al. 1998). After the desired length has been achieved, the neutralization phase begins with callus formation (Giebel 1992). During the dynamization phase, there is a gradual conversion of the lengthening system from a static to a dynamic mode, to permit micromovement under weight-bearing conditions (Giebel 1992).

The molecular signals that drive the regenerative process during distraction osteogenesis are similar to the signals in fracture healing and include the pro-inflammatory cytokines (Cho et al. 2007), the BMPs (Sato et al. 1999), and angiogenic factors (Choi et al. 2002). A difference between the two processes of new bone formation can be seen in the relative levels of expression of individual mediators and their timing of expression (Ai-Aql et al. 2008). The angiogenic response in callus distraction has been shown to be directly related to the rate of distraction (Lewinson 2001 et al.; Meyer et al. 2001).

There are three basic types of callus distraction: longitudinal, angled and lateral callus distraction (Giebel 1992). Mechanical strain during callus distraction stimulates osteogenesis. In animal models of distraction osteogenesis Yasui et al. describes the ossification during the early stages of distraction as endochondral with callus formation, and this cartilaginous callus is replaced by bone (Yasui et al. 1997). The process that takes place during the distraction phase also resembles the endochondral bone formation based on the findings of the chondrocyte-like cells and fibroblasts. During the neutralization phase, when the callus formation is

increased, the fixation is not likely to be so rigid that intramembranous bone formation will occur as it would in a stress-shielded mechanical environment as in a plated fracture. During the distraction the mechanical strain is applied to the bone and to the callus, in a predetermined direction as the patient is weight-loading. According to Wolff's law, bone structure, growth and repair are influenced by the functional demands, and the way in which bone responds to a variety of pathological conditions is called adaptation. Early during the distraction and loading, the bone cells get the information about which direction the predominant load will be in, and deposit osteoid accordingly along the collagen bundles in the regenerate.

During the dynamization phase the callus volume increases, is gradually mineralized and finally remodeled. Intermittent distraction and compression of a diaphyseal osteotomy leads to higher bending stiffness and larger callus (Claes et al. 2008) and it may be speculated that it is the same in a metaphyseal osteotomy. Too early dynamization does not improve healing (Claes et al. 2009), perhaps due to the tissue deformation and microcracks that would occur in the fracture gap if the strength of the tissue is insufficient. If so, osteoclastic resorption would be initiated and the catabolic response would even exceed the anabolic response, leading to delayed healing. A technically suboptimal osteotomy with severed periosteum or thermally injured bone ends due to blunt saw blades (Toksvig-Larsen 1992), causing necrosis, frame instability (Aronson 1994) or a too high distraction rate (Ilizarov 1989; Choi et al. 2004) may disturb the vascularization and local blood supply to the regeneration tissue, thereby also causing delayed bone healing (Choi et al. 2002). However, in the metaphyseal bone, the site where the osteotomy was performed in our randomized study (III and IV), the vascular supply and a cell-rich marrow is abundant and the risk of delayed healing and non-union will be lower than in the diaphyseal bone.

### **Pin fixation and loosening**

Stable pin fixation is a prerequisite for successful treatment by external fixation to minimize the risk of complications such as pin loosening, delayed

healing, non-union and pin site infection (Moroni et al. 2001). The stable fixation of non-cemented orthopedic implants is a result of a direct contact of living bone with the implant surface; bone ingrowth into a porous surface of the implant; biological bonding between bone and a bioactive surface; or a combination of these phenomena (Dhert et al. 1998).

Various pin and screw designs have been compared to optimize the thread and tip design in order to enhance the fixation (Asnis et al. 1996; Rovinsky et al. 2000; Gausepohl et al. 2001). In several studies, both animal and clinical, improved fixation and reduced rates of pin site infection have been described with the use of hydroxyapatite (HA) coated pins in both cancellous and cortical bone (Magyar et al. 1997; Moroni et al. 1998; Moroni et al. 2001; Moroni et al. 2008). In some situations the HA-coating by itself seems to be more important than the pin design (Moroni et al. 2008) but the effect differs greatly between cancellous and cortical bone. In our clinical model the pin fixation is satisfactory with HA-coated pins in the metaphyseal/cancellous bone and standard pins in the diaphyseal/cortical bone. HA-coated pins in the diaphyseal/cortical bone can be too well fixed and thereby painful and difficult to remove in the out-patient clinic (Moroni et al. 1998). With insertion of conical pins, each screw thread cuts a new, slightly larger path in the bone. This contrasts with bicylindrical pins, where each successive screw thread occupies the grooves cut in the bone by previous threads. In an animal study, both conical and bicylindrical HA-coated external fixation pins proved to be well fixed and osseointegrated, but the conical pins showed higher extraction torque – an important factor in treatment using external fixation (Moroni et al. 2002).

During insertion there is a risk of micro-cracks (Verborgt et al. 2000) and thermal injury (Wikenheiser et al. 1995), which can lead to resorption around the pins. The design of conical pins has been shown to generate less heat in bone during insertion (Wikenheiser et al. 1995), to optimize bone grip (Lavini et al. 1994) and reduce stress on the pin during dynamization in fracture treatment (Aro et al. 1993). Pre-drilling reduces the forces required for pin insertion, while self-drilling pins may require additional force to insert them

**Table 3. Clinical studies on local or systemic bisphosphonate effect on implant fixation**

Author (year)	Model	Bisphosphonate, type and administration	Dose	Effect
Friedl et al. 2009	Cementless total hip arthroplasty, human	Zoledronate i.v.	4 mg, single infusion	Minimized migration of the cup
Hansson et al. 2009	Uncemented knee prosthesis, human	Alendronate p.o.	70 mg/week, 6 months	No decreased migration of the implants
Hilding and Aspenberg 2007	Cemented knee prosthesis, human	Ibandronate local	1 mg into tibial bone surface	Improved fixation
Moroni et al. 2007	Hip fracture, external fixation, human	Alendronate p.o.	70 mg/week, 3 months	Improved fixation in cancellous bone

**Table 4. Experimental studies on local or systemic bisphosphonate effect on implant fixation**

Author (year)	Model	Bisphosphonate, type and administration	Dose	Effect
Miettinen et al. 2009	Titanium implant femur, rat	Zoledronate lavage in medullary canal	10 ml/min, lavage time 1 min, concentration 20 µM	Elevated perimplant bone volume
Jakobsen et al. 2009	Titanium implants tibia, dog	Alendronate local	2 mg/ml, 5 ml into drill hole	Increased peri-implant bone volume fraction. Increased fixation
Suratwala et al. 2008	HA-coated intramedullary nail femur, rat	Zoledronate local	50 µM, soaked for 5 min	Increased pull-out force. Higher bone mass
Eberhardt et al. 2007	HA- and non-coated titanium implant femur, rat	Ibandronate s.c.	1.0; 2.5 or 5.0 µg/kg daily	Accelerated osseointegration of HA-coated implants
Jakobsen et al. 2007	Titanium implants and morzelised allograft humerus, dog	Alendronate local	2 mg/ml, soaked not rinsed	Decreased implant fixation
Wermelin et al. 2007	Stainless-steel screw tibia, rat	Pamidronate and ibandronate coating		Increased pull-out force
Miyaji et al. 2005	External fixator femur, rat	Alendronate s.c.	350 µg/kg once a week, 5 weeks	Reduced loosening
Bobyn et al. 2005	Porous tantalum implant ulna, dog	Zoledronate i.v.	0.1 mg/kg, one dose postop.	Increased ingrowth
Skoglund et al. 2004	Stainless-steel screw tibia, rat	Ibandronate systemic and local	3 µg/day, 14 days 0.1 mg into drill hole	Enhanced extraction force and pull-out strength
Mochida et al. 2002	Hip arthroplasty, titanium, HA-coated, dog	Alendronate p.o.	10 mg daily	No difference in radiology or histological findings

(Wikenheiser et al. 1995). Predrilling is necessary for certain pins in certain bones and unnecessary for others. Hutchinson showed significantly elevated temperatures with self-cutting pins when

treating distal radial fractures with external fixation, but the predrilling did not decrease the incidence of pin track infections or other pin problems (Hutchinson et al. 2000).

The principal mechanisms underlying the osseointegration process around implants are similar to those occurring during fracture repair and remodeling (Fini et al. 2004). Good implant stability tends to minimize distortional strains in the regeneration tissue facilitating osteogenesis and bone ingrowth. On the contrary, motion or poor implant stability which result in tensile and shear motions, stimulate fibrous tissue formation (Carter et al. 1998).

During the postimplantation period, when the bone that originally was in contact with the implant is being remodeled, a situation may develop where implant integration in bone is less than it was initially (Brånemark et al. 1997). The heat necrosis in the bone that can appear during the insertion of the pin (Wikenheiser et al. 1995), as well as microcracks due to fatigue (Verborgt et al. 2000), lead to remodeling of the bone at the bone-pin interface. If an imbalance occurs between the anabolic and catabolic local factors acting on bone formation and remodeling, the risk of pin loosening increases.

Initially fibroblasts and chondrocytes proliferate in the gap between the implant and bone and collagenous matrix fills the gap (Prendergast et al. 1997). When the fixation is sufficiently stable, the mechanical environment would stimulate osteoblast proliferation (Prendergast et al. 1997). If the early pin fixation fails, this would lead to prolonged micromotion at the pin bone interface and there would be no mechanically stable situation that stimulates osteoblast proliferation. This can result in further loosening of the pin. On the other hand, too low a load on the pin, minimizing the deformation of the bone adjacent to the pin, would create a stress-shielded environment with a high catabolic response in the bone, leading to resorption. Also, for pin fixation there is a thin balance between insufficient mechanical stimulation and overload, both stimulating bone catabolism and thereby possible to be moderated by bisphosphonate treatment.

# Materials and methods

## Animal model

### *The bone conduction chamber*

In paper 1 and 2 we used the bone conduction chamber (BCC) (Figure 9). The BCC consists of a threaded titanium cylinder, formed out of two half-cylinders held together by a hexagonal closed screw cap. The overall length is 13 mm and the screw cap is 7 mm, leaving 6 mm of the implant to be screwed into the bone. The bone ingrowth chamber has an inside diameter of 2 mm and an inside length of 7 mm. The outside diameter is 3.5 mm. The BCC is screwed into the proximal tibia of a rat. There are two bone ingrowth openings located at the bone end of the chamber. Thus, the ingrowing tissue enters the cylindrical space from the bone compartment. The chamber space extends far out into the subcutaneous region and the ingrowing bone-derived tissue can grow into the chamber without competition with other tissues. Ingrowing tissue will fill a portion of the chamber but not reach to the far end of the cylinder with the normal experiment time of 6 weeks. The interior can be left empty or an osteoconductive material, such as bone graft can be inserted right from start, and the chamber fills with mesenchymal tissue, which gradually differentiates into bone.

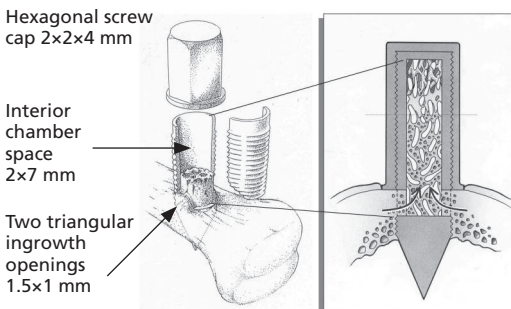


Figure 9. The bone conduction chamber in situ in the proximal tibia. The graft is placed in the chamber and mesenchymal tissue grows from the bottom upwards into the bone graft, which subsequently remodels. Arrows point at ingrowth openings (Aspenberg and Wang 1993).

In the present studies, we used cancellous bone grafts. Pairs of structurally intact cancellous bone grafts were obtained from female Sprague-Dawley rats. A cylindrical 2 × 6 mm bone rod was resected in the axial direction from the knee joint with a hole cutter. The epiphysis and the growth plate were excised. The grafts were kept sterile and freeze-dried at  $-70^{\circ}\text{C}$ . At surgery the grafts were inserted into the chambers, which then were screwed into the proximal tibiae of recipient male Sprague-Dawley rats.

Under aseptic conditions the medial proximal tibial metaphysis was exposed with a longitudinal incision. After incising and raising of the periosteum, the medial and posterolateral cortices were pierced with a 1 mm awl just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was enlarged manually with a 2.7 mm drill. The chamber was then screwed into position so that the bone ingrowth holes were placed at the level of the cortical bone. In the first paper we used unilateral chambers and in the second paper bilateral chambers.

### *Administration of drugs and proteins*

In the first paper 0.5 mL subcutaneous injections of zoledronate 1.05  $\mu\text{g}$  were given at day 4 postoperatively and then weekly until harvest. The controls were given the same amount of saline solution at the same regime.

In the second paper one subcutaneous injection of zoledronate 0.1 mg/kg was given at day 14. The controls were given a subcutaneous injection of 0.4 mL NaCl. The graft in the chamber at the right side was soaked with 8  $\mu\text{L}$  of a dilution corresponding to 1  $\mu\text{g}$  of BMP-7 per graft just before implantation. The control grafts at the left side were soaked with 8  $\mu\text{L}$  buffer alone.

### *Evaluation*

The specimens were fixed in 4% formalin, decalcified, dehydrated and embedded in paraffin. The specimens were cut with a microtome parallel to the long axis of the chamber and were stained with



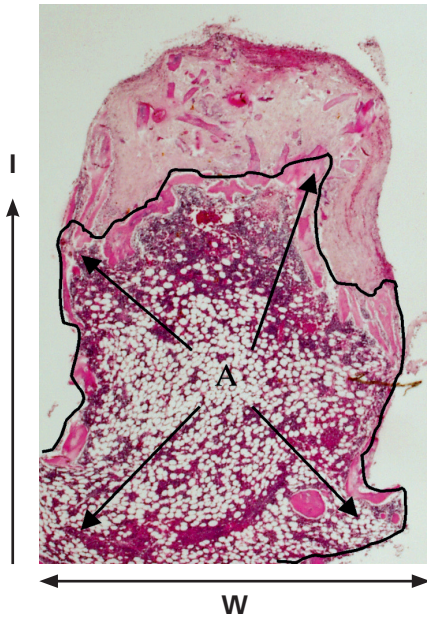


Figure 10. The mean ingrowth distance (I) in each slide is calculated by dividing the new bone area (A) with the width of the specimen (W). The mean of the three sections yields as a value of the specimen.

hematoxylin and eosin. Three sections from the middle of the specimens, each 300  $\mu\text{m}$  from the next slide, were used for histological and histomorphometric analysis. All slides were investigated in random order. The area of the new ingrown bone was measured by circumscribing it on a digitizing table using the Videoplan (Kontron GmbH, Germany) equipment at 40 $\times$  screen magnification. This area includes marrow cavities and graft remnants that had been surrounded by new bone. The mean ingrowth distance in each slide was calculated by dividing the new bone area by the width of the specimen (Figure 10). In all cases, fibrous tissue had penetrated into the chamber ahead of the new bone. The total tissue ingrowth distance, that is, the distance from the ingrowth end of the chamber to the fibrous ingrowth border, was measured in the same way. We measured the bone density of the remodeled graft by manual point counting of an area of interest ranging from the bottom of the chamber (the ingrowth end) to the advancing new bone formation frontier, but comprising only the central third of the bone so that bone close to the titanium side walls was excluded. Points superimposing new bone or dead graft were counted and

recorded as “bone points”. The total number of “bone points” on the slide was divided by the sum of total measured points, and the mean for the slide calculated. The mean of three slides at a distance of 300  $\mu\text{m}$  was then used to determine the final value for each chamber.

The net amount of retained graft and new-formed bone within the remodeled graft was determined by calculating the volume of the remodeled bone cylinder (the radius<sup>2</sup> of the interior of the chamber  $\times \pi \times$  the ingrowth distance of new bone) times the bone volume/total volume in percentage.

## Clinical model

### *Hemicallotaxis osteotomy (HCO)*

High tibial osteotomy using the hemicallotaxis technique is a treatment option for younger and/or physically active patients with unicompartmental knee osteoarthritis or knee deformities. HCO is an open wedge technique, based on an external fixation, and both HA-coated and uncoated pins are used (Magyar et al. 1998). The angle deformity is successively corrected postoperatively under radiographic control. HCO is a procedure with high demands on pin fixation, due to early weight bearing combined with forces necessary for the angular correction. All this makes the HCO an appropriate and repeatable clinical model for studying bone and fracture healing including pin fixation, and to investigate the effect of a bisphosphonate. The osteotomy is performed in well vascularized metaphyseal bone where access to circulation and cells is high. It resembles a closed fracture animal model and there is no need to bolster the anabolic part of the fracture healing as in an open fracture with compromised bone formation (Ristiniemi et al. 2007; Little et al. 2005). Because the patients are younger (35–65 years) the risk of osteoporosis as a confounding factor is low in this model.

### *Surgical procedure*

The HCO was performed as an out-patient procedure using the Orthofix<sup>®</sup> T-garche as external fixator. The two proximal holes in the metaphyseal bone were made with a 3.2 mm drill and the distal holes in cortical bone with a 4.8 mm drill. Four conical stainless steel pins were inserted (6/5 mm

diameter), two of which HA-coated (Orthofix® Bussolengo, Italy) in the metaphyseal bone and two un-coated pins in the diaphyseal bone (Orthofix®). The HA coating was applied by plasma spraying, with an HA-layer thickness of 45–70 µm (Magyar et al. 1998). The pins must penetrate the posterior cortex with 2–3 threads for best fixation, which was controlled in fluoroscopy. A 5-cm longitudinal skin incision was made ventral to the tibial tuberosity. The osteotomy was done at the level of the distal third of the tibial tuberosity. The osteotomy was tested and judged to be sufficient if the gap could easily be opened 4–5 mm. For valgus deformity, the surgical procedure was identical except that a fibulotomy was performed 10–15 cm below the head of the fibula (Magyar et al. 1998). The patients were allowed free mobilization and full weight bearing after the operation. No prophylactic antibiotic was used. Pin site care was carried out once a week using chlorhexidine-moistened gauzes as dressings in the orthopedic outpatient clinic. In case of pin site infections oral antibiotic treatment (flucloxacillin 1g × 3) was given for 7 days.

#### *Callus distraction*

The distraction started seven to ten days postoperatively. The patient made the correction by adjusting one quarter of a turn 4 times per day on a distractor placed at the external fixator, the first turn in the morning and the last turn not later than 5 pm to avoid pain at night. This corresponds to 1 mm or 1° per day. The correction was measured by the hip-knee-ankle angle (HKA angle) of the knee during the corrective phase until the desired correction, 4° valgus for the varus knee and 0–2° varus for the valgus knee with respect to the error of measurement (2 degrees). When the desired correction was achieved the instrument was locked.

Eight weeks postoperatively, the fixator was dynamized.

#### *Administration of bisphosphonate*

Four weeks postoperatively the patient received an infusion either of zoledronate 4 mg (Zometa®, Novartis Pharma GmbH, Basel, Switzerland) or sodium chloride 9 mg/ml. The infusion of zoledronate was prepared by diluting 4 mg zoledronic acid in 100 ml sodium chloride 9 mg/ml and was given as a 15-min intravenous infusion.

#### *Evaluation*

##### *Time to clinical healing*

Eight weeks postoperatively a radiograph of the lower leg was performed, without loading and 10 weeks postoperatively came the first evaluation of healing on radiographs and ultrasound. The ultrasonic examination shows unmineralized callus formation in the distracted area earlier than conventional radiographs. When the callus was judged to be sufficient by radiographic and ultrasonic examinations, the fixator was removed and the patient was encouraged to practice full weight bearing for 1 or 2 days before the pins were eventually extracted. If the healing of the bone was not clinically satisfactory, the patient was checked every second week radiographically and by ultrasound examination until healing of the bone was satisfactory (Magyar et al. 1998).

##### *HKA angle*

The HKA angle was measured radiographically with anteroposterior and lateral views of the lower limb (hip, knee and ankle) with the patient standing in a weight-bearing position. By drawing a line from the centre of the femoral head to the midpoint of the tibial eminential spine and another line from this midpoint to the centre of the talus surface of the ankle joint, the mechanical axis of the limb can be calculated (Siu et al, 1991). The medial angle between the lines is the HKA angle (varus < 180°). The accuracy and reproducibility of measurement of the HKA angle has been shown to be within 2 degrees (Odenbring et al. 1993).

Radiographs were taken a mean 20 months (SD 2) after surgery with measurement of the HKA angle to evaluate whether the correction was retained. All radiographs were analyzed and measured by one radiologist. The value of the HKA angle was compared to an estimated angle of the correction i.e. 4° valgus for the varus knee and 0–2° varus for the valgus knee. This was used as a measure of the retention of the correction.

##### *DEXA*

Dual Energy X-ray Absorptiometry (DEXA) of the operated lower leg and bilateral proximal femur was performed 10 weeks postoperatively. The fixator was removed but the pins were still in place. The DEXA scans were centered anteroposteriorly

over the osteotomy and 2–3 scans were performed to avoid over-projection of the fibula. Only one of the scans was used for evaluation. The region of interest (ROI) was centered over the osteotomy including the edges. When the osteotomy gap was almost consolidated at the DEXA images, the borders of the osteotomy were measured on the corresponding X-ray and transferred to the DEXA image. The bone mineral density (BMD; g/cm<sup>2</sup>) and the bone mineral content (BMC; grams) were measured. The total T-score of the proximal femur was used to evaluate whether the patients had osteoporosis (<−2.5 SD), osteopenia (−1 to −2.5 SD) or normal bone. All scans were performed using GE Lunar Prodigy 16196 (Lunar BMP Products, Madison, WI, USA).

### *KOOS*

The patient-relevant outcome measure Knee injury and Osteoarthritis Outcome Score (KOOS) was filled in preoperatively, at 8 and 10 weeks and then every second week postoperatively until extraction of the external fixation, as well as at the 20-week and 1½-year follow-up. KOOS is a 42-item self-administered knee-specific questionnaire based on the WOMAC index (Roos et al. 1998) and comprises five subscales: pain, symptoms, activities of daily living (ADL), sports and recreation function and knee-related quality of life (QOL). Standardized reply options are given in 5 Likert boxes and each question is scored 0 to 4. A percentage score from 0 to 100 is calculated for each subscale 100 points representing the best possible results. A dif-

ference of 8 points was considered a clinically significant difference (Roos and Lohmander 2003).

### *PPI*

Both the insertion and extraction torque forces (Nm) of the pins were measured using a torque force screwdriver (range 0–1100 Ncm; Orthofix® SRL, Italy). The pins were inserted by the surgeon in a slow clockwise turn. The torque was measured during the whole insertion procedure and the peak torque was registered as the pin engaged the second cortex. The extraction was done in a slow counterclockwise turn and the maximum peak torque recorded immediately as the screw loosened. The pins were removed in the out-patient clinic.

The pin performance index (PPI) was calculated from the insertion and extraction torque. PPI is the ratio of extraction to insertion torque and expressed as percentage (Lawes et al. 2004). An equal insertion and extraction torque would result in a PPI of 100%. The PPI gives the information on how the fixation is proceeding over time.

### *Pin site infection*

The clinical symptoms of a pin site infection are redness of the skin, discharge from the pin site, pain and tenderness in the soft tissues, alone or in different combinations. Tenderness and pain around the pin site often occur one or two days before other signs of infection (Gordon et al. 2000; Hedin 2003). The need (number of patients) and use (days/treated patient) of antibiotics were used as outcomes of pin site infection.

# Methodological considerations

## Animals

The use of laboratory animals in medical experiments allows large series to be performed and thereby the outcome to be treated statistically, all with the intention to change only one factor at a time. Surgical intervention and drug delivery are controlled and local conditions are consistent, which provides relatively reproducible and quantifiable information. The animals are generally obtained from the same breed and living environment.

The adult male rat weighs 450–520g and the female 250–300g. Their general food intake is 10g/100g bodyweight per day and they mainly eat during the night. Rats normally have a life of 3–4 years, are sexually mature at 7–8 weeks and suited for breeding at 9–16 weeks of age. Rats are popular for animal experiments as homogeneous populations are available at low costs, but their physiology differs from that of humans. In rat bone, collagen constitutes only approximately 60% of their organic matrix compared to 90% in humans (Jorgensen et al. 1991), thereby influencing the mechanical properties of the bone. The cortical bone is subdivided into an outer zone of concentric lamellar bone and an inner zone of more irregularly oriented non-lamellar bone, with the outer lamellar zone not occurring until after the age of 3 months (Danielsen et al. 1993). Rats in our two experimental studies were male Sprague-Dawley rats weighing 382–425g and 320–360g respectively. The rationale for using male rats in the experiments was to avoid the effect of the polyestral cycle in the female rat and also because the male rat is larger and its tibia is more suited for the BCC. The rat tibia undergoes remodeling due to growth, which might influence the results. The cellular mechanism involved in bone loss or gain is much the same as in humans, and rat skeleton provides an appropriate model when evaluating the principal effects of therapeutic agents on bone mass and bone structure (Frost and Jee 1992; Sandhu and Khan 2002). These rats have bone with

a healing and remodeling capacity much larger than in humans.

## The bone chamber

The measurement error, methodological or analytical, of new bone ingrowth into a graft in the BCC, i.e., the intraindividual error with repeated measurements, has been reported to be 6% (Thorén 1994) and the interindividual error has been reported to be 8% (Tägil 2000). By these standards the method has a high reliability. The bone density measurement by manual point counting was made on the central third of the specimens. The ingrowth distance varies in different parts of the specimen and thus the mean of all three slides was used to determine the final value for each chamber. All measurements were made blinded by one laboratory technician.

## Dose of BMP and bisphosphonate

The BMP treated grafts in study 2 were soaked in a dilution corresponding to 1 $\mu$ g BMP-7 per graft. The same dose of BMP-7 has been used in earlier studies with the BCC in rats and has shown to increase the ingrowth into impacted graft and thereby overcome the decreased ingrowth caused by impaction (Tägil et al. 2000). The dose was also used in a study where BMP-7 was combined with local treatment of the graft with clodronate (Jeppsson et al. 2003). In a rat critical defect model both 11 and 50  $\mu$ g, i.e. significantly higher doses, induced significant osteogenic response (Chen et al. 2002; Little et al. 2005). In our first study the rats were given a weekly dose of 1.05  $\mu$ g of zoledronate. The dose was calculated by the manufacturer of the drug to correlate to the dose of alendronate given in preceding experiments by our group. In the second study the bisphosphonate was given as a single injection of zoledronate 0.1 mg/kg. This would correspond to the human dose of 5 mg dose

of the zoledronate dose approved for humans in osteoporosis.

### Pin Performance Index

Both the insertion and extraction torque forces of the pins were measured using a torque force screwdriver. Before measuring, the torque force screwdriver was reset to zero and if needed the instrument was adjusted so that the starting point for the measurement always was zero. We had one torque force screwdriver in the operating theater and one in the outpatient clinic which was considered more convenient and we did not change during the study time. All patients were randomized so any measurement error would be equally distributed between the two groups.

The pins were inserted in a slow clockwise turn. The torque was measured during the whole insertion procedure and the peak torque was registered as the pin engaged the second cortex. The extraction was done in a slow counterclockwise turn until the pin suddenly released and the torque measured at this point was recorded as the extraction torque. This corresponds to the yield point in a stress/strain diagram. This method of measuring the insertion and extraction torque force has been used in many clinical studies. In studies with the same operation technique and the same type of pins, Moroni used an electronic torque wrench connected to the pin. A comparison of the results from these studies with our results using the torque force screwdriver yielded comparable values (Moroni et al. 2001).

The ratio of extraction and insertion torque is expressed as a percentage and defined as PPI. An equal insertion and extraction force would result in a PPI of 1 or 100% (Lawes et al. 2004). The PPI gives the information about how the fixation is proceeding over time. We are interested in how the contact area between pin and bone develops over time as a reaction to the surgical trauma, HA coating, correction, weight bearing etc. Altogether 15/184 single pin measurements are missing. We did not exclude these patients in the calculation of insertion and extraction forces, but it was not possible to calculate PPI.

### Fracture healing (outcome)

In the clinical situation, the decision as to when a fracture is healed is often based on a combination of clinical and radiographic criteria. In the scientific studies too, the combination is most often (62%) used (Corrales et al. 2008) but in 37% the decision was based on the radiographic criteria only and in 1% on clinical criteria only. The most common clinical criteria were absence of pain or tenderness during weight bearing, the absence of pain or tenderness on palpation or physical examination, and the ability to bear weight. The most common radiographic definitions of fracture healing in studies involving the use of plain radiographs, were bridging of the fracture site by callus, or signs of trabeculas or bone (53%); bridging the fracture site at three cortices (27%); and/or obliteration of the fracture line and/or cortical continuity (18%). When both orthopedic surgeons and radiologists are involved in the judgment, the radiologists often give a more conservative measurement. In the literature there is a lack of consensus about to the definition of fracture healing (Corrales et al. 2008). Richardson et al. measured the stiffness of tibial fractures to define a biomechanical healing time. They suggested that 15 Nm/degree is a reasonable, if slightly conservative, definition of healing of the tibia (Richardson et al. 1994). The method may be more suitable for external fixation in diaphyseal fractures than for external fixation in HCO.

At ultrasound examination of the fracture site the unmineralized callus can be visualized and echogenic foci representing early ossification foci are seen. These echogenic areas are often not detected radiographically (Maffulli and Thornton 1995). Ultrasonography can be used, at least in animals, to document complete fracture healing earlier than conventional radiography (Risselada et al. 2005).

In our clinical studies the first evaluation of healing on radiographs and ultrasound was performed 10 weeks postoperatively. When the callus was judged to be sufficient by radiographic and ultrasonic examinations, the fixator was removed and the patient was encouraged to practice full weight bearing for 1 or 2 days before the pins were eventually extracted. If the healing of the bone was not clinically satisfactory, the patient was checked every second week radiographically

and by ultrasound examination until healing of the bone was satisfactory (Magyar et al. 1998). An earlier first evaluation of the healing might possibly have revealed a difference between the groups, as some patients had sufficient healing at 8 or 9 weeks. More frequent examination could also have revealed a difference between the groups, but there is a limit to how often we can radiograph the patients. We believe a two-week interval is a reasonable interval as this difference in time to healing was what we deemed clinically relevant.

### Statistical analysis

In the experimental studies the number of animals used in the studies was based on previous experience, with 10 rats needed in each group to obtain a power of 80% at  $p=0.05$  in a two-sided test and with an estimated reduced ingrowth of 50%. In the first article the results were tested for significance using unpaired Student's t-test and because the response is continuous (numerical), the data has a normal distribution in relation to mean and standard deviation, and the standard deviations in

the two groups are comparable, we believe we can justify the use of a parametric test.

For the clinical studies a power analysis was performed based on the data from a pilot study as well as previous pin design studies. Twenty-five samples were needed in each group to obtain a power of 85% at  $p=0.05$  in a two-sided test, with an estimated mean difference of insertion and extraction torque force of 1.00 Nm (SD 1.5) between the bisphosphonate and control group and an estimated mean difference of healing time of 20 days between the bisphosphonate and control group. For each patient the mean value of the two proximal pins and the mean value of the two distal pins were calculated and used for the statistical analysis. For bilateral procedures, the index operation was used for statistical analysis. Unpaired Student's t-test was performed to test the differences between the groups. In the clinical studies, the sampled size of more than 20 samples in each group and again the fact that the response is continuous, the data have a normal distribution in relation to the mean and standard deviation, and the standard deviations in the two groups are comparable allow us to use a parametric test.

## Results of our own studies and discussion

### Experimental studies

#### *Manipulating bone density and ingrowth distance in the bone conduction chamber*

The bisphosphonate alendronate has previously been shown to be effective in preventing or delaying resorption of bone grafts. In previous studies, bone grafts were pretreated with alendronate, or alendronate was given systemically in different doses (Åstrand and Aspenberg 2002; Aspenberg and Åstrand 2002). In both modes of administration, the bisphosphonate treatment reduced the resorption of necrotic bone. A more potent bisphosphonate, zoledronate, also decreases the bone resorption during graft remodeling and when given systemically has the advantage of having to be administered less frequently. Zoledronate was prepared and commercially available as a solution, in contrast to alendronate.

Bone healing and bone graft remodeling can be influenced by adding both an anabolic and an anticatabolic drug like BMP and bisphosphonate. In bone chambers, BMPs increase bone ingrowth rate in non-vital bone grafts, but also cause an almost simultaneous resorption of almost all of the newly formed bone. BMPs can compensate for the decreased ingrowth caused, for example, by impaction of a graft (Tägil et al. 2000). The combination of a local bisphosphonate (clodronate) and BMP-7 caused both increased ingrowth and increased bone density in impacted grafts in the bone conduction chamber model (Jeppsson et al. 2003).

We investigated zoledronate in the BCC to see if it could be administered less frequently than alendronate and at the same time reduce the resorption to the same extent. We also investigated whether the combination of local BMP-7 and zoledronate given systemically as a single dose could increase the ingrowth distance into a bone graft and at the same time increase total bone volume.

The first study showed that zoledronate was equally effective in weekly injections as the thrice-weekly alendronate treatment. The total distance of the soft tissue ingrowth, which corresponds to

the revascularized but not remodeled parts of the graft, did not differ between the treated rats and the controls. A difference was found in bone density in the remodeled bone. In the controls, the frontier of active bone formation was thin and both the graft and the new-formed bone underneath this primarily formed bone appeared to be immediately resorbed and replaced by hematogenous or fatty bone marrow (Figure 11). In the grafts in rats treated with zoledronate, the graft and new-formed bone underneath the active bone formation front remained intact with new bone lining the graft trabeculae, leaving little space for the marrow (Figure 12). The bone density in the remodeled bone was 35% in the zoledronate-treated grafts compared to 19% in the controls ( $p=0.01$ ). (Table 5)

In the second study, the combination of a single injection of the potent nitrogen containing bisphosphonate, zoledronate and local BMP-7 was superior to zoledronate or BMP-7 alone. With the combination, both the BMP-induced increased ingrowth distance and the bisphosphonate-induced increased bone density were affected and the net amount of bone formed and retained in the BMP-7 + zoledronate group was increased five times compared to the untreated controls (Figures 13 and 14).

The bone chamber model is a stress-shielded model. The resorptive stimulus is high due to the stiff chamber walls protecting the graft from any stress leading to a mechanically triggered resorption. Despite this absence of strain in the ingrowing tissue, the default mode of tissue healing in close contact and within chemical reach of living bone is bone formation. Normally, in the stress-shielded chamber, all graft and new formed bone are resorbed as the remodeling is finished, and an anticatabolic drug such as a bisphosphonate is probably more effective than in a mechanically loaded environment. The results show not only a decreased resorption as expected, but also an increased amount of new bone in the remodeled area. This is probably the result of a delay in the resorption of the newly formed bone rather than an increased formation. However, the ingrowth distance of new bone into

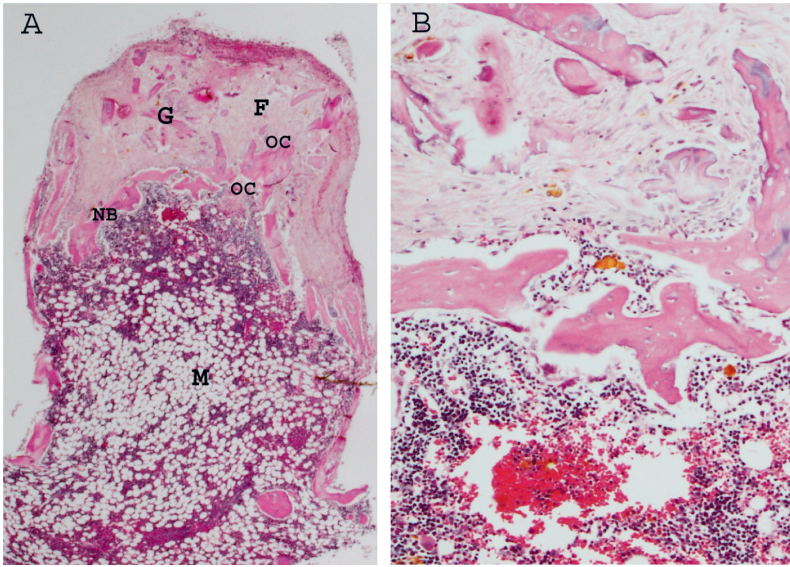


Figure 11. (A) Untreated control graft. The fibrotic tissue (F) is revascularized but the graft trabeculas (G) have not remodeled or started to resorb. The frontier of new formed bone (NB) advances as a thin rim into the graft, preceded by osteoclasts (OC). The new formed bone is resorbed immediately and replaced by haematogenous or fatty bone marrow (M). (B) Magnification of the borderzone between the remodeled and resorbed bone, the front of new formed bone.

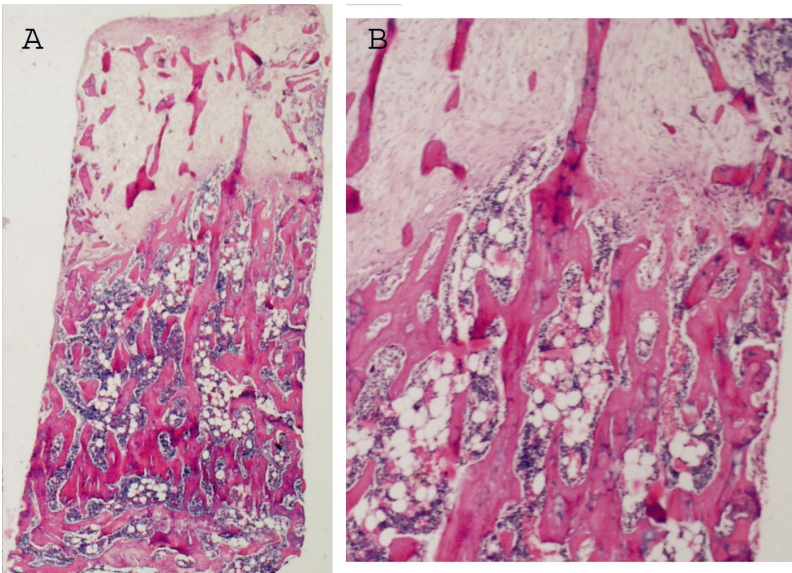


Figure 12. (A) Zoledronate treated graft. No marrow cavity has formed behind the ossification front and the graft trabeculas below are largely retained. (B) Magnification of the border zone with retained trabeculas in the remodeled area.

the graft is a measure which we consider correlates to bone anabolism. Although non-significant in the separate studies, the bone ingrowth distance

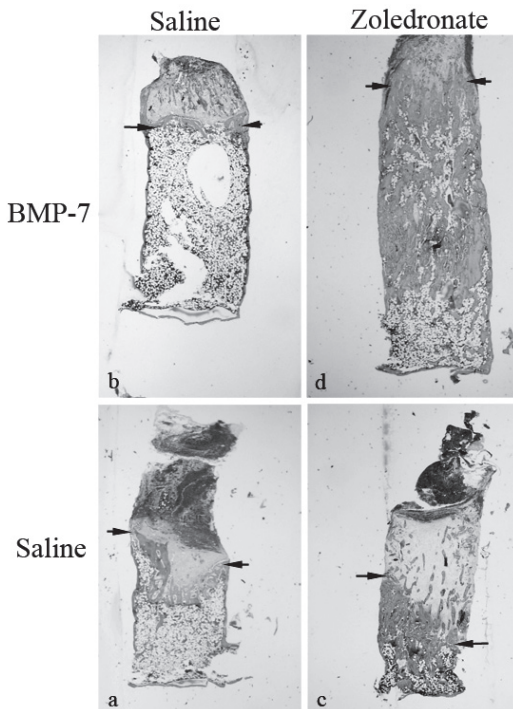
becomes significantly increased when all series are pooled, with a bisphosphonate-treated chamber group being compared with a saline group (unpub-



**Table 5. Bone volume in the remodeled area and ingrowth distance of new bone into the graft. Bone volume fraction expressed in percent of total tissue volume. Values are mean (standard deviation)**

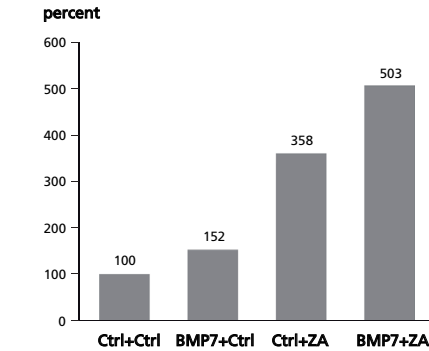
	Zoledronate	Control	P-value
New-formed bone (%)	22 (7)	14 (9)	0.03
Remaining graft bone (%)	13 (6)	5 (5)	0.008
Total bone (%)	35 (0.12)	19 (0.13)	0.01
Bone ingrowth distance into graft (mm)	2.65 (0.48)	2.12 (0.63)	0.052 ns
Vasculature ingrowth distance (mm)	4.2 (0.9)	3.9 (1.0)	ns

ns = not significant



**Figure 13.** (a) Untreated chamber specimen with remodeled bone graft after 6 weeks in the chamber. The bone ingrowth front is marked with arrows. (b) In the BMP-7 treated specimens, the bone ingrowth front (arrows) reached 50% to 100% further into the graft compared to controls. Most newly formed bone below the bone ingrowth front has already been resorbed and replaced by fatty marrow. (c) In the zoledronate treated specimen, the remodeled bone below the ingrowth front contained three to four times more bone than in the controls. (d) When both BMP-7 and zoledronate were given both an increased ingrowth distance and increased bone retention occurred.

lished data). One could interpret these results as showing that bisphosphonates are not only anti-catabolic drugs but also function as an anabolic



**Figure 14.** The net amount of bone in the remodeled part of the graft as a function of the ingrowth distance of new bone  $\times$  radius<sup>2</sup>  $\times$   $\pi$   $\times$  BV/TV for the drug treated grafts relative the untreated controls.

substance, as hypothesized in some in vitro (Im et al. 2004, Wedemeyer et al. 2005) and in vivo studies (Reinholz et al. 2000). Bisphosphonates, for example, have an antiapoptotic effect in osteocytes and osteoblasts (Plotkin et al. 2005). Several other explanations for the findings of an increased amount of newly formed bone can be discussed. In contrast to cortical bone, remodeling in cancellous bone does not require cutting cones to make space for new bone. In cancellous bone there is sufficient space for new bone to form, and often the new bone formation appears as appositional growth, covering the dead bone graft, which is not resorbed because it is bisphosphonate-treated. In consequence, a larger surface area exists to lay down new forming bone. The fact that we find an increased amount of newly formed bone does not necessarily mean that more bone has formed or that zoledronate is by any means anabolic. New-formed bone might simply just prevail for a longer period if bone

resorption is reduced. Before resorption starts in untreated grafts, due to the stress shielding, bone is gradually formed inside the chamber. We believe that the chemical signals, first from the damaged but living surrounding bone, and later from the bone entering/gradually growing in through the chamber openings, are responsible for the biologically driven bony ingrowth into the chamber. If we impede the mechanically driven resorption by an anti-catabolic drug, the bone front grows further into the chamber and we speculate that this could be a result of elevated “auto-signaling” of BMP from the osteoblasts and the retained bone.

In the BMP-7 treated graft, the newly forming bone grows into the dead graft at almost twice the speed of untreated controls (Tägil et al. 2000). However, increasing the anabolism pharmacologically leads to unexpected consequences. BMPs are differentiating paracrine proteins acting on a variety of cells in bone remodeling, not only bone-forming cells but also osteoclasts (Kaneko et al. 2000; Wutzl et al. 2006; Jensen et al. 2009). In the chamber, the results of the osteoclast activity can clearly be seen as the ingrowth front sweeps into the dead graft (Figure 13 b). The newly formed bone and the graft are immediately resorbed if not treated by an antiresorptive agent. In a weight-bearing non-cemented knee prosthesis model in rabbits, treatment of impacted bone grafts by BMP-7 did not increase bone density, probably because instability caused increased resorption of both old graft and newly formed bone (Tägil et al. 2003).

In experimental fracture models, postponing the catabolic response and in consequence the resorption of the new-forming callus leads to a stronger callus (Amanat et al. 2005; Li et al. 2000). In osteonecrosis of the femoral head this inhibition of bone resorption prevents collapse of the femoral head (Ramachandran et al. 2007; Aya-ay et al. 2007). In the clinical situation of a healing fracture or bone graft, the balance between catabolism and anabolism is modulated by the mechanical situation. In analogy to our stress-shielded chamber, a low stress environment such as a rigidly fixed fracture would cause a resorptive stimulus, which would then be further boosted by the BMP-7. In another bone chamber study in rabbits allowing motion, the response to BMP-2 switched from net

resorption to net formation when micromotion was added (Bostrom et al. 1998). Fixation of a fracture or pseudarthrosis using an intramedullary nail, especially if locked and non-dynamized, would increase the catabolic stimulus. In fractures with already adequate anabolic drive, BMP-7 might only boost catabolism, and the addition of an anti-catabolic drug would hypothetically increase the proportion of healed fractures. At the other end of the spectrum of mechanical stimulation, a high or too high stress environment, for example, in the vicinity of a semistable prosthesis or an unstable fracture, also leads to an increased resorptive stimulus. Similarly, too high or too low mechanical stimulation allows the resorption to be further boosted by BMP-7.

Open tibial fractures treated with intramedullary nail fixation and an implant containing BMP-2 have reduced the frequency of secondary interventions, accelerated fracture and wound healing, and reduced infection rate compared to fractures treated with intramedullary nail fixation and routine soft-tissue management. The rate of union, however, is similar to autograft (Govender et al. 2002). In tibial non-unions, the healing rate was 85% when BMP-7 was used, which is on par with but not superior to autograft (Friedlaender et al. 2001). In distal tibial pilon fractures treated by external fixation, BMP-7 has been found to accelerate the healing in a non-randomized but paired study (Ristiniemi et al. 2007).

In conclusion, the experimental studies showed that zoledronate given in weekly injections was equally effective as the thrice-weekly alendronate treatment. Increasing the dose, zoledronate was found to be effective also given as a single injection for the whole study period of 6 weeks. Given as a single dose, the combination of zoledronate with BMP-7 increased new bone formation and at the same time protected the graft from premature catabolism. We believe that the combination of an anabolic and an anti-catabolic drug might be a potent bone graft enhancer and a powerful tool to treat non-unions or to prophylactically prevent delayed or non-union in some difficult, compromised fractures. With the combination of BMP with an anti-resorptive drug, BMP can perhaps be made to perform better than autograft.

## Clinical studies

### *Bisphosphonate effect on fracture healing and pin-fixation*

The HCO is a human, relatively standardized and relatively frequent model of bone healing in a non-compromised environment. As in the bone graft remodeling in the BCC, both anabolic and anticatabolic drugs, separately, can affect fracture healing. The efficacy and mode of action of the drugs appear to differ between open and closed fractures (Tägil et al. 2009). In open or in other ways compromised fractures, a specific anabolic drug like a BMP sometimes appears to be necessary (Little et al. 2005; Ristinieni et al. 2007), whereas for a fracture in a cell-rich, well vascularized environment, an anti-catabolic drug might be sufficient (Little et al. 2005). In our study, the osteotomy is performed in well vascularized metaphyseal bone where access to circulation and cells is high. It resembles a closed fracture animal model and there is no need to bolster the anabolic part of the fracture healing as in an open fracture with compromised bone formation.

The healing time in our study was not affected by a single infusion of zoledronic acid. The healing time was the same in both groups, but a true shortening of the healing time in both groups compared to previous studies was achieved, unrelated to the drug treatment. Also, the bone mineral density and bone mineral content were not affected by the treatment. A fear that a too early extraction of the external fixator would cause a loss of the achieved correction could not be verified at the late radiographic control, the correction was maintained over time (Table 6). The result of the KOOS questionnaire improved over time and there was no difference between the groups.

The bisphosphonate was given four weeks postoperatively. The choice of time point to administer the drug was based on the animal experiments. Bisphosphonates bind to bone mineral, and when given at the time of ongoing fracture healing, a larger proportion binds to the fracture site than, for example, at the time of the osteotomy. In animals, delayed administration of zoledronate has been shown to have a superior effect compared both to saline and to zoledronate administered immediately at the time of the fracture (Amanat et al.

**Table 6. DEXA 10 weeks postoperatively. Change in HKA 20 months postoperatively compared to achieved HKA angle at the primary healing time point. Values are mean (standard deviation)**

	Zoledronate	Control	P-value
DEXA	n = 24	n = 20	
BMD (g/cm <sup>2</sup> )	1.14 (0.27)	1.01 (0.18)	0.1
BMC (g)	6.2 (2.3)	5.2 (2.3)	0.2
HKA change (degrees)	n = 25 0.3 (3.3)	n = 19 -1.0 (3.3)	0.2

2007). A second reason to administer the bisphosphonate at four weeks was to avoid disturbing the initial bone formation, which has been feared in both in vitro (Im et al. 2004) and in vivo studies (Jacobsen et al. 2007).

The pin-bone interface is important and maybe the most critical for the treatment by external fixation. Stable pin fixation minimizes the risk of complications such as pin loosening, delayed healing, non-union and pin site infection (Moroni et al. 2001). By reducing the resorption of the bone surrounding an implant, increased fixation can theoretically be achieved. Bisphosphonate treatment of the bone adjacent to a screw has been shown to be mechanically effective in experimental pull-out tests of screws. By both local and systemic treatment, an enhanced extraction torque and pull-out strength was found (Skoglund et al. 2004). For HA-coated (Eberhardt et al. 2007) or porous implants (Bobyn et al. 2005) the osseointegration was also accelerated by administration of bisphosphonates.

In our clinical study, we were able to show improved fixation, in patients operated on by the HCO and treated with a single dose of zoledronate, of the non-coated pins in the cortical diaphyseal bone distally but not of the HA-coated pins in the cancellous metaphyseal bone proximally (Table 7). Zoledronate almost doubled the fixation of the non-coated pins.

In high tibial osteotomy using the hemicallotaxis technique, both HA-coated and standard pins are used. The method makes high demands of pin fixation due to early weight bearing combined with large forces at the angular correction (Magyar et al. 1997). A high load is carried by the pins and the contact pressure at the interface between the pin

**Table 7. Insertion/extraction torque and Pin Performance Index (PPI) in metaphyseal and diaphyseal bone. Values are mean (standard deviation)**

	Zoledronate n=25	Control n=21	P-value
Metaphyseal bone			
Pin insertion torque (Nm)	2.0 (0.6)	1.5 (0.6)	0.02
Pin extraction torque (Nm)	4.7 (1.3)	4.0 (1.3)	ns
PPI (%)	257 (106)	289 (115)	ns
Diaphyseal bone			
Pin insertion torque (Nm)	7.0 (2.0)	7.0 (1.7)	ns
Pin extraction torque (Nm)	4.5 (2.1)	2.4 (1.0)	<0.0001
PPI (%)	62 (23)	35 (14)	<0.0001
ns = not significant			

and bone might be high enough to induce resorption around the pin, especially during the hemicallosis procedure/correction phase, and could lead to pin loosening. Magyar et al. (1997) compared HA-coated and standard pins and found that standard pins in cancellous bone all became loose in contrast to HA-coated ones. In the cortical bone no such loosening was found, but the standard pins lost about 40% of their grip during the fixation time. In contrast, the HA-coated pins increased the fixation/insertion torque (Magyar et al. 1997). The increase in fixation in the cortical bone in our study, caused by the zoledronate treatment, is on a par with the effect of the HA coating.

In another study in patients with hip fractures treated with external fixation, the extraction torque force of the pins in the cancellous bone was almost doubled, but not for the pins in the cortical bone in those given bisphosphonates. HA-coated pins were used in both the femoral neck and the femoral shaft (Moroni et al. 2007). Compared to our study these patients were older and the rate of osteoporosis probably higher. Considering this, the pin fixation in our study might be better already at insertion, and the insertion torques were found to be higher in the present study of osteoarthritis patients than

in the hip fracture patient study. In hip fracture fixation, the pins in the femoral head rely on cancellous bone fixation only, whereas in the knee each cancellous pin still has a bicortical grip. The single dose systemic mode of application may not be the final solution to manipulate pin fixation. Perhaps local application or coating of the pins with bisphosphonates (Skoglund et al. 2004; Wermelin et al. 2007) will be more effective, thereby avoiding the well-known side effects of the drug when given intravenously.

With the experience from our animal experiments, we used a potent third-generation bisphosphonate, zoledronate, which was administered as a single infusion four weeks after surgery. A single infusion of zoledronate has an effect on the bone remodeling since it almost doubled the pin-fixation in the cortical diaphyseal bone. As regards the fracture healing time no such effect could be detected. Perhaps a higher dose, a different time of administration or a continuous administration would have an effect. However, a higher dose could elevate the risk of side effects and unwanted effects such as local toxic effects in the osteoblasts, and continuous administration can lower compliance with the treatment.

## Conclusions

1. Zoledronate given in weekly injections is equally effective as alendronate daily or three times a week in decreasing the resorption of graft or new-formed bone.
2. The combination of BMP-7 and a single dose of zoledronate increases new bone formation and protects at the same time protects the new-formed bone from premature catabolism.
3. Zoledronate increases fixation of non-coated pins in diaphyseal bone in humans.
4. Zoledronate, as a single dose, does not decrease fracture healing time in humans.

## Future studies

Bisphosphonate treatment affects bone remodeling and thereby the bone-pin interface. The treatment with one single infusion of zoledronate improved the fixation of non-coated pins in diaphyseal bone, but no effect was seen on the fixation of the HA-coated pins in metaphyseal bone. This single dose systemic mode of application may not be the final one, but we believe the concept is an interesting way to improve pin fixation. We are aware of other studies in progress, for example with local application or direct coating of the pins with bisphosphonates. Since the study was designed for fracture treatment and therefore a delayed administration was chosen, one can only speculate about what would have happened if we had given the BP immediately postoperatively. Would it be possible to replace the HA pins by uncoated pins in the metaphyseal bone as well? We know we cannot do so without treatment. The method of using bisphosphonates to improve pin fixation is definitely not finalized for clinical application, and more studies are necessary.

Bisphosphonates are disputed at the present due to the problem of ONJ, and alternative anticata-

bolic substances would be welcome. Drugs other than bisphosphonates are in the pipeline for introduction into the field of osteoporosis, such as OPG, denosumab and sclerostin. When treating fractures and non-unions, a long-term effect of anti-catabolism is unnecessary, in contrast to osteoporosis, and short-acting alternatives would be attractive. Several alternatives are about to be launched as osteoporotic treatment and should be attractive to test regarding fracture healing as well.

BMPs have been tested in clinical studies of fractures and non-unions, but the combination of BMP with a bisphosphonate has never been investigated in a clinical randomized study. Animal studies by ourselves and others show a superior effect with the combination, and it would be thrilling to see if this combination affected the healing rate of non-unions in humans and proved better than autograft. Finally, the two tested drugs in this study have their downsides. BMP is expensive and in its current form difficult to handle. Maybe other substances can be tested also in fractures and non-unions such as bone active peptides, which are much cheaper to manufacture than the recombinant proteins.

## Summary

Drug treatment is one of the most successful methods to affect the course of disease in modern medicine, but in orthopedic surgery it is used surprisingly seldom. A better understanding of the biological prerequisites of fracture healing or other diseases or conditions in orthopedic surgery, together with the research and development of bone modulating pharmaceuticals in other areas, especially osteoporosis, has given us new tools to work with. Healing and normal physiological skeletal repair involve a complex set of regulated signaling pathways that control the formation of new bone matrix and the resorption of damaged bone at the disease or injury site. Pharmaceutical substances, both anabolic and anticatabolic, have been used to modulate fracture healing. Anabolic drugs such as PTH or BMP influence the osteoblasts and thereby increase the bone formation. Anti-catabolic drugs, such as bisphosphonates, reduce osteoclastic activity and in fracture healing this leads to increased strength of healing by retaining the new-forming callus. In the present study we address two separate tasks in fracture healing: (1) if fracture healing can be faster or rather achieved earlier; and (2) if fracture healing can be better, i.e. decrease the incidence of pseudarthrosis/delayed healing and offer a solution to pseudarthrosis/delayed healing as they occur. Four hypotheses were suggested to address these questions:

1. Is it possible to prolong the time between the doses with a bisphosphonate, also in fracture healing as the trend has been in osteoporosis treatment?
2. Is it possible to improve the net biological effect of the BMPs by controlling the resorption, thereby increasing the amount of callus?
3. Is it possible to replace the expensive, hard-to-handle anabolic drugs with an anti-catabolic drug such as a bisphosphonate?
4. Is it possible to use the biologic effect of the bisphosphonate to improve pin fixation?

In the first two studies, bone chambers were implanted in rats and the distance of new bone ingrowth into the graft and bone density were mea-

sured. In Paper I, subcutaneous injections of zoledronate 1.05  $\mu\text{g}$  were given at day 4 postoperatively and then weekly until harvest. The controls were given the same amount of saline solution with the same regime. We concluded that zoledronate was equally effective in weekly injections as the thrice-weekly alendronate treatment. The bone density in the remodeled bone was 35% in the zoledronate-treated grafts compared to 19% in the controls.

In Paper II, one subcutaneous injection of zoledronate 0.1 mg/kg was given at day 14. The controls were given a subcutaneous injection of 0.4 mL NaCl. The graft in the chamber at the right side was soaked with 8  $\mu\text{L}$  of a dilution corresponding to 1  $\mu\text{g}$  of BMP-7 per graft just before implantation. The control grafts at the left side were soaked with 8  $\mu\text{L}$  buffer alone. The results show that the combination of zoledronate and BMP-7 was superior to zoledronate or BMP-7 alone. Both the ingrowth distance and the bone density were increased and the net amount of bone formed and retained in the BMP-7 + zoledronate group was increased five times compared to the untreated controls.

In the two subsequent clinical studies, high tibial osteotomy by the hemicallotaxis technique was used as a clinical model to study fracture healing and pin fixation. Four weeks postoperatively the patients were randomized to receive either an infusion of zoledronate 4 mg or sodium chloride 9 mg/ml. In Paper III the insertion and extraction torque forces (Nm) of the pins were measured and the pin performance index was calculated from the insertion and extraction torque. Zoledronate almost doubled the fixation of the non-coated pins in the diaphyseal bone, but no effect was seen on the fixation of the HA-coated pins in the metaphyseal bone.

In Paper IV, we evaluated whether a single infusion of zoledronate can decrease the time to clinical osteotomy healing. Dual Energy X-ray Absorptiometry was performed 10 weeks postoperatively. Radiographs were taken at the same time and then every second week until clinical healing. After 18 months, an additional radiograph was performed

and the hip-knee-ankle angle measured to evaluate whether correction was retained. The healing time was not affected by a single infusion of zoledronate and there was no difference in bone mineral density or bone mineral content between the groups. Both groups had retained the acquired correction.

In conclusion, zoledronate given in weekly injections is equally effective as the thice-weekly

alendronate treatment. Given as a single dose, the combination of zoledronate with BMP-7 increases new bone formation and at the same time protects the bone from premature catabolism. A single infusion of zoledronate has an effect on bone remodeling since it almost doubled the pin-fixation in the cortical diaphyseal bone. As regards the fracture healing time, no such effect could be detected.



## Summary in Swedish

Läkemedelsbehandling är en av de mest framgångsrika metoderna för att påverka sjukdomsförlopp inom modern medicin men har använts förvånansvärt lite inom ortopedi. En ökad förståelse för de biologiska förutsättningarna för frakturläkning eller andra sjukdomar och tillstånd inom ortopedin tillsammans med framtagande av benaktiva mediciner inom andra områden, framförallt osteoporos, ger oss nu helt nya verktyg att arbeta med.

Frakturläkning men även normal fysiologisk benomsättning innefattar komplexa välreglerade signalvägar som kontrollerar både bildandet av ny benmatrix och resorption av skadad benvävnad på fraktur stället. Både anabola och anti-katabola läkemedel har använts för att påverka frakturläkning. Anabola läkemedel, som PTH och BMP, påverkar osteoblasterna och ökar därigenom bildandet av ny benmatrix. Anti-katabola läkemedel, som bisfosfonater, minskar osteoklasternas aktivitet. I samband med frakturläkning leder detta till att den nybildade kallusen inte resorberas och därigenom starkare läkningsvävnad. I denna avhandling inriktar vi oss på två separata frågor inom frakturläkning: (1) om frakturläkning kan ske snabbare eller uppnås tidigare och (2) om frakturläkning kan förbättras dvs minska incidensen av pseudartros/förlängd läkningstid men också att kunna erbjuda en lösning när det förekommer. Fyra hypoteser föreslogs:

1. Är det möjligt att förlänga tiden mellan doserna av en bisfosfonat i samband med frakturläkning på samma sätt som det har visat sig vara möjligt i samband med behandling av osteoporos?
2. Är det möjligt att förbättra den biologiska effekten av BMP genom att farmakologiskt kontrollera resorptionen och därigenom öka mängden kallus?
3. Är det möjligt att ersätta de dyra, svårhanterbara anabola läkemedlen med anti-katabola läkemedel som t ex en bisfosfonat?
4. Är det möjligt att använda den resorptionshämmande effekten av en bisfosfonat för att öka pinnfixation vid extern fixation?

I de två första studierna implanterades benkon-

duktionskammare i råttor och inväxtdistansen av nytt ben och bendensiteten mättes. I studie I gavs 1.05 µg zoledronat subkutant på 4:e dagen efter operationen och sedan en gång per vecka fram tills avlivning efter 6 veckor. Kontrolldjuren gavs på samma sätt motsvarande volym saltlösning. Zoledronat givet som injektion en gång per vecka visade sig lika effektivt som alendronat givet tre gånger per vecka. Bendensiteten i det remodelerade området var 35 % i den zoledronatbehandlade gruppen jämfört med 19 % i kontroll gruppen.

I studie II gavs 14 dagar efter operationen 0.1 mg/kg zoledronat som subkutan injektion. Kontrolldjuren gavs en subkutan injektion av 0.4 ml NaCl. Bengraften i kammaren på den högra sidan var indränkta i 8 µL av en lösning motsvarande 1µg BMP-7/graft före implantationen. Kontrollgraften på vänster sida var indränkta i 8µL av enbart buffertlösning. Resultaten visar att kombinationen av zoledronat och BMP-7 är överlägsen zoledronat eller BMP-7 ensamt. Både inväxtdistansen och bendensiteten ökade och den totala mängden nybildat och bevarat ben ökade femfalt i zoledronat+BMP-7-gruppen jämfört med obehandlade kontrollerna.

I de två följande kliniska studierna användes hög tibiaosteotomi med hemikallotasisteknik som en klinisk modell för att studera frakturläkning och pinnfixation. Fyra veckor postoperativt randomiserades patienterna till antingen en infusion av 4 mg zoledronat eller 9 mg/ml saltlösning. I studie III mättes insättnings- och utdragskraften (Nm) av pinnarna och pin performance index räknades ut. Zoledronat nästan fördubblade fixationen av obehandlade pinnar i diafysärt ben, men ingen effekt sågs på fixationen av hydroxyapatitbehandlade pinnar i metafysärt ben.

I studie IV utvärderades om en injektion av zoledronat kan minska tiden till klinisk läkning av osteotomi. Tio veckor postoperativt genomfördes DEXA-mätning. Röntgen genomfördes samtidigt och varannan vecka fram tills klinisk läkning. Efter 18 månader genomfördes röntgen med mätning av HKA-vinkel för att se om korrektionen var bibehåll-

len. Läkningstiden påverkades inte av en infusion av zoledronat och DEXA-mätningen visade ingen skillnad mellan grupperna av benmineraldensitet eller benmineralinnehåll. Båda grupperna bibehöll sin korrigering i lika stor utsträckning.

Sammanfattningsvis fann vi att zoledronat givet som veckoinjektion är lika effektivt som alendro-

nat givet tre gånger per vecka. Kombinationen av en dos zoledronat och BMP-7 ökar benbildningen samtidigt som benet skyddas från förtidig nedbrytning. En infusion av zoledronat påverkar benremodelleringen eftersom den nästan fördubblar pinnfixationen i kortikalt diafysärt ben. Någon liknande effekt på frakturläkningstiden kunde inte ses.

## Acknowledgments

I would like to express my sincere gratitude to:

Magnus Tägil, my mentor, whose encouragement, supervision and support from the preliminary to the concluding level enabled me to develop an understanding of the subject. Without his guiding, patience, advice, support and pushing, this work would not have been realized.

Annette W-Dahl, my assistant mentor, for always having the time to explain and share her knowledge.

Sören Toksvig-Larsen, co-author who performed all the HCO surgery, for valuable advice.

My co-authors Jörgen Åstrand and Mats Geijer.

Mats Christensson for making the chambers.

Research colleagues in the "Tägil group", for inspiring discussions and support.

Inger Mårtensson for technical support and Monica Lilja for DEXA support.

Friends and colleagues at the eighth floor for all

support, especially the girls in the "crowded room" and Emily Styring for her good advice.

Peter Abdon for introducing me into the world of orthopedics, giving me the best possible start and for encouraging me to start working in Lund.

Kaj Knutson for typesetting the thesis.

My parents, Annie and Staffan Kaping, for encouragement and support.

Last but not least my beloved husband Hans for always being there and our darling children Alma and Truls.

This study was supported by grants from:

The Faculty of Medicine, Lund University, The Swedish Research Council-Medicine (09509), Stiftelsen för bistånd åt rörelsehindrade i Skåne, Greta och Johan Kocks Stiftelser, Alfred Österlunds Stiftelse, Stiftelsen Konsul Thure Carlssons Minne, and Maggie Stephens Stiftelse.

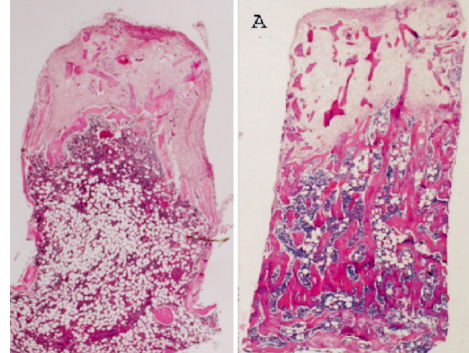
# Comparisons in this thesis

## Paper I

**Hypothesis:** Zoledronate (ZA) prevents resorption of a bone graft during remodeling and weekly dosing is sufficient.

**Method:** Bone conduction chambers with cancellous graft. ZA or saline subcutaneous injections starting day 4 postoperatively and then weekly until harvest at 6 weeks.

**Conclusion:** ZA given in weekly injections are equally effective as the thrice-weekly alendronate injections.



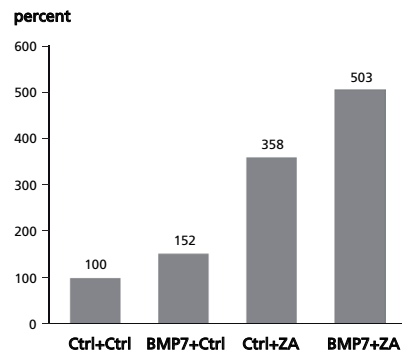
Untreated graft to the left and ZA treated to the right after 6 weeks in the chambers. In the untreated grafts the trabeculae are resorbed and replaced by fatty tissue. By ZA treatment the bone is retained during remodeling.

## Paper II

**Hypothesis:** The combination of local BMP-7 and ZA will increase ingrowth and prevent resorption.

**Method:** Bone conduction chamber with cancellous graft treated with BMP-7 or saline combined with one subcutaneous injection of ZA or saline at day 14.

**Conclusion:** The combination of locally applied BMP-7 and ZA increases new bone formation and prevents resorption.



The net amount of bone in the remodeled part of the graft.

### Paper III

**Hypothesis:** Bisphosphonate increases pin fixation in external fixation.

**Method:** Tibia osteotomy by the hemicallotaxis technique. Hydroxyapatite-coated and standard pins. Randomization to either a single infusion of ZA or saline at 4 weeks postoperatively.

**Conclusion:** A single dose of ZA increases fixation of standard pins in diaphyseal bone.

**Pin Performance Index (PPI) in metaphyseal and diaphyseal bone. Values are mean (standard deviation)**

	Zoledronate n = 25	Control n = 21	P-value
Metaphyseal bone PPI (%)	257 (106)	289 (115)	ns
Diaphyseal bone PPI (%)	62 (23)	35 (14)	<0.0001

ns = not significant

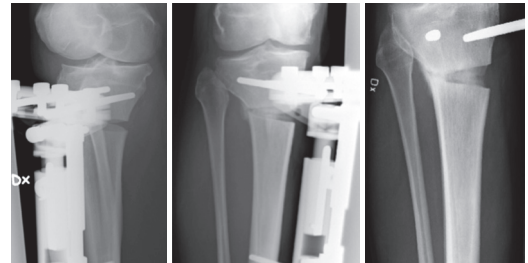
The ratio of extraction and insertion torque expressed as a percentage and defined as PPI (pin performance index). ZA doubled the fixation of the non-coated pins.

### Paper IV

**Hypothesis:** A single dose of ZA decrease fracture healing time.

**Method:** Tibial osteotomy by the hemicallotaxis technique as a clinical model of fracture healing. Randomization to either an infusion of ZA or saline at 4 weeks postoperatively in 48 patients.

**Conclusion:** A single dose of ZA does not decrease fracture healing time.



After a tibial osteotomy successive lengthening by 1 mm a day (left). The HKA angle is slowly normalized and the frame locked at the desired angle and kept until bone healing (middle). Sufficient callus has formed and the frame is removed (right).

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