Effect of combined anabolic and anti-catabolic therapy on initial implant fixation

Canine studies with BMP-2 and bisphosphonate

PhD thesis

Rasmus Cleemann



Faculty of Health Sciences University of Aarhus 2017

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Preface

This PhD thesis is based on scientific work conducted during my enrolement as a PhD student at the Faculty of Health at Aarhus University, from 2013-2017. The experimental work and analysis was performed at the Orthopedic Research Laboratory, Aarhus University Hospital, and the Department of Research at Elective Surgery Center, Silkeborg Regional Hospital. The experimental surgeries were performed at North American Science Associates (NAMSA), Minneapolis, MN (Study I and II), and at the Orthopedic Biomechanics Laboratory, University of Minnesota, Minneapolis Medical Research Foundation, Minneapolis, MN (Study III). I owe sincere gratitude to everyone involved in this project without your help this work would not have been possible. I am sincerely grateful to my main supervisor Kjeld Søballe and Silkeborg Elective Surgery Center for their continuous support and confidence in me. Research is challenging, and my project supervisors Jørgen Baas and Mette Sørensen have provided me with direction and guidance. I appreciate your knowledge, advice and continuous questioning that have helped me perform these studies. My american Professor Joan E. Bechtold deserves gratitude and thanks for providing research facilities at her institution, and for guidance and counsel in the field of orthopedic research. These studies could not have bee performed without the help knowledge and skills of the laboratory technicians Jane Pauli, Anette Battrup and Anna Bay Nielsen. I wish to thank all of my many wonderful present and former colleagues at The Orthopaedic Research Laboratory in Aarhus for their help, support and advice. Finally, to all of my family and friends, I would like to thank you for your unconditional support

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List of papers:

This thesis is based on the following papers:

- Cleemann R., Bechtold J.E., Sorensen M., Soballe K., Baas J.
 Dose dependent resorption of allograft by rhBMP-2 uncompensated by new bone formation - A canine study with implants and zoledronate.
 Manuscript accepted for publication in Journal of Arthroplasty, November 2017.
- II. Cleemann R., Sorensen M., Bechtold J.E., Soballe K., Baas J.
 Healing in peri-implant gap with BMP-2 and systemic bisphosphonate is dependent on BMP-2 dose - a canine study.
 J Orthop Res. 2017 Oct 4. doi: 10.1002/jor.23766. [Epub ahead of print]
- III. Cleemann R., Sorensen M., West A., Bechtold J.E., Soballe K., Baas J. Augmentation of implant surfaces with BMP-2 in a revision setting: Effects of local and systemic bisphosphonate. Manuscript submitted to Bone, November 2017

The papers are referred to in the text by their Roman numerals (I-III).

Abbreviations

BMP	Bone morphogenetic protein
BMU	Bone multicelluar unit
BP	Bisphosphonate
BRC	Bone remodeling compartment
CHO	Chinese Hamster Ovary cells
ll-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
M-CSF	Macrophage colony stimulating factor
MMA	Methylmethacrylate
OPG	Osteoprotegrin
PMMA	Polymethylmethacrylate
PE	Polyethylene
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
rh	Recombinant
RSA	Radiostereometric analysis
SEM	Scanning electron microscopy
TGF-β	Transforming growth factor beta
TAR	Total ankel replacement
THA	Total hip arthroplasty
TKA	Total knee arthroplasty
TNF-α	Tumor necrosis factor alfa
VEGF	Vascular endothelial growth factor

Table of Contents

English summary2
Danish summary3
Background4
Implant failure4
Implant fixation4
Osseointegration5
Cementless implant fixation
Grafted implant fixation6
Impaction grafting6
Bone grafts6
Bisphosphonates7
Pharmacokinetics of BPs8
Biological action of bisphosphonates 8
Effects on bone9
Bisphosphonates in the context of total
ioint replacements and bone grafts 9
Bone morphogenetic protein
BMP structure and signaling
rhBMP in the context of bonehealing 11
Combined anabolic and anti-catabolic
therapy
17 17
AIM
Hypotheses for Study I-III
Study I
Study II
Study III13
Methodology14
Study design14
Implant models14
Implant specifications16
Implant cleaning and sterilization 16
Animal model 17
Ethical considerations17
Sample size17
rhBMP-2 delivery18
Zoledronate delivery 18
Allograft preparation18
Surgery - Study I-III19
Study I 19
Study II 19
Study III 20
Observation time20
Observation period21
Adverse events 21
Specimen preparation 21
Mechanical testing22
Biomechanical parameters
Limitations - mechanical test

Histomorphometric analysis	24
Embedding	24
Stain	24
Microscopy	24
Regions of interest	24
Histological morphology	25
Stereology	25
Stereological design	26
Histomorphometric bias	21
Specimen preparation bias	27
Sampling blas	0∠
	29
Statistical analysis	30
Summary of studies	31
Study I	31
Study II	32
Study III	33
Discussion	34
BMP-2 dose	04 34
Bisphosphonates and allograft	34
rhBMP-2 and bisphosphonates in	-
grafted gaps	36
rhBMP-2 and bisphosphonates in e	mpty
defects	37
Limitations	38
Animal model	00 38
Implant model	
Observation time	
rhBMP-2 delivery	39
Distant and local effects	40
Conclusion	41
Future perspectives	42
References	43
Appendix	. I-XIV
Papers	
- Paper I (Manuscript)	
Paper II (Proof)	
Paper III (Manuscript)	

English summary

Total joint arthroplasties are a good and efficient treatment for alleviating the symptoms of end stage arthritis.

Implants failure is primarily due to aseptic loosening caused by osteolysis or poor implant osseointegration. Lost bone can be replaced with allograft to provide initial stability to implants. Combined anabolic and anticatabolic treatment can potentially improve allograft incorporation and implant osseointegration.

Bone morphogenetic protein 2 (BMP-2) stimulates osteoblast formation and activity, but also invokes an indirect catabolic response by stimulating osteoclast activity. Nitrogen-containing bisphosphonates are potent inhibitors of osteoclast activity.

The aim of the present thesis was to investigate, whether the initial implant fixation and osseointegration of experimental orthopedic implants could be improved by combining a bone anabolic agent (BMP-2) with an anti-catabolic agent (zoledronate). All studies with BMP-2 were conducted on an anti-catabolic background.

All three studies in this dissertation were conducted in a canine animal model and covered an observation period of 4-12 weeks. Mechanical and histomorphometric evaluation were used to measure the effect of the treatment.

Study I investigated unloaded BMP-2 coated (15 μ g, 60 μ g and 240 μ g) and untreated implants surrounded by a 2.5-mm gap impacted with allograft. All animals received zoledronate (0.1 mg/kg) IV 10 days postoperatively. The untreated control implants had the best mechanical fixation, best osseointegration, and largest volume of retained allograft in the peri-implant gap. Mechanical fixation, osseointegration, and volume of allograft decreased as the BMP-2 dose increased.

Study II investigated unloaded BMP-2 (15 μ g, 60 μ g and 240 μ g) coated and untreated implants surrounded by an empty 0.75 mm gap. All animals received zoledronate (0.1 mg/kg) IV 10 days postoperatively. Implants coated with 15 μ g of BMP-2 had the best mechanical fixation. This was corroborated by

histomorphometric findings of best osseointegration and highest volume of new bone in the peri-implant gap.

Study III investigated loaded intraarticular revision implants surrounded by a 1.1-mm gap with allograft. Each animal received 2 implants. One implant was implanted into each medial femur condyle in each knee. Each animal received one implant coated with rhBMP-2 (5 μ g) and an untreated control implant was implanted in the contralateral knee. Half of the animals received allograft soaked in zoledronate at the revision surgery and the other half received zoledronate (0.1 mg/kg) IV 10 and 20 days after the revision surgery. BMP-2 did not improve implant osseointegration or fixation. Local zoledronate showed the best results for retaining allograft, whereas systemic zoledronate accrued new bone.

The studies demonstrate that BMP-2 within a relative narrow dose range exhibits significant and different catabolic and anabolic effects depending on the implant bone interface conditions. BMP-2 showed no positive effect to augment implants with allograft (Study I and III). In contrast, BMP-2 improved implant fixation and osseointegration of implants without direct bone contact (Study II). Study III confirmed that local zoledronate decreased allograft resorption, and that systemic zoledronate accrued a larger volume of new bone compared to local zoledronate.

Danish summary

Kunstige led proteser er en god behandling af smerter og bevæge indskrænkning forårsaget af slidgigt. Når en led protese går løs skyldes det primær aseptisk løsning. Aseptisk løsning skyldes hovedsageligt osteolyse og dårlig osseointegration af protese overfladen. Manglende knogle kan erstattes med donor der giver knogle (allograft) umiddelbar mekanisk støtte til protesen. Kombineret anabol og anti-katabol behandling kan potentielt forbedre integreringen af knoglegraft osseointegration af ortopædkirurgiske og proteser.

Bone morphogenetic protein 2 (BMP-2) stimulerer modning og differentiering af osteblaster men øger samtidig indirekte osteoklast aktiviteten. Nitrogenholdige bisphosphonater, som zoledronat, hæmmer effektivt osteoklaster. Formålet med denne afhandling var at undersøge om den tidlig fiksering og osseointegration af eksperimentelle ortopædkirurgiske proteser kunne forbedres ved at kombineret anabol anti-katabol (BMP-2) oq (zoledronat) behandling. Alle studier med BMP-2 var på en anti-katabol baggrund.

I alle 3 studier anvendtes en hundemodel og en observationstid på 4 (Studie I og II) eller 12 (Studie III) uger. Effekt af behandling blev evalueret ved mekaniske og histomorphometriske evaluering.

I studie I undersøgte vi ubelastede ekstraartikulære primære proteser coatede med en af tre BMP-2 doser (15 μ g, 60 μ g, 240 μ g) eller ubehandlet omgivet af en 2.5 mm defekt pakket med allograft. Alle dyr fik zoledronate mg/kg) IV 10 dage postoperativt. (0.1)Ubehandlede proteser havde den bedste forankring, mekaniske bedste osseointegration og største volumen af bevaret allograft. Mekanisk fiksering og volumen af allograft faldt med stigende BMP-2 dosis.

I studie II undersøgte vi ubelastede ekstraartikulære primære proteser coatede med en af tre BMP-2 doser (15 μ g, 60 μ g, 240 μ g) eller ubehandlet omgivet af en tom 0.75 mm bred defekt. Alle dyr fik zoledronate (0.1 mg/kg) IV 10 dage postoperativt. Proteser coated med 15 μ g BMP-2 havde den bedste mekaniske fiksering. Det kunne forklares med den bedste osseointegration og det største volumen af ny knogle omkring protesen.

I studie III undersøgte vi belastede intraartikulære revisions proteser omgivet af et 1.1 mm bred defekt pakket med allograft. Alle dyr fik isat 2 proteser. En protese coatet med BMP-2 (5 μ g) blev indsat i det ene knæ, og en ubehandlet protese blev isat i det modsatte knæ. Halvdelen af dyrene modtog allograft med zoledronat ved revisions vædet proceduren; den anden halvdel modtog zoledronate (0.1 mg/kg) IV 10 og 20 dage efter revisions proceduren. BMP-2 forbedrede osseointegration eller ikke fiksering af proteserne uanset zoledronat behandlingen. Lokal zoledronat beskyttede allograft bedst hvorimod systemisk zoledronat resulterede i det største volumen af ny knogle.

Studierne demonstrer at BMP-2, indenfor en relativ smal dosis række, har signifikant forskellige anabole og katabole effekter afhængig af protese-knogle grænsefladen. BMP-2 havde ingen positiv effekt på proteser pakket med allograft (studie I og III). I modsætning hertil forbedrede en lav BMP-2 dosis den mekaniske fiksering og osseointegration af proteser omgivet af en knogledefekt.

Studie III bekræftede at lokal zoledronat behandling af allograft mindsker resorptionen af allograft, hvorimod systemisk zoledronat resulterer i en større mængde af ny knogle.

Background

Total joint replacements, such as total hip arthroplasties (THA) and total knee arthroplasties (TKA), are some of the most successful orthopedic interventions. A THA, TKA or TAR is an effective treatment for alleviating pain and regaining ambulation for people with degenerated joints because of osteoarthritis, osteonecrosis and rheumatoid arthritis. The total numbers of both THAs and TKAs have steadily increased since the introduction of these procedures^{1; 2}. Initially, total joint replacements where not expected to be worn out prior to the recipient's death³. Today, because of improvements in general health, the average life expectancy has increased and morbidity decreased in the elderly recipients. Compared to earlier, younger patients are now being offered total joint replacements. It has been projected that in the United States by 2030, more than 50% of both THA and TKAs will be performed in patients younger than 65 years³. Implant failure rates are higher in younger recipients, which is likely caused by increases in both cumulative and peak strains inflicted upon the bone-implant interface⁴. High activity levels have also been linked to increased wear of liner material⁵. With demographic changes and the projected increase in the number of both THA and TKAs, the demand for revision procedures in the future are expected to increase⁶. The survival of revision implants is inferior to primary implants and is even lower for re-revision implants^{7; 8}.

According to the Danish Hip Register, 17.042 THAs were revised in Denmark between 1995-2015. The Implants primarily failed because of aseptic loosening (51%), with other reasons being dislocations (17%), deep infections (9%), and peri-prosthetic fractures $(8\%)^7$.

Elderly recipients can now expect to outlive their implants, and younger recipients are expected to outlive their implants at least once and potentially twice⁶. This underlines the significance of continued research on ways to improve implant osseointegration of both primary and revision implants.

Implant failure

The primary reason for hip implant failure is aseptic loosening, which is the result of insufficient implant osseointegration or periimplant osteolysis.

The etiology of osteolysis is diverse, and clinical loosening likely is the sum of contributing factors both in primary and revision implants⁹⁻¹¹. Osteolysis is an aseptic inflammatory response leading to resorption of bone by osteoclasts. The decreasing contact between host bone and the implant surface ultimately compromises implant fixation.

Initially, this was attributed to MMA particles in cemented total hip prostheses and termed cement disease because fibrous tissue was formed at the bone-cement interface. Later, it was demonstrated that polyethylene wear particles from implant liners are strongly associated with osteolysis.

Implant micromotion also induces a fibrous membrane and causes fluid flow and fluid oscillations¹². Fluid pressure pressure oscillations and flow promotes osteolysis¹³ and can transport particulate material to distant sites along the implant surface and induce wear debris osteolysis¹⁴. Securing intimate contact between the host bone and implant will secure an initial good implant fixation and decrease the effective joint space¹⁵. Sealing off the peri-prostetic area from the effects of particulate wear debris and stresses from fluid flow and pressure will likely protect the bone-implant interface^{12; 14; 16}.

Implant fixation

Initial implant stability is important in securing long-term implant survival. Multiple RSA studies have documented early implant migration as a predictor of later implant failure in both femoral stems^{17; 18}, acetabular cups¹⁹ and TKAs²⁰. Implants are inserted and anchored to the surrounding bone by cementless or cemented technique. Initial cementless implant fixation depends on mechanical press-fit seating into host bone that, in time, is converted into a biological fixation by implant surface osseointegration. Cemented implants depend on their tapered stem shape to seat into a cement mantle that interdigitates with surrounding bone and secures mechanical fixation.

Osseointegration

Brånemark et al. described the term osseointegration in 1977²¹. In 1981 Albrektson et al.²² demonstrated direct adhesion of living bone cells to the surface of titanium implants via collagen filaments resembling Sharpey's fibers to bone. Osseointegration was defined as direct contact between living bone and the implant at the light microscopic level²². In the same paper, six pre-requisites to enable implant osseointegration was listed:

- 1. Implant material should be biocompatible, nontoxic and load resistant.
- 2. Implant design should allow an intimate contact with the host bone
- 3. Finish of the implant surface should be attractive to osteoblasts
- 4. Acknowledge status of bone tissue
- 5. Apply careful surgical technique to avoid inflicting unnecessary trauma.
- 6. Patients should defer from initial loading.

In 1993 Albrektson et al. suggested a modification to the histological definition to the biomechanical definition: "A process whereby asymptomatic clinically rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading"23. The last pre-requisite from 1981

has thus been abandoned, and the inclusion of functional loading has since been corroborated by experimental²⁴ and clinical results²⁵. Implant osseointegration is conditioned by а variety of factors. Osteoconduction describes a surface that is amiable for osteoblasts to attach to, take residence on, and form bone on through appositional growth. Autograft, allograft, and calcium phosphate ceramics are passive osteoconductive surfaces. Implant surface topography and the choice of implant material are also important to make surfaces attractive osteoblasts, and this signifies to the of a biocompatible implant importance surface, as suggested by Albrektson et al.²⁶. Osteogenesis is the ability to provide living mesenchymal progenitor cells or mature osteoblasts capable of forming new bone. Only fresh autografts have osteogenic capabilities, and host osteogenic potential also dependents on age. Osteoinduction has been defined as the process by which osteogenesis is induced. It stimulates pluri-potent primitive mesenchymal cells into an osteoblastic lineage. The presence of committed osteoblasts or pre-cursor cells is necessary for growth factors such as BMP to induce bone formation.

Cementless implant fixation

The objective when inserting a cementless orthopedic prosthesis is to achieve initial stability and, in time, surface osseointegration. The regenerative process around orthopedic implants somewhat resembles fracture healing. With stable interface conditions, bone forms by the intramembranous pathway without a cartilage intermediate as in enchondral ossification²⁷. The regenerative process is described as a sequential threestep process of inflammation. tissue regeneration, and remodeling. The process starts with the formation of a hematoma around the implant, due to disruption of the vasculature, and pro-inflammatory cytokines

(TNF- α , IL-1 β , IL-6 and M-CSF) are released and attracts immune-cells. Within 24 hours, the pro-inflammatory response is converted to an anti-inflammatory response (II-10 and TGFβ and VEGF). The conversion initiates the regenerative phase, where vasculogenesis and angiogenesis of the hematoma starts to supply oxygen and nutrients for tissue regeneration²⁸. Multipotent mesenchymal stemcells are attracted by chemotaxis and initiate proliferation. and maturing into osteoblasts that form woven bone. The formed woven bone undergoes primary mineralization within 5-10 days²⁹. Secreted M-CSF and RANKL from the osteoblasts attracts mononuclear hematopoietic cells to form preosteoclasts that invade the hematoma and stimulate them to fuse into mature osteoclasts, which then, resorb necrotic bone³⁰. The final phase is the remodeling, where BMUs in a defined sequence resorb woven bone and replace it with lamellar bone. The BMU activation frequency is increased in areas with damaged bone. Ideally, orthopedic implants inserted in press-fit conditions will have a uniform and even contact with the surrounding host bone. Despite adherence to meticulous surgical technique, a uniform and intimate implant-to-bone contact is not achieved. Areas of close, but not intimate, contact are present initially³¹, and areas without bone persists after even several years in-situ as demonstrated with retrieved acetabular sockets³².

Grafted implant fixation

When implants fail because of osteolysis, bone stock is often compromised. Lost host bone stock can be substituted with a bone graft. Clinically, impaction grafting has produced good results both in femur and acetabular components ³³⁻³⁶.

Impaction grafting

The modern impaction grafting technique is the result of a development that started with Hasting and Parkers in 1975. They reconstructed acetabular defects in patients suffering from rheumatoid arthritis, by using a Vitallium mesh and cement fixation of the acetabular component ³⁷. Sloof et al. modified this technique and characterized it as impaction grafting due to an intended compression of the bonegraft with the trial socket prosthesis ³⁸. Ling and Gie of the Slooff group later adapted the technique for use in femur revisions ³⁹. The graft impaction technique consists of a stepwise impaction of morselized allograft into а contained acetabular defect or neo-medullary femoral canal. Cement is applied topically and pressurized into the allograft before an acetabular or femoral implant is inserted, respectively.

Bone grafts

Biological bonegrafts vary depending on their structure, and are categorized as structural, cortical, corticocancellous, cancellous, or osteochondral.

Autograft refers to bone that is harvested from and implanted into the same patient and is most often harvested from the iliac crest. Autograft is considered the gold standard because it is not immunogenic and has good osteoinductive and osteoconductive capabilities, and is the only bone graft material with osteogenic potential⁴⁰. Autograft supply is limited, and harvest is associated with donorsite morbidity such as nerve damage, infection, pain, and persistent discomfort ⁴¹.

An allograft refers to bone where the graft donor and graft recipient both are of the same species. Allograft primarily originates from excised femur heads harvested at primary hip surgery. Allografts are pretreated before use; fresh-frozen allograft is the most commonly used graft. Other procedures include rinsing, chemomodification, irradiation, and freezedrying^{42; 43}. A simple procedure, such as rinsing morselized allograft prior to impaction, displaces marrowfat from the graft. This

allows for better allograft chip interdigitation and increases implant fixation⁴⁴. Rinsing also reduces the immunogenic load and improves allograft incorporation⁴⁵. Nibbling of bone by hand or using a bone mill can produce morselized allograft. Chip size is recommende to be large on the acetabular side (8-10 mm) and smaller on the femoral side (2-4 mm)⁴⁶. Use of a size-graded bone chip mix produces an allograft aggregate more resistant to shear forces⁴⁴. The primary objective in the revision procedure is to achieve an initial stable implant surrounded by impacted bone graft that subsequently will remodel into host bone ⁴⁷. Long-term implant fixation depends on allograft incorporation. This is histologically defined as the revascularization of all tissue surrounding the allograft and new bone apposition to the necrotic allograft fragments ⁴⁸. The grafted gap is necrotic, and remodeling starts by invasion of a fibrovascular front. The allograft is resorbed by osteoclasts, and new bone is formed on the surface of the allograft. Incorporation is variable though; retrieved biopsies of impacted allograft revealed extensive replacement of allograft with host bone over time in acetabular components⁴⁹. In biopsies from femoral stems contrast. demonstrate a variable incorporation of the impacted allograft, with bone chips often encapsulated in fibrous tissue rather than being in contact with or replaced by host bone ^{50; 51}. The incorporation of allograft depends on extrinsic factors such as mechanical loading and intrinsic allograft properties²⁴. New bone formation in the grafted gap depends on the allograft both to provide an osteoconductive lattice, but also indirectly by releasing embedded growth factors when resorbed by osteoclasts. Combined, the allograft is incorporated and replaced in time with host bone through creeping substitution^{51; 52}. If bone resorption in the process of graft incorporation surpasses bone formation and maturation, this could lead to the allograft aggregate or composite becoming mechanically incapacitated.

Bisphosphonates

Bisphosphonates are synthesized, chemically stable analogues of naturally occurring inorganic pyrophosphates (PPi). Bisphosphonates dates back to the late 19th century, where they were used in industrial production as a water softener. Naturally occurring PPi prevent desorption of hydroxyapatite and is a regulator of bone mineralization, and can prevent soft tissue calcification. The effects that bisphosphonates exert on osteoclasts were discovered in the 1960s and are classified as an anti-resorptive drug used to treat osteoclast-mediated bone loss⁵³. Naturally occurring PPi is characterized by a central oxygen atom, with two phosphate groups attached (P-O-P), whereas bisphosphonates have a central carbon (P-C-P) (Figure 1). The carbon atom renders bisphosphonates as a stable compound, contrary to PPi, which guickly undergoes



PYROPHOSPHATE

GEMINAL BISPHOSPHONATE



Figure 1. Chemical structure of pyrophosphate, geminal bisphosphonate and zoledronate

hydrolysis into inactive metabolites. Two side chains, designated R1 and R2, are attached to the central carbon atom in bisphosphonates (Figure 1). The R1 side chain and the two phosphate groups define a moiety responsible for the compound's affinity to bone mineral. The R2 side chain determines the biological effect and potency. The Addition of an amino group increases the biological effect and binding affinity hydroxyapatite. to Bisphosphonates are classified as nonnitrogen-containing and nitrogen-containing depending on the R2 side chain 54; 55.

Pharmacokinetics of BPs

Clinically, bisphosphonates are administered orally or IV. The bioavailability of orally administered bisphosphonates is only 0.5-2%. Upon entry into the circulatory system, bisphosphonates are quickly adsorbed by exposed bone mineral. Bisphosphonates have a half-life of 1/2 -2 hours in humans, and with systemic zoledronate administration, 50-60% of the administered dose is retained in the skeleton. Unbound bisphosphonate is not metabolized in the organism and is excreted unaltered in the urine⁵⁶. The half-life of bonebound zoledronate in humans equals bone turnover and is estimated to be 10 years. The skeletal distribution of bisphosphonates is not uniform; it has a predilection for metaphyseal bone and areas of increased bone turnover⁵⁷. This can potentially be explained by the increased number of hydroxyapatite epitopes that are exposed in areas of increased bone turnover and the trabecular structure of metaphyseal bone constituting a larger surface area relative to cortical diaphyseal bone. Low-affinity bisphosphonate analogues have been shown to penetrate deeper into both cortical osteons and trabecular hemiosteons when compared to high affinity bisphosphonate analogues, and this is why bisphosphonate potency also affects distribution within bone⁵⁸. The bond between high-affinity bisphosphonates and bone

mineral is virtually permanent until the bone is resorbed and constitutes an anti-resoptive reservoir. Physiochemical-dependent aspects, such as desorption and the diffusion of nitrogen containing bisphosphonates are thus less relevant while the reattachment of osteoclast-released bisphosphonate is high.

Biological action of bisphosphonates

Bone resorption starts when osteoclasts forms a ruffled border on top of bone surfaces, effectively sealing off a resorption pit with a local acidic environment. The low pH and released proteolytic enzymes dissolve bone mineral and collagen. Bisphosphonates' affinity to bone mineral decreases in an acidic environment, and free bisphosphonates are actively internalized osteoclast. by the Bisphosphonates interfere with distinct biochemical processes in the osteoclast depending on the type of bisphosphonate⁵⁹. Non-nitrogen-containing bisphosphonates are incorporated into non-hydrolyzable ATP analogues in the osteoclast's mitochondria. This depletes the osteoclasts of functional ATP, disabling them from maintaining normal



Figure 2. The mevalonate pathway leading to posttranslational phrenylation of GTP binding proteins. Inhibitory step of nitrogen-bisphosphonate (BP) on Farnesylpyrophosphate Synthase (FPPS).

cellular functions. Nitrogen-containing bisphosphonates inhibits the enzyme Farnesyl Pyrophosphate Synthase (FPPS) in the mevalonate pathway. FPPS is a critical enzyme in the mevalonate pathway, and is a pivotal step in the formation of the isoprenoid compounds: farnesylpyrophosphate and geranylgeranylpyrophosphate. These two proteins are necessary in the posttranslational phrenylation of GTP binding proteins (Ras, Rho, Rac and Rab). GTPbinding proteins are essential for intracellular signaling and the regulation of core osteoclast cellular activities, such as membrane ruffling, differentiation. survival and vesicular trafficking. Both modes of action lead to an impaired osteoclast function and ultimately to osteoclast apoptosis, which reduces bone resorption^{53; 54}.

The biological effects of bisphosphonates have been viewed as reserved for osteoclasts, because osteoclasts were considered the only cells able to internalize the compound. Both in vivo and in vitro studies have demonstrated inhibitory effects of nitrogen-containing bisphosphonates osteoblast on and angiogenesis⁶⁰⁻⁶⁴. And in vivo studies showed bone decreased amount of new with increasing bisphosphonate dose⁶⁵⁻⁶⁸.

Effects on bone

In vivo, bisphosphonates directly preserve bone's mineral content by preventing dissolution of hydroxyapatite and secondly by apoptosis55; osteoclast 69 inducing decreases Bisphosphonates the BMU activation frequency and reduces both the number and size of remodeling sites. Similarly to the heterogeneous skeletal distribution, bisphosphonates also demonstrate sitespecific reduction in remodeling. In time, this allows for a net increase in bone formation and a more extensive mineralization to occur, increasing bone mineral density (BMD)⁷⁰. In postmenopausal women, an annual IV administration of 5 mg of zoledronate was shown to decrease the risk of low-energy vertebral and femur neck fractures⁷¹, likely caused by an increase in trabecular number and volume⁷². Treatment with monthly zoledronate suppressed bone remodeling by 95% after 3 months⁷³. The reduced bone remodeling and site-dependent bone repair by bisphosphonates have raised concerns regarding the accumulation of microfractures and adverse skeletal events, such as atypical femur fractures⁷⁰. The mechanical properties of bone are affected by bisphosphonate treatment. Canine studies have demonstrated that prolonged high alendronate exposure decrease the resistance of bone to dynamic loading⁷⁴ and a clinically relevant alendronate regime increases cortical brittleness⁷⁵. The benefits for the prevention of low-energy fractures likely outweighs the occurrence of a rare skeletal event, and no clear causal relation ship has of yet been established between prolonged bisphosphonate treatment and atypical femur fractures⁷⁶. Although considered a safe drug, the results underline continue the need to monitoring bisphosphonates' long-term effects and further explore pharmacodynamics and effects.

Bisphosphonates in the context of total joint replacements and bone grafts

Anti-resorptive treatment as adjuvant treatment to arthroplasties has demonstrated encouraging results in relation to total joint replacements.

Register studies have shown that bisphosphonate users have a decreased risk THAs⁷⁷ revision of and TKAs⁷⁸. for Randomized RSA studies have demonstrated decreased migration of acetabular sockets zoledronate⁷⁹ with systemic and local bisphosphonate in TKAs⁸⁰, primary acetabular components⁸¹ and in grafted hip revision implants⁸². Experimentally, svstemic bisphosphonates' were shown to protect necrotic autograft in a piglet model of osteonecrosis⁸³, cancellous allograft in a rodent bone chamber model⁸⁴⁻⁸⁷, newly formed bone in a rabbit model of distraction osteogenesis⁸⁸, increase callus size and strength in a rodent femur fracture model ^{89;90} and increase implant fixation⁹¹ (Appendix: Table XIII). Local bisphosphonates can be detrimental for initial implant fixation and osseointegration, despite excellent allograft protection^{66;67} and the inhibitory effects seems related to bisphosphonate dose^{65; 92-94} (Appendix: Table XIII). Despite good clinical and experimental results, bisphosphonates can only help to maximize the effects of a system's intrinsic bone-forming capacity.

Bone morphogenetic protein

In 1965, Urist demonstrated, that pieces of lyophilized demineralized bone matrix could induce ectopic bone formation in the muscle of a rabbit. He attributed this to the presence of a protein with osteoinductive capabilities⁹⁵. Wozney and Wang characterized and cloned the first BMPs in 1988⁹⁶ and currently, 20 bone morphogenetic proteins (BMPs) have been identified⁹⁷. Nineteen of the BMPs belong to a large family of structurally related signaling molecules called TGF- β . BMP-2, and 7 and 9 have osteoinductive potential⁹⁷. BMPs are pivotal signaling molecules during embryonic development and normal cell



Figure 3. Activation of osteclasts (OC) by osteoblasts via RANK/RANKL/OPG pathway signaling.

homeostasis⁹⁸. BMPs induce boneformation by stimulating the differentiation and the proliferation of osteoblast precursor cells and the activity of mature osteoblasts. BMP-2 can indirectly increase osteoclast activity via the RANK/RANKL/OPG signaling pathway⁹⁹ (Figure 3).

BMP structure and signaling

Active BMPs are composed of two inactive monomeric BMP-molecules connected by a covalent disulfide bond, called a cysteine knot. Two additional cysteine knots, along with heparine binding sites, are responsible for BMP binding to cell surfaces and the extracellular matrix. Described simply, BMPs exert their biological effects upon binding to a transmembrane surface receptor and Smad-dependent activates Smador independent intracellular pathways (Figure 4). Activated Smad proteins are translocated into and the cell's nucleus stimulate BMP genes⁹⁸. responsive Reality is more complicated, because each surface receptor is comprised of two dimeric receptors (BMPR type I and BMPR type II), and the biologic effects not only depend on receptor and pathway activation, but also on concepts such as receptor recruitment and crosstalk with other signaling pathways, such as Wnt signaling¹⁰⁰. BMP-2 is commercially available as human recombinant BMP (rhBMP-2), which is produced by Chinese hamster ovary cells (CHO). rhBMP-2 is delivered as a lyophilized powder that is reconstituted to a concentration of 1.5 mg/ml, and the dose is applied by volume to an absorbable collagen sponge (ACS). Clinically used rhBMP-2 doses are 6-12 mg, which are supraphysiological doses compared to the nanograms present in normal bone¹⁰¹.



Figure 4. BMPs bind to a transmembrane surface receptor and activate intracellular signaling pathways. Three different BMPR type I receptors and 4 different BMPR type II receptors have been identified. The surface receptor is a heterotetrameric complex comprised 4 single receptors (2xBMPR type I and 2xBMPR type II). The dimeric active BMP molecule binds to the heterotetrameric BMP-receptor and forms an active heterohexametric receptor complex. The attachment of BMP to the receptor initiates phosphorylation of down-stream signaling proteins, activating Smad-dependent or Smad-independent signaling pathways.⁹⁹ Eight different Smads proteins have been described and are categorized as regulatory (R-Smads: Smad -1/5/8), accompanying Smads (co-Smads: Smad-4) or inhibitory Smads (I-Smads: Smad 6/7). R-Smads are activated by phosphorylation and interact with co-Smads and translocate into the cell's nucleus and stimulates BMP responsive genes such as Runx2 and Osterix. I-Smads at the same time regulates the effect of BMP signaling via negative feedback on the BMPR type I receptor. Smad-independent pathways activate other downstream signaling pathways such as nuclear factor kappa beta (NKκβ). Besides intracellular negative feed-back by I-Smads, BMPs are extracellularly regulated by antagonist molecules such as noggin, CAN, twisted gastrulation and chordin.¹⁰⁰

rhBMP in the context of bonehealing

The FDA has approved rhBMP-2 for maxillofacial reconstruction, open tibia fractures, and in lumbar spinal fusion. Adverse events in elective spine surgery can be linked to BMP dose¹⁰² and BMP-7 provided no benefit when used to augment allograft in cemented hip revisions¹⁰³ and increased bone fractures¹⁰⁴. resorption in spine Experimentally, BMPs have improved bone healing¹⁰⁵⁻¹¹¹, but they also increased the resorption of graft material^{67; 112-114} and produced cystic bone in high doses^{115, 116}. It has been documented that transient bone resorption precedes new bone formation in metaphyseal trabecular bone^{117; 118} (Appendix: Table XIV).

Combined anabolic and anticatabolic therapy

Combining anabolic therapy with anti-catabolic therapy may be beneficial when attempting to harvest a net benefit from anabolic therapy with an rhBMP. Combined anabolic and anticatabolic treatment have produced favorable results in rodent critical defects and fracture models^{119; 120} ^{121; 122}, rodent bone chamber models with local or systemic bisphosphonate ^{123; 124} ¹²⁵. In a piglet model of osteonecrosis, the femoral head sphericity was preserved while bone composition was normalized when alone126-128 compared to ibandronate (Appendix: Table XV). The body of evidence behind the efficacy of combined treatment is increasing when it comes to fracture healing and as an adjuvant to allo- and autografts; but a synergistic effect of implant osseointegration has not been demonstrated^{67; 129} (Appendix: Table XV). Dose for Study I-III were determined based on a review of previous studies on bone healing with BMPs with or without bisphosphonate treatment (Appendix: Table XIV-XV). The dose and effect of rhBMP-2 is species specific, rodents being highly susceptible, humans and higher primates

being resistant, and canines being of intermediate sensibility¹¹⁵.

To allow for a comparison between studies, BMP dose was normalized to the estimated defect or gap volume, if one was present. BMP dose could have been evaluated in relation to graft weight. But graft material was not universally used, and the type of graft varied: autograft, allograft and ceramic graft substitutes; interface conditions varied: empty defects. grafted defects. and press-fit conditions; implant surface: plasma-sprayed or porous; material: tantalum or titanium; and implant coating: hydroxyapatite, tri-calcium phosphate, or untreated. All mentioned variables that potentially affect the outcome of reported data and blur an optimal BMP-2 dose. An optimal BMP-2 dose with implants was estimated to range between 10 and 100 μq in canines. Because rhBMP-2 was administered along with anti-catabolic treatment, we expected to be able to increase the rhBMP-2 dose and still harvest a net gain of new bone, so we settled on 15 μ g, 60 μ g and 240 μ g of rhBMP-2 in Study I and II.

Aim

The general purpose of this thesis was to investigate if combined anabolic and anticatabolic treatment in different implant models (grafted gap, empty gap, and grafted revision) improve the initial fixation could and osseointegration of experimental orthopedic implants. All studies were performed under the assumption of bone resorption as a given when using rhBMP-2, and this is why only combined anabolic and anti-catabolic treatments were investigated. Systemic zoledronate was administered to bypass the potential negative effects on initiating an anabolic response, protect the allograft, and accrue new bone.

In Study III, combined anabolic and anticatabolic treatment were re-investigated along with a comparison of systemic and local zoledronate, in the more austere healing environment of a revision cavity.

Hypotheses for Study I-III

Study I

Hypothesis: In an allograft-filled 2.5-mm gap with zoledronate IV, coating the implant surface with one of three rhBMP-2 doses (15 μ g; 60 μ g; 240 μ g) will improve initial fixation and osseointegration of implants compared to untreated implants.

Study II

Hypothesis: In an empty 0.75-mm gap model with zoledronate IV, coating the implant surface with one of three rhBMP-2 doses (15 μ g; 60 μ g; 240 μ g) will improve initial fixation and osseointegration of implants compared to untreated implants.

Study III

Hypotheses a-b: In grafted revision implants with local zoledronate (a) or zoledronate IV (b), coating the implant surface with rhBMP-2 (5 μ g) will enhance initial implant fixation and osseointegration compared to untreated implants.

Hypothesis c: In grafted revision implants, systemic zoledronate treatment will be comparable to local zoledronate treatment, on initial implant fixation and osseointegration.

Methodology

Study design

studies (I-III) were All conducted on orthototopic implant models using canines as experimental animals. Study I and II were paired studies, where each animal functioned as its own control by having both untreated control and intervention implants inserted. The design of Study III enabled an unpaired comparison between local and systemic zoledronate and paired studies with comparing combined anabolic and anticatabolic treatment to anti-catabolic treatment alone.

In different interface conditions, implants coated with one of three rhBMP-2 doses (15 μ g, 60 μ g and 240 μ g) and untreated control implants, where investigated in Study I and II, with zoledronate IV. rhBMP-2 treatment was allocated in blocks with untreated and 15 μ g implants in one limb and 60 μ g and 240 μ g implants contralaterally, to avoid a potential neighboring effect by dose or agent.

Treatment allocation blocks were assigned with random start and systematically alternated between the left and right limb. Within treatment allocation block implant position was alternated systematically with random start. Study I and II were performed in the same group of animals. A third implant study, investigating rhBMP-2 coated press-fit seated implants, was performed in the proximal tibias.

Study III investigated pairs of revision implants coated with rhBMP-2 (5 μ g) or untreated with local or systemic zoledronate. Within each zoledronate group (Local or Systemic), the treatment was assigned with random start and systematically alternated between the left and right stifle joint. Additionally, the untreated implants in each zoledronate group enabled an unpaired comparison of local versus systemic zoledronate exposure in the implant

revision model. No additional studies were conducted on the animals in Study III.

Because treatment was assigned systematically by alternating between sides with a random start (Studies I-III) and position (Study I and II), a potential bias, from site and position and an asymmetrical loading pattern, was minimized.

Implant models

Three different implant models were used in this thesis, with their characteristic feature of being implanted into cancellous bone. All models evaluated initial osseointegration (with a 4 weeks observation period after final surgery) and were intended to mimic various aspects of implant osseointegration. Implants in Study I and II were non-loaded implants and placed transcortical and extra-articular. The models investigated the initial osseointegration of implants independent of the hydrodynamic forces from joint fluid in mechanically unloaded conditions.

Grafted gap model (Study I):

Two implants were placed in each *proximal humeri* using drill holes with a diameter of 11 mm. Nominel dimensions of implants were 6 mm in diameter and 10 mm length, with 11 mm endplates mounted on each end to secure concentric implant placement. The 2.5 mm circumferential gap was filled with impacted morselized allograft.



Figure 5. Grafted implant (Study I) with dimensions indicated. Upper left: X-ray with implants in-situ.



Figure 6. Empty gap implant (Study II) with dimensions indicated. Upper left: X-ray with implants in-situ.

Two implants were inserted in each distal femur at the epicondylar level using drill-holes with a diameter of 7.5 mm, one from the medial side and one from the lateral side. Nominel dimensions of implants were 6 mm in diameter and 10 mm in length, with 7.5 mm endplates mounted to secure concentric implant placement. The 0.75 mm concentric peri-implant gap was left empty. The 0.75 mm peri-implant gap, mimics a difficult but notdefect. and is critical-sized suited for potential anabolic evaluating bone compounds.

The implants in study III were loaded and placed intra-articular. The model evaluated the osseointegration of intra-articular loaded revision implants after loosening of a cemented primary implant. The revision protocol was developed by Professors Soballe and Bechtold¹³⁰⁻¹³² and consists of a two-stage procedure (Figure 6 and 7). The first stage imitates unstable conditions with relative implant movement. The second stage imitates post-revision conditions with a stable implant. The protocol consistently produces a revision cavity similar to clinical implants with a loose cement mantle. The microenvironment in the revision cavity is composed of a tri-laminar structure with а sclerotic bone rim. interposition of a dense fibrous tissue membrane and a synovial-like membrane towards the PMMA implant. At the primary surgery, an unstable PMMA implant (6 mm x 10 mm) was inserted in the stifle joint. At the revision procedure 8 weeks later, the cavity



Figure 8. Time-line of revision protocol with transverse section depiction of micromotion device in distal medial femur condyle with PMMA implant (0-8w) and titanium-implant (8-12w).

Revision model (Study III):



Figure 7. Micromotion device (center) with a PMMA implant (right) and a titanium-implant (left). Upper left: X-ray with micromotion devices and titanium-implants insitu.

was mechanically debrided by over-reaming the cavity (7.5 mm \rightarrow 8.2 mm) removing the fibrous tissue and sclerotic bone rim. The unstable PMMA implant was replaced with a stable revision titanium implant (6 mm x 10 mm), and the peri-implant cavity was impacted with allograft and a PE end-plug was mounted to level just above the articular surface to ensure loading of the implant; the animals were observed for an additional 4 weeks before euthanasia and harvest of bones with implants in-situ. The model depended on specific hardware, such as the micromotion device and two implants with different micromotion device interlock designs. The micromotion device consists of an anchor house and a piston (Figure 7). A spring is located inside the anchor house, with the piston suspended on top enabling axial piston movement (500 μ m ± 15 μ m) (Figure 8). The piston has 2 sections; a short cylindrical base that slides within the anchor house and protrudes slightly above the top of the anchor house and an upper thin-threaded section for mounting PMMA and revision titanium implants. The base of the PMMA implants rests on the base of the piston and allows for axial movement. The base of the revision titanium implant has an internal recess larger than the piston base's diameter and rests directly on the anchor house, disabling axial piston movement.

Implant specifications

All implants used in this thesis (Study I-III) share the same basic construction: a cylindrical titanium alloy (Ti6AI4V) core with a diameter of 4.4 mm and a length of 10 mm. The cylinder has a hollow-threaded inner (size: M3; pitch: 0.5), allowing for mounting endplates (Study I and II) or for attaching to a micromotion device (study III). The porous titanium coating is commercially available (Gription[®], DePuy-Synthes, Warsaw, IN) and is used on acetabular components. The coating is constructed from commercially pure



Figure 9. SEM image of Gription[®] implant surface (x100).

titanium (CP-Ti) with an inner layer of spherical beads (diameter: $150 \ \mu m$ - $300 \ \mu m$) and an outer layer of highly irregular shaped particles sintered onto the titanium-alloy cylindrical core. The coating porosity is 63% (±3%), as reported by the manufacturer. The implants used in Stud I-II and Study III were from the same production batch (Figure 9). All components in the micromotion device (Study III) were made from titanium-alloy (6AI-4V) to avoid galvanic corrosion. The micromotion spring was preoperatively adjusted to a stiffness of 14 N/mm and a 0.5 N preload.

Implant cleaning and sterilization

Implants (Study I-III), micromotion device (Study III), and end-plates (Study I and II) were defatted in an ultrasonic bath with trichlorethylene (TCE) for 10 minutes, double rinsed in 70% denaturized ethanol and put for 10 minutes in an ultrasonic bath with 70% denaturized ethanol, left to air dry, and then sterilized in a steam autoclave for 20 min at 121°C. The micromotion devices, PMMA implants, PE end-plugs, and particulate PE used in Study III were gas sterilized using ethylene oxide.

Animal model

Choosing the correct animal model is important and depends on the question examined. The experimental animal needs to be a good surrogate for humans and share characteristics with the subject. Here, we investigated osseointegration of implants in a trabecular environment. Canine physes have large amounts of trabecular bone and the composition is very similar to human bone when evaluated based on the content of collagen and extractable proteins¹³³. The implantation sites in the distal femora and proximal humeri are easily accessible and have a size that allows for paired comparisons of several interventions in one animal. Besides decreasing variance in data, this also contributes toward reducing the number of animals needed to conduct an adequately powered study. The research group had a good understanding of the specific bone characteristics associated with the canine animal model. due to their extensive experience with the animal both in the surgical and analytical settings^{65-67; 92; 129}. All things considered canines were chosen as a surrogate for human bone (Figure 10).



Figure 10. Experimental canine animal model. Surgical sites indicated by a red circle (Study II) and a blue circle (Studies I and III).

Ethical considerations

Study I and II were approved and monitored by the local Animal Care and Use Committee (IACUC) at North American Science Associates Inc. (NAMSA[®]), MN, (Submission ID: 288-01). Study III was approved and monitored by the IACUC at the Minneapolis Research Foundation Medical (MMRF), Minneapolis, MN, (Submission ID: A-3875-01). All animals in all three studies, including allograft donors (study I and III), were specifically breed for scientific purposes. The attending veterinarian at both NAMSA and MMRF supervised and directed the surgeries and observation periods.

Studies at both experimental facilities were conducted in accordance and adherence to both institutional guidelines for the treatment and care of experimental animals and The National Institutes of Healths "Guide to the care and Use of Laboratory Animals"¹³⁴.

Sample size

Study I and II were paired studies and performed in the same group of animals. Sample sizes were estimated using the following equation for paired studies:

Equation 1

$$N = (C_{2\alpha} + C_{\beta})^{2} \times \frac{CV_{diff}^{2}}{\Delta^{2}}$$
$$= 9.9; \begin{cases} C_{2\alpha} = 2.262 \ (p = 0.05) \\ C_{\beta} = 0.883 \ (p = 0.2) \\ CV_{diff} = 50\% \\ \Delta = 50\% \end{cases}$$

Where *N* is the total number of animals to be included, $C_{2\alpha}$ is the 2α fractile in the tdistribution at two sided testing, and C_{β} the β fractile in the t-distribution at two-sided testing, CV_{diff} the coefficient of variance of paired tests, and Δ the minimal difference to be detected. Full filled assumptions for the paired t-test (two-tailed) were assumed. P≤0.05 was considered significant.

Study III was designed to enable both *paired* and *unpaired* comparisons between treatments and sample size was estimated using the following equation for unpaired studies:

Equation 2

$$N = 2 \times n = 2 \times (C_{2\alpha} + C_{\beta})^{2} \times \frac{2 \times CV^{2}}{\Delta^{2}}$$
$$= 22.1 ,; \begin{cases} C_{2\alpha} = 2.080 \ (p = 0.05) \\ C_{\beta} = 0.859 \ (p = 0.2) \\ CV_{diff} = 40\% \\ \Delta = 50\% \end{cases}$$

Where N is the total number of animals to be included, $C_{2\alpha}$ is the 2α fractile in the tdistribution, C_{β} the β fractile in the tdistribution, CV_{diff} the coefficient of variance of the two groups (assumed to be similar in both groups), and Δ the minimal difference to be detected. We assumed that conditions for both the paired and unpaired t-test (two-tailed) were full filled. P≤0.05 was considered significant. The assumptions for estimating the sample sizes were based on the results (histomorphometry and mechanical) from previous studies performed in the group using the same models and evaluation endpoints^{67;} ^{92; 111; 135}. Two additional animals were added to the sample size to prevent loss of power if animals or implants should be lost during the observation, preparation or analysis. A total of

	Study I - II	Study III	
Age (months)	17.6 (16.2-18.7)	14.1 (12.9-15.2)	
Weight (kg)*	29.7 (26.8-34.7)	25.4 (20.6-29.8)	
Weight (kg)**	-	27.1 (23.0-32.0)	
Weight (kg)***	29.2 (25.3-33.4)	26.5 (21.0-30.6)	
Sex	Male (n=12)	Male (n=24)	

 Table 1. Animal baseline characteristics presented as mean (range). *: Primary surgery; **: Revision surgery;

 ***: Necropsy.

12 and 24 animals were assigned to Study I-II and Study III respectively.

rhBMP-2 delivery

To ensure delivery of rhBMP-2 to the implant gap, the porous implant surface coating served as the delivery vehicle. The volume of retained fluid in the implant porous coating was determined (Appendix: Table II) and corresponding rhBMP-2 solutions to final implant coating doses of 15 μ g, 60 μ g, 240 μ g (Study I and II) and 5 μ g (Study III) were produced (Appendix: Table III). rhBMP-2 was applied directly to the implant surface (Appendix: Figure I); a separate release study confirmed presence and release of agent in the expected range from the implant surface (Appendix: Figure II and Table IV).

Zoledronate delivery

Zoledronate IV (0.1 mg/kg) was administered 10 days after surgery in Study I and II. In study III, zoledronate IV (0.1 mg/kg) was administered 10 and 20 days after revision surgery. In Study III, local zoledronate was administered peri-surgically by soaking the allograft in a zoledronate solution. Two containers with morselized allograft were thawed for 15 minutes before being soaked under gentle irrigation in a 0.005 mg/ml zoledronate solution (Zolendronic Acid, Actavis, Iceland) for 3 minutes; the allograft was then rinsed 1 minute in isotonic saline to remove any unbound zoledronate. The zoledronate soaking procedure was identical to Sorensen et al.65.

Allograft preparation

The allograft was obtained from two animals not included in any of the studies. The allograft donor animals were sedated with acepromazine IM (0.1 mg/kg) and propofol IV (4 mg/kg) and euthanized with hypersaturated barbiturate (Socumb, Med-Pharmex Inc, CA). The proximal humeri, distal femora, and proximal tibias were harvested using sterile technique. Bones were wrapped in moist sterile gauze, sterile surgical cotton drapes and then placed in two sealed sterile industrial grade plastic bags and stored at -80°C.

In Study I, prior to surgery, bones were thawed, debrided for connective tissue and cartilage and milled on a standard bone mill (Biomet, Warsaw, IN). This produced a morselized allograft with bone chips in the range of 1-3 mm. Morselized allograft from both animals and all anatomical sites were mixed into one batch. Bone chips larger than 2 mm were removed manually. The allograft was packed separately in 1 cm³ PE containers with an average of 1.27 g (range: 1.24 - 1.29) allograft and stored at -20°C. Four containers where allowed to thaw for 15 minutes before each surgery.

Study III was conducted 12 months after Study I and II. To adhere to the reduction in the three Rs (Replacement, Reduction, Refinement), un-milled bones acquired for Study I were used. Bones were from different animals and included one proximal humeri, one distal femur, and two proximal tibias. Bones were prepared as described in Study I, washed three times in 37°C isotonic saline, gently squeezed in a surgical cotton drape to remove excess water, divided into aliquots, and finally portion packed in 1 cm³ PE containers with an average weight of 0.57 g (range: 0.51-0.61) and stored at -80°C.

Surgery - Study I-III

Surgery was performed on animals in general anesthesia, attending sterile surgical technique and with perioperative antibiotics. Anatomical landmarks were identified, skin incised with cautery and sharp dissection to implantation site. Start side and site was determined, as described under the study design. In Study I and II, the periosteum was removed from the implantation site, and a 2.5 mm k-wire was inserted perpendicular to the bone's surface to serve as a drill guide. Drilling was performed at 2 rotations per second to avoid thermal trauma to the bone. The drill hole edges were trimmed for periosteum and the cavity cleaned for debris and irrigated with saline. After implant insertion and hemostasis, fascia and skin tissue were closed in layers with absorbable sutures. Afterwards an identical procedure was performed at the second ipsilateral implantation site and contralaterally.

Study I

The greater tubercle of the humerus was identified along with the supraspinatus tendon. Two 2.5 mm k-wires were inserted perpendicular to the bone's surface, with the most cranial k-wire at the level of the greater tubercle. A constant distance of 17 mm was secured using a designated guide. Next, 12 mm deep holes were drilled with an 11 mm cannulated drill, securing subcortical implant placement. Implants were inserted in a fixed order with untreated implant, 15 μ g implant, 60 μ g implant, and 240 μ g implant to avoid contamination from agent or dose. Morselized allograft was stepwise impacted into the 2.5 mm concentric gap with a tamper. Endplates secured the implant's concentric placement and containment of the impacted allograft (Figure 5).

Study II

The epicondyle of the distal femur was identified. A 2.5 mm k-wire was inserted and a 12 mm deep hole was drilled with a 7.5 mm cannulated drill to secure subcortical implant placement. Implants were inserted in a fixed order with untreated implant, 15 μ g implant, 60 μ g implant and 240 μ g implant to avoid contamination from agent or dose. Endplates at each end of the implant secured the concentric implant placement (Figure 6).

Study III

The revision procedure involved two separate surgeries 8 weeks apart (Figure 8). The first surgery created a revision cavity with an unstable micro-motion device and a PMMA implant. At the second surgery, the created revision cavity was debrided, and a stable implant was inserted and augmented with impacted allograft.

With the animal in the supine position and a maximally flexed stifle joint, a medial arthrotomy was made. A 2.1 mm k-wire was inserted into the central portion of the medial weight-bearing surface. condyles' А cannulated step-drill created a 30 mm deep cavity at the speed of 2 rotations per second, consisting of a deep cavity (6 mm x 10 mm) and an upper cavity (7.5 mm x 20 mm). The most superficial 3 mm of the upper cavity (7.5 mm in diameter) was tapped for later insertion of a titanium centralizer ring. The cavity was irrigated with saline and debris removed. The unstable micro-motion implant was inserted (Figure 7 and 8). A titanium centralizer ring was mounted and the PMMA implant was inserted and locked onto the piston base. An estimated 5 x 10^7 particulate polyethylene suspended in synthetic hyaluronic acid (LifeCore BioMedical, Minneapolis, MN) was injected around the implant. The PE mix was composed of 15% ultra-high molecular weight PE with a mean size of 30 μ m (range 10-50 μ m) and 85% high-density PE with a mean size of 4.0 μ m (range 0.4-11 μ m). A PE endplug was threaded on the unstable rod tip and adjusted to secure the minimal protrusion, which allowed for full piston movement (500 μ m). Implant displacement and free range of motion of the stifle joint was affirmed before tissue and skin were closed in layers with absorbable sutures. Eight weeks after the primary surgery, all animals underwent a revision procedure. Using the same medial arthrotomy, the PE plug, PMMA implant, and centralizer Visual ring were removed. inspection affirmed the presence of excess synovial fluid, synovitis, and a fibrous membrane in the superficial cavity in 48 out of a total of 48 revision cavities, and an 8.2-mm reamer debrided the sclerotic bone rim in the superficial cavity to bleeding cancellous bone. The most superficial 3 mm of the upper cavity (8.2 mm in diameter) was tapped for a larger titanium revision-centralizer ring. The cavity was debrided and irrigated with saline. An untreated titanium revision implant was threaded onto the implant piston until a secure lock at the anchor house stabilized the implant and prevented further axial motion. The Allograft was impacted in the peri-implant gap. In the local zoledronate group the allograft was soaked in zoledronate prior to impaction. A titanium revision-centralizer was mounted with a revision PE end-plug. The PE end-plug was adjusted to a minimal protrusion to secure load transfer to the revision titanium implant during each gait cycle. Tissue and skin were closed in layers. An identical procedure was performed on the contralateral stifle with an rhBMP-2 coated implant. At the revision procedure, five micromotions devices had their piston thread stripped because of a sharp reamer edge. This made threading and locking of the titanium revision implants impossible and the damaged micro-motion devices were replaced with new functional micro-motion devices. The failures happened in three animals. Two animals had both the left and right micro-motion device replaced, and one animal had only the left replaced. A separate statistical analysis, omitting the replaced micro-motion devices, revealed no effect on endpoints. Which is why all implants were included in the final analysis. To reduce variability and increase reproducibility, one surgeon performed all surgeries in each study

Observation time

The general aim of the studies was to evaluate treatments to improve initial implant fixation and osseointegration. Studies on initial implant fixation investigate, if a treatment has a potentially positive effect and simultaneously function as a screening procedure that uncover early negative effects. Evaluation time point is critical and difficult to choose.

Choosing an earlier time point, may be to soon for the regenerative processes to have started, and at a later evaluation point, the differences may have evened out between the groups. Either situation demonstrates the importance of choosing the correct evaluation point. Studies of growth factors in similar implant models showed effects at 3 weeks¹³⁶; ¹³⁷, 4 weeks^{67; 129; 138}, 6 weeks¹³⁹ and 12 weeks¹⁰⁶. Overall, 4 weeks were considered the most optimal evaluation time.

Observation period

Dogs were housed in cages (4 ft x 6 ft) with two (Study I and II) or one (Study III) animal in each and no restrictions on activity. In Study III hind limb function was assessed daily to ensure loading of the implants. Animals were exercised outside their cage for 2 hours per day without restrictions.

Adverse events

Study I-II: In Study II, one animal sustained a clinical superficial infection at the right stifles lateral aspect, which was treated with 5 days of PO antibiotics. Cultures taken at bone harvest were negative for all animals. One animal sustained a hematoma at the right stifles lateral aspect that resolved with a light compressive dressing. All animals completed the studies, and all specimens were available for mechanical and histomorphometric evaluation. In Study I, no clinical signs of infection were present at any time.

Study III: Two animals sustained superficial wound ruptures due to chewing (primary and revision procedure) and a fall (revision). Both animals were treated with a collar and 10 days of antibiotics, and the wounds granulated from the bottom without sign of infection. A third animal (local zoledronate group) spared the right stifle after the revision surgery. X-rays confirmed correct implant placement and no sign of fracture. All animals completed Study III and all specimens were available for mechanical and histomorphometric evaluation.

Specimen preparation

Animals were euthanized after 4 weeks of observation (Study I-III). Immediately, postmortem bones were harvested under aseptic conditions, assigned a harvest ID label, and stored at -21°C before and during transportation to Denmark for processing and Orthopaedic analvsis at the Research Laboratory, Aarhus, DK. Proximal humeri and distal femora were harvested in Study I and II. Only distal femora were harvested in Study III. Specimen preparation was performed blind to treatment. Bones were picked at random from the freezer and designated a consecutively study ID. A chart was kept, linking harvest ID with the study ID. After thawing, whole-bone specimens were mounted in the vice of a water-cooled Exakt[®] diamond band saw. Bone cubes with single implants in-situ were cut from whole-bone specimens. Before sectioning, the outermost endplate in Study I and II and the articulating PE end-plug in Study III was removed. A thin metal rod with a threaded tip was inserted into the hollow



Figure 11. Implant with sections indicated. Diameter (A), cortical discarded cut-off (B), section for mechanical test (C) and section for histomorphometry (D).

threaded implant core to align the implant perpendicular to the blade of the Exakt[®] saw blade, and the most cortical 1 mm of the implant was cut off and discarded. The remaining 9 mm thick implant and bone block was cut into a superficial section (~3 mm) and stored at -21°C until mechanical testing. The innermost section (~5.5 mm) was stored in 70% ethanol for processing. Approximately 400 μ m was lost per cut due to the width of the saw blade (Figure 11).

Mechanical testing

To thaw, specimens were placed at room temperature 1 hour before testing. The examiner was blinded to treatment, and tests were performed in one continuous session per study. Bone-implant specimens were placed on a metal support jig with the cortical side facing up (Figure 12). Implants were placed centrally over a 7.4 mm opening, securing a clearance of 0.7 mm between the implant surface and jig-edge as recommended¹⁴⁰. A 5.0 mm cylindrical solid steel probe was attached to a MTS 858 mini bionics Test Machine (MTS Systems Corporation, Eden Prarie, MN, USA). A preload of 2 N defined contact between probe and implant, after which the probe was displaced at a continuous rate of 5 mm/min until failure of the bone-implant interface, producing a loaddisplacement curve (Figure 13). Load was



Figure 12. Mechanical push-out test. Bone-implant specimen (left) with test set-up (right).



Figure 13. Stress-displacement curve with derived mechanical parameters.

recorded for every 10 μ m by a load cell (2.5 kN load cell, MTS Systems Corporation, Eden Prarie MN) and stored using the designated software (MTS Test Star 790.00 Version 4.00). Implant surfaces were visuallv inspected immediately after the push-out test. Only small islets of bone were present on the porous implant surface and deep into the porous coating. No delamination of the sintered titanium particles or breakage of the titanium beads was observed. The line of failure between implant and the surrounding bone was regarded as being at the boneimplant interface.

Biomechanical parameters

Bone-implant specimens for mechanical testing were of variable thickness due to unavoidable variations in performing specimen sectioning (Table 2). To enable comparisons, force-displacement data were normalized to an approximated implant *surface area* (A_s), and calculated with the following equation:

Equation 3

$$A_s = 2 \times \pi \times L \times (\frac{D}{2})$$

Where D is implant diameter and L implant length. Normalization transformed load-data into stress-data and 3 biomechanical parameters were calculated (Figure 13).

Maximum shear strength (strength: Pa) was derived from the maximum load (F) prior to failure of the bone-implant interface. A few specimens displayed a later additional force peak; this was attributed to the wedging of bone spikes distal to the primary site of failure. The first peak was considered to be the point of failure of the bone-implant interface. Shear strength in material science describes the point of stress where plastic deformation begins to occur in a material. Here, it describes the point where the bone implant interfaces failed in shear and where contact was lost between implant and bone.

Total energy absorption (energy: J/m²) was calculated as the area under the stress/displacement curve until failure (Figure 13 blue area). Total energy absorption (energy) describes the amount of energy the bone-implant interface can store until failure and is a measure of the resilience or toughness of the bone-implant interface.

Apparent shear stiffness (stiffness: Pa/mm) was the maximum slope of the load/displacement curve. Apparent shear stiffness or rigidity describes the ability the bone-implant interface to resist deformation in response to an applied force. Stiffness is a good parameter to describe the material surrounding an implant because different

Study	Height (mm)	m) Diameter (mm)	
1	3.30 (0.29)	5.88 (0.18)	
П	3.28 (0.34)	5.85 (0.09)	
III (LZ)	3.08 (0.10)	5.80 (0.09)	
III (SZ)	3.13 (0.09)	5.80 (0.09)	

Table 2. Diameter and height of mechanical specimens.(mean (sd)).LZ:LOcal zoledronate,SZ:Systemiczoledronate

tissues have different stiffness or elastic modulus. A low stiffness points to poor bone on-growth or fixation in fibrous tissue, whereas a high stiffness indicates the presence of mineralized tissue. The three biomechanical parameters correlates well with histological findings at the bone-implant-interface¹⁴¹.

Limitations - mechanical test

A push-out test is, by nature, a destructive and definite test and voids any test replication. No reproducibility measurements were performed on the 3 biomechanical parameters, because they are automatically calculated from predefined equations in an excel spreadsheet. The push-out test only measures the load needed to induce failure of the boneimplant interface at a continuous displacement rate. In-vivo, an implant is not subjected to such a uniform destructive stress but rather multiple repetitive loads, comprised of a range submaximal torsional, bendina of and compressive forces. Where accumulated stress at the interface could be imagined to deteriorate the integrity of the bone-implant interface. The assumption of a pure shear stress failure at the bone-implant interface may be true for a polished implant, but the implants used in the current studies, had a rough porous surface that allow bone ingrowth. The push-out test was chosen because axial loading is the most common clinically encountered stress on an implant. Second, the push-out test is simple and easy to replicate, and we used similar implants and coatings meaning the requisite for control over the test conditions were met¹⁴². The estimated implant surface area used to normalized loaddisplacement data was a pure cylindrical shape. The implants used had a highly porous coating with a large surface area, given the same implant height and diameter. This resulted in a general unknown overestimation relative to the true unknown values.

Histomorphometric analysis

Information of the structural composition of the tissue at the implant surface and in the periimplant gap was obtained by histomorphometric evaluation of specimens cut from the innermost 5.5-mm section of the implant-bone specimen (Figure 11).

Embedding

Sections were cut using vertical sectioning technique. The 5.5 mm implant-bone block was sequentially dehydrated in graded ethanol (70%-96%),100% iso-propyl alcohol, defatted with xylene and finally embedded in methyl methacrylate (MMA, product no. 800590; Merck, Darmstadt, Germany) in a cylindrical plastic mold, attending alignment of the vertical axes of the implant and mold. Embedded bone-implant specimens were randomly rotated around their vertical axes followed by sectioning in parallel to the vertical axis of the implants (Figure 14 and 16). Sections were cut with a hard tissue microtome (KDG-95, MeProTech, Heehugowaard, Holland). Sectioning started with a 1 mm offset from the implants vertical axis, producing four consecutive sections from the central part of the implant, as described by Overgaard et al¹⁴³. The 20-30 μ m thick sections were cut at a distance of 400 μ m, corresponding to the width of the microtome



Figure 14. Principle of random rotation of first section plane, resulting in random orientation in 3D space of plane histological sections.

saw blade.

Stain

Sections were stained with toluidine blue 0.1%, pH 7 (Sigma-Aldrich, St. Louis, MO), rinsed, and mounted on glass. The stain penetrated an estimated 4.1 μ m (±0.56 μ m) into the MMA embedded specimens¹⁴⁴.

Microscopy

Quantitative histomorphometric evaluation was performed using a light microscope at a (Olympus, magnification x10 Ballerup, Denmark) with examiner blinded to treatment. Fields of vision were digitally transmitted from the microscope by a camera to a computer Histomorphometric analysis was screen. performed using a dedicated stereological software screen where predefined regions of interest (ROIs) were superimposed onto the transmitted images (NewCast. Version 3.0.9.0. Visiopharm Integrator System, Hørsholm, Denmark).

Regions of interest

All ROIs were defined from a line through the median thickness of the porous implant coating, referred to from here on out as the reference line. All ROIs spanned the length of the implant axially, except for 500 μ m at either end (Figure 15).

Study I (2.5-mm grafted gap):

ROI 1 began at the reference line and extended 500 μ m into the peri-implant gap.

ROI 2 began 500 μ m from the reference line and extended 2000 μ m into the peri-implant gap.

ROI 3 began 3000 μ m from the reference line and extended 1000 μ m into host bone.



Figure 15. A schematic histomorphometric specimen of an empty gap (0.75-mm) implant with peri-implant ROI I (red) and off-gap ROI II (blue). ROIs are defined from the reference line.

Study II (0.75-mm empty gap):

ROI 1 began at the reference line and extended 1000 μ m into the peri-implant gap. ROI 2 began 1500 μ m from the reference line and extended 1000 μ m into host bone.

Study III (1.1-mm grafted revision gap):

The ROI began at the reference line and extended 1000 μ m into the peri-implant gap.

The peri-implant ROI width was chosen to be shorter than the concentric peri-implant gap to ensure only tissue in the peri-implant gap was evaluated. A median line through the implant coating was chosen because of variations in coating thickness along the implant surface (Figure 19, 21, 23). If either the "valleys" or "peaks" of the porous implant were used, it would induce sideways variation of ROI positioning inside and outside the peri-implant gap.

Histological morphology

Staining with toluidine blue enables differentiation of the various tissues of interest based on the observed morphology (Figure 19, 21 and 23). Toluidine blue is a cationic dye and binds to negatively charged areas in DNA, RNA, and collagen, and presents the tissues in the chromatic spectrum of blue to purple.

New bone or woven bone appears as a disordered structure with dark blue, round cells surrounded by a purple dense structure.

Less mineralized new bone appears more transparent in a light blue shade with large dark nuclei. Lamellar bone appears as pale purple lamellar structures separated by cement lines with elongated lacuna with small Allograft purple cells. has а similar appearance as lamellar bone but with empty lacunae. Bone marrow is characterized as loose, disordered cell rich areas with large empty fat vacuoles defined by thread like structures. Fibrous tissue appears as cell rich, spindle-shapede structures in well-organized bundles or fibers. Implant osseointegration was determined as bone-to implant contact and peri-implant bone density. Direct bone-toimplant contact and peri-implant bone density were used to evaluate implant osseointegration. Bone-to-implant contact was defined as bone in direct contact with the implant surface and estimated as surface area Peri-implant bone density fraction. was estimated as volume fraction. Surface coating with rhBMP-2 was not visible at the magnification level (x10) used for histomorphometric evaluation.

Stereology

We were interested in quantifying the osseointegration of implants and getting unbiased estimates of fractions of ongrowth of tissue to the implant surface and volume fractions of tissue around the implants. For this thin-plane purpose we produced implants histological sections of with bone from harvested bonesurrounding implant specimens. Osseointegration of an implant occurs in three dimensions (3D): along the length, around the circumference and outwards to the surrounding host bone. The 3D structure of bone-implant specimens is a plane reduced to structure in two dimensions when bone-implant (2D), specimens are sectioned to plane histological sections. 3D estimates of parameters of interest can be obtained by application of

stereological analysis principles to structures present in 2D. Stereology is the mathematical science of obtaining three-dimensional data from two-dimensional sections. Stereology combines statistical sampling principles with the appropriate geometric probes to estimate geometric quantities, such as surface area and volume. Each geometric probe has an associated geometric quantity.

The sum of dimensions must adhere to the dimensionality rule, which states that the sum of dimensions of the probe and the geometric quantity, must equal at least three, as the geometric probe and the geometric quantity interacts in 3D-space¹⁴⁵. This implies that volume fractions (3D) can be estimated using points (0D) as a probe, and that surface fractions (2D) can be estimated using lines (1D) as a probe.

practice. stereology performed In is superimposing a uniformly random-shifted grid with probes onto sample windows of a plane section, and then by counting the number of intersects between the probe and structure in question in each sample. With an appropriately selected probe, the number of intersections between the probe and the structure in question, translates directly to the quantity of the structure in 3D as a fraction. For the estimates to be unbiased, the applied probe must be isotropic uniform random (IUR) in 3D space, as well as the plane section of the structure, or the structure itself must be statistically isotropic.

Isotropy means that all directions are possible or that an object has no preferred direction in space. Surface area (2D) as a geometric quantity can have a preferred orientation in 3D space, as well as for the corresponding probe (e.g., line (1D)).

Baddeley et al.¹⁴⁶ developed the vertical sectioning technique (VST) to deal with these problems. The technique describes four requirements that must be fulfilled, as follows:

- 1) Identification of a vertical axis
- 2) Plane sections are cut in parallel to the vertical axis
- First plane section is cut with random orientation to the vertical axis and succeeding plane sections systematically with respect to the first
- The test lines are given a weight proportional to the sine of the angle between the test line and the vertical axis

The first three requirements adhere to the practical sectioning of the bone-implant specimen; and the fourth applies to the stereological sampling used.

The estimation of volumes is excepted from the presumption of isotropy, and the probe (point) is dimensionless with no preferred orientation in space.

Adherence to the above requirements, secures that both the acquired plane sections for histomorphometric evaluation and the applied test probe indeed are IUR, and that the derived surface-area fraction estimates are unbiased.

The total number of intersects for surface area and volume in each region of interest were calculated as the sum of intersects of similar probes across all tissues. Tissue surface and volume fractions in each region of interest were calculated as the proportion of specific tissue intersects relative to the total number of intersects of the corresponding probes.

Stereological design

In each bone-implant specimen, a vertical axis was defined. Bone-implant specimens were embedded in cylindrical plastic molds to enable uniform random rotation around the implants vertical axis (Figure 13). Finally, the vertical plane sections were cut and sampled using stereological software (NewCast,

Study	Points	Lines	Fraction
1	4 x 4	10	100%
П	5 x 4	15	100%
III	5 x 4	15	100%

Table 3. Sampling intensity for Study I-III.

Visiopharm a/s, Hørsholm, Denmark). Four plane sections from each implant had ROIs superimposed on to each side of the implant (Figure 15). Regions of interest were sampled at a 100% with systematic uniform random sampling in 2D space (Meander sampling principle). Sine weighted test line probes and point probes were used to register tissue intersects in the superimposed sampling windows (Table 3). Probe intensity was adjusted by test-counts on four randomly chosen implants in each study, too optimize sampling efficiency and obtain precise, reproducible results. The required sampling intensity in each study depended on the presence of the tissue of interest within a group and between groups. A tissue of interest that is highly frequent needs to be sampled at a lower intensity relative to a tissue with infrequent presentation. The same applies if large differences exist between groups. Based on previous experience from similarly designed studies, the "rule of thumb" indicates that the tissue fraction should be based on 100 counts of the tissue of interest (e.g., bone). Sampling intensity can accordingly be adjusted to minimize the workload (e.g., increase efficiency)

Histomorphometric bias

The principles and techniques of histomorphometric stereology were applied to enable us to obtain unbiased estimates efficiently. Despite scrupulous adherence to these techniques and principles, bias can be introduced at multiple levels in the analysis process and can result in a systematic deviation from the true value. Potential sources of bias and their influences are addressed below.

Specimen preparation bias

Sampling width and section sampling *coverage:* Tissue in relation to the implant was of interest, which is why only the central part of the bone-implant specimens with the implant was sampled. Four sections covering 1320 μ m were serially cut from the central part of the implant in the presented studies. It is technically possible to cut a 6 mm implant into 14 sections, covering a total of 5930 μ m¹⁴³. Overgaard et al. demonstrated that the increase in observed¹⁴³ variance is negligible when decreasing the number of plane sections evaluated from 14 to 4, because the primary cause of variance is biological and not due to sampling. By reducing the number of evaluated plane sections the workload is decreased significantly (cut. stain. and sampling procedures) and work efficiency is increased. Bias could potentially be introduced by reducing the number of evaluated plane sections, because the evaluated fraction of the implant's circumference is reduced from almost 100% to 22% in the present study^{143;} ¹⁴⁷. A better representation of the peri-implant gap could be achieved by increasing the intersectional distance. Multiple cuts using the 400 μ m Exakt[®] microtome blade would increase the intersectional distance and increase the covered area for sampling. But



Figure 16. Illustration of increase in central section sampling width by expanding the intersectional distance.

the third VST requirement dictates that succeeding plane sections must be cut systematically with respect to the first section. In practice, performing multiple cuts would result in a variable intersectional distances and increase the workload considerably. A practical, easy solution to increase the intersectional distance would be to increase the microtome blade's thickness. Application of an 800 μ m microtome blade would increase the sampled area by 92% (from 1320 μ m to 2520 μ m) but also increase the possibility of central section bias (addressed below). The potential bias of narrow sampling width or small intersectional distance is considered to be small, because it is fair to assume relative homogeneity of the peri-implant gap tissue¹⁴⁷ and the application of VST omits the requirement of isotropy of the sampled tissue when estimating surface area coverage, and the estimation of volumes does not require isotropy¹⁴⁶.

Section offset bias: Applying VST to a cylindrical implant, results in a reduction in the apparent implant diameter and an increase in apparent gap size for a plane section cut more tangential to the implant surface when compared to a plane section cut through the implant's vertical axis (Figure 17). The ROI starts at the median implant line and extends



Figure 17. Section off-set bias. A peripheral cut section (upper right) and a central cut section (lower right).

a fixed distance out into the peri-implant gap. As a consequence of fixed ROI dimensions, an increasing volume in the perimeter outside the ROI, but within the peri-implant gap, is not peripheral evaluated in increasing cut sections. This area is of potential interest, because new blood vessels and appositional bone growth emanate from the host bone. In the most peripheral cut sections the gap increases by 3-4 % depending on the drill hole's radius^{147; 148}. Because an increase in the peri-implant gap is minor, and because the relative volume fractions are estimated on accumulated intersect counts across all plane histological specimens, it seems reasonable to conclude that tissue estimates are minimally affected by section offset bias.

Central section bias: A theoretical source of bias in the used implant model is central section bias¹⁴⁷. Tissue near the implant surface has an increased probability of being sampled relative to tissue far from the implant surface. This potential bias stems from the fact that tissue volume increases with the distance away from the implant's vertical axis, which is why each probe count (point) represents proportionally different volumes near and far from the implant surface. Tissue far from the implant surface is thus theoretically inclined to be underestimated whereas the opposite applies to tissue near the implant surface. The influence of central section bias has been assessed to be within a low range and acceptable¹⁴⁷.

Sampling bias

Projection: Plane sections used for histomorphometric evaluation were approximately 30 μ m thick. This is a relatively thick section for histomorphometric evaluation. Attempts to produce a thinner section will result in separation at the bone-implant interface or the histological specimen simply breaking during the sectioning procedure. Ideally, when performing a histomorphometric

evaluation, only intersections between the probe and tissue on the plane section surface, a true 2D surface, should be registered. Due to the thickness of the plane section, structures positioned below the surface, deeper into the plane section, can be projected to the plane section surface. Toluidine blue has an estimated penetration depth of 4 μ m, which is why projection bias can be considered negligible ¹⁴⁴.

Shrinkage: During preparation of the boneimplant specimens, artifacts can be induced to the specimen, due to tissue shrinkage. Shrinkage is of particular interest at the implant surface, because it has a similar morphological appearance to bone marrow. Mineralized bone does not shrink, but an effect will likely be proportional to the area covered with bone and thus not contribute to an increase in the difference between implant groups.

Overall impact of bias

We have adhered meticulously to studies stereological principles in the presented (Study I-III) when estimating tissue ongrowth to the implant surface and volumes inside and outside of the peri-implant gap. The presented sources of bias can make the seem faultv sampling procedure and inaccurate. But bias is, to some extent, certain and is acceptable as long as its effect is small. We used a paired design and relative estimates of tissues, which is why a given bias only will have a minor impact on the results. From the discussion above, it seems reasonable to assume that the effect of bias in the studies was small and acceptable.

Reproducibility

In the context off histomorphometric evaluation, reproducibility is the ability to categorize distinct morphological appearances in a uniform manner independently of time.

	New bone	Allograft bone	Bone marrow	Fibrous tissue	Lamellar bone
Study I					
Surface	6.7	31.6	4.0	31.6	-
Gap	3.9	5.3	1.6	10.5	0.6*
Study II					
Surface	3.7	-	12.8	n/e	-
Gap	0.9	-	31.6	n/e	1.0*
Study III					
Surface	3.8	12.6	1.4	15.6	-
Gap	1.8	5.9	0.6	17.7	-

 Table 5: Inter-observer reproducibility, CV (%). *:

 Outside the peri-implant gap.

Reproducibility can be expressed as *coefficient of variance (CV)*, as follows:

Equation 4

$$CV = \frac{s}{\bar{x}}, \ s = \sqrt{\frac{1}{2 \times k} \times \sum d^2}$$

Where \bar{x} is the mean value of the first and second estimate, k is the number of double estimates, and d is the differences between the first and second estimate¹⁴⁹. To determine inter-observer reproducibility, five implants, four specimens each, were chosen in Study I-III. using a random integer generator (www.random.org), and two separate measurements were performed by one examiner (Table 5). Intra-observer reproducibility was performed on the same implants by a second examiner (Table 6). Reproducibility measurements were performed using the same equipment with a delay of 3-15 months after the initial evaluation. Examiners were blinded to treatment and specimens were evaluated in random order. A CV below 10% is considered a high degree of reproducibility. In general, the studies were below 5%, but a high interobserver variance was seen in the estimated surface area of fibrous tissue and allograft
	New bone	Allograft bone	Bone marrow	Fibrous tissue	Lamellar bone
Study I					
Surface	4.4	23.6	3.8	20.0	-
Gap	1.8	3.7	2.3	14.8	0.9*
Study II					
Surface	2.7	n/e	2.9	n/e	-
Gap	1.7	n/e	3.0	0.0	1.9*
Study					
Surface	0.7	9.3	0.5	4.2	-
Gap	1.8	4.6	1.8	13.3	-

 Table 6. Intra-observer reproducibility, CV (%). *:

 Outside the peri-implant gap.

(Study I) and volume of fibrous tissue (Study I and III). Intra-observer variance mirrored the inter-observer variance with the addition of surface fibrous tissue (Study II and III) as well as volume fibrous tissue (Study II). The variances seen in these studies are in accordance with previous studies in the implant models^{147; 148}. The inter- and intravariance calculations demonstrate that the CV for a specific tissue needs to be accessed along with its proportion to the total count. If a tissue is only represented scarcely, its reproducibility becomes comparably lower, and the CV increases.

A high CV may indicate that a chosen sampling intensity is not sufficient to evaluate a given tissue. But if specific tissue is only present in minute amounts in the selected region of interest, increasing the sampling intensity to enhance precision would not necessarily decrease the variance.

Statistical analysis

Study I and II were paired studies, with four treatment groups per study. Data were primarily evaluated parametrically by one-way repeated measures ANOVA and Post-hoc test. Mean estimates and mean differences are presented with 95% confidence intervals. Models were checked by evaluating residuals for normality by QQ and scatter plots.

If non-normal, data were evaluated with Friedman repeated measures analysis of

variance followed by Wilcoxon Signed ranktest. Median estimates are presented with inter-quartile range. All mechanical data as well as most histomorphometric data could be assumed normally distributed. Α few histomorphometric variables needed nonparametrically evaluation since normal distribution of data could not be assumed despite logarithmic transformation.

In study III, paired and un-paired comparisons were made. Effects of treatment on mechanical and hitomorphometric endpoints were estimated using a mixed model, adjusting for implant position, age and weight of animal, and taking the between animal and between side variations into account.

The probability of type-1 error was not adjusted for multiple comparisons. Mean estimates and differences are presented with 95% confidence interval.

Histomorphometric variables with low intersection or count values were evaluated using a two-step analysis. Data were dichotomized to indicate the presence or absence of tissue. The probability of presence or absence of a variable was modeled using logistic regression. For variables present measurements were further analysed using a logistic regression model to estimate the median effect. The median treatment effect is presented with 95% confidence interval. In all studies two-tailed p-values below 0.05 for overall and pairwise comparisons were considered statistically significant. The statistical analysis was carried out in StataCorp. 2013 (Stata Statistical Software: Release 13, College Station, TX).

Summary of studies

Study I

Hypothesis:

In an allograft-filled 2.5-mm gap with zoledronate IV, coating the implant surface with one of three rhBMP-2 doses (15 μ g; 60 μ g; 240 μ g) will improve initial implant fixation and osseointegration compared to untreated implants.

Comments: We cannot conclude on an rhBMP-2 dose optimum, because none was found. There was no evident benefit to augment implants with rhBMP-2; contrarily, the addition of rhBMP-2 was detrimental for mechanical fixation, osseointegration, and retention of allograft. A single systemic exposure of zoledronate seems inferior in countering the catabolic stimulus induced by a narrow range of rhBMP-2 (Figure 18-19 and Appendix: Table V-VI)



Figure 18. Grafted gap model: Mechanical (left) and histomorphometric (right) results presented as mean (95% CI). Intergroup comparisons: *(p < 0.05), **(p < 0.01), ***(p < 0.001).



Figure 19. Grafted gap model: Representative histological sections from different animals. Untreated implant (middle left; x1.25) and 240 μ g implant (middle right, x1.25). White squares specify position of magnified sections close to the implant surface (upper image; x10) and further away from the implant surface (lower image; x10). Bar (x1.25/x10) = 1.0 mm/0.1 mm).

Hypothesis disproved: Yes

Study II

Hypothesis:

In an empty 0.75-mm gap model with zoledronate IV, coating the implant surface with one of three rhBMP-2 doses (15 μ g; 60 μ g; 240 μ g) will improve early implant fixation and osseointegration compared to untreated implants.

Hypothesis disproved: No

Comments: With delayed systemic zoledronate exposure, 15 μ g implants had

significantly better mechanical fixation on all three mechanical parameters compared to untreated implants and implants with higher doses of rhBMP-2. The 15 μ g implants had the largest amount of new-formed bone in continuity from the implant surface to pass over the peri-implant gap to secure anchorage into adjacent host bone. Of importance, the results demonstrate opposite effects on mechanical and histomorphometric endpoints, within a narrow range of rhBMP-2 doses when used to improve the healing of empty defects around cementless implants (Figure 20-21 and Appendix: Table VII-IX).



Figure 20. Empty gap model: Mechanical (left) and histomorphometric (right) results presented as mean (95% Cl). Intergroup comparisons: *(p < 0.05), **(p < 0.01), ***(p < 0.001).



Figure 21. Empty gap model: Representative histological sections from the same animal. Center images (x1.25): Untreated implant (upper left); 15 μ g implant (upper right); 60 μ g implant (lower right); 240 μ g implant (lower left). White squares specify position of magnified section (x10) close to the implant surface. Bar (x1.25/x10) = 1.0 mm/0.1 mm.

Study III

Hypotheses a-b: In grafted revision implants with local zoledronate (a) or zoledronate IV (b), coating the implant surface with rhBMP-2 (5 μ g) will enhance initial implant fixation and osseointegration compared to untreated implants.

Hypothesis a disproved: Yes Hypothesis b disproved: Yes

Hypothesis c: *Hypothesis c*: In grafted revision implants, systemic zoledronate treatment will be comparable to local zoledronate treatment, on initial implant fixation and osseointegration.

Comments: rhBMP-2 did not improve or enhance fixation and osseointegration of revision implants irrespective of zoledronate treatment. Systemic zoledronate was comparable to local zoledronate on mechanical fixation. But systemic zoledronate treatment was inferior to protect allograft compared to local zoledronate, but superiorly accrued more new bone than local zoledronate treatment (Figure 22-23 and Appendix: Table X-XII). The results prompt further investigations to elucidate if an additive effect exists when combining local and systemic zoledronate treatments, and if other rhBMP-2 doses can augment the impacted allograft in revision implants with adequate anti-catabolic control.





Figure 22. Revision model: Mechanical (left) and histomorphometric (right) results presented as mean (95% CI). Intergroup comparisons: (p < 0.05), **(p < 0.01), ***(p < 0.001). Local zoledronate (LZ) and systemic zoledronate (SZ).



Figure 23. Revision model: Representative histological sections from systemic zoledronate (far left; x1.25) and local zoledronate (far right; x1.25). White squares specify magnified regions in the peri-implant gap (x10). Bar (x1.25/x10) = 1.0/0.1 mm.

Discussion

The general aim of this thesis was to investigate if the combined augmentation of implants with a bone anabolic stimulus (rhBMP-2) and concomitant anti-catabolic treatment would improve the osseointegration of primary and revision implants, addressing defect healing and allograft incorporation in increasingly tenuous healing environments.

More specifically, the aim of Study I and II were to investigate, if one of three rhBMP-2 doses on a background of systemic zoledronate, would improve the osseointegration of allograft impacted or empty gaps around primary implants.

Study III investigated the differences in anticatabolic control between local and systemic administration of zoledronate in impaction grafted revision implants. Second, we investigated if the addition of rhBMP-2 would improve implant osseointegration with exposure of implants to either local or systemic zoledronate.

BMP-2 dose

In preparation for Study III, we were aware of the negative catabolic effects of 15 μ g of rhBMP-2 on the grafted defect model (Study I) and the positive effect on empty defect healing (Study II). The peri-implant gap volume-ratio between the implant models in Study I and III is 2.6 (Appendix: Table I), hence only a third of the lowest rhBMP-2 dose used in Study I was applied in Study III (Appendix: Table III).

The most interesting aspect in Study I and II was not the specific rhBMP-2 doses used, but rather that within a narrow rhBMP-2 dose range, significantly different catabolic and anabolic effects were exerted. The effect seemed to depend on the interface conditions because 15 μ g of rhBMP-2 improved induced mechanical fixation and bone formation in implants with empty defects (Study II), whereas 15 μ g of rhBMP-2 increased bone resorption (Study I and III) and weakened mechanical fixation in grafted primary implants (Study I).

different The effect between implants impacted with allograft and implants surrounded by an empty defect could have been caused by a local increase in rhBMP-2 concentration at the implant surface, as the implant was encased behind the impacted allograft. Or, the regenerative processes facilitating healing of empty peri-implant defects and grafted peri-implant defects differ.

Bisphosphonates and allograft

Impacted allograft was used in Study I and III, to augment fixation of cement-less porouscoated titanium implants.

Incorporation of the impacted allograft into host bone involves graft resorption and new bone formation. Bone resorption can leave the allograft construct mechanically incapacitated and reduce implant fixation.

Local bisphosphonate treatment of bone graft ^{65-67; 150-153} and pre-emptive systemic bisphosphonate ^{83; 86; 87} administration could protect the bone graft against resorption.

In the canine studies with allografted implants listed above, local zoledronate treatment seemed to block new bone formation, resulting in inferior mechanical fixation⁶⁵⁻⁶⁷. In a canine revision study by Sorensen et al.⁹², soaking the allograft in zoledronate protected the allograft against resorption compared to the untreated allograft, but demonstrated no accrual effect of new bone ongrowth or contrast, delayed volume. In systemic bisphosphonate has proven to both preserve allograft and not stall new bone formation ⁸³⁻⁸⁵.

The use of systemic zoledronate in a canine implant model with allograft, can potentially simultaneously protect the allograft against resorption, not interfere with the formation of new bone and help to accrue newly formed bone.

In Study III, no difference was detected between local and systemic zoledronate exposure on any mechanical fixation parameter. The similar mechanical fixation contrasted with an almost reciprocal periimplant gap composition: Local zoledronate exposure was superior to systemic zoledronate exposure to protect the allograft in the peri-implant gap, with volume fractions of 26% and 15%, respectively. In contrast, systemic zoledronate exposure was superior to local zoledronate exposure for the accrual of new bone in the peri-implant gap, with volume fractions of 34% and 25%, respectively. Unexpectedly, the ongrowth of new bone with systemic zoledronate exposure was inferior to local zoledronate exposure, with surface fractions of 15% and 23%, respectively. The histomorphometric results with local zoledronate exposure in Study III corroborates fully with Sorensen et al.'s previously reported results on volumes allograft and new bone ⁹². Relating the results from systemic zoledronate exposure in Study III to the control implants in the Sorensen et al. study ⁹², indicate that repeated systemic zoledronate have exposure may а preservative effect on allograft with a volume fraction of 15% and 9%, respectively.

In Study I, only a single zoledronate dose (0.1 mg/kg) was administered IV 10 days postsurgery. The untreated implants had on average volume fractions of allograft and new bone of 26% and 31%, respectively, in the peri-implant gap. In previous canine studies with allograft, volume fractions of allograft (7-20%) and new bone (12-23%) were lower. This could indicate a protective effect of a single zoledronate administration on allograft compared to allograft not exposed to anticatabolic treatment ⁶⁵⁻⁶⁷.

Graft incorporation in necrotic defects begins with the ingrowth of a fibrovascular front, closely followed by an increase in osteoclast activity that is suggested to be uncoupled from new bone formation^{50; 51; 154}. Soaking the allograft in zoledronate theoretically secures a uniform, thin protective coating around the entire graft particle. For systemic zoledronate to be effective, the allograft needs to be encircled by blood vessels prior to zoledronate administration. Otherwise, the zoledronate cannot be transported by the bloodstream, reach and bind to the exposed bone mineral on the allograft's surface. Potentially, the vascular net was not extensive enough, or resorption had preceded the first administration time point. In both events, the allograft would be left unprotected to resorption by osteoclasts. The new bone is a living and highly vascularized tissue, which is why systemic zoledronate likely will penetrate the depth of new bone and return a more homogenous anti-catabolic protection⁵⁸. Local zoledronate can protect the allograft against resorption, but will very unlikely protect layers of new bone deposited on top of it. Fluorochrome labeling would have helped to shed light on the temporal and spatial differences on new bone formation between local and systemic zoledronate.-The lower volume of new bone on the implant systemic surface with zoledronate is counterintuitive, not expected, and difficult to explain. Comparing systemic zoledronate administration in Study I and III with untreated controls in previous studies, could indicate some protective effect of zoledronate on allograft. Study III demonstrated that systemic zoledronate is inferior to local zoledronate in a revision setting when it comes to protecting the allograft against resorption, but it can accrue peri-implant new bone. A comparison of single versus repeated IV zoledronate or local zoledronate exposure would be of interest, but implant models are essentially different regarding the type, (primary vs. revision), position (extra-articular vs. intraarticular), load (unloaded vs. loaded), and gap size (2.5 mm gap vs. 1.1 mm gap).

Study I investigated the effect of coating implant surfaces with three doses of rhBMP-2 (15 μ g, 60 μ g and 240 μ g) or left untreated, and zoledronate IV (0.1 mg/kg) 10 days post surgery. We anticipated some allograft resorption in Study I, but not to the extent seen with any of the rhBMP-2 groups. Mechanical fixation decreased with rhBMP-2 dose increments, which can be explained by decreased volumes off allograft, whereas the volume of new bone was comparable between the implant groups. Because bone formation in a grafted gap depends on osteconduction and osteoinduction, the increasing new boneto-allograft ratio could imply that the relative contribution from osteinduction increased with rhBMP-2 dose increments. An osteoinductive stimulus could also stem from the release of growth factors embedded in resorbed bone, as well as from rhBMP-2 coated onto implant surfaces; but separate contributions are indistinguishable.

Our results contrast a previous rodent study with combined local anabolic and systemic anti-catabolic therapy in a bone conduction chamber model ¹²⁵. A bone conduction chamber is unloaded and regarded as a high catabolic environment; but combined anabolic and systemic anti-catabolic therapy increased bone ingrowth and bone content. Implants in study I were unloaded, which can increase bone metabolism, as explained by Wolff's law of adaption to strain.

A reason could be that Study I used impacted allograft compared to structural cancellous allografts in the rodent studies.

Impaction of bone grafts has been shown to decrease bone ingrowth,^{48; 155; 156} which is why the relative density in our grafted defect could decrease vascular ingrowth. This would leave the allograft unprotected from IV administered zoledronate. Similar arguments apply to the dependency of systemic zoledronate on

vascularization regarding its ability to exert an anti-catabolic effect, as outlined in the previous section. Most likely, the administered dose of zoledronate was not proportional to the indirect catabolic stimulus from any of the rhBMP-2 doses used.

Study III investigated the effect of coating revision implant surfaces with rhBMP-2 (5 μ g) with either local or systemic zoledronate. A lack of catabolic control with systemic zoledronate was evident from Study I, which is why the rhBMP-2 dose was decreased and administration points of zoledronate IV were doubled in study III.

Combined anabolic and anti-catabolic therapy have merit (Appendix: Table XV); the application of rhBMP-7 in a previous study revealed opposite effects of rhBMP-7, with a positive effect in revision implants but being inferior when used in primary implants.¹¹¹

In Study III, no difference on mechanical fixation was detected between the implants with local or systemic zoledronate and rhBMP-2. The resemblance on mechanical fixation covered a histological discrepancy in the composition of the peri-implant gap and mirrored nicely the difference between local svstemic zoledronate and exposure (Appendix: Table XI). The addition of rhBMP-2 to either type of zoledronate exposure seemed to attribute synergistically and re-enforce anabolic and catabolic effects. The inability of systemic zoledronate to protect the allograft became clearer with the introduction of rhBMP-2, whereas local zoledronate exerted a sufficient anti-catabolic effect.

The surface area and volume of new bone increased in implants coated with rhBMP-2 relative to untreated implants although this was not statistically significant. The dose of rhBMP-2 could potentially be too low to induce a clinical significant anabolic effect. As in the other un-paired comparisons in study III (local zoledronate vs. systemic zoledronate), detectable effects were likely caused by zoledronate exposure. Osteoclasts have been viewed as the only cell able to internalized nitrogen containing bisphosphonates and be affected by the inhibition of FPPS in the mevalonate pathway (Figure 2). But in-vitro studies have demonstrated that osteoblasts differentiation and activity can be affected by bisphosphonates^{60; 62}. Bisphosphonate dose been suggested to condition how has angiogenesis is inhibited. FPSS dependent inhibition required high doses of bisphosphonates, whereas non-FPSS dependent inhibition was shown with low bisphosphonate doses in an in-vivo and exstudy⁶³. vivo The bisphosphonate concentrations required to decrease endothelia cell viability and tubule formation in-vitro are high compared to the bisphosphonate concentrations reached clinically with а single systemic administration¹⁵⁷. An inhibitory effect on nonbone cells was demonstrated with colorectal adenoma cells and CHO cells grown on bone disks impregnated with bisphosphonate. Cell number and growth decreased with increasing potency of the bisphosphonate ⁶⁴. Allograft bound zoledronate may potentially exert a lower grade but persistent inhibition on endothelial cells and thus affect vascularization of an impacted peri-implant gap. An approximated sustained zoledronate with multiple exposure systemic administrations of zoledronate (20 μ g of zoledronate SC three times a week for three weeks) in mice decreased the number of new vessels¹⁵⁷. Whereas blood а clinical resembling single zoledronate administration of 120 μ g/kg did not affect blood vessel formation or bone healing in a rat calvaria bone chamber study⁶⁸. A potential negative effect on endothelial cells from local bisphosphonate may be part of the reason for retained allograft with local zoledronate and greater amount of new bone with systemic zoledronate, because both osteoclast and osteoblast cells precursor depends on transportation by the bloodstream to enter the

grafted gap. The results from Study I and III indicate that a positive effect of rhBMP-2 may be harvested with sufficient anti-catabolic control. The presented data warrants further research on combining local and systemic anti-catabolic therapy.

rhBMP-2 and bisphosphonates in empty defects

Study II demonstrated that 15 μ g of surfaceeluted rhBMP-2 increased mechanical fixation due to superior amounts of new bone from the implant surface in continuity with surrounding cancellous host bone. Higher rhBMP-2 doses decreased the amount of new bone in the peri-implant defect. Interestingly, the formation of new bone outside the peri-implant defect in the host bone increased with rhBMP-2 dose increments (Figure 20 and Appendix: Table VIII). Outside the peri-implant defect, bone formation appeared primarily to happen by appositional growth on pre-existing trabecular bone. A similar appositional healing pattern was described by Sumner et al¹¹⁰ in a canine study on empty defect healing with rhBMP-2, where inter-trabecular spacing in the host outside peri-implant bone the defect decreased with rhBMP-2 dose increments; although, this was only significant for a high rhBMP-2 dose (Appendix: Table XIV), whereas all rhBMP-2 groups in study II where superior to untreated implants (Figure 20 and Appendix Table VIII).

Chen et al¹⁵⁸ and Aspenberg et al¹⁵⁹ have described that metaphyseal fractures heal by a combination of appositional growth from preexisting trabecular bone and from what appears to be condensation of osteoblast precursor cells in fracture adjacent bone marrow. Bragdon et al. noted a similar inter-trabecular healing pattern in the host bone outside the defect, in a study on backside defect healing of acetabular components in a canine model investigating rhBMP-2 and ceramic graft ¹⁰⁶. Some specimens in Study II had what appeared to be similar new bone islet- and peninsula-like structures of new bone in between the host trabecular bone, resembling inter-trabecular bone formation (Figure 21; "15 μ g implant" (x1.25)). In contrast to Bragdon et al¹⁰⁶, who only noted inter-trabecular bone formation in animals treated with rhBMP-2, we also noted an inter-trabecular healing pattern in some untreated histological specimens.

We observed only direct new woven bone formation inside and outside the peri-implant defect without any cartilage intermediates, which is in accordance with Sumner et al.¹¹⁰. The healing of peri-implant defects has been described to happen by appositional unidirectional bone growth to titanium surfaces or appositional bi-directional bone growth with hydroxyapatite coated implant surfaces surfaces ¹⁴¹. With the close proximity and timelv coincidence of bone with the morphological appearance of inter-trabecular bone formation in host bone in Study II, the healing of empty peri-implant defects could potentially also happen by inter-trabecular healing on top of uni- and bi-directional bone growth. Chen et al.¹⁵⁸ and Aspenberg et al.¹⁵⁹ described that condensation the of osteoprogenitor cells happen within one or two weeks, why our evaluation time point of four weeks is beyond this point. Further studies are needed to investigate this suggestion. studies³¹ Experimental and retrieved acetabular components³² demonstrated that areas of close but not intimate contact are present initially and after considerable in-situ time. The results from Study II could be relevant when investigating optimized healing of empty defects around press-fit seated implants. The presented results with rhBMP-2 needs to be seen in the context of delayed systemic zoledronate. Why the effects of rhBMP-2 between grafted and empty defects differ, could be because of the direct access to bone marrow with abundant anabolic cytokines and osteoprogenitor cells in the empty defect implants.

Limitations Animal model

Canines were chosen as experimental animals due to their resemblance with human bone regarding bone biology and mineral and non-mineral composition. But the bone turnover rate is considerably higher in canines compared to humans, with a factor of 2-3¹⁶⁰. The inter-individual variation in bone turnover rate in a group of female beagles has been described to be in the range of 16-300%¹⁶⁰. The potential contribution to total variance was addressed by the paired design to minimize a contribution from inter-individual variance.

The age of age of the animals used in Study I-III was comparable to young adults as to the more senior disposition of the average recipients of hip replacements. ¹⁶¹ Age has been demonstrated to affect interface shear strength negatively in greyhounds. ¹⁶² Despite the species difference, this may limit the generalizability, and our results need to be evaluated from this perspective. All animals were skeletally mature male mongrel dogs of a similar breed and had comparable age and weight within each study (Table 1). This reduced the biological inter-variability of bone structure and helped to minimize confounders affecting the results.

Implant model

The applied implant model bear no resemblance to functional arthroplasties. The implants were of a simple cylindrical shape that simplified evaluation of the bone-implant interface and gave a large degree of variable control. The surface coating was commercially available and represented the bone-implant interface of cementless arthroplasties. In Study I and II, the implants were placed extraarticular and orthogonal to the load direction inside cancellous bone. Implants were thus not subjected to either direct mechanical load or effects from joint fluid pressure or flow. A more clinical relevant model was applied in

In Study III was formed over only 8 weeks. This is a short period compared to the in-situ time of clinical-loosened implants, but the revision protocol consistently produces the trilaminar revision cavity of an aseptically loose implant^{131; 132}.

Observation time

A 4-week observation period was chosen based on previous experience with the implant models. In the applied experimental animal, 4 weeks optimally displays initial bone formation before remodeling is completed; here, we would be able to distinguish the effects of therapeutic treatments relevant for initial implant fixation. With only a single time point at 4 weeks being evaluated, extrapolation of the results to earlier or later time points is not possible and would be speculative. Choosing a later evaluation time point may have revealed different results, and an initial positive effect of an intervention may have been evened out. An early evaluation time point could also have been relevant, because negative short-term effects likely not will translate into positive long-term effects, especially in the field of joint arthroplasty, where initial fixation is paramount to ensure implant survival.

rhBMP-2 delivery

In all three studies, the surface was coated with varying doses of rhBMP-2 and solvent constituents, except for the untreated control implants.

Clinically, rhBMP-2 (InductOS[®]/Infuse[®]) is delivered as a lyophilized powder with additives that are reconstituted prior to use into a stock solution of 1.5 mg/ml in a 5 mM glutamic acid buffer, 2.5% (w/v) glycine, 0.5% sucrose, and 0.01% (w/v) polysorbate 80. The dose is applied by volume on a collagen sponge (bovine collagen type 1) to ensure a gradual release and act as a scaffold for new bone formation. Both a titanium and titaniumalloy surface consists of an oxide layer with a thickness of 3-5 μ m. The oxide layer is described amphoteric, as hydrophobic, mechanically stable, and readily able to adsorbs proteins, and is thus theoretically suitable to give footing for rhBMP-2 at the implant surface and not directly be repelled or slide off ²⁶. TCE cleaning does not affect albumin binding to titanium surfaces ¹⁶³ and should not affect protein adherence to the implant surface. Direct implant surface application of growth factors 110; 164; 165 or peptides have been demonstrated practically feasible ^{166; 167}. rhBMP-2 is, as other proteins, highly sensible to alterations of the solution pH. We did not investigate pH changes in reconstituted rhBMP-2 below or above the recommended concentration of 1.5 mg/ml. In Study I and II, to allow for 240 μ g in a 60 μ I volume, rhBMP-2 was reconstituted to 4 mg/ml. Second, rhBMP-2 was reconstituted with saline in Study I and II and saline and buffer (5 mM glutamic acid buffer, 2.5% (w/v) glycine, 0.5% sucrose, and 0.01% (w/v) polysorbate 80) in Study III. We took great care in handling rhBMP-2 during the separate coating procedures in all 3 studies. To avoid binding of rhBMP-2 to plastic surfaces, we used low protein binding laboratory utensils and blocked our coating wells with bovine serum and immediately transferred the coated implants to a freezer until implantation. We did not perform a bio-activity assay of eluted rhBMP-2 in the release study. Despite deviations from manufacturers' recommendations, the presence of detectable rhBMP-2 in the intended range combined with the dose-related biological response in the experimental studies indicated the release of functional rhBMP-2 from inserted implants (Appendix: Figure II and Table IV).

Distant and local effects

In Study I and II, multiple paired studies were performed on each animal (proximal humeri, distal femurs, and proximal tibias), and in Study III, the animals' stifle joints were operated on bilaterally. Multiple surgeries in each animal could potentially introduce bias and distort results and can be divided into local and distant effects. In Study I and II, paired four-armed studies were conducted on each animal. Two implants in relative proximity to one another were inserted in each proximal humerus (Figure 5) and distal femur (Figure 6). Performing surgery at immediate adjacent sites can be viewed as a noxious stimulus and spur a Regional Acceleratory Phenomenon (RAP), as described by Frost et al in diaphyseal bone¹⁶⁸. In a sheep study with press- fit seated implants in cancellous bone, RAP was considered to have a negligible to no effect on the host bone 3 mm from the inserted implant surfaces¹⁶⁹. The sum of total surgical trauma from non-neighboring sites can potentially induce a systemic metabolic effect on distant sites. By default, this is relevant for all 3 studies due to the paired design, but the heavier total surgical load inflicted upon animals in Study I and II could exaggerate a potential distant metabolic effect. Einhorn et al.¹⁷⁰ demonstrated that complete bone marrow ablation can induce distant bone anabolic effects, but to a lesser degree when only cortical bone was traumatized. Gazit el al.171 reported that no bone anabolic response was raised if the marrow was not allowed to regenerate by physical blockage of the medullary cavity and that periosteal injury did not exert an effect.

Our studies were conducted with implants in metaphyseal cancellous bone in contrast to the studies on diaphyseal bone without implants by Einhorn et al. and Gazit et al.. Metaphyseal bone injury does raise an inflammatory response, both locally and at distant sites, as recently demonstrated by Tätting et al.¹⁷². In another rodent study by Sandeberg et al.¹⁷³, on metaphyseal and diaphyseal fracture healing, ablating the inflammatory response with glucocorticoids did not affect the healing of stable metaphyseal fractures, but in contrast, it impaired the healing of diaphyseal fractures. If local inflammation in metaphyseal bone injury is not important for local metaphyseal bone healing, it is feasible that the bone anabolic effect would be minor in distant trabecular bone sites. Last, the injury inflicted upon the metaphyseal bone marrow was more than a periosteal and cortical injury alone, but less severe than eradication of the diaphyseal bone marrow, as described by Einhorn et al. and Gazit et al.. With a potentially minor important role of inflammation in metaphyseal bone healing, blockage of the drill-hole with an implant and a lesser surgical insult, systemic effects from parallel metaphyseal studies could be minor. Growth factors, although administered locally, will likely desorb from the implant surface and enter the circulation. Sumner et al., demonstrated distant bone anabolic effects after administering TGF-B2 on implants inserted in the proximal humeri in canines¹⁶⁴.

In summary, the single animal was exposed to a similar stimulus in both degree and magnitude to the effects from a local RAP and a systemic contribution from distant surgical insult or applied bone anabolic growth factors. Recognizing these potential confounders, we used a within-animal/within-site design where the animal functioned as its own control. Potential confounders were, by this design, equally and uniformly distributed and should not have introduced bias in the results. If present, we expect an effect to be moderate and not be able to change the relative ranking of implant fixation.

Conclusion

This thesis primarily investigated the effects of combined anabolic and anti-catabolic therapy with rhBMP-2 and zoledronate on early implant fixation in primary and revision implants. Secondly, we investigated the effects on allograft incorporation with different exposures to anti-catabolic treatment by applying local and systemic zoledronate. Thirdly, we verified the ability of delivering rhBMP-2 locally without carrier by surface elution from the implants used in Study I-III.

The results demonstrate that rhBMP-2 exerts significantly different catabolic and anabolic effects within a narrow dose range and it seems to depend on bone-implant interface conditions.

Two studies showed, irrespective of mechanical loading, no positive effect of augmenting implant surfaces with rhBMP-2 in allograft-impacted peri-implant defects; but in contrast, improved healing of unloaded implants surrounded by an empty peri-implant defect via direct bone formation was found.

All studies demonstrate that rhBMP-2 can induce bone resorption, and concurrent anticatabolic therapy appears indispensable if rhBMP-2 is used with orthopedic implants in cancellous appendicular bone. The use of rhBMP-2 with allograft seems questionable based on Study I and Study III.

Local anti-catabolic control with zoledronate in impaction-grafted peri-implant revision defects was superior to systemic zoledronate when protecting allograft, but new bone accrued superiorly with systemic zoledronate treatment. The present studies are limited by the observation period, the applied rhBMP-2 and zoledronate doses, the delivery of rhBMP-2, and the number and administration time points of zoledronate which all warrants further pre-clinical investigation.

Future perspectives

In Study II, combining anabolic and anticatabolic therapy to heal empty peri-implant defects, demonstrated a positive effect on implant osseointegration within a narrow rhBMP-2 dose range. In press-fit seated acetabular components, backside defects are often present, which is why it could be of benefit to further explore on anabolic dose with anti-catabolic therapy in the healing of empty peri-implant defects. The surface release of rhBMP-2 demonstrated a positive dose-dependent effect of bone formation outside empty gap implants. Such augmentation of the host bone without compromising the implant-bone interface with combined therapy could be of interest and potentially re-enforce peri-implant support. In Study III, the separate results of local and systemic zoledronate treatment to protect the allograft and accrue new bone, respectively, warrants exploring an eventual additive effect of combining local and systemic zoledronate therapy.

Bisphosphonates as anti-catabolic therapy are intriguing. They are incorporated into the host bone and act like an anti-catabolic reservoir. Therefore, the effects are not restricted to the evaluation point applied in these studies, which is why evaluation of later time points could be of interest.

All studies demonstrated that seeking an anabolic effect from rhBMP-2 while balancing the indirect catabolic drive is difficult. Combining local and systemic anti-catabolic therapy could potentially allow for the use of higher doses of rhBMP-2 and enable harvesting an increase in bone formation in grafted implants.

Bisphosphonate treatment affects bone remodeling, which is why the accumulation of microfractures could impede achieving longterm benefits from short-term gains on initial implant fixation. Bisphosphonates are not anabolic and attribute to implant fixation by preserving an osteoconductive lattice. Extending treatment with an anabolic stimulus has also proven its merit in the healing of empty defects in this study. But bone formation is a sequentially ordered process that depends on correct spatial and temporal expression of a range of growth factors and cytokines to reach an optimum. Combining rhBMP-2 with other anabolic growth factors, could potentially improve implant osseointegration instead of relying on a single anabolic entity such as rhBMP-2. The presented studies demonstrate that animating bone formation is difficult, and that the therapeutic window for rhBMP-2 is narrow. The study results underline that combined anabolic and anti-catabolic therapy with implants is not ready to be tested clinically. More protocolled animal studies are needed to explore potential beneficial effect on initial implant fixation and osseointegration. Both on combined local and systemic anti-catabolic therapy and rhBMP-2 with sufficient anticatabolic therapy.

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Figures:

Figure 1: Rendered from: "Novel insights into actions of bisphosphonates on bone: differences in interactions with hydroxyapatite"; Nancollas, G.H. ⁵⁹

Figure 4: Rendered from: "Bone Morphogenetic Protein (BMP) signaling in development and human diseases"; Wang,R.N.⁹⁸

Figure 10: Courtesy of Andreas West

APPENDIX

Table of Contents

Volume of implant coatingII Implant coating procedureIII IMPLANT RELEASE STUDYIV rhBMP-2 release procedureIV rhBMP-2 quantificationIV rhBMP-2 release kineticsIV RHBMP-2 release GRAPHSV IMPLANT SEMVI IMPLANT SEM IMAGESVII STUDY RESULTSVIII Study IVIII
Implant coating procedureIII IMPLANT RELEASE STUDYIV rhBMP-2 release procedureIV rhBMP-2 quantificationIV rhBMP-2 release kineticsIV RHBMP-2 RELEASE GRAPHSV IMPLANT SEMVI IMPLANT SEM IMAGESVII STUDY RESULTSVIII Study IVIII
IMPLANT RELEASE STUDY IV rhBMP-2 release procedure IV rhBMP-2 quantification IV rhBMP-2 release kinetics IV RHBMP-2 RELEASE GRAPHS V IMPLANT SEM VI STUDY RESULTS VIII Study I VIII
rhBMP-2 release procedure
rhBMP-2 quantificationIV rhBMP-2 release kineticsIV RHBMP-2 RELEASE GRAPHSV IMPLANT SEMVI IMPLANT SEM IMAGESVII STUDY RESULTSVIII Study IVIII
rhBMP-2 release kinetics
RHBMP-2 RELEASE GRAPHSV IMPLANT SEMVI IMPLANT SEM IMAGESVII STUDY RESULTSVIII Study IVIII
IMPLANT SEMVI IMPLANT SEM IMAGESVII STUDY RESULTSVIII Study IVIII
IMPLANT SEM IMAGESVII STUDY RESULTSVIII Study IVIII
STUDY RESULTS
Study IVIII
Study IIIX
Study IIIX
BP STUDIESXI
BMP STUDIESXII
BP + BMP STUDIESXIII
THESES FROM THE ORTHOPAEDIC
RESEARCH GROUPXIV
DOCTORAL THESES XIV
PHD THESES XIV

Implant coating

The initial intention was to drip coat the implants with the rhBMP-2 solution, but drops slid off because of the solution's surface tension. We opted instead for desiccation to enable the liquid to saturate the porous surface coating. To determine the amount of retained liquid per implant, we performed an implant soaking study. The experimentally measured porosity volume was used to calculate the rhBMP-2 solution concentration for implant coating (Appendix: Table III). To document the coating procedure a separate release study (Appendix: Figure II and Table IV) and a SEM scan (Appendix: Figure III) were performed. The relase study and SEM scan combined, confirmed the presence and release of agent in the intended range from rhBMP-2 coated implants.

Volume of implant coating

Ten porous-coated implants were mounted on stainless-steel rods and were fully submerged in separate wells in a well-plate (96-Well, Standard F, Sarstedt AF & Co., Nümbrecht, Germany) with 150 μ l of sterile PBS (Sigma-Aldrich Chemie Gmbh., Steinheim, Germany) per well. Following the well-plate with implants were placed in a standard glass desiccator with a vacuum for 5 minutes. Implants with rods were measured dry and after desiccation on a scale (AG204 Deltarange[®], Metler

Implant data	Study	Study	Study
	I	П	Ш
Length (mm)	10	10	10
Outer diameter (mm)	5.8	5.8	5.8
Core diameter (mm)	4.4	4.4	4.4
Drill hole volume (ml)	0.950	0.442	0.528
Implant volume (ml)	0.264	0.264	0.264
Coating volume (ml)	0.112	0.112	0.112
Porosity volume (ml)	0.071	0.071	0.071
Gap volumen (ml)	0.686	0.178	0.264

Appendix Table I. Dimensions for implant and periimplant gap. Coating volume = Implant core volume -Implant volume. Gap volume = Drill hole volume -Implant volume. Porosity volume = Coating porosity (63%) x Coating volume.

10	59.9 (0.03)
5	59.9 (0.04)
Time (min)	Weight (µg)

Appendix Table I	I. Mean (sd)	average PB	S uptake pr.
implant. Desiccatio	on time (Tim	e).	

Toldeo, Singapore). The soaking procedure was repeated 3 times on separate days using the same implants (Appendix: Table II). Differences were calculated and averaged per implant. The weight per volume for PBS (1.00142 μ g/ μ l at 22°C) is nearly identical to pure water (1.00 μ g/ μ l at 20°C) and the weight of contained PBS was converted to volume. Average implant coatings contained 60 μ g of PBS corresponding to a volume of 60 μ l. This was 18% lower compared to manufacturer specifications (Appendix: Table I and II).

Volumes of cylindrical implants and drill-holes were calculated using the equation for volume of a cylinder:

Equation 5:

Implant volume =
$$\pi \times (\frac{D}{2})^2 \times L$$

Where D is implant diameter and L is implant length.

Implant coating procedure

The coating of implants with rhBMP-2 was performed in a laminar airflow bench with sterile technique. In Study I and II, rhBMP-2 powder was reconstituted with kit diluent (sterile saline). In study III, the rhBMP-2 powder was reconstituted with kit diluent to a concentration of 1.5 mg/ml. Further dilution was performed using a buffer (5.0 mg sucrose, NF; 25 mg glycine, USP; 3.7 mg Lglutamic acid, FCC; 0.1 mg sodium chloride, USP; 0.1 mg polysorbate 80, NF pr. ml; pH=7.4). Upon reconstitution. visual inspection of the rhBMP-2 solution ensured that all of the rhBMP-2 powder was dissolved, and liquid appeared completely the transparent. Implants were mounted on a threaded stainless-steel rods and placed into individual wells in a well-plate (96-Well, Standard F, Sarstedt AF & Co., Nümbrecht, Germany) and positioned in a standard glass desiccator (Appendix: Figure I). Each well contained 150 μ l of rhBMP-2 solution, and the fluid surface was below the top edge with fully submerged implants. Vacuum was applied for 5 minutes to saturate the porous coating with the rhBMP-2 solution. Implants were air-dried in vacuum and packed in separate sterile tubes (Eppendorf LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and stored at -21°C (Study I and II) and -80°C (Study III) until implantation. Prior to the coating procedure, well surfaces were blocked with 0.5% BSA (Bovine Serum Albumin, Sigma-Aldrich, Saint Louis, MO) in PBS (Sigma-Aldrich Chemie Gmbh., Steinheim, Germany), for 24 hours at room temperature, rinsed, and air-dried before coating procedure to minimize rhBMP-2 adsorption to the well



Appendix Figure I. Implant coating with rhBMP-2. A: A well is filled with the rhBMP-2 solution, B: An untreated implant mounted on a threaded rod is submerged into the well, C: Desiccation D: Evaporation of solute.

surfaces. Pipette-tips were 100% polypropylene (Finntip, Thermo Fischer Finland)¹⁷². Scientific Ov. Vantaa, The stainless-steel rod spaced out the hollow implant core and prevented excess rhBMP-2 from adsorbing to the hollow implant core and aided in the ease of handling.

		rbPMP 2 solution (us/ml)	Peri-implant gap rhBMP-2 conc. (µg/ml)		
Dose (µg)	volume (μ)	IIIBMF-2 Solution (µg/III)	Study I	Study II	Study III
5	60	0.84	-	-	19
15	60	0.25	22	84	-
60	60	1.00	87	338	-
240	60	4.00	350	1352	-

Appendix Table III. Estimated rhBMP-2 implant dose (μ g), rhBMP-2 solution concentration (μ g/ml) used for implant coating and estimated peri-implant gap concentration (μ g/ml).

Implant release study

rhBMP-2 release procedure

In vitro release was performed with two rhBMP-2 coated implants from each group and two untreated implants. Implants were placed in separate wells in a lid covered wellplate (24-well, Standard F, Sarstedt AF & Co., Nümbrecht, Germany), blocked with 0.5% BSA/PBS and filled with 5 ml of PBS on a stable plate in a heating cabinet (37°C). Samples were collected in duplicates from each well, and immediately replenished with fresh sterile PBS.

Sampling points:

Study I and II: 0h, 6h, 12h, 24h, 48h and 72h.

Study III: 0h, 1h, 6h, 12h, 24h, 48h, 72h, 96h and 120h.

Sampling fraction was 20% (2 x 0.5 ml) in Study I and II and 40% in study III (2 x 1 ml). Samples were stored in sterile Eppendorf tubes (Eppendorf LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and kept frozen (-20°C) until quantification. The different sampling fractions were due to a procedural error in Study III.

rhBMP-2 quantification

Released rhBMP-2 was quantified using a quantitative sandwich enzyme immunoassay specific for CHO-cell produced rhBMP-2 (BMP-2 Quantikine Elisa kit DBP200, R&D systems, Minneapolis, MN) according to per manufacturer instructions. Readings were done with a microplate reader set to 450 nm with a wavelength correction set to 540 nm to compensate for optical imperfections in the microreader plate. Results from each rhBMP-2 group and controls were separately averaged.

rhBMP-2 release kinetics

Average released dose per sample point were used to calculate cumulative rhBMP-2 release kinetics, as described by Strobel et al ¹⁷³ using the equation:

Equation 6

$$\left[(C_{cs} \times V_e) - \left((C_{ps} \times V_e) \times F_d \right) \right]_{current}$$

+
$$\left[(C_{cs} \times V_e) - \left((C_{ps} \times V_e) \times F_d \right) \right]_{prior}$$

Where:

 C_{cs} : rhBMP-2 concentration current sample (μ g/ml)

 C_{ps} : rhBMP-2 concentration prior sample (μ g/ml)

V_e: Elution volume (ml)

F_d : Dilution factor of sampling (Study I-II: 0.8; Study III: 0.6)

All rhBMP-2 coated implants demonstrated an initial concentration increase in the release solute that subsequently decreased. In Study I and II, the total rhBMP-2 release (96 h) was 26% lower (15 μ g implants) or 11-12% higher (60 μ g and 240 μ g implants, respectively), compared to the estimated rhBMP-2 doses. In Study III, total release (120 h) was 12% higher compared to the estimated rhBMP-2 dose (Appendix: Figure II and Table IV).

Implant (µg)	Released (μ g)	Difference (%)
5	5.6 (± 0.25)	12
15	12 (± 1)	- 20
60	67 (±3)	12
240	272 (± 7)	13

Appendix Table IV. Mean	(sd) release of rhBMP-2 vs.
estimated rhBMP-2 dose.	



rhBMP-2 release graphs

Appendix Figure II. Average rhBMP-2 release from rhBMP-2 coated implants. Study I and II (15 μ g, 60 μ g and 240 μ g) and Study III (5 μ g).

Implant SEM

Implant surfaces of one untreated implant, one 5 μ g rhBMP-2 implant and one 5 μ g rhBMP-2 implant after release procedure were visualized by scanning electron microscopy (Nova NanoSEM 600, FEI Company, Hillsboro, OR). Please refer to Appendix: Figure III.

The untreated control implant surface resembled the manufacturer's description. Outermost a highly irregular and textured layer on top of a layer of spherical beads.

A similar dual surface structure was recognized in the 5 μ g implants as in the untreated control implant, but the 5 μ g implant contained areas spread along the length of the implant with a glacier like crystalline appearance. These areas appeared significantly decreased in area and thickness on the post-release 5 μ g implant and were not seen on the untreated implant.

Only the 5 μ g implants were SEM scanned. SEM evaluation is only "skin deep" on a porous surface structure; any rhBMP-2 adsorbed to the implant core or titaniumbeads would not be visualized.

Implant SEM images



Appendix Figure III. SEM images of an untreated implant (A), a 5 μ g rhBMP-2 coated implant (B1-3) and a 5 μ g rhBMP-2 coated implant after release (C1-2). A: Minor impurities on the implant surface (dark areas) but without elevation from the surface. B1: Multiple areas with glacier like structure scattered on the implant surface. B2-3: "Glacier structure" up close. C1-2: A decreases in area, number and thickness of coated areas compared to B1-3. Areas of impurities as seen on untreated implants were also found on both the coated and released implant.

Study results

Study I

	Strength	Energy (kJ/m ²)	Stiffness (MPa/mm)
Untreated	4.6 (3.8 - 5.4)	0.7 (0.5 - 0.8)	24.0 (19.6 - 28.4)
15 µg	2.6 (1.8 - 3.4)	0.4 (0.2 - 0.5)	14.0 (9.6 - 18.4)
60 µg	1.7 (0.9 - 2.5)	0.3 (0.2 - 0.4)	7.2 (2.7 - 11.6)
240 µg	0.9 (0.1 - 1.6)	0.1 (0.0 - 0.3)	4.4 (0.0 - 8.8)
ANOVA*	p = 0.0000	p = 0.0000	p = 0.0000

Appendix Table V. Biomechanical results Study I. Results are presented as mean (95% CI) per treatment group. Strength: maximum shear strength (MPa); Energy: total energy absorption (KJ/m²); Stiffness: apparent shear stiffness (MPa/mm). *Repeated measures ANOVA.

	New bone			
	Surface	In gap	Off gap	
Untreated	0.21 (0.17 - 0.25)	0.31 (0.28 - 0.33)	0.03 (0.02 - 0.05)	
15 µg	0.16 (0.12 - 0.20)	0.29 (0.27 - 0.32)	0.04 (0.02 - 0.05)	
60 µg	0.07 (0.04 - 0.11)	0.28 (0.25 - 0.31)	0.05 (0.04 - 0.07)	
240 µg	0.06 (0.02 - 0.09)	0.26 (0.24 - 0.29)	0.03 (0.02 - 0.05)	
ANOVA*	<i>p</i> = 0.0000	<i>p</i> = 0.1415	<i>p</i> = 0.2015	
		Allograft	Lamellar bone	
		In gap	Off gap	
Untreated	-	0.23 (0.21 - 0.26)	0.34 (0.30 - 0.37)	
15 µg	-	0.14 (0.12 - 0.17)	0.36 (0.33 - 0.40)	
60 µg	-	0.07 (0.05 - 0.10)	0.36 (0.33 - 0.40)	
240 µg	-	0.03 (0.00 - 0.05)	0.33 (0.30 - 0.37)	
ANOVA*	-	<i>p</i> = 0.0000	<i>p</i> = 0.4211	

Appendix Table VI. Histomorphometric results Study I. Results are presented as mean fraction (95% CI) of surface area and volume per treatment group. *: Repeated measures ANOVA. "In gap" and "Off gap" designates in- and out-side the allografted peri-implant gap. Significant results are marked in bold.

Study II

	Strength	Energy	Stiffness (MPa/mm)
Untreated	1.2 (0.5 - 1.9)	0.2 (0.0 - 0.3)	6.5 (3.3 - 9.7)
15 µg	3.1 (2.4 -3.8)	0.6 (0.4 - 078)	14.4 (11.2 - 17.6)
60 µg	1.4 (0.8 - 2.1)	0.3 (0.2 - 0.4)	6.5 (3.2 - 9.7)
240 µg	1.2 (0.5 - 1.9)	0.3 (0.1 - 0.4)	4.8 (1.5 - 8.0)
ANOVA*	p = 0.0004	p = 0.0014	p = 0.0006

Appendix Table VII. Biomechanical results Study II. Results are presented as mean (95% CI) per treatment group. Strength: maximum shear strength (MPa); Energy: total energy absorption (KJ/m²); Stiffness: apparent shear stiffness (MPa/mm). *Repeated measures ANOVA.

	New bone			Lamellar bone
	Surface	In gap	Off gap	Off gap
Untreated	0.10 (0.06 - 0.13)	0.22 (0.19 - 0.25)	0.09 (0.07 - 0.12)	0.25 (0.20 - 0.29)
15 µg	0.15 (0.11 - 0.18)	0.23 (0.20 - 0.26)	0.14 (0.12 - 0.17)	0.23 (0.18 - 0.27)
60 µg	0.09 (0.06 - 0.13)	0.19 (0.16 - 0.22)	0.13 (0.10 - 0.15)	0.24 (0.19 - 0.28)
240 µg	0.07 (0.04 - 0.11)	0.17 (0.14 - 0.20)	0.17 (0.15 - 0.20)	0.19 (0.15 - 0.24)
ANOVA*	<i>p</i> = 0.0298	<i>p</i> = 0.0145	<i>p</i> = 0.0006	<i>p</i> = 0.304

Appendix Table VIII. Histomorphometric results Study II. Results are presented as mean fraction (95% CI) of surface area and volumes in- and out-side the peri-implant gap per treatment group (referred to as "In gap" and "Off gap respectively). *Repeated measures ANOVA.

	Strength	(Mpa)	Energy (ł	<j m²)<="" th=""><th colspan="3">Stiffness (Mpa/mm)</th></j>	Stiffness (Mpa/mm)		
	Spearman	<i>p</i> -value	Spearman	<i>p</i> -value	Spearman	<i>p</i> -value	
Surface - New bone	0.58	0.0000	0.53	0.0001	0.60	0.0000	
Gap - New bone	0.47	0.0009	0.42	0.0029	0.48	0.0006	
Off gap - New bone	0.16	0.28	0.27	0.06	0.05	0.75	

Appendix Table IX. Correlation between histomorphometric and mechanical results Study II. Results are presented as Spearman Rho with corresponding *p*-value.

Study III

	Strength (MPa)	Energy (kJ/m2)	Stiffness (MPa/mm)
LZ	10.5 (9.5-11.6)	1.5 (1.4-1.7)	55.3 (48.4-62.2)
LZ+rhBMP-2	10.3 (9.2-11.4)	1.5 (1.3-1.6)	54.6 (47.7-61.5)
SZ	8.6 (6.7-10.6)	1.2 (0.8-1.5)	45.9 (35.7-56.2)
SZ+rhBMP-2	8.2 (6.3-10.2)	1.1 (0.8-1.5)	42.7 (32.5-53.0)

Appendix Table X. Biomechanical results Study III. Results are presented as mean (95% CI) per treatment group. Strength: maximum shear strength (MPa); Energy: total energy absorption (KJ/m²); Stiffness: apparent shear stiffness (MPa/mm). SZ: Systemic Zoledronate. LZ: Local Zoledronate.

	Sur	face	Gap				
	New bone	Allograft	New bone	Allograft			
LZ	0.23 (0.18-0.28)*	0.007 [0.004-0.01]*	0.25 (0.21-0.29)**	0.26 (0.20-0.31)**			
LZ+rhBMP-2	0.27 (0.22-0.32) ^{††}	0.01 [0.006-0.016]	0.29 (0.25-0.33)	0.28 (0.23-0.33) ^{†††}			
SZ	0.15 (0.10-0.20)	0.002 [0.001-0.005]	0.34 (0.30-0.38) ^{††}	0.15 (0.10-0.21) ^{††}			
SZ+rhBMP-	0.16 (0.11-0.21)	n/e	0.36 (0.32-0.40)	0.07 (0.02-0.12)			

Appendix Table XI. Histomorphometric results Study III. Results are presented as mean fraction (95% CI) or median fraction [95% CI] of surface area and volumes inside the peri-implant gap per treatment group. n/e: not estimable. SZ: Systemic Zoledronate. LZ: Local Zoledronate. p-values (<0.05; <0.01; <0.001) when compared to SZ (*;**;***) and rhBMP-2+SZ ([†]; ^{††}; ^{†††}).

		Surfa	ace			Vol	ume	
	New bo	one	Allograft		New bo	one	Allograft	
	Spearman	<i>p</i> -value	Spearman	р-	Spearman	<i>p</i> -	Spearman	<i>p</i> -
Strength	0.43	0.003	0.39	0.006	0.19	0.20	0.41	0.004
Energy	0.37	0.009	0.22	0.140	0.15	0.33	0.29	0.045
Stiffness	0.42	0.003	0.39	0.006	0.14	0.33	0.46	0.001

Appendix Table XII. Correlation between histomorphometric and mechanical results Study III. Data are presented as Spearman Rho with corresponding p-value.

Species	Period	Model	Graft	BP	Route	Administration	BP dose	Result	Reference
Rabbit	6w	DO	-	ZOL	IV	0w, 0/2w	0.1 mg/kg	Strength↑ New bone↑ Mineral↑	Little et al. ⁸⁸ 2003
Rat	6w	IPC	ALG CLO	ALE	SC	x3/w/5w x3/w/5w	4 - 205 μg/kg 0.1 - 21 μg/kg	High > Low Total bone ↑	Astrand et al. ⁸⁴ 2002
Rat	6w	BCC	ALG	ALE	S	0w	1.0 mg/ml	Total bone ↑ Graft ↑	Aspenberg et al. ⁸⁷ 2002
Piglet	8w	ON	ABG	IBN	SC	Pre or Post	1.5 mg/kg	Total bone ↑ vs. saline	Kim et al. ⁸³ 2005
Canine	6w	IMP	-	ZOL	IV	Ow	0.1 mg/kg	New bone ↑	Bobyn et al. ⁹¹ 2005
Rat	6w	BCC	ALG	ZOL	SC	x1/w/5w	1.05 <i>µ</i> g	New bone ↑ Graft ↑	Astrand et al. ⁸⁵ 2006
Rat	6w	BCC	ALG	ZOL	SC	Pre 24h	0.7 mg/kg	New bone ↑ Total bone ↑	Tagil et al. ⁸⁶ 2006
Rat	6w	FX	-	ZOL	D/IV	0w,1w, 2w	0.1 mg/kg	1-2w > 0w Strength ↑ New bone ↑	Amanat et al. ⁸⁹ 2007
Canine	4-12w	IMP	ALG	ALE	S	Ow	2 mg/ml	Strength ↓ New bone ↓ Graft ↑	Jakobsen et al. ⁶⁶ 2007
Canine	4w	IMP	ALG	ZOL	S	Ow	0.5 mg/ml 0.05 mg/ml 0.005 mg/ml	0.005 best Strength ↑ New bone ↑ Graft ↑	Jakobsen et al. ⁶⁵ 2010
Rat	1-26w	FX	-	ZOL	SC	1w x1/w/5w	0.1 mg/kg 0.02 mg/kg	1w > 5w Strength ↑	McDonald et al. ⁹⁰ 2008
Canine	4w	IMP	ALG	ZOL	S	Ow	0.005 mg/ml	Strength \uparrow New bone \rightarrow Graft \uparrow	Sorensen et al. ⁹² 2013
Canine	12w	IMP	-	ALE	D	Ow	0.2 mg 1.0 mg	1.0 > 0.2 Total bone ↑	Bobyn et al. ⁹³ 2014
Canine	12w	IMP	-	ALE	D	Ow	0.02 mg/cm ² 0.06 mg/cm ² 0.18 mg/cm ²	0.06 > 0.02 0.02=0.18 Total bone ↑	Pura et al. ⁹⁴ 2016

Table XIII: ALE: alendronate; ALG: allograft; BCC: bone conduction chamber; BP: bisphosphonate; D: direct administration
(adm.); DO: distraction osteogenesis; FX: femur fracture; IBN: ibandronate; IMP: implant; IPC: instable plate chamber; IV:
intravenous; ON: osteonecrosis; Post: after harveste/procedure; Pre: before harvest/procedure; S: soak; SC:sSubcutaneous; w:
week; ZOL: zoledrabbitronate; \downarrow : decrease; \uparrow : increase; \rightarrow : unchanged.

BMP studies

Species	Period	Model	Graft	GF	Carrier	Dose	GF concentration	Result	Reference
Rabbit	2-6w	BHC IM	None	rhBMP- 2	СМ	0.6-12 μg	0.12-2.4 μg/mm ³	BHC: New bone ↓ IM: New bone ↑	Jeppson et al. ¹¹² 1996
Canine	12- 24w	CD - radius	ABG	rhBMP- 2	ACS	150-2400 μg	50-800 µg/ml	rhBMP-2 = ABG Strength Total bone	Sciadini et al. ¹¹⁶ 2000
Canine	6w	IMP	None	rhBMP- 7	СМ	325 µg	492 µg/ml	Strength ↑ New bone ↑ CM induce bone	Lind et al. ¹¹³ 2000
Canine	12w	THA	CPC	rhBMP- 2	Graft	160 µg	64 µg/ml	New bone \rightarrow Total bone \rightarrow	Bragdon et al. ¹⁰⁶ 2003
Canine	4w	IMP	ALG	rhBMP- 7	Graft	400 - 800 μg	2.5-5.0 mg/ml	Revison: Strength ↑ New bone ↑ Primary: Strength↓ New bone →	Soballe et al. ¹¹¹ 2004
Sheep	6-26w	THA	ALG	rhBMP- 7	СМ	2500 μg	n/e	Resorption ↑ Remodeling ↑	McGee et al. ¹¹⁴ 2004
Canine	4w	IMP	None	rhBMP- 2	Surface	100-800 µg	35 - 283 µg/ml	New bone ↑	Sumner et al. ¹¹⁰ 2004
Canine	12w	THA	CPC	rhBMP- 2	PLA-PEG	200 µg	n/e	New bone ↑	Hoshino et al. ¹⁰⁵ 2007
Canine	6w	OT-Tibia	CPC	rhBMP- 2	Graft	576 µg	1.1 mg/ml	rhBMP-2 > ATG Strength ↑ New bone ↑	Schaefer et al. ¹⁰⁹ 2009
Canine	12w	CD- Ulnae	ABG ALG CPC	rhBMP- 2	ACS	210-650 <i>µ</i> g	37.5-116 <i>µ</i> g/ml	rhBMP-2 + graft Strength ↑ Healing ↑	Jones et al. ¹⁰⁷ 2008
Sheep	1-8w	D-Femur condyle	None	rhBMP- 2	ACS	0.43-3.0 mg	82-569 μg/ml	Resorption ↑ /wid New bone ↑	Toth et al. ¹¹⁸ 2009
Cyno	1-24w	D-Femur neck	None	rhBMP- 2	ACS	360 µg	n/e	Resorp.↑ New bone ↑	Seeherman et al. ¹¹⁷ 2010
Canine	12w	CD- Ulnae	CPC	rhBMP- 2	Graft (S)	35-2240 µg	32-2028 μg/ml	New bone ↑ Healing w/ high dose	Harada et al. ¹⁰⁸ 2012
Rat	8w	CD- Femur	None	rhBMP- 2	PLGA	0.4-45 µg	5-600 µg/ml	2.25 µg best Callus ↑/wid Cyst ↑/wid	Zara et al. ¹¹⁵ 2011

Table XIV: ABG: Autograft; ACS: absorbable collagen sponge; ALG: allograft; BCD: bone conduction chamber; BHC: bone harvest chamber; CD: critical defect; CM: Ccllagen matrix; CPC: calcium-phosphate-ceramic; Cyno: cynomolgus monkey; EO: ectopic ossification; GF: growth factor; IM: intra-muscular; IMP: implant; n/e: not estimable; OT: osteotomy; PLA-PEG: poly-d-lactic acid-polyethylene glycol; PLGA: poly(lactic-co-glycolic acid; S: soak; TGF- β 1: tissue growth factor beta-1; THA: total hip arthroplasty; wid: With increasing dose; \downarrow : decrease; \uparrow : increase; \rightarrow : unchanged.

	m					l. ¹²⁶		54	0		28		
Reference	Jeppsson et al. ^{12:} 2003	Little et al. ¹²⁰ 2005	Baas et al. ⁶⁷ 2008	Harding et al. ¹²⁵ 2008	Belfrage et al. ¹²⁴ 2011	Vandermeer et al 2011	Doi et al. ¹²² 2011	Bosemark et al. ¹² 2013	Mathavan et al. ¹¹¹ 2013	Kim et al. ¹²⁷ 2014	Aruwajoye et al. ¹ 2017	Baas et al. ¹²⁹ 2017	osphonate; CLO: ellous bon; IO: ↑: increase: →:
Result	rhBMP-7 CLO Total bone ↑ Ingrowth ↑	rhBMP-7 Healing ↑ rhBMP-7 ZOL Strength↑ Callus ↑	rhBMP-2 Graft ↓ PAM Graft ↑	rhBMP-2 ZOL Bone ingrowth ↑ Total bone ↑	rhBMP-2 ZÓL Bone ingrowth ↑ Total bone ↑	rhBMP-2 IBN Total bone ↑ Ectopic IA bone	rhBMP-2 ZOL Healing ↑ Strength ↑	rhBMP-7 ABG ZOL Callus↑ Total bone↑	GF ALG ZOL > ABG Callus ↑ Strength ↑	rhBMP-2 IBN Total bone ↑	rhBMP-2 IBN Total bone ↑ Mineralization →	rhBMP-2 ZOL Bone resoption∱	hamber; BP: bispho MP: implant in canco mate: ⊥: decrease: `
concentration	46 mg/ml	2653 mg/ml	674 mg/ml	46 mg/ml	92 mg/ml	n/e	n/e	n/e	n/e	n/e	n/e	225 mg/ml	ie conduction c ibandronate; II < ZOI : zoledro
Dose GF	1.0 µg	50 µg	0.45 mg	1.0 <i>µ</i> g	2.0 µg	1 mg	1 µg	50 µg	50 µg	0.5 mg	0.5 mg	0.15 mg	nate; BCC: bon -articular; IBN: naous: w: waal
Carrier	Graft	ACS+ CMC	Graft	Graft	CM	Graft	Hydrogel	ACS+ CMC	ACS+ CMC	Graft	Graft	Graft	P: bisphospho factor; IA: intra k: SC: subcuta
GF	rhBMP-7	rhBMP-7	rhBMP-2	rhBMP-7	rhBMP-7	rhBMP-2	rhBMP-2	rhBMP-7	rhBMP-7	rhBMP-2	rhBMP-2	rhBMP-2	: allograft; B GF: growth
BP Dose	60 mg/ml	0.1 mg/kg	9 mg/m	0.1 mg/kg	4 µg	0.56 mg	0.1 mg/kg	0.1 mg/kg	0.1 mg/kg	0.6 mg	0.6 mg	0.005 mg/ml	Idronate; ALG: emur fracture; DAM: nemidro
Time point	Pre: n/a Peri: 0w	0-2w	MO	Σw	MO	1w	Σw	2w	Σw	1-2w	1w	OW	nge; ALE: aler sellulose; FX: fe
Route	Pre: IV Peri:L	SC	w	sc	ა	0	≥	sc	sc	<u>0</u>	<u>0</u>	S	llagen spo :y methyl c blo: ON: o
ВР	CLO	ZOL	PAM	ZOL	ZOL	IBN	ZOL	ZOL	ZOL	IBN	IBN	IBN	able co : carbox
Graft	ALG		ALG	ALG	ALG	ABG		ABG	ALG ABG	ABG	ABG	ALG	: absort x; CMC
Model	BCC	CD Femur	IMP (D)	BCC	BCC	NO	ΕX	ΕX	Ϋ́	NO	NO	IMP	lograft; ACS Ilagen matri
Period	бw	5-8w	4w	6w	6w	8w	6w	бw	6w	8w	8w	4w	ABG: all ; CM: co
Species	Rat	Rat	Canine	Rat	Rat	Piglet	Rat	Rat	Rat	Piglet	Piglet	Canine	Table XV: Clodronate

BP + BMP studies

THESES FROM THE ORTHOPAEDIC RESEARCH GROUP

Doctoral and PhD Theses from the Orthopaedic Research Group, www.OrthoResearch.dk, Aarhus University Hospital, Denmark

Doctoral Theses

- Hydroxyapatite ceramic coating for bone implant fixation. Mechanical and histological studies in dogs Kjeld Søballe, 1993 Acta Orthop Scand (Suppl 255) 1993;54
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- 10. The influence of hydroxyapatite coating on the peri-implant migration of polyethylene particles Ole Rahbek, October 2002 *www.OrthoResearch.dk*
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- On the longevity of cemented hip prosthesis and the influence on implant design Mette Ørskov Sjøland, April 2007 www.OrthoResearch.dk
- Combination of TGF-β1 and IGF-1 in a biodegradable coating. The effect on implant fixation and osseointegration and designing a new in vivo model for testing the osteogenic effect of micro-structures in vivo Anders Lamberg, June 2007 www.OrthoResearch.dk

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1	Dose dependent resorption of allograft by rhBMP-2 uncompensated by
2	new bone formation - A canine study with implants and zoledronate
3	
4	Abstract
5	Introduction: Impacted bone allograft is used to restore lost bone in total joint
6	replacements. Bone morphogenetic proteins (BMPs) can induce new bone formation to
7	improve allograft incorporation, but they simultaneously invoke a seemingly dose-
8	dependent allograft resorption mediated by osteoclasts. Bisphosphonates effectively
9	inhibit osteoclast activity. Predicting allograft resorption when augmented with Bone
10	morphogenetic protein 2 (BMP-2), we intended to investigate whether a balanced bone
11	metabolism was achievable within a range of BMP-2 doses with systemic zoledronate
12	treatment.
13	Methods: Implants were coated with one of three BMP-2 doses (15 μ g, 60 μ g and 240 μ g)
14	or left untreated. Implants were surrounded by a 2.5 mm gap filled with impacted
15	morselized allograft. Each of the 12 dogs included received 1 of each implant (15 μ g, 60
16	μ g, 240 μ g and untreated), 2 in each proximal humerus. During the 4-week observation
17	period, zoledronate IV (0.1 mg/kg) was administered to all animals 10 days after surgery
18	as anti-catabolic treatment. Implant osseointegration was evaluated by histomorphometry
19	and mechanical push-out tests.
20	Results: Untreated implants had the best mechanical fixation and superior retention of
21	allograft as compared to any of the BMP-2 implants. Both mechanical implant fixation
22	and retention of allograft decreased significantly with BMP-2 dose increments.
23	Surprisingly there was no difference among the treatment groups in the amount of new
24	bone.
25	Conclusion: The use of BMP-2 to augment impaction grafted implants cannot be
26	recommended even when combined with systemic zoledronate.

28 Manuscript

29	Introdu	uction:

30	Impacted bone allograft is an established procedure to replace lost bone and secure initial
31	implant fixation in revisions of total hip replacements [1, 2]. Good initial implant fixation
32	is important, because initial implant subsidence or instability predicts later implant failure
33	[3]. Histological evaluations of retrieved biopsies of impacted allograft and prosthesis
34	components have demonstrated a variable incorporation of the impacted allograft. Often,
35	the allograft is found encapsulated in fibrous tissue rather than being in contact with or
36	replaced with host bone [4]. The desired outcome, allograft incorporation, is
37	histologically defined as revascularization of all tissue surrounding the allograft and new
38	bone apposition to the necrotic allograft fragments [5].
39	
40	Allograft incorporation begins with invasion of a fibrovascular front from host bone into
41	the impacted morselized allograft [4]. This vascular invasion is followed by resorption of
42	the allograft by osteoclasts recruited from hematopoietic precursor cells. Unlike normal
43	coupled bone remodeling, in graft remodeling, graft resorption appears not to be coupled
44	spatially or temporally to new bone formation by osteoblasts [4]. This can potentially
45	leave the graft material mechanically incapacitated, with the graft composite collapsing.
46	Causing loosening of the implant. Increasing the formation of new bone while negating
47	bone resorption can potentially improve the clinical outcome of implants inserted with
48	impacted allograft.
49	
50	Bone morphogenetic protein 2 (BMP-2) is a bone anabolic protein that stimulates the
51	proliferation and maturation of mesenchymal-derived osteoprogenitor cells into mature
52	osteoblasts capable of producing bone [6].

Recombinant human BMPs (rhBMPs) have variable clinical results when used with bone graft: in a study on staged reconstruction of tibia fractures with segmental defects, the use of freeze dried-allograft augmented with rhBMP-2 resulted in similar healing as autograft [7]. In a revision arthroplasty study, accelerated allograft resorption was observed with rhBMP-7 augmentation, resulting in 4 of 10 acetabular components being classified as loose [8].

60

Experimental studies have demonstrated increased graft resorption in impaction-grafted
implants [9-14] and transient cancellous bone resorption in metaphyseal bone [15, 16]
when combined with a BMP. The studies indicated no optimal BMP dose, but they
indicated that accompanying anti-catabolic therapy seemed necessary when using a BMP
in a cancellous-bone environment.

66

Bisphosphonates are effective anti-catabolic agents that bind to exposed bone mineral and 67 68 remain inactive until the bone and bisphosphonate are actively resorbed and internalized 69 by osteoclasts. Nitrogen containing bisphosphonates (N-BPs) disable integration of 70 proteins into the cell membrane by inhibiting protein prenylation leading to osteoclast 71 apoptosis [17]. In clinical trials, systemic bisphosphonate treatment has proven effective 72 in decreasing the migration of acetabular components and knee prosthesis [18, 19]. 73 Experimentally, systemic administration of zoledronate at one or two weeks improved 74 fracture healing in rodents [20]. Local anabolic (BMP) therapy combined with systemic 75 anti-catabolic (zoledronate) therapy at 2 weeks, have in rodent studies using allograft 76 demonstrated to retain bone in a bone conduction chamber study [21] and improve 77 fracture healing [22]. The anabolic potential of BMPs motivated the present study to 78 investigate if a balanced net positive response was possible with combined anabolic and 79 anti-catabolic therapy.

81 We hypothesized, that in an allograft-filled 2.5 mm gap with zoledronate IV, coating the 82 implant surface with one of three rhBMP-2 doses (15 µg, 60 µg and 240 µg) would 83 improve the initial fixation and osseointegration of those implants compared to untreated implants. Increased osseointegration was defined as increased mechanical fixation, 84 85 increased new bone formation, and decreased amounts of fibrous tissue and retention of 86 allograft. 87 88 Materials and methods: 89 Experimental design: 90 The experiment was designed as a paired randomized controlled study in 12 canines. Each 91 animal was used as its own control by comparing the 4 implants within each animal with 92 one another, constituting a paired design.

93

94 Four non-loaded implants were inserted in each animal, 2 in each proximal humerus

95 (Figure 1). Implants were cylindrical and had nominal dimensions of 6 mm (diameter) by

96 10 mm (length). The drill holes were 12 mm deep, ensuring subcortical implant

97 positioning, and 11 mm in diameter, giving a peri-implant gap of 2.5 mm.

98

99 To minimize a potential neighboring effect of rhBMP-2 by agent or dose, treatment was

assigned in 2 blocks. Untreated and 15 µg implants were assigned on one side; 60 µg and

101 240 µg implants were assigned contralaterally . Side was assigned with a random start and

102 changed systematically between left and right as well and between proximal and distal

103 sites within treatment blocks.

104

105 We wanted to detect a minimal difference of 50% on the primary and secondary

106 endpoints between groups. Based on previous studies using the same model, we assumed

107 a coefficient of variance of 50% between paired differences, and a 2-sided α and β of 5%

and 20% respectively. This resulted in a sample size of approximately 10 animals. In
anticipation of loss of power due to exclusion of animals, or loss of implants during
preparation and testing, 2 additional animals were assigned.

111

112 Animals served simultaneously in 2 parallel studies in anatomically distant sites (distal

113 femurs and proximal tibias) that investigated the effect of combined anabolic (rhBMP-2)

and systemic anti-catabolic treatment (zoledronate) on implant osseointegration during a

similar observation period (4 weeks). Animals received only a single zoledronate

116 administration.

117

118 Graft material:

119 Under sterile conditions, the proximal humeri, tibias, and distal femurs were harvested

120 post-mortem from 2 dogs not included in the study and stored at -80°Celsius (C). Before

121 surgery bones were thawed and debrided of all soft tissue and cartilage. Bone physes were

122 morselized using the finest setting on a standard bone mill (The Mill - Bone Mill System,

123 Biomet, Warsaw, IN), producing bone chips of 1-3 mm in size. Morselized cortico-

124 cancellous graft from all bones and both animals were mixed into 1 batch, and bone chips

larger than 2 mm were removed manually. The allograft was divided into aliquots and

126 firmly compressed into 1.0 ml standard sterile polyethylene containers (mean weight 1.27

127 g; range 1.24-1.29) and stored at -80°C.

128

129 Implants:

130 We used 48 cylindrical implants consisting of a cylindrical titanium alloy (Ti6Al4V) core

131 with a commercial porous coating (Gription[®], DePuy-Synthes, Warsaw, IN). The porous

- 132 coating was composed of a dual layer consisting of inner spherical beads covered by
- 133 small, highly irregular particles, sintered onto the central titanium-alloy cylinder. The

134 coating was made from commercially pure titanium with a volume porosity of 63% ($\pm 3\%$)

as specified by the manufacturer. Ninety-six (96) titanium alloy (Ti6Al4V) endplates witha diameter of 11 mm were used.

137

138 Coating procedure:

rhBMP-2 (InductOs[®] 12 mg, Medtronic BioPharma, B.V. Holland) was reconstituted with 139 140 kit solute (sterile saline) to a concentration of 4 mg/ml. A well plate (96-Well, Standard 141 F, Sarstedt AF & Co., Nümbrecht, Germany) was blocked with a solution of 0.5% bovine 142 serum albumin (Bovine Serum Albumin, Sigma-Aldrich, Saint Louis, MO)/phosphate 143 buffered saline (PBS) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) before 150 µl 144 of rhBMP-2 solution was filled into the individual wells. Implants were mounted on 145 stainless-steel rods and were fully submerged into the rhBMP-2 solution and then 146 subjected to vacuum in a desiccator to suck the viscous rhBMP-2 solution into the porous 147 coating. The solute was evaporated by vacuum in a desciccator leaving rhBMP-2 on the 148 porous coating. Coated implants were packed individually in separate tubes (Eppendorf 149 LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and stored at -21°C until use. 150 After submersion and desciccation the porous implant coating retained an average of 60 151 µL of fluid, resulting in an estimated total dose of 240 µg of rhBMP-2 using a 4 mg/ml 152 rhBMP-2 solution. Accordingly diluted rhBMP-2 solutions were used to functionalize the surface of 60 µg (1.0 mg/ml) and 15 µg (0.25 mg/ml) implants using the same procedure. 153 Adhesion of rhBMP-2 to the implant surface after submersion depended on the adsorption 154 155 of protein directly to the titanium surface [23] and the retention of the rhBMP-2 solution in the porous implant coating. In-vitro elution confirmed that rhBMP-2 was released from 156 157 the implants within the expected range (Figure A1). Release kinetics was calculated as 158 described by Strobel et al [24].

159

160 Animals:

The animals were purpose-bred skeletally mature mongrel/mixed-breed canines. Their
mean weight was 29.7 kg (range 26.8-34.7) with a mean age of 18 months (range 16-19).
Two comparable animals served as allograft donors and were not included in the study.
Skeletal maturity was confirmed by closed humeral epiphyseal plates on plane X-rays.
The study was approved by the Institutional Animal Care and Use Committee (IACUC) at
North American Science Associates (NAMSA), Minneapolis, MN, (study ID 288-01).

168 Surgery:

169 Operations were performed under sterile conditions and general anesthesia. Peri-

170 operatively 15 mg/kg of cefazolin IV and 1 gram of ceftriaxone SC for 5 days post-

171 operatively were used as antibiotic prophylaxis. Anesthesia was induced with

acepromazine IV (0.05 mg/kg) and propofol IV (1.5 mg/kg) and sustained with isoflurane

173 (1-5% inhalant). The proximal humerus was exposed through a lateral extra-articular

174 incision. A 2.5 mm k-wire was inserted at the level of the greater tubercle, and a second

k-wire was inserted 17 mm more distally. A 12 mm deep hole was drilled with an 11 mm

176 cannulated drill bit at 2 rotations per second. The cortical edge was trimmed for

177 periosteum, the bone debris was removed, and the cavity irrigated. An implant with an 11

- 178 mm endplate attached was inserted endplate first. Morselized allograft was stepwise
- 179 impacted into the 2.5 mm peri-implant gap with a hollow cylindrical impaction tool. To

180 contain allograft and maintain concentric implant placement, a second 11 mm endplate

181 was mounted on the implant's cortical end. Tissue was closed in layers using absorbable

182 sutures. Analgesia was obtained using buprenorphine SR (0.03 mg/kg), marcaine with

183 epinephrine (0.5%) at the incisions, and acetaminophen (15 mg/kg daily).

184

The animals were allowed full weight bearing immediately after surgery. All animals
received a single IV administration of 0.1 mg/kg of zoledronate (Zoledronic Acid Actavis
4 mg/mL, Actavis Group, Iceland) 10 days after surgery. After 4 weeks of observation the

animals were sedated with acepromazine IV (0.5 mg/kg), anesthetized with propofol IV

189 (4 mg/kg), and euthanized with a dose of hyper-saturated barbiturate IV (Socumb, Med-

190 Pharmex Inc., CA).

191

192 Specimen preparation:

193 After euthanasia, the proximal part of each humerus was harvested and immediately

stored at -21 °C (Figure 1, upper left). After thawing, a bone cube with implant in-situ

195 was cut from the proximal humerus using a water-cooled diamond-band saw (Exact

196 Apparatebau, Nordstedt, Germany). The implant with surrounding bone was divided into

197 3 traverse sections perpendicular to the long axis of the implant (Figure 2A). The most

198 cortical 1 mm of the outermost section was discarded and the next 3 mm were stored at -

199 21° C for mechanical testing. The remaining innermost 5.5 mm were used for

200 histomorphometric evaluation and gradually dehydrated in graded ethanol (70-96 %),

201 100% 2-propanol, defatted in xylene, and finally embedded in methyl methacrylate

202 (MMA, product no. 800590; Merck, Darmstadt, Germany). Then, four 30-µm slides were

203 cut from each specimen with a hard-tissue microtome (KDG-595, MePro Tech,

204 Herhugowaard, Holland) as vertical uniform random (VUR) sections from the central part

205 of the implant (Figure 2C) [25]. The distance between each section was 400 μ m,

206 corresponding to the width of the blade. Specimens for histomorphometric evaluation

207 were stained with 0.1% Toluidine blue (pH 7, Sigma-Aldrich, St. Louis, MO, USA),

208 rinsed, and mounted on glass.

209

210 Mechanical testing:

211 Prior to blinded testing, specimens were thawed. Using a MTS Bionics servo hydraulic

212 test rig (MTS Systems, Inc., Eden Prairie, MN), implants were loaded by axial push-out

213 until failure of the bone-implant interface. The bone-implant specimen was positioned on

top of a test support jig. The implant was centered over the hole in the support jig with a

215	distance of 0.7 mm from the implant surface to the edge of the hole on the support jig. A 5
216	mm cylindrical metal test probe was used to apply force to the implant-bone specimen at a
217	constant displacement rate of 5 mm/min. Load (N) was recorded for every 10 μm of
218	displacement by a 2.5 KN load cell. Data points were transferred and stored on a PC using
219	designated software (MTS Test Star 790.00, version 4.0). Derived mechanical endpoints
220	were normalized to the surface area of the implant section tested, producing a stress-
221	displacement curve (Figure 2B). Maximum shear strength (T_u ; Strength: Pa) was derived
222	from the maximum load (F) prior to failure of the bone-implant interface and calculated
223	by $T_u = F/\pi DL$, where D is the implant diameter and L is the implant length. Total energy
224	absorption (Energy: J/m^2) was calculated as the area under the load/displacement curve
225	until failure. Apparent shear stiffness (S; Stiffness: Pa/mm) was the steepest slope of the
226	load/displacement curve and was calculated as $S = (\Delta F / \pi DL) / \Delta L$.

227

228 Histomorphometry

229 Blinded quantitative evaluation of non-decalcified histological specimens was performed 230 in a random sequence using an optical microscope (Olympus, Ballerup, DK) and 231 stereological software (newCAST version 3.0.9.0, Visiopharm, Hoersholm, DK). Four 232 vertical sections of each implant were analyzed and cumulated. The stereological software 233 was used to superimposed 3 regions of interest (ROIs) onto the histological specimens 234 (Figure 3). Zone 1 began at the median thickness of the implant coating and extended 500 235 µm outwards. Zone 2 began at the peripheral border of Zone 1 and extended 2000 µm out 236 in the peri-implant gap. Zone 3 began 3000 µm from the median implant surface and 237 extended 1000 µm out into the host bone. This assured that only the grafted gap was 238 evaluated in Zone 1-2 and that host bone was evaluated in Zone 3. Zone 1 and 2 are 239 referred to as "in-gap" and Zone 3 as "off gap" (Figure 3). The implant surface coverage 240 (surface-area fraction) and peri-implant volumes (volume fraction) of new bone, allograft

- 241 (in gap), lamellar bone (off gap), marrow, and fibrous tissue were assessed by line-
- intercept technique [26], and point-counting technique [27] respectively.
- 243

244 Statistical analysis:

245 Statistical analysis was performed using STATA software (version IC 13.1, StataCorp,

Lakeway Dr., TX,). Data were evaluated by repeated measures ANOVA and hypothesis

- 247 were tested using post-hoc test. Model assumptions were diagnosed by visual inspection
- of residuals and fitted values using QQ and scatter plots. Mean estimates and mean
- 249 differences are presented with 95% confidence interval (CI). Non-normally distributed
- 250 data were evaluated with Friedman repeated measures analysis of variance followed by
- 251 Wilcoxon Signed rank-test. Median and inter quartile range (IQ) of the data are presented.
- 252 Only data that passed repeated measures ANOVA or Friedman repeated measures
- analyses were analyzed with post-hoc test or Wilcoxon signed-rank test. A p < 0.05 was
- considered significant.
- 255

256 Results:

- 257 Observation of animals
- All animals recovered within 2 days postoperatively and completed the observation
- 259 period. No adverse events were recorded. All specimens were available for mechanical
- and histomorphometric evaluation.
- 261

262 Mechanical results (Table 1 and Figure 4):

263 The untreated implant group had significantly better mechanical fixation than any of the

rhBMP-2 implant groups. Within the rhBMP-2 implant groups, the mechanical-implant

265 fixation decreased as the rhBMP-2 dose increased.

267	Relatively, the untreated implant group had 78 - 430% better fixation than the rhBMP-2
268	implant groups by strength to failure ($p \le 0.003$); 87 - 397% better fixation as measured
269	by energy to failure ($p \le 0.001$) and 71 - 451% better fixation as measured by stiffness (p
270	\leq 0.001). Relatively, within rhBMP-2 implant groups, 15 μg implants had 51 - 197%
271	better fixation than the 60 µg and 240 µg implants ($p \le 0.03$), 95 - 221% better fixation as
272	measured by apparent shear stiffness ($p \le 0.03$), and 9 - 166% better fixation as measured
273	by energy absorption, although this was significant only when compared to the 240 μg
274	implants ($p = 0.01$).
275	
276	Histomorphometry (Table 2 and Figure 5)

as

277 On the implant surface, the untreated implant group had 21% of the surface covered with 278 new bone, an area relatively 189 - 285% larger than those in the 60 μ g and 240 μ g 279 implant groups (p < 0.001).

280

292

281 The untreated implant group had a median 4.1% (0.0 - 7.6%) surface area fraction with 282 fibrous tissue, and although no fibrous tissue was detected in any of the rhBMP-2 implant 283 groups, this was not significant (data not shown, Friedman repeated measures: p = 0.07). 284

285 Farther away from the implant surface, no significant difference in volumes of new bone 286 was detected among the implant groups. In contrast, the retention of allograft in the peri-287 implant gap depended on the rhBMP-2 status and dose. Relatively, the volume of allograft 288 in the untreated implant group was 65 - 749% higher than in any of the rhBMP-2 implant groups (p < 0.001). Within the rhBMP-2 implant groups, a clear, significant inverse 289 290 relationship between increasing rhBMP-2 dose and decreasing volume of retained 291 allograft was evident ($p \le 0.018$).

Among implant groups, no difference was detected outside the peri-implant gap (off-gap)in the volumes of new bone or lamellar bone.

295

296 Discussion:

The purpose of this study was to investigate if surface eluted rhBMP-2, within a dose range, had a positive effect on initial implant fixation, while attempting to balance the growth factors inherent indirect catabolic drive on bone remodeling using delayed systemic zoledronate.

301 No benefit of adding rhBMP-2 to the implant surface was detected. Instead,

302 rhBMP-2 dose dependently decreased mechanical fixation and osseointegration of

303 implants with increasing rhBMP-2 dose. The allograft was well-retained in the untreated

group, and it appeared as rhBMP-2 did not promote formation of new bone in the peri-implant gap.

306 The used canine implant model is a well-established experimental model [9-13, 307 28], that investigates initial implant fixation and osseointegration of porous coated 308 cementless implants impacted with morselized allograft. The applied model is 309 experimental; implants are cylindrical, have no morphological resemblance to clinical 310 implants, and are not subjected to stresses from repetitive cyclic loading or oscillations in 311 joint-fluid pressure. This standardized experimental model has a high degree of variable 312 control that ensures consistent, reproducible results, which compensates for the trade-off 313 in clinical resemblance.

Canines where chosen as the experimental animal because canine bone is a good surrogate for human bone in terms of mechanical properties and composition [29]. Canine metaphysis are rich in cancellous bone, easily accessible, and large enough to

317 accommodate several treatment groups in a paired design.

318 Observation time was based on previous studies, that had examined BMPs and 319 impacted allograft over observations periods of three to six weeks [9, 11, 13]. An 320 observation period of four weeks was chosen because it was judged to optimally display321 the formation of new bone before remodeling begins.

Our intention in administering systemic zoledronate was to enable protection of both the allograft and the new bone. However, the study's results demonstrated a clear mismatch between the undesired, indirect catabolic stimulation by rhBMP-2 and the anticatabolic restraint on osteoclast activity produced by a single systemic administration of zoledronate.

327 In contrast to our expectation, the implant surface area covered with new bone 328 decreased dose dependently as rhBMP-2 doses increased incrementally. Surface release of 329 rhBMP-2 from a surface encased behind a wall of impacted allograft might have resulted 330 in a high local concentration of rhBMP-2, diverting the net metabolic response in a 331 catabolic direction by stimulating osteoclast activity. For systemically administered 332 zoledronate to have an anti-catabolic effect at, or near, the implant surface, the peri-333 implant gap must have been extensively vascularized and new bone must have undergone 334 some degree of primary mineralization. If neither of these processes is sufficiently 335 advanced, zoledronate is not transported to or retained in the central parts of the peri-336 implant gap, leaving them unprotected from osteoclast activity. 337 Augmenting the implants with rhBMP-2 was expected to increase graft resorption 338 to some extent, which is why a single dose of zoledronate was administered. However, the 339 statistically significant, stepwise decrease to the nearly complete resorption of all allograft 340 as was observed in the 240 µg implants, was unexpected. The excessive allograft 341 resorption was in contrast to the results of rodent studies using a bone conduction 342 chamber model, in which a single systemic exposure to zoledronate [30] also with the 343 addition of a BMP [21] increased bone ingrowth and protected both the allograft and the 344 new bone.

In a canine implant model, similar to the one used in the present study, soakingallograft with bisphosphonates before impaction proved to effectively protect the allograft

347 against resorption. The volumes of the retained allograft that had been soaked in 348 zoledronate ranged from 32-44% [9, 31, 32] compared to 26% in the present study, which 349 used systemic zoledronate. Theses differing results indicate that zoledronate administered 350 locally is superior to that administered systemically for protecting impacted allograft. 351 In the present study, the administration point for the zoledronate was based on 352 rodent graft studies that conducted administrations at one to two weeks [20-22] and re-353 vascularization studies of cancellous and intercalary allografts in canines that 354 demonstrated extensive new vessel ingrowth at one [33] and two weeks [34], respectively. 355 Considering the depth of the allograft-impacted gap, the vascularization, and the initiation 356 of primary mineralization, an administration point of 10 days was assessed as optimal. 357 The dose in the present study was based on previous rodent studies [20-22] and clinical 358 application [17, 19].

359 Soaking the allograft with bisphosphonate before impaction likely results in a 360 homogenous, and even surface coating of the morselized graft. A similar homogenous 361 exposure of bisphosphonate to the allograft surface was likely not achieved using 362 systemic administration of zoledronate. Possibly, the shortcoming of the systemic 363 administration of zoledronate was due to hampering of the vascular ingrowth caused by 364 the increased density of the impacted graft, as demonstrated by Tagil et al. [5]. Increasing 365 the frequency or dose of zoledronate administration could potentially improve the anti-366 catabolic control.

In contrast to the area of new bone on the implant surfaces, the volume of new bone in the peri-implant gap was not significantly different across all four implant groups. This was unexpected, but may potentially be explained by the concepts of osteoconduction by allograft [35] and osteoinduction by growth factors [36]. The ratio between the volumes of new bone and retained allograft increased in parallel with the increments of the rhBMP-2 dose. This could indicate that the formation of new bone in the grafted peri-implant gaps also relied on an osteoinductive stimulus from implant-

eluted rhBMP-2 and/or on growth factors released from the resorbed allograft in addition
to the osteoconduction from the allograft. However, separate osteoinductive contributions
from implant-eluted rhBMP-2 and allograft-embedded growth factors would be
indistinguishable.

In the present study, the volume of new bone in untreated implants was 31%, which is higher than the 20-26% seen in implants that received zoledronate-soaked allograft in previous canine studies [10, 32]. This could indicate that systemic zoledronate protects new bone better than local zoledronate. New bone is a living tissue and thus vascularized, which explains why systemic zoledronate should result in a more uniform zoledronate exposure compared to surface bound zoledronate.

384 The use of growth factors can potentially induce an anabolic spillover effect on 385 adjacent implants. In the present study, no difference in volume of new bone or lamellar 386 bone was detected between implant groups in ROI III, which is why a spill over effect 387 from rhBMP-2 must be considered unlikely. Recognizing that use of a soluble anabolic 388 agent is a potential confounder, we applied a within-animal/within-site design in which 389 each animal functioned as its own control. When using this paired design, potential 390 confounders become, by the paired design, equally and uniformly distributed and should 391 not introduce bias into the results. In addition, the paired design reduces total variation by 392 eliminating the contribution of inter-individual variance and returns more accurate results. 393 Along with insufficient anti-catabolic control, the surface delivery of rhBMP-2, 394 was likely to be part of the explanation for why augmenting the surface was detrimental to 395 implant osseointegration. This delivery method secured a uniform delivery of rhBMP-2 in 396 the peri-implant gap. However, entrapment of the rhBMP-2 behind the impacted allograft, 397 likely resulted in a high local concentration of rhBMP-2, diverting the net metabolic result 398 in a catabolic direction, which was not negotiable using a single administration of 399 systemic zoledronate.

400	It is important to note that rhBMP-2 doses are not transferable to humans because
401	of different interspecies susceptibility. If a therapeutic window exists for rhBMP-2 in the
402	context of impaction grafting, it appears to be very narrow, perhaps too narrow a dose
403	range for practical clinical use. However, a benefit from rhBMP-2 in the context of
404	impaction grafting could potentially be harvested using better anti-catabolic control.
405	
406	Conclusion:
407	The present study signifies no clinically useful effect of rhBMP-2 on promoting
408	osseointegration of impaction-grafted implants even when combined with an anti-
409	catabolic agent. We cannot conclude an optimum rhBMP-2 dose as none was found.
410	However, a lower rhBMP-2 dose may have yielded better results, and we cannot exclude
411	that a higher dose or multiple administrations of zoledronate would have provided better
412	anti-catabolic control. The results prompt further investigation with adequate anti-
413	catabolic control to elucidate whether rhBMP-2 can have a role in augmenting impaction-
414	grafted implants
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	Strength (Mpa)	Energy (kJ/m ²)	Stiffness
Untreated	4.6 (3.8 - 5.4)	0.7 (0.5 - 0.8)	24.0 (19.6 - 28.4)
15	2.6 (1.8 - 3.4)	0.4 (0.2 - 0.5)	14.0 (9.6 - 18.4)
60	1.7 (0.9 - 2.5)	0.3 (0.2 - 0.4)	7.2 (2.7 - 11.6)
240	0.9 (0.1 - 1.6)	0.1 (0.0 - 0.3)	4.4 (0.0 - 8.8)
ANOVA*	p=0.0000	p=0.0000	<i>p</i> =0.0000

Table 1. Biomechanical results

Results are presented as mean (95% CI) per treatment group. Strength: maximum shear strength; Energy: total energy absorption; Stiffness: apparent shear stiffness. Significant results are marked in bold.

	New bone		
	Surface	In gap	Off gap
Untreated	0.21 (0.17 - 0.25)	0.31 (0.28 - 0.33)	0.03 (0.02 - 0.05)
15 µg	0.16 (0.12 - 0.20)	0.29 (0.27 - 0.32)	0.04 (0.02 - 0.05)
60 µg	0.07 (0.04 - 0.11)	0.28 (0.25 - 0.31)	0.05 (0.04 - 0.07)
240 µg	0.06 (0.02 - 0.09)	0.26 (0.24 - 0.29)	0.03 (0.02 - 0.05)
ANOVA	p = 0.0000	p = 0.1415	p = 0.2015
		Lamellar bone	
		In gap	Off gap
Untreated	-	0.23 (0.21 - 0.26)	0.34 (0.30 - 0.37)
15 µg	-	0.14 (0.12 - 0.17)	0.36 (0.33 - 0.40)
60 µg	-	0.07 (0.05 - 0.10)	0.36 (0.33 - 0.40)
240 µg	-	0.03 (0.00 - 0.05)	0.33 (0.30 - 0.37)
ANOVA		n = 0.0000	n = 0.4211

Table 2. Histomorphometric results

Results are presented as mean fraction (95% CI) of surface area and volume per treatment group. *: Repeated measures ANOVA. "In-gap" and "Off-gap" designates in- and out-side the peri-implant gap respectively. Lamellar bone "In gap" designates allograft. Significant results are marked in bold.

Figure 1.



Figure 1.

A porous coated implant and endplates with the dimensions indicated. Embedded picture in upper left corner: X-ray of a pair of proximal humerei with 2.5 mm impaction graftedimplants in-situ. Right: R, Left: L. Figure 2



Figure 2.

Bone-implant block before sectioning with implant in-situ. A whole implant with most cortical of the end plate removed (A). Dotted lines indicate section planes of the perpendicular cuts. The top 1mm was discarded, the middle section was used for push-out test and the bottom section was used for histomorphometric analysis. The stress (y-axis) - displacement (x-axis) curve from implant-surface normalized push-out data (B). Four central VUR sections from the central aspect of the implant (C).



Figure 3.

Representative histological specimens of an untreated implant (3a-c) and 240 µg implant (3d-f) from the same animal (toluidine blue stain) divided in half along the implant axis. Magnification = x1.25 (3a and 3d) / x10 (3b-c and 3e-f). Scale bar (x1.25 / x10) = 1 mm / 0.1 mm. The vertical dotted line marks the original drill-hole border. The dotted square brackets in section 3a represents ROI I-III. Bold white squares mark the magnified (x10) areas of interest. New bone appears as dark violet with round lacuna with large nuclei embedded. Unmineralized immature bone is stained light blue and is distributed as a band on mineralized bone or onto the implant's surface. Graft is stained light violet and has a lamellar appearance with small empty lacunae. Bone marrow is a loosely disorganized tissue with few cells. Fibrous tissue is not present but would appear as organized structures with spindle-shaped cells. In the untreated implant allograft was retained with new bone formation throughout the peri-implant gap (3a). Extensive new bone formation on the implant surface and deep into the porous coating (3b). Retained allograft was present in the peri-implant gap and appeared with resorptions lacunae and extensive layers of new bone (3c). Implants coated with 240 µg of rhBMP-2 had thin strands of new bone and almost all allograft resorbed and remodeled into new bone in the peri-implant gap (3d). The surface had low amounts of immature new bone (3e). Thin strands of darker and lighter blue new bone was seen the peri-implant gap (3f).



Figure 4.

Mean (95% CI) for the derived mechanical fixation parameters. Maximum shear strength (Strength), total energy absorption (Energy) and apparent shear stiffness (Stiffness). Square brackets indicate significant inter-group comparisons. *(p < 0.05), **(p < 0.01), ***(p < 0.001) indicate the significance level.



Figure 5.

Mean fractions (95% CI) of implant surface-area coverage (Surface) and volumes of new and allograft in the peri-implant gap per treatment group. Square brackets indicate significant inter-group comparisons. *(p < 0.05), **(p < 0.01), ***(p < 0.001) indicate the significance level.

Figure A1







Figure A1

Graphs with rhBMP-2 implant release kinetics per treatment group. A release study was performed with two implants per rhBMP-2 group and the untreated group. The implants were placed individually in separate wells in a lid covered well plate (24-Well, Standard F, Sarstedt AF & Co., Nümbrecht, Germany) on a stable plate in a heating cabinet (37°C). Each well was filled with 5 ml of phosphate buffered saline (PBS). The samples were collected in duplicates at time points: 0h, 6h, 12h, 24h, 48h and 72h. The sample volume (1 ml) was replaced immediately with fresh PBS. The samples were stored in sterile Eppendorf tubes (Eppendorf LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and kept frozen (-20°C) until quantification with an rhBMP-2 detection kit (Quantikine ELISA kit DBP200, R&D Systems, Minneapolis, MN). The results from similar implants were averaged, and the mean release kinetics was estimated as described by Strobel et al. [24]. The 15 µg and 60 µg implants demonstrated an initial burst release within 24 hours. In contrast the 240 µg implants demonstrated a more prolonged release profile. The average rhBMP-2 release (mean (sd)) were: 240 μ g implants: 272 μ g (\pm 7); 60 μ g implants: 67 μ g (±3); and 15 μ g implants: 12 μ g (±1). The implants used for the elution study were from the same implant batch and the same rhBMP-2 coating procedure as used in the present study. All rhBMP-2 handling was performed using polypropylene pipette tips (Finntip, Thermo Fischer Scientific Oy, Vantaa, Finland), the well plate was blocked with a 0.5% BSA/PBS solution prior to release and samples were stored in protein lowbind tubes to avoid rhBMP-2 adsorbing to the sides of the equipment. The samples from untreated implants were negative and are not depicted.

Healing in Peri-Implant Gap With BMP-2 and Systemic Bisphosphonate Is Dependent on BMP-2 Dose—A Canine Study

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ABSTRACT: The bone–implant interface of cementless orthopedic implants can be described as a series of uneven sized gaps with discontinuous areas of direct bone–implant contact. Bridging these voids and crevices by addition of an anabolic stimulus to increase new bone formation can potentially improve osseointegration of implants. Bone morphogenetic protein 2 (BMP-2) stimulates osteoblast formation to increase new bone formation but also indirectly stimulates osteoclast activity. In this experiment, we investigate the hypothesis that osseointegration, defined as mechanical push-out and histomorphometry, depends on the dose of BMP-2 when delivered as an anabolic agent with systemic administration of the anti-resorptive agent zoledronate to curb an increase in osteoclast activity. Four porous coated titanium implants (one with each of three doses of surface-applied BMP-2 ($15 \mu g$; $60 \mu g$; $240 \mu g$) and untreated) surrounded by a 0.75 mm empty gap, were inserted into the distal femures of each of twelve canines. Zoledronate IV (0.1 mg/kg) was administered 10 days into the observation period of 4 weeks. Bone–implant specimens were evaluated by mechanical push-out test and histomorphometry. The $15 \mu g$ implants had the best fixation on all mechanical parameters and largest surface area covered with new bone compared to the untreated, 60 and 240 μg implants, as well as the highest volume of new bone in the implant gap compared to 60 and 240 μg implants. The results in a canine implant model demonstrated that a narrow range of BMP-2 doses have opposite effects in bridging an empty peri-implant gap with bone, when combined with systemic zoledronate. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: osseointegration; arthroplasty; BMP-2; bisphosphonate; adjuvant therapy; histomorphometry; mechanical test

Orthopedic implants primarily achieve initial fixation by press-fit seating into the cancellous bone bed. Ideally, this ensures an even and uniform boneimplant contact. In actual practice, implants are wedged into the bone bed, leaving areas of close but not intimate contact between implant and bone.¹ Even after several years, areas exist where in- and ongrowth of new bone will be absent or limited,² effectively reducing implant osseointegration and implant fixation.^{3,4} This can leave implants at an increase risk for early subsidence and later failure.^{5,6} Introduction of a bone anabolic stimulus to increase new bone formation, in areas of close but not intimate bone implant contact, should in theory improve osseointegration and thus implant fixation.^{5,6} Several bone morphogenetic proteins (BMPs) belonging to the transforming growth factor-Beta (TGF-B) superfamily are known. Some have osteoinductive potential such as BMP-2, BMP-7, and BMP-9 and stimulate recruitment, proliferation, and differentiation of osteoprogenitor cells to mature osteoblasts and partake in normal bone homeostasis.⁷ BMP-2 is commercially available as recombinant bone morphogenetic protein 2 (rhBMP-2) and is approved for use in alveolar ridge and sinus floor augmentation, open tibia fractures in the appendicular skeleton, and lumbar spinal fusion in the axial skeleton. Preclinical studies of BMPs have demonstrated its ability to heal critical sized defects in canines⁸ and clinically to be as efficacious as autograft in staged reconstruction of diaphyseal tibia fractures.⁹

However, the use of BMPs with allograft in revision hip surgery or as a graft substitute in healing of metaphyseal defects after corrective osteotomies demonstrated inferior results because of bone resorption.^{10,11}

Gap healing in cancellous bone with rhBMP-2 is preceded by bone resorption,^{12,13} why addition of an anti-resorptive drug to balance the anabolic and catabolic response to improve osseointegration of implants may be attractive. The approach has been proven in an experimental canine implant model¹⁴ and demonstrated effective in healing of open long bone fractures in a rat model.¹⁵

The anti-resorptive drug zoledronate is a nitrogencontaining bisphosphonates and a potent inhibitor of osteoclast activity. Zoledronate is clinically used to treat diseases with increased bone turnover such as Paget's disease, myeloma, and bone metastases.¹⁶ Zoledronate binds to exposed bone mineral and remains inactive until internalized by osteoclasts through active bone resorption.¹⁶ Systemic zoledronate exposure has proven effective in decreasing migration of acetabular sockets of total hip replacements in humans.¹⁷ Delayed zoledronate administration decreased resorption and postponed remodeling of new bone in animal studies on

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fracture healing,¹⁸ distraction osteogenesis,¹⁹ and healing into porous implants.²⁰

An optimal rhBMP-2 dose to improve healing of implants surrounded by an empty gap is not clear from previous animal studies. Positive histological and radiological results were not supported by mechanical testing. ^{8,21,22} Which is why further knowledge regarding rhBMP-2 dose effect on healing of empty periimplant gaps is needed.

Therefore, our aim was to evaluate the use of a local anabolic stimulus (rhBMP-2) in combination with an anti-resorptive agent (zoledronate), at a bone-implant interface where a gap exists immediately after implantation. We hypothesized that with a background of delayed systemic zoledronate exposure, in experimental implants surrounded by a 0.75 mm empty gap, coating the implant surface with one of three rhBMP-2 doses (15 µg; 60 µg; 240 µg) would improve implant osseointegration compared to an untreated control implant, based on our primary endpoint: Mechanical push-out until failure of the implant-bone interface (strength; energy to failure; stiffness) and secondary endpoint: Histomorhometric fractions of tissues (new bone; lamellar bone; marrow; fibrous tissue) at the implant surface (area) and in the peri-implant gap (volume).

METHODS

The study was approved by the Institutional Animal Care and Use Committee (study ID 288-01) at North American Science Associates (NAMSA) Inc., Minneapolis, MN. Surgery, observation and bone harvest was performed at the NAMSA facility and in accordance with National Institute of Health regulations.²³

Twelve purpose-bred, skeletally mature, male mongrel/mixed breed canines with a mean weight of 29.7 kg (range: 26.8– 34.7 kg) and mean age of 17.6 months (range: 16.2–18.7 months) were used. Plane X-rays of the distal femure confirmed closed epiphyseal plates indicating growth arrest and skeletal maturity.

The experiment was designed as a paired randomized controlled study in 12 animals, where each animal functioned as its own control. We wished to detect a minimal difference of 50% on our primary and secondary endpoints between groups. We assumed a coefficient of variance of 50% between paired differences, a two-sided α and β of 5% and 20%, respectively. This resulted in a sample size of approximately 10 animals. An additional two animals were included to prevent loss of power if animals were excluded or implant-specimens lost during evalution.

Each animal received one implant from each group, a total of four implants. The implants were inserted in the medial or lateral epicondyle of each distal femur. Through surgical overdrilling, a peri-implant gap of 0.75 mm surrounded the implant after insertion, and was maintained with endplates on deep and superficial ends of the implants. No adjuvant material was added to the gap, which is referred to here as an empty gap (Fig. 1).

Implants

Forty-eight (48) porous coated titanium alloy (Ti-6Al-4V) implant cores and ninety-six (96) titanium alloy (Ti-6Al-4V) 7.5 mm endplates were used. The implant coating (Gription^{*}; DePuy-Synthes, Warsaw, IN) consists of commercially pure titanium CP-Ti) titanium. With a volume porosity of 63% ($\pm 3\%$)



Figure 1. A 0.75 mm empty gap porous coated implant and endplates with the dimensions indicated. Embedded picture upper left corner: X-ray of a pair of distal femurs with the 0.75 mm empty gap implants in situ.

as specified by the manufacturer. The implants were cylindrical with a nominal height of 10 mm and diameter of 6 mm.

Functionalization of the implant surface with rhBMP-2 was performed using sterile technique with a commercially available rhBMP-2 kit (InductOs[®] 12 mg, Medtronic Bio-Pharma, B.V. Holland). rhBMP-2 was reconstituted to a concentration of 4 µg/µl rhBMP-2 with supplied kit diluents and 150 µl of the rhBMP-2 solution was put into a wellplate (96-Well, Standard F, Sarstedt AF & Co., Nümbrecht, Germany) blocked with a 0.5% bovine serum albumin (Bovine Serum Albumin, Sigma-Aldrich, Saint Louis, MO)/ phosphate buffered salien (PBS) (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) solution. All handling of the rhBMP-2 solution was performed using polypropylene pipette tips (Finntip, Thermo Fischer Scientific Oy, Vantaa, Finland). Implants were mounted on stainless steel rods and fully submerged in wells containing the rhBMP-2 solution, placed in a desiccator and subjected to vacuum to saturate the porous coating. Implants were air-dried in a vacuum, packed separately in tubes (Eppendorf LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and stored at -21°C.

The porous coating on average retained $60 \,\mu l$ of fluid, resulting in an estimated total dose of $240 \,\mu g$ rhBMP-2 with a 4 mg/ml rhBMP-2 solution. The 15 and $60 \,\mu g$ implants were processed similarly, with an accordingly diluted rhBMP-2 solution. A separate in-vitro elution study confirmed release of rhBMP-2 from coated implants²⁴ in within expected range (Supplementary Material; Figs. S1–S3).

To minimize a potential neighboring effect of rhBMP-2, treatments were block-allocated with untreated and $15 \,\mu g$ implants on one limb and 60 and $240 \,\mu g$ implants contralaterally. To avoid a potential bias from site and obtain a uniform distribution, treatment was assigned with random start and systematically altered between sides of femurs and epicondyles within and between block-allocations. Animals served at the same time in two other studies in anatomical separated sites; which investigated the effects of rhBMP-2 with anti-resorptive treatment, on implants surrounded by bonegraft (proximal humeri) or direct bone contact (proximal tibias). A possible interference with the present study is addressed in the Discussion section.

Statistical Analysis

Statistical analysis was performed using STATA software (StataCorp, Lake Way Dr, TX, USA, version IC 13.1). Data were evaluated by repeated measures ANOVA and hypothesis were tested using post hoc test. Model assumptions were diagnosed by visual inspection of residuals and fitted values using QQ and scatter plots. Mean estimates and mean differences are presented with 95% confidence interval (CI). Non-normal distributed data were evaluated with Friedman repeated measures analysis of variance followed by Wilcoxon Signed rank-test. Median and inter quartile range (IQ) of the data are presented. Only data that passed repeated measures ANOVA or Friedman repeated measures analysis were analyzed with post hoc test or Wilcoxon signed-rank test. p values <0.05 was considered significant.

Surgery

Surgeries were undertaken using sterile technique and general anesthesia. Anesthesia was induced with acepromazine IV (0.05 mg/kg), propofol IV (1.5 mg/kg) and sustained with isoflurane (1-5% inhalant). With the animal in a supine position, at the level of the femur condyle, a 4 cm incision exposed the apex of an epicondyle. Periosteum was removed and a 2.5 mm Kirschner-wire was inserted perpendicular to the bone at the apex of the epicondyle. A 12 mm deep hole was drilled with a 7.5 mm cannulated drill, at a speed of 2Hz to avoid inflicting thermal trauma. Debris was manually removed and remaining periosteum at the cortical edge was trimmed off, before irrigating the cavity with saline. An implant with attached endplate was inserted endplate first into the cavity and a second endplate was mounted at the superficial, cortical level (Fig. 1). This secured concentric implant placement in a 0.75 mm empty gap between the cancellous bone and implant surface. Soft tissues were closed in layers with absorbable sutures. An identical procedure was performed at the opposite ipsilateral epicondyle and in the epicondyles of the contralateral femur. The procedure was performed in a fixed order starting with the untreated implant, followed by 15, 60, and $240\,\mu g$ implants (to avoid contamination from rhBMP-2 by agent or dose).

Peri-operatively, animals received 15 mg/kg of cefazolin IV, and post-operatively 1 g of ceftrixone SC for 5 days as infection prophylaxis. Analgesia was obtained with marcaine with epinephrine (0.5%) applied at the incisions at the time of closure and SR buprenorphine SC (0.03 mg/kg) and acetaminophen (15 mg/kg) daily. Ten days after surgery, all animals received 0.1 mg/kg of zoledronate IV (Zolendronic Acid, Actavis Group, Iceland). Animals were allowed full weight bearing immediately after surgery. After a 4-week observation period, animals were sedated with acepromazine IV (0.5 mg/kg), anesthetized with propofol IV (4 mg/kg), and euthanized with a dose of hyper-saturated barbiturate IV (Socumb, Med-Pharmex Inc., CA).

Specimen Preparation

The distal femurs with implants in situ were harvested and stored at -21° C until specimen preparation. All specimen preparations were performed blinded. After thawing, bone cubes with single implants were cut from each femur condyle using a water-cooled and lubricated diamond band saw (Exakt Apparatebau, Nordstedt, Germany). The most-cortical 1 mm of the implant was discarded. The remaining implant was divided into two parts by a cut perpendicular to the long axis of the implant. The most-superficial 3 mm section was stored at -21° C for later mechanical testing. The remaining 5.5 mm innermost section was used for histomorphometric evaluation.

Histomorphometry

Specimens were gradually dehydrated in graded ethanol (70-96%), 100% iso-propyl alcohol, defatted with xylene, and finally embedded in methyl methacrylate (product no. 800590; Merck, Darmstadt, Germany). Four 30 µm slides were cut from each specimen with a hard tissue microtome (KDG-595, MePro Tech, Herhugowaard, Holland) into verical uniform random sections parallel to the central part of the axis of the implant.²⁵ The distance between each section was 400 µm, corresponding to the width of the blade. The specimens were stained with 0.1% toluidine blue (pH 7. Sigma-Aldrich, St. Louis, MO), rinsed, and mounted on glass. Blinded histomorphometric evaluation was performed using stereological software (new CAST version 3.0.9.0, Visiopharm Integrator System, Hoersholm, DK) and a lightmicroscope (Olympus, Ballerup, DK). The stain and technique²⁶ allows for differentiation of mature bone, woven bone, fibrous tissue, and marrow (Fig. S4). Ongrowth of tissue was defined as tissue in direct contact with the implant surface. The surface area fraction of tissue coverage was performed assessing the number of intersections at the bone/implant interface by line-interception technique.27

And tissue volumes fractions were determined by pointcounting technique.²⁸ Application of these techniques secures highly reliable estimates with negligible bias.²⁹ Histomorphometry was evaluated in two regions of interest (ROIs), one in the peri-implant gap (ROI 1) and one outside the implant gap (ROI 2). Both ROI's ranged along the length of the implant axis excluding 500 μ m at the ends. ROI 1 began at the median thickness of the implant coating and extende 1,000 μ m out in the peri-implant gap. ROI 2 began 1,500 μ m from the median thickness of the implant coating and extende 1,000 μ m out into host bone.

Mechanical Testing

Using a MTS Bionics test rig (MTS Systems Inc., Eden Prarie, MN) implants were tested by axial push-out to failure of the bone-implant interface. Thawed bone-implant specimens were centered over a 7.4 mm opening, ensuring 0.7 mm distance between the support jig and implant surface. A 5 mm in diameter cylindrical test probe applied an axial force at the upward facing cortical side of the bone-implant specimen. A 2N preload defined contact position. Displacement rate was 5 mm/min. Load (N) and displacement (mm) were recorded every 10 µm, producing a load/displacement curve. Load-displacement curves were normalized to implant surface area to counter for differences in specimen height. Maximum shear strength (MPa) was defined as the peak of the stress-displacement curve at failure of the implant-bone interface, total energy absorption (J/m^2) as the area under the curve until failure of the bone-implant interface, and apparent shear stiffness (MPa/mm) was the maximum slope of the stress-displacement curve before the first peak.

RESULTS

All animals were fully ambulatory and weight bearing within 2 days postoperatively. One animal sustained a superficial infection at the incision site on the stifle joint and was treated with 5 days of antibiotics. A
second animal developed a hematoma at the stifle incision site at the 2nd postoperative day that resolved with light compression. All animals completed the study and all specimens were available for mechanical and histomorphometric analysis. No signs of infection were present at termination and swabs taken from all implants at bone harvest were negative.

Mechanical

On a background of systemic zoledronate exposure, $15 \,\mu\text{g}$ implants had significantly better mechanical fixation on all measured parameters compared to any group. Maximum shear strength was 115-163% higher compared to the other groups. Total energy absorption was 91-235% higher and apparent shear stiffness was 122-203% higher compared to the other groups. No significant differences were detected between the untreated control, 60 or $240 \,\mu\text{g}$ implants (Fig. 2 and Table 1).

Histology

All implants had on-growth of new bone onto the implant surface and into the peri-implant gap but there were clear and distinct variations (Fig. 3). The 15 μ g implants had the largest implant surface area of new bone (Fig. 3c and d). The proportion of unmineralized bone to new bone appeared to increase with rhBMP-2 dose (Fig. 3e-h). The formation of new bone in host cancellous bone was primarily appositional with occasional inter-trabecular bone formation.

Implant Surface

With systemic zoledronate exposure, $15 \,\mu g$ implants had a statistically significantly higher bone ongrowth compared to any group, with a mean difference ranging from 5% to 8%. Ongrowth with other doses did not demonstrate significant differences (Fig. 3 and Table 2).

Implant Gap

In the peri-implant gap, $15 \,\mu\text{g}$ implants had a higher volume fraction of new bone compared to any other group, with a mean difference ranging from 1% to 6%. The mean difference was statistically significant when compared to 60 and 240 μg implants. No significant difference was detected between 15 μg implants and untreated control implants (Fig. 3 and Table 2).

Bone Outside Drill Hole

Outside the peri-implant gap the volume of new bone increased with rhBMP-2 dose. The 240 μ g implants had the highest volume of new bone compared to any other group, with a mean difference ranging from 3% to 8%. Within the rhBMP-2 implants a significant mean difference of 4% was detected only between the 240 μ g implants and 60 μ g implants. All rhBMP-2 implants had a statistical significant higher volume of new bone when compared to the untreated control implants (Fig. 4 and Table 2). A reverse trend was



Figure 2. Mean (95%CI) for derived mechanical fixation parameters. Maximum shear strength (strength), total energy absorption (energy), and apparent shear stiffness (stiffness). Square brackets imply a significant difference between treatments with *(p < 0.05), **(p < 0.01), ***(p < 0.001) describing the significance level.

seen with decreasing volumes of lamellar bone with rhBMP-2 dose increments; however, this was not statistically significant (Fig. 4 and Table 2).

Fibrous tissue was predominantly present in the untreated control group (median fraction <4% in terms of both surface area coverage (Wilcoxon signed rank test: p = 0.3173) and volume fraction (Friedman: p = 0.07). The median value of the untreated control group implants was primarily caused by two implants with abundant fibrous tissue formation (surface area: >68%; volume fraction: >11%).

All mechanical parameters were positively and significantly correlated with surface area coverage of new bone and volume of new bone in the peri-implant gap (Table 3).

DISCUSSION

The purpose of the study was to investigate if implant osseointegration depended on dose of a local anabolic stimulus (rhBMP-2) with accompanying systemic antiresorptive therapy.

No evidence on any clear dose-related response between the different rhBMP-2 doses applied was detected on any of the primary mechanical endpoints or secondary histomorphometric endpoints. However, the mechanical results mirrored the observed formation of new bone well. The 15 μ g implants had the best overall combination of new bone formation in three sections (surface, in gap, and outside gap) in continuity with one another, translating with statistical significance into the best mechanical fixation on all mechanical parameters.

Histology confirmed a large ongoing intra-membraneous bone formation and lesser resorption of newly formed bone in the peri-implant gap, indicating that remodeling is occurring but has been slowed down (Fig. S4).

Bone healing around stable implants occurs by intramembraneous bone formation. Metaphyseal fracture healing is suggests to also rely on condensation of osteoblast progenitor cells between existing host trabeculae termed inter-trabecular healing.³⁰ Intertrabecular healing in host bone was also seen in a

	Control	15 µg	60 µg	240 µg
Strength	1.2(0.5-1.9)	3.1 (2.4–3.8)	1.4 (0.8–2.1)	1.2(0.5-1.9)
Energy	0.2 (0.0-0.3)	0.6 (0.4–0.7)	0.3 (0.2–0.4)	0.3 (0.1-0.4)
Stiffness	6.5(3.3-9.7)	14.4 (11.2 - 17.6)	6.5 (3.2–9.7)	4.8 (1.5-8.0)

 Table 1.
 Mechanical Results

Data are presented as mean (95%CI) per treatment group. Strength (MPa): maximum shear strength; Energy (KJ/m²): total energy absorption; Stiffness (MPa/mm): apparent shear stiffness.

canine study on back-side defect healing in pressfit seated acetabular components with ceramic graft putty and rhBMP-2.³¹ With the morphologic resemblance and timely proximity to inter-trabecular bone healing in adjacent host bone see in this study (Fig. 3c); healing of a peri-implant defect could also depend on inter-trabecular bone formation besides uni- and bi-directional appositional bone growth.

The model used in this experiment is simple, surgically controlled, and reproducible and has a reduced number of variables potentially affecting the outcome (thereby reducing variance). The model's simplicity results in limitations, which must be kept in mind when interpreting the results. This model specifically addresses healing around primary unloaded implants with a surgically created gap adjacent to the cancellous bone bed, and with no added auto- or allograft. Impaction grafted or revision implants could demonstrate different findings. The 0.75 mm gap enables us to evaluate critical distances of close but not intimate contact between implant surface and bone bed. Since the model is unloaded and not subjected to synovial fluid nutrition and hydrodynamic pressure, it does not expose the implant to all the environmental influences seen clinically.

A 4-week observation period was chosen since it displays initial bone formation before remodeling starts and can distinguish effects of therapeutic treatments relevant for initial implant fixation.^{3,29,32–35} Extrapolation to effects outside this timepoint are speculative and beyond the study scope.

Canines have served as a valuable experimental model for human bone due to their close resemblance in composition and remodeling to human bone. 36,37



Figure 3. Representative histological sections of the 3 rhBMP-2 groups and the untreated control group. Untreated control implant (a and b); 15 μ g implant (c and d); 60 μ g implant (e and f); 240 μ g implant (g and h). Toludine blue stain; magnification: ×1.25 (a, c, e, and g)/×10 (b, d, f and h); Scale bar (×1.25/×10) = 1 mm/0.1 mm. The dotted line designate the original drill-border. Untreated control implant: Thin strand of new bone in the peri-implant gap (a) with some fibrous tissue on the implant surface (b). Fifteen microgram implant: Large amount of new bone out side the drillborder by appositional and intertrabcular formation (c), and large amounts of new bone in the peri-implant surface without fibrous tissue (d). Sixty and two-hundred and-forty microgram implants: Only thin strands of new bone with a low degree of interconnectivity is present in the peri-implant gap (e and g). Low amounts of new bone on the implant surface (f and h). Resorption of host lamellar bone has occured at the drillborder in 60 and 240 μ g implants (e and g). High amounts of new bone is present outside the peri-implant gap in the 240 μ g group.

	New	New Histomorphometric Results Bone			
	Surface	In gap	Off gap	Off gap	
Control	0.10 (0.06-0.13)	0.22 (0.19-0.25)	0.09 (0.07-0.12)	0.25 (0.20-0.29)	
15 µg	0.15(0.11-0.18)	0.23 (0.20-0.26)	0.14(0.12-0.17)	0.23(0.18-0.27)	
60 µg	0.09(0.06-0.13)	0.19(0.16-0.22)	0.13 (0.10-0.15)	0.24 (0.19-0.28)	
240 µg	0.07(0.04-0.11)	0.17(0.14-0.20)	0.17(0.15-0.20)	0.19(0.15-0.24)	
ANOVA ^a	p = 0.0298	p = 0.0145	p = 0.0006	p = 0.304	

Table 2. Fractions of Bone on Implant Surface and in Peri-Implant Gap

Data are presented as mean fraction (95%CI) of implant surface area coverage (surface) and volume in- and out-side the peri-implant gap per treatment group (referred to as "In gap" and "Off gap," resp.). ^aRepeated measures ANOVA. Significant results are marked with bold.

The higher remodeling rate present in young canines with good bone quality, warrants caution when interpreting the results, because normal recipients of joint arthroplasties are older and have poorer bone stock.³⁷ The rhBMP-2 doses used are not interchangeable with human clinical use, because interspecies differences on dose-effect exists, with higher doses required in both non-human primates and humans compared to canines, ovines, rabbits, and mice in descending order.^{13,21}

It can be considered a weakness of the study that a control group without zoledronate exposure was not included. We chose to do so based on previous results obtained from the implant model. In two previous studies, using a similar empty gap model, systemic alendronate administration had no effect on implant fixation compared to control implants.^{34,38} Because zoledronate is a more potent bisphosphonate than alendronate, only a single zoledronate (0.1 mg/kg) dose was administered, compared to previous studies 0.5 mg/kg oral alendronate daily for 10 weeks. In two other canine implant studies investigating BMP-2 the initial implant fixation decreased if an anti-resorptive agent was not used.^{14,39} This necessarily requires our results with rhBMP-2 to be interpreted in the context of systemic zoledronate.



Figure 4. Mean fractions (95%CI) of implant surface area coverage (Surface) and volumes of new and lamellar bone in- and out-side the peri-implant gap per treatment group (referred to as "In gap" and "Off gap," resp.). Square brackets imply a significant difference between designated treatments with *(p < 0.05), **(p < 0.01), ***(p < 0.001) describing significance level.

Zoledronate was chosen because it is the most potent bisphosphonate and dose was based on clinical application.¹⁶ Local zoledronate exposure has a positive effect on mechanical fixation and osseointegration when applied to press-fit seated implants.^{33,40} However, bisphosphonates remains highly localized after administration.⁴¹ We aimed for a uniform antiresorptive exposure of the implant gap, which is why systemic zoledronate exposure was used.

Concern on effects from other implant sites as well as unrelated studies performed in the same animals are relevant. Effects can be divided into local effects due to the Regional Acceleratory Phenomenon (RAP) and systemic effects from distant sites. Two drill holes in each femur could influence osseointegration by RAP as described in diaphyseal bone healing, where a regional increase in remodeling rate away from the injured site occurs due to a noxious stimulus.⁴² A study in sheep with implants seated in cancellous bone in the distal femur revealed no effect on bone 3 mm from the surface of the implants.⁴³ Implants in the presented study were separated by approximately 1 cm of cancellous bone and partly separated by the intercondylar notch (Fig. 1).

A systemic generalized bone anabolic response to distant sites have been described with local application of the growthfactor TGF-B2 in a canine implant study⁴⁴ and a study with surgical injury induced to diaphyseal bone in rats.⁴⁵ The systemic anabolic response to injury in rats was demonstrated to depend on the magnitude of the surgical insult. Bone marrow ablation, but not injury limited to the periosteum, induced distant bone anabolic effects. A later study in rats⁴⁶ demonstrated that the bone anabolic effect induced from distant site injury, is caused by regeneration of the bone marrow in the affected limb. If the marrow was not allowed to regenerate, by physical blocking the marrow cavity, the anabolic response failed. In our study, the drill holes were filled with implants, and the magnitude of surgical injury was less than bone marrow ablation but more than periosteal injury alone. The resulting effect from distant surgical sites both in relation to this study and from other studies performed at the same time cannot be

	Strength		Energy		Stiffness	
	Spearman Rho	<i>p</i> -Value	Spearman Rho	<i>p</i> -Value	Spearman Rho	<i>p</i> -Value
Surface, new bone	0.58	0.0000	0.53	0.0001	0.60	0.0000
In gap, new bone	0.47	0.0009	0.42	0.0029	0.48	0.0006
Off gap, new bone	0.16	0.28	0.27	0.06	0.05	0.75

 Table 3.
 Correlation Between Histomorphometric and Mechanical Parameters

Data are presented as Spearman Rho with corresponding p-value. Strength (MPa): maximum shear strength; Energy (KJ/m²): total energy absorption; Stiffness (MPa/mm): apparent shear stiffness. Histomorphometric parameters are implant surface area coverage and volume of new bone in- and out-side the peri-implant gap per treatment group (referred to as "In gap" and "Off gap," resp.).

excluded. If present, we expect an effect to be moderate. Importantly, it would contribute in a similarly degree and magnitude at all sites and only abate differences between groups. In summary, all implants were subjected to an equal stimulus from both RAP and distant application of growth factors, as well as injury in number and magnitude. Potential confounding influences were thus equally and uniformly distributed among all implants, which is why RAP and systemic effects from distant sites were not expected to induce confounding bias into the results.

Clinically, rhBMP-2 is applied to an absorbable collagen sponge (ACS) and used as a graft substitue.⁴⁷ We aimed for a fast release of rhBMP-2 and because collagen has bone anabolic effects,³⁵ which is why an ACS was omitted and rhBMP-2 was applied directly to the implant surface as proven feasible in previous studies.²² Pure titanium is covered with a thin oxide layer that is highly biocompatible and readily adsorbs proteins to its surface.⁴⁸ Functionalization of the implant surface with rhBMP-2 thus depended on a combination of direct adherence to the titanium surface and physical restraint of the rhBMP-2 solution in the porous implant coating. Both release and dose of rhBMP-2 was confirmed by a release study (Supplementary Material; Figs. S1–S3).

Anti-resorptive control seems to be pivotal when applying rhBMP-2 to implants in a cancellous bone bed to protect newly formed bone from resorption. The enlarged gap boundary observed in 240 µg implants (Fig. 3g) coincides with a lower volume of lamellar bone outside the original drill boundary. Even with systemic zoledronate, the original cavity enlarged over 4 weeks due to bone resorption. This is consistent with other studies reporting lower bone volume with higher doses of rhBMP-2 without anti-resorptive therapy.^{13,39} High doses of rhBMP-2 without adequate anti-resorptive control can thus be considered as potentially detrimental for the initial fixation of press-fit implants, since the areas of contact between a press-fit implant and the surrounding cancellous bone bed are subjected to an increase in osteoclast activity.

In summary, at an early time point of 4 weeks in an experimental implant with an unaugmented periimplant gap and addition of the anti-resorptive agent zoledronate by systemic exposure, within a narrow rhBMP-2 dose range our results demonstrate opposite effects on osseointegration, as evaluated by mechanical fixation and new bone formation. Whether different administration time points, dosages and exposure routes of zoledronate in combination with rhBMP-2 would have different results is unknown. Further pre-clinical investigation is needed before determining if this combination therapy approach (systemic anti-resorptive and local anabolic) is beneficial to use in clinical settings where fixation is expected to be tenuous.

AUTHORS' CONTRIBUTIONS

Rasmus Cleemann: Design, surgery, data acquisition, analysis, author. Mette Sørensen: Design, surgery, analysis, manuscript review. Jørgen Baas: Design, surgery, analysis, manuscript review. Joan E. Bechtold: Design, surgery, analysis, manuscript review. Kjeld Søballe: Design, analysis, manuscript review. All co-authors have reviewed and agreed on the content of the manuscript. The work is not submitted or published elsewhere.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

1	ļ	Augmentation of Implant Surfaces with BMP-2 in a Revision Setting: Effects of
2		Local and Systemic Bisphosphonate
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49 Abstract

Local bisphosphonate treatment of allograft can improve fixation of impaction grafted 50 51 experimental revision implants by preserving allograft without having a positive effect on 52 formation of new bone. On the other hand, bone morphogenetic proteins (BMP) can stimulate new bone formation but decreases mechanical fixation of implants because of 53 54 increased bone resorption. We wanted to investigate if this bone resorption could be 55 counteracted by local or systemic zoledronate when combined with a BMP. We 56 hypothesized that initial fixation of impaction grafted revision implants with a systemic anti-57 catabolic agent (zoledronate) would be comparable to treatment with a local anti-catabolic 58 agent (zoledronate), and that initial fixation would improve with addition of an anabolic 59 stimulus from bone morphogenetic protein-2 (BMP-2) irrespective of anti-catabolic 60 treatment. Our standardized implant revision protocol was performed bilaterally in the stifle joint of 24 canines. After 8 weeks of observation, the unstable primary implants were 61 62 removed, the revision cavity debrided and stable titanium implants with impacted allograft 63 were inserted. The stable revision implants were either untreated or functionalized with 64 BMP-2 (5 μ g) and animals were observed for an additional 4 weeks before euthanasia. 65 Twelve animals received zoledronate IV (0.1 mg/kg) 10 and 20 days after revision surgery, 66 and 12 animals received allograft soaked in zoledronate (0.005 mg/ml) at the time of 67 surgery. No difference in mechanical implant fixation was detected between the 68 zoledronate groups with or without BMP-2 or within the zoledronate groups. The implants 69 with zoledronate soaked allograft had 53% more new bone on the implant surface (p=0.03) 70 and 65% more allograft in the peri-implant gap (p=0.007) relative to implants exposed to systemic zoledronate. In contrast, the volume of new bone in the peri-implant gap was 71 72 34% higher when implants were exposed to systemic zoledronate relative to implants with

73 zoledronate soaked allograft (p=0.003). Addition of BMP-2 to the implants exposed to 74 systemic zoledronate decreased the volume of allograft relatively by 47% (p=0.017), 75 whereas no decrease was seen when BMP-2 was added to the implants with zoledronate soaked allograft (p=0.468). The study results confirmed that soaking allograft in 76 zoledronate locally prevented allograft resorption and demonstrated that revision implants 77 78 accrued new bone with systemic zoledronate. We were not able to demonstrate increased 79 implant fixation with BMP-2 combined with local or systemic anti-catabolic therapy. The 80 results from this experimental model show that BMP-2, even in a relatively low dose, is a 81 potent compound that significantly increases allograft remodeling when effective anti-82 catabolic therapy is not applied. It would be of interest to explore further if an additive 83 effect on implant fixation can be achieved by either combining local and systemic anti-84 catabolic treatment, and whether a mechanically effective anabolic effect from BMPs 85 would be possible with targeted anti-catabolic control.

86

87 Keywords:

88 Revision; Bone morphogenetic protein 2; Local bisphosphonate; Systemic

- 89 bisphosphonate; Implant; Revision
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97 1. Introduction

98 Primary total joint replacements is a successful treatment for osteoarthritis and has 99 excellent clinical outcomes and a high degree of patient satisfaction [1]. Revision implants 100 have a higher failure rate than primary implants [2], and good initial fixation of revision 101 implants is important to reduce the risk of implant failure [3]. Revision surgeries are often 102 complicated by a deficient bone stock due to osteolysis, and impacted bone graft is widely 103 used to restore lost bone [4]. Impacted allograft provides initial mechanical support and 104 function as a lattice for new bone to form and remodel into host bone over time. 105 Histological specimens retrieved from patients have demonstrated variable incorporation 106 of graft, which is often encapsulated in fibrous material and not in contact with the host 107 bone [5]. Allograft is less osteoconductive and osteoinductive compared to autograft [4], 108 and the challenged healing environment around a loose implant that has sclerotic bone, 109 deficient vascularity, and increased levels of inflammatory cytokines reduce the healing 110 capacity[6]. In theory, the addition of a bone anabolic substance could improve integration 111 of the implant surface with the host bone.

112

Bone morphogenetic protein 2 (BMP-2) is an anabolic osteoinductive protein that directly
stimulates bone formation by recruitment, proliferation and differentiation of
osteoprogenitor cells into osteoblasts [7]. Due to the coupling of osteoblast and osteoclast
activity in normal bone, osteoclast activity is then also increased, via the RANK/RANKL
signaling system [8].

Experimentally, implant fixation has been improved using BMP augmented allograft in a canine revision model [9]. In contrast, no favorable effect of BMP augmented allograft has been detected in hip revisions clinically, a result attributed to increased allograft resorption

[10]. Anti-catabolic treatment that consisted of soaking allograft in a bisphosphonate prior
to impaction has mitigated allograft resorption both experimentally [11] and clinically [12].
Although protective, bisphosphonates cannot induce a tissue to reach its full anabolic
potential.

125 The concept of combining anabolic and anti-catabolic therapies to induce and preserve bone has experimental merit. Local ibandronate and BMP have preserved autograft and 126 127 increased bone formation in a piglet model of osteonecrosis of the hip [13]. In a rodent 128 bone conduction chamber model, systemic zoledronate and local BMP have increased 129 bone ingrowth and total bone [14]. However, combined anabolic and anti-catabolic therapies in relation to the fixation of implants impacted with allograft have proven less 130 131 fruitful. A canine study that used local BMP-2 (450 μ g) and allograft soaked with the bisphosphonate pamidronate (9 mg/ml) resulted in extensive allograft resorption induced 132 by BMP-2 and no new bone formation, which was attributed to blockage of osteoblast 133 134 function by pamidronate [15]. These results motivated the present study to investigate the 135 effects of BMP-2 coated implants in combination with local or systemic administration of 136 anti-catabolic treatment, zoledronate, on the initial implant osseointegration of impaction-137 grafted revision implants. We hypothesized that osseointegration of implants would 138 improve with BMP-2 in combination with zoledronate, local or systemic, compared to anti-139 catabolic treatment alone. We defined increased osseointegration as improved mechanical 140 fixation (as evaluated by push-out test) and increased formation of new bone and retention 141 of allograft (as evaluated by histomorphometry).

142

143 2.1 Materials and Methods

All animals were bred for scientific purposes and the study was approved by the
Institutional Animal Care and Use Committee (IACUC). Twenty-four (24) skeletally mature
male mongrel canines were included. The animals had a mean weight of 25.4 (range 20.629.8) kg and a mean age of 14 (range: 13-15) months. Plane X-rays of the distal femurs
showed closed epiphyseal plates and confirmed skeletal maturity.

149

150 2.2 Sample size

151 An a priori sample sized calculation was performed to allow for unpaired comparisons between systemic and local zoledronate exposure, and for separate paired comparisons of 152 systemic or local zoledronate with the addition of BMP-2. In the unpaired study, we 153 154 wished to detect a relative minimal difference of ≥50% on endpoints and assumed a coefficient of variation of 40% in the unpaired differences, an alpha of 0.05 and a beta of 155 0.8. This resulted in a sample size of 22 animals in the unpaired study. To counter the loss 156 157 of power in the advent of animal exclusions or implants lost during analysis, an additional 2 158 animals were included. Assuming a similar coefficient of variation in the paired differences, 159 an alpha of 0.05, a beta of 0.8, resulted in a sample size of 12 animals for each of the 160 paired studies.

161

162 2.3 Treatment groups:

Animals were randomly allocated into 2 groups that either received systemic (n=12) zoledronate IV 10 and 20 days after the revision procedure or zoledronate-soaked allograft at time of the revision surgery (n=12). Each animal received 2 implants, 1 implant in each medial femoral condyle (Figure 1). The implants were either coated with 5 μ g of

recombinant BMP-2 (rhBMP-2) or left untreated. rhBMP-2 treatment was assigned with
 random start and systematically altered between the left and the right femurs.

169

Our established revision protocol using a micromotion device (Figure 2) was implemented to produce a peri-prosthetic tissue reaction similar to the milieu around a mechanically loose implant in the presence of particulate polyethylene [6, 9, 11, 16, 17]. Adaption of the revision protocol consistently produces a tri-laminar structure around the implant, with synovial-like cells adjacent to the implant being surrounded by a thick dense fibrous membrane encapsulated by a sclerotic shell of dense cortical-like bone.

176

177 2.4 Implants

The primary surgery used 48 poly-methyl-methacrylate (PMMA) implants and the revision surgery used 48 porous coated titanium-alloy (6AI-4V) implants (Figure 1). Implants were cylindrical and had nominal dimensions of 10 mm in length and 6 mm in diameter. The titanium implants had a commercially available porous coating (Gription[®], DePuy-Synthes, Warsaw, IN) that had a volume porosity of 63% \pm 3% as specified by the manufacturer.

184 2.5 Implant coating with rhBMP-2

185 rhBMP-2 (Infuse[®] 12 mg, Medtronic, Memphis, TN) was reconstituted with kit dilutes and

186 buffer (5.0 mg sucrose, NF; 25 mg glycine, USP; 3.7 mg L-glutamic acid, FCC; 0.1 mg

sodium chloride, USP; 0.1 mg polysorbate 80, NF; pH=7.4) to a concentration of 84 μ g/ml.

188 Then, 150 μ l of the rhBMP-2 solution was placed in each well of a well-plate (96-Well,

189 Standard F, Sarstedt AF & Co., Nümbrecht, Germany) blocked with 0.5% bovine serum

albumin (BSA) (Sigma-Aldrich, Saint Louis, MO) in phosphate buffered saline (PBS)
(Sigma-Aldrich Chemie Gmbh, Steinheim, Germany).

192

193 Titanium implants mounted on stainless steel rods were fully submerged into the rhBMP-2 194 solution. To saturate the porous implant coating with the rhBMP-2 solution, the well-plate 195 and implants were placed in a standard glass desiccator and subjected to a vacuum for 5 196 minutes. Then, the rhBMP-2 solute was evaporated in a vacuum, leaving rhBMP-2 on the 197 implant surface. The rhBMP-2 coated implants were packed in sterile tubes (Eppendorf 198 LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and stored at -80°C until surgery. 199 All handling of the rhBMP-2 solution was performed using polypropylene pipette tips 200 (Finntip, Thermo Fischer Scientific Oy, Vantaa, Finland). The implants' porous coating 201 retained an average of 60 μ L of fluid, resulting in an estimated dose of 5 μ g of rhBMP-2 left 202 on the implant surface.

203

In-vitro release using an rhBMP-2 Quantikine ELISA kit (R&D Systems, Minneapolis, MN)
 confirmed the presence and release of rhBMP-2 within the expected range (Figure 3).

206

207

208 2.6 Graft material

209 Cortico-cancellous allograft was obtained from 2 animals not included in the study. Bones 210 from the proximal humeri, distal femora and proximal tibias were harvested under sterile 211 conditions and stored at - 80° C. Prior to surgery, the bones were thawed, thoroughly 212 debrided of connective tissue and cartilage and the physes were milled on the finest 213 setting on a standard bone mill (The Mill - Bone Mill System, Biomet Inc., Warsaw, IN) to produce morselized allograft with bone chips of 1-3 mm in length. Bone chips larger than 2mm were manually removed. The milled bone from both dogs and all sites was mixed into a single batch, rinsed 3 times for 1 minute in 0.5 L of fresh saline ($37^{\circ}C$), squeezed in gauze to remove excess saline, and manually compressed into 1.0-mL sterile vials containing 0.57 g (±0.02 g) of allograft and were stored at - 80°C until being used within 1 week of preparation.

220

221 2.7 Zoledronate administration

In the local zoledronate group, immediately prior to revision surgery, allograft was thawed
and soaked in a solution (0.005 mg/ml) of 5 ml of zoledronate (Zoledronic Acid Actavis 4
mg/mL, Actavis Group, Iceland) for 3 minutes under gentle stirring, followed by 3
consecutive rinses in fresh saline for 1 minute each to remove excess zoledronate [18]. In
the systemic zoledronate group, zoledronate IV (0.1 mg/kg) was administered 10 and 20
days after revision surgery.

228

229 2.8 Surgery

230 All surgical procedures were performed on animals using general anesthesia, sterile 231 technique and infection prophylaxis using cefazolin IV (22 mg/Kg) preoperatively and 232 cefuroxime SC (50 mg/Kg) for 9 days postoperatively. Surgery was performed as described previously [6, 17] by a surgeon experienced in the procedure. At time of the 233 234 revision procedure, animals allocated to receive systemic zoledronate were operated on 235 before animals allocated to receive local zoledronate. To avoid contamination with rhBMP-236 2, untreated implants were inserted first followed by an identical contralateral procedure 237 with an rhBMP-2 coated implant. Postoperative analgesia was provided using 3-4 mL of

0.5% bupivacaine administered at the incision site and transdermal fentanyl (Recuvyra[®]
2.7 mg/Kg). Four weeks after the revision procedure, all animals were sedated with
acepromazine IM (0.1 mg/Kg) and propofol IV (4 mg/Kg) and euthanized with

241 hypersaturated barbiturate IV.

242

243 2.9 Observation period

244 Animals were housed one per run (4 ft. by 6 ft.) and allowed unlimited pen activity. Animals 245 were exercised 2 hours per day to ensure loading of the implants, and hind limb function 246 was assessed daily. Two animals sustained superficial wound ruptures because of a fall (revision procedure) and chewing (primary and revision procedure). Animals were treated 247 248 with 10 days of antibiotics and a collar. The wounds healed nicely without sign of infection. 249 A third animal spared the right stifle after the revision procedure. An X-ray confirmed 250 correct implant placement and no fracture. All animals completed the observation period. 251 No implants were damaged and all specimens were available for both mechanical and 252 histomorphometric evaluation.

253

254 2.10 Specimen preparation

After euthanasia the distal femurs with implants in-situ were harvested and stored at - 21° C. A bone cube with implant in-situ was cut from the medial femur condyle using a watercooled diamond band saw (Exakt Apparatebau, Nordenstedt, Germany). The outermost 1 mm of the implant-bone specimen closest to the subchondral bone was discarded. The remaining implant-bone specimen was divided in two by a cut perpendicular to the axis of the implant. The 3.5 mm closest to the subchondral bone was stored at -21°C for later

261 mechanical testing. The remaining 5.5 mm of the bone-implant section was used for262 histomorphometric evaluation.

263

264 2.11 Histomorphometry

Specimens for histomorphometric evaluation were sequentially dehydrated in graded 265 ethanol (70-96%) and 100% 2-propanol, defatted in xylene and embedded in methyl 266 267 methacrylate (MMA Product no. 800590, Merck, Darmstadt, Germany) in a cylindrical 268 mold; taking care that the vertical axis of the mold and implant were parallel. The 269 embedded specimen was rotated uniform random along its vertical axis before 4 serial 270 sections that were 400 μ m apart (corresponding to the width of the saw blade) were cut 271 from the central part of the implant using a hard-tissue microtome (KDG-95; MeProTech, 272 Heerhugowaard, Holland). The specimens were stained using 0.1% toluidine blue ((pH=7) 273 Sigma-Aldrich, St.Louis, MO), rinsed and mounted on glass. The staining method enabled 274 different tissues to be distinguished on the basis of morphological appearance (Figure 4). 275 Using an optical light microscope (Olympus, Ballerup, Denmark) and associated software 276 (NewCast software, version 3.0.9.0, Visiopharm Integrator System, Hørsholm, Denmark), 277 quantitative histomorphometric evaluation was performed by a reviewer blinded to 278 treatment, with specimens in random order. The surface area-fraction of new bone, 279 allograft, marrow and fibrous tissue were estimated by line intersection technique [19] using a probe intensity of 15 lines. The volume fraction of new bone, allograft, marrow, and 280 281 fibrous tissue were estimated by point counting technique [20] using a probe intensity of 5 282 x 4 points. Histomorphometry was performed at x10 magnification using meander 283 sampling (100% fraction). The technique applied returns a relative estimate using fractions 284 of area coverage and volumes and provides reliable results with minimal bias [21]. To

estimate surface area fractions, a mean of 556 (\pm 110) intersections were counted, and to estimate peri-implant volume fractions, a mean of 334 (\pm 38) points were counted. Intraand inter-observer reproducibility was performed (Table 1).

288

289 2.12 Mechanical testing

290 Thawed specimens were tested in random order by an examiner blinded to treatment. The 291 specimens were placed on a metal support jig over a 7.4-mm hole, ensuring 0.7 mm of 292 clearance between the implant and the edge of the support jig [22]. Implants were tested 293 until failure of the bone implant interface by applying a continuous force axially onto the 294 implant with a cylindrical metal test probe connected to a servohydraulic test rig (MTS 858 295 Mini Bionics Test Machine, MTS Systems Inc., Eden Prarie, MN). Before loading, a 296 preload of 2 N was applied as standard contact condition. Force was applied at a constant 297 displacement rate of 5 mm/min, recording displacement (mm) and load (N) for every 10 298 μ m to produce a load/displacement curve.

299

300 2.13 Statistical analysis

Statistical analysis of data was carried out in StataCorp. 2013 (StataCorp, Lakeway Dr,
TX, USA, version IC 13.1). Effects of treatment on mechanical and histomorphometric
endpoints were estimated using a mixed model adjusting for implant position, age and
weight of the animal and taking the between animal and between side variations into
account. The probability of Type-1 error was not adjusted for multiple comparisons. Mean
estimates and differences are presented with 95% confidence interval.
Histomorphometric variables with low intersection or count values were evaluated by a

308 two-step analysis. Data was dichotomized to indicate the presence or absence of tissue.

309 The probability of presence or absence of a variable was modeled using logistic 310 regression. For variables present measurements were further analyzed using a mixed 311 model to estimate the median effect. The median treatment effects are presented with 95% 312 confidence interval. In all studies, two-tailed p-values below 0.05 for overall and pairwise 313 comparisons were considered statistically significant. 314 3. Results 315 316 3.1 Mechanical results No significant differences were detected on any mechanical parameter between local and 317 318 systemic zoledronate exposures with or without the addition of rhBMP-2. (Figure 5 and 319 Table 2). 320 321 3.2 Histomorphometric results 322 3.2.1 New bone on implant surface 323 Implants with zoledronate soaked allograft had 53% more new bone on the implant 324 surface relative to implants exposed to systemic zoledronate alone (p=0.03). Adding 325 rhBMP-2 to implants with zoledronate soaked allograft increased the surface area of new 326 bone by 70% relative to rhBMP-2 coated implants exposed to systemic zoledronate 327 (p=0.002) (Figure 6 and Table 3). 328 329 3.2.2 Graft at the implant surface

330 Only a small area of the implant surface was in contact with allograft. When graft was

331 present, implants with zoledronate soaked allograft had a 165% greater surface area

332 covered with allograft (p=0.03) relative to implants exposed to systemic zoledronate alone.

Addition of rhBMP-2 to systemic zoledronate exposure resulted in virtually no graft
remaining in contact with the implant surface (Table 3).

335

336 3.2.3 New bone volume

Implants exposed to systemic zoledronate had a 34% higher volume of new bone in the peri-implant gap relative to implants with zoledronate soaked allograft (p=0.003). Adding rhBMP-2 to implants exposed to systemic zoledronate resulted in a 26% higher volume of new bone relative to rhBMP-2 coated implants with zoledronate soaked allograft (p=0.009) (Figure 6 and Table 3).

342

343 3.2.3 Graft volume

Implants with zoledronate soaked allograft had a 65% higher volume of allograft relative to 344 implants exposed to systemic zoledronate alone (p=0.007). Adding rhBMP-2 to implants 345 346 exposed to systemic zoledronate decreased the volume of allograft by 47% relative to 347 implants exposed to systemic zoledronate alone (p=0.017). In contrast, adding rhBMP-2 to 348 implants with zoledronate soaked allograft only decreased the volume of retained allograft 349 by 10% relative to implants with zoledronate soaked allograft alone (p=0.47). Adding 350 rhBMP-2 to implants exposed to systemic zoledronate decreased the volume of allograft 351 by 74% relative to rhBMP-2 coated implants with zoledronate soaked allograft (p=0.000) 352 (Figure 6 and Table 3).

353

3.3 In all specimens, fibrous tissue was present only in very low fractions, both on the
implant surface and in the peri-implant gap. No significant differences were detected
between either zoledronate exposure and rhBMP-2 status.

357

358 3.4 The histomorphometric variables and mechanical results had a medium but significant
 359 correlation (Table 4). Inter- and intra-observer reproducibility were acceptable (Table 1).
 360

361 4 Discussion

The purpose of this study was to increase the mechanical fixation of impaction grafted orthopedic revision implants. Bone morphogenetic protein 2 (BMP-2) was used to stimulate formation of new bone and was administered as an implant surface coating. BMP-2 also indirectly increases osteoclast mediated bone resorption, which is why the bisphosphonate zoledronate was administered to all animals either by systemic administration or as a local treatment of the impacted bone allograft.

368

369 Even though mechanical fixation was similar between groups the histomorphometric 370 analysis revealed a statistically significant dissimilar composition of the impaction grafted 371 peri-implant gap depending on zoledronate exposure and BMP-2 status. Local zoledronate protected allograft bone from resorption, but not the resorption of newly formed bone. With 372 373 local zoledronate allograft bone chips had few resorptions lacuna whereas they were 374 abundant in areas with new bone (Figure 4). Systemic zoledronate exposure protected 375 newly formed bone against resorption, but not the allograft. With systemic zoledronate the 376 new bone had few resorptions lacune whereas the few remaning allograft chips had 377 resorptions lacuna littered on the surface (Figure 4). Addition of rhBMP-2 did not 378 significantly increase new bone formation, but increased bone graft remodeling 379 significantly when combined with systemic zoledronate.

381 Canines as an experimental model were chosen because their bones are a good 382 representation of human bone in regards to bone density, remodeling and collagen and 383 mineral composition [23, 24]. In this experimental model, the revision cavity consists of a 384 cylindrical defect and does not represent the wide array of manifestations encountered in 385 clinical revision arthroplasties. The revisions implants bears no morphological or functional 386 resemblance to clinical implants, but are intra-articular, cyclically loaded, subjected to 387 oscillating fluid pressure and have a porous coating identical to commercially used clinical 388 implants. The lack of morphological similarity to clinical implants is compensated for by the 389 high degree of control of variables, which ensures a high degree of reproducibility with low 390 variation.

391

392 Interestingly, implants with zoledronate soaked allograft had a 53% greater surface area of 393 new bone coverage relative to implants exposed to systemic zoledronate. This result is in 394 line with the results of a previous revision study that investigated allograft soaked in 395 zoledronate [11]. That study detected no difference on surface area of new bone between 396 implants exposed to local zoledronate and control implants [11]. In the present study, the 397 lower surface area of new bone on implants exposed to systemic zoledronate is difficult to 398 explain from the evidence on the inhibitory effects of bisphosphonates on osteoblasts [15, 399 18, 25]. Studies using rodent bone chamber models have investigated new bone ingrowth 400 into cancellous allografts using local or systemic bisphosphonate exposure and have 401 suggested that the increased amount of new bone is caused by preserved allograft acting 402 as an osteoconductive scaffold [26-28]. Potentially, the higher volume of retained allograft 403 in the peri-implant gap of implants exposed to local zoledronate functioned as a scaffold 404 for appositional bone formation by helping new bone to reach the implant surface.

405

406 In the present study, local zoledronate exposure was superior to systemic zoledronate 407 exposure in protecting allograft against resorption. Theoretically, exposure of allograft to 408 local zoledronate leaves only a thin layer of bone with zoledronate, which can be removed over time by osteoclasts. Exposing implants and graft to zoledronate IV (0.1 mg/kg) twice 409 410 was not equivalent to soaking the allograft in zoledronate (0.005 mg/ml). The delivery of 411 systemic zoledronate to the peri-implant gap depends on formation of new vessels in the 412 peri-implant gap. In a necrotic graft, vascularization is closely followed by increased 413 osteoclast activity that is often uncoupled from formation of new bone. Potentially, at the 414 time of the systemic zoledronate administration, the vascular network was not yet 415 extensive enough or the vascular ingrowth into the grafted gap had preceded the 416 administration time point. In both events, allograft would have been left unprotected from 417 resorption by osteoclasts.

In a revision study by Sorensen et al. [11], the volumes of retained allograft were 27% and 9% for the zoledronate and control groups respectively. The present study's results for implants exposed to local zoledronate were consistent with these results, but they could also indicate that systemic zoledronate does have a minor protective effect of impacted allograft compared to no treatment (Table 2).

423

In contrast to the volume of retained allograft, the implants exposed to systemic
zoledronate had a significantly higher volume of new bone in their peri-implant gap relative
to the implants exposed to local zoledronate. This was not unexpected, as new bone is a
living tissue and highly vascularized, as opposed to impacted allograft which is necrotic.
Thus, by default new bone is more susceptible to systemic anti-catabolic therapy than is

429 necrotic bone graft, where an anti-catabolic effect of systemic zoledronate depends on 430 graft particles being encircled by new vessels. Potentially the difference between local and 431 systemic anti-catabolic therapy may be attributed to the endothelial cells or the osteoblasts 432 to not being directly exposed to zoledronate in the peri-implant gap and thus not interfering 433 with vascularization or bone formation [25, 29]. The resorbed allograft could also contribute 434 to the formation of new bone, resulting in a larger volume of new bone. When osteoclasts 435 resorb bone, embedded osteoinductive growth factors are released and attract and 436 stimulate osteoprogenitor cells to differentiate and form new bone.

The double systemic zoledronate administrations also theoretically contribute to retaining
new bone by forming a layered structure of protected bone and by providing a second
dose to the osteoid not mineralized at the time of the first zoledronate administration.
Administration of a fluorochrome label could have helped elucidate the temporal and
spatial relationship of bone resorption and new bone formation, but it was not performed in
the present study.

443

444 In contrast to local zoledronate exposure, the distribution of zoledronate after systemic 445 administration is less controlled. When soaking allograft in nitrogen-containing 446 bisphosphonates only low doses are needed to protect the allograft against resorption, as 447 they remain highly localized and have a minimal systemic spillover after administration [30]. When systemic administration is used, only 50% of the administered dose is taken up 448 449 by the bone. But bisphosphonates have a predilection for areas that have increased bone 450 turnover, and they demonstrate a heterogenic skeletal distribution, accumulating in the physes of appendicular bone [30, 31]. The peri-implant bone in the systemic group is likely 451 452 to be exposed to zoledronate, but the extent to which it is depends on the vascularization

and the degree of primary mineralization of new bone. Systemic zoledronate exposure is
seemingly beneficial to protect and accrue new bone, but two administrations of systemic
zoledronate in quick succession may pose problems. A single administration of systemic
zoledronate delays bone remodeling only slightly [32], whereas regimes that include
multiple administrations of zoledronate decrease bone remodeling [33] and alters the
mechanical properties of the host bone [34].

459

460 In the present study, adding rhBMP-2 to implants either exposed to local or systemic zoledronate increased both the surface area and the volume of new bone. However, no 461 statistically significant advantage of adding rhBMP-2 to implants exposed to local or 462 463 systemic zoledronate was detected in terms of improvement of mechanical implant fixation or increased new bone formation. This result contrasted with that of a previous study in 464 which adding rhBMP-7 to impacted allograft in revision implants increased both the 465 466 mechanical fixation and the total bone volume [9]. The use of a different delivery method 467 other than the one recommended by the manufacturer could contribute to the absence of an anabolic effect on formation of new bone. It should be noted that in the present study, 468 469 biological activity was confirmed by the increased resorption of allograft that was seen with 470 exposure to systemic zoledronate. It is possible that the rhBMP-2 dose applied was too 471 small to cause an anabolic response. A higher dose of rhBMP-2 in combination with 472 adequate anti-catabolic treatment could potentially balance the anabolic and catabolic 473 response in a grafted revision setting, but this is speculative.

474

475 5 Summary

476	In summary, the results of the study supported that local zoledronate treatment can protect
477	necrotic bone graft against resorption and demonstrated that systemic zoledronate protect
478	and accrue vascularized new bone. The results also support that effective anti-catabolic
479	control can prevent bone resorption when rhBMP-2 is combined with allograft.
480	
481	6 Conclusion
482	Additional studies are needed to investigate whether combined local and systemic
483	zoledronate exposures or in combination with an anabolic agent, can improve
484	osseointegration of impaction-grafted revision implants.
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Table 1. Histomorphometric Reproducibility

	New bone	Allograft bone	Bone marrow	Fibrous tissue	
	Inter-observer				
Surface	3.8	12.6	12.6 1.4 15		
Gap	1.8	5.9	0.6	17.7	
	Intra-observer				
Surface	0.7	9.3	0.5	4.2	
Gap	1.8	4.6	1.8	13.3	

Inter- and intra-observer variability presented as coefficient of variation (CV) in percentage (%).

 Table 2. Mechanical Results

	Strength (MPa)	Energy (kJ/m2)	Stiffness (MPa/mm)
LZ	10.5 (9.5-11.6)	1.5 (1.4-1.7)	55.3 (48.4-62.2)
LZ+rhBMP-2	10.3 (9.2-11.4)	1.5 (1.3-1.6)	54.6 (47.7-61.5)
SZ	8.6 (6.7-10.6)	1.2 (0.8-1.5)	45.9 (35.7-56.2)
SZ+rhBMP-2	8.2 (6.3-10.2)	1.1 (0.8-1.5)	42.7 (32.5-53.0)

Results are presented as mean (95% CI) per treatment group. Mechanical fixation parameters were normalized to the implant surface area to compensate for differences in specimen thickness. Based on the load-displacement curve ultimate shear strength (MPa; Strength) was calculated as the maximum value of the load-displacement curve before failure of the bone-implant interface; Total energy absorption (kJ/m²; Energy) was calculated as the area under the curve until failure of the bone-implant interface. Maximum shear stiffness (MPa/mm; Stiffness) was calculated as the steepest section of the load-displacement curve before failure of the bone-implant interface. SZ: Systemic Zoledronate; LZ: Local Zoledronate

Table 3. Histomorphometric Results

	Sur	face	Gap		
	New bone Allograft		New bone	Allograft	
LZ	0.23 (0.18-0.28)*	0.007 [0.004-0.01]*	0.25 (0.21-0.29)**	0.26 (0.20-0.31)**	
LZ+rhBMP-2	0.27 (0.22-0.32) ^{††}	0.01 [0.006-0.016]	0.29 (0.25-0.33)	0.28 (0.23-0.33) ^{†††}	
SZ	0.15 (0.10-0.20)	0.002 [0.001-0.005]	0.34 (0.30-0.38) ^{††}	0.15 (0.10-0.21) ^{††}	
SZ+rhBMP-2	0.16 (0.11-0.21)	n/e	0.36 (0.32-0.40)	0.07 (0.02-0.12)	

Results are presented as mean fraction (95% CI) or median fraction [95% CI] of surface area and volumes inside the peri-implant gap per treatment group. n/e: not estimable. SZ: Systemic Zoledronate. LZ: Local Zoledronate. P-values (<0.05; <0.01; <0.001) when compared to SZ (*;**;***) and SZ+rhBMP-2 ([†]; ^{††}; ^{†††}).

Table 4. Correlation between Biomechanical and Histomorphometric Results (Spearman rho)

	Surface					Volume			
	New bone		Allograft New bo		one	Allogr	aft		
	Spearman	<i>p</i> -value	Spearman	<i>p</i> -	Spearman	<i>p</i> -	Spearman	<i>p</i> -	
Strength	0.43	0.003	0.39	0.006	0.19	0.20	0.41	0.004	
Energy	0.37	0.009	0.22	0.140	0.15	0.33	0.29	0.045	
Stiffness	0.42	0.003	0.39	0.006	0.14	0.33	0.46	0.001	

Mechanical fixation parameters are Maximum shear strength (Strength), total energy absorption (Energy) and apparent shear stiffness (Stiffness). Surface parameters are surface area fraction of new bone and allograft, and peri-implant gap volume fraction of new bone and allograft.

Figure 1.



Figure 1.

Micromotion device (center) with a titanium implant (left) and a PMMA implant (right).

Upper left: X-ray with in-situ micromotion devices and titanium-implants.



Figure 2.

Illustration of the revision protocols time sequence (right) and the surgical procedure (left). The protocol includes 2 separate surgeries 8 weeks apart and consistently produces a revision cavity as encountered in revision joint arthroplasties under unstable conditions in the presence of particulate polyethylene. At the primary surgery (t = 0 weeks), the medial condyle of each stifle joint had inserted into it a pistoning micromotion device that had attached to it a poly-methyl-methacrylate (PMMA) implant with particulate polyethylene particles that represented a loose cement mantle and wear particles. At the revision procedure (t = 8 weeks), the cavity was reamed and the PMMA implant replaced with a stabilized titanium revision implant impacted with allograft. The animals were observed for an additional 4 weeks before euthanasia (t = 12 weeks). The micromotion device consists of a self-tapping anchor house to secure its fixation in the subchondral cancellous bone of the medial condyle. A threaded rod sits on top of a spring located inside the body of the anchor house, allowing for 500 μ m (± 15 μ m) of rod movement axially. The spring returns the threaded rod to its original position after the compression that occurrs during the standing phase and flexion of the stifle joint. The unstable PMMA implant engages the base of the suspended rod enabling axial piston movement relative to the anchorhouse. The stabilized titanium-implant engages the top of the anchor house and prevents the rod from moving axially.

Figure 3.



Figure 3.

Result of in-vitro elution study with sampling points (0 h, 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 120 h). Two rhBMP-2 coated and 2 untreated control implants were placed in individual wells in a lid-covered BSA-coated well-plate (24-Well, Standard F, Sarstedt AF & Co., Nümbrecht, Germany) with 5 ml of PBS as the release medium in a 37°C heating cabinet. Samples were collected in duplicate and PBS was replenished accordingly using a 40% sampling fraction. Samples were stored in sterile Eppendorf tubes (Eppendorf LoBind Tube 1.5 ml, Eppendorf AG, Hamburg, Germany) and kept frozen (-20°C) until analysis with an rhBMP-2 detection kit (Quantikine ELISA kit DBP200, R&D Systems, Minneapolis, MN). Cumulative release was calculated as described by *Strobel et al.* [35] and averaged. Average cumulative release was 5.8 μ g of rhBMP-2 per implant. No rhBMP-2 activity assay was performed on released rhBMP-2. Control implants were negative and are not depicted.
Figure 4.



Figure 4.

Representative histological sections of the zoledronate-treated groups (toluidine blue), with an implant from the systemic zoledronate group (SZ) to the far left and an implant from the local zoledronate group (LZ) to the far right. Sections are cut longitudinally to the long axis of the implant. The dotted line marks the original drill-border, and the square, solid white frames in the 2 peripheral images (x1.25) marks the positions of the 2 central images (x10). The region of interest (ROI) is marked with a dotted, white rectangle in SZ (x1.25). The ROI began at the median surface line and extended 1000 μ m into the peri-implant gap. Allograft appears as a lightly stained lamellar structure with empty fusiform lacuna. Lamellar bone appears as allograft having fusiforme lacuna with cells. New bone presents as a disorganized, dark-stained structure having round lacuna with cells. SZ (x1.25): extensive remodeling has occurred, with a few small pieces of allograft in the peri-implant gap being covered with new bone, which exhibits high interconnectivity in the peri-implant gap and into the porous surface coating. SZ (x10): allograft with a thick layer of new bone and few resorptions lacunae. The surface of the remaining allograft is covered with resorption lacunae, giving it a serrated appearance. LZ (x1.25): large pieces of allograft with thin layers of new bone displaying low interconnectivity in the peri-implant gap and into the porous implant surface. LZ (x10): allograft with ragged and intact edges, noncircumferential new bone coverage, and extensive number of resorption lacuna.

Figure 5.



Figure 5.

Mean (95% CI) for derived mechanical implant-fixation parameters per treatment group. Maximum shear strength (strength), total energy absorption (energy) and apparent shear stiffness (stiffness). SZ: systemic zoledronate. LZ: local zoledronate. Figure 6.



Figure 6.

Mean fractions (95% CI) per treatment group of implant surface area and volumes in the peri-implant gap per treatment group. SZ: systemic zoledronate. LZ: local zoledronate. P-values (<0.05; <0.01; <0.001) when compared to SZ (^{*}; ^{**}; ^{***}) and SZ+rhBMP-2 ([†]; ^{††}; ^{†††}).