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Manipulation of the mevalonate pathway in the bone-implant interface

PhD dissertation

Mette Sørensen

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Preface

This thesis is based on the scientific studies conducted during my enrolment as a PhD student at the Graduate School of Health Sciences, Aarhus University from August 2009 through July 2012. The experimental surgeries were performed at Orthopaedic Biomechanics Lab, University of Minnesota, Minneapolis Medical Research Foundation, Excelen Center for Bone and Joint Research and Education, Minneapolis, MN, USA while the subsequent analyses were carried out at the Orthopaedic Research Laboratory, Aarhus University Hospital, Denmark.

This PhD would never have become reality for me had I not been introduced to orthopaedic research by my main supervisor, Professor Kjeld Søballe and project supervisor Jørgen Baas. Kjeld Søballe has provided excellent research facilities at the Orthopaedic Research Laboratory -a great place to conceive ideas. Jørgen Baas has been my daily supervisor and he has guided me towards the right direction when I have been lost. I admire and appreciate him greatly as both a colleague and a friend.

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- ISurTec, Saint Paul, MN, USA donated the PLGA-based coating for study II
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This thesis is based on the following papers:

I. **Effect of poly (D,L-lactide), with and without simvastatin, on fixation of orthopaedic implants. A study in 12 canines.**
Mette Sørensen, Jørgen Baas, Joan E. Bechtold, Marianne T. Vestermark, Jeppe Barckman, Rasmus Cleemann, Kjeld Søballe

II. **Lactic acid-based polymers for drug delivery can negatively affect the bone-implant interface.**
Mette Sørensen, Jørgen Baas, Jeppe Barckman, Joan E. Bechtold, Kjeld Søballe

III. **Pre-clinical evaluation of zoledronate to maintain bone allograft and improve implant fixation in revision joint replacement.**
Mette Sørensen, Jørgen Baas, Jeppe Barckman, Joan E. Bechtold, Kjeld Søballe.

Contents

Abbreviations

ATP	Adenosine triphosphate
BMP	Bone Morphogenetic Protein
BP	Bisphosphonate
CV	Coefficient of variance
EtO	Ethylene oxide
GTPase	Guanosine triphosphatase
HMG	3-hydroxy-3-methylglutartyl
M-CSF	Macrophage colony-stimulation factor
N	Nitrogen
OPG	Osteoprotegerin
PBS	Phosphate buffered saline
PDLLA	Poly (D,L-lactide acid)
PE	Polyethylene
PLGA	Poly (lactic-co-glycolic acid)
PMMA	Polymethylmethacrylate
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
RSA	Radiostereometric analysis
SEM	Scanning electron microscopy

1. English summary

Orthopaedic implants that are not securely fixed in the bone during the early post-operative period (early instability) are at increased risk of later aseptic loosening. Revisions of loose implants often have inferior functional outcomes, which may be complicated by loss of bone stock. This can be compensated for by bone allograft. The allograft provides immediate stability for the revision implant. Local administration of augments to the bone-implant interface may increase the bioactivity of the bone graft, provided that the augment provides proper stimulus and that the delivery vehicle does not interfere with the local environment. Statins and bisphosphonates both inhibit the mevalonate pathway and have been shown to have an effect on bone. The statin, simvastatin, which is used to treat hypercholesterolemia, has been shown to stimulate bone formation. Local delivery of simvastatin may provide a signal for bone formation, thereby improving early osseointegration and implant fixation. The bisphosphonate, zoledronate is used to treat conditions with increased bone resorption and has been shown to reduce resorption of bone allograft after both local and systemic administration.
This dissertation consists of three studies. For all studies we used a canine model of implant fixation. All implants were surrounded by a concentric gap, which was either left empty or impacted with bone allograft. All studies were evaluated by push-out test assessing mechanical implant fixation and by histomorphometrical analysis of the osseointegration.

Study I compared untreated porous coated titanium implants with implants that had been coated with poly (D,L-lactide) (PDLLA) alone, or in combination with two different doses of simvastatin (0.1 mg or 1.0 mg). The PDLLA coating impaired the mechanical fixation and reduced bone formation on the implant surface. Likely due to the delivery vehicle, we were not able to detect any positive or negative effect of simvastatin.

Study II compared a PDLLA coating or poly (lactic-co-glycolic acid) (PLGA) microparticle coating ± 1.0 mg simvastatin with untreated titanium implants. The objective of this study was to confirm that PDLLA as a delivery vehicle impaired implant fixation and to investigate if this was also the case when using a PLGA-based delivery vehicle. Both types of coating impaired implant fixation and reduced bone formation on the implant surface.

In study III we used a 12-week revision model to investigate if zoledronate-impregnated bone allograft could improve early implant fixation and osseointegration of revision implants without impairing new bone formation compared to untreated bone allograft. The treatment resulted in increased mechanical fixation explained by increased preservation of the bone allograft. New bone formation was not impaired.

These studies show the importance of evaluating known materials used for new applications prior to clinical use. Polymers are widely used in the clinical setting for instance as suture materials and orthopaedic devices for fracture fixation. Studies I and II indicate that the lactic acid-based polymers as prepared in this thesis disturbs early implant fixation, and are therefore unsuitable for local drug delivery to the bone-implant interface. Study III shows that local delivery in morselized bone allograft of a drug that is often used as systemic anti-resorptive therapy may work well in the novel application to revision implant fixation and protocolled trials are warranted and needed before implementing this as a standard regimen.

2. Danish summary

Udskiftning af hofteled med hofteproteser (hoftealloplastik) er en succesfuld behandling, der resulterer i forbedring i af patientens smerter, mobilitet og livskvalitet. Den tidlige knogle forankring er afgørende for protesens levetid. Proteser, der ikke forankres i knoglen hurtigt efter isættelse har øget risiko for senere løsning fra knoglen. Den eneste behandling af dette er udskiftning af protesen med en ny, en revisions alloplastik. Revisioner har ofte dårligere prognose og kortere holdbarhed end en primær hoftealloplastik. Ydermere kan kvaliteten af knoglen, hvor revisions protesen skal forankres, være af dårligere kvalitet og mængde. Derfor kan være nødvendigt at anvende donorknogle til at stabilisere revisions protesen. Lokal administration af knogle stimulerende faktorer til knogle-implantat grænsefladen kan være fordelagtigt, hvis administrationsformen ikke påvirker det lokale miljø omkring protesen.
Statiner og bifosfonater er farmaka, der begge påvirker den samme intracellullære signalvej, mevalonat vejen, der er vigtig for dannelse af kolesterol og prenylering af små GTPaser. Begge farmaka kan påvirke knoglerne.
Statinet, simvastatin, et middel mod hyperkolesterolæmi, har i eksperimentelle studier vist at stimulere dannelse af ny knogle og kan ved lokal administration muligvis stimulere dannelse af ny knogle. Derved opnås en hurtigere og bedre osseointegration og forankring af knogle implantater. Bisfosfonatet, zoledronsyre, bruges til at behandle tilstande med øget knogle resorption og eksperimentelle studier har vist at det kan være med til at forhindre resorptionen af donorknogle. Nitrogenholdige bisfosfonater hæmmer enzymet farnesyl purofosfat syntase hvorved prenyleringen proteiner hæmmes og aktiviteten af osteoklasterne nedsættes.

Denne afhandling består af tre studier, der alle er evalueret histologisk og mekanisk. Vi har anvendt en klinisk relevant dyre model til at evaluere forankringen af eksperimentelle ortopædkirurgiske titanium implantater.

Studie I sammenligner ubehandlede titanium implantater med implantater, der er coatet med PDLLA ± simvastatin (0.1 mg eller 1.0 mg). PDLLA coatningen nedsatte den mekaniske forankring og hæmmede dannelsen af knogle på overfladen af implantatet. PDLLA, der blev brugt til lokal administration af simvastatin, medførte at vi ikke kunne konkludere om simvastatin havde positiv eller negativ effekt på forankringen af implantatet.

Studie II sammenligner PDLLA coatede implantater, implantater behandlet med PLGA partikler (± 1.0 mg simvastatin) og ubehandlede titanium implantater. Begge typer coatning nedsatte forankringen af implantatet og hæmmede dannelsen af knogle på implantat overfladen.

I studie III anvendte vi en 12-ugers revision model til at undersøge om zoledronsyre behandling af donorknogle, pakket omkring et revisions implantat kunne forbedre forankringen. Vi fandt at donorknoglen i højere grad blev bevaret og det resulterede i en forbedret stabilitet af protesen sammenlignet med ubehandlet donorknogle. Nydannelsen af knogle omkring implantatet blev ikke hæmmet af zoledronsyre behandlingen.

Overordnet viser de tre studier i denne afhandling, vigtigheden af at teste velkendte behandlinger inden de anvendes til nye indikationer. Polymerer som PDLLA and PLGA anvendes ofte i klinikken som bl.a. suturmaterialer, skruer og andre materialer til stabilisering af frakturer. Studie I og II indikerer, at PDLLA og PLGA, som de er anvendt her, ikke er hensigtsmæssige som leveringsmetode, når det er til knogle-implantat grænsefladen. Studie III viser at zoledronate måske kan anvendes på en ny indikation, men randomiserede kliniske studier skal gennemføres før behandlingen kan implementeres som standard behandling i klinikken.

3. Introduction

Total hip replacement is a successful treatment for degenerative joint-diseases such as osteoarthritis and rheumatoid arthritis and also is an option for treatment of femoral neck fractures. Total hip replacement is a cost-effective treatment to regain patient mobility. In addition, after surgery, quality of life for patients diagnosed with osteoarthritis approximates that of a healthy reference population (1) and the procedure is considered to be one of the most successful orthopaedic interventions (2, 3). Because of the high success rate with this procedure, the number of primary total hip replacements is increasing (4). In addition, younger patients with higher levels of physical activity are being offered this treatment. These factors contribute to higher demands for mechanical load and implant longevity, and therefore both the absolute and relative number of revisions is expected to rise (5). According to the Danish Hip Arthroplasty Register approximately 9,000 primary total hip replacements was performed in Denmark in 2010. Overall, the mean survival rate was reported to be 92% after 10 years and 87% after 15 years (6). The revision rate is even higher in patients younger than 50 years and approximately 20% of the primary total hip replacements in this patient group are revised within 14 years (6). The higher revision rate in the younger patients is partly related to their increased physical activity compared to the elderly patients. For example, high impact activities such as running, skiing or martial arts practice increases wear rate and adversely affects implant survival (7). The treatment of a failed primary total hip replacement is to surgically remove the loose implant and insert a new (revision) implant. Revision implants often have inferior functional outcome, and are complicated by an increased risk of dislocation, infection and a decreased bone quality or loss of bone stock at the implantation site. Besides the complications related to revision implants, they also have higher failure rates than primary implants and the failure rate increases with subsequent revisions (8). For these reasons, it is desirable to avoid revision.

Aseptic loosening is the leading cause of revision (6). Loosening of the prosthesis not only causes severe pain and disability for the patient and thus a major reduction in quality of life, but it is also an economic burden for society with increased costs for surgery, longer hospital stays, care of the patients and lost tax money because of the patient's inability to work.

Implant failure

According to the Danish Hip Arthroplasty Register 57.8% of all revisions from 1998-2010 were because of aseptic loosening, making this the leading cause of revision. The second and third causes were dislocation of the total hip arthroplasty (16.8%) and infection (8.1%) (6).

Aseptic loosening

Aseptic loosening seems to have a multifactorial etiology. Several contributing factors play a complex and not fully understood role in this scenario (9, 10).

Different theories for aseptic loosening have been suggested such as implant micromotion, stress shielding, unsealed bone-implant interface, fatigue failure at the bone-implant interface, inappropriate mechanical load of the prosthesis and low-grade infection. In addition to these theories, patient-related factors may also play an important role in aseptic loosening.

Implant micromotion

Goodman (11) defined micromotion of the implant as: "Small movements between a prosthesis (whether cemented or uncemented) and the surrounding bone, that are not detectable with conventional radiographic methods." Failure for the implant to gain initial stability predicts later aseptic failure (12-15). Early migration can be detected by radiostereometric analysis, which is the most precise method (14). Conventional x-ray is a less sensitive but less expensive method to detect migration of the implant (14). Movement of the implant results in bone resorption in the immediate vicinity of the implant and leads to formation of a fibrous membrane encapsulating the implant and thereby preventing the necessary osseointegration that would stabilize the implant (16). Under

experimental conditions, repetitive displacement of the implant with as little as 150 μm may cause formation of a fibrous membrane preventing osseo-integration of the implant (17).

Fatigue failure at the bone-implant interface

Repetitive loading of the implant may cause mechanical damage to the implant material or the bone-implant interface. This results in accumulation of mechanical damage that long-term may lead to failure of the bone-implant interface resulting in micromotion of the implant, formation of a fibrous membrane and later failure (18).

Stress shielding

Insertion of a prosthesis in the hip leads to new load conditions in the proximal femur. In intact femurs without implants, strain is most pronounced in the upper portion of the femur and decreases progressively towards the diaphysis. This pattern is reversed after insertion of a prosthesis, resulting in less strain at the proximal part of femur, especially at the calcar femorale and increased strain distally with maximum at the tip of the prosthesis (19). The altered loading conditions depend on implant shape and material and can lead to peri-prosthetic bone remodeling with net bone loss in areas with reduced strain. These areas are visible on conventional radiographs as thinning of the bone and decreased density of the cortices (20). As a consequence, the bone-implant interface may become open to joint fluid and wear particles may access.

Effective joint space and wear-particle induced osteolysis

Wear of the articulating prosthesis components generates wear debris particles of different materials and sizes depending on which component is subject to wear (9). The most important source of wear debris seems to be the polyethylene lining of the acetabular component. Wear debris dispersed in joint fluid can be distributed to the bone-implant interface via the effective joint space (21). The effective joint space is determined by how intimate the contact is between prosthesis and bone. The potential space between bone and prosthesis represents the effective joint space (21). When the bone-implant interface is not sealed by sufficient osseointegration of the prosthesis, joint fluid may access the effective joint space and transport wear debris particles to the surface of the implant. The particles may initiate an inflammatory response and activate macrophages, which may result in resorption of the peri-implant bone and thus increase the risk of implant loosening. The peri-implant bone resorption may increase the size of the effective joint space, further facilitating the access of joint fluid and wear particles, which in turn will increase the macrophage activation and bone resorption. A vicious circle!

All the above-mentioned factors may contribute to the process of implant loosening by increased access of joint fluid to the bone-implant interface and increase instability of the implant.

Early osseointegration stabilizes the implant, prevents formation of a fibrous membrane and seals the bone-implant interface, thereby preventing wear debris particles from reaching the bone-implant interface (22).

Implant osseointegration

In 1981 Albrektsson defined implant osseointegration as "a direct -on the light microscopic level- contact between living bone and implant" (23). In the same paper, Albrektsson described six factors important for osseointegration of implants (Table 1). Several prosthesis-related factors influence osseointegration of implants such as biocompatibility of the implant material, geometry and design and surface texture. The implant material should be non-toxic as well as biocompatible and not trigger an inflammatory reaction. In addition, the material should have the necessary strength and endurance to withstand load. The geometry of the implant (femoral stem) is important both in terms of a tight fit between implant and bone to facilitate osseointegration, and in the distribution of stress along the femoral stem. The distribution of stress plays an important role in stress shielding, which is desirable to minimize. Stress shielding may lead to excessive bone loss and compromise long-term implant stability. Furthermore, bone loss may complicate subsequent revision surgery because loss of bone stock.

The technical aspects of insertion of a hip arthroplasty are equally important and related to the skills of the surgeon, surgical

Factors for reliable osseointegration according to Albrektsson et al.	
Implant material (biocompatibility)	Biocompatible Non-toxic Withstand load
Implant design and geometry	Tight fit Distribution of stress
Implant surface	Texture of surface Micro structure Nano structure Facilitate adherence of cells
State of host bone	Healthy bone facilitates osseointegration
Surgical technique	Minimal trauma to the bone
Implant loading conditions	Stable implant Adequate load to secure remodeling

Table 1. Factors important for osseointegration

technique and the stability of the implant. A skilled surgeon combined with a delicate technique and a carefully prepared cavity for the implant provide the most optimal conditions for implant osseointegration with the smallest possible necrotic border zone in the host bone to facilitate early revascularization and bone formation (24). Although implant osseointegration depends on controllable technical- and implant-related factors, patient-related factors must also be taken into account. These factors are not easy to control and include status of the bone in which the implant is inserted and how the patient loads the implant after insertion. The patient may have osteopenia or even osteoporosis at the time of surgery, compromising initial stability of the implant as described by Aro et al. (25). Albrektsson et al. recommended that immediate loading of implants should be avoided until the necrotic border zone in the implant cavity is completely remodeled (23). Although this may seem reasonable, bone remodeling is stimulated by weight bearing of the limb and may enhance implant osseointegration if the implant is stable immediately post-surgery. Woolson et al. confirmed this and reported that bone ingrowth fixation occurred both after partial or full weight bearing immediately after surgery (26). Early weight bearing and mobilization after total hip arthroplasty is a common regimen today and may not only stimulate bone remodeling but also prevent post-operative complications such as thrombosis and pneumonia.

Besides the six factors important for osseointegration reported by Albrektsson et al. other factors influencing osseointegration should be mentioned. Implant wear producing wear debris particles could compromise implant fixation long-term if the bone-implant interface is not sealed. Also both local and systemic drugs may affect implant fixation.

Another important point to consider is that osseointegration is characterized as a direct contact between bone and implant histologically, total (100%) bone contact does not occur immediately after implantation and the initial bone-implant contact may be as little as 10-20% (27).

The delivery and effect of local augments for improving initial implant fixation and osseointegration (of both primary and revision implants) is the main focus of the studies in this dissertation.

Implant endosseous healing

Insertion of an implant -a total hip replacement- inflicts injury to the bone surrounding the implant. Repair of the bone is necessary for osseointegration of the implant to occur. By stimulating bone ingrowth to the implant surface, a sealed interface may be achieved by a tight bond between bone and implant surface thus making the bone-implant interface less susceptible to wear particles and joint fluid (22).

Implant fixation is also dependent on both osteoinduction and osteoconduction. Osteoinduction is a perquisite for osteoconduction and describes the recruitment of primitive, undifferentiated

and pluripotent cells and the stimulation of these cells to develop into the bone-forming linage and ultimately into osteoblasts. Osteoconduction means that bone grows on a surface. The surface can be an implant or bone graft material. The osteoconductivity is also influenced by the properties and chemical composition of the surface (biocompatibility) (28).

Preparing the implant cavity for implantation requires rasping and reaming and causes injury to the bone. Fracture healing and peri-implant healing have many similarities but in the presence of an implant the mode of healing is characterized by intramembranous ossification (29).

The healing and osseointegration of implants consists of several phases. Initially, a hematoma forms at the implantation site followed by an inflammatory phase with recruitment of cells. Then, the reparative phase with formation of woven bone and finally, the remodeling phase where woven bone is remodeled into mature trabecular bone.

A hematoma arises as a consequence of the bleeding caused by preparation of the implant site. A number of growth factors are released at the site such as platelet-derived growth factor, transforming growth factor beta and bone morphogenetic proteins (BMPs). These factors have chemotaxic and osteoinductive properties and aid in the recruitment of cells to initiate bone healing around the implant (30, 31).

In the inflammatory phase activated macrophages secrete cytokines that recruit inflammatory cells. In addition, BMP is secreted. This protein recruits and induces mesenchymal stromal cells to differentiate towards osteoblasts for formation of woven bone (32). Finally, the remodeling phase initiates. Bone remodeling reflects the functional adaption of the bone structure after insertion of an implant. Basic multicellular units resorb the woven bone and replace it with new lamellar bone on the resorbed surface (33).

Bone allograft

Bone allograft was used in study III in the peri-implant gap to stabilize a revision implant. Revision surgery is often complicated by reduced bone stock, which can be compensated for by the use of bone graft. Several types of grafts exist (Table 2) and autograft is regarded the gold standard in bone grafting. This type of bone graft can be harvested from the patient's iliac crest or from the costae. The autograft is completely histocompatible and does not trigger a foreign-body response. The use of autograft also eliminates any potential risks of disease transfer from one patient to another. The disadvantages of autograft include the need for an additional incision, increased blood loss, prolonged time in surgery and the quantity of the bone autograft is limited and may not be sufficient for larger defects. In addition, donor site morbidity and pain is a considerable problem in autograft harvest. Bone allograft is used more frequently than autograft (34). Bone allograft is harvested from a donor either post mortem or in relation to total hip replacements where the femoral head is removed. Although sufficient in quantities, this type of graft may elicit a foreign-body response since the allograft seldom is completely histocompatible. This may trigger an immune response towards the allograft that enhances its resorption (34). Furthermore, there is a risk of infectious

Types of graft	
Autograft	Donor and recipient same individual E.g. bone harvested from the patient
Allograft	Donor and recipient same species but not same individual E.g. bone harvested from another individual than the recipient
Xenograft	Donor and recipient not same species E.g. calcified matrix from bovines
Synthetic graft	Artificial bone Eg. hydroxyapatite, tricalciumphosphate

Table 2. Types of graft.

disease transfer using fresh allograft. Bone allografts can be divided into different types depending on their origin: cortical, cancellous, cortiocancellous or osteochondral. Several events occur during graft incorporation (35):

1. Formation of hematoma and release of cytokines and growth factors.
2. Inflammation, migration and proliferation of mesenchymal cells and development of fibrovascular tissue around the graft.
3. Invasion of vessels into the graft from adjacent tissues.
4. Focal osteoclastic resorption of graft.
5. Bone formation on graft surfaces.

Besides serving as a scaffold for new bone formation the bone allograft has osteoinductive properties because growth factors such as BMPs are incorporated in the graft material (31, 35). Presently, morselized cortiocancellous bone can be used during revision surgery to restore the patient's bone stock, which frequently is of poorer quality than at the primary surgery. The morselized bone graft is tightly impacted to create a stable bed for the revision implant (36). Early stability of the implant facilitates osseointegration by which long-term stability can be obtained. During this period, the bone graft is resorbed and remodeled. The resorption may exceed new bone formation, leading to a net bone loss and potentially transient weakening of the stability and thereby risk of prosthesis failure. Delaying resorption of the bone graft might aid in ensuring early implant stability and improving the long-term outcome of revision hip arthroplasties.

Bisphosphonates are potent anti-resorptive agents used for the treatment of bone diseases having increased bone resorption such as osteoporosis. Several studies have investigated bisphosphonate-treated bone allograft (37-42). These studies conclude that bisphosphonate-treatment of allograft reduces allograft resorption. In canine studies by Jakobsen et al. (38, 41) and Baas et al. (39) the allograft was retained while only the lowest doses of bisphosphonate did not block new bone formation. This was in contrast to the rodent studies (37, 40, 42) where new bone formation was not affected by the bisphosphonate treatment. It seems, that there is a therapeutic window for bisphosphonate-treatment of bone allograft at least in higher order animals, such as the canine. In study III of this dissertation we address the effect of low-dose local bisphosphonate treatment of bone allograft in a canine model of revision that produces an environment and tissue response representative of aseptic implant loosening (43, 44).

Main actors in osseointegration

Three different cell types with distinct functions reside in bone. It is the matrix-producing osteoblast, the matrix-resorbing osteoclast and the osteocyte, trapped in calcified matrix. Remodeling is a task for osteoclasts in collaboration with osteoblasts. Osteoclasts resorbs bone matrix leaving lacunaes that osteoblasts fill with new uncalcified bone matrix, osteoid. Under normal circumstances this process is balanced and adapts to changes in mechanical load and metabolic demand. Osteocytes are fully differentiated osteoblasts embedded in bone matrix. The osteocytes are interconnected with cytoplasmatic extensions enabling them to communicate with each other and also other cell types. They are thought to orchestrate the recruitment of resorptive and formative cells, i.e. remodeling (45).

Osteoblasts

Osteoblasts origin from pluripotent mesenchymal stem cells, which also gives origin to chondrocytes, adipocytes and muscle. Besides being bone-forming cells, osteoblasts and their precursors have an important role in regulating osteoclast differentiation and proliferation. They secrete macrophage colony-stimulation factor (M-CSF), which stimulates osteoclast precursor proliferation (46) and may also stimulate osteoclast-mediated bone resorption (47). In addition, osteoblasts and pre-osteoblastic cells express receptor activator of nuclear factor κB ligand (RANKL) on their surface. RANKL binds to the osteoclast precursor via receptor activator of nuclear factor κB (RANK) and thereby stimulates osteoclastogenesis. To further modulate bone resorption by osteoclasts, osteoblasts secrete osteoprotegerin (OPG), a glycoprotein that functions as a decoy for RANKL preventing RANK from binding to its ligand, RANKL. This results in reduced activity of osteoclasts and reduced bone turnover since osteoclastogenesis is inhibited (48).

Differentiation of mesenchymal stem cells into osteoblasts is regulated by several hormones and cytokines. BMPs promote bone formation by stimulating osteoprogenitor cells to develop into osteoblasts (49). Wnt signaling also seems to play a role in promoting osteoblast differentiation. Sclerostin expressed by osteoblasts, is a Wnt signaling inhibitor suppressing osteoblast proliferation and function (50).

Osteoclasts

Osteoclasts are bone-resorbing cells originating from the hematopoietic progenitor cells. Mature osteoclasts are large multinucleated cells formed by fusion of mononuclear monocytes/macrophages able to resorb bone matrix. Both M-CSF and RANK-RANKL are essential for the development of osteoclasts. While M-CSF stimulate osteoclast precursors to proliferate, RANK-RANKL interaction is crucial for the differentiation, activation and survival of osteoclasts (46). At present, the RANK-RANKL-OPG triad seems to be the most important in regulating bone hemostasis.

Manipulation of osteoblasts and osteoclasts to stimulate osseointegration

Several approaches can be taken in order to improve early osseointegration of orthopaedic implants. The interventions can either be anabolic, stimulating osteoblasts, or anti-catabolic, inhibiting or even killing osteoclasts (51).

Stimulation of osteoblasts results in an accelerated formation of bone. Several growth factors and drugs have been reported to have bone anabolic properties. BMPs are potent growth factors that induce bone formation in vivo by osteoinduction (52). A study investigating the effect of BMP on early implant fixation of an allografted implant reported that although new bone formation was increased, implant fixation was impaired because bone turnover was accelerated which caused increased resorption of the bone allograft and thus reduced stability of the implant (39).

Mundy et al. reported that statins, a group of drugs used for lowering serum cholesterol, enhanced the expression of BMP-2, increased new bone formation *in vitro,* and increased trabecular volume *in vivo* (53). Although simvastatin and other statins have been examined in the context of bone regeneration and fracture healing the effect on implant fixation remains unclear (54, 55-60). Other growth factors that have been shown to stimulate bone formation include but are not limited to transforming growth factor-B (TGF- B), insulin-like growth factor 1 (IGF 1) and fibroblast growth factors (FGFs) (31, 61). Despite new bone formation is the primary goal, stimulating bone formation may lead to increased bone turnover. This may not be advantageous in settings where bone graft provides immediate support for the implant, as the bone graft may be resorbed and lead to a net bone loss thus impairing implant stability as seen in the study by Baas et al. (39). Modulation of Wnt signaling pathways is an additional way to promote osteoblast proliferation and differentiation. This signaling pathway is suppressed by sclerostin, secreted by osteocytes, and by dickkopf-1 that binds to Lrp5/6, a receptor for Wnt. This interaction competitively inhibits Wnt signaling and thus proliferation and differentiation of osteoblasts. By introducing monoclonal antibodies towards sclerostin and dickkopf-1, these inhibitors of Wnt can be blocked resulting in increased osteoblast activity (62).

Another approach to increase peri-implant bone volume and osseointegration is by inhibiting or inactivating osteoclasts. Bisphosphonates (BPs) are anti-resorptive agents with high affinity for bone mineral. They are used for the treatment of osteoporosis and bone diseases with increased resorption. After administration they bind to exposed bone mineral and are internalized by resorbing osteoclasts. BPs inactivate and may induce apoptosis in osteoclasts thereby reducing resorption and resulting in an increase in bone mass density (63). To decrease the differentiation and activation of osteoclasts the RANK-RANKL-OPG triad is a potential target. This is possible either by increasing OPG or decreasing RANK. A study by Zhang et al. suggested that a peri-prosthetic increase in OPG could prevent osteolysis around the implant and thus improve implant fixation (64). While this may seem as a practical approach, OPG may facilitate survival of multiple myeloma cells thereby acting as a cancer survival factor (65). Another option when manipulating the RANK-RANKL-OPG

triad, is to decrease the availability of RANKL thus reducing or eliminating the possibility for RANK to bind to its ligand thereby hindering activation of osteoclasts. A drug with this function has been developed, Denosumab (Prolia®, Amgen Inc, CA, USA). It is a human monoclonal anti-body targeting RANKL, currently used for the treatment of osteoporosis.

In studies I and II of this dissertation we investigate the potential anabolic effect of simvastatin on peri-implant bone and the delivery of simvastatin to the bone-implant interface. In study III we address the bisphosphonate, zoledronate, and its potential anti-catabolic effect in reducing resorption of bone allograft in a revision setting.

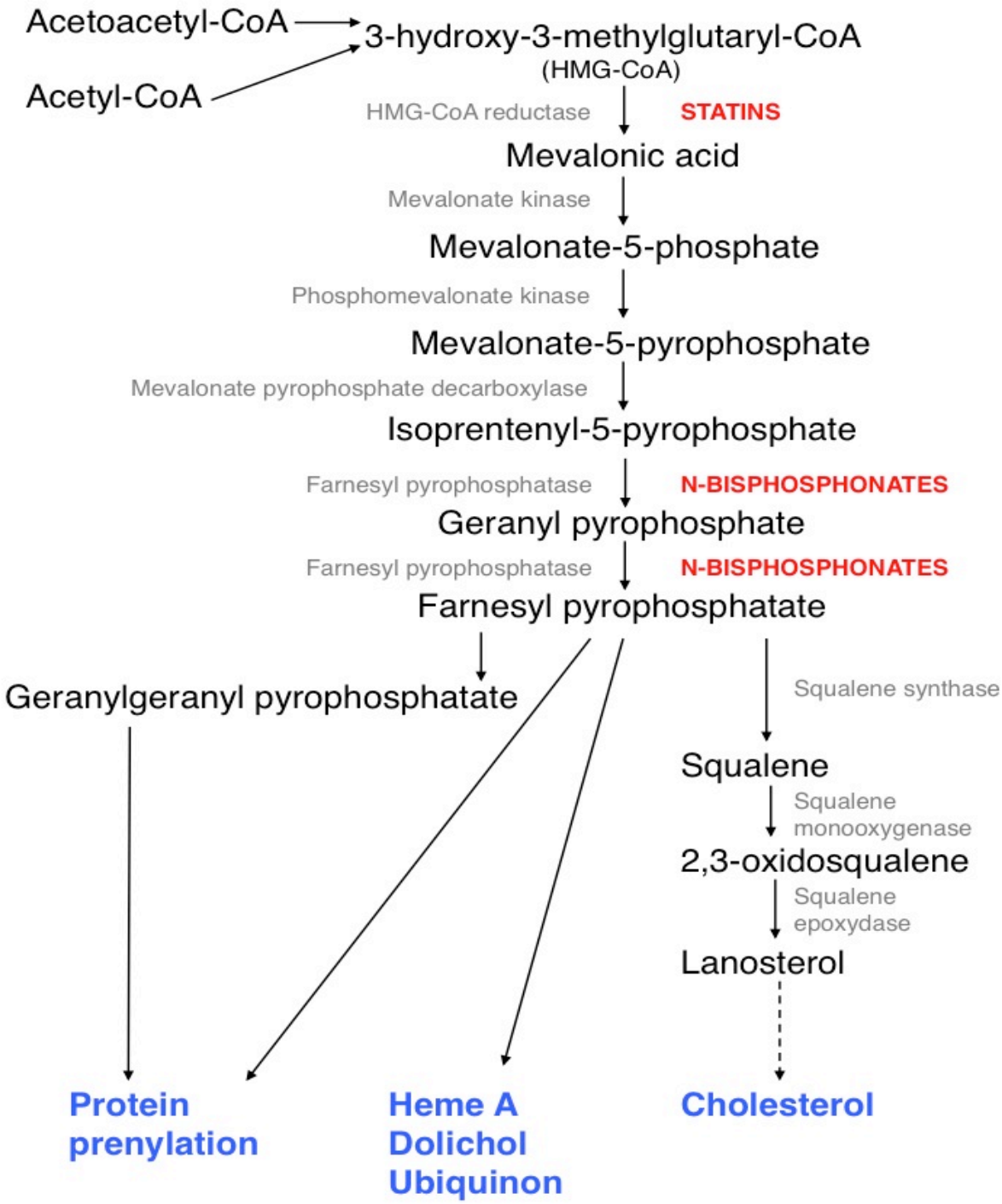


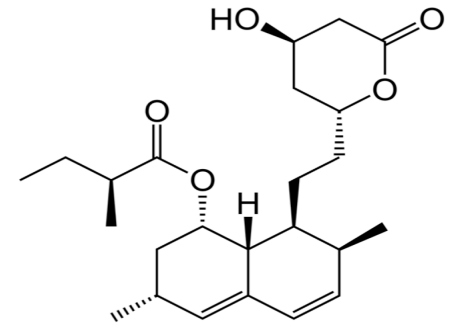
Figure 1. The mevalonate pathway. Adapted from Wikipedia and Buhaescu et al. (66).

The mevalonate pathway

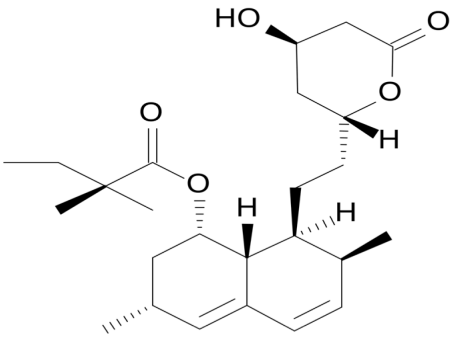
The mevalonate pathway is a pivotal metabolic pathway present in all human cells. It provides the cells with essential molecules involved in cell maintenance, hormone regulation and membrane-protein anchoring. This pathway converts mevalonate into cholesterol, which is a precursor for bile acids, lipoproteins and steroid hormones. In addition, several hydrophobic molecules that play an important role in post-translational modification of proteins crucial for intracellular signaling and are essential in cell growth and differentiation are generated (66) (Figure 1). The metabolic pathway can be manipulated by both statins and nitrogen-containing bisphosphonates both of which inhibit different enzymes in this pathway.

Statins

In the late 1970s Akira Endo identified competitive inhibitors of HMG-CoA reductase derived from the mold *Penecillium citrinum*. Later lovastatin was developed by Merck Research Laboratories and approved for clinical use. Subsequent statins, including simvastatin, were developed (Figure 2). These statins are either semi-synthetic derivatives of lovastatin or completely synthetic products (67).



LOVASTATIN



SIMVASTATIN

Figure 2. Lovastatin and simvastatin.

Conventionally, statins are used for the treatment of hypercholesterolemia or dyslipidemia to reduce elevated levels of plasma cholesterol and benefit in both primary and secondary prevention of coronary heart disease. In recent years, statins have been reported to have pleiotropic effects. In addition to the cholesterol lowering effect, statins are reported to reduce smooth muscle proliferation, a central event in the pathogenesis of vascular lesions; reduce platelet reactivity, limit inflammation associated with atherosclerosis and stabilize atherosclerotic plaques reducing the risk of rupture. Furthermore, statins may have a positive effect on the myocardium and the central nervous system (68). Besides the effects on the cardiovascular and the central nervous system statins may also influence bone by inducing the expression of BMP-2 (53).

Mundy et al. first discovered the statins potential effect on bone (53). They examined more than 30,000 compounds to identify those that activated the BMP-2 gene. In the same study they reported simvastatin and lovastatin to increase bone formation when injected locally over mice calvaria. Later both simvastatin and lovastatin was reported to enhance fracture healing in rodents (56, 69, 70) and Moriyama and colleagues reported local delivery of fluvastatin to enhance bone healing around titanium implants (55).

Statins –pharmacodynamics and pharmacokinetics

Statins are inhibitors of the rate-limiting enzyme in the cholesterol biosynthesis pathway, the mevalonate pathway. They are competitive inhibitors of 3-hydroxy-3-methylglutaryl(HMG)-CoA reductase and inhibit the conversion of HMG-CoA to mevalonic acid. Furthermore, statins up regulate low-density-lipoprotein receptors resulting in increased clearance of cholesterol from the circulation.

Usually statins are administered orally, since they exert their main function in the liver inhibiting endogenous cholesterol synthesis. The delivery of statins to bone depends on their bioavailability. Orally administered simvastatin has a first pass metabolism of 95% resulting in a low bioavailability with only 5% entering the systemic circulation (72). Furthermore, simvastatin is highly bound to plasma proteins rendering the systemic exposure

even lower (72). Since simvastatin does not have a particular affinity for bone, a very small fraction of the administered dose will be available at the bone sites. In order to achieve a higher dose at the bone sites, an extensive dose of simvastatin would have to be administered, increasing the risk for adverse effects such as myopathy or even rhabdomyolysis and asymptomatic increase in hepatic transaminases.

Simvastatin is administered as an inactive pro-drug and must be converted to its active form. Usually this conversion from the inactive lactone form to the active β-hydroxyacid is in the liver by cytochrome p450 3A4 when administered orally. If simvastatin is delivered locally, the hydrolysis of the inactive form occurs at the site of administration.

Bisphosphonates

Bisphosphonates (BPs) are analogs of pyrophosphate that contain a carbon atom instead of an oxygen atom resulting in a P-C-P backbone, resistant to hydrolysis. BPs also have two side chains, both attached to the central carbon atom, R1 and R2 (Figure 3). These side chains determine the affinity to bone mineral and the anti-resorptive properties. R1 substitutes enhance the adsorption to bone mineral, while the R2 substitutes results in differences in anti-resorptive potencies. In general, BPs can be classified as non-nitrogen(N)-containing and N-containing BPs. The N-BPs have a N-containing R2 substitute and are more potent than the non-N BPs (Figure 3).

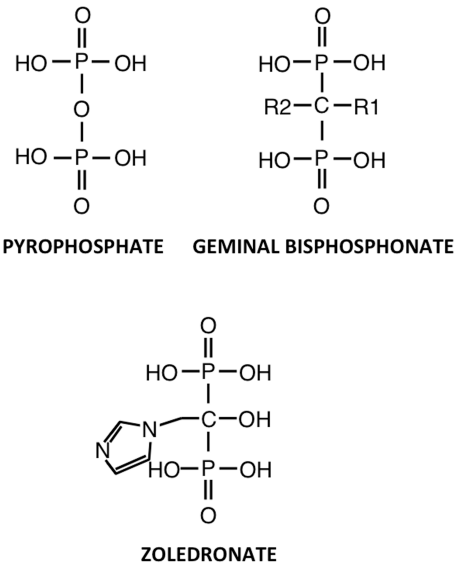


Figure 3. Pyrophosphate, geminal bisphosphonate and zoledronate.

Bisphosphonates -mechanisms of action

Whereas the two types of BPs have different modes of action they both adsorb to the bone mineral surface from where osteoclasts internalize them by endocytosis. BPs are released from the bone mineral surface under the acidic conditions in the resorption pit during osteoclastic resorption. The non-N BPs, the earliest developed and least potent, are incorporated into adenosine triphosphate (ATP) resulting in non-hydrolysable cytotoxic ATP analogs. This inhibits all ATP-dependent processes in the osteoclasts ultimately leading to osteoclast apoptosis. N-BPs interfere with the mevalonate pathway by inhibiting the enzyme farnesyl pyrophosphate synthase. This leads to a reduction in the metabolites required for post-translational lipid-modification (prenylation) of the signaling proteins, GTPases that are essential for cell growth, differentiation, cell survival, vesicular trafficking and organization of the cytoskeleton. The result is impairment of osteoclast function thus reducing the osteoclast mediated bone resorption (63, 73, 74).

In vitro and *in vivo* studies have also suggested an osteoanabolic effect of BPs. Im and colleagues suggested that the two N-BPs, alendronate and risendronate were promoters of osteoblast proliferation and maturation as they detected enhanced gene expression of BMP-2, Type I collagen and osteocalcin in cultures of human osteoblast-likes cells (75). Similar results were later published by von Knoch and colleagues (76) who also reported both alendronate and zoledronate to have anabolic effects on peri-implant bone stock using a rabbit model (77).

Bisphosphonates –pharmacokinetics and effects on bone

Usually BPs are administered either orally or intravenously. Orally administered BPs are poorly absorbed resulting in a low bioavailability of 0.5-2%. BPs are rapidly cleared from the systemic circulation due

to their high affinity to bone mineral and bind preferentially to sites with high bone turnover with exposed hydroxyapatite. About 60% of a single dose of zoledronate is sequestered in bone while the rest excreted un-metabolized in urine. Similar to other growth factors, BPs are incorporated into the bone matrix over time making the skeleton a reservoir for BPs. Bone remodeling will slowly release retained BP back into the systemic circulation from where the drug will either bind to bone mineral at another site or be excreted (74, 78, 79).

As a result of the anti-resorptive function of BPs, osteoclasts are inhibited, resulting in decreased activation of osteoblasts due to their coupling in the basic multicellular unit. This results in decreased bone turnover and over time increased bone density. The decreased bone turnover may lead to accumulation of micro damages of the trabecular bone, which may increase fracture risk (80). Although long-term results with systemic BP treatment seem encouraging (81) and have been suggested to decrease long-term fracture incidence in osteoporotic post-menopausal women (82, 83) recent reports have raised concern that BP therapy may severely suppress bone turnover leading to atypical fractures especially of the proximal femur (84-86). Atypical fractures of the femur seem to be a very rare complication and large clinical trials are needed to clarify this.

Manipulating the mevalonate pathway to improve early implant fixation

Statins

Since Mundy et al. first discovered the effect of simvastatin on bone (53) several studies have been performed investigating the effect of statin treatment on bone. Results from several observational studies are not in concordance whether statins are beneficial in terms of decreased fracture risk and increased bone mineral density (87, 88). Despite these studies could not report any beneficial effect on bone of orally administered statins, Thillemann et al. reported that statins users had decreased revision risk after primary total hip arthroplasty (89). A number of experimental studies have investigated the effect of different statins on implant fixation and fracture healing after oral, parenteral or local treatment with statins. While Oxlund and colleagues reported an increase in vertebral cancellous bone mass and increased compression strength after high-dose oral statin treatment (90), Maritz and colleagues could not confirm these results (91). The inconsistency of orally administered statins to exert an effect on bone may be due to the pharmacokinetic and pharmacodynamic properties of statins and the fact that they do not have inherent affinity for bone. Parenteral or local administration of statins may be an option to circumvent the high first pass metabolism after oral administration. Yin et al. reported subcutaneous injections of simvastatin to promote implant osseointegration in a canine model of total hip arthroplasty (92). Several studies have used local delivery of statins to improve fracture healing (69, 70) and implant osseointegration (55), mainly in rodent models. Provided that simvastatin and other statins can be delivered to the bone-implant interface, these studies suggest that statins may be beneficial in fracture repair and implant osseointegration.

In studies I and II we evaluate two different local delivery vehicles and investigate if simvastatin offers any benefit in early implant fixation.

Bisphosphonates

The anti-resorptive properties of this group of drugs have already shown promising results in the context of total hip arthroplasties and implant fixation with and without bone allograft. Hilding and co-workers reported that oral administration of clodronate, a non N-BP, could reduce migration of total knee replacement components 1 year postoperatively (93). This is in accordance with the results published by Thillemann et al., where they reported long-term users of BPs to have decreased risk of revision of primary hip arthroplasties (94). Kesteris and Aspenberg reported that the bone allograft around revision hip implants was preserved after the bone graft had been treated with BP (95). Several experimental studies have examined the effect of both systemic and local treatment with BPs on bone allograft (37-39, 41, 42, 96, 97). These experimental rodent or canine studies agree that systemic or local BP treatment

prevents resorption of bone allograft. However, despite the preservation of allograft, not all studies report a positive effect on mechanical implant fixation (38, 39). In these studies, they used local treatment of the bone allograft and the authors reported that the BP treatment blocked new bone formation resulting in impaired mechanical implant fixation. Recently, Jakobsen et al. published at dose-response study where they treated bone allograft with different doses of zoledronate (41). At higher doses, bone formation was completely blocked and the allograft preserved. In the low-dose group bone allograft resorption was delayed without impairing new bone formation. Local treatment of bone allograft with BP is a method to selectively inhibit resorption of bone allograft and may work well in the clinical setting provided that the optimal dose and type of BP for local treatment is identified.

In addition to being anti-resorptive several authors have suggested that BPs may also have osteoinductive properties (75, 77). This means that BPs may have the potential to both decrease resorption, while increasing new bone formation thereby retaining the bone allograft until new bone is formed and has secured osseointegration after implantation. von Knoch and colleagues suggested BPs may have their anabolic effect through stimulatory effects during the early stage of osteoblast differentiation during the commitment of the pluripotent mesenchymal stem cell towards the osteoblast linage (76). They also reported an increase in BPM-2 gene expression after treatment with BPs, which is similar to what Mundy et al. observed after treatment with statins (53). The mechanism for the potential anabolic effects of BPs is not yet fully understood but may be similar to statins, as both statins and BPs inhibit enzymes in the mevalonate pathway

In study III we address the effect of local treatment with zoledronate on preservation of bone allograft and its effect on new bone formation. This is done in a revision setting where reduced bone stock is present and bone allograft is used to stabilize the revision implant. The dose-response study by Jakobsen et al. formed the basis for our choice of zoledronate dose.

Polymer drug delivery to the bone-implant interface

Synthetic degradable polymers, including the polymers PDLLA and PLGA, are widely used in the clinical setting for example as suture materials and fracture fixation devices. These polymers are large molecules, that consist of chains of smaller repeating units such as lactic and glycolic acid. These polymers, alone or in combination, can be used to coat implant surfaces, thus the implant serves as a joint replacement or a fracture fixation device and a local drug delivery system to stimulate implant osseointegration or fracture healing.

The polymer, PDLLA consist of lactic acid linked in chains. Lactic acid is a chiral molecule and exists in two isomers, D-lactic acid and L-lactic acid and PDLLA is a mixture of the two isomers. PDLLA is mainly degraded by hydrolysis and the released lactic acid is eliminated via the citric acid cycle and is ultimately excreted by the lungs as carbon dioxide (98).

PLGA has a similar structure to PDLLA, but is made of lactic acid and glycolic acid. The degradation is also by hydrolysis resulting in lactic acid and glycolic acid. The lactic acid is eliminated as described for PDLLA, while the glycolic acid either excreted by the kidneys or is first transformed into glycoxylate by glycolate oxidase and the reacts with glycine transaminase to form glycine. Glycine may take part in protein synthesis or the citric acid cycle (98). Ideally these coatings are present initially to deliver augments to the bone-implant interface while they are degraded and ultimately completely eliminated.

Overall, lactic acid based polymers are reported to have acceptable biocompatibility (99-101) and several authors have reported that these polymers have osteogenic potential (102, 103). Various degrees of foreign-body reactions and osteolysis around implants made of lactic-acid-based polymers have been described (104-106). During hydrolytic degradation of lactic-acid-based polymers, lactic acid is released. The capacity to eliminate degradation products may depend on the vascularization and metabolic activity of the peri-implant tissue, and the amount and chemical composition of the degradation products (98). Due to the relatively closed environment of the effective joint space,

this may cause a drop in local pH if the fluid exchange (vascularization) is not sufficient and the produced acid exceeds the buffer capacity of the local host tissue. The result may be a disturbance in the normal metabolic activity and bone formation during implant osseointegration.

For study I we chose a PDLLA coating of the implants as our simvastatin delivery vehicle because positive results using this coating as local drug delivery vehicle has been reported (56, 103). For study II we used a new PLGA-based coating to investigate if this formulation would be appropriate for delivery of simvastatin to the bone-implant interface and to assess if this coating would have any positive or negative effect on peri-implant bone formation.

4. Aims of the studies

The overall aim of the studies included in this thesis is to contribute to an increase in the longevity of both primary total hip replacements and revision hip replacements thus reducing the risk of one or several revision hip arthroplasties. The goal is that these studies contribute to the knowledge of what compounds may stimulate the bone-implant interface to a swift and stable osseointegration and how these compounds may be delivered to the bone-implant interface.
More specific, in this thesis we focus on two compounds that interfere with the mevalonate pathway and the delivery of these compounds to the bone-implant interface. In studies I and II we evaluate different delivery vehicles for simvastatin to the bone implant interface and assess if simvastatin may be beneficial in orthopaedic implant fixation. In study III we investigate local treatment with zoledronate in the setting of a revision implant where bone allograft in needed to restore bone stock.

Hypotheses for the studies

Study I
PDLLA coated onto experimental orthopaedic implants will not influence early implant fixation and simvastatin released from this coating will stimulate bone formation and enhance early implant fixation.

Study II
Implants coated with either a PDLLA coating or PLGA microparticles will not negatively influence early implant fixation and simvastatin delivered in PLGA microparticles will improve implant fixation compared to untreated titanium implants and implants coated with empty PLGA microparticles.

Study III
Zoledronate-treatment of bone allograft impacted around stable loaded revision implants will retain the graft without impairing new bone formation thus improving early implant fixation

All three studies were conducted within a paired study design and with non-loaded implants in studies I and II and loaded implants in study III. Implants were inserted into the proximal humerus (studies I and II) or the distal femur (study III).
The studies were evaluated mechanically and histologically. Mechanical implant fixation was assessed by mechanical push-out test to failure while quantitative histomorphometry was used to assess implant osseointegration, gap healing and ongrowth at the light-microscopic level. All analyses were performed blinded.

5. Methodological considerations

Experimental model

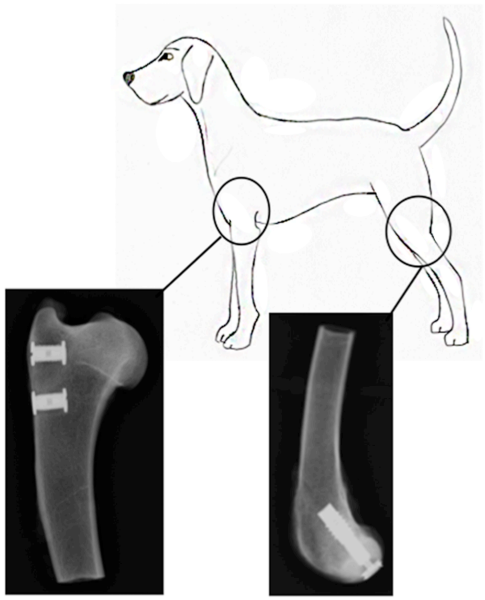


Figure 4. Implant positions.
Proximal humerus and distal femur.

Study design

All three studies were conducted as experimental animal studies with canines as our experimental animal. The studies were designed as paired studies, where the animal serves as its own control. This paired design reduces the inter-animal variation and strengthens the statistical power enabling us reduce the number of animals included in the studies. Studies I and II were both four-armed studies with non-loaded implants situated in the proximal humerus, while study III was a two-armed study with weight-bearing implants in the distal femur (Figure 4). Implantation sites were alternated systematically between left and right limb with a random start. In study I two groups included simvastatin and these implants were always on the same side to avoid contamination of the non-simvastatin groups but the proximal and distal position was alternated bilaterally. In study II implantation was alternated from left to right and in neighbourship on each side to avoid bias from potential contamination from the treatment of the neighboring implants.
To conserve animal resources, unrelated studies were performed simultaneously in the animals (Table 3). Study I was conducted in the first experimental series while studies II and III both were performed in the second experimental series. The unrelated studies included in these series all evaluated different local treatments as their intervention. Blood samples from the tobramycin study in the first experimental series documented that the systemic distribution of tobramycin was less than we were able to detect using a standard lab analysis. No systemic measurements on the bisphosphonate in the first series were preformed, however, because BPs have high affinity to bone mineral we estimated that the systemic distribution was very limited. Overall, we estimated the potential influences from the additional studies included in the two series to be negligible.

Experimental animals

Animal experiments are important in developing new treatments and are an important link between *in vitro* studies and clinical trails on humans. To gain detailed knowledge on mechanisms and tissue response, new treatments should be evaluated in clinically relevant animal models prior to clinical testing of the treatments. If pre-clinical evaluation of new treatments and devices could be performed in cell- or tissue cultures the use of experimental animals could be limited substantially. However, it is not possible to create a laboratory setting that imitates the complex mechanisms of a live organism and thus it is necessary to use experimental animals in order to estimate the exact interaction between implant and bone. This can be done using an animal model that provides a standardized setting with high reproducibility.
Several species can be used for orthopaedic implant research such as rodents, canines, sheep and non-human primates. Despite rat bone has low resemblance to human bone, the rat is the most commonly used animal in bone research (107). This is due to several practical advantages such as availability, low costs and easy accommodation and care. Larger animals such as sheep, dogs and non-human primates have larger bones that allow evaluation of larger implants. This allows extensive mechanical and histomorphometrical

analysis of the samples. Especially canines, and to some extent sheep, have bone structure and bone quality that resembles human bone in regards to bone mineral density, biochemical composition and mechanical endurance. Although canine bone remodeling occurs at a faster rate, skeletally mature canine bones undergo remodeling similar to humans (108, 109).
We chose canines as our experimental animal because of their skeletal resemblance to humans. This species along with non-human primates are regarded the most optimal experimental animals for orthopaedic research (108, 109). Although the animals were skeletally mature, they were healthy and had healthy bone, which may not reflect the bone quality of most elderly orthopaedic patients. These patients are expected to have lower bone quality and their response to a treatment may not be as pronounced as in younger healthy canines. Shaw and colleagues reported a good potential for bone ingrowth in canines independent of ovarian function. Furthermore, they reported that female dogs and elderly (postmenopausal) non-human primates appeared to be equivalent animal models for bone growth into porous titanium surfaces (108).
In addition, canines have several advantages. The animals are easy to handle and implantation sites are easy accessible with minimal invasive surgery causing minimal trauma to the animal. The cancellous bone sites at the proximal humeri and the distal femora allow implantation of two implants bilaterally making it possible to conduct four-armed studies. According to the past experience with sheep in our research group, only one implant can be inserted bilaterally in the proximal humeri eliminating the possibility for a four-armed study within the paired design.
Disadvantages with canines include their availability, costs and ethical issues because these animals are used as pets.
We consider the canine model used for the present studies a valid animal model for investigating orthopaedic implant fixation. All animals included for the studies in this thesis, were raised for scientific purposes. The local Animal Care and Use Committee, Minneapolis Medical Research Foundation, Minneapolis, MN, USA approved and monitored all studies

Sample size

The number of animals needed for the studies in this thesis was based on sample size estimations for paired study groups. We performed the calculations in order to ensure sufficient number of animals to detect the minimal relevant difference between the study groups. The sample size estimation was also performed for ethical reasons to prevent excess animals from being included in the studies. The sample size calculations was based on the following equation:

$$n \geq (u + v)^2 \, x \, \frac{CV_{diff}{}^2}{\Delta^2}$$

n is the number of animals to be included
u is the one-sided percentage point of the normal distribution corresponding to the power of the studies. This was set to 80% yielding $u = 0.876$ (from t-table).
v is the two-sided significance level and was set to 0.05 yielding v = 2.201 (from t-table).
CV_{diff} is the coefficient of variance of the paired difference and Δ the minimal relative difference to be detected. Both values were set to 30%.

$$n \geq (0.876 + 2.201)^2 \, x \, \frac{0.3^2}{0.3^2} \approx 9.5$$

	Experimental series 1	**Experimental series 2**
Humerus	Study I	Study II
Femur	Tobramycin study Periost study	Study III
Tibia	Bisphosphonate and cement study	Allograft wash study

Table 3. Studies included in experimental series 1 and 2.

The assumptions for this sample size estimation were based on previous studies using the same models and same endpoints (110-112).
Ten animals were needed for each experimental series. Two additional animals were added to the estimated sample size to counteract loss of power in case animals or implants were lost for follow-up and subsequent analysis. The bone allograft used in study III was harvested from animals that had been included in non-orthopaedic studies and the animals were not included in the sample size estimation.

Model for early implant fixation

The model

The implant models used for the studies in this thesis are based on the models developed by Professor Kjeld Søballe and Professor Joan E. Bechtold (43, 44, 113). We used two different types of models. For studies I and II we used a non-loaded implant model with two implants placed in each proximal humerus. The implants were cylindrical with a diameter of 6 mm and a height of 10 mm. On each end an end washer of 8 mm diameter was mounted. Implants were inserted into 8-mm drill holes of 12 mm depth. End washers secured central placement of the implants in the drill hole and a uniform concentric 1-mm defect around the implants. The defect was left empty.
In study III we employed a two-step surgical revision protocol in the distal femur with unstable primary implants that were revised and replaced by stable revision implants. This model will be described in detail later.
Both models are designed to study early implant fixation and osseointegration of an uncemented implant component in the setting of primary (studies I and II) and revision implants (study III). To standardize conditions and ensure high reproducibility we used simple basic models where implants are not functional arthroplasties, but implants with a simple cylindrical shape. In addition, implants used studies I and II were subject to indirect load and not direct axial load and the bone-implant interface was not affected by oscillating joint fluid pressure. The revision model used in study III has additional limitations. Even though the implant is subjected to direct axial load and oscillating joint fluid pressure, the revision cavity is created over a short period of time (8 weeks) compared to clinical implants with aseptic loosening. Furthermore, the revision model does not reflect the entire spectrum of revision settings encountered by orthopaedic revision surgeons. However, this experimental revision protocol does produce an environment and tissue response representative of aseptic loosening and represent the mechanical conditions at a clinical-bone-implant interface and the intra-articular loading conditions that implants can undergo (43, 44).
Besides the design of the implant model, choice of experimental animals also influences interpretation of results. As mentioned, we chose canines because their bone structure and quality resembles human bone (107). In addition to this, canines have a higher bone turnover than humans and the animals we used were young and healthy with no degenerative conditions or fractures in their bones. This is in contrast to most human recipients of joint replacements who may have poor bone quality.
The simplifications in these models make them highly reproducible, minimizing the variability in the studies. Furthermore, the revision model that is more complex than the model used for studies I and II produces an environment and tissue response representative of aseptic implant loosening and the conditions represent the mechanical conditions at a clinical bone-implant interface and the intraarticular loading conditions implants can undergo (43, 44).

Observation period

The overall purpose of these studies was to improve early implant fixation both in a primary implant setting and in a revision setting. As mentioned earlier early implant stability is crucial for the long-term survival of the implant. Several clinical RSA studies have reported that early subsidence of the implant predicts late implant loosening (12, 13). The studies included in this thesis all investigated early implant fixation and thus a relatively short observation period of 4 weeks was chosen. Had we chosen a shorter period, interventions may not have had enough

time to exert an effect, and a with longer observation period, differences between the groups might have leveled out, and it would not have been possible to detect any early improvement or impairment of implant fixation. The choice of observation period was based on previous studies using similar models (61, 112).

Surgical procedure

All surgeries were performed with the animals under general anesthesia and under sterile conditions. After implant placement and hemostasis the incisions were closed in layers. Preoperatively and three days postoperatively 1 g of ceftriaxone was administered. As postoperative analgesic treatment 2-3 mL of bupivacaine, 0.5% was administered at the incision sites and a fentanyl transdermal patch (75 μg/hour) was applied immediately post surgery. After completion of the observation period the animals were sedated with acepromazine 0.5 mg/kg, anaesthetized with proporfol, 4 mg/kg, and euthanized with an overdose of hypersaturated barbiturate (Beuthanasia-D Special, Shering-Plough Animal Health Corp., Union, NJ).

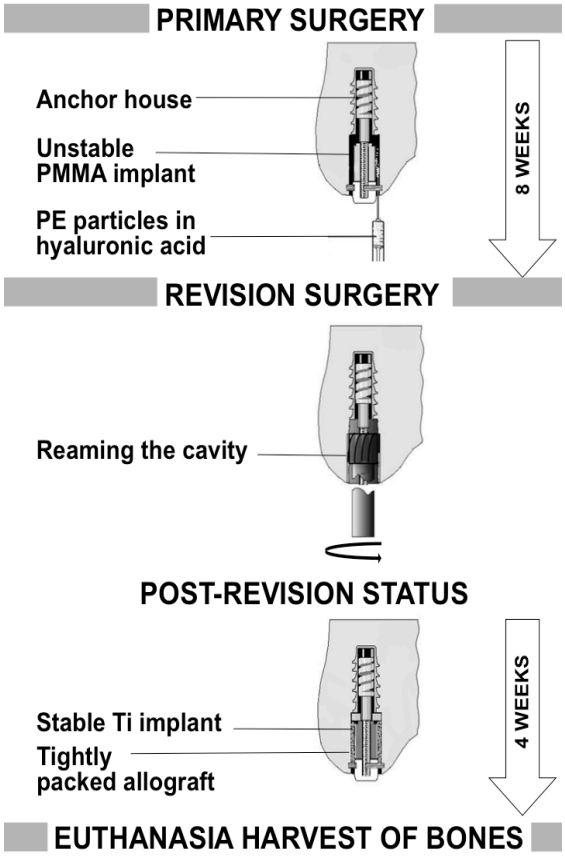


Figure 5. Overview of the revision protocol.

The surgery protocol for studies I and II were identical while the surgery for study III followed our two-step revision protocol.

All surgeries were conducted at Excelen Center for Bone and Joint Research and education (formerly Midwest Orthopaedic Research Foundation), Hennepin County Medical Center, Minneapolis, MN, USA and all studies were approved and monitored by the local Animal Care and Use Committee, Minneapolis Medical Research Foundation, Minneapolis, MN, USA. NIH guidelines for the care and use of laboratory animals were observed (NIH publication #85-23 Rev. 1985).

Surgery protocol, studies I and II

Implants were inserted into cancellous bone in the proximal humerus. A skin incision was made on the lateral proximal humerus and the bone was accessed through blunt dissection. We inserted a 2.1-mm guide wire perpendicular to the surface antero-laterally and distal to the insertion of the supraspinatus muscle. 17 mm distal and parallel to the first guide wire, we inserted another 2.1-mm guide wire. Over each wire, an 8-mm cannulated drill bit was used to drill two 12-mm deep cavities. To avoid thermal damage to the surrounding bone, the maximum speed of the drill was two rotations per second. Using a scalpel, the edge of each cavity was trimmed removing excessive periosteum. Afterwards, each cavity was irrigated with 10 ml sterile saline for removal of loose bone chips and the implants with both endplates were inserted into the drill holes. Finally, hemostasis was secured and the soft tissue closed in layers. The same procedure was repeated for the opposite side. The same surgeon inserted all implants in both studies.

Revision protocol, study III

The revision protocol was initiated at the first (primary) surgery. This protocol uses a pistoning micromotion device inserted into the medial condyle of each distal femur. A PMMA implant, placed in an overdrilled cavity, was attached to the micromotion device and mimics a loose cement mantle. PE particles applied in the implant-bone gap represent wear debris. A PE plug attached to the piston articulates with the tibial plateau, thereby inducing controlled micromotion of the

intra-articular PMMA implant with each gait cycle (500 μm) (Figure 5).
A 2.1-mm guide wire was placed in the central portion of the medial condyle perpendicular to the weight bearing articulating surface, with the knee in full flexion. A 30-mm deep cavity was made using a cannulated step drill, thereby creating a superficial cavity with a diameter of 7.5 mm and a depth of 20 mm and a deeper cavity 6 mm diameter and 10 mm depth. The outermost 3 mm of the superficial cavity was tapped for later placement of a subcortical centralizing ring. The anchor section of the micromotion device was placed in the deep cavity. The centralizing ring was screwed into the subchondral bone of the superficial cavity, and the PMMA implant was mounted onto the piston of the micromotion device. The internal spring of the micromotion device controlled the movement of the PMMA implant. During knee loading, the implant was displaced 500 ± 15 μm in axial direction, and when unloaded, the implant pushed back to its initial position by the internal spring. The 0.75 mm concentric gap surrounding the implant was filled with polyethylene particles (0.5-50 μm; 0.5 x 10E8 particles; 85% less than 12μm) administered in 0.2 ml hyaluronic acid (Lifecore Biomedical, Minneapolis, MN, USA). Finally, a PE plug was mounted onto the distal portion of the threaded piston superficial to the PMMA implant. This PE plug articulated with the proximal tibia, thereby providing input force during knee motion. This procedure was repeated for both knees.
Eight weeks after the primary surgery, the second (revision) surgery was performed using the same approach. At the revision, the PMMA implant and the centralizing ring were removed and the fibrous membrane was meticulously cleared with a curette. Then the superficial cavity was reamed with an 8.2-mm cannulated reamer, removing the neocortex. A new thread for a new revision-centralizing ring was tapped, and the cavity irrigated with saline. A cylindrical porous coated revision implant was screwed onto the piston. This revision implant incorporates a flange that prevents further micromotion and ensures stability. Bone allograft from the control or intervention group was impacted in the reamed 1.1-mm gap surrounding the implant. The revision-centralizing ring was mounted, and finally, the revision polyethylene plug (0.25mm shorter than the primary plug) was mounted on the distal part of the piston and manually adjusted to minimal protrusion into the joint space to secure loading during each gait cycle.

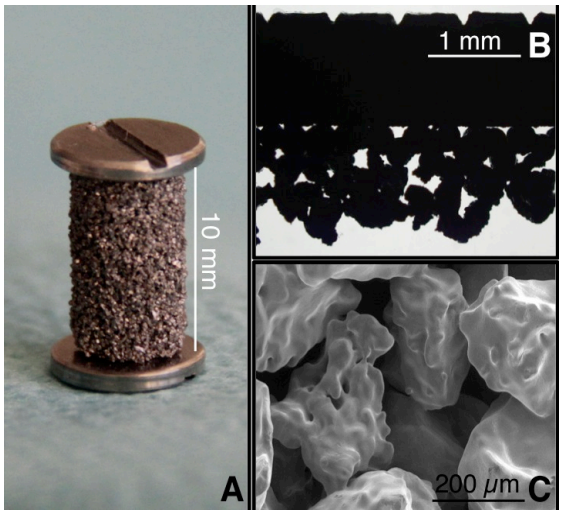


Figure 6. Porous coating of implant. **A:** macroscopic structure. **B:** Light microscopic profile of porous coating. Magnification x4. **C:** SEM of surface. Magnification x400.

Implant specifications

All implants used for the studies in this thesis consisted of a solid titanium alloy (Ti6A14V) core with an additional porous coating. The porous coating consisted of an inner layer of spherical unalloyed titanium beads and an outer layer of non-spherical unalloyed titanium powder (150-300 μm) (Figure 6). These layers were sintered onto the surface of the solid core. The porous coating had an average volume porosity of 64 % ±3 % (114). The final dimensions of the implants were about 6 mm diameter (Table 4) and 10 mm height.
The porous coating of the implants was donated by DePuy, Inc., Warsaw, IN, USA and is similar to commercially available porous coatings of orthopaedic implants.

Study	Diameter (mm)		Height (mm)	
	Mean	(sd)	Mean	(sd)
I	5.85	0.16	3.35	0.12
II	5.77	0.09	3.14	0.15
III	5.85	0.16	3.04	0.05

Table 4. Diameter and height of mechanical specimens [mean (sd)].

Groups	Study I	Study II	Study III
1	Untreated titanium	Untreated titanium	Saline treated allograft (control)
2	PDLLA empty	PDLLA empty	Zoledronate soaked allograft
3	PDLLA + simvastatin 0.1 mg	PLGA microparticles empty	-
4	PDLLA + simvastatin 1.0 mg	PLGA microparticles + 1.0 mg simvastatin	-

Table 5. Treatment groups included in studies I, II and III.

The porous coating was not the subject investigated in this thesis. The surface merely served as a substrate for other interventions. In study I, three of the four groups had an additional surface coating with PDLLA ± simvastatin 0.1 mg or 1.0 mg. In study II, three of the four implant groups also had additional coating, one with an empty PDLLA coating and two with PLGA microspheres ± simvastatin 1.0 mg. In study III, there was no additional surface coating of the implants (Table 5).
Although the cylindrical shape of the implants is a major simplification of clinical implant surgery, the surgery for these implants is simple, uncomplicated and highly reproducible. Even though the surgery required for insertion of the primary and revision implants in study III is more complex compared to the surgery needed for studies I and II, the model still a simplified version of clinical revision surgery and offers the same advantages for the trained surgeon as the model used in studies I and II. The cylindrical design of the implants also enables an easy, standardized and reproducible sectioning of the implants into well-defined bits for mechanical test and histomorphometry (Figure 7).

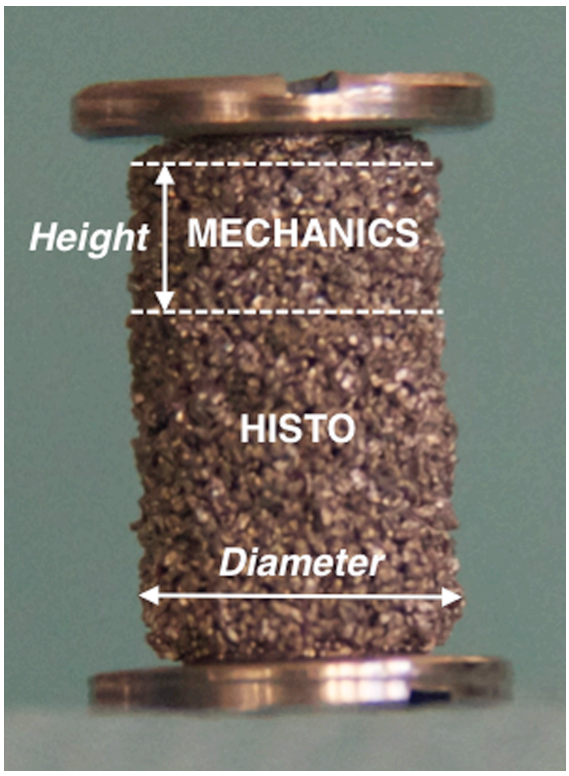


Figure 7. Sectioning of implants for mechanical and histomorphometrical evaluation.

Additional surface coating, studies I and II

Study I
Three quarters of the 48 implants were additionally coated with PDLLA Resomer 203 (Bhoringer Ingelheim GmbH, Ingelheim am Rhein Germany) ± simvastatin (Teva Denmark A/S, Kgs. Lyngby, Denmark). We used the protocol previously described by Schmidmaier et al. to coat experimental implants in orthopaedic applications (115).
PDLLA ± simvastatin was dissolved in volatile solvent, chloroform, at room temperature resulting in either an empty PDLLA coating containing no simvastatin or a PDLLA coating containing approximately 0.1 mg or 1.0 mg simvastatin.
The implants were dipped twice in the coating solution and dried overnight under laminar airflow conditions. The coating procedure was performed under sterile conditions. The amount of PDLLA coated onto the implants was determined with an electronic balance (AG 204 DeltaRange, Mettler Toledo, Greifensee, Switzerland). The average weight of the coating was 8.76 mg (range 8.37- 9.2 mg) (Figure 8).
Release of simvastatin was confirmed by in vitro release studies.

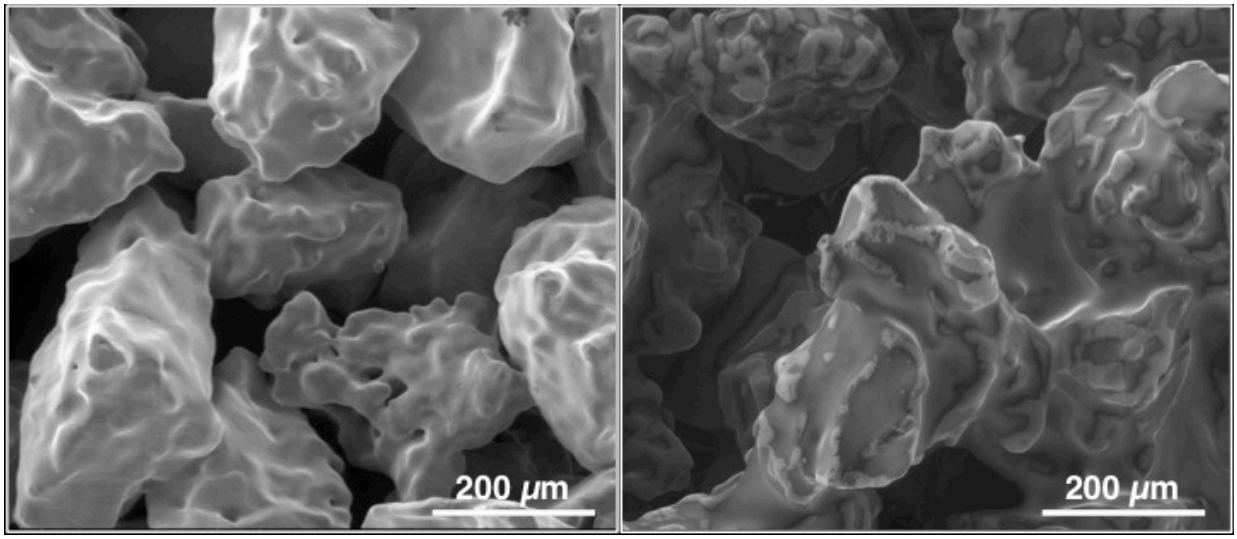


Figure 8. SEM of implant surfaces from study I. **Left:** Untreated. **Right:** Coated with PDLLA ± simvastatin. There was no visual difference between the coated groups (PDLLA ± simvastatin 0.1 mg or 1.0 mg).

The remaining quarter of the implants were untreated and served as controls. Please see table 5 for groups included in study I.

Study II

Half of the implants used in this study were coated with a PLGA microparticle formulation ± 1.0 mg simvastatin. The coating procedure was as follows. The PLGA microparticles with simvastatin was prepared by dissolving 700 mg PLGA and 70 mg simvastatin in 6 ml dichlormethane. Then 1 % polyvinyl alcohol was added and the mixture was homogenized for one minute using a PRO Scientific Homogenizer (Oxford, CT, USA). The emulsion was poured into 75 ml 0.3 % polyvinyl alcohol solution and stirred for 40 minutes. The drug-loaded microparticles were collected by centrifugation at 4000 rpm for 5 minutes and washed with water three times. Finally, the microparticles were re-suspended in 15 ml water and lyophilized. The empty microparticles were prepared using an identical approach, except simvastatin was not included. To assess the simvastatin loading of the microparticles 10.8 mg simvastatin/PLGA microparticles were dissolved in 100 ml acetonitrile and the ultraviolet absorption at 240 nm was measured. PLGA and acetonitrile solution was used as blank and a standard curve was established using a series of standard simvastatin solutions. The drug loading was determined to be 8.99 %. Prior to coating the coating procedure the titanium implants were treated with 30µl 100 mg/ml polyvinlypyrrolidon (K30) in isopropyl alcohol. After 10 minutes, 30µl aqueous suspension containing either 11 mg empty PLGA microparticles or 11 mg simvastatin/PLGA microparticles was spot coated on the implant using a pipette. After 30 minutes of air-drying, 30 µl Lubricent 475 (ISurTec, St. Paul, MN, USA) was applied as a topcoat. The coating was dried for an additionally 20 minutes followed by 5 minutes of ultraviolet irradiation (Harland Medical UVM400, Eden Praire, MN, USA). The distance from the light source to the implants was 12 cm. Finally, the coated implants were individually packed and EtO sterilized. IsurTec (St. Paul, MN, USA) donated the microparticle coating of the implants.

An additional quarter of the implants were coated with PDLLA as described above for study I. The remaining quarter of the implants were untreated and served as controls (Figure 9). Please see table 5 for groups included in study II.

Characterization of surface coatings

To characterize the coatings used for studies I and II scanning electron microscopy of the uncoated implant surface and the coated surfaces was done. We also performed *in vitro* release studies to verify that simvastatin was indeed released from the coating and finally, we assessed peri-implant pH for untreated and coated implants in an *in vitro* model.

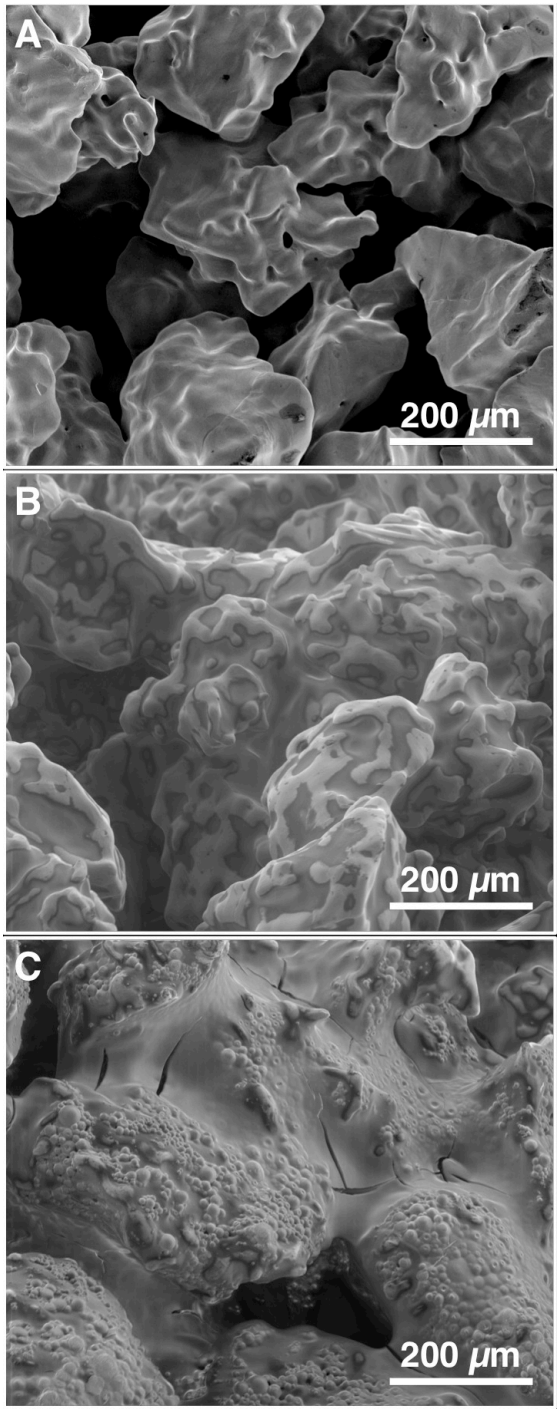


Figure 9. SEM of implant surfaces from study II. A: Untreated. B: PDLLA coated. C: PLGA-microparticle coated. There was no visual difference between thr PLGA-microparticle coating ± simvastatin.

Scanning electron microscopy

To visualize the implant surface we used a scanning electron microscope (Nova NanoSEM 600, FEI Company, Hillsboro, OR, USA). The uncoated implant surface was highly porous and covered with irregular shaped particles. At several locations the base layer consisting of spherical beads could be visualized. The particles on the surface of the PDLLA coated implants appeared to be less irregular and the surface seemed to have lost some of its porosity because of the coating. We could not see any difference between the implants coated with only PDLLA and implants coated with PDLLA and simvastatin (0.1 mg or 1.0 mg) (Figure 8). Implants coated with the PLGA-microparticle formulation had a more pronounced loss of irregular surface structure and porosity in the coated areas. In areas without coating, the implant surface was identical to the surface of the uncoated implants. There was no visual difference of the PLGA-microparticle coating with or without simvastatin (Figure 9).

In vitro release studies

Implants from all groups included in study I and II were used for *in vitro* release. We used three implants from each group and used the average of the measurements for the subsequent calculations. Prior to the release studies a standard curve was made from a stock solution with simvastatin (Figure 10).

Implants were submerged in PBS (study I: 7mL; study II: 8 mL) and placed at 37 °C while rotated horizontally. At several time points 10 % of the PBS (pH = 7.2) was removed and immediately replaced with fresh PBS. On each sample the absorbance was measured three times at 238nm (116) with a spectrophotometer (CE 2012, 2000 Series, Cecil Instruments Ltd., Cambridge, England) and an average absorbance was calculated. Coatings without simvastatin were used as blanks to omit interference form the polymers used and the spectrophotometer was reset at the absorbance measured for the empty coating. Using the average absorbance and the standard curve the amount of drug released was estimated.

From the PDLLA coating, simvastatin was released with an initial burst. Implants coated with PDLLA + 0.1 mg simvastatin had a faster release than implants coated with PDLLA + 1.0 mg simvastatin (Figure 11). A similar release pattern was seen for the PLGA microparticle + simvastatin coated implants (Figure 12). We did not include the PLGA microparticle + 0.1 mg simvastatin in the experimental series.

Simvastatin was released from the coatings during the in vitro release study. The coatings with 1.0 mg simvastatin did not release the drug 100 % during the observation period.

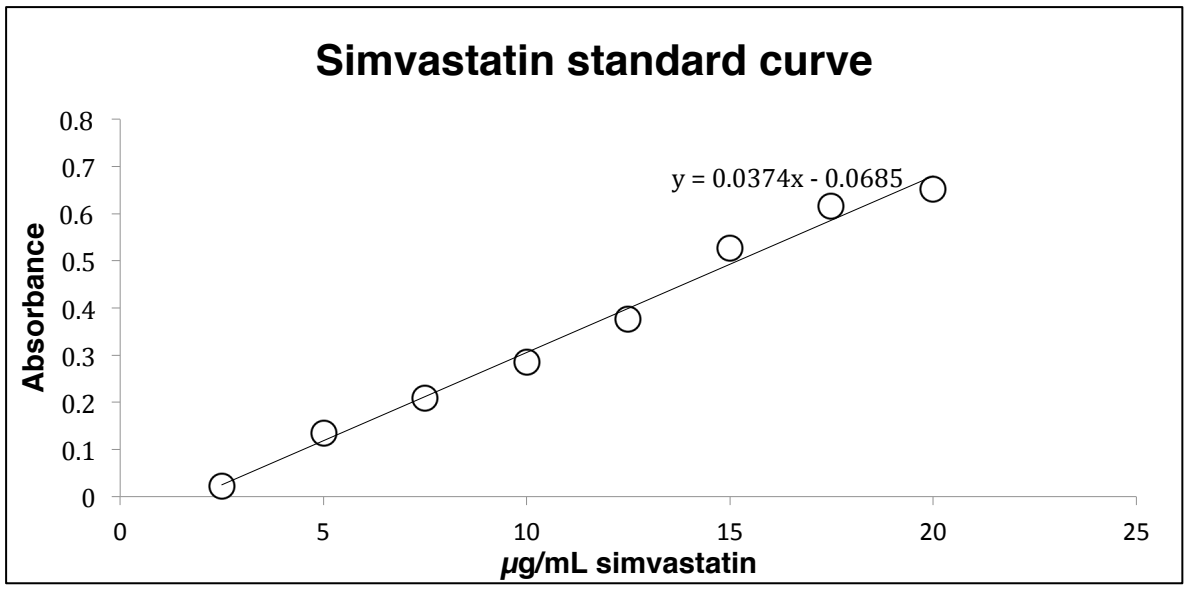


Figure 10. Standard curve for simvastatin measured at 238 nm.

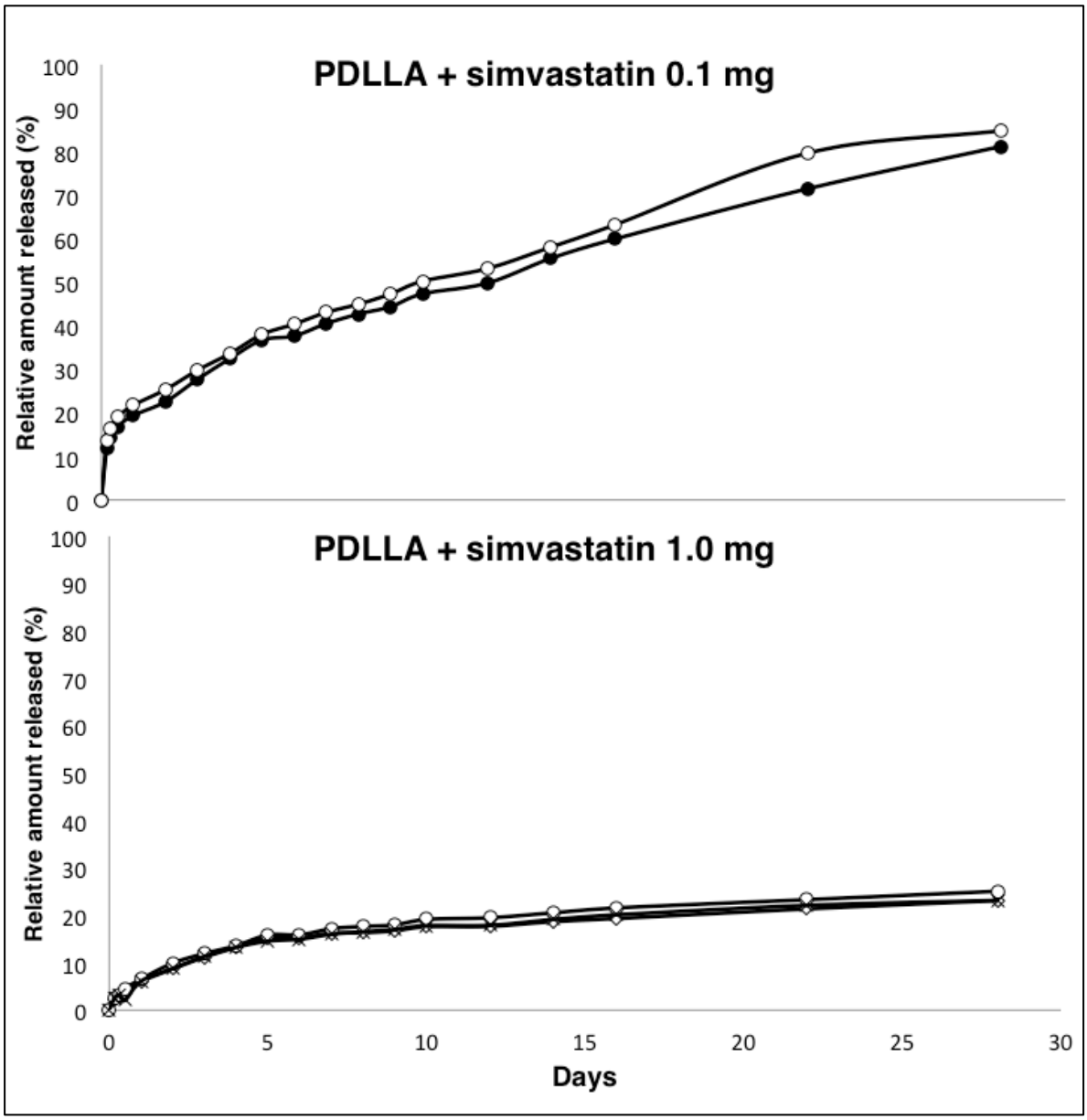


Figure 11. *In vitro* release from the PDLLA coating.

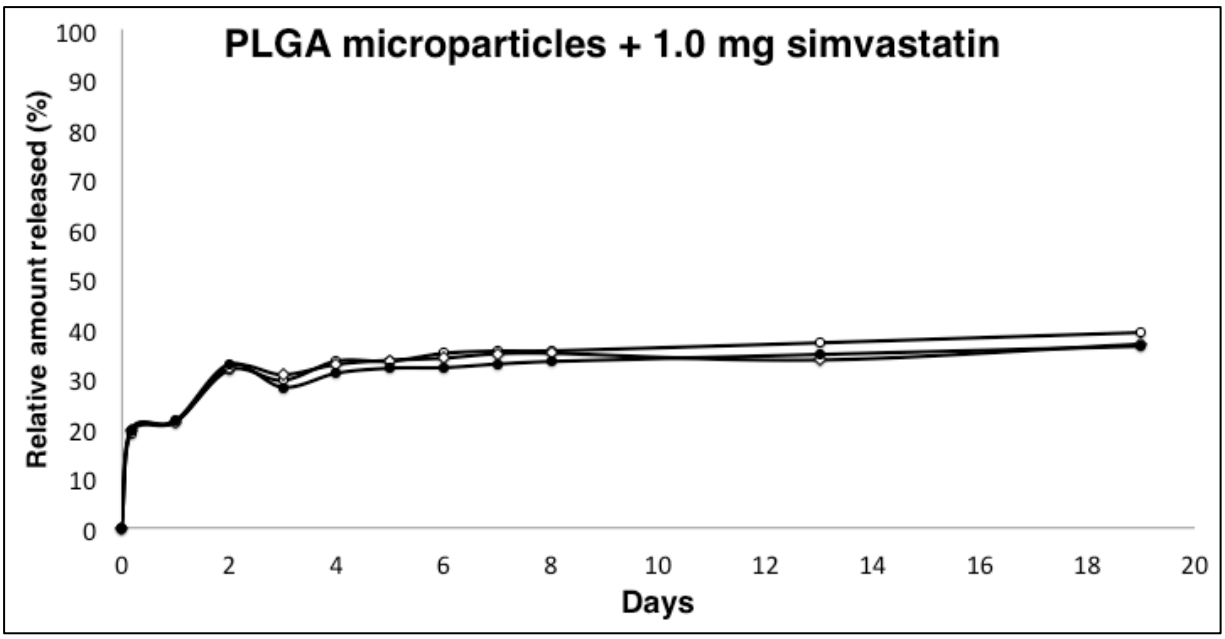


Figure 12. *In vitro* release of simvastatin from the PLGA-microparticle coating.

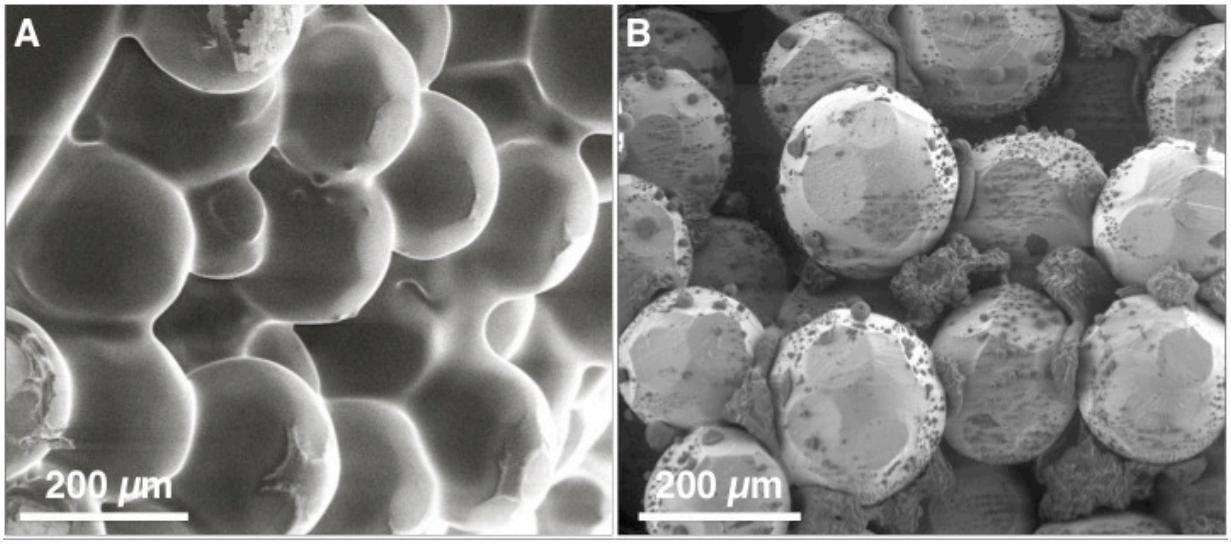


Figure 13a. PDLLA coating before **(A)** and after **(B)** *in vitro* release.

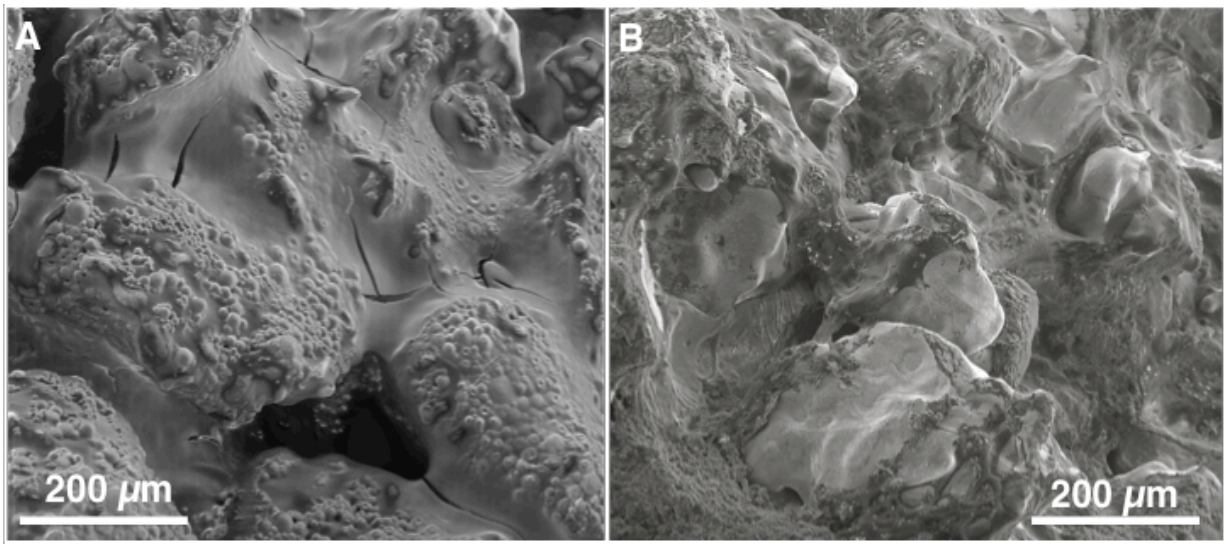


Figure 13b. PLGA-microparticle coating before **(A)** and after **(B)** *in vitro* release.

SEM images after the in vitro release studies revealed that some of both the PDLLA and the PLGA-microparticle coating were present after four weeks (Figures 13a and 13b).
The method used here is a simple way to estimate if simvastatin is released from the coatings. We chose to remove and replace only 10 % of the PBS volume to avoid extensive dilution of simvastatin that would make it impossible for us to detect simvastatin. However, we may have replaced too small a volume resulting in a saturation of the PBS with simvastatin thereby limiting further release from the coating. These release studies confirm that simvastatin indeed is released from the coating, but we are not able to make any firm conclusions on the amount released.

Peri-implant pH
To assess the effect of the coating on the peri-implant pH, implants from the groups included in studies I and II were submerged into saline or PBS. Ideally, the volume of saline or PBS should be similar to the volume of the peri-implant gap.

$$V_{gap} = V_{cavity} - V_{Implant}$$
$$V_{cavity} = (\pi\ x\ (0.4\ cm)^2\ x\ 1.0\ cm)$$
$$V_{implant} = (\pi\ x\ (0.3\ cm)^2\ x\ 1.0\ cm)$$
$$V_{gap} \sim 0.22\ mL$$

In this setup and with the equipment we used to assess peri-implant pH, a volume of 220 μL was too small to measure pH and we used a total volume of 1800 μL saline or PBS. Implants in saline or PBS were placed at 37°C while rotating horizontally.
In un-buffered saline, untreated implants and implants coated with PDLLA + 0.1 mg simvastatin roughly stayed at initial pH, while all other implants had a substantial drop in pH (Figure 14a).
In PBS, uncoated implants and implants coated with PDLLA ± simvastatin 0.1 mg, caused no reduction in pH, while the PLGA microparticle coating ± simvastatin caused a substantial reduction in pH. This was most pronounced for the group with simvastatin (Figure 14b).
For this in vitro study we used spare implants from the surgeries and there was only one implant left with PDLLA + simvastatin 1.0 mg. For that reason we were only able to include implants coated with PDLLA + simvastatin 1.0 mg in the saline group.
These measurements of peri-implant pH demonstrate that acid is released from the coatings into the surroundings. For most implants in PBS, the release of acid did not exceed the buffer capacity. Implants coated with the PLGA-microparticle formulation caused a drop in pH. For the implants in un-buffered saline all implant coatings (except PDLLA + simvastatin 0.1

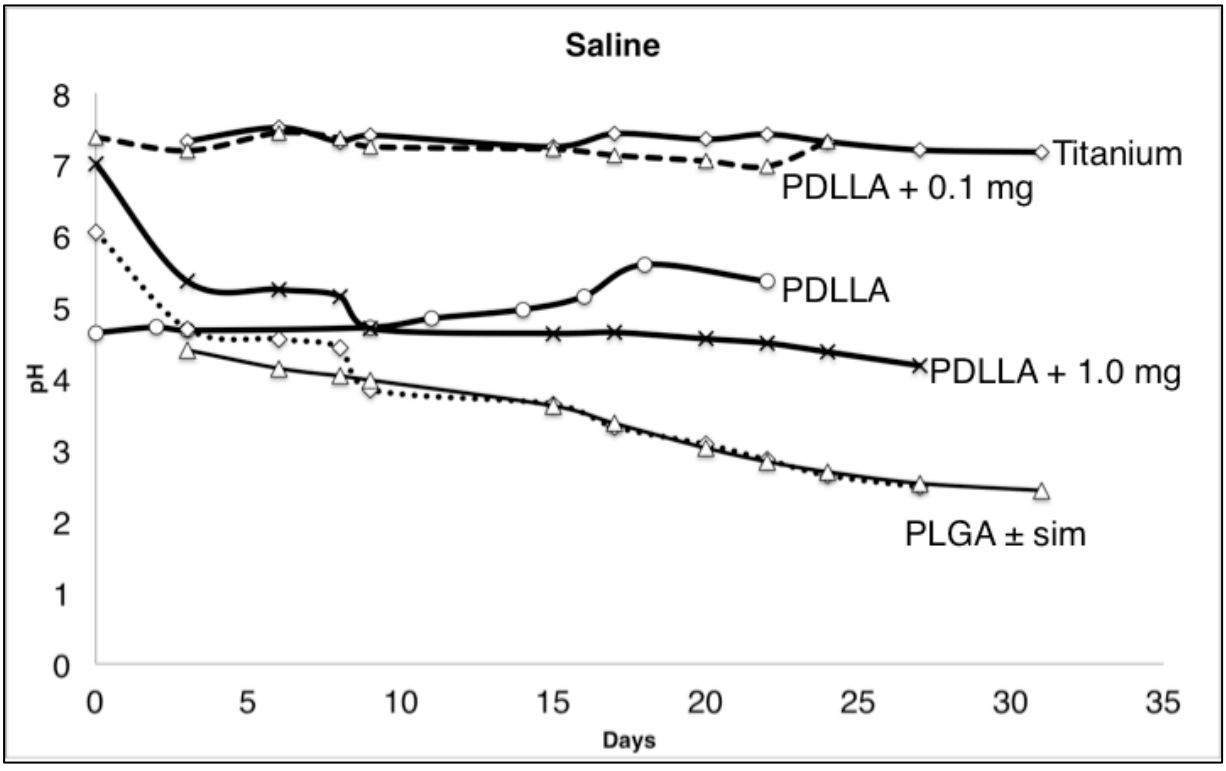


Figure 14a. Peri-implant pH in saline.

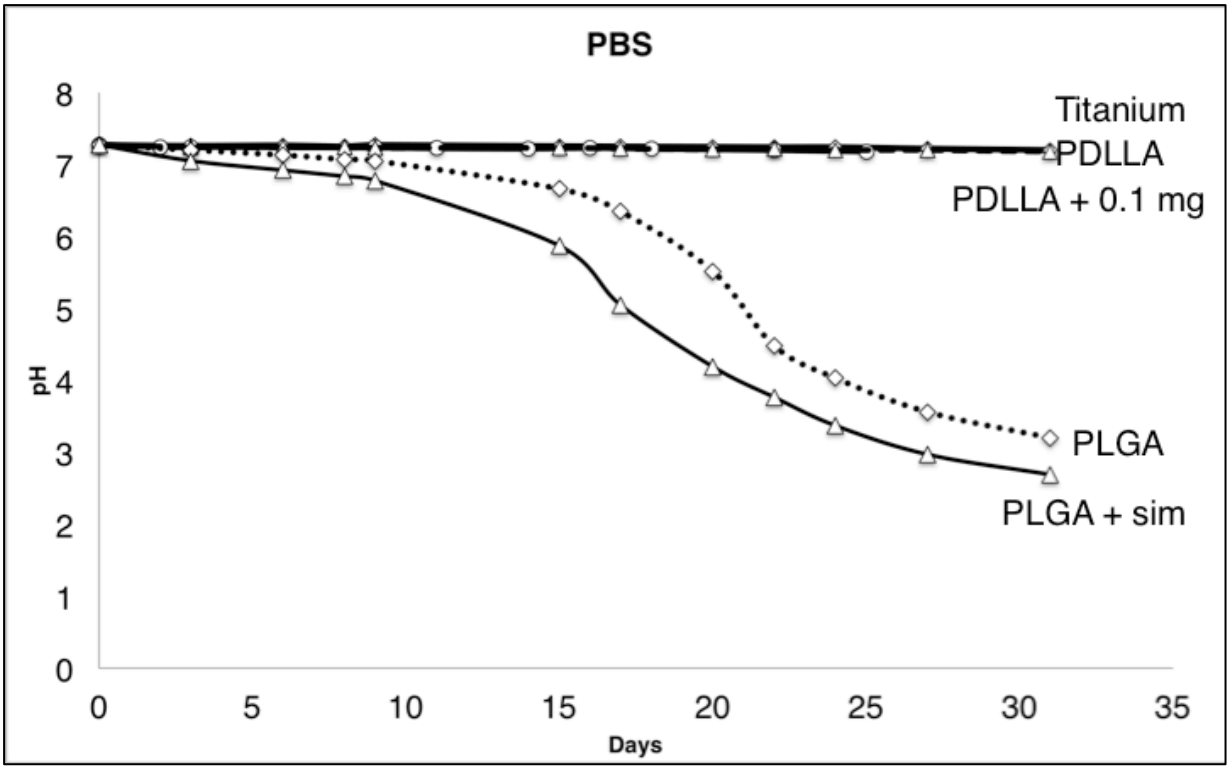


Figure 14b. Peri-implant pH in PBS.

mg) resulted in decreased pH. These *in vitro* measurements do not completely imitate the peri-implant conditions. First, the volume surrounding the implants is not the same. Second, the *in vivo* peri-implant volume is not absolutely static as this setup suggests. Overall, these measurements point toward an acidic milieu in the implant vicinity, which may affect peri-implant bone formation if the release of lactic acid exceeds the buffer capacity of the tissue.

Bone allograft preparation, study III

The bone allograft used in study III was harvested from two animals not included in any of the studies in this thesis. The harvest was performed immediately post mortem and under sterile conditions. Bones were stored at -80°C until processing. After complete removal of soft tissue and cartilage, the proximal humerei, distal femora and the proximal tibiae were morselized using a standard bone mill on fine setting (Biomet, Warsaw, IN, USA). This resulted in bone chips with the size of 1-3 mm. The morselized bone allograft from the two donors was mixed to ensure a uniform foreign body response in the recipient animals. Afterwards, the bone allograft was rinsed in 0.5 L 37°C sterile saline for one minute. This procedure was repeated three times. Finally, the bone allograft was divided into twenty-four portions and stored in sterile containers at -80°C.

At the time of surgery, two portions of bone allograft were thawed for 10 minutes prior to soaking in either sterile isotonic saline or zolendronate 0.005 mg/mL (Zometa, Novartis, basel, Switzerland). Diluting Zometa in sterile saline made the zoledronate solution. For the intervention group, the bone allograft was soaked in 5 mL of zoledronate solution (0.005 mg/mL) for three minutes. Afterwards, the bone allograft was rinsed in saline for one minute while gently stirring. This was repeated three times to ensure complete removal of unbound zoledronate. The allograft in the control group underwent the same soaking and rinsing procedure, but with saline only.

During the revision surgery, the bone allograft was impacted around the stable revision implants and surgery was always conducted on the control side first, to avoid contamination with zoledronate.

Specimen preparation

Following euthanasia the bone-implant specimens were removed *en bloc* and stored at -20 °C immediately after retrieval. The specimens from both set of surgeries had to be transported from USA to Denmark for processing and analysis.

The specimens were kept frozen during transportation and were frozen upon arrival in Aarhus, Denmark. Preservation of bone tissue by freezing has been reported not to have any adverse effects on the mechanical properties of bone (117). Freezing of tissue may not preserve cells as well as other methods for preservation. In the studies of this thesis the histomorphometrical analysis was based on tissue morphology rather than cell morphology. Prior to preparation, the specimens were thawed briefly. In study III the articulating PE plug was removed prior to sectioning. Afterwards, the outermost 1 mm of the implant-bone specimen was cut off and discarded. The remaining implant with surrounding bone was divided into two sections perpendicular to the long axis of the implant (Studies I and II: Struers Accutom 50, Ballerup, Denmark; Study III: Exact Apparatebau, Nordenstadt, Germany).
The outer section was cut to a thickness of approximately 3.5 mm and refrozen to -20 °C until mechanical testing leaving an inner section with a thickness of approximately 5.5 mm prepared for histomorphometry (Figure 7).

Biomechanical testing

The primary goal of the studies included in this thesis was to improve early implant fixation. We evaluated fixation biomechanically. Biomechanical fixation was determined by loading implant specimens until failure by mechanical push-out test. The specimens from all three studies were tested on an MTS 858 mini Bionics Test Machine (MTS Systems Corporation, Eden Prairie, MN, USA). Prior to testing, the specimens were thawed and placed with the cortical side facing upwards on a metal support jig with a 7.4-mm opening. A flat-ended cylindrical test probe with a diameter of 5 mm was used to apply load directly to the implant. Testing was performed blinded and the same person tested each study in one continuous session. To define contact with the implant and initiate the test, a preload of 2 N was applied. Then axial push-out of the implant from the surrounding bone was performed at a speed of 5 mm/min. Load versus implant data were continuously recorded for every 10 μm of implant displacement and stored on a personal computer. Following the test, we measured diameter and height of the specimens (Table 4).

Test parameters

Using specimen-thickness and implant diameter, load-displacement data were converted and strength, stiffness and energy were derived. This accounted for small variation in specimen thickness and implant diameter, thereby facilitating comparison between specimens with slightly varying dimensions (Table 4). Because implants were cylindrical, the surface of a smooth cylinder shape was used as a surrogate to estimate the area in contact with bone.
After conversion of units, the derived data points were plotted, generating the strength-displacement curve (Figure 15). The mechanical parameters were derived from the curve using a Microsoft Excel spreadsheet. The calculations were performed as described by Baas (118).
We assessed mechanical implant fixation using three standard parameters: *Ultimate shear strength*, *apparent shear stiffness* and *total energy absorption*.

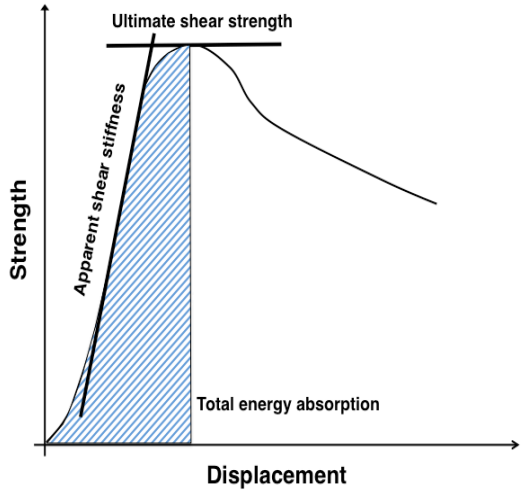


Figure 15. Mechanical parameters derived from the strength-displacement curve.

Ultimate shear strength (MPa)
This parameter was determined as the maximum force at implant failure, defined as the value of the first peak of the strength-displacement curve.
Occasionally, late peaks occurred. These were not included in the analysis and were regarded as post-failure interlocks of the bone trabeculae and the implant surface. This single exposure to high forces may not reflect the loads that

clinical implants are subjected to as these implants are loaded repetitively with lower forced during each gait cycle. The distribution of load in clinical implants is also multi-directional and not in a single direction as it is in the type of test we used. However, shear is a primary load direction that causes implant subsidence, and therefore was chosen for the one load direction possible in these destructive tests.

Apparent shear stiffness (MPa/mm)
This parameter is a measurement of rigidness or stiffness with which the implant is anchored in the surrounding tissue. The parameter is determined from the steepest part of the strength-displacement curve. The value of this parameter is dependent on the type of tissue that surrounds the implant, meaning that different types of tissues have different rigidity. Mineralized bone tissue have higher stiffness than fibrous tissue and other non-mineralized tissue, and hence a higher value indicates more mineralized tissue, while a low value point towards a soft tissue anchorage or only sparse bone-implant contact.

Total energy absorption (kJ/m^2)
This parameter is defined as the area under the strength-deformation curve until failure. It reflects the total amount of energy absorbed in the peri-implant tissue before failure. The parameter depends on both strength and stiffness as similar values for total energy absorption can be obtained with completely different values of ultimate shear strength and apparent shear stiffness. Thus the implant may be anchored in different tissues and tissue combinations and as a consequence this parameter may not completely reflect the type of tissue that the implant is anchored in. It can reflect the resilience of the tissue, or the amount of energy it can store before it fails.

Limitations and strengths

The push-out test only considers shear load in the axial direction and does not completely represent the spectrum of forces that clinical implants undergo, such as bending and compressive forces. Although this test is a gross simplification of the clinical reality, it represents a small section of the surface of a clinical implant. The push-out test is a destructive test since the implant is pushed out of the surrounding tissue, thereby destroying the interface. This makes it impossible to make double measurements and evaluate reproducibility of the test. As an additional test parameter, ideally a non-destructive test would be followed by the destructive test, but this scenario requires validation using actual implant specimens. In non-destructive testing a high frequency and low amplitude cyclic load can be applied to the implant. High frequency dynamic testing evaluates the viscoelastic properties of the tissue in the bone-implant interface. Prior to implementing this test in the experimental models used in this thesis, extensive pilot work must be conducted to determine the frequency and magnitude of load and to verify that the test truly is non-destructive. This type of test increases the requirements to equal handling of the specimens as freezing and thawing may affect the viscoelastic properties more than the mechanical properties of bone (117).
Due to the destructive nature of the push-out test, the same specimen cannot be used for histomorphometrical evaluation. This requires us to make the necessary assumption that the superficial part of the bone-implant specimen used for mechanical testing is comparable to the profound part used for histomorphometry in terms of distribution and density of the different tissues present in the peri-implant area and that these two sections have the same mechanical characteristics.
Despite the implants had a porous surface, a smooth cylinder was used as a model for approximation of the surface area used to normalize data even though the undulating porous surface would have a larger surface area. We assumed equal porosity of the implants and thus this simplification aided the normalization of the mechanical data by diameter and height of the specimen.
The push-out test is a simple analysis of the bone-implant interface applied to a simple model of early implant fixation. This introduces less variation, as the specimens are identical in shape and the cylindrical shape is easy to evaluate by push-out test. Although simple, the specimens must be meticulously placed on a metal support jig with the implant centered over the opening that must be slightly larger than the implant diameter as reported by Dhert et al (119). To further reduce variation and eliminate bias from

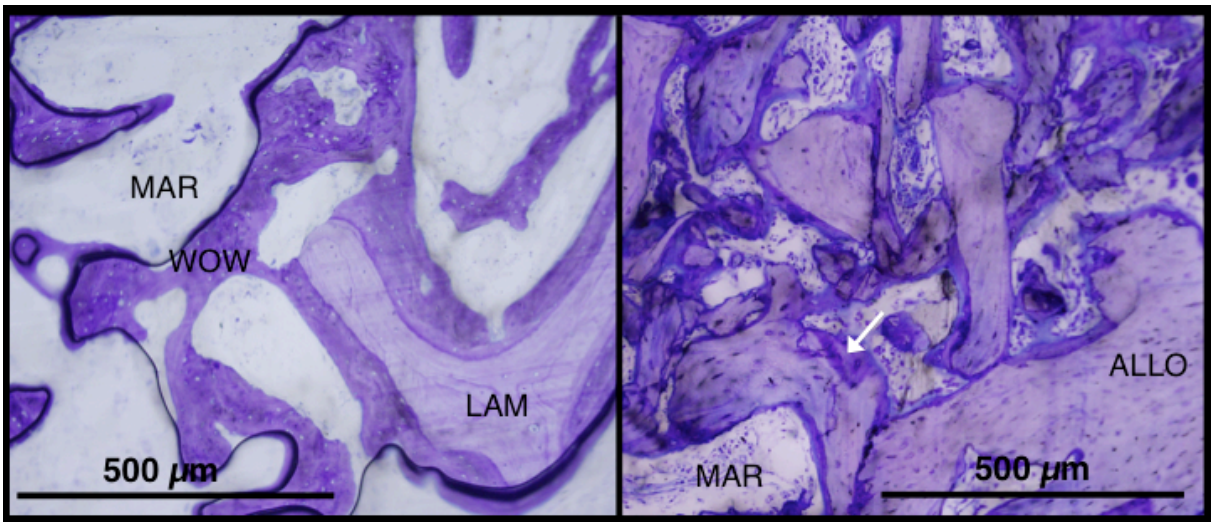


Figure 16. Tissue morphology. **Left:** Implant section from study I; **Right:** implant section from study III. MAR: marrow space; WOW: new (woven) bone; LAM: lamellar (old) bone; ALLO: bone allograft. White arrow: new bone.

the test the specimens were blinded and all specimens from each study were tested in one session and by the same operator.

Histological evaluation

Several sections from each bone-implant specimen were evaluated using histomorphometry. The aim of this analysis was to acquire a quantitative and unbiased estimate of the osseointegration of the implant and the peri-implant tissues.

The 5.5-mm inner sections for histomorphometry were dehydrated in graded ethanol (70-100%) (study I) or 96 % ethanol for two days, 100 % 2-propranolol for one day and finally, xylene for two days (studies II and III). Then the specimens were embedded in methylmethacrylate (MMA, art. 800590, Merck, Darmstadt, Germany) in a cylindrical mold with the vertical axis of the implant parallel to the vertical axis of the mold. Before sectioning the embedded specimens were randomly rotated around their vertical axis and four serial sections of approximately 30 µm were produced from the central part of the implant using a microtome (KDG-95, MeProTech, Heerhugowaard, Holland). The approximate thickness of each specimen was 30µm and about 400 µm was lost to the saw. Each section was surface stained with toluidine blue 0.1 %, pH 7 (Sigma-Aldrich, St. Louis, MO, USA), rinsed and mounted on a glass.

Several tissues on the implant surface and in the peri-implant gap were quantified: new woven bone, fibrous tissue, bone marrow, and bone allograft (study III only). The different types of tissue were distinguished based on their morphological characteristics. The staining method aided in classification of the different types of tissue. Newly formed bone was characterized as dense dark purple disorganized substance with embedded cells in large lacunae. Fibrous tissue was identified mainly as well-organized bundles of fibers with a low cell density and spindle shaped nuclei but it was also seen as a less organized loosely interconnected fibrous network. Bone marrow was a less dense, cell-rich disorganized conglomerate with empty areas representing dissolved fat. The bone allograft was assumed to consist of lamellar bone and was identified as lightly stained lamellar structures with empty lacunae (Figure 16). Less than 1% of the counts were recorded as unknown. The number was so low that the counts were not included in the final analysis and statistical evaluation.

During the histomorphometrical evaluation of the three studies, the specimens were blinded to the examiner. At the light microscopic level it was not possible to visualize the additional surface coating in studies I and II and the zoledronate treatment of the bone allograft in study III, and thus the blinding of the examiner was complete throughout the histomorphometrical analysis.

Stereological histomorphometry

Stereology provides techniques for extracting quantitative information about a three-dimensional material from measurements made on two-dimensional sections. Specifically, the bone-implant specimens from the three studies of the present thesis have three-dimensional volume fractions and area fractions covering a cylindrical implant. These fractions are quantified on several two-dimensional histological sections from the same bone-implant specimen. Applying these techniques we estimated implant osseointegration by assessing bone ongrowth (surface area fraction of tissues in contact with the implant) and bone ingrowth (bone volume in the peri-implant gap). Volume and surface fractions can be quantified using different test probes superimposed on the object investigated. The sum of dimensions must add up to three. Hence volume fractions are three-dimensional and must be assessed using a zero-dimensional probe while surface area fractions are two-dimensional and must be evaluated using a one-dimensional probe.

To estimate surface area fractions we used line-interception technique where a line-probe (one-dimensional) is randomly superimposed onto the section. When a line intercepts with the surface of the implant, the tissue at that point is determined and recorded. For this purpose the material must be isotropic, meaning that the probability of an interception between the material and the line probe is the same regardless of the orientation of the probe. Due to its porous coating the implant surface cannot be considered as completely isotropic and sine-weighted grid-lines were applied to compensate for this.

On the contrary to the surface fraction estimations, the volume fraction estimations are independent of orientation and it is not necessary to consider if the material is isotropic or not. This is because the point counting probe is without dimensions (zero-dimensional) and thus without any preferred direction. The result is that volume fractions estimates can be conducted without any specific requirements for isotropy.

We used the vertical sectioning technique thoroughly described by Baddeley et al. (120). This technique allows surface area estimation without assuming isotropy. Four requirements must be met: 1) an identifiable vertical axis, 2) sections are cut parallel to the vertical axis, 3) sections are placed after random rotation around the vertical axis, 4) sine-weighted line-probe. The first three requirements ensure that the specimens are cut into vertical uniform sections, while the fourth requirement ensures that the line probe is isotropically distributed in the three-dimensional space. An additional advantage of the stereological principles used in this thesis, is that they enable us to limit the workload of the tissue analysis while still achieving an unbiased estimation of volume and surface area fractions. Based on previous experience from studies with a similar design, we aimed at a minimum of 100 positive hits (from both point counting and from the line-intercepts) in the most important tissue parameter, new bone. Since we analyzed four sections from each bone-implant specimen we aimed at an average around 30 hits of the parameter "new bone" per section. To determine the sampling intensity, a blinded test counting of four to five implants per study was made. This allowed us to adjust the number of points and lines needed for the final evaluation of the specimens.

In studies I and II most of the groups had sparse amounts of new bone, which forced us to increase the intensity of the sampling, i.e. increase the number of points in the probe for volume fraction estimation and increase the number of lines in the probe for surface area estimation. This resulted in a high number of counts per bone-implant specimen but ensured that the estimates were more reproducible and allowed us to rule out the potential that poor sampling intensity could be a reason for lack of difference in any of the groups (Table 6). Intra-observer (studies I, II, and III) and inter-observer (studies I and II) reproducibility were

Study	Surface		Volume	
	Mean	(sd)	Mean	(sd)
I	923	187	2683	408
II	1498	222	1982	238
III	583	103	397	54

Table 6. Mean counts for volume and surface fractions (mean [sd]).

estimated for the studies and will be discussed later.
Point counts or line-intercept counts were each accumulated and tissue volume and surface area fractions were calculated for each histomorphometrical parameter as follows:

$$Fraction = {}^{counts}/_{total\ counts}$$

Total counts equal the number of counts made for the surface area estimation or volume estimation. Fractions derived from the histomorphometrical analysis were used in the subsequent analysis.

Regions of interest

In all three studies of this thesis a single region of interest was applied to the histomorphometrical analysis. The peri-implant gaps were empty 1.0-mm gaps (studies I and II) or a grafted gap of 1.1 mm (study III). These gaps were analyzed as a single region where volume fractions were estimated. These volume parameters represented ingrowth while the surface area fractions were interpreted as ongrowth onto the implant surface. Since the porous coating of the implant varied slightly in thickness and porosity we were not able to use the solid implant core as reference for the region of interest. For that reason we decided to use a median line between the solid core and the outermost part of the porous coating. From this line the width of the region of interest was measured. In all studies of this thesis, the region of interest ranged from the median line of the porous coating and 1000 μm into the gap. In the end with the remaining end washer the region of interest started 500 μm central to the end of the solid implant core (Figure 17).
We performed blinded quantitative histomorphometry using an Olympus light microscope (Olympus, Ballerup, Denmark) with Visiopharm Integrator System (NewCast version 3.0.9.0, Visiopharm, Hoersholm, Denmark). The software aided in defining the region of interest, applied the probes used for the analysis and collected the data.

Strengths and limitations

Bias in specimen preparation
The sections made from the bone-implant specimens were cut on a heavy-duty microtome because the implants were made of titanium. We made sections as thin as possible resulting in a thickness of approximately 30 μm. Sections were produced from the central part of the implant aiming at perpendicular intersections between implant and bone. Because of the section thickness and because approximately 400 μm was lost to the blade of the microtome the last section may have been more than 1 mm away from the vertical axis, depending of the offset of the first section. The more peripheral sections are cut more tangential to surface of the implant. The result is that the implant appears to have a smaller diameter and a wider peri-implant gap

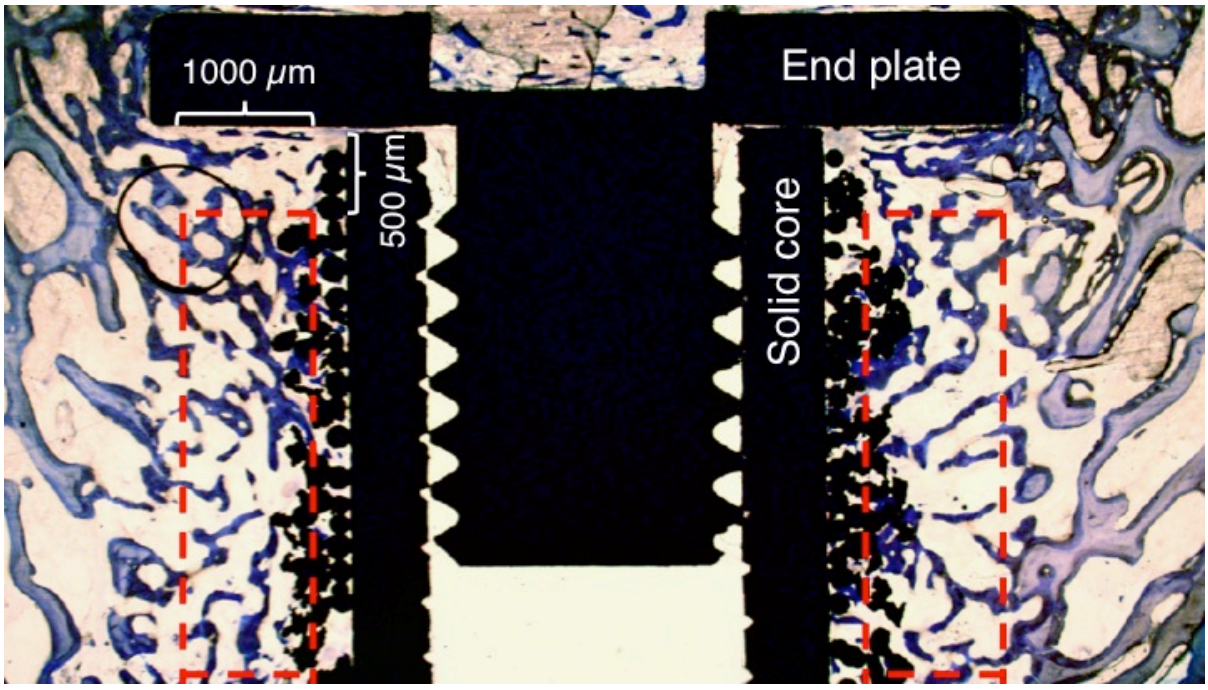


Figure 17. Region of interest.

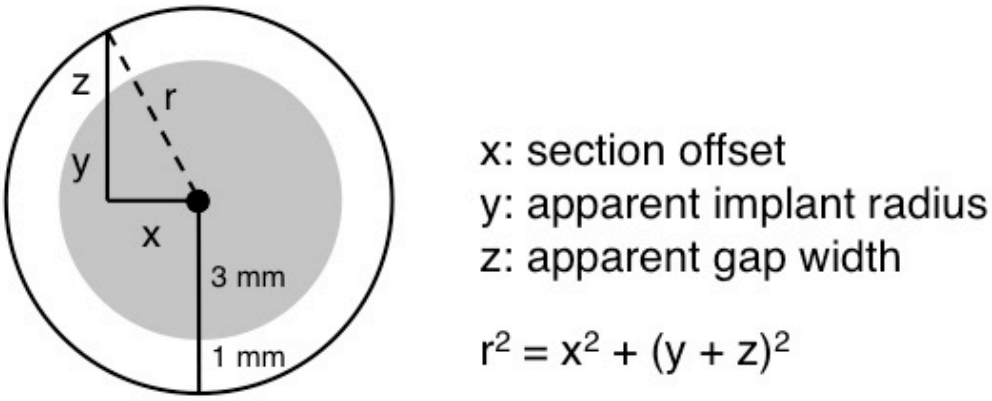


Figure 18. Section offset bias.

compared to sections cut through the center of the implant and thus the predefined region of interest will cover less of the peri-implant gap compared to central sections since the region is defined from the implant surface as mentioned earlier. This may result in a systematic difference in what part of the peri-implant defect that has actually been sampled between the sections of each implant. Because recruitment of osteoprogenitor cells and ingrowth of new bone is expected to occur from the drill hole border towards the implant surface, we cannot assume that the distribution of tissues from the implant surface towards the gap is homogenous. The section offset bias can constitute a problem as the tissues in the periphery of the gap (especially new bone) may be underestimated. Since these variations in sampling occurred within the four sections from each implant and the data for each implant was accumulated this would probably not introduce any systematic bias, but rather increase variation of the data. Baas used the Pythagorean theorem to estimate the impact of section offset bias and found the apparent gap width to increase with only 3.2% with maximal offset for implants with a diameter of 6 mm in 11 mm diameter drill holes (118).

Using the same approach we estimated the impact of maximal section-offset bias for a vertical section with the offset x from the vertical implant axis. The implant will have the apparent implant radius y, a drill hole radius r, and the true implant radius $r_,$. The Pythagorean theorem gives the following relation between the apparent gap width z and the section's offset x:

$$z = \sqrt{r^2 - x^2} - y$$

$$z = \sqrt{r^2 - x^2} - \sqrt{r,^2 - x^2}$$

For implants with 6 mm diameter in 8 mm diameter drill holes, the apparent gap with will increase with 4.0% (0.04 mm) at maximum offset (x = 1 mm) (Figure 18). Because this increase was only minor, we considered the section-offset bias to be of negligible importance.

Another possible source of bias to consider is the central-section bias where volume counts close to the implant surface represent smaller volumes than counts towards the periphery of the peri-implant defect. This means that the probability of a tissue or structure appearing in a central vertical section decreases towards the drill hole border and thus the tissues in the implant vicinity are at risk at being overestimated compared to the more peripheral tissues.

The impact of central-section bias has been investigated thoroughly by Bass, who reported it to have minimal influence on the results (118).

Section thickness bias

The sections used for histomorphometry were thick sections because of the metal implant in the center of the bone-implant specimen. This introduces the risk of over-projection of implant material and tissue in the deep parts of the specimen. Because the implant surface is highly porous, implant surface over-projection may result in a decreased ability to determine whether or not the tissue is in direct with the implant because the deeper parts of the implant surface may cast shadows. The result is that areas with only a thin layer of tissue may be underestimated while tissue close to but not in direct

contact with the surface may be overestimated.
Another shortcoming of the section thickness is that tissue profound of the surface of the specimen may be overestimated. This is mainly a problem in specimens that have been infiltrated with stain, where fibrous tissue below the focus plane may be projected onto marrow space that is more transparent than the fibrous tissue, causing overestimation of the fractions of fibrous tissue. For the studies in this thesis, bone-implant specimens were not infiltrated with stain. The sections were surface stained, and therefore not stained until after they were produced. As mentioned earlier, we used toluidine blue to stain our specimens. This only stained the most superficial 4 μm of the specimens (121). Using this staining method we were able to limit over-projection of tissue from deeper parts of the sections and obtain a more unbiased estimation of tissue fractions.

The stereological methods used for the studies in this thesis were applied to estimate tissue ongrowth and ingrowth. The mentioned possibilities to introduce bias have previously been investigated (118) and found to only have minor influence on the results.
Histomorphometrical evaluation should always be carefully planned and the sections cut and stained meticulously to introduce the least possible bias.
All analyses were performed on specimens from paired studies, and all implant-bone specimens within each study were subjected to identical processing and analysis. We consider the stereological methods applied in this thesis valid and thus the results presented valid estimates of the peri-implant tissue volume and area fractions.

Histomorphometrical reproducibility

The histomorphometry is based on tissue classification and the distinction is not always completely clear, which may increase variability. Furthermore, the histomorphometrical analysis takes place over a period of time. During this period the investigator may not distinguish the same tissues identically. Also, if the investigator is inexperienced, there may be a learning curve causing the investigator to decrease reproducibility.
These issues were dealt with by making clear morphological descriptions of each tissue represented in the sections. In addition, the analysis was performed over a short period. The histomorphometrical analysis of each study was initiated with a test-count, to adjust the probes used and to standardize the method. The histomorphometry was performed blinded by the same investigator and with the bone-implant specimens in random order.
Reproducibility can be expressed as the coefficient of variance (CV):

$$CV = \frac{s}{\bar{x}} \; ; \; s^2 = \frac{\Sigma (x - \bar{x})^2}{n - 1}$$

$\bar{x}$ is the mean value of the first and second measurement, n is the number of double estimates. The intra- and inter-observer reproducibility were calculated from five (studies I and II) or four (study III) randomly chosen implants using the same equipment. Both the inter-observer and intra-observer reproducibility were good and in general less than 5 % (Table 7). The variation of the measurements decreases as the fraction of a particular tissue increases. This means that the CV not only reflects the investigator's ability to determine the types of tissues present in the region of interest, it also reflects the

Study		Volume				Surface			
		New bone	Fibrous	Marrow	Allograft	New bone	Fibrous	Marrow	Allograft
I	Intra	2.3 %	3.6 %	0.35 %		2.4 %	2.2 %	0.64 %	
	Inter	1.3 %	6.1 %	0.35 %		2.7 %	6.4 %	0.59 %	
II	Intra	0.53 %	1.1 %	0.23 %		4.4 %	1.0 %	0.73 %	
	Inter	0.56 %	2.3 %	0.46 %		3.7 %	1.7 %	1.1 %	
III	Intra	1.2 %	4.0 %	0.64 %	1.4 %	0.48 %	38 %	0.19 %	2.6 %
	Inter	-	-	-	-	-	-	-	-

Table 7. Intra- and inter-observer variation.

sampling intensity. With low fractions of a particular tissue the reproducibility of the measurements may be more dependent on the sampling intensity, as a denser sampling will increase the precision of the estimated tissue fractions present in the peri-implant gap. For tissues with low fractions, CV was higher compared to those types that had higher fractions. CVs for all relevant tissues are displayed in Table 7.

Statistical evaluation

All datasets form the studies in this thesis were paired with either four groups (studies I and II) or two groups (study III) included. Data from the paired, four-armed studies (I and II) for which normal distribution could be assumed were initially evaluated with repeated measures ANOVA followed by paired t-test. The data for which normal distribution could not be assumed were evaluated with Friedman repeated measures analysis of variance by ranks followed by Wilcoxon signed-rank test. In most cases the reason for the non-normally distribution was because of many values close to zero. The paired, two-armed study (III) was evaluated with paired t-test as normal distribution could be assumed for both the mechanical and histomorphometrical dataset. For all datasets, differences between means or medians were considered statistically significant for p-values <0.05.

In each of studies I and II, four groups were compared yielding the possibility to perform six tests on each of the mechanical and histomorphometrical parameters. This extensive testing increases the risk of falsely rejecting the null hypothesis increasing the risk of a type I error where a true null hypothesis is rejected (contrary to a type II error, where a false hypothesis is accepted). To reduce the risk of this, each parameter in studies I and II were tested for equality of means or medians using repeated measures ANOVA if normally distributed or Friedman repeated measures analysis of variance by ranks if not normally distributed. Only if these preliminary test yielded p-values <0.05 rejecting the null hypothesis of equality, post hoc testing was performed using either paired t-test of Wilcoxon signed-rank test.

For the statistical analysis we used STATA 11.2 software (StataCorp, College Station, TX, USA).

Specimens available for evaluation

Study I

In study I, all twelve animals were fully recovered from surgery within three days. Eleven animals completed the observation period without any signs of infection or other complications. One animal died five days prior to the end of the observation period. Autopsy performed by the attending veterinarian revealed three intussusceptions, two in the small bowel (jejunum) and one at the ileo-coecal junction as the cause of death. This was neither related to the implants nor the coating. The animal was not excluded since the observation period was almost completed at the time of death, and it had been in normal health until that time. No specimens were lost during preparation, thus all 48 implants were available for mechanical and histomorphometrical evaluation.

Studies II and III

All animals were fully recovered from both surgeries within three days and all animals completed the observation period. There were no clinical signs of infection neither at the humerus sites nor at the femoral sites. In study II, all 48 specimens were available for both mechanical and histomorphometrical analysis and in study III all 24 specimens were available for mechanical and histomorphometrical analysis.

6. Summary of studies

All studies were paired and conducted using an experimental model for implant fixation. In studies I and II we used an indirectly loaded implant model, where implants were inserted into the proximal part of humerus. In study III we employed a more complex model of revision joint replacement with weight-bearing implants in the articulating portion of the distal femur.

The overall purpose of these studies was to improve fixation of uncemented clinical implant components. All studies investigated local treatment with compounds that interfere with the mevalonate pathway, simvastatin (studies I and II) and zoledronate (study III). In addition, two vehicles for local delivery to the bone-implant interface were evaluated (studies I and II).

In our experimental setup we defined improved mechanical implant fixation as an increase in the mechanical parameters while improvement in implant osseointegration was defined as increased new bone formation and decreased fibrous tissue formation (in volume and surface fractions) and in study III, also increased retainment of bone allograft.

Study I

Hypothesis 1: PDLLA coated onto experimental orthopaedic implants will not influence early implant fixation.

Hypothesis 1 disproved: Yes.

Hypothesis 2: Simvastatin released from a PDLLA coating will stimulate bone formation and enhance early implant fixation.

Hypothesis 2 disproved: No.

Comments: The PDLLA coating used in this study as additional surface coating had a detrimental effect on the mechanical implant fixation and the formation of bone in the implant surface. This detrimental effect of the PDLLA carrier complicated the interpretation of the data from the simvastatin loaded PDLLA implants.

This study indicates that the PDLLA formulation as used in this study may not be appropriate for drug delivery to the bone-implant interface (Tables 8a, 8b, 9a, 9b).

	Shear strength (MPa)	**Shear stiffness (MPa/mm)**	**Energy absorption (J/m^2)**
Ti	4.2 (2.9-5.9)	20 (13-31)	892 (620-1136)
PDLLA	0.77 (0.42-2.88)	4.0(2.0-13)	116 (86-471)
PDLLA + 0.1 mg	1.6 (0.9-2.5)	6.8 (4.3-12)	345 (113-454)
PDLLA + 1.0 mg	2.0 (0.53-3.6)	11 (2.2-18)	271 (126-682)
p-value	0.031	0.107	0.013

Table 8a. Study I, mechanical push-out [median (interquartile range)].

p-value	**Shear strength**				**Energy Absorption**			
	Ti	**PDLLA**	**PDLLA + 0.1 mg**	**PDLLA + 1.0 mg**	**Ti**	**PDLLA**	**PDLLA + 0.1 mg**	**PDLLA + 1.0 mg**
Ti								
PDLLA	0.028				0.010			
PDLLA+ 0.1 mg	0.050	0.530			0.034	0.388		
PDLLA+ 1.0 mg	0.019	0.182	0.583		0.010	0.136	0.695	
	(Friedman, Wilcoxon signed-rank)				(Friedman, Wilcoxon signed-rank)			

Table 8b. Study I, shear strength and energy absorption. Paired comparisons of groups.

	Surface area fractions in %		**Volume fractions in %**	
	New bone % [mean (sd)]	**Fibrous tissue %** [median (iqr)]	**New bone %** [mean (sd)]	**Fibrous tissue %** [median (iqr)]
Titanium	16 (6)	0 (0-0)	22 (6.9)	0 (0-0)
PDLLA	4.8 (4.5)	0 (0-0.04)	16 (6.9)	0(0-0.01)
PDLLA+ 0.1mg	6.8 (6.0)	0 (0-0.10)	16 (5.3)	0(0-0.03)
PDLLA+ 1.0mg	7.0 (4.0)	0 (0-0.12)	18 (9.5)	0(0-0.04)
***p* ANOVA**	<0.001		0.1889	
***p* Freidman**		0.2057		0.1479

Table 9a. Study I. Surface area fractions (ongrowth) and volume fractions (ongrowth). Iqr: interquartile range. Paired comparisons for new bone surface area fractions in Table 9b.

p-value	**New bone surface area fraction**			
	Ti	**PDLLA**	**PDLLA + 0.1 mg**	**PDLLA + 1.0 mg**
Ti				
PDLLA	<0.001			
PDLLA + 0.1 mg	0.005	0.368		
PDLLA + 1.0 mg	<0.001	0.273	0.951	
	(ANOVA, t-test)			

Table 9b. Study I. Paired comparisons for new bone surface area fractions.

Study II

Hypothesis 1: Implants coated with either a PDLLA coating or a PLGA microparticle coating will not negatively influence early implant fixation.

Hypothesis 1 disproved: Yes.

Hypothesis 2: Simvastatin delivered to the bone-implant interface in a PLGA microparticle coating will improve implant fixation compared to untreated titanium implants and implants coated with an empty PLGA microparticle coating.

Hypothesis 2 disproved: Yes.

Comments: Both polymer coatings resulted in inferior mechanical implant fixation and reduced bone formation at the implant surface, most pronounced in the groups with PLGA microparticles ± simvastatin 1.0 mg where bone formation in the peri-implant gap also was impaired compared to the untreated titanium implants. In addition, the polymer coated implants had significantly more fibrous tissue ongrowth on the surface of the implant. The PLGA-based carrier complicated the interpretation of the data from the simvastatin loaded implants and we were not able to determine if simvastatin has any positive or negative effect on implant fixation. This study along with study I suggests that the lactic-acid based polymers used in these formulations are not appropriate for drug delivery to the bone-implant interface (Tables 10a, 10b, 11a, 11b, 11c).

	Shear strength (MPa)	**Shear stiffness (MPa/mm)**	**Energy absorption (J/m^2)**
Ti	0.82 (0.47-1.3)	3.3 (2.0-6.5)	166 (105-278)
PDLLA	0.14 (0.01-0.58)	0.80 (0.08-2.8)	13 (0.43-87)
PLGA	0.15 (0.03-0.43)	1.0 (0.13-1.9)	17 (1.1-76)
PLGA sim	0.02 (0.00-0.14)	0.11 (0.00-0.68)	1.6 (0.00-19)
p-value	0.003	0.003	0.002

Table 10a. Study II, mechanical push-out [median (interquartile range)].
p-values for paired comparisons of groups in Table 10b.

	Ultimate shear strength			**Apparent shear stiffness**			**Total energy absorption**		
	Ti	**PDLLA**	**PLGA**	**Ti**	**PDLLA**	**PLGA**	**Ti**	**PDLLA**	**PLGA**
PDLLA	0.084			0.100			0.015		
PLGA	0.034	0.938		0.034	0.875		0.017	0.638	
PLGA sim	0.002	0.060	0.117	0.002	0.028	0.060	0.003	0.136	0.182

Table 10b. Study II, mechanical parameters. Paired comparisons of groups.

	Surface area fractions in %		Volume fractions in %	
	New bone % [mean (CI95)]	**Fibrous tissue** % [mean (CI95)]	**New bone** % [mean (CI95)]	**Fibrous tissue** % [mean (CI95)]
Titanium	9.6 (5.1; 14)	10 (0.24; 20)	15 (9.1; 21)	1.5 (-0.10; 3.1)
PDLLA	1.4 (0.38; 2.3)	43 (27; 58)	11 (7.1; 15)	9.6 (5.3; 14)
PLGA	1.5 (0.20; 2.9)	47 (27; 67)	9.4 (6.5; 12)	14 (4.4; 23)
PLGA sim	0.55 (-0.01; 1.1)	46 (24; 67)	7.2 (-1.9; 6.1)	22 (4.5; 40)
***p* ANOVA**	0.003	0.003	0.024	0.011

Table 11a. Study II. Surface area fractions (ongrowth) and volume fractions (ongrowth). CI95: 95% confidence interval. Paired comparisons in Tables 11b and 11c.

Surface area fractions								
p-value	**New bone**				**Fibrous tissue**			
	Ti	**PDLLA**	**PLGA**	**PLGA sim**	**Ti**	**PDLLA**	**PLGA**	**PLGA sim**
Ti								
PDLLA	0.002				0.001			
PLGA	0.004	0.830			0.004	0.667		
PLGA sim	<0.001	0.136	0.203		0.003	0.660	0.897	
	(ANOVA, paired t-test)				(ANOVA, paired t-test)			

Table 11b. Study II. Paired comparisons for new bone and fibrous tissue surface area fractions.

Volume fractions								
p-value	**New bone**				**Fibrous tissue**			
	Ti	**PDLLA**	**PLGA**	**PLGA sim**	**Ti**	**PDLLA**	**PLGA**	**PLGA sim**
Ti								
PDLLA	0.269				0.022			
PLGA	0.089	0.235			0.016	0.229		
PLGA sim	0.009	0.111	0.269		0.026	0.092	0.312	
	(ANOVA, paired t-test)				(ANOVA, paired t-test)			

Table 11c. Study II. Paired comparisons for new bone and fibrous tissue volume fractions.

Study III

Hypothesis: Zoledronate-treatment of bone allograft impacted around stable loaded revision implants will retain the graft without impairing new bone formation, and thus improve early implant fixation.

Hypothesis disproved: No.

Comments: Implant fixation was significantly improved without impairing new bone formation. Zoledronate treatment of bone allograft may help maintain the stability of allografted revision implants which can improve the longevity of revision joint replacements and reduce the risk of subsequent revision. Clinical studies are warranted (Figures 19-21, Table 12).

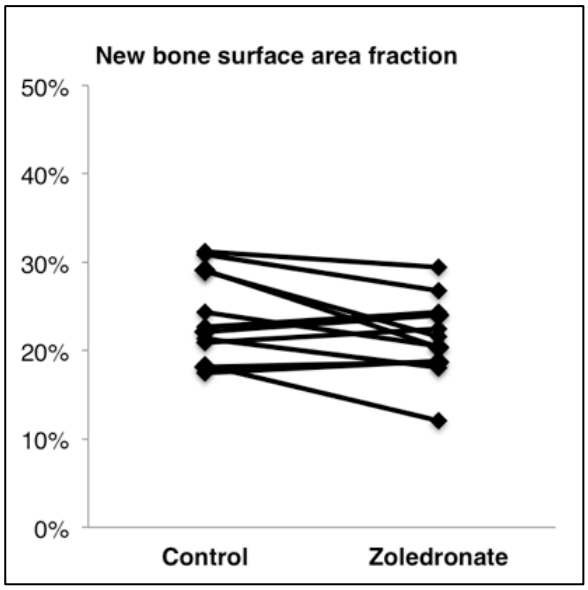


Figure 20. New bone surface area fractions. Implant pairs are connected with a line. Mean (95% CI). Control: 23.80% (20.58-27.02), zoledronate: 21.43% (18.57-24.29).

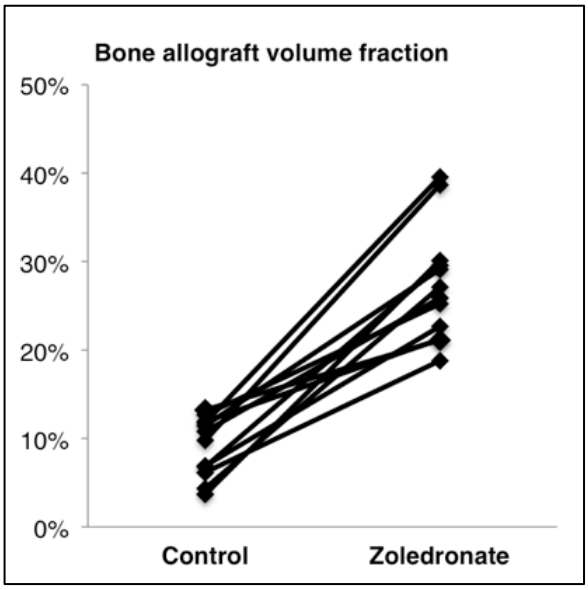


Figure 19. Bone allograft volume fractions. Implant pairs are connected with a line. Mean (95 % CI). Control: 9.11% (6.97-11.24), zoledronate: 27.40% (23.24-31.56%).

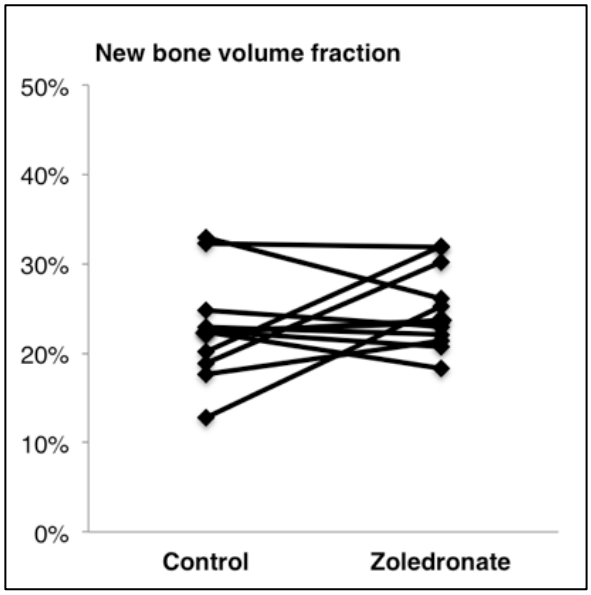


Figure 21. New bone volume fractions. Implant pairs connected with a line. Mean (95% CI). Control: 22.67% (19.10-26.24), zoledronate: 24.82% (21.99-27.64).

	Ultimate shear strength (MPa)	Apparent shear stiffness (MPa/mm)	Total energy absorption (J/m^2)
Control (saline)	2.7 (2.1; 3.2)	13 (11; 16)	426 (302; 549)
Zoledronate	3.5 (3.0; 4.1)	20 (16; 23)	476 (379; 573)
Zoledronate – control*	0.87 (0.14; 1.60)	6.47 (2.9; 10)	50 (-89; 189)
p	0.023	0.002	0.444

Table 12. Results from the mechanical push-out test (mean [95% CI]). *Absolute difference between means.

7. Discussion

The overall purpose of the research conducted for the three studies in this thesis is to improve the osseointegration and mechanical fixation of orthopaedic implants. Ideally, implants obtain a life-lasting anchorage in the surrounding bone, thus reducing the risk of revision. Revision joint replacements often have inferior functional outcomes, increased risk of infection and dislocation, and bone stock may be reduced. These situations all challenge robust osseointegration and implant stability. Notably, these factors contribute to even higher risks of additional revisions following the first revision. More specifically, the aim of this thesis was to investigate if manipulation of the mevalonate pathway in the bone-implant interface with simvastatin (studies I and II) or zoledronate (study III) could improve early implant fixation.

Studies I and II, delivery of simvastatin using different polymer formulations

In studies I and II we investigated if locally delivered simvastatin could enhance implant fixation. Before we could evaluate if simvastatin could benefit orthopaedic implant fixation, the drug had to be delivered to the bone-implant interface by a vehicle that would not affect the bone-implant interface negatively. Therefore, study I was designed to detect any positive or negative effect of PDLLA, which was used as a vehicle for delivery of simvastatin in this study. Furthermore, two different doses of simvastatin (0.1 mg/implant or 1.0 mg/implant) were evaluated. We found that PDLLA in this formulation had a detrimental effect on the mechanical implant fixation and the formation of bone on the surface of the implant (ongrowth). The deleterious effect of PDLLA complicated the interpretation of the data from the simvastatin loaded PDLLA implants. Implants with only PDLLA had approximately an 80% decrease in fixation strength compared to the untreated titanium implants. Implants with PDLLA and simvastatin had roughly a 50-70% decrease in fixation strength compared to the untreated titanium implants. The reduction in fixation strength was statistically significant for ultimate shear strength and total energy absorption. Although a similar trend was evident for apparent shear stiffness it was not statistically significant.

In study II we evaluated a new carrier, PLGA microparticles ± 1.0 mg simvastatin. We also included a group with an empty PDLLA coating, both to confirm the results from study I and to compare the two local delivery vehicles. As a control group, untreated titanium implants were included in the study design. Implants with additional surface coatings showed inferior mechanical implant fixation and less new bone ongrowth compared to the untreated implants. Furthermore, implants with PLGA microparticles and simvastatin had less new bone formation in the peri-implant gap.

Provided that the delivery vehicle does not negatively affect the local environment, local delivery is desirable, ensuring that the augment reaches its target while the risk for adverse systemic effects is minimized. Polymers are widely used in vivo as orthopaedic devices, suture materials and as drug delivery systems. Overall, lactic acid based polymers are reported to have acceptable biocompatibility (99-101) and several authors have reported that these polymers have osteogenic potential (102, 103). Various degrees of foreign-body reactions and osteolysis around implants made of lactic-acid-based polymers have been described (104-106). During hydrolytic degradation of lactic-acid-based polymers, lactic acid is released. Due to the relatively closed environment of the effective joint space, this may cause a drop in local pH if the fluid exchange is not sufficient and the produced acid exceeds the buffer capacity of the local host tissue. Acidosis may affect bone by decreased mineralization (122) as the solubility of hydroxyapatite increases at lower pH. Furthermore, cell-mediated bone-resorption may be increased as pH is reduced (123). Merolli et al. reported that new bone is only formed after complete disappearance of the polymeric material (101). Besides the negative effect on bone formation, the polymeric coating material may also act as a simple barrier for bone ongrowth onto the implant surface, also acting to hinder osseointegration. In addition, SEM images of the coated surfaces revealed reduced surface roughness and porosity, which may have

contributed to reduced ongrowth.
These effects may provide an explanation why the PDLLA- and PLGA microparticle-coated implants had inferior mechanical fixation and decreased ongrowth of bone compared to untreated titanium implants.
The lack of effect of simvastatin on implant fixation and bone formation could be a result of 1) simvastatin does not stimulate bone formation, or does not stimulate bone formation as much in a higher order animal as it does in rodents, 2) simvastatin was inactivated during the coating procedure or the dose was not appropriate, 3) negative influence from the delivery vehicles, their formulation or processing in these studies.
Previous rodent studies in fracture models have reported an increase in biomechanical parameters and bone formation (55, 56, 70) after local administration of statins. We were not able to confirm these effects in our studies. The dose of simvastatin may not have bee appropriate. Other authors have suggested that simvastatin can be overdosed, resulting in a diminished effect (54), or possibly induce inflammation (124, 125). We did not see any clinical signs of inflammation during the observation period or signs of inflammation during the histomorphometrical analysis.
Furthermore, the release of lactic acid during degradation of the polymers may impair the conversion of simvastatin from the inactive lactone form the to active β-hydroxyacid form (126) and both polymer carriers may have blunted the effect of simvastatin. This contrasts with the findings by Pauly et al. who used a similar formulation of PDLLA for local delivery of simvastatin to a fracture site (56). Several studies (56, 70) have reported a local effect of simvastatin indicating that simvastatin was present in its active form. Since simvastatin does not actively target bone, the activation may not be complete and the effect of simvastatin could be impaired.
Although we were not able to make any conclusions on whether or not simvastatin benefits orthopaedic implant fixation, studies I and II suggest that the lactic-acid-based polymers and coating formulations as used in these studies may not be appropriate for delivery of augments to the bone-implant interface.
Polymers are also used for non-orthopaedic applications such as coatings for intra-arterial stents for local drug delivery to prevent restenosis (127) or induce angiogenesis (128). Lactic acid-based polymers may work well at sites like this where there is a continuous dilution of the polymer degradation products resulting a more favorable tissue response. Fracture sites may also have more blood perfusion compared to the peri-implant space and may benefit from local delivery of augments using lactic acid-based polymers as vehicle.

Study III, zoledronate-treatment of bone allograft around a revision implant
This study was conducted to investigate the effect of zoledronate-impregnated bone allograft compared to saline treated bone allograft impacted around revision implants. We hypothesized that the zoledronate treatment would retain bone allograft in a bone site that had been subjected to a loose implant without impairing the formation of new bone around a stable revision implant. We found that implants impacted with zoledronate-treated bone allograft had a statistically significant increase in biomechanical fixation in two out of three parameters (ultimate shear strength and apparent shear stiffness). Furthermore, in the zoledronate group, there was a three-fold increase in retained bone allograft compared to the control (saline) group. The zoledronate treatment did not negatively affect the formation of new bone on the implant surface or in the peri-implant gap.
In this study, the focus was on a problem often encountered by surgeons performing revision arthroplasties: insufficient bone stock. This may be compensated for by the use of bone graft. While the bone graft provides immediate support for the implant, this stability can be challenged when resorption of the bone graft proceeds at a greater pace than the remodeling of newly formed woven bone into structurally strong lamellar bone. The result may be a transient period of mechanical weakening, during which time a fibrous membrane could form, preventing osseointegration and long-term implant stability.
Previous studies with bisphosphonate (BP)-treated bone allograft in both bone conduction chambers in rodents (37, 40) and in non-weight bearing canine models of implant fixation (38, 39) suggest that this strategy can reduce resorption of bone allograft. While the rodent studies

reported BPs to decrease resorption without any negative effect on bone formation, Jakobsen et al. (38) and Baas et al. (39) reported that the mechanical implant fixation was impaired because new bone formation appeared to be sensitive to BPs. In these studies, unbound BPs had not been removed and was reported to be the probable cause of these adverse effects. Since the mevalonate pathway is an important cellular pathway present in all higher eukaryotes, the proapoptotic effects BPs are not restricted to only osteoclasts but they may affect any cell that is able to internalize them including osteoblasts. Idris et al. showed that N-BPs caused osteoblast apoptosis and inhibited protein prenylation in osteoblasts in a dose-dependent manner (129). Although there is no information on the concentrations of systemic administered BPs that osteoblasts, osteoclasts and osteocytes are exposed to in the bone microenvironment, osteoclasts are most likely exposed to higher concentrations than osteoblasts and osteocytes. This is because BPs bind to exposed bone mineral and are released and internalized by the osteoclasts during bone resorption, resulting in higher concentrations in the osteoclast cytoplasm. This also underlines the importance of removal of unbound BP after soaking bone allograft in BP solution asw was done in study III.

A dose-response study (41) with different doses of zoledronate suggested that increasing doses of zoledronate resulted in a higher volume of retained bone allograft but also increasingly blocked new bone formation. The lowest zoledronate dose for soaking bone allograft retained less bone allograft but did not impair bone formation. We chose the lowest dose (0.005 mg/ml) followed by thorough rinsing of the bone allograft to ensure removal of all unbound zoledronate. In our study, bone allograft was retained without impairing new bone formation, indicating an anti-catabolic effect of zoledronate without an inhibitory effect on bone formation. We were not able to demonstrate any anabolic effect of BPs as has been reported by other authors (77).

Although experimental studies have suggested a local effect on bone allograft of systemically administered BP (96, 97), we chose to soak the bone allograft in the zoledronate solution. This ensured that the drug was delivered locally to the non-vascularized bone allograft. Furthermore, by restricting the BP exposure to a local site, undesired effects in individuals not taking BPs for a bone disease would be limited. With the soaking procedure we were only able to control the concentration of zoledronate in the solution and not the exact amount of zoledronate that was adsorbed to the surface. To ensure equal amounts of zoledronate on the bone allograft the soaking and rinsing procedure was performed in a standardized manner. Even though we were not able to quantify the exact dose of zoledronate, we did observe an effect of the zoledronate treatment, suggesting that a sufficient dose of zoledronate was present on the bone allograft in the peri-implant gap.

In clinical practice, zoledronate treatment of bone allograft will be a simple procedure to perform after preparation and prior to impaction of the bone allograft. This may aid in retaining the bone allograft and the structural support of the implant may be sustained until osseointegration is sufficient. Based on previous experiments, topically administered zolendronate seems to have a therapeutic window and may be detrimental to implant fixation and new bone formation if overdosed. For these reasons, clinical trials that incorporate a wider spectrum of conditions present in revision arthroplasty are needed before implementing this promising and practical procedure in the clinical setting.

Strengths and limitations

For the studies included in this thesis, we used well-established experimental models of early implant fixation and osseointegration that mimic the fixation of clinical implant components in cancellous bone. Implants were not functional arthroplasties, but implants with a simple cylindrical shape. The porous surface on the experimental implants was intended to represent the surface uncemented implant components. In studies I and II implants were inserted extra-articularly in the proximal part of humerus and therefore not subjected to direct axial load but only to indirect load. In addition, there was no oscillating joint fluid pressure around the implants. In study III we used a more complex model, in an altered revision environment subjected to implant loosening and reimplantation. The revision cavity was created over a short

period (eight weeks) and does not reflect the complete spectrum of revision settings encountered by surgeons performing revision arthroplasty. However, the experimental protocol in this study does produce an environment and tissue response representative of aseptic implant loosening (43, 44). The conditions represent the mechanical conditions at a clinical bone-implant interface and the intraarticular loading implants can undergo.

In all studies the observation period was four weeks. This is a relatively short observation period but this time was chosen based on experience from previous studies using similar models investigating early implant fixation. Our observations only represent a single time point in the process of implant osseointegration. At later time points, differences between groups may have been leveled out, and any improvement or impairment of early implant fixation may have been impossible for us to detect within the study design and sample size applied for these studies.

Because the two sets of surgeries were conducted at two different time points and because of the paired study design we were not able to compare results between the studies conducted at the same implant sites (studies I and II). In each of the studies twelve pairs of four (I and II) or two (III) samples were compared. The sample size estimation indicated that ten animals were to be included. To counteract power loss if animals or implants were lost to follow-up, we included an additional two animals. Especially in study I, the data revealed a higher variance compared to previous studies using the same model. This may have prevented us from detecting a real difference of simvastatin delivered in a PDLLA coating compared to PDLLA alone. Had we known that the variance of the data would have been higher, we would have performed the sample size estimation accordingly and more animals should have been included.

8. Conclusion

In study I we could not confirm the main hypothesis that PDLLA coated onto the surface of titanium implants do not influence early implant fixation and that simvastatin delivered locally in a PDLLA coating improves the fixation of orthopaedic implants. In study II we investigated two different delivery systems for local delivery to the bone-implant interface, a PDLLA coating, identical to the one used in study I, and PLGA microparticles (empty or with 1.0 mg simvastatin). Both coatings resulted in decreased mechanical implant fixation and reduced bone formation at the surface of the implant. This was most pronounced for the PLGA microparticles. Studies I and II strongly suggest that the formulations of PDLLA and PLGA used in these studies may not be suitable for drug delivery to the bone-implant interface. These polymers may be appropriate in a wide range of other applications, but caution is warranted when choosing delivery vehicle for delivery of augments to the bone-implant interface.

In study III we evaluated the effect of zoledronate-treated bone allograft around revision implants. The treatment resulted in a significant increase in the mechanical implant fixation explained by increased preservation of the bone allograft without impairing new bone formation. In this way the bone allograft may be retained and the structural support of the implant may be sustained until osseointegration is sufficient. Zoledronate treatment of bone allograft may help improve early implant fixation of allografted revision implants, which could improve the longevity of revision joint replacements and reduce the risk of subsequent revisions. Based on previous experiments, topically administered zoledronate seems to have a therapeutic window and can be detrimental to implant fixation and new bone formation if overdosed. Clinical trials that incorporate the spectrum of conditions present in revision arthroplasty are warranted before widely implementing this promising and practical procedure in humans.

9. Perspectives and future research

In this thesis we have attempted to manipulate the mevalonate pathway (with simvastatin or zoledronate) to improve early implant fixation and osseointegration. In study I we were not able conclude if simvastatin offers any benefit in orthopaedic implant fixation. Most likely, this was because the delivery vehicle we used was shown to impair implant fixation. Provided simvastatin can be delivered using a more appropriate vehicle that does not negatively affect the bone-implant interface, we cannot rule out that local delivery of simvastatin to the bone-implant interface may aid early implant fixation. In study II we investigated another vehicle for local delivery, PLGA microparticles. This vehicle had similar detrimental effects on early implant fixation and was not suitable for local delivery to the bone-implant interface either. Local delivery of augments to the bone-implant interface is desirable to stimulate new bone formation and improve osseointegration. At minimum, the delivery vehicle must be neutral in regards to implant fixation and new bone formation and not have any negative effects as the formulations of polymers used for studies I and II. Future research could investigate new formulations of polymers and other types of vehicles for augment delivery to the bone-implant interface.

In study III, the mevalonate pathway was manipulated downstream of where simvastatin acts. We found zoledronate treatment of bone allograft to be an easy and practical method of retaining bone allograft without impairing new bone formation. Goals for future research in this area could be a clinical trial to evaluate if this treatment would benefit patients undergoing revision joint replacement. In the experimental setting, the anti-catabolic zoledronate treatment could be combined with a weak anabolic signal to stimulate bone formation. As bone resorption and bone formation is coupled in the basic multicellular unit, bone formation may be impaired if the osteoclasts are strongly inhibited and a strong anabolic stimulus may also act as a catabolic stimulus initiating bone resorption. The challenge is to balance the stimuli so the net result is bone formation and not bone resorption.

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Appendix

Doctoral and PhD theses from the Orthopaedic Research Group, Aarhus University Hoapital, Denmark.

Doctoral theses

1. Hydroxyapatite ceramic coating for bone implant fixation. Mechanical and histological studies in dogs
Kjeld Søballe, 1993
Acta Orthop Scand (Suppl 255); 1993; 54

2. Growth factor stimulation of bone healing. Effects on osteoblasts, osteotomies, and implant fixation
Martin Lind, 1998
Acta Orthop Scand (Suppl 283); 1998; 69

3.Calcium phosphate coatings for fixation of bone implants. Evaluated mechanically and histologically by stereological methods
Søren Overgaard, 2000
Acta Orthop Scand (Suppl 297); 2000; 71

4. Adult hip dysplasia and osteoarthritis. Studies in radiology and clinical epidemiology
Steffen Jacobsen, 2006
Acta Orthop Scand (Suppl 324); 2006; 77

5. Gene therapy methods in bone and joint disorders. Evaluation of the adeno-associated virus vector in experimental models of articular cartilage disorders, periprosthetic osteolysis and bone healing
Michael Ulrich-Vinther, 2007
Acta Orthop Scand (Suppl 325); 2007; 78

6. Assessment of adult hip dysplasia and the outcome of surgical treatment.
Anders Troelsen, 2012
Dan Med J. 2012 Jun; 59(6): B4450

PhD theses

1. In vivo and vitro stimulation of bone formation with local growth factors
Martin Lind, 1996
www.OrthoResearch.dk

2. Gene delivery to articular cartilage
Michael Ulrich-Vinther, 2020
www.OrthoResearch.dk

3. The influence of hydroxyapatite coating on the peri-implant migration of polyethylene particles
Ole Rahbek, 2002
www.OrthoResearch.dk

4. Surgical technique's influence on femoral fracture risk and implant fixation. Compaction versus conventional bone removing techniques.
Søren Kold, 2002
www.OrthoResearch.dk

5. Stimulation and substitution of bone allograft around non-cemented implants
Thomas Bo Jensen, 2003
www.OrthoResearch.dk

6. The influence of RGD peptide surface modification on the fixation of orthopaedic implants
Brian Elmengaard, 2004
www.OrthoResearch.dk

7. Biological response to wear debris after total hip arthroplasty using different bearing materials
Marianne Nygaard, 2005
www.OrthoResearch.dk

8. DEXA-scanning in description of bone remodeling and osteolysis around cementless acetabular cups
Mogens Berg Laursen, 2005
www.OrthoResearch.dk

9. Studies based on the Danish Hip Arthroplasty Registry
Alma B. Pedersen, 2006
www.OrthoResearch.dk

10. Reaming procedure and migration of the uncemented acetabular component in total hip replacement
Thomas Baad-Hansen, 2007
www.OrthoResearch.dk

11. On the longevity of cemented hip prosthesis and the influence on implant design
Mette Ørskov Sjøland, 2007
www.OrthoResearch.dk

12. 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, 2007
www.OrthoResearch.dk

13. Evaluation of Bernese periacetabular osteotomy; Prospective studies examining projected load-bearing area, bone density, cartilage thickness and migration
Inger Mechlenburg, 2007
Acta Orthop Scand (Suppl 329); 2008; 79

14. Rehabilitation of patients aged over 65 years after total hip replacement – based on patients' health status
Britta Hørdam, 2008
www.OrthoResearch.dk

15. Efficacy, effectiveness, and efficiency of accelerated perioperative care and rehabilitation intervention after hip and knee arthroplasty
Kristian Larsen, 2008
www.OrthoResearch.dk

16. Rehabilitation outcome after total hip replacement; prospective randomized studies evaluating two different postoperative regimens and two different types of implants
Mette Krintel Petersen, 2008
www.OrthoResearch.dk

17. CoCrMo alloy, *in vitro* and *in vivo* studies
Stig Storgaard Jakobsen, 2008
www.OrthoResearch.dk

18. Adjuvant therapies of bone graft around non-cemented experimental orthopaedic implants. Stereological methods and experiments in dogs
Jørgen Baas, 2008
Acta Orthop Scand (Suppl 330); 2008; 79

19. The Influence of Local Bisphosphonate Treatment on Implant Fixation
Thomas Vestergaard Jakobsen, 2008
www.OrthoResearch.dk

20. Surgical Advances in Periacetabular Osteotomy for Treatment of Hip Dysplasia in Adults
Anders Troelsen, 2009
Acta Orthop Scand (Suppl 332); 2009; 80

21. Polyethylene Wear Analysis. Experimental and Clinical Studies in Total Hip Arthrolpasty
Maiken Stilling, 2009
Acta Orthop Scand (Suppl 337); 2009; 80

22. Step-by-step development of a novel orthopaedic biomaterial: A nanotechnological approach
Thomas H. L. Jensen, 2009
www.OrthoResearch.dk

23. Osteoclastic bone resorption in chronic osteomyelitis
Kirill Gromov, 2009
www.OrthoResearch.dk

24. Use of medications and the risk of revision after primary total hip arthroplasty
Theis Thillemann, 2009
www.OrthoResearch.dk

25. Different fixation methods in anterior cruciate ligament reconstruction
Ole Gade Sørensen, 2010
www.OrthoResearch.dk

26. Postoperative pain relief after total hip and knee replacement; prospective randomized studies evaluating two different peri- and postoperative regimes
Karen V. Andersen, 2012
www.OrthoResearch.dk

27. A comparison of two types of osteosynthesis for distal radius fractures using validated Danish outcome measures
Jesper O. Schønemann, 2010
www.OrthoResearch.dk

28. Optimizing the cementation of femoral component in hip arthroplasty
Jouzas Petruskevicius, 2010
www.OrthoResearch.dk

29. The influence of parathyroid hormone treatment on implant fixation
Henrik Daugaard, 2010
www.OrthoResearch.dk

30. Strontium in the Bone-Implant Interface
Marianne Toft Vestermark, 2010
Dan Med Bull. 2011 May;58(5):B4286

31. The Applicability of Metallic Gold as Orthopaedic Implant Surfaces. Experimental animal studies
Kasra Zainali, 2011
www.OrthoResearch.dk

32. Mobile or Fixed Bearing Articulation in TKA? A Randomized Evaluation of Gait Analysis, Implant Migration, and Bone Mineral Density
Michael Tjørnild, 2011