FACULTY OF HEALTH SCIENCES
AARHUS UNIVERSITY

# The Applicability of Metallic Gold as Orthopedic Implant Surfaces

## Experimental animal studies

**PhD thesis**

**Kasra Zainali**



**Faculty of Health Sciences**
**University of Aarhus**
**2011**

FACULTY OF HEALTH SCIENCES

AARHUS UNIVERSITY

FACULTY OF HEALTH SCIENCES
AARHUS UNIVERSITY

# The Applicability of Metallic Gold as Orthopedic Implant Surfaces

## Experimental animal studies

**PhD thesis**

**Kasra Zainali**


**Faculty of Health Sciences**
**University of Aarhus**
**Orthopedic Dept. E, Aarhus University Hospital**

FACULTY OF HEALTH SCIENCES
AARHUS UNIVERSITY

"Your theory is crazy, but it's not crazy enough to be true" - Niels Bohr

## *Contact info*

Kasra Zainali, MD,
Orthopedic Research Center
Department of Orthopedic Surgery
Aarhus University Hospital
Nørrebrogade 44, 1A
DK-8000 Aarhus C
Mail: kasra.zainali@ki.au.dk

*Thesis defense April 1st. 2011*

FACULTY OF HEALTH SCIENCES
AARHUS UNIVERSITY

# Supervisors

## *Main supervisor*

Prof. Kjeld Søballe, MD, DMSc
Department of Orthopedic Surgery
Aarhus University Hospital, Denmark

## *Project supervisors*

Jørgen Baas, MD, PhD
Orthopedic Research Center
Department of Orthopedic Surgery
Aarhus University Hospital

Thomas V. Jakobsen, MD, PhD
Orthopedic Research Center
Department of Orthopedic Surgery
Aarhus University Hospital

## *Assessment Committee*

Associate Professor Sten Rasmussen – chairman of the committee
Dept. of Orthopedics
Aalborg Hospital,
Aarhus University Hospital, Denmark

Professor Pentti Tengvall, FPSE
Dept. of Biomaterials
Institute of Clinical Sciences
Sahlgrenska Academy
University of Gothenburg, Sweden

Chief Surgeon Ole Simonsen, DMSc
Dept. of Orthopedics
Aalborg Hospital,
Aarhus University Hospital, Denmark

# Preface

This thesis is based upon the experimental studies performed at the Orthopedic Research Laboratory, Department of Orthopedics, Aarhus University Hospital in collaboration with Orthopedic Research Laboratory, Hennepin Medical County Center, Minneapolis, USA, during my enrolment as an MD/PhD student at the Faculty of Health Sciences, Aarhus University 2008 – 2011. The work is a continuation of my work as a research year student 2006 – 2007. The Faculty of Health Sciences, Aarhus University, kindly financed the complete MD/PhD study.

I am greatly indebted to my main supervisor, Professor Kjeld Søballe MD, for involving me into the field of orthopedic research and providing fantastic research facilities and work surroundings. Much appreciation and admiration goes to my project supervisors Jorgen Baas MD, PhD, and Thomas Jakobsen MD, PhD for their enthusiastic and tremendous help, and their endless knowledge and support, that has thought me many aspects of research, helping me focus, and in the end making it all possible.

Also, I would especially like to thank Professor Gorm Danscher for involving me into his world and his ideas. I am very grateful for the many long and creative discussions, we have had. I would also like to thank the highly skilled Professor Per Møller from DTU for his involvement and inputs in these studies and I fondly look back upon many of our long talks, capable of draining any mobile battery. These studies could not have been possible without the help and effort of Professor Joan Bechtold, thank you very much for creating a wonderful time for us every time we meet. I would also like to thank our highly skilled laboratory technicians Anette Baatrup, Jane Pauli, Anette Milton, Anna Bay Nielsen, and Lisa Feng for their expertise and their continuous help, always with a warm smile. Furthermore, I would like to thank my co-workers at Orthopedic Research Laboratory and everyone I ever have shared an office with for creating a wonderful working space and a unique time of my life, which I shall treasure forever.

I would also like to thank my friends and family for their encouragement through the years. Finally, I would like to thank my beloved Rose, for sharing this with me, for her support and comfort in needed times, and the ability to always say the right thing and just being there.

# The PhD thesis is based on the following papers:

I. Effects of gold coating on experimental implant fixation. Zainali K, Danscher G, Jakobsen T, Jakobsen S, Baas J, Møller P, Bechtold J, Soballe K. *J. Biomed Mater. Res. Part A, 2009, Jan. 88(1): 274-280* *

II. Particulate gold as an anti-inflammatory mediator in bone allograft – an animal study. Zainali K, Baas J, Jakobsen T, Danscher G, Soballe K. *J. Biomed Mater. Res. A. 2010, Dec 1;95(3):956-63J.*

III. Partial gold coated implants as anti-inflammatory mediator without disturbing early implant fixation. Zainali K, Danscher G, Jakobsen T, Baas J, Møller P, Bechtold J, Soballe K, *J. Biomed Mater. Res. Part A, submitted 2010, August****

The papers will be referred in the text by their Roman numerals (I-III)

* Presented at the autumn meeting for "The Danish Orthopedic Society," Copenhagen 2006, and annual meeting for "Orthopedic Research Society," San Diego 2007, and the annual meeting for "European Federation of National Associations of Orthopedics and Traumatology," Florence 2007.

** Presented at the annual meeting for "Orthopedic Research Society," Las Vegas 2009, and the spring meeting for "The Danish Orthopedic Society, Odense 2009.

***Presented at the autumn meeting for "The Danish Orthopedic Society," Copenhagen 2009, and the meeting for "Nordic Orthopedic Federation," Aarhus 2010 and "The 7th. Combined Orthopedic Research Society Meeting," Kyoto 2010.

**Awards:**

(I) was awarded the "Best Poster Award" by The Danish Orthopedic Society at the annual autumn meeting, Copenhagen 2006.

(III) was awarded the "Best Poster Award" by The Faculty of Health Sciences, Aarhus University at the annual PhD Day 2010.

# Contents

# Abbreviations

| | |
|---|---|
| *ALP* | Alkaline phosphatase |
| *AMG* | Autometallography |
| *Au* | The periodic symbol for gold. |
| *$AuCl_4$* | Gold chloride |
| *$AuCN_2$* | Gold cyanide complex |
| *AuTG* | Aurothioglucose |
| *AuTi* | Partial gold-coated titanium |
| *BMP* | Bone morphogenetic proteins |
| *DNA* | Deoxyribonucleic acid |
| *EDS* | Energy dispersive X-ray spectroscopy |
| *GST* | Gold sodium thiomalate |
| *GTM* | Gold thiomalate |
| *HLA* | Human leukocyte antigen |
| *$H_2SO_4$* | Sulfuric acid |
| *IKK* | Inhibitors of kappa B kinase |
| *IL-1* | Interleukin - 1 |
| *IL-6* | Interleukin - 6 |
| *IL-8* | Interleukin - 8 |
| *K-wire* | Kirschner wire |
| *mRNA* | Messenger ribonucleic acid |
| *M-CSF* | Macrophage colony-stimulating factor |
| *MTX* | Methotrexate |
| *NaOH* | Sodium hydroxide |
| *NF- k B* | Nuclear Factor kappa-light-chain-enhancer of activated B cells |
| *NSAID* | Nonsteroidal antiinflammatory drug |
| *OA* | Osteoarthritis |
| *OPG* | Osteoprotegrin |
| *$PGE_2$* | Prostaglandin E2 |
| *PMMA* | Polymethyl methaacrylate |
| *RA* | Rheumatoid arthritis |
| *RANK* | Receptor activator of NF-kB |
| *RANKL* | Receptor activator of NF-kB ligand |
| *RGD-integrins* | Arg-Gly-Asp integrins |
| *SEM* | Scanning electron microscope |
| *THR* | Total hip replacement |
| *Ti* | The periodic symbol for titanium |
| *Ti6Al4V* | Titanium-Aluminum-Vanadium alloy |
| *TNF-$\alpha$* | Tumor necrosis factor – alpha |
| *TRAP* | Tartrate-resistant acid phosphatase |
| *UHMWPE* | Ultra high molecular weight polyethylene |

# English summary

Total hip arthroplasty is a successful form of surgical intervention offered to patients with severe end-stage degenerative joint diseases. Although considered successful, with an impressive increase in mobility, improved quality of life, and satisfactory pain relief, improvements are still needed, as serious problems regarding long-term implant longevity still exist. Today, aseptic implant loosening is considered to be the main reason for revision surgery. It is regarded as a low-grade inflammatory process, initiated by activated macrophages, which phagocytizes wear particles, leading to periprosthetic osteolysis and bone defects. The anti-inflammatory capabilities of gold ions have been established and used in inflammatory joint diseases for decades. However, the recent finding that metallic gold implants liberate gold ions in living tissue, a treatment termed *auromedication*, creates an opportunity to locally counter-act the inflammatory process that occurs around an implant.

The aim of the present experimental studies was to investigate whether metallic gold has a clinical role to play in an orthopedic context. The possibility to suppress periimplant inflammation by adding metallic gold to the implant surface facing the bone was studied in short-term experimental studies. These were conducted to address the initial osseointegration of two different gold-coated implants and their mechanical fixation. In addition, gold particles were added to bone allograft to reduce allograft reabsorption, increase total bone stock, and thereby increase initial mechanical fixation.

The results showed that gilded implants and gold particles release gold ions in bone. They also showed that complete gold-coated implants have reduced mechanical strength due to reduced implant osseointegration. By reducing the thickness of the gold coating and the amount of the gold-coated surface area, a mechanical fixation was achieved that was equal to that of control titanium implants. However, no inhibitory effect was found on fibrous tissue formation, as we had hypothesized, and mixing gold particles with the allograft did not give rise to measurable anti-resorptive effects.

In conclusion, the studies done in connection with this thesis shows that gold ions are released from gilded implants and gold particles. Additionally, they show that auromedication has no observable effects on early fibrous tissue formation or allograft reabsorption but does have an inhibitory effect on bone formation resembling that caused by other anti-inflammatory drugs administered during or after surgery. The results also suggest that metallic gold, although having no role in the early phase of implant surgery, may have a clinical effect if applied to only part of the implant surface by adding long-term anti-inflammatory capabilities to the implant surface. The possibility of enriching implants with dots of metallic gold in order to obtain an inbuilt permanent suppressor of aseptic peri-prosthetic inflammation along the bone-implant zone is interesting and warrants further investigations.

# Danish summary

Degenerative ledsygdomme er en hyppig bevægeapparats lidelse, som både har store personlige og samfundsøkonomiske konsekvenser. En indsættelse af kunstige ledproteser, som f.eks. hofte-alloplastik, er en kirurgisk behandlingsform, der, med en klar forbedring i bl.a. livskvalitet, mobilitet samt ved smerter, betragtes som meget succesfuld. Men på trods af protesekirurgiens enestående succes har implantaterne et svagt punkt med hensyn til deres langtidsholdbarhed. I dag skyldes langt den største del af revisionskirurgien en aseptisk implantat-løsning, forårsaget bl.a. af cellulære reaktioner på partikler, som er produceret ved almindeligt slid og kroppens reaktion på fremmedlegemer.

Guldioners anti-inflammatoriske egenskaber er blevet påvist og udnyttet i årtier, især ved inflammatoriske ledsygdomme. I 2002 blev det påvist, at metallisk guld angribes af makrofager ved anbringelse i en organisme. Ved denne proces, som nu er døbt dissolucytosis, frigøres guldioner fra den forgyldte overflade. Den nylige forskning, om metalliske guldoverfladers tilsvarende anti-inflammatoriske effekter i væv, har medvirket til at iværksætte dette Ph.d. studium, der undersøger, om metallisk guld og dets frigjorte ioner kan have en fremtidig rolle indenfor ortopædkirurgien. Metallisk guld og dets virkning på den inflammatoriske proces, og dermed knogleintegrationen af et implantat, er nødvendig at undersøge.

Studiets formål har været at undersøge, om dissolucytosis finder sted i knoglevæv, og hvorvidt metallisk guld kan have klinisk relevans for ortopædkirurgien. I korttidsstudier blev to forskellige guldbelagte implantater studeret, et totalt forgyldt og et delvist forgyldt, for at kortlægge, om der blev frigjort guldioner, om de påvirkede den mekaniske stabilitet, og om mængden af dissolucytotisk frigjorte guldioner var signifikant. I et forsøg med guldpartikler iblandet allograft blev guld-ionernes evne til at reducere knoglemodellering undersøgt. Netop ved at hæmme allograft-reabsorptionen kan den totale mængde knogle i den tidlige fase øges, hvorved den mekaniske stabilitet styrkes.

Resultatene viser: 1) Metallisk guld frigiver guldjoner ved dissolucytosis, når det anbringes i relation til knoglevæv. 2) Komplet forgyldte implantater fremkalder væsentligt nedsat mekanisk styrke. 3) Dosisreduktion, dvs. reduktion i det implantatareal, der er dækket af guld, giver uændret mekanisk styrke. 4) Hæmning af fibrøs vævsdannelse ses ikke ved blanding af guldpartikler med allograft, ligesom blandingen heller ikke påvirker den mekaniske stabilitet eller histologiresultaterne.

Konkluderende kan det siges, at guldioner frigives ved dissolucytosis fra metallisk guld, anbragt i relation til knoglevæv. Et komplet guldbelagt implantat nedsætter knogle-nydannelse i tråd med andre anti-inflammatoriske behandlinger. Disse resultater viser, at metallisk guld ikke har en favorabel rolle i den tidlige fase i implantatkirurgien. Men ved at kombinere guld og titanium som overflademateriale kan sammenlignelige resultater i stedet opnås. Dette sandsynliggør, at en moderat tilførsel af metallisk guld til implantatoverflader kan tilføre varige inflammationsdæmpende kvaliteter uden at påvirke implantat-knogle zonens mekaniske styrke. Det vil kræve et betydeligt viden-skabeligt arbejde at verificere denne hypotese.

# Introduction

Total hip replacement (THR) is a common treatment of end-stage degenerative joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA.) THR is regarded as the most cost-effective treatment to regain patient mobility with high patient satisfaction. Over 800,000 THRs are carried out annually worldwide [1], and in Denmark, approximately 8,500 THRs are performed each year. The success of the procedure, with improvements in infection risks, material properties, and surgical techniques in combination with the implementation of fast-track surgery, has led to an increase in the number of surgically treated patients, and the incidence is expected to increase dramatically during the coming years [2].

The successful outcome of the procedure has made surgical intervention in young patients with degenerative joint diseases more rational. Young patients, who are more active and have a longer life span, have higher demands regarding their THRs. Unfortunately, a decrease in stability and longevity has been observed in most young and active patients. The annual Danish hip Register report states that approximately 20% of THR patients younger than 50 years will be in need of revision surgery within 14 years [3]. In view of the fact that implant number two has a significantly lower success rate because of decreased bone quality at the implant site, revision surgery is indeed something to be avoided. Several authors have documented the relationship between increased physical activity and implant wear, and the influences of these factors on implant longevity [4-6]. It is also well accepted that late implant loosening occurs primarily due to aseptic loosening, which also is the major concern in younger, more active patients [7].

Several studies show that early mechanical fixation of an implant increases the lifetime of the prosthesis, but the exact mechanisms are still uncertain [8, 9]. The supposition is that instability allows implant micro-motion that results in the formation of a fibrous layer [10]. The fibrous layer between the implant and the bone leaves room for wear particle distribution around the implant. This will subsequently initiate a particle-induced, periimplant osteolysis after the activation of macrophages and osteoclasts. Today, tremendous effort is being put into the development of new materials that can increase early implant fixation and into the development of newer, stronger, frictionless acetabular components that reduce wear debris. However, aseptic loosening of THR implant, defined as long-term periimplant osteolysis due to an inflammatory process in the periimplant tissue [5], is still the main reason for revision surgery [3].

## Aim

The overall aim is to contribute to an increase in the longevity of THR implants and thereby minimize the need for revision surgery. The thesis will focus on the effect of metallic gold on the early stages following implant insertion.

The specific aim with this thesis is to investigate the use of metallic gold in orthopedic context. Previous studies have shown both a local and a systemic up-regulation of the inflammatory response after orthopedic surgery [11]. It is responsible for bone healing and implant integration and any anti-inflammatory intervention can influence the early osseointegration and periimplant bone remodeling. Furthermore, anti-inflammatory drugs (e.g. NSAIDs) have shown a significant negative effect on fracture healing such as reduced bone healing [12, 13]. This in combination with the recent understanding of metallic gold's anti-inflammatory capabilities made it interesting to investigate how metallic gold would affect implant fixation and stability. The studies presented in this thesis all focus on the effects of metallic gold on early implant osseointegration and mechanical stability. We imagined that sufficient early mechanical fixation needed to be addressed, because increased mechanical stability eventually leads to an increase in implant longevity [8, 9]. Additionally, the effect of metallic gold on peri-implant bone remodeling is investigated. Finally gold's ability to inhibit fibrous tissue formation is investigated, and a new implant surface model is suggested.

All experiments are conducted in a well-established animal model. Mechanical strength and stiffness are measured with biomechanical push-out tests, stereological histomorpho-metrical analysis is performed to quantify bone volume and bone in contact with the implant, and gold ion release is monitored with the highly sensitive technique termed autometallography.

## Hypothesis

This PhD thesis consists of several hypotheses:

1. The dissolucytosis of gold ions from solid metal implants described in soft tissue also takes place in bone tissue as well (I + II).

2. Metallic gold coating increases early osseointegration, seen as increased bone ongrowth, and thereby strengthens mechanical fixation (I).

3. Gold particles reduce bone graft reabsorption, thereby increasing total bone stock and subsequently implant strength and fixation (II).

4. Partial metallic gold coating increases osseointegration and mechanical fixation, whilst fibrous tissue formation is decreased (III)

# Background

## The story of gold

### Introduction to gold

Gold, with the periodic symbol "Au," derived from the Latin word *"Aurum"* meaning "shining dawn," has the atomic number 79. It is soft, shiny, dense and the most ductile pure metal known. Its bright yellow color, which does not fade due to oxidization with air or water, has made it into a symbol of status and wealth. Throughout history, gold has been highly valued and is related to positive symbolic meaning, ideologies, and religious beliefs. In sport events or other competitive contests, winners are awarded gold medals, gold prizes, and golden statuettes. Aristotle most famous philosophic tenant is referred to as *"the golden mean*" and is about the desirable middle path between two extremes. In Christianity, the halos of Christ, Mary and the Saints are always pictured golden. Today, gold has a positive symbolic meaning. Companies give their highly valued customers *"gold cards"* and phrases such as *"heart of gold"* and *"the golden years"* referring to the wisdom of aging are still used.

The chemical properties of gold are well established. It is a transition metal, and it forms trivalent and univalent cations in solutions. Gold is relatively non-reactive, but different mixtures of hydrochloric acid, nitric acid, and also alkaline cyanide solutions can dissolve it. Although gold is a noble metal, it can form many diverse compounds with different oxidation states. The most common oxidation state is the Au(I) that is found as $Au(CN)_2$ when gold is extracted from low-grade gold ores. Gold cyanide is also the electrolyte used in the electroplating of other base metals. Different solution also exists, and each dissolution form is used for its specific purpose.

### Gold in medicine

Gold is invaluable in modern medicine because of its unique physical and chemical properties. Its biocompatibility combined with its high ductility allows its usage in many different settings, such as an implant in the reconstruction of the upper eye lid after lagophthalmus and its use in the restoration of the ear function after chronic otitis media or trauma, as a coating of endovascular stents, as marker in catheters, and a coating of parts of guide wires, not to mention its use as dental prostheses such as crowns, inlays, and bridges [14].

The use of gold in different therapeutic models has been known since ancient times. Metallic gold plates were used to reconstruct skull defects over 3000 years ago. In ancient China, gold was used to treat smallpox and measles, and in Japan, eatable, thin gold foil was, and still is, ingested for health benefits. In the sixteenth century, it was used as a remedy to treat epilepsy, melancholia, and other nervous disorders. Furthermore, gold preparations during the 19th century were administered as a treatment for drug addiction and alcoholism [15]. Because dementia was one of the more frequent, serious syphilic symptoms, the Frenchman Jean André Chrestien published in 1821 his research about the use of gold in the treatment of syphilis entitled *"Recherches et observations sur les effets des préparations d'or*." His interest came from the observation that gold had fewer side effects than mercury, which was the more common treatment at that time. He also reported that in occasional cases, vitality and intellectual functions improved. Medical doctor and homeopath James C. Burnett published his treatise in 1879, in which he discussed Chrestien's findings. Apparently, the usage of

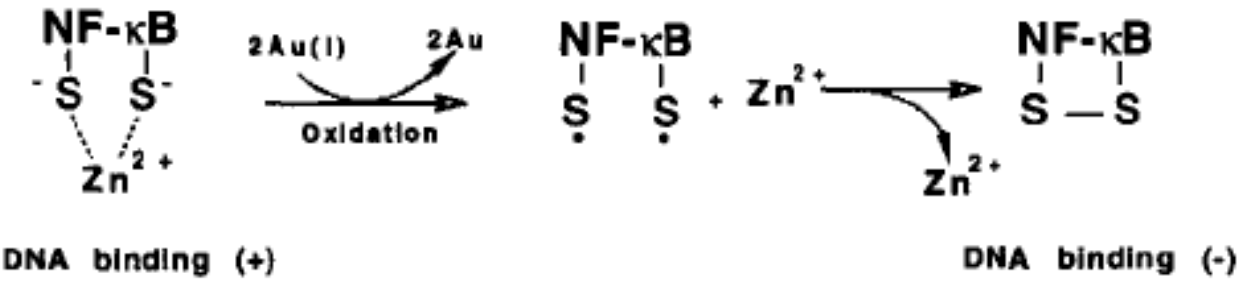



**Figure 1 - From Yang et al. [21]. Oxidation of NF-κB by gold cation: The active NF-κB complex is hypothesized to contain Zink. Gold eventually oxidizes the thiolate anions and the DNA binding is prevented.**

**Parenteral gold – Action mechanisms**

- Inhibited **lymphocyte** proliferation and activation
- Inhibited immunoglobulin production in **B lymphocytes**
- Shift from proinflammatory **Th1** to anti-inflammatory **Th2** cells
- **Macrophages**:
  i. Reduction of infiltrating macrophages
  ii. Inhibition of antigen uptake, antigen processing, and antigen presentation.
  iii. Reduction of proinflammatory cytokines
- Reduction of **adhesion molecules** → reduced cell infiltration
- **Fibroblasts**:
  i. Inhibition of formation
  ii. Toxic deformation
  iii. Lysis of fibroblasts
- **Leukocytes:** chemotaxis and phagocytosis are inhibited
- NF-$\kappa$B DNA binding inhibited
- Inhibition of IKK activation
- Inhibition of I$\kappa$B degradation
- Angiogenesis: Inhibition of vascular endothelial growth factor
- **Lysosomal enzyme release** is inhibited
- **Complement activation** is inhibited

**Table 1. A list of several documented and suggested gold compounds' action mechanisms. Modified from Rolf Rau [17]**

gold had been abandoned by the medical profession for a period of time, but Chrestien's publication made gold popular again, and by the end of the 19th century, and the first half of the 20th century, gold was listed as an approved treatment for nervous disorders.

Robert Koch, medical Nobel Prizewinner and the founder of modern microbiology, discovered the bacteriostatic effect of gold salts in an in vitro setting with mycobacterium tuberculosis [16]. At that time, it was believed that bacteria caused rheumatoid arthritis (RA), and gold compounds were therefore administered to these patients [17]. Further investigations distinguished tuberculosis and RA as different diseases, but the clinical impact on rheumatoid arthritis could not be ignored. The earliest work on gold's effect on RA is by J. Forestier in the early 1930s, who demonstrated gold compounds effectiveness by observing and documenting remission or at least a temporary inactivation of the disease for 2 to 3 years in 70%–80 % of 550 gold-treated RA patients [17, 18]. The therapy became known as *chrysotherapy*. After Forestier's study, which displayed the therapeutic indication and positive clinical effect of gold compounds, several other authors have investigated gold's therapeutic effect on RA. At first, gold was introduced due to its bacteriostatic effects, also called its oligodynamic properties. But as knowledge of the inflammatory aspect of the disease accumulated, gold compounds' different effects on different locations in the immune system became better illustrated. However, the emergence of newer biological anti-inflammatory drugs has displaced gold compounds as the primary therapy. This is in particular due to the high nephrotoxicity of gold compounds. Still, when all medication fails, gold salts are used for these chronic patients [17].

## The anti-inflammatory aspects of gold ions

With progress in understanding the pathophysiology of RA, investigations of parenteral gold compounds, such as gold sodium thiomalate (GST), gold thiomalate (GTM), and gold thioglucose (AuTG), had an important impact on the treatment of rheumatoid patients. It was early accepted that the clinical effects did not rely on a single inhibitory effect and soon, gold's diverse functions were documented [19, 20]. More recent studies suggest that gold salts' anti-inflammatory capabilities are based on cellular transcription factors.

The cellular transcription factor nuclear factor-kappa B (NF-$\kappa$ B) is a protein complex that controls DNA transcription. NF-κB is a so-called "rapid-acting" transcription factor that is present in all cells in an inactive form. This makes it possible to react quickly to any relevant stimuli. Activation of NF-$\kappa$ B and its binding to relevant DNA will allow a quick alteration in gene expression, production of relevant mRNA sequences, and subsequently protein production. NF-$\kappa$ B is inactive in un-stimulated cells and is found sequestered by inhibitory proteins, also known as I-$\kappa$B (inhibitors of $\kappa$B). The activation of NF-$\kappa$ B is caused by the degradation of these inhibitory proteins. This is done through activation of the kinase called the I$\kappa$B kinase (IKK). When a sufficient stimulus is registered, IKK is activated and the phosphorylation of IKK leads to the degradation of the inhibitory proteins

and thereby the release of the NF-κ B-complex, which again will quickly bind to specific DNA binding sites, the so-called κB motifs.

Jian-Ping Yang et al. showed that the binding between κB motifs and the NF-κ B-complex is regulated by a redox mechanism, and zinc ions were suggested to play an essential role. They found that AuTG inhibited NF-κ B-DNA binding in vitro, as illustrated in Figure 1. It was suggested that gold oxidized the thiol groups of the NF-κ B-complex and thereby prevented its direct DNA binding activity [21]. However in 2000, Jeon et al. showed that gold compounds reduced NF-κ B activity owing to their ability to inhibit IKK and the degradation of I-κB [22]. Furthermore, in 2003, Yamashita et al. published a study testing lower doses of gold compounds, which they termed as more clinically relevant. The study found no direct inhibitory effect on NF-κ B DNA-binding, but could still show that macrophages pre-incubated with auranofin, GTM, or AuTG reduced NF-κ B translocation [23]. Subsequently, specific end-results such as nitric oxide and PGE2 levels were also reduced.

At present, the exact mechanisms behind the therapeutic effects of gold compounds are not clarified, but its effects on the inflammatory response are well established. Likewise, the connection between gold compounds and the NF-κB transcription factor axis is quite evident. In vitro studies show that gold compounds are able to reduce release of inflammatory cytokines such as TNF-α, IL-6, and IL-8 [24-26]. Other studies illustrate that gold salts in the form of GST reduce lysosomal enzyme release [20]. GST has later been found to inactivate the C1 component of the complement in rheumatoid synovial fluid [27], inhibit monocyte chemotaxis, and reduce differentiation of monocytes to macrophages [28]. Furthermore, several randomized clinical studies show that parenteral gold treatment with substances such as AuTG and GST is more effective than placebo, more effective than auranofin (oral gold treatment), and as effective, or in some cases more effective, than anti-inflammatory treatment with methotrexate (MTX) [17, 29], which is an appreciated first choice treatment of RA.

However, at present, gold treatment, or crysotherapy, is somehow overlooked due to its adverse reactions. The side effects of parenteral and oral gold treatment are well documented and listed in Table 2. Although the list seems quite long and formidable, Rolf Rau advocates, through his comparative review of disease-modifying anti-rheumatic drugs, that the most common side effects are the mild ones (skin and mucosa lesions). These are harmless and occur most often during blinded clinical trials in which there were no possibility to adjust the dosage. Furthermore, the patients with the mild adverse reactions also seem to be the patients who have the best long-term response. As the list displays, quite severe adverse effects have also been observed and documented. In our time, crysotherapy has slowly been replaced by more modern (and more expensive) anti-inflammatory treatments because of its adverse reactions, although close monitoring of treated patients can prevent severe side effects.

| Adverse reactions due to chrysotherapy | Parenteral gold | Oral gold |
|---|---|---|
| **Dermatological** | | |
| Dermatitis | 39 % | 26 % |
| Stomatitis | 18 % | 13 % |
| Pruritus | 21 % | 17 % |
| Alopecia | <1 % | < 1 % |
| Chrysiasis | < 1 % | NR |
| **Hematological** | | |
| Thrombocytopenia | <3 % | 1 % |
| Granulocytopenia | 2 % | 1 % |
| Anemia | 3 % | 3 % |
| Aplastic anemia | < 1 % | NR |
| **Renal** | | |
| Proteinuria | 5 – 10 % | 2 % |
| Nephrotic syndrome | < 2 % | < 1 % |
| **Pulmonary** | | |
| Diffuse fibrosis | < 1 % | < 1 % |
| Bronchiolitis | < 1 % | < 1 % |
| **Gastroinstestinal** | | |
| Diarrhea | 13 % | < 50 % |
| Enterocolitis | < 1 % | < 1 % |
| Cholestatic jaundice | < 1 % | < 1 % |
| Elevated liver tests | 2 % | 2 % |
| **Cardiovascular** | | |
| Nitritoid reactions | a | NR |
| Syncope | < 1 % | NR |
| **Ophthalmic** | | |
| Corneal/lens chrysiasis | < 1 % | NR |
| Conjunctivitis | < 1 % | 10 % |
| Iritis | < 1 % | NR |
| Corneal ulcer | < 1 % | NR |

a = Sodium aurothiomalate
NR = not reported

**Table 2 - List of adverse reactions, modified from Tozman et al. [30]**

## Auromedication

Gold treatment has until recently focused on various diluted gold-thio solutions or the newer orally administered auranofin. In 1968, Kanabrocki could report that eight patients

with gold wedding bands had significantly higher traces of gold in their fingernails compared to patients without gold rings [31]. Mulherrin in 1997 reported that RA patients with gold rings on their ring finger had reduced progression of their disease on that particular finger and suggested gold could accumulate in the nearby joint in low but sufficient amounts [32]. Both authors were unable to explain their results. They suggested that gold ions could somehow be transported through the skin and perhaps have an effect on a nearby joint. However, their suggestion contradicted the belief that gold is an inert material that does not oxidize in physiological settings, not to mention on the skin.

Gorm Danscher, in 2002, documented that solid metallic gold implants inserted in vivo liberated ions that could be traced and visualized in macrophages, mast cells, and fibroblasts [33]. Furthermore, the uptake of these ions has recently been described and termed dissolucytosis [34], a process defined as an extracellular dissolution of metallic ions from metallic surfaces. The process is initiated immediately after implantation of a metallic implant by macrophages attaching to the "foreign body" and occurs due to the creation of a thin biofilm around the implant [35]. The biofilm attracts the dissolucytes [36-38], primarily monocytes and macrophages, which are believed to monitor and regulate the dissolucytosis. Macrophages have previously been shown to release cyanide, regulate oxygen tensions, and regulate pH values in their surroundings [39]. It is suggested that the liberation of gold ions occurs due to the release of cyanide and thereby the formation of aurocyanide ($Au(CN)_2$) [40], the same aurocyanide used in the mining industry to extract gold from low-grade ores. Macrophages, mast cells, and other cells adjacent to gold or gilded implants take up the Au ions. In addition, urine from patients treated with chrysotherapy has shown traces of $AuCN_2$ [41], consolidating the concept that cyanide is essential in this process.

The opportunity to treat locally with gold is now termed auromedication [33]. This treatment can bypass the systemic spread of gold compounds and thereby reduce the possible serious adverse effects mentioned previously (Table 2). With this in mind, Larsen et al. showed that gold particles too large to be phagocytosized could reduce several inflammatory processes in the central nervous system after traumatic focal brain injury in mice [42]. This was further acknowledged in the recent work of Pedersen et al., who could document that TNF-α secretion, oxidative stress, and inflammatory cell proliferation, among other factors could be reduced by the same model [43-45]. Auromedication as a small release of gold ions by the dissolucytosis of inserted gold implants or gold particles has a significant impact on the inflammatory response after a traumatic injury, or as seen in the "wedding band cases," an effect on RA. The use of auromedication is still in its early phase but its apparent possibilities leaves room for further investigations.

## Reconstructive joint surgery

### Introduction to reconstructive joint surgery

Total joint replacement, especially THR, is known to relieve pain, increase patient mobility, and increase quality of life dramatically. The most common indication is osteoarthritis, but THR is also indicated for the treatment of RA, avascular necrosis, bone tumors, and other variations of arthritis such as juvenile RA and arthritis after Paget's disease. Modern reconstructive joint surgery is based upon Sir John Charnley's (1911-1982) revolutionary work with the novel 3-way designed joint replacement:

1. Metallic femoral component (at first stainless steel, but later replaced by other bulk materials such as titanium alloys)
2. An acetabular component (Teflon, later changed to more wear-resistant ultra high molecular weight polyethylene (UHMWPE).
3. Bone cement (PMMA)

This became known as Charnley's "Low-Friction Arthroplasty," and with further improvements, such as the replacement of the stain-less steel femoral component with titanium stems and the introduction of new acetabular surfaces, the cemented THR is still the gold standard to which all new procedures are compared with [46].

Peri-implant osteolysis and loosening of the cemented implants were observed approximately 10 years after the first THRs and among the younger patients, even earlier. This process became known as the "cement disease," and therefore new uncemented femoral stems were investigated [47]. This finally led to the development of the rough surfaced, porous surfaced, or bioactive coatings intended to stimulate bone ingrowth and bone ongrowth. Today uncemented femoral stems are widely used especially among the younger patient groups [48].

## Uncemented implants

The first uncemented implants were smooth surfaced, but the observed high failure rates were unacceptable. Today uncemented implants consists of rough surfaces such as porous-coated surfaces, grid-blasted surfaces, or etched surfaces. These techniques increase the implant's surface area in contact with the surrounding bone (bone-implant interface). They are designed to promote bone ongrowth and increase the interlock between the implant and the periimplant bone, factors that increase early mechanical fixation. Studies show that enhanced initial implant fixation improves implant longevity [8, 9]. It is believed that without a sufficient firm mechanical fixation, implant micro-motion allows joint fluids to accumulate in the bone-implant interface and thereby permit fibrous tissue formation [49]. In the effort to increase the implant's mechanical fixation, various methods and techniques (e.g. use of growth factors, special surface (nano) textures, osteoconductive coatings) have been and still are being investigated in an effort to increase osseointegration.

Implants coatings such as the hydroxyappatite coating has shown positive clinical results [50]. The layer is applied to the implant surface, and bone is quickly formed on the surface, enhancing early mechanical stability. Hydroxyappatite-coated implants have strengthen short-term mechanical fixation and thereby shown positive long-term results. These implants are therefore a reliable alternative to uncoated porous implants. "The hydroxyappatite coating story" makes it realistic to investigate other implant surfaces that can increase initial fixation by means of local treatment. Initial sufficient fixation is vital for longevity because early implant micro-motion above 150 μm impairs osseointegration and allows fibrous tissue ingrowth [49]. After sufficient early fixation is achieved, long-term results should then be investigated, as was done in the case of hydroxyappatite coatings.

## Early implant fixation and osseointegration

The fixation of uncemented implants is primarily dependent on bone ongrowth and periimplant bone quality. Insertion of an orthopedic implant is a traumatic, invasive procedure, and the early regeneration of bone around an uncemented implant is much like fracture healing. The integration of the implant can be described as taking place in three phases:

- Inflammatory phase
- Reparative phase
- Remodeling phase

Immediately after implant insertion, the implant surface is covered by a thin biofilm [35]. This protein layer attracts inflammatory cells, and the first cells to respond are macrophages. The attraction is performed through several complex pathways. It is suggested that the complement system is activated, and C3 is cleaved to C3b and attaches to the implant surface where it binds the macrophage surface receptor (CD35). Proteins such as fibronectin and vitronection also attach to the implant surfaces and attract macrophages. This is done through the RGD-integrin receptor domains [38]. These ligand-interactions are the primary and the most important interactions between the inserted implant's surface and the periimplant tissue. Activated macrophages secrete cytokines that attract inflammatory cells, and they in turn secrete bone morphogenetic proteins (BMP) [51]. Tremendous effort has been put into the study of BMPs, and BMP-2 is shown to have important osteogenic capabilities, such as proliferation of osteoprogenitor cells toward mature osteoblasts [52]. The reparative phase starts with the differentiation of precursor cells to osteoblasts. They start the formation of new woven bone through the process of intramembraneous ossification or through the remodeling of already attached bone at the bone-implant interface. Necrotic bone after the operative procedure will simultaneously be reabsorbed by activated osteoclasts. The final phase is the remodeling phase, in which multicellular units reabsorb the newly produced woven bone and stronger lamellar bone is produced [53].

The initial implant fixation is dependent on osseointegration that can be described as the direct contact between bone and implant surface. It is dependent on several factors; the most important is osteoinduction. The term describes the stimulation and differentiation of primitive osteoprogenitor cells toward bone forming osteoblasts, primarily influenced by BMPs. Implant fixation is also dependent on osteoconduction. This describes the ability of the implant's surface to serve as a scaffold for new bone formation and bony anchorage [53]. The interaction is influenced by the chemical composition of the surface (biocompatibility), the surface topography, and possibly other yet unknown factors. The implants used in this thesis were porous-coated titanium alloys as used in the clinical setting. The intervention was metallic gold in the form of surface coatings or as pure metallic gold particles.

Previous biocompatibility tests of metallic gold compared to titanium showed no difference or any toxicity toward osteoblast cell proliferation, growth, and morphology [54]. It was therefore concluded that the use of metallic gold would not influence implant biocompatibility.

### Bone allograft

Allograft was used in study II in the periimplant gap. A bone graft is used in revision surgery or in other situations in which it would be advantageous to increase the total bone stock, such as in older patients and/or in patients with osteoporotic bone [55]. The objective with the use of bone graft material in THR is to increase total bone stock and thereby provide mechanical stability while the inserted implant integrates with the living bone [56]. As new bone is produced, the bone graft is also integrated into the periimplant bone [57]. This bone graft incorporation is an interaction between the graft material and the host bone. Long-term biocompatibility is dependent on bone formation and graft reabsorption.

Several non-synthetic bone grafts exist. A bone graft harvested from the same individual is referred to as autograft, while a bone graft from another donor is referred to as allograft. A bone graft from another species is a xenograft. Furthermore, bone grafts can be categorized as to bone type: cortical, cancellous, corticocancellous, or osteochondral. The allograft used in study II was frozen cancellous graft material from animals from the same litter.

Allograft incorporation can take several different pathways:

1. Hematoma formation with the release of cytokines and growth factors.
2. Inflammation and proliferation of mesenchymal stem cells and the formation of fibrous tissue in the graft.
3. Vessel invasion from adjacent tissues.
4. Focal osteoclastic reabsorption of the graft.
5. Intramembraneous ossification.

An allograft is said to have osteoinductive capabilities because essential growth factors such as BMPs are present in the graft material [58]. These are responsible for the formation of new bone. The graft material also contributes to osteoconduction because the bone chips act as a scaffold for bone formation. The combination of osteoinduction and osteoconduction results in the incorporation of the graft. This process is an interaction between osteoblasts and osteoclasts. Furthermore, macrophages monitor and control the interaction with the implant and the immunological response against the foreign bone. The immunogenic properties of allograft have been thoroughly investigated. The immunological response to the allograft is reduced as a consequence of HLA-matching of both donor and recipient and is further decreased by cryopreservation of the graft material [59, 60]. In study II, the material was cryopreserved. Although the allograft was not HLA matched, the donor and the recipient animals were from the same litter, which presumably reduced any significant mismatches.

## Cells involved in implant osseointegration

### Macrophages

Macrophages play a key role in the cellular response toward surgically implanted materials. Macrophages can be stimulated by the initial surgical trauma, by their physical attachment to an implant surface, or by the uptake of wear-particles produced later in the process. The initial response of macrophages plays a dual role in early implant fixation as they master the inflammatory response to the surgical trauma, and they determine biocompatibility by being the first cells to attach to the implant surface [38]. In vivo studies have confirmed that macrophages interact markedly with the inserted implants during the first few weeks [61]. In addition, macrophages have an essential role in wear debris-induced inflammation, osteolysis, and subsequent aseptic loosening of the implant [4, 62, 63]. Pro-inflammatory cytokine secretion (TNF-α, IL-1, IL-6, IL-8, M-CSF and several more) leads to monocyte attraction, osteoclasts proliferation (osteoclastogenesis), and prolonged survival of already activated osteoclasts, which in turn leads to increased bone reabsorption. Especially up-regulation of TNF-α has proven essential in particle-induced osteolysis [64].

A previous in vitro study showed that metallic gold in contact with macrophages liberates gold ions, which can be traced inside the macrophages [34]. Furthermore, as also highlighted previously, in vivo studies suggest that when macrophages are in contact with metallic gold, secretion of inflammatory cytokines, such as TNF-α, is reduced and inflammatory cell proliferation is diminished [44]. Therefore, macrophages may also be involved in bone repair when metallic gold is used as either an implant surface (I, III) or as gold particles in allograft (II).

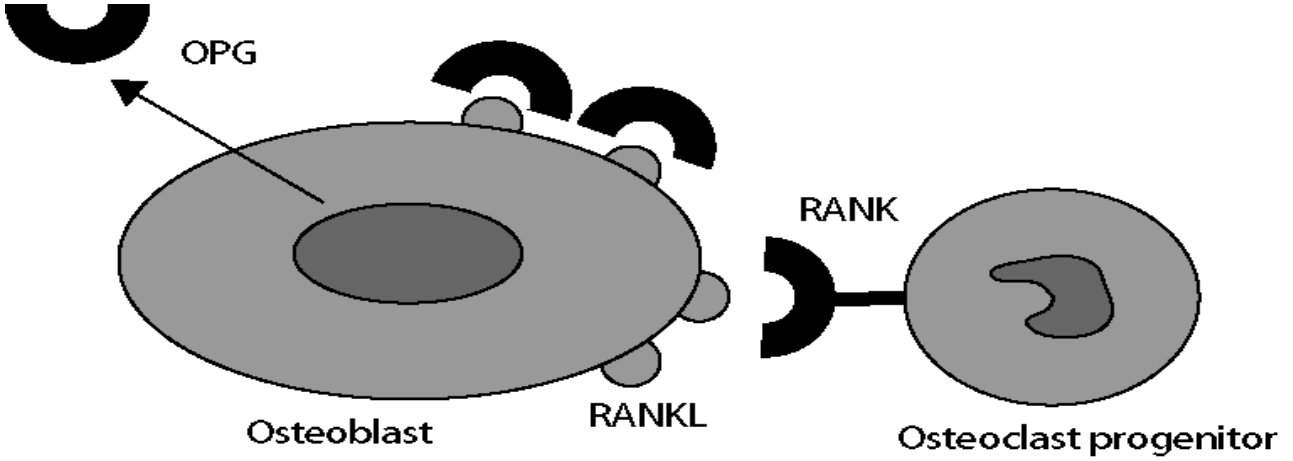



**Figure 2 - A schematic model of the interactions between osteoblasts and pre-osteoclasts. OPG operates as a decoy protein, reducing the osteoblasts' proliferating effect on osteoclast progenitors, modified from Ingham et al. [62]**

## Osteoblasts

Osteoblasts are stromal cells derived from mesenchymal stem cells (MSC), and their role in bone mass regulation is as important as that of osteoclasts. They are bone-forming cells, but their role in the regulation of osteoclast proliferation is particularly essential. They produce macrophage colony-stimulating factor (M-CSF), which participates in the differentiation of premature monocytes/macrophages into osteoclasts. Simonet et al. showed that osteoblasts also secrete a glycoprotein termed osteoprotegrin (OPG) that inhibits osteoclastogenesis [65], see Figure 2. It was later confirmed that osteoblasts have a membrane-bound surface protein, which OPG can bind to and thereby inhibit osteoclast activation [66]. This membrane protein was initially termed osteoprotegrin ligand (OPGL). OPGL is now named RANKL (receptor activator of nuclear factor kappa B (NF-κB) ligand), because the ligand binds to RANK (receptor activator of nuclear factor kappa B) on osteoclast surfaces and stimulates the cells through their NF-κB pathways. RANKL has been shown to not only participate in osteoclastogenesis, but also to stimulate already mature osteoclasts to reabsorb bone [67]. Hence osteoblasts' essential role in bone mass regulation lies in OPG secretion that works as a decoy protein and binds to RANKL, thereby regulating the amount of RANKL expressed.

Osteogenic differentiation of MSCs is controlled by interactions between hormones and transcription factors. Early markers of osteoblast differentiation are alkaline phosphatase (ALP), BMP-2, and many more. Although biocompatibility tests do not show any toxicity of metallic gold toward osteoblast-like cells, such as changes in cell growth, cell morphology, and ALP activity [54], osteogenic differentiation of MSCs could be influenced. This is not addressed directly in this thesis, but discussed later.

## Osteoclasts

Osteoclasts are the bone resorbing cells that play a major role in the regulation of bone mass. The fusion of giant cells results in multinucleated cells described as a specialized monocyte/macrophage polycaryon. The RANK receptor proteins on osteoclasts and osteoclast precursors are TNF-receptor-like surface proteins. RANK activation by RANKL has been shown to be essential in the proliferation of osteoclasts, because macrophages have been shown to fully differentiate into osteoclasts when only exposed to M-CSF and RANKL [68]. Additionally, it has been shown that TNF-α can augment osteoclastogenesis [69]. This means that if TNF-α is available, lower amounts of RANKL are needed to induce osteoclast differentiation and activation (osteolysis) [62] than if TNF-α is not present. (The presence of TNF-α facilitates the action of RANKL on osteoclast differentiation and activation (osteolysis) [62]). Studies have shown that osteoclastogenesis and bone reabsorption are primarily mediated through the NF-κB pathway [70, 71].

Metallic gold's effect on osteoclasts has not been investigated previously. But studies investigating already dissolved gold compounds' inhibitory effects on tartrate-resistant phosphatase (TRAP) activity have shown interesting results [72]. This mechanism is addressed, as bone resorption is evaluated.

## NF-κB pathway in an orthopedic context

The transcription factor NF-κB and the binding to the so-called κB motifs activate mRNA production in cells through several pathways. Several authors have investigated and fully reviewed the NF-κB pathway. Research in this field is increasing because activated T cells have been shown to express RANKL. The link between osteolysis and inflammatory diseases such as RA has made use of the NF-κB factor an interesting way not only to modulate the inflammatory response toward osteolysis but also toward other inflammatory disease, such as autoimmune diseases.

Mbalaviele et al. has shown that by inhibiting the kinase IKK, osteoclast differentiation can be inhibited [73]. The possibility that osteoclast differentiation can be inhibited thereby reducing the osteolysis that occurs because of an inflammatory response, whether it is in the early stage of an implant insertion or late in conjunction with aseptic implant loosening, needs to be investigated. The documentation of the effect of gold ions on NF-κB is substantial. As documentation regarding auromedication increases, it becomes necessary to study metallic gold as an anti-inflammatory mediator in clinically relevant animal models and to investigate whether gold metal has a role in an orthopedic context.

# Materials and methodological considerations

## Experimental models

### Study design

All three studies were conducted as experimental animal experiments. Each study was designed as a paired study, with the intervention implant and the control implant on either side of the same animal. To avoid systematic bias from undetected side-dependent differences, the intervention and control sides were alternated between animals. This was done systematically after a random start to secure uniform distribution. The design allowed paired comparison and also eliminated the variance between animals from the total of variance. This reduced the number of total animals needed.

All implants were cylindrical in shape. The cylindrical shape has several advantages. The implantation site is easily prepared by drilling a hole, and the conditions for all implants were therefore standardized. The shape is also suitable for mechanical push-out testing, and furthermore, it allows parallel vertical sections of the implant's central part, which enables stereological sampling, as described later.

Unrelated studies with similar models were performed simultaneously in all three studies in this thesis. They investigated surgical techniques on loaded implants in the femoral condyle and different surface coatings in unloaded models placed in the humerus and the tibia. One study investigated local bisphosphonate treatment of surgical cavities.

Study I, animals were operated on in the proximal part of the tibia (Fig. 3). In studies II and III operations were in the proximal part of the humerus (Figs. 4 and 5). Study II was conducted in sheep, while studies I and III were performed in dogs. This has scientific shortcomings, as the presented results cannot simply be compared across the studies. Each specific implant model differs from the others and the models used were: press fit I, allograft in 2.5 mm gap II, empty periimplant gap 1.5 mm III. The use of the same surgical site would not have shown any differences in our final conclusions anyhow, as the studies do not share any common control reference. Furthermore, different models were used to address different problems. Most orthopedic implants are inserted press-fit in the clinic. However, only as little as 10-20% of the surface is in actual contact with bone right after implant insertion [50], due to the trabecular structure, anatomical variations, and traumatic handling. The implant-tissue interface can be described as a series of gaps with variable sizes combined with local bone-implant adhesion sites. When periimplant gaps are introduced, osseointegration is further weakened, since bone ongrowth must bridge the gap and is subsequently very limited. Therefore fibrous tissue formation early in the process can be investigated with the use of periimplant gaps [74].

### Experimental animals

The animals used in this PhD thesis were canines (I and III) and sheep (II). Other research groups have used different species in orthopedic research. Larger animals, such as sheep and dogs, have large long bones that allow meticulous mechanical and histological evaluation of an only 10-mm long cylindrical structure. This allows the examination of implant surfaces that mimic clinically relevant settings. Furthermore, especially dogs, but to some extent also sheep, have a bone structure and bone quality that resembles human bone [75]. The metaphyseal parts of their long bones also allow easily accessible and reproducible surgical intervention.

Surgery of large animals is time consuming, and animal handling during and after surgery is expensive, which necessitates multi-study animals. The negative aspect of this is the theoretical contamination or bias from other intervention groups. However, all studies conducted simultaneously with the studies in this thesis were local surface treatments or surgical techniques. A different study using bisphosphonate was conducted in the sheep used in study II. Local bisphosphonate was applied in the femur, whilst (II) was conducted in the humerus. Although systemic contamination is not likely, a possible bias will be eliminated due to the relative paired comparison of the results. However, the effect modification of one group cannot be ignored and must be taken in consideration.

All animals were skeletally mature. The dogs were bred specifically for scientific purpose. Studies I and III were conducted at the Midwest Orthopaedic Research Foundation, Hennepin County Medical Center (Minneapolis, MN, USA). Study II was conducted at the Clinical Institute, Skejby (Aarhus University Hospital, Denmark). The local Animal Care and Use Committee approved all experiments in this

thesis, and ethical and institutional guidelines for treatment and care of experimental animals were followed.

## Sample size

The number of animals needed was based on a sample size calculation for paired study groups using the following:

$$n \geq (t_{1-\alpha/2} + t_{1-\beta})^2 \times \frac{SD^2}{d^2}$$

n = numbers of animals

$t_{1-\alpha/2}$ = the (1-α/2) quantile in the t-distribution at two-sided tests

$t_{1-\beta}$ = the (1-β) quantile in the t-distribution at two-sided tests

SD = square of the standard deviation on the paired differences

$d^2$ = square of the minimal relevant difference.

The quantiles listed in the t-distribution are dependent on the degrees of freedom. The risk of type I error (α) was set to 0.05 and the risk of type II error (β) was set to 0.20. The assumptions for sample size calculations were based upon previous studies with the same model. They suggested that the standard deviation on the relative difference was set to 50%. The minimal relevant difference (d) was set to 50% change

$$n \geq (t_{1-\alpha/2} + t_{1-\beta})^2 \times \frac{SD^2}{d^2}$$

$$= (2.262 + 0.883)^2 \times \frac{0.5^2}{0.5^2}$$

$$= 9.9$$

in biomechanical results.

Ten animals were needed in each study. Two additional animals were used in study II as allograft donors.

## Observation time

The overall aim was to increase early implant fixation, as clinical studies have found a significant correlation between the early implant migration and late implant loosening. It is important to note that early fixation was investigated, and short observation times were used in this thesis. Study I and study III investigated surface coatings and observation time was set to four weeks. This was based on previous experiences from similar models, where differences in fixation and osseointegration could be detected within three to six weeks [74, 76-78]. Study II investigated the effect of metallic gold particles on periimplant allograft remodeling and allograft reabsorption. Previous allograft studies, using 12 weeks of treatment time, show that allograft reabsorption can be reduced, and total bone stock can be found higher [79, 80]. The same time point was used in the present allograft study.

## Implant model and surgery

The implant models used in this thesis are based upon the well-proven implant model from our institution, which is designed to imitate the noncemented part of the THR in cancellous bone. It is a simple basic experimental animal model, which has shown important clinically relevant results in past publications [74, 77-84]. Although the three studies differ in their detailed specifics, such as set-ups, observation times and animals used, they have common features. Cylindrical plasma sprayed porous-coated Ti implant were used in all three studies. One implant was inserted in the cancellous bone in the extremity and the control implant was inserted into the contra-lateral extremity. Each implant was inserted by an extraarticular approach with the use of surgical anatomical landmarks and K-wire guidance that assured standardized implantation sites. However, the model has limitations. It does not address the clinical important joint fluid pressures or the impact of direct weight bearing on bone healing. Furthermore, bone turnover and remodeling are different in animals compared to humans [85]. The animals are young and healthy and do not represent the majority of orthopedic patients, who are old and have osteoporotic bone quality. The strength of the study lies in its paired and simple and highly reproducible model, minimizing variability in the studies.

Although the studies differ in location and animals used, the surgical procedure was generally the same in all three studies. The implants were inserted under general anesthesia and using sterile technique. Implant sites were located and periosteum was removed carefully. A K-wire was used to guide a cannulated drill while creating the drill cavity. All drilling was performed at 2-3 rps to avoid thermal trauma to the bone. After implant insertion, the fascia and skin were closed in layers and prophylactic antibiotic was administered. Full weight bearing was allowed postoperatively.

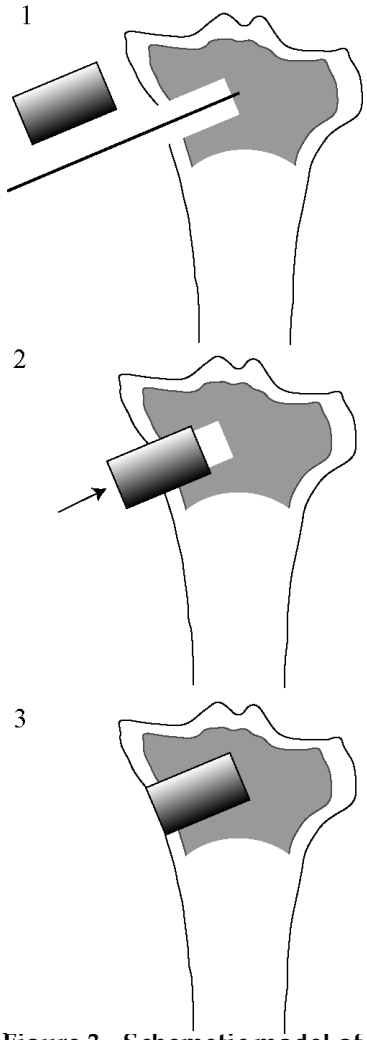



**Figure 3 - Schematic model of the press fit insertion of the implant in the antero-medial side of the tibia in (I)**

***Study I***

The proximal part of the canine tibia was used. A K-wire was inserted 18 mm distal to the tibia plateau, and a 5.6 mm wide hole was drilled with a length of 10 mm. Control or complete gold-coated implants were inserted press fit with repeated firm hammer blows. The operator was not blinded during surgery. Observation time was 4 weeks.

***Study II***

Two additional sheep were used as allograft donors and sacrificed prior to surgery. A bone mill was used to produce fine bone chips sized between 1 mm – 2.5 mm. Half of the bone graft was thoroughly mixed with an exact known amount of gold particles and stored in 1 mL syringes for surgery.

An 11-mm wide hole was created in the proximal part of each humerus with a depth of 12 mm. Each implant had a profound end-washer attached, which centralized the implant

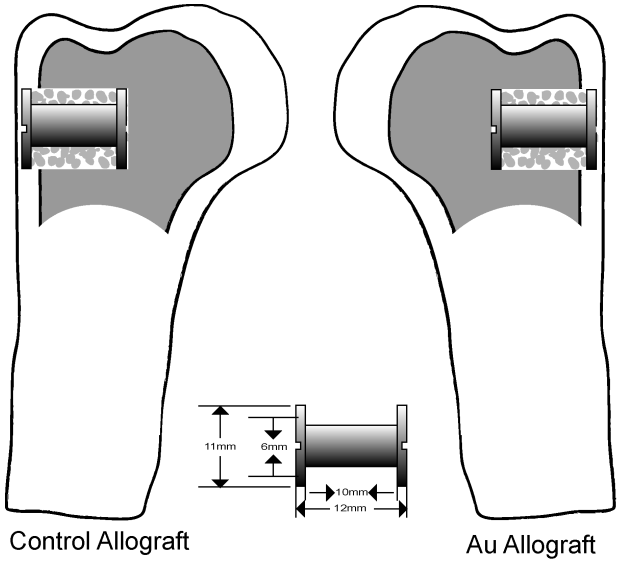



**Figure 4 - Schematic overview of implants inserted in the prox. part of the humerus in (II). Approximately 1mL allograft was inserted in each gap.**

in the cavity. The 2.5-mm gap was filled with control or intervention allograft, and the gap was closed with an end-washer. The operator was not blinded. Observation time was 12 weeks.

***Study III***

In dogs, an 11-mm wide hole was created in the proximal part of each humerus with a depth of 12 mm depth. Each implant was 8 mm in diameter and was centralized in the gap by a profound end-washer. A superficial end-washer closed the empty cavity, and a 1.5-mm empty gap around the implant was maintained. The operator was not blinded. Observation time was 4 weeks.

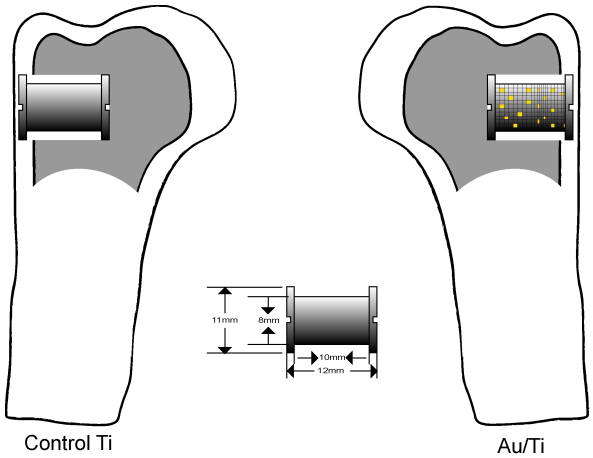



**Figure 5 - Schematic overview of implants inserted in the prox. part of the humerus in (III).**

## Implant characteristics

### *Plasma-sprayed titanium implants*

Custom-made 10-mm cylindrical titanium alloy (Ti-6Al-4V) implants were used in all three studies. All implants consisted of a core with an additional porous-coated titanium alloy (Ti-6Al-4V) surface that was deposited by plasma spray technique. Plasma spray coating is performed in a vacuum chamber to avoid a reaction between oxygen and titanium in the surrounding atmosphere. Thermal sprayed coatings are often characterized by a multilayered heterogeneous structure of pancake-shaped particles with pores or open pore structures. This rather complicated structure is caused by the deformation of the incoming coating material, which forms melted or partially melted droplets combined with some degree of plastic deformation as the incoming material hits the implant surface. This specific titanium alloy is also used in clinical settings and was selected because of its high mechanical strength. All implants were kindly donated by Biomet Inc. (Warsaw, IN, USA).

The surface coating topography was not investigated, as equivalent implants have previously been used and characterized. The mean pores size was approximated to 300 μm (200 μm at substrate core and 1000 μm at surface of the coating). The average surface roughness ($R_a$) was determined to 47 μm, and the profile depth $P_t$ was determined as 496 μm [74].

In (I) and (III), the intervention implants were further coated with metallic Au. The coatings were performed at the Technical University of Denmark. Porous measurement was not performed, but scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) showed that the additional Au coatings were 20-30 μm (I) and 40-80 nm thick (III). For all purposes the porosity was considered unaltered compared to control porous Ti implants.

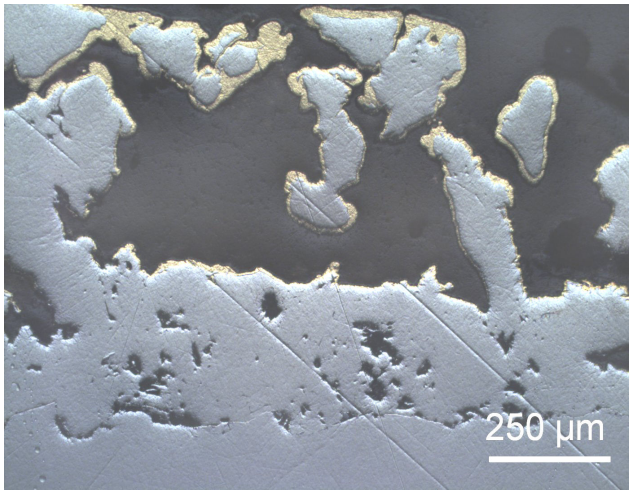



**Figure 7 - A representative cross-sectional view of porous implant surface coated with pure Au. The Au thickness is relatively low and the porosity was not disturbed.**

### *Electroplating (study I)*

The implant surface was coated with metallic gold using electroplating [86, 87]. Electroplating is the coating of an electrically conductive object with a layer of metal with the use of electrical current. This reduces the amount of cations of a desired solution and uses this to coat an object with a thin layer of the specific material, in this case gold. Initially,

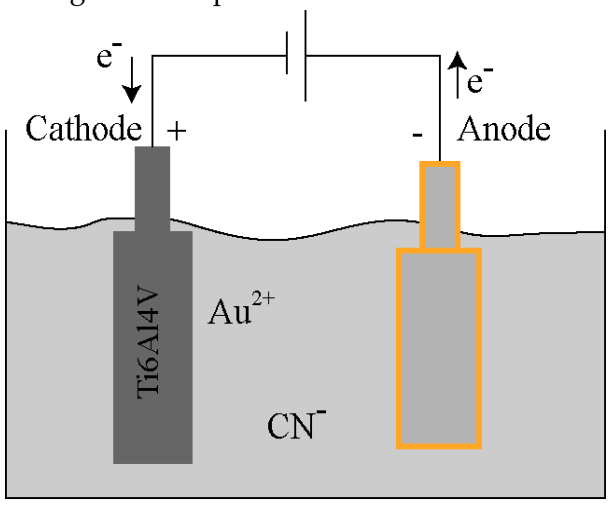



**Figure 6 - a schematic model of the electroplating process of the implant in the AuCN bath. AuCN is complex bound and the Au ion level is low in the bath. This assures a slow release of ions from the AuCN complex and a thin surface coating on the implant**

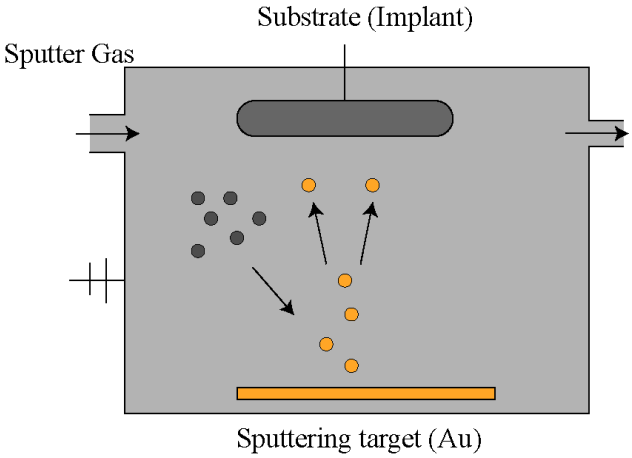
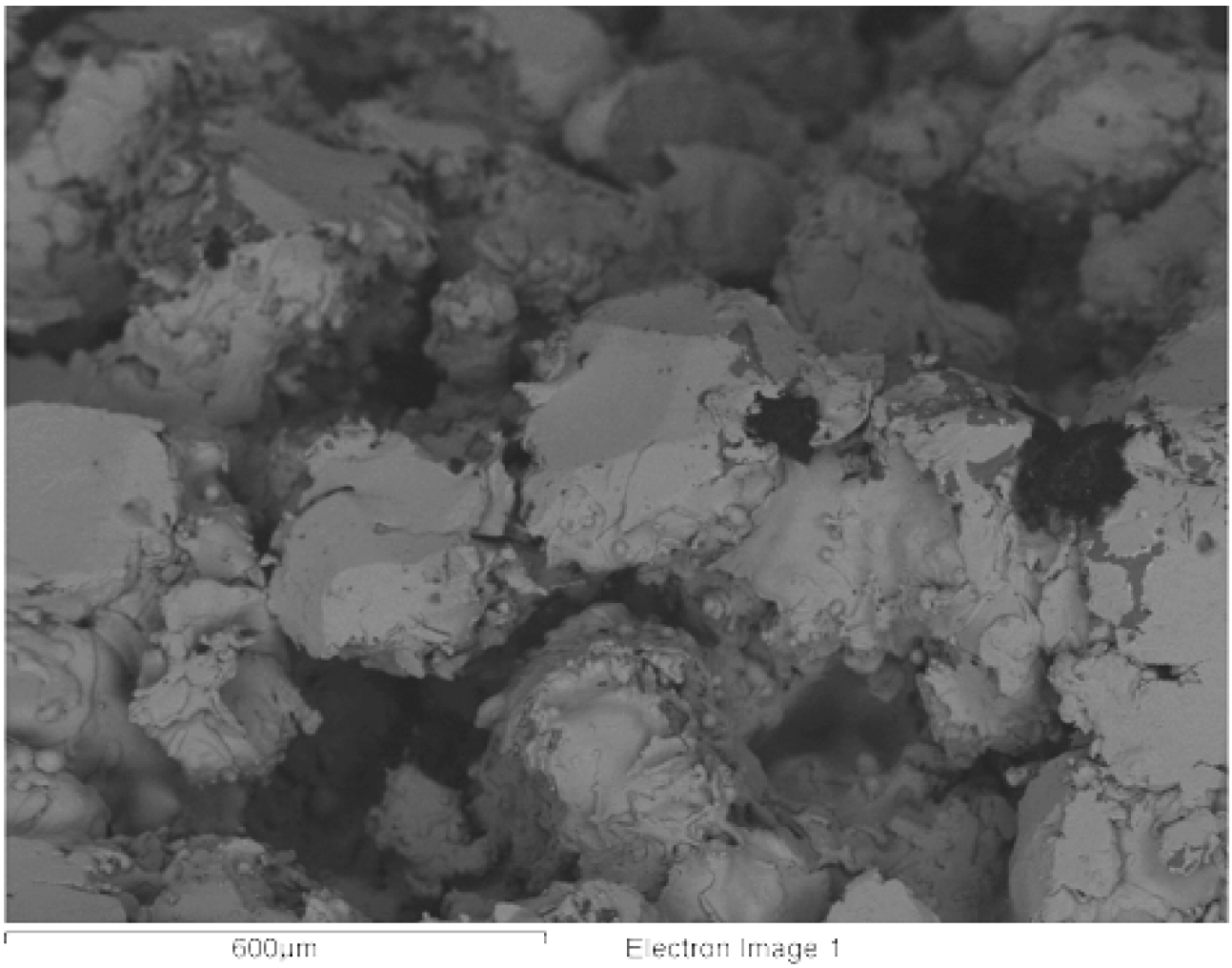



**Figure 8 - Simple model of the sputtering process. Gold (Au) is released after the target is bombarded with sputter gas (Argon)**

the implants were electrolytically cleaned in a strong alkaline solution containing NaOH. The process is performed by cathode polarization of the implant, and hydrogen gas is produced at the implant surface. The implant was dipped carefully into a weak acidic solution (5% $H_2SO_4$) to neutralize the implant surface pH. Then, initial strike plating, based on $AuCl_4$, was performed. The initial strike plating was under 0.5 μm thick. This improves the adhesion of the following gold layer. After assuring that the initial gold surface was sufficiently applied on the titanium (Ti) surface, a 100% 3- to 4-μm-thick pure gold coating was applied by immersion in a weak acid gold bath containing $AuCN_2$. Contamination was avoided by rinsing the implants carefully between each step with pure water.

***Magnetron sputtering coating (study III)***

Magnetron sputtering is a modification of the sputter coating technique, which is frequently used in industry to create various materials, such as semi-conductors used in microchips. It is a non-reactive physical vapor deposition method that can be used to deposit thin films on any surfaces needed [88]. In a gas chamber, atoms are ejected from a solid target material due to the bombardment of the target by energetic particles from the sputtering gas. Argon, which is an inert gas, is usually used as sputtering gas. The released atoms hit the substrate with high energy levels and create a thin film on the implant surface, as illustrated in Figure 8. Magnetron sputtering is used to increase the adhesion of the released atoms to the substrate's porous surface. The superimposed magnetic field induces an




| Spectrum | Al | Ti | Au | Total |
|---|---|---|---|---|
| Sum Spectrum | 3.24% | 64.19% | 32.57% | 100% |

**Figure 9 - SEM picture with EDS retrieved information regarding the surface substrate. 32.57% of the surface is Au**

additional force on the charged particles, creating a spiral pathway for the released atoms instead of a straight line. The atom's longer pathway enables a lower working gas pressure, which again facilitates higher collision energy. The gold layer was applied with a thickness of 40 to 80 nm on the porous coated cylindrical Ti implants, and as the procedure is a line of sight process, the gold layer did not cover the deep structures of the surface. Hence, the partial gold-titanium (AuTi) coating is created.

***Gold particles (study II)***

The intervention allograft was mixed with 99% pure spherical metallic gold particles (Alfa-Aesar, Germany) 45 μm – 63 μm in diameter. Two aluminum sieves were used to obtain these sizes. These particle sizes were suggested, because phagocytes, such as macrophages, are not capable of engulfing large particles. First particles sized less than 63 μm were collected. These were again sieved, and particles above 45 μm were collected. Each cavity was calculated to have the room for 668 $mm^3$ allograft since

$$V_{implant} = \pi \times r^2 \times h$$

$$= \pi \times 3\ \text{mm}^2 \times 10\ \text{mm}^2 = 282.74\text{mm}^3$$

$$V_{cavity} = \pi \times r^2 \times h$$

$$= \pi \times 5.5\ \text{mm}^2 \times 10\ \text{mm}^2 = 950.33\text{mm}^3$$

$$V_{cavity} - V_{implant} = 950.33 - 282.74\text{mm}^3$$

$$= 667.59\text{mm}^3$$

129 mg of gold particles was sieved for each intervention allograft site. This meant that if the particles had a mean diameter of 50 μm, and they were evenly distributed into the allograft, there would be approximately 400 μm between each particle. The particles were sterilized by gamma radiation.

## Specimen preparations

After the observation time had ended, the animals were euthanized with a single overdose of hypersaturated intravenous barbiturate injection. The relevant bones with the incorporated implants were excised, cleaned, and finally stored at -20°C. All further preparations were performed blinded. Two separate specimens were cut with the use of a water-cooled diamond band saw (Exakt-Cutting Grinding System, Exakt Apparatebau, Nordenstedt, Germany) from each bone-implant specimen. The cutting was performed perpendicular to the implant's long axis. Initially, 0.5 mm of the cortical end was cut off. The first specimen, sized 3.0 mm (II) and 4.5 mm (I) and (III), was stored in -20°C and used for biomechanical push-out testing. The remaining inner bone-implant specimens, sized 6.5 mm (II) and 5 mm (I) and (III), were used for histology.

The histology tissue sections were dehydrated gradually in ethanol (70-100%) containing 0.4% basic fuchsin (I) and (III) or nothing (II) and embedded in polymethyl-metaacrylate (PMMA) in circular jars. Four vertical uniform random sections were cut with a hard tissue microtome (KDG-95; MeProTech,

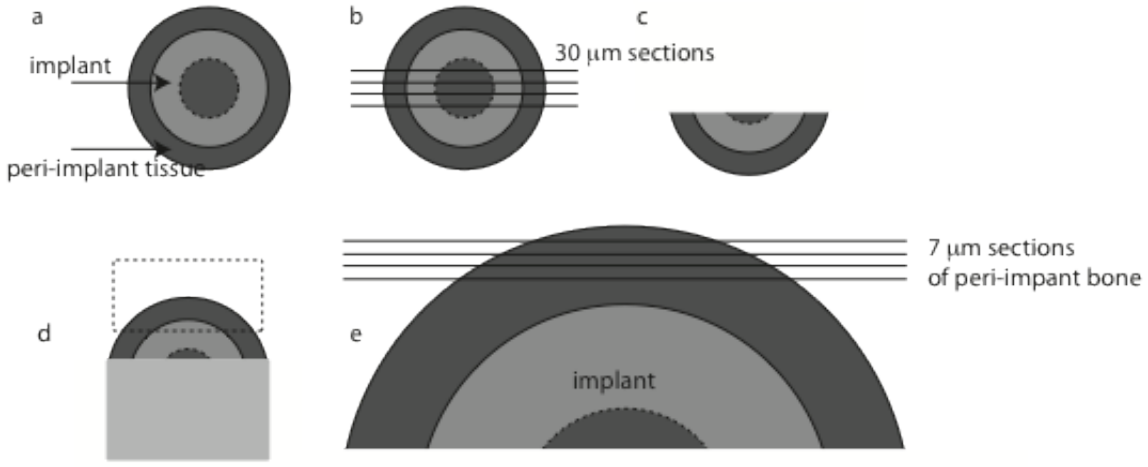



**Figure 10 - A schematic overview of the sectioning and re-embedding of the bone-implant specimen. After initial sectioning (b) the remaining specimen (c) was re-embedded (d) and further cut on the polycut machine into thin sections (e), which were stained with GT.**

Heerhugowaard, The Netherlands) from the central part of each implant, as described by Overgaard et al. [89]. The sections were cut after random rotation of the specimen around the vertical axis of the implant to fulfill the requirements of the vertical sectioning technique, as described in *"stereological methods"*. The four 30-μm sections were surface stained with 2% light green (BDH Laboratory Supplies, Poole, England). This procedure stains mineralized tissue green and non-mineralized tissue red (I) and (III). These sections were used for stereological histomorphometrical analysis. In (II), thin 7-μm sections were cut from the bone gap next to the implant parallel to the previous sections. The bone gap next to the implant was divided into four sections and sections were cut with a polycut machine at 400-μm intervals. These were used for autometallography. In study III, the remaining tissue with the implant was re-embedded in PMMA, and the gap not including the implant was divided into four different levels 250 μm apart (Fig. 10). From each level, two parallel 7-μm sections were cut with the polycut machine and stained with Goldner Trichrome (GT). This stains bone green and osteoid surfaces red and is used for measurement of the bone remodeling activity, such as bone formation and bone reabsorption.

The mechanical tests were performed from the more superficial end of the implant, whereas the histology section was from the deep end of the implant. Although mechanical and histological data was achieved from different parts of the implant, the close distance between these parts, and the fact that the implant is unloaded, made comparison between histology results and mechanical results reasonable.

# Biomechanical testing

## Push-out testing

The mechanical tests were performed as destructive load tests, and the implants were tested once until failure by means of push-out tests. All the mechanical tests were performed on our MTS Bionics Test Machine (MTS, Eden Prairie, MN, USA). Each bone-implant specimen was thawed and placed centrally on a metallic support jig with an opening 1.2 mm – 1.4 mm larger than the implant diameter. The push-out test was performed from the cortical side and inwards. The same person performed the testing, which was completed in one continual session. 0.5 kN load cell (III), 2.5 kN load cell (II), and 10kN load cell (I), were used. A preload of 2 N was used to standardize the start of the test, and the test was conducted with a slow constant displacement rate of 5 mm/min, and force versus displacement data were recorded onto a computer. The data was later used to calculate mechanical fixation parameters. After the test, the height and diameter of each implant was measured. Furthermore, the surface coatings in (I) and (III) were investigated for eventual macroscopically visible delaminating of the gold coating, and each implant was examined to see whether any bone remains were left on the extracted implant surface.

Three general approaches to mechanical tests, pulling, torsions, and pushing, are available for implant fixation measurements. The push-out test was preferred due to its advantages in standardization, as the implants are alike. Early implant fixation is influenced by the up and down micro-movement that occurs during each gait cycle, and the push-out test imitates, in part, the mechanical load applied to a clinical implant in a clinical setting.

## Biomechanical test parameters

To improve comparability between the implants, the collected specimens' heights and diameters were used to normalize the surface diameters. Since the implants were cylindrical, the cylinder was used as a model to calculate the surface area in contact with bone.

$A_{surface} = d \times h \times \pi$

$d$ = implant diameter.

$h$ = implant height.

| Study | Diameter | Height |
|---|---|---|
| | Mean (mm) | Mean (mm) |
| I | 5.70 (0.26) | 4.67 (0.13) |
| II | 5.16(0.26) | 2.95 (0.42) |
| III | 8.80 (0.17) | 5.27 (0.11) |

**Table 3 - Implant sizes for normalization of push-out data. Data are presented as mean (SD)**

Three mechanical parameters were calculated using exact algorithms in a spreadsheet (Microsoft Excel) from the collected force versus displacement data. These were

- Ultimate shear strength (MPa)
- Maximal shear stiffness (MPa/mm)
- Total energy absorption (kJ/m$^2$)

Ultimate shear strength was defined as the first peak on the force-displacement curve. This is interpreted as the maximal force the implant fixation could endure before failure, and it was

found at the maximal point of the graph as the curve became vertical. Ultimate shear stiffness was defined as the maximal slope between ten successive points on the elastic part of the force-displacement curve before the peaking of the force value. Total energy absorption was thereafter calculated as the area under the curve until peak value. These parameters depict different aspects of the mechanical fixation. Furthermore, they have been shown to correlate well with histological interpretation of well-integrated implants [74]. Ultimate shear strength is calculated as one single load that displaces the implant. It could be discussed whether this is a clinically relevant parameter, as clinical implants are loaded repetitively and constantly with lower forces during each gait cycle. In a clinical perspective, hardly any implants are exposed to such high forces that fracture the bone-implant interface. One can argument that sufficient strength is already applied using Ti and improving this parameter is not a priority. However, ultimate shear strength values illustrate the maximal stress the bone-interface can face given a constant displacement rate. This parameter is a reasonable and important factor, when two implant groups are compared in a standardized setting.

Different tissues and different materials have different elastic moduli. Maximal shear stiffness can be interpreted as the specific value for the rigidity with which the implant is anchored in the tissue. Fibrous tissue and non-mineralized bone are more elastic, while mineralized bone is more rigid. A high stiffness value could indicate a fixation in calcified tissue, whereas a low value could indicate an anchorage in fibrous tissue. A change in shear stiffness could also illustrate a possible change in the amount of tissue in contact with the implant surface. Furthermore, as discussed previously, early implant instability leads to micro-motion, early implant migration, and subsequently decreased implant longevity in clinical praxis. An increase in maximal shear stiffness would be interpreted in decreased periimplant tissue elasticity and thereby an implant less prone to micro-motion.

Total energy absorption illustrates the maximal energy needed before the specific implant fails. The tissue's specific ability to absorb energy before implant failure is also the so-called toughness, and the higher the value, the more resilient the tissue. This parameter should be evaluated with care, as different tissues can have equal toughness, although they have different ultimate shear strength and maximal shear stiffness values.

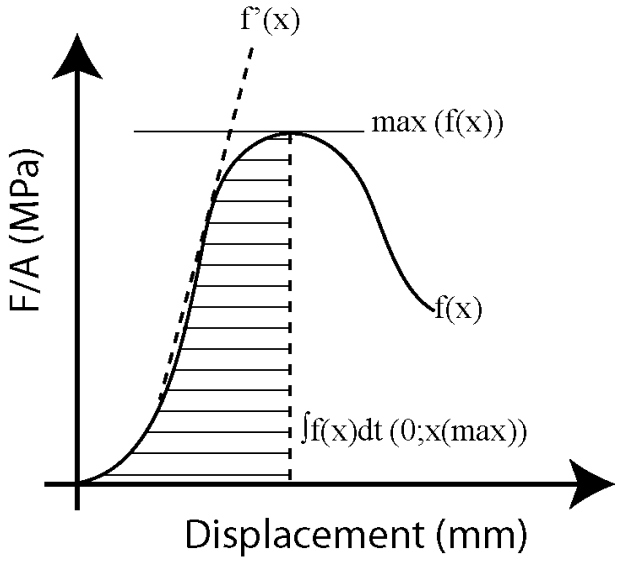



**Figure 11 – Force/displacement curve becomes a stress-displacement curve f(x), when normalized for surface area. The mechanical parameters are illustrated.**

The three mechanical factors are important in the overall discussion of the mechanical fixation. Although it can be discussed whether they are all clinically relevant, it is reasonable not only to look at one mechanical parameter but also to include all results in any needed discussions in the following.

## Considerations

The purpose of the mechanical test was to quantify the strength of the interface between the implant and the surrounding tissue. Push-out testing was used due to the model's simplicity, which allowed standardized and reproducible tests. This model has some critical points. It is not a clinically relevant test, as the clinically mechanical forces, such as bending, shearing, and compression that occur through daily life during each gait cycle, were not investigated. Instead, the test investigated and highlighted any possible immediate differences between two groups, and these could subsequently be explained on the basis of histological findings. The use of cylindrically shaped implants allowed this simple test in which implants could be standardized. The calculations used a smooth cylinder as an approximation of the implant surface, all of which were porous-coated with a relatively higher surface area than a smooth cylinder. As the porosity is assumed equivalent in all implants, the standardization of the surface area by measuring height and diameter enables paired comparison. To minimize variation in experimental conditions, all implants for each separate study were tested on the same day by

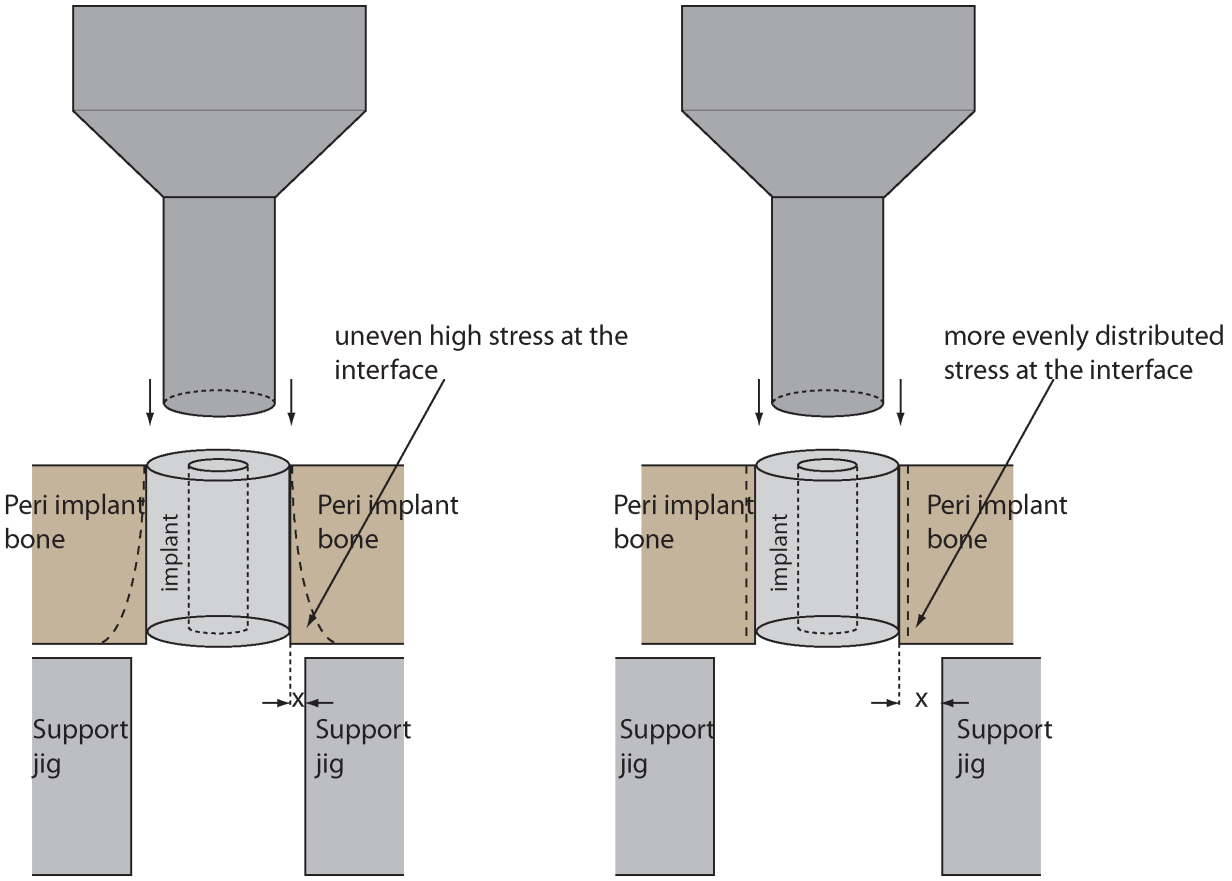



**Figure 12 - schematic overview of the push-out mechanism. A small lateral distance (x = 0.1 mm) creates uneven high stress at the interface, while larger jig hole sizes (x = 0.7 mm) are essential for even representative shear measurements**

the same investigator. The tests were all performed blinded.

Push-out testing is a destructive test, and double-measurements were, as a result, not possible. In addition, the remaining tissue could not be used for histomorphometrical analysis. Push-out testing was performed on the outer superficial part of the bone-implant specimen, whilst histomorphometrical analyses were performed on the part below the mechanical specimen. The conclusions drawn in this thesis are based upon the assumption that these specimens are comparable. This is not necessarily accurate. Nonetheless, it is an essential assumption, which can be reasoned by the fact that the distance between the two specimens is small, and former experiences from our institution using this model supported this method. A nondestructive test would allow double measurements and histological investigations from the same specimens used for mechanical testing. Nondestructive tests exist but require an increased number of specimen subjects [90].

The mechanical results are obtained from bone-implant specimens, which were frozen after bone harvesting, partly thawed during water-cooled saw cutting, refrozen, and finally thawed, before push-out testing was performed. Freezing can have an effect on the viscoelastic properties of cancellous bone, as shown by Linde et al. [91]. The cutting, freezing, and thawing times were therefore kept the same for all specimens in each study. To avoid freezing or re-freezing, it would be necessary to test the specimens immediately after extraction. This would have prolonged the test times and created uneven experimental conditions. In addition, comparison of relative paired changes instead of absolute values reduces any potential bias that would have occurred due to the freezing of specimens.

The push-out test is performed with the bone-implant specimen placed centrally on a metallic support jig with a slightly larger opening than the implant. Dhert et al. reported that the size of this opening is important in the push-out procedure [92].

A small oversize of the hole by a value of 0.1 mm resulted in high stresses at the site where the jig edge supported the bone, meaning that the shear stress at the tissue-implant interface was unevenly distributed [92]. By increasing the jig hole size, the shear stress became more

uniformly distributed, and the data became more representative for the actual stress at the implant surface. They concluded that the distance between the jig and implant edge should be at least 0.7 mm. The presented studies used a jig distance of 0.6 mm (II) and 0.7 in (I) and (III) close or equal to the recommended. The push-out testing investigated shear stress at the implant-tissue surface. The hole created by the jig is relatively small and used to optimize the shear stress measurements. Figure 12 illustrates that the model investigates the weakest link in the fixation. Failure of implant can occur either due to poor bone-implant contact or poor bone quality in the periimplant tissue. This should be considered in the overall conclusions. It is especially important in (II) in which allograft was used. A high periimplant bone quality with strong mechanical properties is as important as a strong interface adhesion. High implant shear stress parameters are not sufficient if breakage occurs in periimplant tissue at lower load forces. A detailed investigation of the specimens needs an inspection of not only the implant-tissue surface but also the periimplant tissue. This is addressed here and further enlightened by stereological histomorphometry.

## Histological analysis

### Histomorphometry

Histomorphometry was used to quantify the tissues in contact with the implant and also quantify the periimplant tissue adjacent to the implant. These measurements were interpreted as data that represent the implant's osseointegration. Relevant tissues were new woven bone, old lamellar bone, allograft (only (II)), fibrous tissue, and bone marrow cells. Tissues were distinguished by their morphological appearances. The 2% light green surface staining, performed during specimen preparations, stained bone green. New bone looked like immature woven bone with less organized large round cells with visible nucleic material. Old bone appeared as more organized lamellar bone with the cells stretched out or oval shaped. The allograft material used in (II) appeared as lamellar bone but without nucleic material. Fibrous tissue was identified as clearly visible fibril complexes with low cell density. The fibrous tissue appeared well organized, dense, and oriented parallel to the implant surface. The identification was made more obvious in study I and study III by use of the 0.4% basic fuchsin-colored PMMA, in which the specimens were embedded. This stained bone marrow cells red and fibrous tissue pink. Basic fuchsin was not used in (II) to allow clearer autometallography visualization of sections from the same specimens. In (III), additional sections were used to measure the bone remodeling in the bone gap. Surfaces with bone formation were identified by osteoid surfaces, which were stained red because of the Goldner Trichrome preparation. Bone reabsorptive surfaces were identified as osteoclast-covered surfaces or reabsorptive lacunae on the trabecular surfaces.

Histomorphometry was performed using a light microscope linked to a computer, fields of vision being captured on screen. The stereological evaluation was performed using a software program (CAST-grid Olympus Denmark A/S, Ballerup, Denmark –(I); newCAST, Visiopharm A/S, Horsholm, Denmark – (II) and (III)). Tissue ongrowth was defined as tissue in direct contact with the implant as determined at the light microscope level, and tissue ingrowth was defined as periimplant tissue density not including the implant. Ongrowth was determined using line intercept technique. The implant surface was easily recognized, and each intersection at the implant surface was categorized and counted in successive adjacent field of views followed by data accumulation. The percentage of each tissue ongrowth was then calculated as the fraction of the specific tissue divided by the

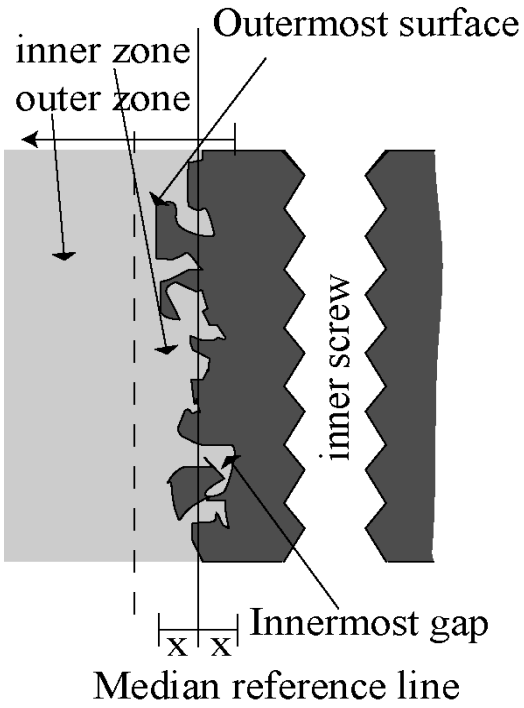



**Figure 13 - a schematic model of the projection of the median line prior histomorphometry**

sum of all intersections at the tissue-implant interface. Tissue ingrowth was determined using point counting technique in different regions of interests. Again, each specific tissue was determined as the fraction of total points counted in each region of interest. In study III, with the use of line intercept technique, the total bone surface, the fraction of bone formation surface, and the fraction of bone resorption were estimated from the thin Goldner Trichrome-stained sections collected from the periimplant gap. Bone formation was recognized as osteoid surfaces, and bone resorption was recognized as osteoclast cells or reabsorbed lamellar bone. These two parameters were quantified as the percentage of total bone surface area. All counting was performed blinded in (I) and (III), whereas blinded counting could not be done in (II), as the gold particles were visible in the fields of vision.

## Regions of interest

As the implant models differ in all three studies, the regions of interests also differ. Two regions were established in all cases: an inner zone next to the implant and an outer zone more distant from the central implant. It was found unfeasible to define standardized regions of interest in a fixed distance to the implant surface due to the roughness of the porous coating. Therefore, a reference line, defined as a median line between the innermost porous depth and the outermost top, approximated by the cross-sectional view of the rough surface on the implant coating, was projected on the specimen. This line was used as reference line for distances measured from the implant surface. A region spanning from the implant center, thereby including the entire surface porosity, and 500 μm lateral from the reference line was defined as "the inner zone." An outer zone was defined using the 500 μm and out ((I) = (500 μm; 1000 μm), (II) = (500 μm; 2000 μm), (III) = (500 μm; 1500 μm)). This ensured that sampling occurred in relevant tissues, such as tissue adjacent to the surface coating (I) or tissue produced in the drill hole (II) and (III).

In (III), the thin Goldner Trichrome sections were used for total bone surface measurement, the quantification of bone formation, and bone reabsorption. Region of interest was applied differently here. The former drill hole was easily discriminated from the intact bone. A custom made 2500 μm x 4500 μm zone was drawn in the visible bone gap, and sampling was performed in this zone. Each implant gap had eight sections, and all data were accumulated and thereafter calculated.

## Stereological methods

Histomorphometrical analysis is performed to quantify the implant's osseointegration represented by bone ingrowth (bone volume) and bone ongrowth (the bone-to-implant surface area). Stereological principles are applied to reduce the amount of workload during tissue estimation. Furthermore, stereological sampling is necessary because flat two-dimensional (2D) sections are used to estimate three-dimensional (3D) parameters, such as surface area and tissue density in a 3D specimen. In short, stereology is the science of 3D interpretation of lower dimensional objects, in this case 2D sections. The parameters wanted can be quantified with the use of test probes superimposed on the investigated object. Requirements for stereological quantification are that the sum of dimensions for the probe and the object must be three, and the object and/or test probe needs to be isotropic. This denotes that the likelihood of intersection between the test probe and the investigated parameter is independent of orientation in space. Unbiased estimates are achieved with uniform random sampling of the sections.

Tissue volumes are 3D and can be quantified using the zero-dimensional probe of point counting. As points are dimensionless, tissue volume quantification becomes independently orientated without any preferred direction in space. Therefore, no requirements are necessary of the object regarding isotropy. On the other hand, surface areas are 2D and require 1-dimensional test probes, such as lines. Unlike points, lines are directional and thereby anisotropic. In addition, the implant surface cannot, due to its rough porous coating, be regarded as completely isotropic. Therefore the vertical sectioning technique, thoroughly described by Baddeley et al. [93], is considered necessary. It allows surface area quantification without assuming isotropic orientation. Vertical sectioning technique requires the following:

1. Identification of the vertical axis.
2. Cutting of sections parallel to the vertical axis.
3. Random orientation of the sections around the vertical axis.
4. Sine-weighted lines used as test probes.

When these requirements are fulfilled, uniform random sections are ensured, and the intersection lines are isotropically distributed in the 3-dimensional space.

The vertical sectioning technique was implemented in the specimen preparation. The method requires that an arbitrary vertical axis be set which the specimen can freely rotate around. The histological preparation was performed in cylindrical plastic jars, in which the long axis of the implant was used as the vertical axis. The cylindrical plastic jars allowed uniform random rotation of each implant around the vertical axis, and the cutting could be performed parallel to this axis. The stereological program allowed the insertion of sine-weighted lines as test probes. With the requirements met, histomorphometry of the surface area became possible.

## Considerations

Stereological histomorphometry was performed to attain unbiased histological results efficiently. Unbiased specifies that the results have no systematic deviation from the true value. Efficiency means that the estimate has a low variability. Any bias can be introduced during the specimen preparation and during the analysis, and one should be aware of the possible influences.

***Specimen preparation bias***

Due to the centrally placed metallic implant, 30-μm sections were the thinnest sections possible to cut on the heavy-duty microtome. Sections were selected from the central part of the bone-implant specimen, since the implant surface area was of interest. If more peripheral sections were cut, the intersections between implant and bone would become tangential and create analyzing barriers. However, since it can be argued that central sections are representative for the entire specimen, central sections introduce increased variability. It is unlikely that the periimplant tissue is homogenously distributed in the entire region of interest. The central sections were cut after an initial cut-off had been made parallel to the central axis. The procedure made way for two sections on one side of the exact central axis and the remaining two sections were on the other side of the central axis. Each 30-μm specimen was cut successively, and approximately 400 μm of tissue was lost to the saw. This makes the width of the sections at least 1320 μm. More representative sections could be created if the distance between sections or the number of sections were increased. However, Overgaard et al. have implied that biological variance is the main contributor to total variance, whereas the intersectional variance is almost negligible, and therefore advocate for a central section technique to maintain a reasonable workload [89].

It is reasonable to assume that bone formation and neo-vascularization originate from the intact, healthy trabecular bone tissue adjacent to the drill hole. This is especially the case in (II) and (III), where a larger bone defect around the implant was created. Therefore, as the sections are cut, one should be aware of the fact that the central sectioning technique introduces a possible systematic measurement bias. The random orientation is performed around the vertical axis. As Figure 15 shows, there is an increased risk that tissues adjacent to the central implant are overestimated, while tissues far away in the "outer zone" are underestimated, all depending on the orientation of the implant prior sectioning. When regions of interest are applied on the vertical sections at microscopy, the regions stretch out 1000 μm (I), 2000 μm (II), and 1500 μm (III), using the implant surface as reference line. The central section biases have been investigated [94] and found to have a very small influence on the histological parameters and therefore found acceptable.

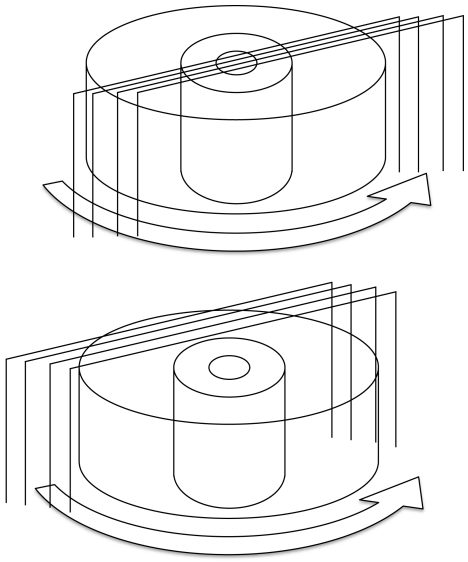


**Figure 14 - schematic model of specimen preparations. After random rotation around the vertical axis, four 30-μm central sections were cut for tissue ongrowth and ingrowth measurement (above) and 2 x 7-μm sections (III) were cut from four different levels in the bone gap for bone surface activity measurement (below)**

*Sampling bias*

To reduce any influences during the analysis procedure, the sampling was done by the same operator and on the same microscope. Furthermore, the operator was blinded in studies (I) and (III), whereas blinding was impossible due to the obvious appearance of the gold particles in the periimplant gap. To avoid further operator-based bias, data were accumulated during a short time period. Prior to data accumulation in each study, the first section samples were used as trial data and recounted.

The thick sections were superficially stained with 2% light green. To avoid tissue over-projection, the green mineralized tissue was kept in focus while sampling. If this had not been done, there would be an oversampling of the more dense tissues such as bone lying deeper in the sections, but not stained by the green surface color, whereas bone marrow would be underestimated because of its less dense structure. The depth of the superficial staining is considered to be approximately 5 μm [95], reducing the possible bias substantially.

During analysis, the implant surface could be found in contact with nothing. This is due to tissue shrinkage and thereby a preparation artifact. The resemblance between the artifacts and bone marrow tissue could lead to overestimation of bone marrow-implant surface area and underestimation of bone-implant surface contact (bone ongrowth). To avoid this, blank surface areas were counted as "not able to discriminate" and not included in the data set. This does not, of course, remove the possible bias from artifacts. Although shrinkage is present regardless of the tissue being bone or bone marrow, the possibility of bias exists, but for practical purposes was considered negligible.

The possibility of tissue overestimation is also introduced in the discrimination between fibrous tissue and bone marrow tissue. In (I) and (III) basic fuchsin was used to highlight the non-mineralized tissue. In (II), basic fuchsin was not used, since the specimens were also used for autometallographic staining. Therefore the morphological appearances of fibrils were used to differentiate fibrous tissue from the bone marrow cells. Fibrous tissue is more dense than bone marrow. Therefore, it is obvious that even though bone marrow cells are located at the same focus level as the green mineralized bone and thereby above fibrous tissue, there is a systematic overestimation of fibrous tissue during histomorphometrical sampling. Fibrous tissue was only estimated in substantial amounts in (III). In (II) the amount of fibrous tissue was small and only located at a close distance to the implant. Nonetheless, the amount of fibrous tissue should be considered with this in mind.

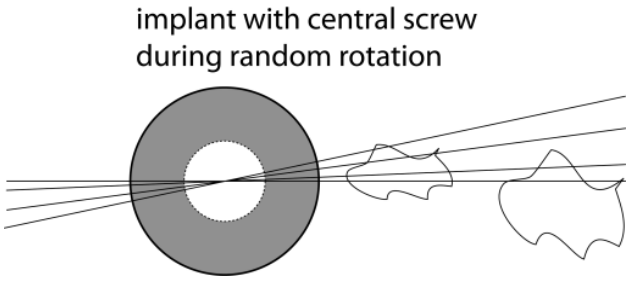



**Figure 15 - Central section technique: Model showing the central section and the subsequent underestimation of peripheral tissue**

*Importance of histomorphometrical bias in this thesis*

The stereological histomorphometrical analyses in the three studies were performed to evaluate the tissue ongrowth, the tissue ingrowth, and, additionally, in (III) the bone formation and bone resorption activity in the periimplant tissue. Above-mentioned possibilities to introduce any systematic bias should be considered, and all sampling should be planned carefully to reduce and control all factors. Mostly biases presented are considered small and acceptable. Furthermore, the conclusions made in this thesis are based upon the evaluation of paired specimens, which all were put through the same procedure. Finally, the estimates compared to each other give relative changes in tissue fractions, instead of conclusions based upon absolute values. It seems reasonable to consider the presented biases as acceptable and our estimates valid for the study design.

*Stereological efficiancy*

Histomorphometrical analysis is the estimation of entire tissues based upon a limited number of samples. Stereological principles are applied to reduce the amount of workload during tissue estimation. Each analysis was performed using meander sampling and based upon previous studies, it was estimated that approximately 100 positive hits of the sought after parameters were the optimal sample design. As there were four sections, sampling of each section was optimized to include approximately 30 positive "new bone" hits.

The applied "rule of thumb" regarding approximately 100 hits of new bone divided between four sections decreases the workload and thereby the time used on each sampling.

The intensity of sampling is based upon the specific tissue. If the tissue is highly represented in the material investigated, a lower sampling intensity can be used, whereas tissue rarely represented needs to be sampled more intensively. When new bone is used as the reference line, less represented tissues, such as allograft in (II) or fibrous tissue in (II) and (III), are in risk of being sampled with a high variability. The rule of thumb assured that relevant tissues were estimated as reproducibly as possible. The reproducibility of the histomorphometry was measured with double measurements using four random specimens for each separate study.

## Histomorphometrical reproducibility

The same investigator performed the histomorphometrical analysis, but intra-observer variation exists, and double measurements were therefore used to address this issue. Reproducibility can be expressed as the coefficient of variance (CV):

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

Where,

$\bar{x}$ = Mean value of first and second estimate

$k$ = number of double estimates

$d$ = difference between first and second estimate.

CV for each relevant tissue is illustrated in study-separated tables. The non-available CVs are due to a lack of the specific tissue in the selected specimens, used for double measurement.

The illustrated CVs showed that the intra-observer reproducibility was sufficient in all studies. We aimed at keeping the CVs under 20%, and a CV under 10 % was considered as a high reproducibility. The CVs were in accordance with other studies, using the same models, and the high 23% CV in (III) was due to the small area fractions of new bone on the implant surface. The CV is not only dependent on the intra-observer variation, but also the meander sampling intensity; a more intensive sampling increases reproducibility but also

| Study I | New Bone | Fibrous Tissue | Marrow |
|---|---|---|---|
| Surface area | 17% | N/a | 2% |
| Volume | 19% | N/a | 1% |

| Study II | New Bone | Fibrous Tissue | Total Bone | Marrow |
|---|---|---|---|---|
| Surface area | 9% | N/a | N/a | 2% |
| Volume inner zone | 10% | N/a | N/a | 9% |
| Volume outer zone | N/a | N/a | 9% | 4% |

| Study III | New Bone | Fibrous Tissue | Marrow |
|---|---|---|---|
| Surface area | 23% | 16% | 3% |
| Volume inner zone | 6% | 6% | 8% |
| Volume outer zone | 3% | N/a | 7% |

**Table 4 a, b, c - reproducibility in (I), (II) and (III), CVs are shown in percent**

increases analysis time. When a particular tissue fraction is low in the specimen, the reproducibility of the corresponding sample is further decreased, because variation in intra-observer tissue classification is further augmented.

## Autometallography

### Introduction to autometallography

Tracings of liberated gold ions were conducted in studies (I) and (II). This was done using silver-enhanced autometallography development (AMG), which previously has been used in several publications [96]. AMG is a highly specific way of tracing small amounts of gold present as gold nanocrystals in tissue sections. In short, AMG is a visualization of metallic nanocrystals that makes it possible to observe the grains in electron microscopes and light microscopes. The procedure is a silver-enhancement of either gold and silver atoms, or nanocrystals of metal-sulfur and metal-selenium complexes. The procedure used was dedicated for metallic gold ion tracing and was conducted at Neurobiological Department, Institute of Anatomy, where the technique is well known and much appreciated.

The gold ions, most likely bound to organic molecules in the lysozomes and cyanide in the dissolution membrane, have to be reduced to free gold atoms before being able to catalyze the AMG process of silver enhancement. The gold atoms will accumulate in nanocrystals that catalyze the AMG process when the tissue sections are placed in an AMG developer. There the free silver ions and the reducing molecules of the developer attach to the gold nanoparticles. The attached silver ions are reduced to metallic silver, and the oxidized hydroquinone molecules released. This process continues as long as silver ions and reducing molecules exist in the developer. The result is that gold nanoparticles grow in size and are thus viewable in a microscope.

### The AMG procedure.

The AMG developer (pH = 3.8) consists of a 60-ml gum arabic solution and 10 ml sodium citrate buffer (25.5 g of citric acid 1 $H_2O$ 23.5 g sodium citrate 2 $H_2O$ to 100 ml distilled water), 15 ml reductor (0.85 g of hydroquinone dissolved in 15 ml distilled water at 40°C), and a 15-ml solution containing silver ions (0.12 g silver lactate in 15 ml distilled water at 40°C) added immediately before use while the AMG solution is thoroughly stirred. AMG procedures and recipes for different metals were found in the thorough description of AMG by Danscher and Stoltenberg [96].

In (I), one complete gold-coated implant-bone specimen was selected to undergo AMG procedure. The specimen was dehydrated gradually in ethanol (70% - 100%) and embedded in PMMA as were the rest of the specimens, but without the basic fuchsin. Vertical sections including the implant (thickness ≈ 30 μm) were cut similar to the other implants and the sections underwent AMG development. In (II), an implant from each group was selected and 7-μm sections were cut parallel to the vertical axis at four levels in the gap. All further processing was analogous in the two studies.

Sections were placed on 10% Farmer-cleaned glass slides and subjected to UV-light for 30 minutes. The slides were placed in Farmer-cleaned jars and covered with newly prepared warm 26°C AMG developer and placed in a water bath at 26°C. A dark hood covered the entire set-up throughout the actual procedure to avoid excess daylight exposure. The AMG development was stopped after 60 min by replacing the developer with a 5% sodium thiosulfate solution for 10 min (AMG stop bath).

### Considerations

The general principle of AMG is to use silver-enhanced development to visualize metallic atoms found in the tissue after catalytic metal ion reduction. The number of ions liberated from metallic implants inserted in vivo is low [33], and the AMG technique is necessarily very sensitive. Cleanliness is of great importance, and therefore all tools, jars, and glass slides used were cleaned with 10% Farmer solution (0.9L 10% sodium thiosulfate, 0.1L 10% potassium ferricyanide). To further avoid unspecific staining, the jars were placed under running ion-exchange water for 5 min after the AMG stop bath. In addition, the glass slides were dipped in a 2% Farmer solution for 10–30 sec to remove excess silver grains on the surface and finally dipped twice in distilled water.

The AMG sections from the two studies were embedded in PMMA. However, some differences still exist. In (I), the sections were thicker than sections cut from the gap in (II), due to the implant. The UV light exposure time is suggested to be longer for thicker sections [96]. The exposure time was, however, kept the same in both studies, as the coated implant is also present at the top of the section. It was considered reasonable to search for AMG-

positive grains on the top of the section and not necessarily in the depths of the section. Thinner sections for AMG were also produced from the remaining bone after push-out testing. These were, however, negative, and it was considered whether the tissue containing gold ions had been destroyed or had followed the implant out during the push-out testing. In addition, the region of interest was the tissue in direct contact with the gold-coated implant surface, while the sections in (II) were taken from the gap with the gold particles. This did not result in any modifying of the development procedure. In both studies, negative control tissues were also investigated to prevent false registration of impurities in AMG sections and to investigate the pure titanium surfaces.

The AMG setup in this thesis created many obstacles for the clear visualization of AMG grains. The obstacles occurred due to the PMMA embedding, the calcified bone, the implant, and the allograft. PMMA sections and especially sections in (II) were fragile, and AMG staining could disintegrate the PMMA embedding. However, positive AMG sections for light microscopy were created, but the quality of these sections was definitely not like other authors' AMG specimens. Nevertheless, the vast publication present regarding AMG's authenticity and gold ions released from metallic implants in combination with the present positive AMG sections are considered more than sufficient in these studies.

## Statistical analysis

The dataset in all studies were compared as paired data consisting of a control and an intervention implant. Statistical analysis was performed using the computer software Stata SE (StataCorp. College Station, TX, USA). All variables were normally distributed after log transformation. Statistical analyses were carried out on the ratios between the paired data [97]. As the ratios were not normally distributed, all variables were log transformed, before Student's paired t-test was executed on the absolute differences between the log transformed paired data. The absolute difference between the logarithms of a paired data set is the same as the logarithm of the ratio within the same pair: "$\ln C - \ln I = \ln (C/I)$"; Two tailed p-values below 0.05 were considered statistically significant. All following results are presented as means of the treatment groups or medians of relative differences between the paired data. The 95% confidence intervals for the medians were calculated as the antilog of the logarithmic bound confidence intervals as described previously [98].

## Exclusions

***Study I***

Ten animals were scheduled for operation, but one had a clinical joint infection before surgery. Puncture showed purulent fluid, and the dog was excluded before any operation was made. Hence only nine animals were used in (I).

***Study II***

One animal was lost immediately after surgery due to respiratory problems. The animal lost was used as reference line and labeled time-point zero. After the study termination, another animal was excluded due to implant migration on one side. Seven animals were used in the histological analysis because one control specimen was cut incorrectly on the Leiden microtome, and the animal had to be excluded from the histomorphometry. The animal was included in the biomechanical testing, because another animal was excluded as one of the push-out specimens broke during push-out testing.

***Study III***

One of the control implants was not centrally placed during the push-out testing, which resulted in extremely high mechanical fixation data. The paired data from this animal were therefore excluded in the push-out dataset and only nine animals were used in the mechanical analysis. The histological specimens from this animal were, however, included in the study.

Two animals were excluded when bone-remodeling activity was analyzed with the use of the 7-µm sections stained with Goldner Trichrome. This was due to preparation problems in the two specimens.

**Table 5**
**Biomechanical results – (I)**

| | Ult. Shear Strength (MPa) | Max. Shear Stiffness (kPa/mm) | Total Energy Absorption ($J/m^2$) |
|---|---|---|---|
| Gold implants | 3.15 (1.49; 4.35) | 16.2 (9.6; 21.9) | 752 (239; 1051) |
| Control implants | 4.75 (2.60; 6.75) | 34.8 (15.3; 51.0) | 651 (324; 931) |
| Gold / Control (%) | 60.8 | 51.8 | 91.4 |
| Significant (*) | *p = 0.045** | *p = 0.017** | *p = 0.808* |

**Table 5 - Mechanical results are shown with absolute mean values for each implant group. The relative median ratio is also shown, and the parentheses show 95% CI**

# Summary of study results.

The studies in this thesis were designed to investigate whether metallic gold had a future role to play in an orthopedic context. Three slightly different models, all of which were paired studies designed for mechanical and histological evaluation, were utilized to investigate this. The clinical objective was to improve fixation of uncemented implants and thereby to improve implant longevity. Metallic gold's possible role in the orthopedic field was based upon its anti-inflammatory capabilities. The inflammatory response's essential role in early bone reparation, as well as its key role in particle-induced macrophage stimulation and osteolysis prior to aseptic loosening, necessitated the use of different models to investigate metallic gold's effect on bone tissue. Gold's impact on new bone formation, allograft reabsorption and fibrous tissue formation were studied. Clinically relevant tissue specific alterations would in any case result in mechanical changes.

## Study (I)

***Hypotheses:***

(1) Complete gilded titanium implants will increase the implant's mechanical strength and stability.
(2) This will be seen histologically by increased new bone formation and thereby increased implant osseointegration.
(3) Complete gilded titanium implants would release gold ions that could be traced in the surrounding bone cells.

***Results in short:***

The gilded implants showed inferior mechanical properties as well as reduced total bony integration with the implant. The periimplant tissue remained unchanged.

Small fractions of gold ions could be visualized with the AMG technique in apparently macrophage-like cells in periimplant tissue.

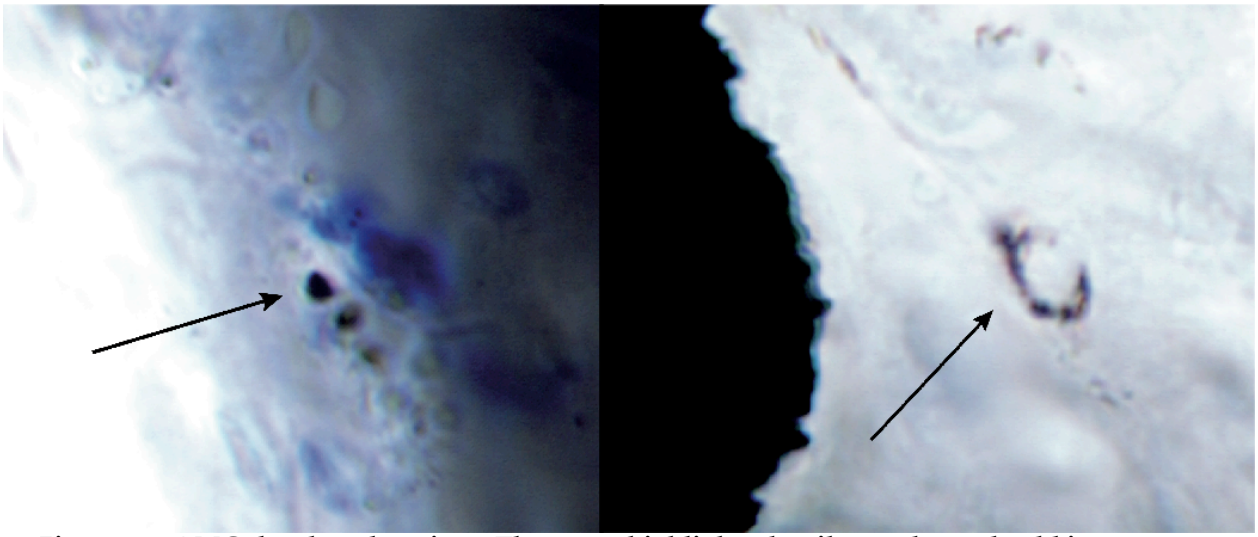


**Figure 16 - AMG developed sections. The arrow highlights the silver-enhanced gold ions**

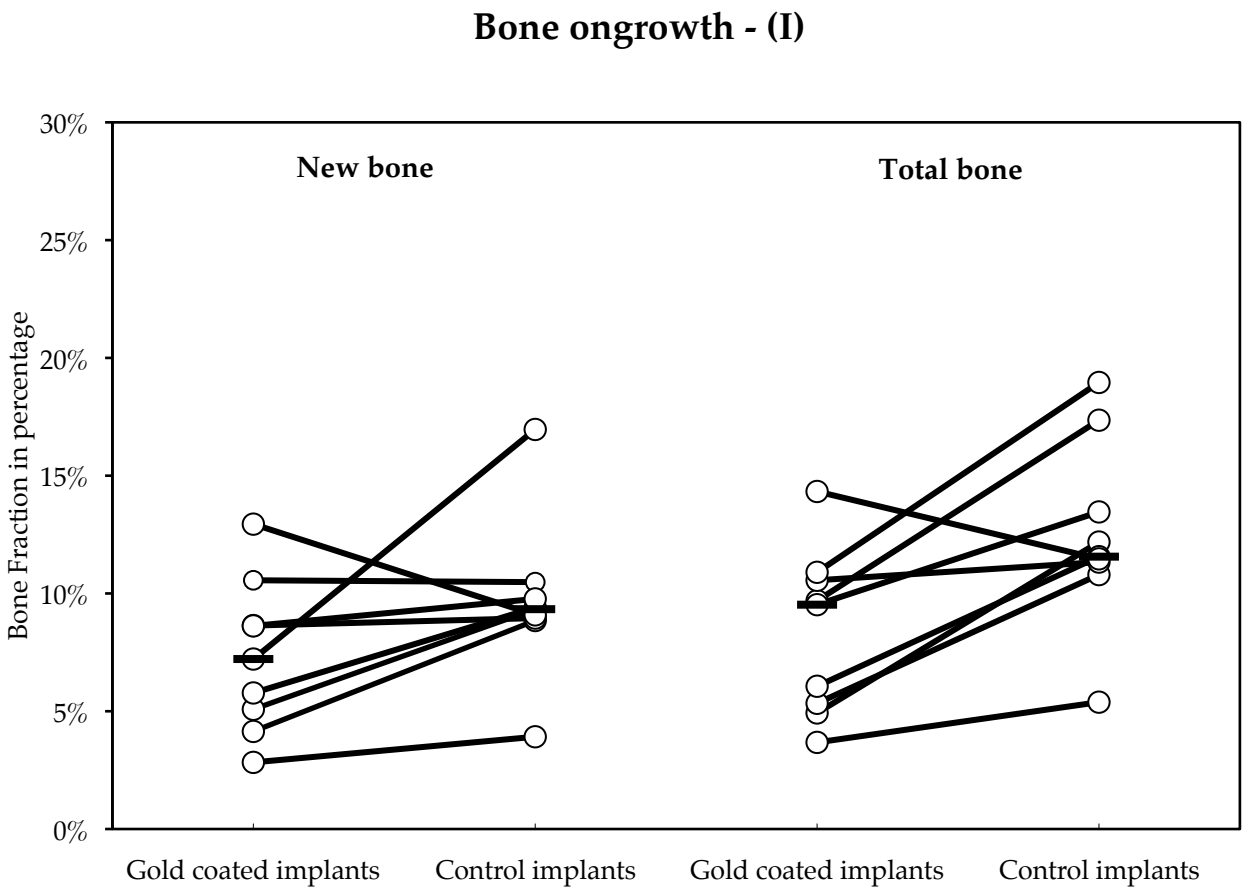



**Figure 17 - New bone and total bone in contact with the implant surface (bone ongrowth). Median value of each group is shown. Paired data are connected with a line**

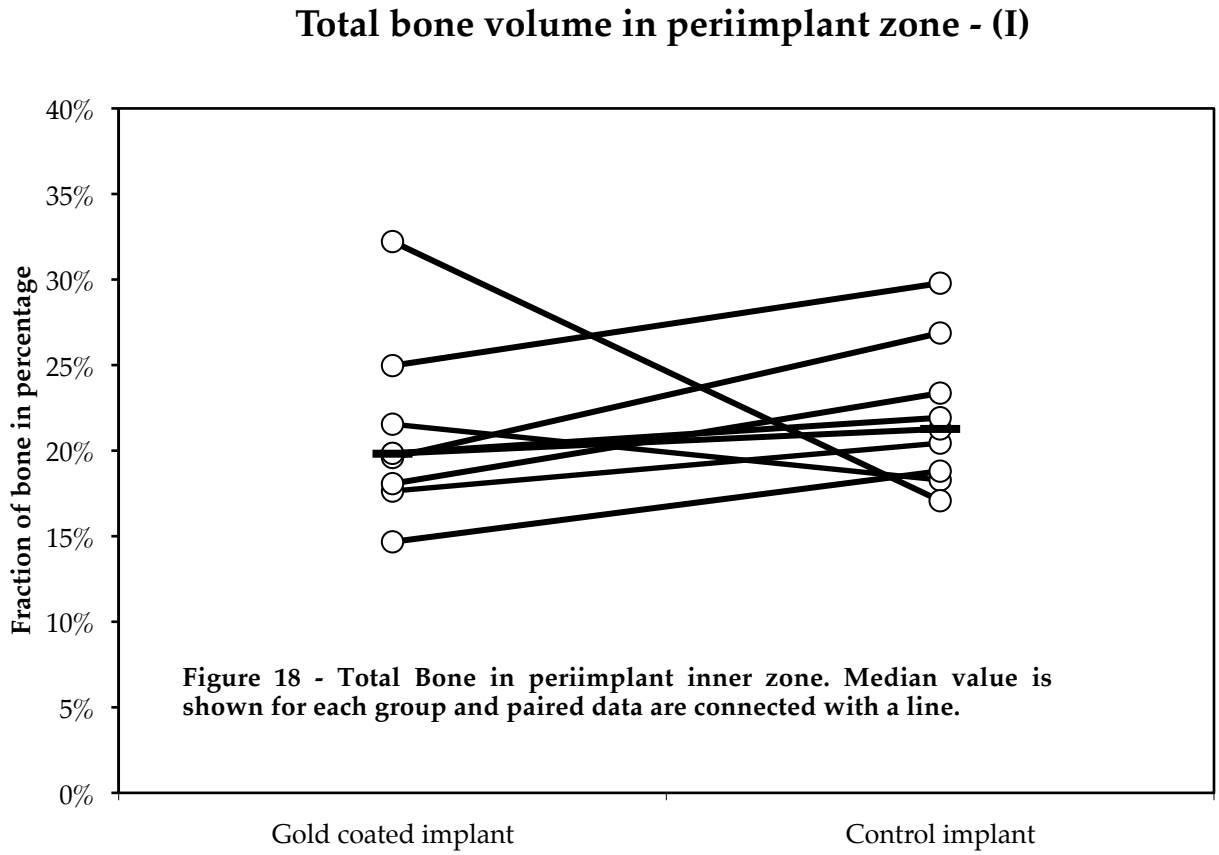



**Figure 18 - Total Bone in periimplant inner zone. Median value is shown for each group and paired data are connected with a line.**

### Biomechanical results

Gilding of the implants reduced the mechanical properties of the implant. Two of three parameters were statistically significantly reduced: Ultimate shear strength was reduced by 40% ($p = 0.045$) and maximal shear stiffness was reduced by 49% ($p = 0.017$). All results are shown in the table above.

### Histomorphometry

Gold coating of titanium implants resulted in a 28% median decrease in the amount of new bone-implant surface area contact ($p = 0.045$) and a 78% decrease in median lamellar bone-implant surface area contact ($p = 0.004$). Since the total amount of lamellar bone was low, all bone hits were accumulated, which resulted in a 35% reduction in total bone-implant contact ($p = 0.005$). There was no fibrous tissue ongrowth. No tissue specific changes could be found in the periimplant zone. The paired results are illustrated in the following graphs.

### Autometallography

The 30-µm sections prevented thorough AMG analysis of the cells adhering to the implant surface. However, AMG development of gold ions could be traced in a small number of scattered cells, most likely macrophages, based on the intracellular alignment of the ions.

## Study (II)

***Hypotheses***

(1) Gold particles can increase mechanical strength and stability of an implant.
(2) This would be due to reduced bone graft reabsorption and thereby increased total bone stock around the implant.
(3) Non-phagocytosable gold particles would be a fitting way to deliver gold ions to the periimplant tissue, and gold ions could be traced by AMG development.

***Results in short***

The study showed no statistically significant differences in implant mechanical fixation. The gold particles did not affect the allograft resorption or amount of total bone stock present around the implant or in contact with the implant.

Silver-enhanced gold ions were found adjacent to the gold particles in the allograft but not further away. The overall spreading of the particles was found uneven in the periimplant gap, and particle delivery in allograft was suggested as inadequate to modulate the whole gap. The local ion releases suggest that a biological effect on the inflammatory response is present, although the consequences are below detectable levels.

### Biomechanical results

Seven animals were used for push-out testing. The use of gold particles in allograft did not result in any statistically significant changes in any of the mechanical parameters. However, it was noticed that the gold particle group had a 27% increase ($p = 0.098$) in shear strength and 47% increase ($p = 0.134$) in total energy absorption. The results should be evaluated with the low number of specimens tested in mind.

## Histomorphometry

The histological results are illustrated in the following figures. No statistically significant differences could be found between the two groups at either the implant surface or the periimplant gap. Analyzed tissues were new woven bone, allograft, fibrous tissue, and bone marrow cells. Histomorphometry of the time-

**Table 6**
**Biomechanical results – (II)**

| | Ult. Shear Strength (MPa) | Max. Shear Stiffness (kPa/mm) | Total Energy Absorption (kJ/m$^2$) |
|---|---|---|---|
| Allograft without gold particles | 7.16 (5.02; 9.30) | 34.0 (14.5; 53.6)) | 1.35 (0.74; 1.96) |
| Allograft with 129 mg gold particles | 9.17 (6.77; 11.56) | 32.4 (23.7; 41.1) | 2.08 (1.2; 3.0) |
| Gold / Control (%) | 127 (94; 171) | 105 (68; 161) | 147 (85; 253) |
| p values | $p = 0.098$ | $p = 0.803$ | $p = 0.134$ |

**Table 6 - Mechanical results are shown with absolute mean values for each implant group. The relative median ratio is also shown, and the parentheses show 95% CI.**

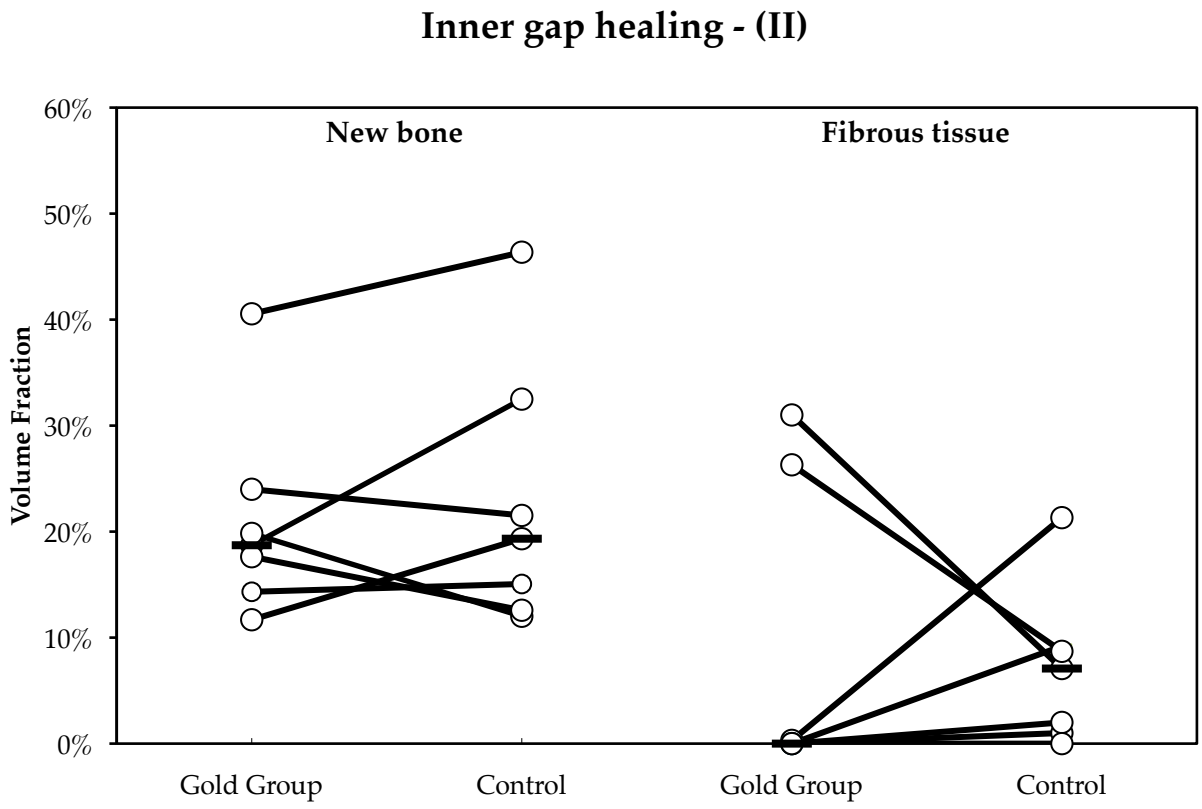



**Figure 19 - Tissue fraction in the inner gap. Median value for each group is shown and paired data are connected with a line.**

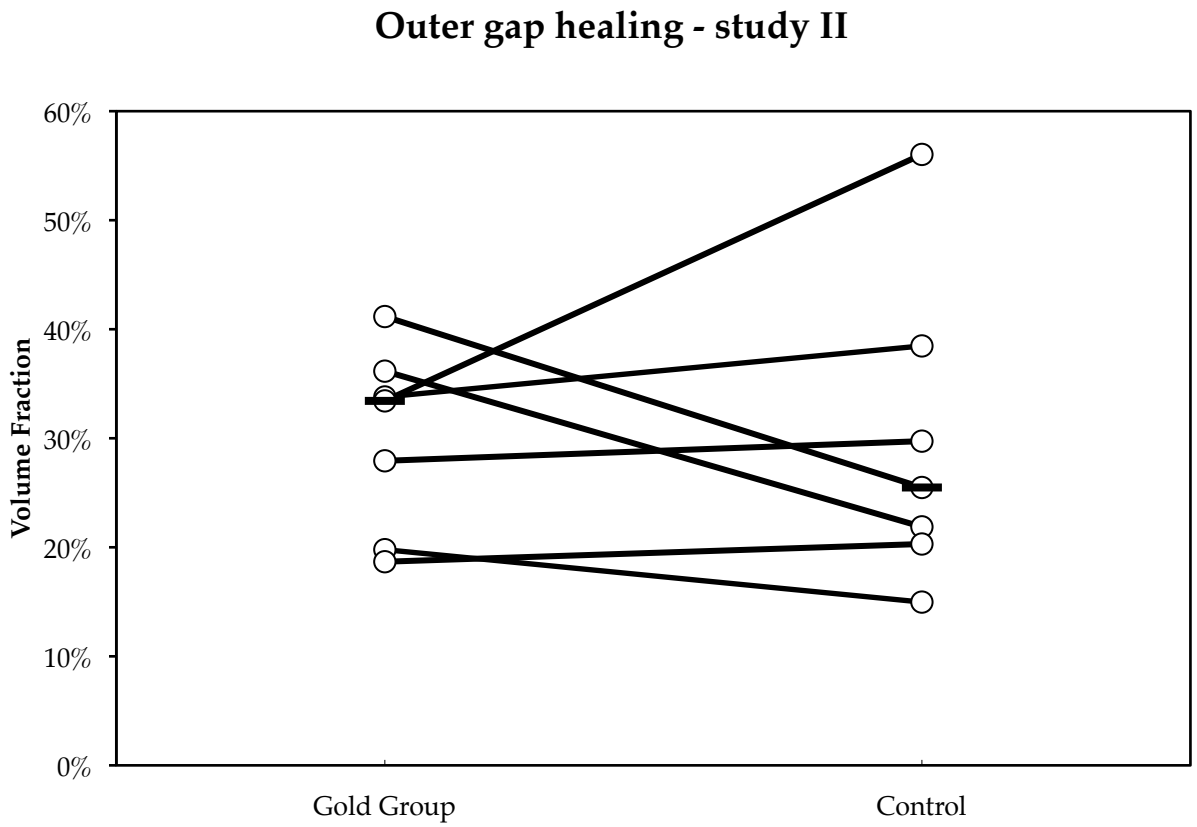



**Figure 20 - Tissue fraction in the outer gap. Median value for each group is shown and paired data are connected with a line.**

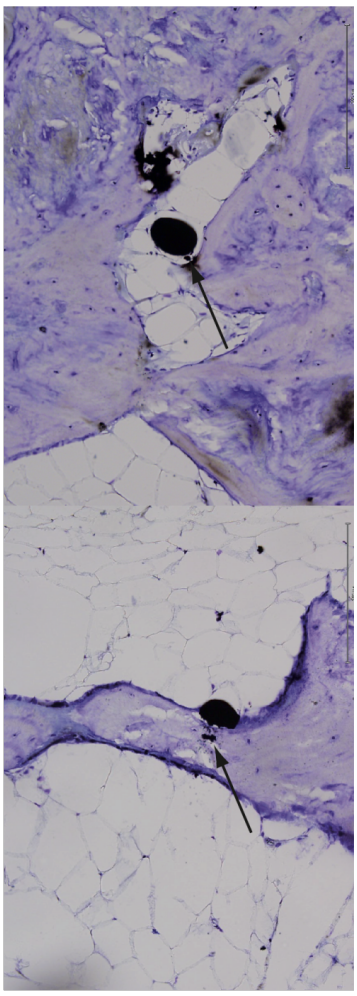


**Figure 21 – Representative sections of AMG-developed clusters in close proximity to the gold particles.**

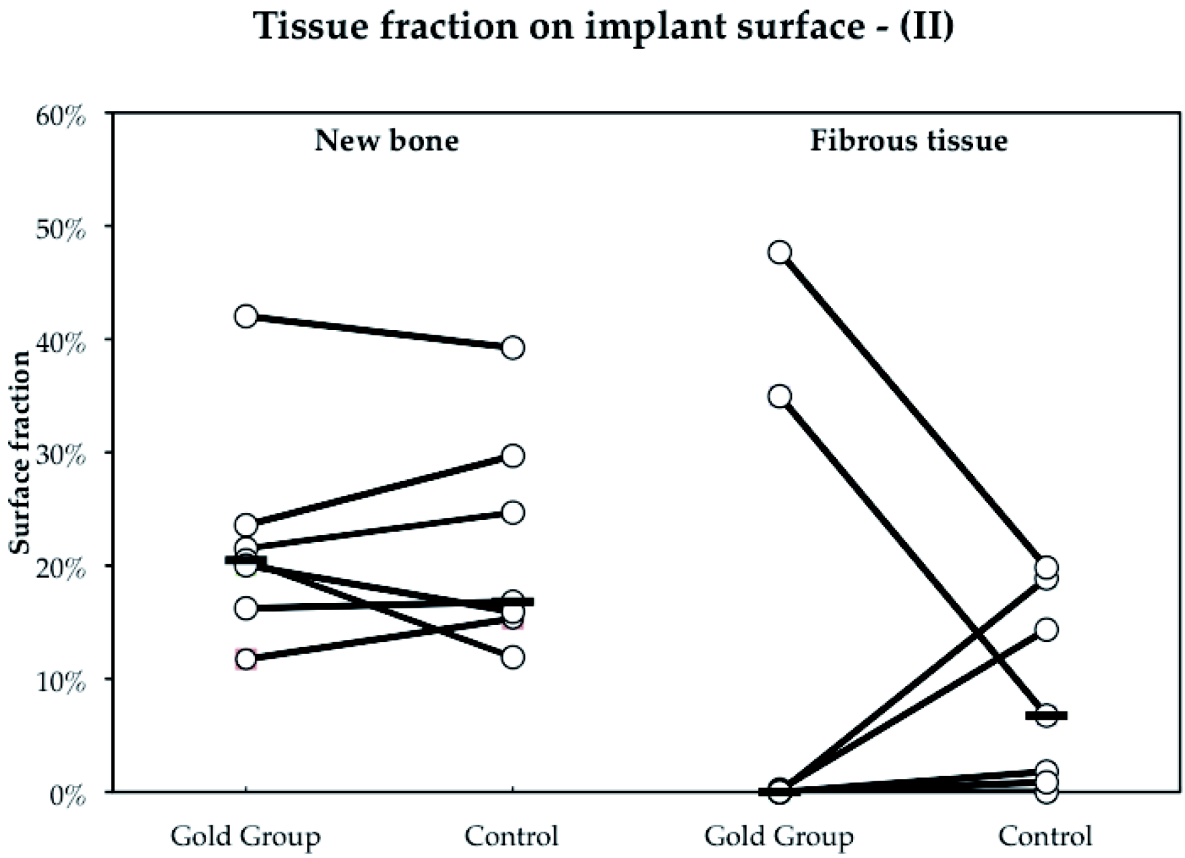



**Figure 22 - Tissue fraction on the implant surface. Median value for each group is shown and paired data are connected with a line**

**Table 7**
**Biomechanical results – (III)**

| | Ult. Shear Strength (MPa) | Max. Shear Stiffness (kPa/mm) | Total Energy Absorption ($kJ/m^2$) |
|---|---|---|---|
| AuTi | 1.94 (1.30; 2.90) | 6.29 (4.18; 9.48) | 452 (302; 678) |
| Ti | 1.87 (1.38; 2.53) | 5.78 (4.33; 7.72) | 504 (338; 752) |
| AuTi/Ti (%) | 104 (67; 160) | 109 (69; 172) | 90 (55; 145) |
| p values | *p = 0.85* | *p = 0.68* | *p = 0.62* |

**Table 7 - AuTi is the partial gold-coated implant. Ti is the control titanium implant. Biomechanical results are shown with absolute mean values for each group. The relative median is also shown and the parentheses show 95% CI.**

point zero specimen (the animal lost during surgery) revealed that approximately 31% of the peri-implant tissue was allograft on both sides. Almost all allograft tissue was found reabsorbed after the 12-week observation time and replaced by either new woven bone or fibrous tissue. Furthermore, the amount of fibrous tissue was at the highest on the implant surface, whilst it was reduced in the inner gap and fibrous tissue was absent in the outer gap.

### Autometallography

The gold particles were clearly visible in the silver-enhanced autometallography sections. They were unevenly distributed and mostly surrounded by bone marrow cells. With the use of higher magnification, numerous small particle-like clusters could be found in cells adjacent to the particles, which corresponded well with gold ion liberation. These findings were only available in some sections, as some specimens were of poor quality due to air between the glass and the tissue. However, the specimens that did reveal positive AMG sections were very illustrative.

## Study (III)

***Hypotheses***

(1) The combination of titanium and gold as an implant surface area (AuTi coating) will improve the mechanical strength and stability of the implant.
(2) AuTi coating will increase bone formation.
(3) Fibrous tissue formation will be reduced in the gap model due to gold's anti-inflammatory capabilities without disturbing the periimplant bone remodeling.

***Results in short***

No statistically significant differences were measured in early mechanical fixation, osseointegration, and periimplant bone density. Bone formation was found to be comparable between the two groups.

The partial gold-coated implants were not able to reduce or inhibit the fibrous membrane that was produced, but the periimplant bone remodeling was not affected by the coating.

### Biomechanical results

Specimens from nine animals were used in the push-out testing. As the table above shows, no statistically significant differences could be found between the partial gold-coated surface and the control titanium implant.

### Histomorphometry

There were no statistically significant differences in bone ongrowth or bone ingrowth between the two groups. Mean bone ongrowth using the AuTi coating was 4.8% compared to 2.8% in the control group ($p = 0.23$). Total bone ingrowth in the gap was found to be 39.6% compared to 34.6% in the control group ($p = 0.14$).

No statistically significant effect could be measured on fibrous tissue formation. Mean amount of fibrous tissue on the implant surface was found to be 20.6% in the AuTi group and 25.0% in the control titanium group ($p = 0.14$). The periimplant fibrous tissue was found to be 5.1% in the AuTi group and 5.0 in the control group ($p = 0.25$) when measuring the entire gap. However, as the amount of fibrous tissue was very low in the outer gap, the inner gaps were analyzed separately and mean fibrous tissue was found to be 11.7% in the AuTi group compared to 14.3% in the control titanium group ($p = 0.13$).

The bone remodeling activity measured in the periimplant gap was found unaltered as can be seen in Figure 25.

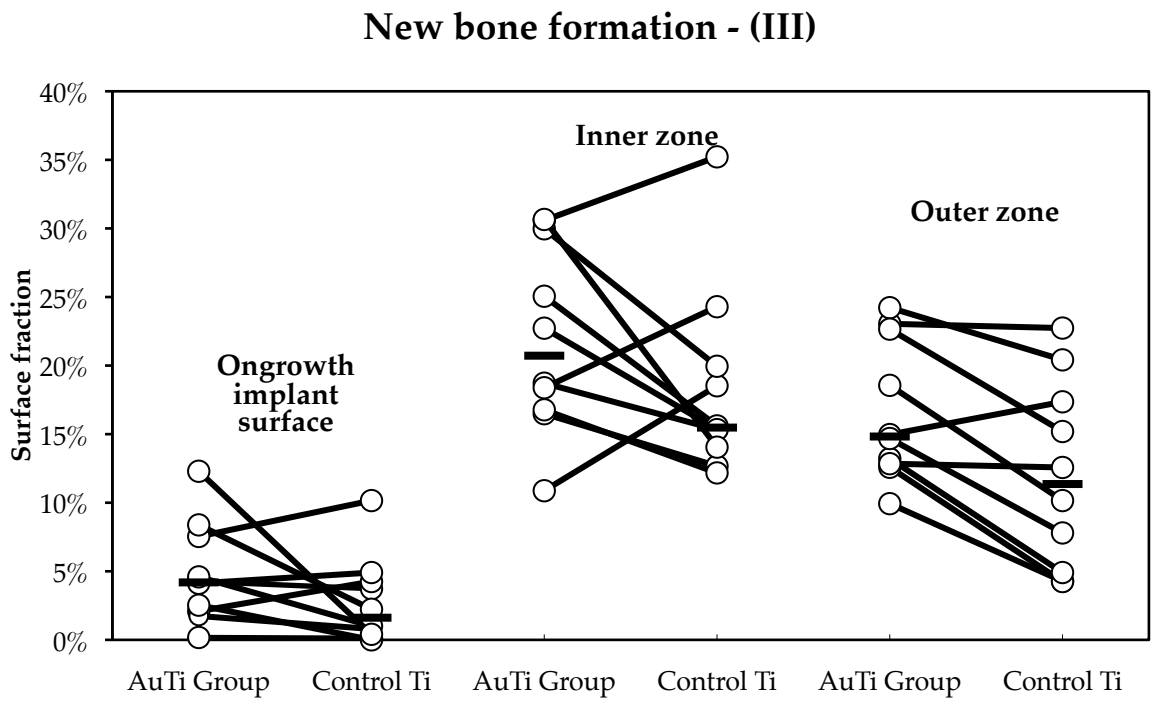



**Figure 23 – New bone formation on the implant surface and the periimplant gap. The median value is shown and the paired data are connected with a line.**

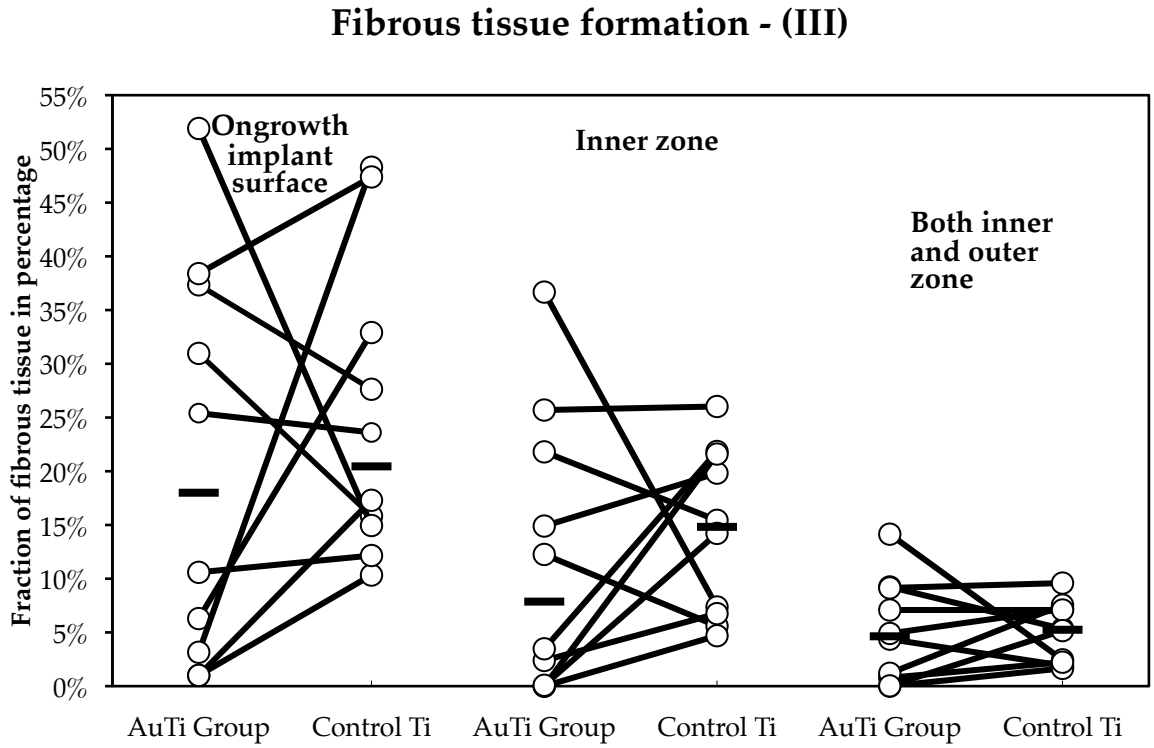



**Figure 24 - Amount of fibrous tissue on the implant surface and the periimplant zone. Amount of fibrous tissue in the outer zone was very low. Median value for each group is shown and the paired data are connected with a line.**

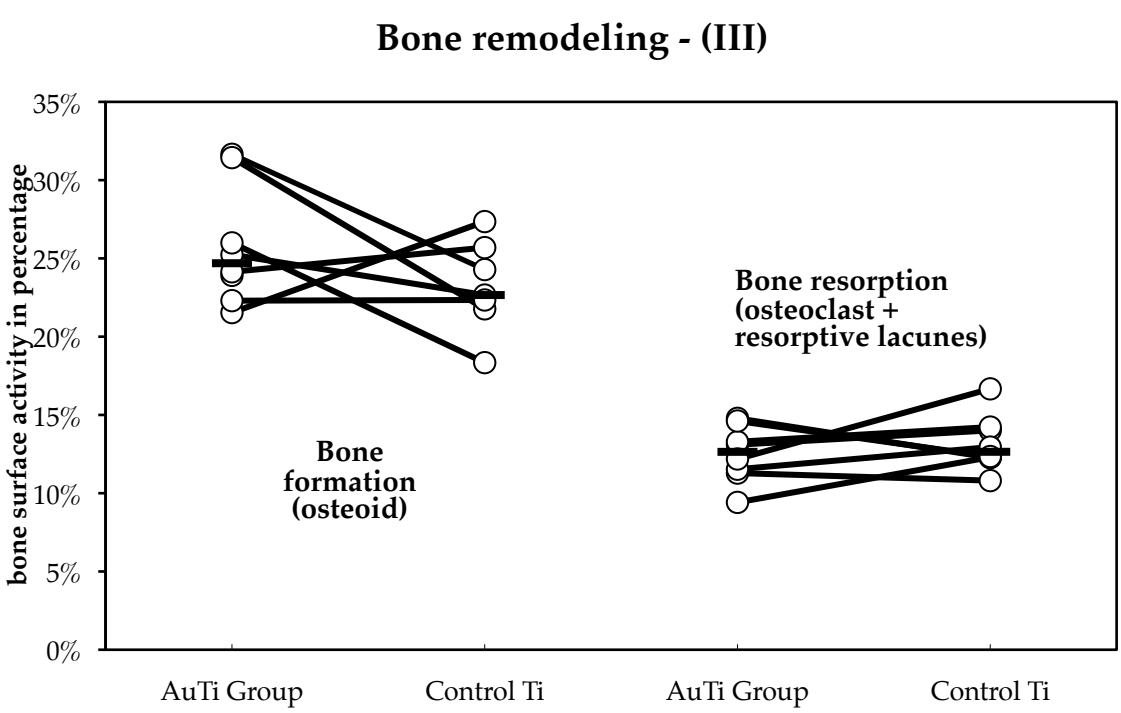



**Figure 25 - Bone formation and bone resorption are illustrated. Median values for each group are shown. The paired data are connected with a line.**

# Discussion

## Key aims and findings

The overall objective in orthopedic research is to enhance implant fixation with the purpose of obtaining a life-long anchorage between bone and implant. Increased implant fixation is achieved by facilitating osseointegration. Several contributing factors exist, including implant design, host bone bed, mechanical stabilization, loading conditions, adjuvant therapies, and remodeling of the periimplant bone [74]. Vast effort, in particular, has been made to increase the implant's early bony anchorage, as this has been correlated with prolonged longevity. Although initial osseointegration is significantly improved with the implementation of hydroxyappatite coatings and the development of newer implant designs, chronic low-grade inflammatory processes with subsequent periimplant osteolysis and aseptic implant loosening are still a major concern in implant surgery, and necessitate continued interest in implant longevity improvement.

The specific aim with this thesis was to initiate an investigation of whether the recently documented anti-inflammatory capabilities of metallic gold implants inserted in vivo could represent a contribution to the field of orthopedic surgery. Short-term experimental studies were initiated to address the magnitude of the, in principal, disadvantageous effects of antiinflammatory effects on early implant integration and ways to bring them to a minimum. Metallic gold particles were incorporated into allograft to see whether immediate graft reabsorption could be reduced. Furthermore, mechanical fixation and the osseointegrative properties of two different gold-coated implants were compared to these factors in control titanium implants.

No measurable effect of the gold particles on allograft reabsorption, bone density, and bone ongrowth was found in (II), and therefore mechanical fixation was unchanged. The release of gold ions was detectable only in tissue in close proximity to each gold particle. Although statistically insignificant, mechanical tests showed an increase in ultimate shear strength and total energy absorption, with p values close to statistically significant values ($p = 0.098$ and $p = 0.134$, respectively). These results should be considered along with the fact that only seven specimens were investigated, and statistically significant results were therefore difficult to obtain. Histological results, however, did not support any mechanical trends. It is plausible that the incorporation of non-soluble particles into the graft by itself increased the mechanical strength of the allograft compared to the allograft alone. This is only speculative, but it resembles the mixing of gravel with cement in the building industry, which strengthens the cement's strength.

(I) revealed that complete gilding of the implant reduced its osseointegrative properties. New bone ongrowth and total bone-to-implant contact were statistically reduced by 28% and 35%, respectively. Histomorphometry revealed only small amounts of fibrous tissue in both group. The reduction in strength and stiffness must therefore have been due to a reduction in the amount of new bone formed and in the degree of mineralization of the bone in contact with the inserted implant.

Consequently, the partial gold-coated implant surface was designed, constructed, and investigated in (III). Biomechanical testing showed no difference between the partial gold-coated implants and the control titanium implants nor did histomorphometrical analysis. However, as seen in Figure 23, bone ongrowth and bone ingrowth in the periimplant gap were slightly increased, with p values close to statistically significant values. Furthermore, the partial gold-coated implants show reduced median values for fibrous tissue ongrowth and reduced median values for periimplant fibrous tissue density, although these values were not statistically significant. It is important to bear in mind that no statistically significant differences were seen between any of the histological parameters in (III). This is, however, considered as an encouraging result, as the partial gold-coated implant is an improvement compared to the complete gold-coated implant. It is also plausible that by increasing the strength of the study, it would have been possible to establish a statistically significant difference between the two groups.

## Limitations

As previously discussed in "Observation time," short study times were used to address the immediate effects of metallic gold in these studies. Positive long-term results are not clinically applicable, if the short-term effect, as seen in (I), is negative. Clinically inserted implants need sufficient initial fixation because patient mobility, which involves weight bearing, is essential right after surgery. In addition, the time points were chosen on the

basis of previous studies using the same models. One should still keep in mind that these observations represent only a brief moment in the entire bone healing process. Any possible unrevealed effects before or after these time points can only be speculative. However, it is reasonable to question whether a shorter observation time than 12 weeks in (II) could have contributed to statistically significant results, as the present study showed that allograft were reabsorbed in both groups. A shorter observation time could in theory have shown that graft reabsorption was significantly slower in the gold group. The hypothesis was that due to gold's anti-inflammatory capabilities, the immune response would be decreased, with a subsequent reduction of osteoclasts and their reabsorptive functions. This is, however, only speculative, as no differences could be registered after 12 weeks.

This thesis does not question the anti-inflammatory capabilities of metallic gold, since recent documentation regarding this is substantial. But it raises the question of whether metallic gold can be used in orthopedic surgery or not. When it comes to gold in general, in vitro studies have illustrated some of the working mechanisms of gold ions [21, 25, 99-101], whereas in vivo studies not only have documented metal ion releases in the form of the previously mentioned dissolucytosis and the subsequent ion uptake in inflammatory cells [33], but also proven inhibitory effect of gold ions on inflammatory cell proliferation [42-45]. These effects and findings have been highlighted earlier on in this thesis. The inflammatory response plays a pivotal role in implant surgery. In short, it augments bone healing and bone regeneration immediately after surgery with the help of fibrous tissue formation and subsequent intramembraneous ossification, but it can also induce unfavorable conditions if a solid fibrous membrane is produced around an implant, leading to a reduced likelihood of osseointegration of the implant. One can argue that even though the working mechanisms considered in this thesis are believed to be inflammatory processes, none of the commonly used inflammatory markers, such as cytokines, were measured. This is not considered a limiting factor, seeing that bone regeneration and implant osseointegration are considered directly linked to the inflammatory process, as described in "Background."

Three slightly different models were chosen to address different aspects of initial implant fixation, with metallic gold as the common denominator: bone ongrowth, (I); effect on the osteoclast-based bone resorption, (II); and possible early inhibition of fibrous tissue formation, (III). Inter-study comparison should only be done with extreme care. Nonetheless, the results from (I) and (III) were compared with each other, as the partially gold-coated implant is a further development from the original complete gilded implant. The results show that bone ongrowth is very low in both groups in (III) compared to those in (I). In (I), bone ongrowth was high, and it was also the parameter mostly influenced by the coating. Previous studies show that with the use of a critical size defect above 1 mm around the implant, bone ongrowth is reduced after four week observation time [83]. The amount of bone in direct contact with the implant is significantly lower in (III) in both groups. It is reasonable to speculate whether the partial gold-coated implant would have behaved differently in a press-fit model than it did in the gap model that was used. The present work is therefore limited in its ability to compare results across studies. However, because (III) created the opportunity to investigate another parameter, e.g. fibrous tissue, it was hypothesized that gold's antiinflammatory effect could be visualized by quantifying its effect on fibrous tissue formation.

In each study, ten paired implants were investigated. The power calculation that was done requested ten animals. There is a possibility that ten animals are insufficient, due to the risk of losing animals. This was made particularly obvious in (II) in which the strength of the study was reduced by the loss of several specimens. This is indeed a limiting factor, and further investigations should include extra animals to prevent this.

## Considerations regarding mechanisms and results

Several studies have dealt with inflammatory response modulation in the context of orthopedic surgery. Several experimental and clinical investigations show increasing evidence of reduced new bone formation after the use of NSAIDs, e.g. the inhibition of spinal fusion after the use of NSAIDs [102]. Long-term studies have shown that the use of NSAIDs postoperatively increases the risk of implant loosening in the long run [12], presumably due to decreased early osseointegration of the implant. All treatments were, however, systemic interventions. The purpose of this thesis was to integrate *auromedication* into orthopedic

surgery, because a local intervention could bypass any systemic adverse effects. To our knowledge no previous in vivo studies have investigated metallic gold as an orthopedic implant surface.

## Study (I)

Cortizo et al. investigated the influence of dental materials' biocompatibility related to metal ion release on osteoblast-like cells in vitro. They studied cell functions such as morphological alterations, cell growth, and osteoblast differentiation and found that pure gold and pure titanium were both biocompatible and not toxic toward osteoblast proliferation. But as (I) shows, the metallic gold surface resulted in a statistically significant reduction in new bone ongrowth, with subsequent reduction of mechanical fixation. The reduced mineralization on gold surfaces corresponds with the work of Thomsen et al. [103], who found reduced bone formation on gold-coated screws. This was however in cortical bone, whereas in the present study implants were inserted into trabecular bone. Thomsen et al. considered gold as a noble metal that does not create surface oxides, and thus does not affect the surrounding tissue, whilst titanium surfaces were active oxidized surfaces. Although metallic gold is not active, we reckon that it interacts with the surrounding tissue due to the organism's always-active inflammatory cells, such as macrophages, and their dissolucytosis of implant surfaces, as illustrated by Danscher and also the present AMG sections. However, gold's effect is not on cell growth or cell differentiation, and the reduced bone formation is not considered to be due to any toxicity or biocompatibility issues. The reason must be found elsewhere.

Gold implants were inserted due to gold ions documented effect on the NF-κ-B-DNA binding in macrophages and macrophages role in osteoclastogenesis [21, 44]. Especially the role of TNF-α in osteoclastogenesis is believed to be very important [104, 105]. The inflammatory process was believed to somehow modulate the reduction of bone reabsorption in the period immediately after implant insertion, whilst bone formation was unaffected, thereby increasing total implant osseointegration and strengthen mechanical stability. Instead, the reduced bone formation and thereby weakened mechanical fixation could be due to an excessive inhibitory function on the inflammatory response after the traumatic insertion of the implant. As described earlier, macrophages are the first cells to be involved in bone repair after bone damage. An overall inhibition of these early inflammatory processes could reduce bone ingrowth. Although gold-coated implants are a local anti-inflammatory treatment, reduced osseointegration was still observed similar to that seen in association with systemic NSAID administration.

An important factor is tartrate-resistant acid phosphatase (TRAP). TRAP containing cells are macrophages, osteoclasts, and dendritic cells, and TRAP is believed to be important for processing osteopontin. Osteopontin is a highly acidic protein secreted by osteoblasts and osteoclasts and is associated with bone formation and mineralization because of its capacity to bind calcium and hydroxyappatite. Hayman et al. showed that TRAP could be modulated by gold compounds such as gold chloride [106]. These findings concerning gold compounds' inhibitory effect on TRAP activity could explain why new bone formation is reduced on the metallic gold-coated implants, although Cortizo showed no difference in osteoblast proliferation. It is possible that gold ions, released through dissolucytosis and found intracellular in macrophages and monocytes (visualized with the applied AMG technique), reduce the TRAP activity and thereby the mineralization process. Macrophages and their precursors are the first cells to arrive at the site of the inserted implant, and after attachment, they initiate bone and tissue regeneration. If macrophages are disabled or are somehow unable to initiate bone formation due to inhibited TRAP activity, new bone formation and mechanical stability will be reduced, as seen in this study. Whether the observed results are due to an inhibition of NF-κ-B-DNA binding in macrophages and the effect of this on bone repair or to an inhibition of TRAP activity and thus a direct effect on bone mineralization, or perhaps to a complex combination of several pathways is uncertain.

## Study (II)

The application of auromedication to the allograft was meant to modify the immediate allograft resorption that occurs. As described earlier, the negative immunological responses can be reduced significantly by using HLA-matched donors and cryopreserving the graft material. Although allograft contains osteoinductive and osteoconductive capabilities, its weakness lies within the possibility that the graft can be reabsorbed before it bridges the gap between healthy bone and the implant, leaving a gap void. In the

present allograft study, only a few animals had fibrous tissue in the periimplant gap, and most of the allograft was reabsorbed and replaced by new bone in both groups studied. As mentioned earlier, it is possible that a shorter observation time could result in more obvious effects of gold particles on allograft resorption. This is because macrophages are believed to have a significant role in the initiation of allograft reabsorption. Furthermore, the osteoclasts responsible for allograft reabsorption could also be affected. They derive from pre-monocytes, which again could have been inhibited with regard to further functioning and proliferation after as a result of the dissolucytosis of gold ions. However, AMG showed that the gold ions were only visible in close proximity to the particles. It is possible that the dosage of the gold particles was insufficient to have any clinical effect. It is also possible that the distribution of the gold particles was not adequate, as most particles were surrounded by bone marrow tissue. Whether the distribution method caused this or whether it was because of the gold particle's capability to suppress osteoclastogenesis and thereby allograft reabsorption, is uncertain.

**Study (III)**

The partial gold-coated implant, referred to as an AuTi coating, was constructed to reduce the gold thickness, to reduce the surface area covered with metallic gold, and to bring the osteoinductive capabilities of titanium to the surface. This was to prevent the negative effects of gold on early bone formation and on the bone mineralization that is essential for osseointegration, and at the same time maintain an anti-inflammatory surface.

As the two previous studies showed, metallic gold can only affect tissue in close proximity to the implant due to the limited range of the dissolucytosis. Bone ongrowth was unchanged in this study. It should however be highlighted that the amount of total bone-in-contact with implant was very low in both the control group and the intervention group; the possibility of an unregistered difference is therefore present (type 2 error). However, looking at the paired results in each animal, it becomes clear that there is no obvious trend in the data. It seems that the AuTi coating does not interfere with the above-mentioned mineralization process. Surprisingly, the AuTi coating did not show any statistically significant effect on fibrous tissue ongrowth either. Perhaps the initial postoperative inflammatory response was so extreme that the metallic gold was unable to modulate it significantly. However, this is in part contradicted by the work of Pedersen et al., who showed that inserted gold particles in the brain could minimize the inflammatory response following focal brain injury [43-45]. It is still reasonable to speculate whether this is the case, because the inflammation and fibrous tissue formation are extreme. With this in mind, it is suggested that the presented AuTi coating could play a role against the more chronic low-term inflammatory response that occurs due to particle-induced macrophage activation. However, it is still uncertain whether the AuTi coating would interact differently if it had been introduced in a different setting such as press-fit insertion. Furthermore, since the reduction of gold thickness and the reduced surface area covered with gold lead to results comparable to those seen with the control titanium implant, it can be speculated whether further reduction of gold would increase the osseointegrative properties of the implant, and whether the results in studies (I) and (III) are merely a dosage-dependent issue.

# Conclusion

This thesis investigated the early functions of metallic gold on implant osseointegration and implant fixation. Additionally, the effect of metallic gold on periimplant bone remodeling was studied. Finally the ability of the AuTi coating to inhibit fibrous tissue formation was addressed. The thesis shows that metallic gold inserted into bone releases gold ions that can migrate intracellular into adjacent boney tissues. It is clear that dissolucytosis of ions has a limited range in bone and the effect is only observed in close proximity to the gold substrate. Metallic gold as an implant surface is inferior compared to titanium due to reduced bone mineralization, implant osseointegration and thus subsequent mechanical strength and stability. For now, metallic gold shows no encouraging results in orthopedics, because we did not find any effect on early fibrous tissue formation or any effect on osteoclastogenesis that could reduce allograft reabsorption. But its inhibitory effect on new bone formation resembles findings with different anti-inflammatory treatments. In conclusion, metallic gold and auromedication have no imperative role in promoting early implant fixation and implant osseointegration in orthopedics.

# Perspectives and future research

The development of the partial gold-coated implant that combines gold and titanium as a surface substrate (AuTi) creates new questions. If sufficient bone mineralization and bone formation together with satisfactory mechanical fixation could be established, the effects on the low-grade chronic inflammation that occurs antecedent to aseptic implant loosening could be addressed. It is feasible that a partial gold-coated surface is capable of suppressing the late particle-induced inflammatory process, without reducing the amount of early fibrous tissue production. Furthermore, the prevention or reduction of osteolysis can be investigated using the micro-motion model [84] in which osteolysis is initiated by creating standardized loading conditions. These studies can eventually lead to prolonged long-term investigations.

To further investigate different implant coatings and their immediate effect on osteoblast morphology and bone mineralization, in vitro studies have been initiated. They focus on early cell attachment on implant surfaces and furthermore, they investigate early osteoblast markers, such as ALP, and bone mineralization, such as the calcium content. Such studies investigating titanium, complete gold, and partial gold with titanium surfaces, similar to the implants used in the in vivo studies, are underway. This setup allows quick investigation of coatings before large and more comprehensive animal models are employed.

# References


1. Konttinen, Y.T., et al., *Cytokines in aseptic loosening of total hip replacement.* Current Orthopaedics, 1997. **11**(1): p. 40-47.
2. Pedersen, A.B., et al., *Total hip arthroplasty in Denmark: incidence of primary operations and revisions during 1996-2002 and estimated future demands.* Acta Orthop, 2005. **76**(2): p. 182-9.
3. Overgaard, S., et al., *Danish Hip Register Annual Report 2009*. 2010.
4. Archibeck, M.J., et al., *The basic science of periprosthetic osteolysis.* Instr Course Lect, 2001. **50**: p. 185-195.
5. Bauer, T.W. and J. Schils, *The pathology of total joint arthroplasty.II. Mechanisms of implant failure.* Skeletal Radiol., 1999. **28**(9): p. 483-497.
6. Holt, G., et al., *The biology of aseptic osteolysis.* Clin Orthop Relat Res, 2007. **460**: p. 240-52.
7. Eskelinen, A., et al., *Uncemented total hip arthroplasty for primary osteoarthritis in young patients: a mid-to long-term follow-up study from the Finnish Arthroplasty Register.* Acta Orthop., 2006. **77**(1): p. 57-70.
8. Karrholm J Fau - Herberts, P., et al., *Radiostereometry of hip prostheses. Review of methodology and clinical results.* (0009-921X (Print)).
9. Ryd, L., et al., *Roentgen stereophotogrammetric analysis as a predictor of mechanical loosening of knee prostheses.* J Bone Joint Surg Br, 1995. **77**(3): p. 377-83.
10. Gretzer, C., et al., *The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials.* J Biomater Sci Polym Ed, 2006. **17**(6): p. 669-87.
11. Buvanendran, A., et al., *Upregulation of prostaglandin E2 and interleukins in the central nervous system and peripheral tissue during and after surgery in humans.* Anesthesiology, 2006. **104**(3): p. 403-410.
12. Persson, P.E., O.S. Nilsson, and A.M. Berggren, *Do non-steroidal anti-inflammatory drugs cause endoprosthetic loosening? A 10-year follow-up of a randomized trial on ibuprofen for prevention of heterotopic ossification after hip arthroplasty.* Acta Orthop., 2005. **76**(6): p. 735-740.
13. Gerstenfeld, L.C., et al., *Differential inhibition of fracture healing by non-selective and cyclooxygenase-2 selective non-steroidal anti-inflammatory drugs.* J.Orthop.Res., 2003. **21**(4): p. 670-675.
14. Demann, E.T., P.S. Stein, and J.E. Haubenreich, *Gold as an implant in medicine and dentistry.* J.Long.Term.Eff.Med.Implants., 2005. **15**(6): p. 687-698.
15. Richards, D.G., et al., *Gold and its relationship to neurological/glandular conditions.* Int J Neurosci, 2002. **112**(1): p. 31-53.
16. Burmester, G.R., *Molecular mechanisms of action of gold in treatment of rheumatoid arthritis--an update].* Z.Rheumatol., 2001. **60**(3): p. 167-173.
17. Rau, R., *Have traditional DMARDs had their day? Effectiveness of parenteral gold compared to biologic agents.* Clin.Rheumatol., 2005. **24**(3): p. 189-202.
18. Kean, W.F., et al., *The history of gold therapy in rheumatoid disease.* Semin Arthritis Rheum, 1985. **14**(3): p. 180-6.
19. Vernon-Roberts, B., *Action of gold salts on the inflammatory response and inflammatory cell function.* J.Rheumatol.Suppl, 1979. **5**: p. 120-129.
20. Persellin, R.H. and M. Ziff, *The effect of gold salt on lysosomal enzymes of the peritoneal macrophage.* Arthritis Rheum, 1966. **9**(1): p. 57-65.
21. Yang, J.P., et al., *Inhibition of the DNA-binding activity of NF-kappa B by gold compounds in vitro.* FEBS Lett., 1995. **361**(1): p. 89-96.
22. Jeon, K.I., J.Y. Jeong, and D.M. Jue, *Thiol-reactive metal compounds inhibit NF-kappa B activation by blocking I kappa B kinase.* J Immunol, 2000. **164**(11): p. 5981-9.
23. Yamashita, M., et al., *Inhibition of TPA-induced NF-kappaB nuclear translocation and production of NO and PGE2 by the anti-rheumatic gold compounds.* J.Pharm.Pharmacol., 2003. **55**(2): p. 245-251.
24. Yanni, G., et al., *Intramuscular gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane.* Ann Rheum Dis, 1994. **53**(5): p. 315-22.
25. Yoshida, S., et al., *Inhibition of IL-6 and IL-8 induction from cultured rheumatoid synovial fibroblasts by treatment with aurothioglucose.* Int.Immunol., 1999. **11**(2): p. 151-158.
26. Mangalam, A.K., A. Aggarwal, and S. Naik, *Mechanism of action of disease modifying anti-rheumatic agent, gold sodium thiomalate (GSTM).* Int Immunopharmacol, 2001. **1**(6): p. 1165-72.
27. Schultz, D.R., et al., *Inactivation of C1 in rheumatoid synovial fluid, purfied C1 and C1 esterase, by gold compounds.* Clin Exp Immunol, 1974. **17**(3): p. 395-406.
28. Littman, B.H. and R.E. Hall, *Effects of gold sodium thiomalate on functional correlates of human monocyte maturation.* Arthritis & Rheumatism, 1985. **28**(12): p. 1384-1392.
29. Felson, D.T., J.J. Anderson, and R.F. Meenan, *The comparative efficacy and toxicity of second-line drugs in rheumatoid arthritis. Results of two metaanalyses.* Arthritis Rheum, 1990. **33**(10): p. 1449-61.
30. Tozman, E.C. and N.L. Gottlieb, *Adverse reactions with oral and parenteral gold preparations.* Med Toxicol, 1987. **2**(3): p. 177-89.

31. Kanabrocki, E., et al., *Neutron-activation studies of trace elements in human fingernail.* J Nucl Med, 1968. **9**(9): p. 478-81.
32. Mulherin, D.M., G.R. Struthers, and R.D. Situnayake, *Do gold rings protect against articular erosion in rheumatoid arthritis?* Ann Rheum Dis, 1997. **56**(8): p. 497-9.
33. Danscher, G., *In vivo liberation of gold ions from gold implants. Autometallographic tracing of gold in cells adjacent to metallic gold.* Histochem.Cell Biol., 2002. **117**(5): p. 447-452.
34. Larsen, A., M. Stoltenberg, and G. Danscher, *In vitro liberation of charged gold atoms: autometallographic tracing of gold ions released by macrophages grown on metallic gold surfaces.* Histochem Cell Biol, 2007. **128**(1): p. 1-6.
35. Futami, T., et al., *Tissue response to titanium implants in the rat maxilla: ultrastructural and histochemical observations of the bone-titanium interface.* J Periodontol, 2000. **71**(2): p. 287-98.
36. Ziats, N.P., K.M. Miller, and J.M. Anderson, *In vitro and in vivo interactions of cells with biomaterials.* Biomaterials, 1988. **9**(1): p. 5-13.
37. Walivaara, B., et al., *Titanium with different oxides: in vitro studies of protein adsorption and contact activation.* Biomaterials, 1994. **15**(10): p. 827-34.
38. Kao, W.J., J.A. Hubbell, and J.M. Anderson, *Protein-mediated macrophage adhesion and activation on biomaterials: a model for modulating cell behavior.* J Mater Sci Mater Med, 1999. **10**(10/11): p. 601-5.
39. Ferre, N. and J. Claria, *New insights into the regulation of liver inflammation and oxidative stress.* Mini Rev Med Chem, 2006. **6**(12): p. 1321-30.
40. Graham, G.G. and A.J. Kettle, *The activation of gold complexes by cyanide produced by polymorphonuclear leukocytes. III. The formation of aurocyanide by myeloperoxidase.* Biochem.Pharmacol., 1998. **56**(3): p. 307-312.
41. Elder, R.C., et al., *Dicyanogold (I) is a common human metabolite of different gold drugs.* J Rheumatol, 1993. **20**(2): p. 268-72.
42. Larsen, A., et al., *Gold ions bio-released from metallic gold particles reduce inflammation and apoptosis and increase the regenerative responses in focal brain injury.* 2008(0948-6143 (Print)).
43. Pedersen, M.O., et al., *Metallic gold treatment reduces proliferation of inflammatory cells, increases expression of VEGF and FGF, and stimulates cell proliferation in the subventricular zone following experimental traumatic brain injury.* Histol Histopathol, 2009. **24**(5): p. 573-86.
44. Pedersen, M.O., et al., *Metallic gold reduces TNFalpha expression, oxidative DNA damage and pro-apoptotic signals after experimental brain injury.* Brain Res, 2009. **1271**: p. 103-13.
45. Pedersen, M.O., et al., *Bio-released gold ions modulate expression of neuroprotective and hematopoietic factors after brain injury.* Brain Res, 2009. **1307**: p. 1-13.
46. Bauer, T.W. and J. Schils, *The pathology of total joint arthroplasty. I. Mechanisms of implant fixation.* Skeletal Radiol., 1999. **28**(8): p. 423-432.
47. Harris, W., et al., *Extensive localized bone resorption in the femur following total hip replacement.* J Bone Joint Surg Am, 1976. **58**(5): p. 612-618.
48. Heisel, C., M. Silva, and T.P. Schmalzried, *Bearing surface options for total hip replacement in young patients.* Instr Course Lect, 2004. **53**: p. 49-65.
49. Pilliar, R.M., J.M. Lee, and C. Maniatopoulos, *Observations on the effect of movement on bone ingrowth into porous-surfaced implants.* Clin Orthop Relat Res, 1986(208): p. 108-13.
50. Geesink, R.G., *Osteoconductive coatings for total joint arthroplasty.* Clin Orthop Relat Res, 2002(395): p. 53-65.
51. Champagne, C.M., et al., *Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2.* Bone, 2002. **30**(1): p. 26-31.
52. Chen, D., M. Zhao, and G.R. Mundy, *Bone morphogenetic proteins.* Growth Factors, 2004. **22**(4): p. 233-41.
53. Jakobsen, T., *The Influence of Local Bisphosphonate Treatment on Implant Fixation*, in *Orthopaedic Research Laboratory*. 2008, Aarhus University: Aarhus. p. 58.
54. Cortizo, M.C., M.F. De Mele, and A.M. Cortizo, *Metallic dental material biocompatibility in osteoblastlike cells: correlation with metal ion release.* Biol.Trace Elem.Res., 2004. **100**(2): p. 151-168.
55. Burchardt, H., *The Biology of Bone Graft Repair.* Clinical Orthopaedics and Related Research, 1983. **174**: p. 28-34.
56. Slooff, T.J., et al., *Bone grafting in total hip replacement for acetabular protrusion.* Acta Orthop Scand, 1984. **55**(6): p. 593-6.
57. Toms, A.D., et al., *Impaction bone-grafting in revision joint replacement surgery.* J Bone Joint Surg Am, 2004. **86-A**(9): p. 2050-60.
58. Bauer, T.W.M.D.P. and G.F.M.D. Muschler, *Bone Graft Materials: An Overview of the Basic Science. [Report].* 2000.
59. Goldberg, V.M., et al., *Bone grafting: role of histocompatibility in transplantation.* J Orthop Res, 1985. **3**(4): p. 389-404.
60. Goldberg, V.M., et al., *Improved acceptance of frozen bone allografts in genetically mismatched dogs by immunosuppression.* J Bone Joint Surg Am, 1984. **66**(6): p. 937-50.
61. Rostlund, T., et al., *Difference in tissue response to nitrogen-ion-implanted titanium and c.p. titanium in the abdominal wall of the rat.* J Biomed Mater Res, 1990. **24**(7): p. 847-60.
62. Ingham, E. and J. Fisher, *The role of macrophages in osteolysis of total joint replacement.* Biomaterials, 2005. **26**(11): p. 1271-1286.

63. Goodman, S.B., *Wear particles, periprosthetic osteolysis and the immune system.* Biomaterials, 2007. **28**(34): p. 5044-8.
64. Merkel, K.D., et al., *Tumor necrosis factor-alpha mediates orthopedic implant osteolysis.* Am.J.Pathol., 1999. **154**(1): p. 203-210.
65. Simonet, W.S., et al., *Osteoprotegerin: a novel secreted protein involved in the regulation of bone density.* Cell, 1997. **89**(2): p. 309-19.
66. Lacey, D.L., et al., *Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation.* Cell, 1998. **93**(2): p. 165-76.
67. Burgess, T.L., et al., *The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts.* J Cell Biol, 1999. **145**(3): p. 527-38.
68. Teitelbaum, S.L., *Bone Resorption by Osteoclasts.* Science, 2000. **289**(5484): p. 1504-1508.
69. Wei, S., et al., *IL-1 mediates TNF-induced osteoclastogenesis.* J.Clin.Invest, 2005. **115**(2): p. 282-290.
70. Clohisy, J.C., et al., *RANKL is an essential cytokine mediator of polymethylmethacrylate particle-induced osteoclastogenesis.* J.Orthop.Res., 2003. **21**(2): p. 202-212.
71. Nakashima, T. and H. Takayanagi, *Osteoclasts and the immune system.* J Bone Miner Metab, 2009. **27**(5): p. 519-29.
72. Hayman, A.R. and T.M. Cox, *Tartrate-resistant acid phosphatase: a potential target for therapeutic gold.* Cell Biochem Funct, 2004. **22**(5): p. 275-80.
73. Mbalaviele, G., et al., *Activation of peroxisome proliferator-activated receptor-gamma pathway inhibits osteoclast differentiation.* J Biol Chem, 2000. **275**(19): p. 14388-93.
74. Soballe, K., *Hydroxyapatite ceramic coating for bone implant fixation. Mechanical and histological studies in dogs.* Acta Orthop.Scand.Suppl, 1993. **255**: p. 1-58.
75. Aerssens, J., et al., *Interspecies differences in bone composition, density, and quality: potential implications for in vivo bone research.* Endocrinology, 1998. **139**(2): p. 663-670.
76. Lind, M., et al., *Transforming growth factor-beta1 adsorbed to tricalciumphosphate coated implants increases peri-implant bone remodeling.* Biomaterials, 2001. **22**(3): p. 189-93.
77. Jakobsen T - Baas, J., et al., *Soaking morselized allograft in bisphosphonate can impair implant fixation.* 2008(0009-921X (Print)).
78. Elmengaard, B., J.E. Bechtold, and K. Soballe, *In vivo study of the effect of RGD treatment on bone ongrowth on press-fit titanium alloy implants.* Biomaterials, 2005. **26**(17): p. 3521-3526.
79. Jakobsen, T., et al., *Local alendronate increases fixation of implants inserted with bone compaction: 12-week canine study.* J Orthop Res, 2006.
80. Soballe, K., et al., *Bone graft incorporation around titanium-alloy- and hydroxyapatite-coated implants in dogs.* Clin.Orthop.Relat Res., 1992(274): p. 282-293.
81. Kold, S., et al., *Bone compaction enhances fixation of hydroxyapatite-coated implants in a canine gap model.* J.Biomed.Mater.Res.B Appl.Biomater., 2005. **75**(1): p. 49-55.
82. Rahbek, O., et al., *Particle migration and gap healing around trabecular metal implants.* Int.Orthop., 2005. **29**(6): p. 368-374.
83. Elmengaard, B., J.E. Bechtold, and K. Soballe, *In vivo effects of RGD-coated titanium implants inserted in two bone-gap models.* J.Biomed.Mater.Res.A, 2005. **75**(2): p. 249-255.
84. Soballe, K., et al., *The effects of hydroxyapatite coating and bone allograft on fixation of loaded experimental primary and revision implants.* Acta Orthopaedica Scandinavica, 2003. **74**(3): p. 239-247.
85. Eitel, F., et al., *Bone regeneration in animals and in man. A contribution to understanding the relative value of animal experiments to human pathophysiology.* Arch Orthop Trauma Surg, 1981. **99**(1): p. 59-64.
86. Schlesinger, M. and M. Paunovic, *Modern electroplating*. Electrochemical Society series. 2000, New York, N.Y.: Wiley.
87. Møller, P., *Overfladeteknologi*. 2003, Kbh.: Ingeniøren/Book.
88. Mattox, D.H., *Handbook of Physical Vapor Deposition (PVD) Processing: Film Formation, Adhesion, Surface Preparation and Contamination Control*. 1998: Noyes Publications. 948.
89. Overgaard, S., et al., *Efficiency of systematic sampling in histomorphometric bone research illustrated by hydroxyapatite-coated implants: optimizing the stereological vertical-section design.* J.Orthop.Res., 2000. **18**(2): p. 313-321.
90. Berzins, A., et al., *Nondestructive measurements of implant-bone interface shear modulus and effects of implant geometry in pull-out tests.* J Biomed Mater Res, 1997. **34**(3): p. 337-40.
91. Linde, F. and H.C. Sorensen, *The effect of different storage methods on the mechanical properties of trabecular bone.* J Biomech, 1993. **26**(10): p. 1249-52.
92. Dhert, W.J., et al., *A finite element analysis of the push-out test: influence of test conditions.* J.Biomed.Mater.Res., 1992. **26**(1): p. 119-130.
93. Baddeley, A.J., H.J. Gundersen, and L.M. Cruz-Orive, *Estimation of surface area from vertical sections.* J.Microsc., 1986. **142**(Pt 3): p. 259-276.
94. Baas, J., *Adjuvant therapies of bone graft around non-cemented experimental orthopedic implants stereological methods and experiments in dogs*, in *Orthopedic Research Department*. 2008, Aarhus University: Aarhus. p. 1-43.
95. Overgaard, S., et al., *The influence of crystallinity of the hydroxyapatite coating on the fixation of implants. Mechanical and*

*histomorphometric results.* J Bone Joint Surg Br, 1999. **81**(4): p. 725-31.
96. Danscher, G. and M. Stoltenberg, *Silver enhancement of quantum dots resulting from (1) metabolism of toxic metals in animals and humans, (2) in vivo, in vitro and immersion created zinc-sulphur/zinc-selenium nanocrystals, (3) metal ions liberated from metal implants and particles.* Prog Histochem Cytochem, 2006. **41**(2): p. 57-139.
97. Bland, J.M. and D.G. Altman, *Transformations, means, and confidence intervals.* BMJ, 1996. **312**(7038): p. 1079.
98. Bland, J.M. and D.G. Altman, *The use of transformation when comparing two means.* BMJ, 1996. **312**(7039): p. 1153.
99. Parente, J.E., et al., *Effects of gold compounds on leukotriene B4, leukotriene C4 and prostaglandin E2 production by polymorphonuclear leukocytes.* J.Rheumatol., 1986. **13**(1): p. 47-51.
100. Bondeson, J. and R. Sundler, *Antirheumatic gold compounds and penicillamine enhance protein kinase C-mediated activation of the arachidonate-mobilizing phospholipase A2 in mouse macrophages.* J.Leukoc.Biol., 1993. **54**(4): p. 329-335.
101. Thunus, L., et al., *Anti-inflammatory properties of copper, gold and silver, individually and as mixtures.* Analyst, 1995. **120**(3): p. 967-973.
102. Glassman, S.D., et al., *The effect of postoperative nonsteroidal anti-inflammatory drug administration on spinal fusion.* Spine (Phila Pa 1976), 1998. **23**(7): p. 834-8.
103. Thomsen, P., et al., *Structure of the interface between rabbit cortical bone and implants of gold, zirconium and titanium.* J Mater Sci Mater Med, 1997. **8**(11): p. 653-65.
104. Abu-Amer, Y., I. Darwech, and J. Otero, *Role of the NF-kappaB axis in immune modulation of osteoclasts and bone loss.* Autoimmunity, 2008. **41**(3): p. 204-11.
105. Clohisy, J.C., et al., *Tumor necrosis factor-alpha mediates polymethylmethacrylate particle-induced NF-kappaB activation in osteoclast precursor cells.* J.Orthop.Res., 2002. **20**(2): p. 174-181.
106. Keller, J.C., et al., *Effects of indomethacin on bone ingrowth.* J Orthop Res, 1989. **7**(1): p. 28-34.

# Appendices

## Thesis from the Orthopedic Research Group

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

***PhD Theses***

1. In vivo and vitro stimulation of bone formation with local growth factors
   Martin Lind, January 1996
   *www.OrthoResearch.dk*
2. Gene delivery to articular cartilage
   Michael Ulrich-Vinther, September 2002
   *www.OrthoResearch.dk*
3. The influence of hydroxyapatite coating on the peri-implant migration of polyethylene particles
   Ole Rahbek, October 2002
   *www.OrthoResearch.dk*
4. Surgical technique's influence on femoral fracture risk and implant fixation. Compaction versus conventional bone removing techniques
   Søren Kold, January 2003
   *www.OrthoResearch.dk*
5. Stimulation and substitution of bone allograft around non-cemented implants
   Thomas Bo Jensen, October 2003
   *www.OrthoResearch.dk*
6. The influence of RGD peptide surface modification on the fixation of orthopaedic implants
   Brian Elmengaard, December 2004
   *www.OrthoResearch.dk*
7. Biological response to wear debris after total hip arthroplasty using different bearing materials
   Marianne Nygaard, June 2005
   *www.OrthoResearch.dk*
8. DEXA-scanning in description of bone remodeling and osteolysis around cementless acetabular cups
   Mogens Berg Laursen, November 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, February 2007
    *www.OrthoResearch.dk*
11. On the longevity of cemented hip prosthesis and the influence on implant design
    Mette Ørskov Sjøland, April 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, June 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, August 2007
    *Acta Orthopaedica (Suppl 329) 2008;79*
14. Rehabilitation of patients aged over 65 years after total hip replacement - based on patients' health status
    Britta Hørdam, February 2008
    *www.OrthoResearch.dk*
15. Efficacy, effectiveness, and efficiency of accelerated perioperative care and rehabilitation intervention after hip and knee arthroplasty
    Kristian Larsen, May 2008
    *www.OrthoResearch.dk*
16. Rehabilitation outcome after total hip replacement; prospective randomized studies evaluating two different postoperative regimes and two different types of implants
    Mette Krintel Petersen, June 2008
    *www.OrthoResearch.dk*

17. CoCrMo alloy, *in vitro* and *in vivo* studies
    Stig Storgaard Jakobsen, June 2008
    *www.OrthoResearch.dk*
18. Adjuvant therapies of bone graft around non-cemented experimental orthopaedic implants. Stereological methods and experiments in dogs
    Jørgen Baas, July 2008
    *Acta Orthopaedica (Suppl 330) 2008;79*
19. The Influence of Local Bisphosphonate Treatment on Implant Fixation
    Thomas Vestergaard Jakobsen, December 2008
    *www.OrthoResearch.dk*
20. Surgical Advances in Periacetabular Osteotomy for Treatment of Hip Dysplasia in Adults
    Anders Troelsen, March 2009
    *Acta Orthopaedica (Suppl 332) 2009;80*
21. Polyethylene Wear Analysis. Experimental and Clinical Studies in Total Hip Arthroplasty.
    Maiken Stilling, June 2009
    *Acta Orthopaedica (Suppl 337) 2009;80*
22. Step-by-step development of a novel orthopaedic biomaterial: A nanotechnological approach.
    Thomas H.L. Jensen, September 2009
    *www.OrthoResearch.dk*
23. Osteoclastic bone resorption in chronic osteomyelitis
    Kirill Gromov, November 2009
    *www.OrthoResearch.dk*
24. Use of medications and the risk of revision after primary total hip arthroplasty
    Theis Thillemann, December 2009
    *www.OrthoResearch.dk*
25. Different fixation methods in anterior cruciate ligament reconstruction
    Ole Gade Sørensen, February 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, June 2010
    *www.OrthoResearch.dk*
27. A comparison of two types of osteosynthesis for distal radius fractures using validated Danish outcome measures
    Jesper O. Schønnemann, September 2010
    *www.OrthoResearch.dk*
28. Optimizing the cementation of femoral component in hip arthroplasty
    Juozas Petruskevicius, September 2010
    *www.OrthoResearch.dk*
29. The influence of parathyroid hormone treatment on implant fixation
    Henrik Daugaard, December 2010
    *www.OrthoResearch.dk*

***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, osteomies, and implants fixation
    Martin Lind, October 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, December 2006
    *Acta Orthopaedica (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, March 2007
    *Acta Orthopaedica (Suppl 325) 2007;78*