Strontium in the Bone-Implant Interface

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This review has been accepted as a thesis together with 3 papers to be published by Aarhus University 28th of October 2010 and defended on 11th of January 2011.

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Dan Med Bull 2011;58: (5):B4286

PAPERS

The dissertation is based on the following papers:

I. Strontium Substituted Hydroxyapatite Coating did not Improve Implant Fixation and Osseointegration. Vestermark MT, Hauge EM, Bechtold JE, Jakobsen T, Gruner H, Soballe K, Baas J. In preparation.

II. Strontium Doping of Bone Graft Extender: Effect on Fixation of Allografted Experimental Implants. Vestermark MT, Hauge EM, Soballe K, Bechtold JE, Jakobsen T, Baas J. Submitted to Acta Orthopaedica.

III. Grit-blasting of Titanium Implants Affects Structure and in vivo Performance of Strontium-substituted Bioactive Glass Coating. Vestermark MT, Brauer DS, Soballe K, Jakobsen T, Hauge EM, Bechtold JE, and Baas J. In preparation.

BACKGROUND

Total hip replacements surgery is performed on an increasingly large part of the population. The reasons are that firstly, the treatment of hip conditions with a hip replacement is overall very successful. As a result of the success, hip replacement is offered to patients with a wide range of hip conditions and at increasingly younger age. Secondly, we live longer and stay physically active at increasingly higher ages. According to the Annual Report 2008 from the Danish Arthroplasty Register, 136 per 100,000 citizens received primary hip replacement surgeries and the number will increase [1]. Unfortunately, the revision rate is unacceptably high, especially if the patient is less than 50 years old at the time of primary surgery, because 20% of these surgeries are revised within 14 years. The high revision rate of prostheses in young patients is related to their high level of physical activity. The survival rate of cemented and cementless implants is the same for patients under 50 years and maybe in favor of the cementless implant in patients 50-60 years of age. Cementless implants seem more easily revised, and the loss of bone around the implant tends to be smaller. Cementless implants are the focus of this dissertation. For the younger patients, the main indication for revision surgery is aseptic loosening of the implant. An aseptically loosened implant is a painful and disabling condition. Clearly, patients with an aseptically loosened implant have a reduced quality of life. Revised implants have an even higher failure rate, which increases with increasing number of re-revisions [2]. Therefore the issue of aseptically loosened implants also constitutes a financial burden for the society in terms of repeated operations, the daily care of the disabled patients, and the disabled patients' inability to work. So, the longevity of both primary and revision implants clearly needs further investigation.

ASEPTIC LOOSENING

The causes and optimal treatment of aseptically loosened implants seem complex and not fully understood.

Instability of the implant is known to induce aseptic loosening. Under experimental settings, micromotions of implants as small as 150 µm inhibit osseointegration of the implant. Instead, a fibrous membrane encapsulates the implant and motion is continuously taking place [3]. Clinically, Kärrholm et al. observed that subsidence of the prosthesis is correlated with an increased risk of the prosthesis becoming aseptically loosened [4].

Inflammation is another well-known aspect of aseptic loosening [5]. Particles of wear debris from the implant materials can induce the inflammation. Subsequently, osteoclasts are differentiated and activated [6]. The consequence of inflammation is bone resorption and loss of bone around the prosthesis [7]. Hereby, instability of the implant is further increased.

Early osseointegration will both stabilize the implant and prevent the wear debris from reaching the bone-implant interface [8].

OSSEOINTEGRATION

During experiments on blood flow in bone, Brånemark found that the titanium oculars placed into bone could not be removed after healing. Brånemark then conducted extensive research into insertion of screw-shaped dental implants. In 1977, Brånemark stated: "The re- and new-formed bone tissue enclosed the implant with perfect congruency to the implant form and surface irregularities, thus establishing a true osseointegration of the implant without any interpositioned connective tissue" [9]. His co-worker Albrektsson defined in 1981 the osseointegration as direct contact between living bone and implant at the light-microscopic level (Table 1) [10]. But histological analysis of the interface could not be performed in vivo.

Table 1
Definitions of osseointegration

Osseointegration
1981, definition by Albrektsson [10]: Direct contact between living bone and implant at light microscopic level 1990, Zarb and Albrektsson added a functional definition [11]: A process whereby clinically asymptomatic, rigid fixation of alloplastic materials in bone during functional loading is achieved and maintained for 80% over 10 years

Therefore, a functional or biomechanical definition was added [11]: "A process whereby clinically asymptomatic, rigid fixation of alloplastic materials in bone during functional loading is achieved and maintained for 80% for ten years." Roentgen stereophotogrammetric analysis can evaluate the functional osseointegration. Thus, simply speaking, osseointegration is a direct structural and functional connection between host bone and the surface of an implant.
The biological process leading to osseointegration can be split into gap healing, when a initial gap between host bone and the implant is present, and ongrowth [12]. The reason for splitting up the process is because the surface of the implant can have or release an agent with bioinert, osteoconductive, osteoproductive, or osteoinductive properties (Table 2).

Table 2
Definitions of bioactive properties [13, 14].

Nearly Bioinert	Formation of a non-adherent fibrous capsule of variable thickness; e.g. Alumina, Zirconia, and Polyethylene.
Osteoconductive	Bone grows on the surface; e.g. HA.
Osteoproductive (osteostimulative)	Biologically active hydroxyl carbonate apatite layer on the glass surface is formed chemically and is colonized with osteogenic cells; e.g. Bioactive Glasses.

Whether the aspects of osseointegration of dental implants can be applied to major arthroplasties in orthopedics has been questioned [14]. Albrektsson objections are firstly that orthopedic implants are less biocompatible, secondly heat during surgery can damage the host bone, and thirdly immediate postoperative loading of the implants does not favor bone formation. On the other hand, hip arthroplasty is quite successful because in 2008 92% of all hip prosthesis had survived for 10 years [15]. Based on roentgen stereophotogrammetric analysis, Kärrholm concluded that painful prostheses are correlated with subsidence of the implants [16]. This finding is in accordance with the definition of functional osseointegration. So it seems that the rules of osseointegration can be applied to arthroplasties. Furthermore, as Albrektsson states, the bone density in the interface must resemble the density of the surrounding bone. Arthroplasties are inserted into cancellous bone, so arthroplasty implants are not to be fully covered by bone. Moreover, great efforts to improve biocompatibility and otherwise promote osseointegration of the arthroplasty implants have been and still are being made [17, 18]. Also, the studies of this dissertation are efforts to improve the osseointegration of arthroplasty implants.
The osseointegration of implants can be influenced by several local factors that can be divided into: 1) implant stability in respect of implant design, implantation technology, surgical technique, and patient variables, such as bone quality and possible bone defects; 2) distance between the host bone and the implant, despite the cavity at surgery being carefully prepared for a tight implant fit; 3) bioactivity of the implant surface and any material in the gap between host bone and implant [19].
Early osseointegration must be established during fracture healing and then maintained during modeling, and remodeling.

FRACTURE HEALING
Fracture healing of long bones is defined as primary and secondary healing [20]. Primary healing can take place in aligned fragments of bone cortex. The fracture is healed by directly coupled removal of and replacement with lamellar bone when the cutting cone moves from one fragment to the opposite fragment across the fracture. Primary healing of the cortex demands stability of the fracture site, so that the fragments stay aligned. Secondary healing is characterized by callus formation, which is replaced by lamellar bone. Secondary healing shows the closest resemblance to the process seen around an inserted implant, because woven bone is abundant and the implantation site is in the cancellous bone.
Secondary fracture healing runs through four overlapping phases: an inflammatory, a resorptive, a formative, and a modeling/remodeling phase [20, 21].
Inflammatory phase: a hema-toma with platelets and inflammatory cell forms immediately at the fracture site. Cytokines and growth factors, including TNFα, IL-1, PDGF, GDF and BMP, with chemotaxic and osteoinductive functions are released from the site [22, 23]. Then mesenchymal stem cells, preosteoclasts, and preosteoblasts are mobilized to the site from the neighboring living tissues and the blood stream. The cells are stimulated to proliferation, differentiation, and activation. Strong signals of resorption and formation are initiated. During the inflammation phase, the hematoma is invaded and replaced by callus. A callus consists of fibrovascular tissue in which abundant amounts of collagen fibers and woven bone matrix is laid down. The callus is anchored to living bone fragments by newly formed bone.
Resorptive phase: Within a week resorption of both necrotic and misplaced bone fragments begins [20]. The necrotic bone fragments can serve as scaffolds for new bone formation and contribute to implant stabilization [24]. In the initial 4 weeks, the effect of high resorption activity and pending effect of new bone formation is seen as a reduction in bone density surrounding implants [25]. Low bone densities have also been correlated with inferior implant fixation [26].
Formative phase: If the implant or fracture site is stable, then after about a week HA is beginning to be precipitated in the collagen [20, 27]. In successful fracture healing, the gap between bone fragments or to the implant surface is entirely bridged by woven bone, which stabilizes the site. Successful fracture healing at the implant site, i.e. osseointegration, can take place if a porous-coated implant is subject to a micromotion of 28 μm. [3]. Then if the micromotions are increased to 150 μm, the implant is not osseointegrated but becomes encapsulated into a fibrous membrane. The volume of the newly formed, woven bone is excessive and needs to be reduced by modeling.
Modeling/remodeling: Over 1-4 years, the last phase of fracture healing occurs. Any excessive or misplace bone tissue is removed, and trabelulae of lamella bone are formed in an architectural pattern that matches the mechanical strain on the bone [28, 29].
Fracture healing of cancellous bone is a little different from healing of cortex because only an internal callus is formed. The cancellous bone is very well vascularized, so only a relative small amount of bone becomes necrotic. A large area of bony contact at the fracture site ensures that a union is rapidly formed be-

tween fragments in direct contact via the endosteal callus. The union of fragments will subsequently spread across the fracture site, unless the distance between fragments is too long [30]. Successful fracture healing results when fractured bone ends are connected without interpositioned connective tissue. Fracture healing can fail if, for instance, the fracture site is infected or subjected to motion. Then connective tissue is positioned between the bone fragments even at a late time point. Failed healing around an implant is characterized by a fibrous encapsulation of the implant. These aspects of fracture healing clearly show similarities to aspects of implant osseointegration. The similarities between osseointegration and fracture healing are perhaps more clear, if a bioactive implant material or implant surface is used because new bone formation proceeds unidirec-tionally, like in fractures, from host bone and from the surface of the bioactive material [12].

BIOACTIVE SUBSTANCES

A bioactive material is defined as: "A bioactive material is one that elicits a specific biological response at the interface of the material, which results in the formation of a bond between the tissue and the material" (Table 3) [13]. Several materials, e.g. HA and bioactive glasses, elicit different but yet bioactive responses in bone, and the only common characteristic feature of these bioactive implant materials is that "a layer of biologically active hydroxyl carbonate apatite forms on the implant surface"[13]. The effect of the bioactive substance can be classified as osteoconductive or osteoproductive [14, 31].

An osteoconductive substance releases calcium and phosphate, mainly by ion ex-change, which forms a biocompatible surface of a biologically active hydroxyl carbonate apatite for bone formation to migrate [14, 32]. HA is an osteoconductive substance, which can be added to the bone-implant interface as a coating or bone graft substitute. HA coatings bond to bone with a shear strength that is comparable with the shear strength of bone [33]. An osteoconductive substance only elicits en extracellular response, so that osteoblasts will have to be present for bone to be formed [13].

The osteoproductive property is only connected with bioactive glass which elicits both an extracellular and intracellular response [13]. The osteoproductive property is defined as the biologically active hydroxyl carbonate apatite layer on the glass surface being colonized with osteogenic cells [34]. The osteogenic cells are recruited from the surgical site. Bioactive glass substance are both osteoconductive and osteoproductive [13].

Table 3

Definition of bioactivity [13].

Bioactive materials elicit a specific biological response at the interface of the material and tissue, which results in the formation of a bond between the tissue and the material

HA

The extracellular part of bone consists of organic and inorganic materials. The organic material is collagen, mainly type 1 and non-collagenous proteins entrapped in the collagen. The inorganic material consists of crystalline apatite compounds, which are precipitated in the collagen. Apatites are a group of calcium phosphate minerals with OH^-, F^-, and Cl^- ions bound in a hexagonal dipyrimal lattice structure. These apatites are referred to as hydroxyapatite, fluorapatite, and chlorapatite. Ideally, the apatite of bone is hydroxyapatite, HA. The apatite is a carbonate hydroxyapatite with the formula $(Ca, Mg, Na)_{10}(PO_4HPO_4CO_3)6(OH)_2$ [32]. It is also possible to substitute some of the calcium with, for instance, strontium and magnesium, strontium hydroxyapatite, and magnesium hydroxyapatite [35]. With substitution of the calcium with magnesium in the HA, the apatite structure is less chemically stabile; in consequence the substituted HA can more easily convert to ß-tricalcium phosphate when heated [36]. ß-tricalcium phosphate is more readily dissolved and subsequently more bioactive especially at low pH, such as during fracture healing [21, 37]. Strictly speaking, ß-tricalcium phosphate is not an apatite.

Apatites are widely formed in nature but can also be synthesized for commercial medical use. HA is usually synthesized by precipitation and subsequently sintering at 1000°-1300° Celsius. HA granules are used as bone graft substitutes, and HA vacuum plasma-spray coatings are used for many types of joint prostheses. Under stable conditions the HA is mainly removed by cell-mediated resorption and dissolution, but under unstable conditions then also by mechanical erosion [37, 38].

HA BONE GRAFT SUBSTITUTES

HA granules are commercially available, like Calcibon®, for use as a bone graft substitute for filling critical bone defects.

When HA is used as bone grafts substitute, several morphological and mechanical aspects influence the bioactive property and clinical application of the material. A certain morphological profile for the HA material is recommended, e.g. porosity of 50-60% for optimizing the bioactivity of the material (Table 4).

Table 4

Recommendations for the morphology of the bone graft substitute material.

Recommended morphological profile
50-60% porosity Minimal interconnection channel diameter size of 50-100 µm Minimum 20% strut porosity

However, the mechanical strength of the material, unfortunately, decreased with increasing porosity [39]. Verdonschot showed that the high total deformation of the HA/TCP (80:20) with 50% porosity is the most important factor for the decreased mechanical property of the bone graft substitute material compared with allografts. The difference in mechanical properties between the synthetic and biological material is especially the lack of viscoelastic properties of the HA. The aspect of low mechanical strength limits the clinical use of bone graft substitutes. The grafted bone site will then have to be mechanically supported by internal or external fixation. Additionally, HA bone graft substitutes are less bioactive than allograft, and an osteoinductive agent often needs to be added to obtain successful healing of the bone defect [40, 41].

HA COATING

Plasma-sprayed HA coating was introduced in the 1980s and is still the most common calcium phosphate coating used clinically [33, 42]. Today, the HA plasma spray coating is performed under vacuum (vps), which gives a denser HA coating with a higher adhesive strength to the underlying metal substrate, a higher crystallinity and purity of HA [43]. The ratio of crystallinity versus amorphous structure of the calcium phosphate is greatly influenced by temperature during plasma spraying, because HA can be transformed by heat to ß-tricalcium phosphate (ß-TCP). When the

plasma spraying is performed under vacuum, then the temperature can be lower while the HA is still viscous. Any amorphous calcium phosphate of the coating is more readily dissolved than HA [44]. A crystalline HA-coated Ti implant surface provides a long-term bioactive surface [45]. Whereas an amorphous calcium phosphate or ß-TCP coated Ti surface will only be bioactive for a short term until the coating has dissolved, which will leave the implant with a raw Ti surface. The performance of the HA coating is greatly influenced by purity, crystallinity, Ca/P ratio, porosity, and mechanical strength [45]. Hence it is advisable to determine and control these factors. The vps HA coating is strongly bonded to a porous Ti surface and delamination rarely happens [46, 47]. In 2003, Rössler et al. published work on HA coating of implants by electrochemically assisted deposition of the HA at 36° Celsius [48].

Clinically, HA-coated implants show less subsidence on roentgen stereophotogrammetric analysis, which subsequently may reduce the risk of developing aseptic loosening of the implant [49]. Initially in the history of HA-coated implants, the survival rate of hip prostheses was as high as 99% and 100% after 6 years [50]. But in a recent study, the superior survival rate of HA-coated implants was not found after 3.5 years [51]. The decline in prognosis is perhaps because the HA-coated implants are chosen for patients with a poor prognosis for the implant survival, like young and physically active patients. Experimentally, osseointegration is enhanced by HA coating [52, 53]. Experimentally, no difference in osseointegration was found between a vps HA coating and a coating of electrochemically assisted HA deposition [54].

Elements like magnesium, strontium, and sodium can substitute calcium in the apatite lattice. Substitution of calcium in HA can influence the bioactivity of the bone graft substitute and the HA coatings two-fold [55]. Firstly, substitution of elements can cause lattice defect or destabilization, so the modified HA dissolves more readily (Fig. 1) [56, 57]. Secondly, the element substituted into the HA can thus be released into the surroundings by dissolution, ion exchange of the ions at the HA surface, or by cellular biodegradation [58]. The substituted HA then has an additional

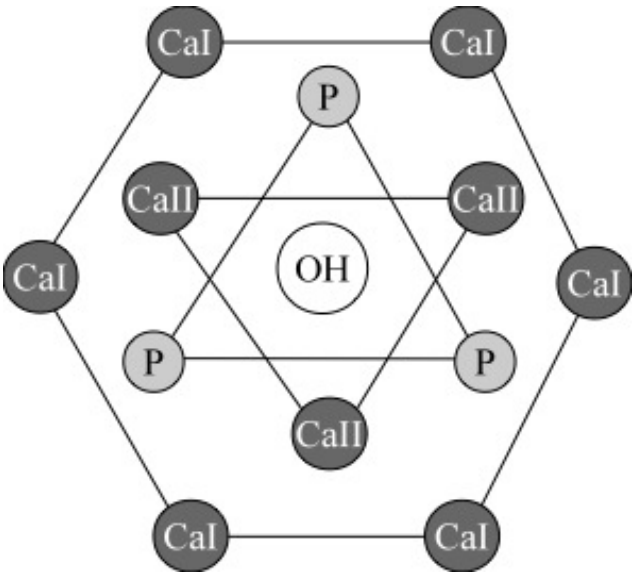

Figure 1
A sketch of the chemical structure of hydroxyapatite. Strontium is preferably incorpo-rated at the CaII position, and this expands the apatite structure and cause destabilization [57].

effect, besides the osteoconductive property, caused by the elements (ions) released [32]. The studies of this dissertation investigate the effects of strontium-doped or -substituted HA and thereby the effect of strontium in the bone-implant interface.

BIOACTIVE GLASS

Bioactive glass was invented during the Vietnam War. An American orthopedic surgeon challenged Larry L. Hench, Florida, USA to invent a biomaterial to help regenerate bone defects. Hench invented 45S5 BioGlass®, and many variants of bioactive glass have since been made. Glass is characterized as an amorphous material during its solid state and transforms from solid state to liquid state via a soften state. Degradation of the bioactive glass is essential for the glass to be bioactive and osteoproductive. Ions, especially of $Si4^{+}$, are released by degradation. The released ions are then exchanged with the ions in the surrounding milieu, and a biologically active hydroxyl carbonate calcium phosphate layer is formed. The layer is at first amorphous and later becomes a crystalline HA layer. The glass-bone interface is strongly bonded by predominantly Si-O-Si bonds [59-63]. Furthermore, at the optimum concentration of ions released, DNA synthesis will be activated and turnover of both osteoclasts and osteoblasts will be regulated [64]. The sum of the intracellular and extracellular responses leads to rapid bone formation at the same rate as the glass is degradated [60, 65].

To date, commercially available bioactive glass particles, such as Biogran® (FBFC International, Dessel, Belgium and Orthovita, Malvern, PA, USA), have been widely used in dentistry as bone grafts extenders or bone grafts substitutes. Under these clinical conditions involving critical bone defects, the bioactive glass performs well, because it induces rapid new bone formation [66, 67]. Orthopedic implants coated with bioactive glass, on the other hand, are not yet commercially available. The reason for this is two-fold. Firstly, implants coated by enameling technique are dipped into a glass suspension and sintered in a furnace at 730°C in order for the glass to become a homogenous adhesive glass coating. When the implants are heated, the materials expand, as characterized by the thermal expansion coefficient (TEC), which is specific for a given material. If there is a mismatch between the TEC of the metal core (e.g. Ti) of the implant and the glass coating material, then delamination of the glass coating take place, especially during the cool down phase. The TEC of the glass is determined by the chemical composition, so by changing the composition, the TEC of the glass can be matched to the TEC of the metal core of the implant. Secondly, chemical composition greatly influences the degradability of the glass and therefore the osteoproductive property of the glass. Summing up, the challenge of bioactive glass-coated implants is to match the TEC of the metal implant core and the bioactive glass coating and at the same time maintain the osteoproductive property of the glass. These chemical properties of the glass have been reported to oppose one another [68] if the glass is not designed correctly [69].

For maintaining the osteoproductive property of the glass, attention must be paid to the sintering window of the glass. The sintering window is the temperature range between glass softening and the onset of crystallization. The glass must also show a large sintering window to prevent crystallization during the firing process. The sintering window of the glass can be increased by increasing the number of components in the glass, which increases the enthalpy of mixing, stabilizes the disordered glass state, and increases the barrier for crystallization. Ideally, the glass should

also show viscous flow sintering behavior in order to obtain a cohesive glass coating [69].
It was decided to study the influence of strontium (Sr) substitution for calcium (Ca), because Sr has shown to increase the degradability and apatite formation of bioactive glasses. While CaO and SrO are both network modifiers, the Sr^{2+} cation is slightly larger than the Ca^{2+} cation (1.16 nm for Sr^{2+} and 0.94 nm for Ca^{2+}), resulting in expansion of the glass network. For this reason, the molar substitution of Ca by Sr in bioactive glasses increases the rate of degradation of bioactive glasses and thereby increases their bioactivity [70, 71]. This means the osteoproductive properties of the glass would also be expected to increase [13, 60, 72, 73].
The issues of bioactive glass coating of Ti implants is investigated in vivo in study I. Bioactive glass-coated implants with strontium substitution of the glass are evaluated by analysis of osseointegration and mechanical fixation.

ALLOGRAFT

In 1975, bone grafts were used for the first time for restoring the bone stock in connection with total hip replacement surgery [74]. There are three types of grafts: autografts, where donor and recipient are the same individual; allograft, where donor and recipient are of the same species; and xenograft, where donor and recipient are of different species. Autografts are regarded the gold standard for achieving osseointegration, but the disadvantages in connection with harvesting of the graft are considerable [75]. Therefore an autograft is often not the first choice clinically. Second best are allografts, but fresh allografts can induce an immunological host-versus-graft response leading to non-union by intervening fibrous tissue. Additionally, fresh allografts can transfer infectious diseases [76]. Freezing at minus 80° C, freeze drying, or irradiation can considerably reduce these disadvantageous effects of fresh allografts [31, 77]. These procedures also preserve the allografts for later use. The graft material can be of structural or morselized cortical or corticocancellous bone, or morselized cancellous bone. The different materials possess different properties in regard to mechanical strength during the replacement by viable bone and the extent to which it is replaced. Today, morselized corticocancellous allografts are often used during revision hip replacement surgery in which a great loss of bone stock has occurred [2]. The allograft is impacted hard around the prosthesis to immediately stabilize the implant at surgery and to restore the bone stock in the long-term [78]. The long-term stability is then obtained by osseointegration of the implant. In that process any intervening graft material gets partially or fully replaced by new living bone [77, 79]. Allografts are an osteoconductive substance. If the site of impacted necrotic allografts becomes vascularized, then bone resorption is intensively stimulated, and, to a less extent, the coupled bone formation is also stimulated [75]. But the quick resorption of the allograft may exceed the slower replacement of new bone [79]. Then the implant may become instable and at risk of becoming aseptically loosened. By regulating the mismatch between fast resorption of the biologic graft and slower new bone formation, the outcome of grafted revision arthroplasty can perhaps be improved. An investigation of the inhibition of the fast resorption of the allograft by bisphosphonates alone and in combination with BMP-2 has been conducted [80, 81]. Both bisphosphonates and BMP-2 are very potent and strong acting agents. In these studies of soaking the allograft with the agents, implant fixation and osseointegration were impaired. The authors concluded that the therapeutic window of the agents is narrow and further studies of the agents at different dosage are needed.
Study II of this dissertation also addresses the issue of the fast resorption of the allograft and slow new bone formation. A strontium-doped HA bone graft extender is mixed with allograft, because strontium is both an anabolic and anti-catabolic agent in bone [82].

STRONTIUM

Strontium is element number 38 of the periodic system. Placed in the second group of earth alkaline metals together with calcium, strontium and calcium have a quite similar kinetic profile in the body [83]. Strontium was found in 1790 in a mine near the Scottish village Strontian. Strontium does not exists freely in nature because it oxides quickly. Strontium can be made radioactive: Sr85, Sr89, and Sr90. Radioactive strontium is used for tracing sites of high bone formation in vivo, studying kinetics of strontium, and treatment of the pain of bone metastases [83, 84]. In nature, strontium is found in the mineral compounds celestite ($SrSO_4$) and strontianite ($SrCO_3$), which are present in soil and drinking water. In a normal diet, strontium is present in vegetables and cereals at 2-4 mg/day. In 2004 strontium, as strontium-ranelate, was introduced to the European market for the treatment of osteoporosis.

PHARMACOKINETICS OF STRONTIUM

In humans, the gastrointestinal tract is the main route of entrance for strontium into the body [85]. The absorption efficiency of strontium is age-dependent and in competition with calcium. Almost all the absorbed strontium (99.1%) is deposited in bone and mainly in newly formed bone [86]. The blood is the second most important location for strontium in the body. A serum strontium concentration of 10,560 ng/ml, after taking 2 g/day strontiumranelate orally, has proven effective in reducing fracture risk in postmenopausal osteoporosis [87]. The single most important excretion route is by the kidneys, and a secondary excretion route is by the intestines [85, 88]. The renal clearance of strontium is about three times higher than that of calcium [83]. The interspecies differences of pharmacokinetics are difficult to clarify, but caution must be made when extrapolating results between species. The majority of animal studies of strontium are made on rodents. Rodents have a high bone formation rate and do not reach a steady-state of remodeling [19]. Therefore results from studies of bone formation and bone resorption performed in rodents must be interpreted with great care and perhaps only be considered preliminary [89]. In a study by Raffalt et al. the content of strontium in bone was increased to 9 mg/g bone, when 3000 mg/kg/day strontium malonate was administrated orally [90]. The calcium content was constant despite strontium administration. In a study in monkeys, Boivin et al. found the average Sr/Ca ratio in bone can be as high as 1:10 after oral strontium ranelate administration for 13 weeks [86]. Boivin et al. also found that strontium is quickly cleared from the bone after treatment. In the studies of this dissertation, the strontium is applied locally and not orally. Therefore the pharmacokinetic aspects of greatest interest are the therapeutic range of strontium concentration in bone, deposition of strontium in the body, and the elimination of strontium.

MECHANISM OF ACTION AT THE MOLECULAR AND CELLULAR LEVELS

As yet, strontium's mechanisms of action on osteoblasts are not fully understood. Strontium is believed to have more than one mechanism of action. Several studies have proved that strontium can stimulate the calcium-sensing receptor, CaSR, situated in the membrane of osteoblasts and osteoclasts [91-93]. Stimulation of the CaSR situated in the osteoblast cell line triggers mitogenic signals leading to proliferation, differentiation, and activation of the osteoblasts [94, 95]. When the CaSR situated in osteoclast cell line is stimulated, the cells retract and bone resorption is inhibited [95]. Via the CaSR, strontium can also suppress the RANKL production by osteoblasts, which leads to diminished proliferation, differentiation, and survival of the osteoclasts [94, 96]. Hurtel-Lemaire has shown that strontium can induce apoptosis of osteoclasts via the CaSR but in a different manner than that which calcium stimulates the CaSR [97]. In short, strontium simulates, together with the normal level of calcium in the bone marrow, a homeostatic hypercalcemia. A statement was made in the 1960s, that strontium is not under homeostatic control of either the total amount in the body or the concentration in blood [83]. As yet, no studies have disproved the statement. Even in mice with the knocked-out CaSR gene, strontium has an effect on osteoblasts. Other proposed mechanisms of action have been suggested, e.g. release of an autocrine growth factor leading to osteoblast replication or activations of Akt pro-survival pathway in osteoblasts, which leads to a higher increase in bone formation than resorption, so the total effect is an increase in bone mass [93] [92].

The effects of strontium on the cellular level are to increase the pool of active osteoblasts and decrease the number of less active osteoclasts (Fig. 2) [98-101].

EFFECT ON BONE TISSUE

When administrated orally as strontium ranelate, the strontium is found incorporated into hydroxyapatite in place of calcium at a maximum Sr/Ca ratio of 1:10 [86, 102]. In old bone, strontium is incorporated by ion exchange on the bone surface and during bone formation by ion substitution. This does not have a deleterious effect on bone mineralization as long as calcium intake is adequate [103-105]. Hypomineralization caused by strontium has been shown in rats by Grynpas et al. [104]. Grynpas et al. have also described how high bone formation, which rats have, can cause hypomineralization of bone, especially if the formation is increased, e.g. by strontium [56]. In another study by Grynpas et al. the rats were feed a normal calcium-containing diet [106]. Then the bone formation was increased by a relative low strontium dosage without causing hypomineralization.

Several studies on humans, monkeys, and dogs show an increase in parameters of bone formation, such as osteoblast surface, mineral apposition rate, and S-alkaline phosphatase [90, 105]. In vitro, strontium increased bone formation in rat calvaria cultures, but 72 hours after removal of the strontium, the effect was no longer detectable [100]. As yet, the anti-catabolic effect of strontium in vivo in large animals has only been shown in one study of monkeys [99].

Ammann et al. have studied the mechanical effects of strontium on bone in rats [107]. A strontium dose-dependent increase in mechanical properties was found, which was associated with the increase in bone volume and improved micro-architecture in terms of trabeculae number and thickness (Fig. 2).

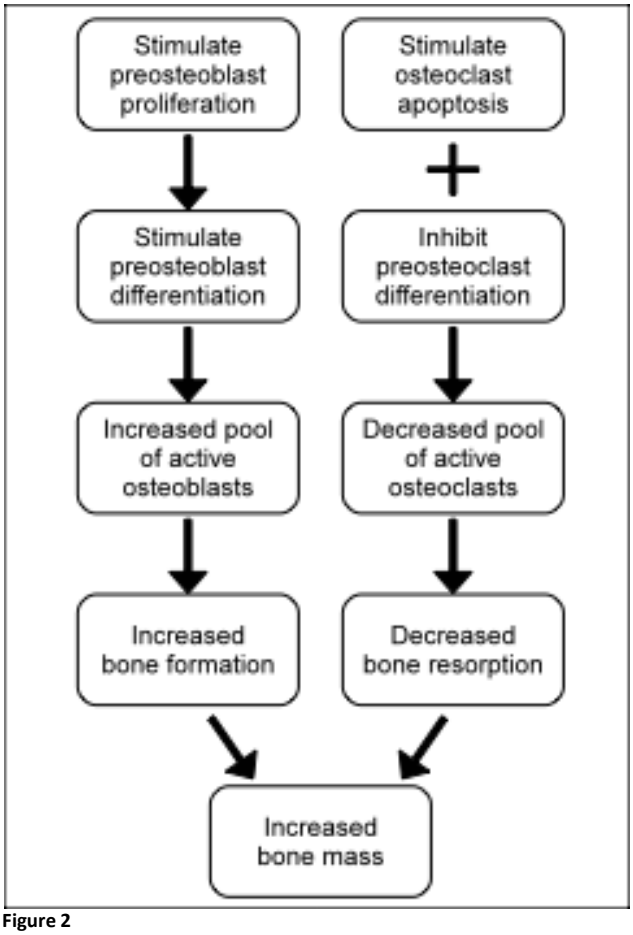

Figure 2
The effects of strontium at the cellular and tissue level.

Clinically, in the treatment of osteoporosis, strontium ranelate has been found to reduce the risk of especially non-vertebral fracture but also vertebral fractures [108-111].

Studies of strontium in connection with cementless arthroplasty are limited and still at the experimental stage. Results are promising but based on studies of rodents [112-114]. Likewise studies of strontium containing bone graft substitutes are promising, but so far only in studies performed on rats [55, 115, 116].

AIMS OF THE STUDIES

In a larger perspective, the aim of these studies is to contribute to a general assessment of whether strontium addition to the bone-implant interface is advisable. To begin with, what is the best method of strontium delivery to the interface; and then, can strontium exercise its dual effects in the bone-implant interface? Before an agent like strontium can be advised for addition to the bone-implant interface, beneficial effects must be evident. At the same time, evidence of no or minimal deleterious effects of strontium must be clarified and estimated.

The aim of the studies in this PhD dissertation was to investigate whether strontium added to the bone-implant interface under various conditions would improve implant fixation.

HYPOTHESES FOR THE STUDIES

Study I
SrHA, strontiumhydroxyapatite, coating on Ti implants will enhance implant fixation both at 4 weeks and 12 weeks.
Theory rationale: Strontium increases bone formation, and SrHA is more bioactive than HA.

Study II
Strontium-doped HA as a bone graft extender mixed with allograft will enhance implant fixation.
Theory rationale: Strontium increases bone formation, SrHA granules are more bioactive, and the anti-catabolic effect of strontium may slow down resorption of the allograft.

Study III
1) Bioactive glass coating of Ti implants will enhance implant fixation compared to HA coating.
Theory rationale: Bioactive glass is osteoproductive, while HA is only osteoconductive.
2) Strontium-substitution of the bioactive glass coating on Ti implants will further enhance implant fixation compared to bioactive glass coating without strontium.
Theory behind: Strontium increases bone formation.

SUB-HYPOTHESES FOR THE STUDIES
Implant fixation was to be investigated histologically and mechanically. Therefore histomorphometrical analysis was chosen for evaluating implant osseointegration, gap healing and ongrowth, at the microscopic level [14]. Mechanical implant fixation was evaluated by biomechanical push-out test to failure.
Several sub-hypotheses, based on several variables, were setup and tested to elucidate the issue of implant fixation in details:

- Gap healing (as volume of new bone in the gap) will be improved by addition of strontium to the interface.
- Ongrowth onto the implant (as surface area of new bone on the implant) will be increased by strontium addition to the interface.
- Ongrowth onto the bone graft extender will be increased by strontium doping of the bone graft extender.
- The allograft will be preserved for longer time when the bone graft extender is strontium-doped.
- Peri-implantary fibrous tissue will be reduced by strontium addition to the interface.
- Apparent shear stiffness will be improved by strontium addition to the interface.
- Ultimate shear strength will be improved by strontium addition to the interface.
- Total energy absorption will be improved by strontium addition to the interface.

All three studies were conducted with a paired study design and with non-loaded implants. The implants were inserted into the metaphysis of the humerus and surrounded by a concentric gap of variable size between studies. All analyses were performed blinded.

METHODOLOGICAL CONSIDERATION
The majority of health research is aimed at gaining knowledge concerning the diagnosis and treatment of human disease. These investigations usually start with in vitro observations, proceed to in vivo tests in animals of increasing size before being applied to humans. In order to apply results from one level to the next, the model and method used must resemble the conditions of the end goal of the diagnosis or treatment as closely as possible [117]. Discrepancies between the experimental study and the clinical endpoint in humans must be clarified and estimated when possible.

DESIGN
All studies are paired, block-randomized intervention studies. The paired study designs eliminated various foreseen and unforeseen variables of inter-individual biological and conditional character. Thus, the statistical power of the studies was strengthened and a lower number of animals could be included.
In the three studies, the implants were positioned in the proximal humerus, and the locations were alternated systematically with random start between right and left limb, and between proximal and distal hole in the same limb in study III. The positioning was alternated in order to rule out bias due to systematic differences between the implantation sites with regards to bone quality or loading pattern [26].
In the two four-arm studies (I and III), the interventions were strontium-substituted coatings. The strontium-substituted coatings were expected to be readily soluble and strontium would be released into the surrounding marrow space. The strontium would then become present in the surroundings of the neighboring implant. To eliminate any risk of strontium contamination of a strontium-free neighboring implant, the implants with strontium-substituted coatings were placed in the same humerus. In study I, the intervention of SrHA coating was investigated at 4 weeks and 12 weeks (Fig. 3). As a consequence, each humerus was operated twice, 8 weeks apart. One potential disadvantage here could be the influence of regional acceleratory phenomenon (RAP) inflicted upon the host bone both at time zero and at time 8 weeks (Fig. 4). For assessment of the effect by RAP, the following questions should be considered:

1. How far from the fracture/drill hole does RAP increase remodeling activity and at which time points?
   In a previous study RAP was not observed in a zone 2-5 mm from the implant after 8 weeks [118]. It is possible that the RAP had already passed at the distance of 2-5 mm so where the RAP effect would be after 8 weeks is uncertain.
2. Does RAP cause improved fracture healing of the neighboring bone?
   I have not found literature on the subject; but if fracture healing of the implants at 4 weeks observation time was improved by a RAP stimulus from the surgery 8 weeks earlier of the neighboring implant, then due to the paired study design the fracture healing of both treatment arms would be equally improved. Additionally the increase in bone turnover activity by RAP must be less than the increase in bone turnover caused by the fracture healing. The reason is that the stimulus of fracture healing gives rise to the RAP and the induced increase in bone turnover spreads out over time like concentric waves forming with fading intensity when a stone falls into water (Fig. 4).

The effect of RAP on neighboring fracture healing has been found to be minimal and of no relevance in previous studies [22, 119].

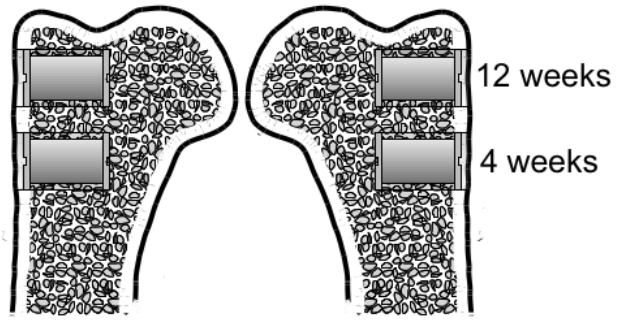

Figure 3
Positioning of the implants in studies I and III. In study II only one implant was inserted at the proximal implantation site, bilaterally.

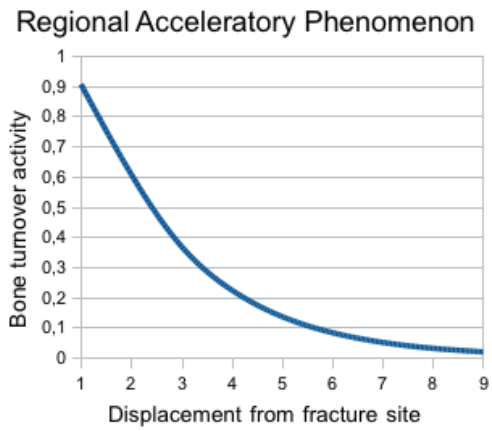

Figure 4
Regional acceleratory phenomenon is an increase in bone turnover activity, which originates from the fracture healing stimulus and fades out over distance from fracture site. The curve is a principal drawing (Y= E (-0.1 · X)) of spreading of a wave for illustrating the aspects of decreasing intensity with increasing displacement.

SAMPLE SIZE

For economical and ethical reasons, the number of dogs needed to be included in the studies were estimated as follows [120]:

Equation 1

$$N = (C_{2\alpha} + C_{\beta})^2 \cdot CV_{diff}^2/\Delta^2$$

$C_{2\alpha} = 2.26$ (p=0.05)

$C_{\beta} = 0.883$ (p=0.2)

$CV_{diff} = 30\%$

$\Delta = 30\%$

Equation 1 is designed for normal distributed data that fulfils the assumption of the paired t-test. The criteria were assumed fulfilled. The risk of accepting a false positive and false negative difference was set at 5%, $C_{2\alpha} = 2.26$ and 20%, $C_{\beta} = 0.883$, respectively. The minimum relative difference in means, Δ, to be detected between intervention and control was set at 30% for any variable in the studies. The estimated value of coefficient of variance was based on previous studies with the same model for the variables of the histomorphometrical analysis and the push-out test [24, 121, 122].

Equation 2

$$N = (2.26 + 0.884)^2 \cdot (50\%)^2/(50\%)^2$$

Equation 3

$$N = 9.88$$

Hence, 10 dogs were included in each study.

EXPERIMENTAL ANIMAL MODEL

The studies in this dissertation were all conducted in skeletally mature American Foxhounds. The implants were inserted into cancellous bone of the metaphysis of the humerus bilaterally. The canine shows great resemblance to humans with regards to bone mineral density, biochemical composition, mechanical quality, and most importantly, bone growth reaches a steady state characterized by remodeling activity [19, 89, 123]. Alongside primates, the canine is regarded as the best experimental animal model for orthopedic research [123, 124]. However, the bone turnover time of the remodeling activity is complete on average of approximately 2.5 times as fast as in humans [125]. In opposition, rodents have a high bone formation rate and do not reach a steady-state of remodeling [19].

When studying bone biology in an animal model, it could be suggested to use rodents for studying conditions and fracture healing in humans between 0 years and 25 years of age; dogs for studying conditions and fracture healing between 25 and 60 years of age; and sheep for studying conditions and fracture healing in humans over 60 years of age. Therefore, the results of the studies in this dissertation may be a little too positive and show a greater effect of strontium under certain conditions than would be expected clinically in elderly humans. On the contrary, a study by Shaw et al. has indicated equally good potential of implant ingrowth between younger dogs and postmenopausal monkeys [123]. Yet, this choice of animal model is acceptable for a first line of experimental studies since any positive effect will be magnified. Any future studies of strontium can be targeted toward its main field of effect.

Canines are also easy to handle and the large size of their bone makes it possible to conduct four-arm, paired studies in cancellous bone, which reduces the number of animals used for research. The implantation site is easily accessible so the implants were inserted with minimal cause of trauma.

The dogs included in the studies were bred for research purposes. Minneapolis Medical Research Foundation, and the Animal Care and Use Committee approved the protocol of the study. The surgeries were carried out at AAALAC-approved animal care facility and NIH guidelines for care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) were observed.

IMPLANT MODEL

Direct versus indirect loading of the implant

In humans, remodeling of the bone from the waist down is balanced by the stimulus of weight loading which helps to maintain bone mass; as a consequence, prolonged bedrest will reduce bone mass [126]. Direct load on allografts has also been found to increase the area of active graft incorporation but not to increase the area of new bone [127]. Clinically the hip implant is directly loaded with body weight during gait. The direct load is then transferred from the implant to the host bone. If the femoral implant is large, the direct load is often not evenly distributed e.g. femoral implant will be inserted at press-fit at least in the distal area, which facilitates bone ingrowth and stress-shielding takes place more proximally [128]. In this case, the areas with high transferral of the direct load usually at the distal tip of the femoral implant, Gruen zone 3 and 5 (Fig. 5), bone formation is increased and osseointegration of the implant is achieved [129, 130]. At areas of low or no transferral of direct load, usually at the proximal part of the femoral implant, Gruen zone 1 and 7, bone mass is lost and the implant is not osseointegrated. Subsequently, in a situation of

pronounced stress-shielding the implant is not well anchored in bone and instability can develop ultimately resulting in aseptic loosening. The distribution of transferral of direct load is mainly dependent on the size, but also on the shape, material, and design of the implant, which is outside the scope of this dissertation. Therefore, the test implants were placed in the humerus of a fully loaded forelimb but were not subject to direct loading.
Clinically it is not possible to control conditions in this manner. Still it was relevant to control the conditions in these studies in order to investigate the isolated effect of strontium in the implant-bone interface.

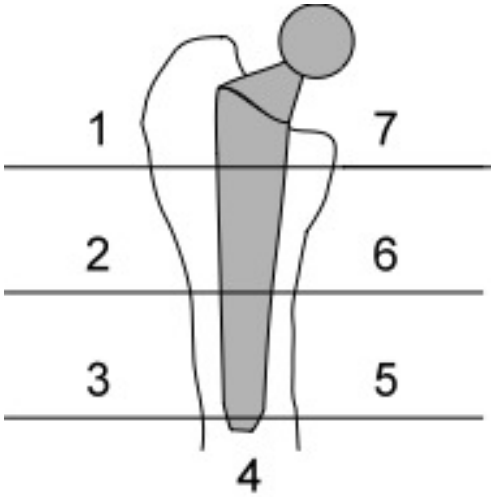

Figure 5
Gruen zones in relation to a femoral component.

Table 5
Anabolic and anti-catabolic effects of agents or coatings can be investigated with the implant model dependent on the implant type, insertion technique and observation time

	Observation time	
Implant model	4 weeks	12 weeks
Gap	Anabolic effect	Anti-catabolic effect on the modelling activity
Inserted by compaction technique	Anti-catabolic and anabolic effect	Anti-catabolic effect on the modelling activity
Allografted implant	Anti-catabolic and anabolic effect	Anti-catabolic effect on the modelling activity

Gap versus press-fit
During hip replacement surgery, a cavity in the host bone is carefully prepared to closely fit the implant. Even so, Geesink has described that the surface of the implant is separated from the bone by a series of small gaps. Therefore, it is important that the implant surface is bioactive to facilitate the healing of the intervening gap.
Hence, the anabolic effect of an implant surface or an agent added to the bone-implant interface is more clearly seen when a gap is introduced between the implant and the host bone [131, 132]. This gap magnifies the anabolic effect (Table 5).
Additionally, the gap model has an advantage during evaluation because only the relevant new bone is present in the interface and will influence the results of the mechanical test and, during histomorphometrical analysis, no mistakes can be made concerning whether the mineralized tissue is newly formed or old, necrotic bone originating from insertion of the implant.

The anti-catabolic effect of an implant surface or agent added to the bone-implant interface is most clearly seen if implant is inserted by the compaction technique or if an implant model with an allograft in the gap between the implant and the host bone is used [24, 121].
A gap model was used for all three studies of this dissertation, which allowed investigation of a possible anabolic effect of strontium (study I and III) (Table 6). A possible anti-catabolic effect of strontium was investigated in an allografted implant model (study II).

Table 6
The size of the gap varied between studies.

Study	**Gap size**
I, Strontium-substitutes HA coating	1.3 mm (± 0.1 mm)
II, Strontium-doped HA bone graft extender	2.8 mm (± 0.2 mm)
III, Strontium-substituted bioactive glass	1.1 mm (± 0.1 mm)

OBSERVATION TIME
Early implant fixation is essential for longevity of the implant (see section “Aseptic loosening”). Therefore, it must be ensured that any new intervention first secure early implant fixation. This is also the case when substances of a proposed long-term effect such as anti-catabolic interventions are investigated, where these interventions also must perform as well as the gold standard (control) in the field. Early implant fixation is established during the formative phase of fracture healing and the effect of anabolic interventions can become evident with an observation time at the end of the formative phase (Table 5). When early implant fixation is confirmed, investigations must then be extended to include the modeling and remodeling phase. To investigate the anti-catabolic effect of new interventions an observation time well into the modeling phase is also needed. Based on previous studies of this particular implant model, the formative phase of the fracture healing is usually well established after 4 weeks in dogs [133, 134]. Factors like size of the intervening gap at the bone-implant interface, motion at the fracture site, and general or local delayed bone biological activity influences the fracture healing time. These factors need to be taken into account when determining the observation time for a study.
Modeling at the fracture site starts after the formative phase and takes one to four years in humans, slowing down with time [22]. In the proximal humerus of beagles, Kimmel determined the annual bone turnover rate to be between 156-220% faster than in humans [125]. A previous study using the same model as the studies in this dissertation showed that fracture healing is in the modeling phase 12 weeks after implantation [81].
For all three studies, our hypotheses that strontium substitution or doping of HA improves implant fixation when applied as a coating or bone graft extender were tested for early implant fixation at 4 weeks. In study I the hypothesis of improved late implant fixation by strontium substitution of the HA coating at 12 weeks was also tested.

IMPLANT SPECIFICATIONS

Core
The implants used in all three studies of this dissertation were made of a 10 mm high cylindrical Ti alloy (Ti6Al4V) core with a

Table 7
Specifications of the coatings investigated or used in the studies.

HA and SrHA **Study I**	An innermost Ti-bond coating: Vacuum plasma sprayed 50µm thick In the middle a Ti-structured coating: Vacuum plasma sprayed 250-300µm thick Ra < 25µm On top a HA or SrHA coating: Vacuum plasma sprayed 60-80 µm thick Specifications of the HA and SrHA spray powder: HA or SrHA purity of 95% Ca/P or CaSr/P: 1.667 ± 0.004 4.86% Sr atoms in SrHA Particle size distribution: 75 (45-125) µm Bulk density: 1.16 g/cm3
Ti **Study II**	Sintered beads 40-50% porosity Average pore size of 250-300 µm
Bioactive Glass **Study III**	Grit-blasted implant cores 0%, 10% and 50% of calcium oxide were replaced by strontium oxide in the glass system: SiO_2-Na_2O-CaO-SrO-K_2O-MgO-ZnO-P_2O_5 (Table 8) Glass powder produced by melt-quench route Dispersion of polymethylmethacrylate, chloroform, and particle smaller than 38 µm. Implant cores dipped four times in the glass- containing dispersion Implants with dip coating were sintered at 750˚C Glass particles in the dip coated layers melted into a cohesive glass layer and the PMMA depolymerized and the monomer evaporated

smooth surface to which a rough surface texture was applied. The mean final outer diameter of the implants were 5.7 mm (± 0.1 mm) in study I, 5.7 mm (± 0.2 mm) in study II, and 5.9 mm (± 0.1 mm) in study III. End-cap screws were then mounted on the implants creating a gap of various sizes; see section "Gap versus press-fit".

Surface texture and coatings

The surface of commercial orthopedic joint implants is fully or partly rough-textured. The rough texture can be grit-blasted, sintered beads, Ti vacuum plasma sprayed, etc. The idea behind the rough textured surface is that bone grows into the porosity, improving the anchorage of the implant [135]. It is a matter for debate which surface texture is most ideal for experimental research. Smooth textured implants perhaps give a clearer picture of the effect(s) of the intervention. Yet smooth surfaced implants may not be able to withstand even small loads of force during the push-out test, regardless of the amount of ongrowth. Additionally, experimental implants with a rough surface texture show greater resemblance to the clinically used orthopedic implants. But most importantly, since the metal surface of the implant is not the subject of these investigations, the key issue with the surface is that it is kept constant because it serves only as a substrate. In the studies of this dissertation three different rough surfaces were chosen: *Ti VPS surface with SrHA/HA coating, a sintered bead surface, and a grit-blasted surface with bioactive glass coating* (Table 6).

SrHA/HA coating: The studies in this dissertation were inspired by the use of strontiumranelate as a treatment for osteoporosis, due to strontium's incorporation into the hydroxyapatite of bone. By mimicking the non-harmful products of the body, the first barrier of unfamiliarity of the substance is usually bypassed. We decided to use a 5% substitution of calcium, based on the limited literature at the time in the field of strontium's effect on bone metabolism, mainly investigated in rodents [99, 100, 103, 104, 106, 107, 136-138]. Prof. Marc Grynpas, Toronto, Canada, was also consulted on the issue.

A precipitate of the 5% strontium substituted hydroxyapatite and pure HA was produced and donated by Osteologix Aps, Denmark, to be used for the SrHA coating and as bone graft extender. The rest of the coating procedure was performed and generously donated by Medicoat AG, Mägenwill, Switzerland. The precipitate SrHA and HA were converted to powder suitable for vacuum plasma spraying. This powder was characterized by x-ray powder diffraction and inductively-coupled plasma mass spectrometry (Table 7).

The Sr-content was uniformly distributed and morphology of the particles was visualized by SEM (Fig. 6).

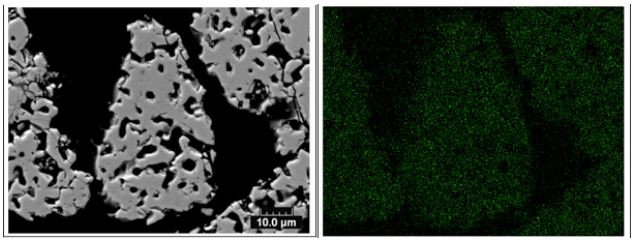

Figure 6
SEM image of the SrHA spray powder particles show morphology and Sr distribution.

A mechanical test of the tensile bond strength and shear stability was not performed. The spraying conditions and specifications of the powder were identical for SrHA and HA and furthermore identical with powder and conditions of the commercially available HA coatings of endoprosthesis for total hip replacement. For commercially available HA coatings, analyses have been made of

both the HA spray powder and HA after VPS coating. The analyses confirmed that the specification of the HA powder listed above (Table 7) is similar to the specification of the HA coating. Similar analysis like XRD has not been done for the SrHA VPS coating, so whether the SrHA underwent any phase transformation during VPS is not known.
Implants with these HA and SrHA coatings were investigated in study I.

Table 8
Chemical composition of the bioactive glass with 0%, 10%, and 50% of calciumoxide substituted by strontiumoxide, presented as mol %.

Oxide	Sr0	Sr10	Sr50
SiO2	49,46	49,46	49,46
Na2O	3,30	3,30	3,30
CaO	32,62	29,36	16,31
SrO	0,00	3,26	16,31
K2O	3,30	3,30	3,30
MgO	7,25	7,25	7,25
ZnO	3,00	3,00	3,00
P2O5	1,07	1,07	1,07
Total	100,00	100,00	100,00

Sintered bead surface: The coating was donated by DePuy Inc, Warsaw, IN, USA. The surface of the test implant resembles the surface of commercially available orthopedic implants. Implants with the sintered beads were used in study II.
Grit-blasted: Plasma Biotal Ltd performed the grit-blasting of the implants for study III. The bioactive glass was expected to dissolve in vivo in approximately 3 weeks leaving a commercially available implant surface.
Bioactive glass coating: Production of the strontium-substituted powder and dip coating was performed by Bioceramic Therapeutics Ltd, London, UK (Table 7 and 8).
A VPS coating of the glass was also considered. The VPS coating method may minimize interactions between the grit-blasted Ti core implant and the bioactive glass yet still provide a good bond. The dip coating method was selected because this method allowed for the bioactive glass to be deposited at surfaces and spaces not accessible by the VPS method.

BONE GRAFT EXTENDER
Solid, crystal precipitate of calciumhydroxyapatite (HA BGE) was studied as a control bone graft extender. In the intervention treatment arm, 4.93% of the calcium had been substituted by strontium (SrHA BGE). The synthetic HA BGE or SrHA BGE material only possess osteoconductive properties and an agent or material with osteoinductive signal needs to be added for gap healing to be successful [31]. Therefore the bone graft extender material was mixed with allograft at a 50:50 volume ratio. Additionally, it was hypothesized that the strontium-doped bone graft extender was ideal for mixing with allograft because of the proposed dual acting properties of released strontium which would regulate the mismatch of fast allograft resorption and slow new bone formation.
A HA bone graft extender was chosen over a TCP. HA is present in bone for longer than TCP [37], which is desirable in many clinical settings especially since the bone graft extender serves as a osteoconductive scaffold, promoting new bone formation. The disadvantage of HA compared to TCP is the lower osteoconductive activity. Therefore, the greatest challenge and interest was to improve the performance of HA as bone graft extender. Our hypothesis was that strontium doping of the HA bone graft extender would improve implant fixation.
The granules of the bone graft extender ranged between 0.6 to 2 mm in diameter (Fig. 7). The size of the granules was within the range recommended for well-graded particle-size, but it may have been beneficial for the size range to also have gone below 0.6 mm [139]. However, in this model, granules of 2 mm can fill out the bone-implant interface in the full height of the specimen block for the mechanical push-out test, which was noted during the test. If or when this happens, the mechanical properties of the interface can be compromised in a matter of no clinical relevance. This issue also might have caused an increase in variation of the data, as well, contributing to non-significant differences between the treatment arms.
The issue of porosity of the bone graft extender material was not included in this investigation.

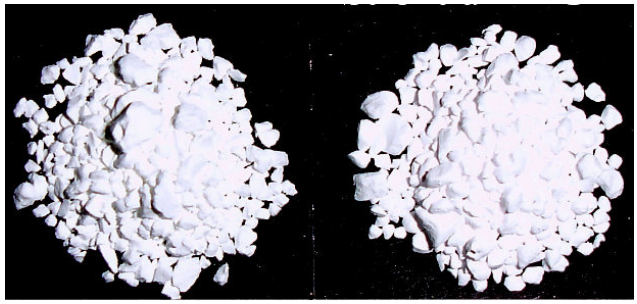

Figure 7
Bone graft extender material HA on the left and SrHA on the right.

SURGERY
Surgery was performed under sterile conditions, and the dogs were fully anaesthetized during the procedure. A 7-cm long skin incision was made with cautery on the lateral proximal humerus. The deltoid muscle was bluntly dissected to expose the humerus. To match the clinical conditions of hip replacements, the test implants were inserted into cancellous bone. The surgical procedure is relatively small and well tolerated by the dogs with complications like infections and fractures rarely observed.
In study I, a 2.5-mm guide wire was inserted anterolaterally at the level of the greater tubercle and oriented perpendicularly to the surface. Another 1.5-mm guide wire was inserted 17 mm distally and parallel to the first one. The distal guide wire was cut off approximately 2 mm above the bone surface. With a cannulated drill (∅ 8.0mm), a 12-mm cavity was drilled over the proximal guide wires at a maximum speed of two rotations per second. The edge of the hole was trimmed, and the cavity irrigated with 10 ml saline for removal of periosteum and loose bone chips. One implant was inserted into the cavity, and after securing hemostasis, the soft tissue was closed in layers. This procedure was repeated for the opposite humerus.
After 8 weeks, a second surgery was performed with the same surgical procedure as just described. At this second surgery, an implant with the same coating as in the proximal implant position was inserted at the position of the cut off 1.5 mm wire.
In study II, one surgery as described above was performed and implants were only inserted into the proximal implantation site, which was created with a cannulated drill (∅ 11.0mm). An implant with a mounted bottom screw was inserted into the cavity. A mixture of 1 mL allograft and 1mL SrHA or HA, was tightly

packed around the implant, and the top screw was mounted. One surgeon impacted the graft mixture for all the implants.
In study III the surgery was performed as described above for study I. The only difference between the surgeries was that in this study all four implants were inserted during one surgery since observation was the same for all treatment arms (Fig. 8).
All dogs were given ceftriaxone (1 g, i.v) and buprenorphine hydrochloride (0.0075 mg/kg/day, i.m) administered immediately before surgery and 3 days postoperatively.
The dogs were given 30 mg/kg tetracycline i.m. day 18 and 20 mg/kg calcein i.v. day 25, for fluorochrome labeling of the mineralization front [140, 141]. After 28 days, the dogs were sedated and killed with an overdose of hyper-saturated barbiturate. Using these dogs, unrelated studies were conducted in the distal femur and proximal tibia. In the dogs of study II, a study of partial gold coating of implants was examined in the distal implant site of the humerus. Only studies with no systemic effects were carried out in the same series of animals.

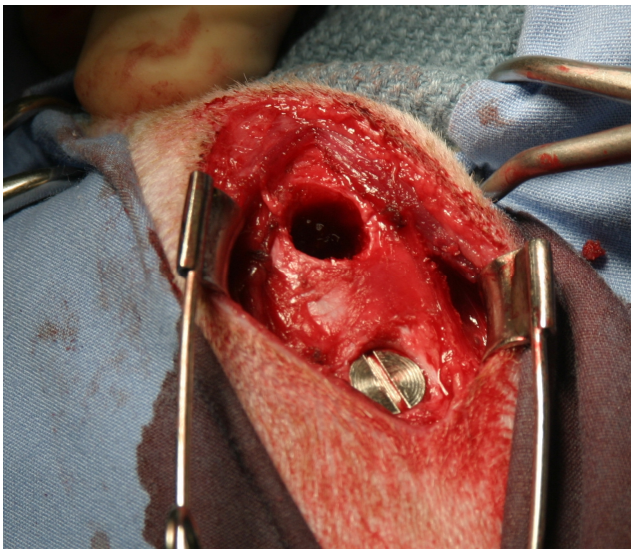

Figure 8
An implant with mounted end screw has been inserted in the distal implantation site while the proximal implantation site is ready for insertion of implant. The observation time for both implants is 4 weeks.

The studies within these dogs were of implant surface modifications, topical short-lived growth factors [142], and topical bisphosphonates, which have a high affinity for bone and therefore do not become systemically available [143].

PREPARATION OF SPECIMENS
The bone cells in the specimens were not the main target of the analyses but it was advantageous to preserve them because they could become useful for morphologically determining the tissue type in the histomorphometrical analysis. On that account, formalin was the optimal fixation for these specimens. However, the lamellae of bone tissue are best preserved in ethanol. Unfortunately, the specimens had to be transported from the USA to Denmark for further preparation and evaluation. Ethanol and formalin are classified as dangerous goods and are therefore not to be transported by airplane, so fixation in ethanol was found to be not feasible. Instead the specimens were retrieved en bloc, each containing two frozen implants which were kept frozen during transportation. This preserved the tissue until its fixation in ethanol in Denmark.

Freezing can cause cells to autolyse because the membrane becomes destabilized. Consequently, fewer cells will be available for morphological determination of the tissue type in the histomorphometrical analysis. Preservation by freezing, however, has been shown not to have adverse effect on the mechanical properties of cancellous bone [144, 145].
The en bloc proximal humeri specimens were cut to two approximately 2.5 x 2.5 x 2.5 cm cubes, each containing an implant and surrounding tissue. The implant specimens were randomly allocated a code number which was unknown to the observer for the treatment of the specimen during tests and analyses. The specimen with the 10 mm high implant was subsequently cut transversely using a water-cooled Accutom-50 wheel diamond saw (Struers A/S, Roedovre, Denmark) (Fig. 9). Each block was cut into two pieces: 1) a 3.0 mm high block for mechanical test closest to the surgical entry site, and 2) a 6 mm high block for histomorphometrical evaluation furthest away from the surgical entry site. The mechanical block was then refrozen while the histological block was submerged in ethanol, initiating fixation and dehydration.

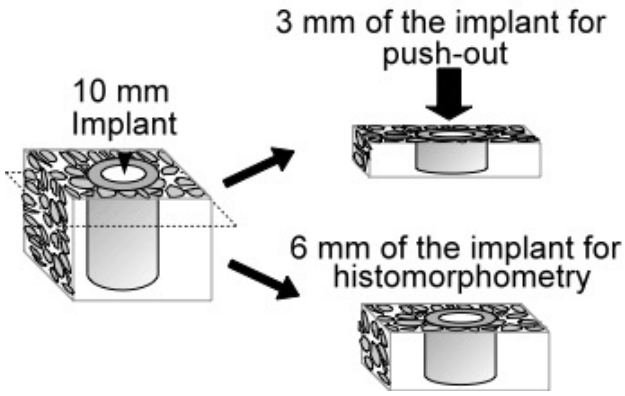

Figure 9
Specimen block cut in two for push-out test and histomorphometry.

In general, these methods of preparation and preservation of the specimens are gentle and do not adversely affect the parameters of later measurements and evaluations that were performed in these studies.

BIOMECHANICAL TEST
For all three studies, the primary goal was to improve mechanical and histological implant fixation. Preferably the test would closely resemble the nature of the mechanical force and load that a clinical implant is subject to. Clinical implants are subject to a simultaneous mixture of non-destructive compressive, shearing and bending forces in a hysteresis-like pattern. A test mimicking these forces is difficult to set up and carry out on a specimen block of 3 mm height and 2.5 x 2.5 cm base.
A destructive push-out test of the implant in the longitudinal axis was selected because the hip replacement prosthesis is subject greatly to axial load, especially during the gait cycle. The Ultimate Shear Strength of the interface found at a push-out test was expected to reflect the upper limit for the load of a given hysteresis-like loading pattern. During push-out of the implant to failure, the inter-digitating interface of the porous implant material and ingrowth of bone is subject to a simultaneous mixture of tensile, shearing and compressive forces.
For the results to be comparable between specimens, the shape and size of the implant, together with the test procedure, had to

be similar [146, 147]. Preparation of the specimens as well as the test itself was standardized and the test parameters were normalized in accordance with the surface area of the implant.
The push-out test was performed with a MTS Bionics Test Machine (Eden Prairie, MN, USA) with 5.0 mm diameter probe.
The specimens were thawed before testing and all were tested in one session. The flat surface of specimen block was placed on a flat support jig with a 7.4 mm diameter central opening [147]. The implant in the specimen block was placed centrally over the hole in the support jig, which assured a distance of 0.7 mm between the implant and the support jig [148]. The direction of loading was from the cortical surface inward. A custom made program kept the test standardized: A preload of 0.5 N was applied to standardize contact conditions before initiating loading. The displacement rate was 5 mm/min with a 500 N load cell. Data points for every 10 µm of displacement were entered into an excel spreadsheet and normalized in accordance with the surface area of the implant. The normalized force-displacement curve was then plotted (Fig. 10) and calculations of the mechanical parameters, *Apparent Shear Stiffness, Ultimate Shear Strength, and Total Energy Absorption* [43, 147], were auto-generated in the spreadsheet based on a PhD dissertation by Baas [146].

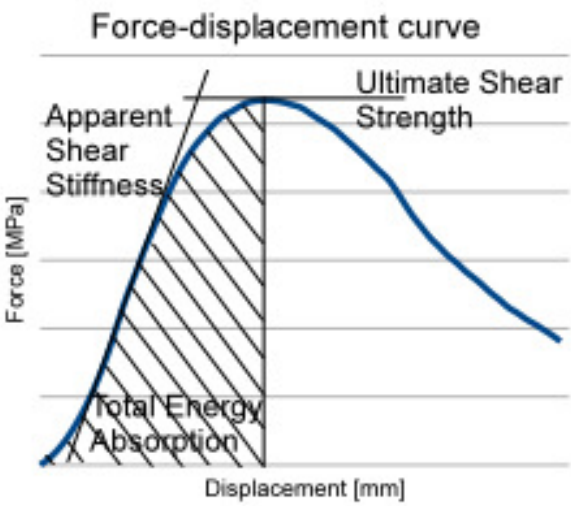

Figure 10
The force-displacement curve was normalized with respect to the surface area of the implant.

Apparent Shear Stiffness (MPa/m): The steepest slope on the force-displacement curve was calculated. The parameter characterizes the deformation property of the interface material and tissue. Note despite the parameter is named apparent shear stiffness then the interface is not only exposed to shear force. A high stiffness indicates bone anchorage of the implant because bone is rigid. A low stiffness indicates fibrous anchorage because fibrous tissue is more elastic than bone.
Ultimate Shear Strength (MPa): The first, highest point on the force-displacement curve defines the maximum force applied until failure. The largest shear strength of the interface is characterized by this parameter. The pushed out implant sections were inspected macroscopically; mineralized tissue was often observed on the HA coated implants. Thus, it was the shear strength of the tissue in the interface that was determined. Again note despite that the parameter is named ultimate shear strength, the interface is not subject to shear force alone. The strength of bone is usually higher for bone than fibrous tissue.
Total Energy Absorption (J/m): This is defined as the area under the force-displacement curve until failure. It characterizes the ability of the interface to absorb energy but this parameter is not characteristic for a given tissue type.
Together these parameters provide a picture of the mechanical implant fixation, which correlates well with desired osseointegration of the implant [43].

HISTOMORPHOMETRY
The osseointegration of the implants was evaluated at the microscopic level in accordance to Albrektsson's definition (Table 1, page 2) [10]. The aim was to obtain a quantitative, unbiased and representative estimation of the osseointegration. Histomorphometrical analysis provides a quantitative evaluation of the tissue in contact with and surrounding the implant. Various stereological designs exist and can provide unbiased estimates, but to adopt the method of a design in practice is not always possible and issues can arise.

Preparation
The 6 mm specimen block for histomorphometrical analysis was fixed and dehydrated in graded ethanol (70-99%). Specimens were then placed in 99% propanol before they were embedded in

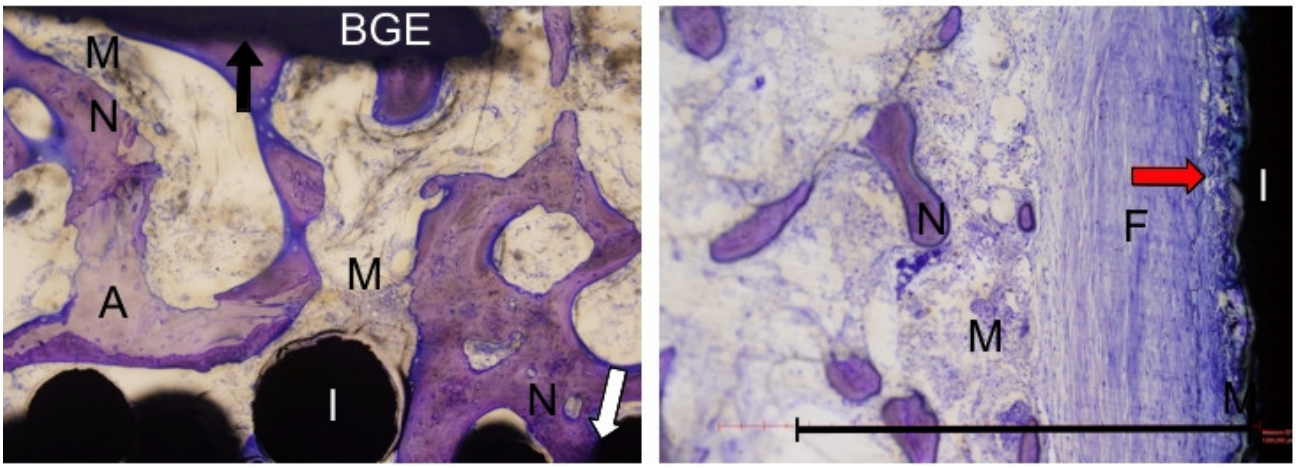

Figure 11
Morphology of the different tissue types: new bone (N), allograft (A), bone marrow (M), and fibrous tissue (F). Implant (I) and bone graft extender material (BGE) is also seen and the bar indicates the initial surrounding gap. Ongrowth onto the BGE (black arrow), ongrowth onto implant (white arrow) and fibrous tissue in contact with bioactive glass coating (red arrow) is also seen.

cold poly(methylmethacrylate), PMMA. Cold PMMA was chosen because the temperature up to 45° Celsius during hardening of ordinary PMMA polymer can damage cells and cause coagulation of tissue. Cold PMMA is equally good in regard to the quality of bone sections produced [149].
MMA embedding material is also a good choice for specimens to be surface stained because the polymer can be removed completely by H_2O_2, allowing the stain to bind to the tissue.
Sections were stained with toluidine blue at pH 7. Toluidine blue is a cation dye, which binds to negatively charged tissues and cells, and can be controlled by regulating the pH in the milieu. At pH 7, collagen, hyaluronic acid, DNA, and RNA are all negatively charged and get dyed in a purple-blue color. For histomorphometrical analysis, tissue classification was based on morphology (Fig. 11): new bone appears as a disorganized, dense substance with embedded cells colored relatively dark purple; while allograft is a dense substance with empty cell lacunae and clear cement lines colored relatively pale purple. Bone marrow is a cell rich conglomerate with intervening empty areas from dissolved fat and a few scattered thread-like structures. Fibrous tissue appears dense, with well-organized bundles of fibers with sparsely intervening small cells. BGE is identified as coarsely profiled shadows.
Toluidine blue does not fluoresce; hence the sections could simultaneously be evaluated by light microscopy and ultraviolet microscopy. This simultaneous evaluation was especially useful for the descriptive study in study III.
The dogs were given tetracycline and calcein for fluorechrome labeling of the mineralization front to alleviate any suspicion of a mineralization defect caused by strontium [141]. No suspicion of mineralization defect arose. Instead, in the bioactive glass coating study, the fluorechrome labels supported the theory that the bioactive glass coating had chemically induced the formation of HA and furthermore allowed us to estimate the time point of mineralization of the induced HA.
Surface staining is superior to infiltration staining of the whole specimen block. First, with a surface stain it is possible to strain almost any specific tissue type and cell of interest. Second, and of greater importance, evaluating the surface of the section is easier for the observer. In contrary to infiltration stains, bone situated deep inside the section is easily visualized due to the nature of light microscopy, which is based on trans-illumination of the section. If more than the tissue at the surface of the section is evaluated then the estimates of bone can be overestimated. With the specific toluidine blue surface staining used in the studies of this dissertation, the depth of staining was estimated to be a mean of 4.1 µm (± 0.56 µm s.d.).

STEREOLOGY

The fundamental idea behind stereology is to gain unbiased estimates of number, length, surface, and volume. The estimates are obtained in a two-dimensional material such as histological sections and anisotropy of the features must be considered and dealt with accordingly. The reason for isotropy is that the probability of interceptions between the test probe and the structure must be independent of the orientation of the structure and the probe. Only when the intercepts are isotropic and sampled at uniform random, the estimates can be unbiased. Trabeculae of bone are anisotropically orientated for cancelling the anisotropy of mechanical stress [28].
In the studies of this dissertation, stereological software (Visiopharm Integrator system, NewCast ver. 3.0.9.0 Visiopharm, Hoersholm, Denmark) superimposed the test probes onto a picture of the field of view from the microscope.
In general the number of dimensions for the probe plus the number of dimensions for the structure to estimate must add up to 3. For volume density estimates, the probe is a point: zero-dimensional and without orientation. Unbiased volume estimates are obtained by Cavalieri's principles described by Gundersen [150]. The method is easily carried out and used in all three studies of this dissertation. For surface area density estimates, the probe is a one-dimensional lineprobe, which therefore has an orientation and the issue of isotropy becomes relevant. For obtaining isotropic uniform random intercepts, either the lineprobe or the structure (the interface) must be isotropic. The issue of isotropy is ensured in stereological designs by keeping the lineprobe isotropic and in stereological models by assumptions defined in regard to the structure of interest, respectively.
The surface of the bone graft extender granules used in study II was isotropic (Fig. 7).
The Vertical Uniform Random, VUR, design was used in the study of bioactive glass coatings (III). A cylindrical model was used in studies of strontium-substituted HA coating and of strontium-doped bone graft extender (I and II, respectively). Unless stated otherwise, the sections of the dissertation concerning stereology are based on the book by Howard and Reed [151].

Vertical uniform random

The VUR design is based on a combination of three sampling techniques but no assumptions are made: firstly, sections must be obtained by uniform random sampling in the predefined vertical axis. In this case, naturally assigned to the longitudinal axis of the implant. Secondly, the defined vertical axis is recognized in the microscope and aligned prior to histomorphometrical sampling. Thirdly, the line-probes are sine-weighted.
This method is highly useful for analyzing biological structures that do not resemble a geometrical shape. In 2000 Overgaard applied this method to the gap implant model (also used in the studies of this dissertation) and optimized the sampling of the VUR design [152]. He found that the number of sections per implant could be reduced to every fourth, which in total were 3-4 sections of the 14 exhaustively cut serial sections. The reduction in sections could be done without compromising the quality of estimates of the surface area density and volume density by increased variance of the data. In the study by Overgaard, the exhaustive cutting of serial sections does not include the gap surrounding the implant at the beginning and end of the specimen block, which contains the Region Of Interest (ROI) for the only volume estimates (Fig. 10). Even if these parts of the specimen block were cut, the ROI is difficult to determine because it is defined in relation to the implant. Usually, the end screw securing the gap at surgery will be present but only in one end because the superficial part of the implant has been cut off for the mechanical analysis. Despite careful efforts to cut parallel to the vertical axis of the implant the sections may be slightly tilted, which on the micron-scale can be considerable. Therefore, the ROI cannot be drawn solely on the presence of one end screw. As a result of these issues, the optimized VUR method was modified for practical purposes so four sections were cut at the central part of the implant (through the plane of the internal thread for the end screw) (Fig. 12). Consequently the rule for uniform sampling was not followed in regard to the volume estimates at the sectioning level. In 2008, Baas calculated that in worst case the bias inflicted upon the volume densities was up to 7.6% underestimation of the

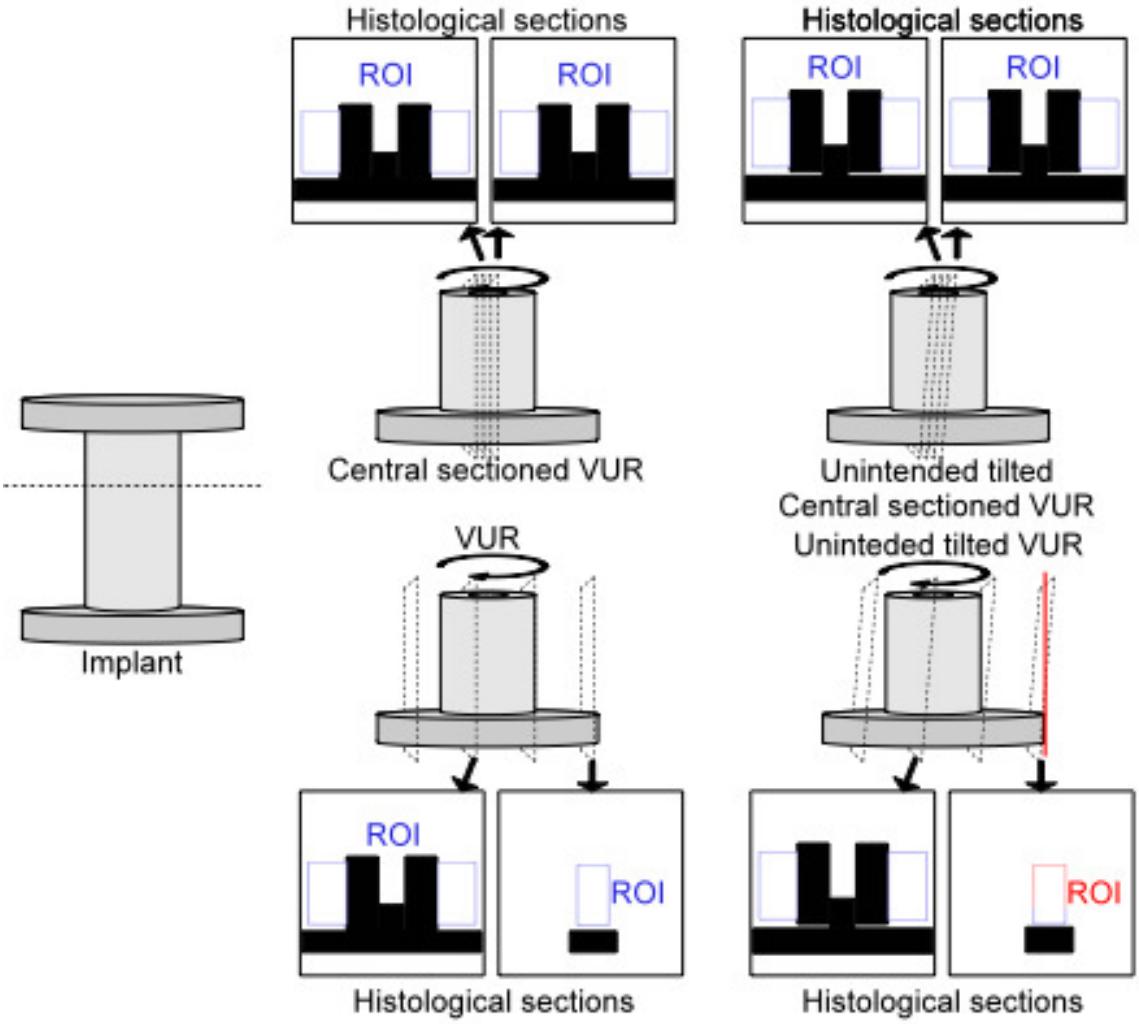

Figure 12
Sketch of the histology sections and the region of interest (ROI). Position and orientation of the sections influence the position of ROI drawn during histomorphometrical analysis. By the VUR technique there is a risk of wrongfully drawing the ROI outside the gap of implant model, indicated by ROI in red down right side corner.

volume of new bone and fibrous tissue in the gap and up to 6.3% overestimation of the volume of allograft in the gap [146]. In practice the bias was estimated to 1% systematic over- and underestimation for all parameters. This bias is negligible. For the central sectioned VUR method another issue - how well the biology is represented - has to be considered. The issue of biological representation will be discussed below. An alternative and more biologically representative stereological method was desired, especially for study II because the main focus in that study was the volume of tissue in the gap.

Central sectioned VUR
The longitudinal axis of the implant was defined as the vertical axis of the specimen. The specimen block was randomly rotated around the vertical axis before sampling of the sections was started (Fig. 12). The first section was placed one-third into the implant and subsequently four consecutive sections were sampled from the central part of the implant at the level of the inner tread for the end screws.
Evaluation of the implant osseointegration was divided between two criteria: gap healing and ongrowth and the two components were estimated in a ROI. The ROI was manually drawn from the implant surface and 750 µm into the initial surrounding gap. At the top and bottom of the implant, 300 µm were excluded due to artifacts from the cutting and disruptive effects caused by the screw at the end.
Gap healing was defined as the volume of new bone, so every grid point was counted as either new bone or non-mineralized tissue (Table 10).
Ongrowth was defined as new bone in contact with the implant surface. Intersections between the implant surface and sine-weighted gridlines were counted as either new bone or non-mineralized tissue (Table 9).
All parameters were estimated at ×250 magnification in randomly sampled fields of view in 100% of the ROI.
The described techniques and preparation of specimens, together with the stereological software, made it possible to obtain estimates with negligible bias as described above [146].

Stereological model for a cylinder
Stereological models for estimating surface area density are based on assumptions, which for a cylinder includes three assumptions: First, that the structure to be estimated is a geometric cylinder. Second, that the orientation of the uniform random

sampling of the histological sections is horizontal in order to present the surface of the cylinder as an isotropic circle in the two-dimensional material (Fig. 13). Third, the interface between the bone and the surface of the implant must be isotropic.

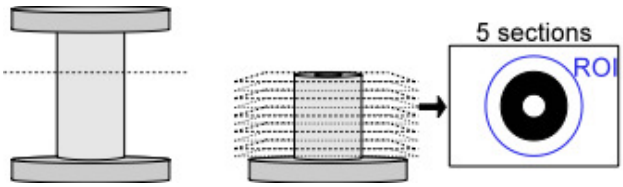

Figure 13
Horizontal sections and the ROI applied.

Stereological models are mainly applicable to man-made, engineered structures because they often are a geometric shape. For geometric structures, equations for calculations of surface area are often known. For example, the equation for the surface area of a cylinder:

Equation 4

$$A = 2r\pi \cdot h$$

-is the circumference of the horizontal circle multiplied by the height of the cylinder.
Therefore, the sampling orientation of the sections of the cylinder must be horizontal; which makes the two-dimensioned presentation of the implant a circle (Fig. 13). The circumference of the circle can be estimated based on Buffon's needle' relationship. In 7, he described that the probability of a needle intercepting a gridline is dependent on the length of the needle and the distance between the gridlines. Based on Buffon's needle relationship, the length of any arbitrary curve, a boundary, in two-dimensions can be estimated by a grid with isotropic direction, which is positioned over the object. T is the distance between gridlines, I is the number of intersections between the grid and the boundary:

Equation 5

$$B\_estimated = \pi/2 \cdot T \cdot I$$

The distance between gridlines, T, represents a straight line but the equation is for estimating any arbitrary curve. π/2 is the average length correction factor between a straight and a curved line (Fig. 14):

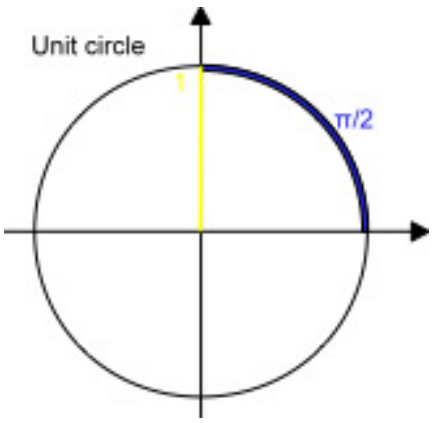

Figure 14
The unit circle shows the average correction factor between a straight and a curved line or arch.

Therefore, by multiplying the length of the straight lines by π/2 the distance between two gridlines, T, is corrected to be the average curved distance between two gridlines. By equation 5, the circumference is estimated as the addition of arches. Finally, the circumference of the circle, the two-dimensional representation of the implant, is multiplied by the height of the cylinder:

Equation 6

$$A = (\pi/2 \cdot T \cdot I) \cdot h$$

The height of the cylinder is the distance between two consecutively cut sections.
Equation 6 calculates the absolute surface area density but in the studies of this dissertation only the relative surface area densities for specific tissue types are of interest, because the total surface area of the implant (cylinder) can differ due to the preparation. Overall, when relative estimates for the surface area density are used, all the constants equal one which leaves the ratio between the specific tissue type divided by the total number of intercepts (the sum of intercepts for all tissue types estimated). For instance:

Equation 7

$$S\ new\ bone = I\ new\ bone / I\ total$$

Average h was estimated by evaluating block advance, where consecutive cuts were made of decreasing depth into a specimen block which were not to be included in the studies. Secondly, sections were cut in the perpendicular plane to the first cuts (Fig. 15) and h was measured from the surface of one section to the surface of what would be the next section. Mean t was 493µm (± 45 µm).

In general for the cylindrical model
Osseointegration, gap healing and ongrowth, was estimated as described for study III of the bioactive glass coatings.
The tissue types estimated in the study of strontium-substituted HA coatings (I) were: new bone, fibrous tissue, or bone marrow tissue (Table 9-10). In the study of strontium-doped bone graft extender (II), allograft and bone graft extender was also estimated (Table 9-10).
In the study of strontium-substituted HA coatings (I), the ROI was manually drawn from an applied grid of two centralized circles: an inner circle 2.9 mm in diameter to centralize the ROI with regard

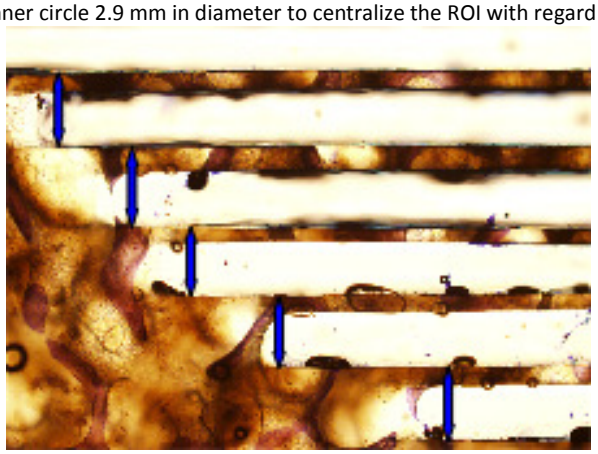

Figure 15
To estimate the bloc advance steps of cuts was made in a specimen bloc. Perpendicular to the first cuts, sections were made like the one on the picture. The distance from an upper surface of one section to the upper surface of the consecutive section was measured, blue arrows.

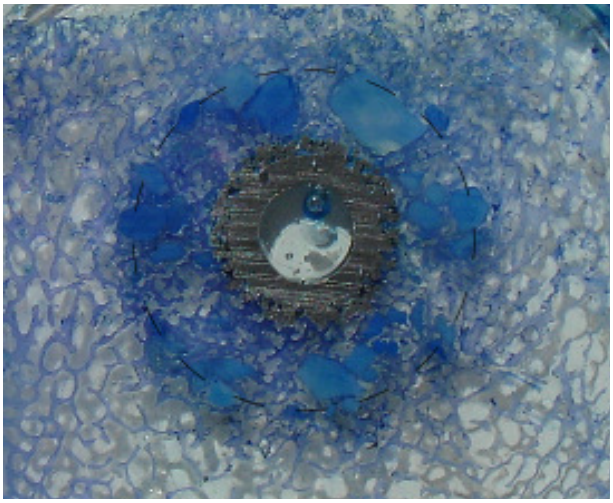

Figure 16
ROI manually drawn on the histology section.

Table 9
The total number of intersections between gridline and implant surface counted for each specimen. Presented as mean (±sd).

Surface Area Density	
Study	**Number of intersections counted**
I, Stronitum-substituted HA coating	443 (± 56)
II, Strontium –doped Bone Graft Extender	756 (± 54)
III, Strontium-substituted bioactive glass coatings	145 (± 24)

Table 10
Total number of grid points counted for each specimen. Presented as mean (±sd).

Volume Density	
Study	**Number of grid points counted**
I, Stronitum-substituted HA coating	946 (± 116)
II, Strontium –doped Bone Graft Extender	1539 (± 68)
III, Strontium-substituted bioactive glass coatings	314 (± 25)

to the implant and an outer circle 7.5 mm in diameter to outline the ROI at a distance of 0.75 mm out into the surrounding gap of the implant (Fig. 13). In the strontium-doped bone graft extender study (II), the outer circle was 10.5 mm in diameter to outline the ROI at a distance of 2.45 mm out into the surrounding gap of the implant (Fig. 16).
The implant was also included in the ROI but the size in area and volume was roughly the same within the group and between groups of each study, which equalizes the effect.
The volume and surface area estimates were presented as relative differences in terms of the number of hits or intercepts for a given tissue type divided by the total number of hits or intercepts. The fraction was multiplied by 100 and presented as the percent of the volume in gap occupied by the given tissue type and percent of the implant surface in contact with the given tissue type, respectively.

Overprojection
The issue of overprojection of the implant surface, such as the beads (250-300 µm in diameter) of the porous coating in study II, is considered negligible. Because in the worst cases of study II, 20 µm of the interface was in the shadow and could not be analyzed. To put this in perspective, an osteoclast measures 50-100 µm. Besides this, very thin layers of bone ongrowth onto the implant will not withstand a clinically relevant mechanical load.

Intra-observer variation
One observer performed the histo-morphometrical analysis. The observer was blinded to the treatment of the specimens except when the difference between treatment groups was visually clear, for example with glass coating versus HA coating. One randomly selected implant from each of the treatment groups was chosen for intra-observer variation analysis. The intra-observer variation was determined as coefficients of variation (CV) on double measurements of the selected implants.

Deviations from design and model
Any deviation from the stereological design or the assumptions of the model is likely to cause bias of the estimates.
Deviation from the design: In VUR, the sampling of the material must be uniform random after the vertical axis is assigned. In the central sectioned VUR method, sampling is systematic not uniform. This is an important issue when the material of the ROI is not homogenous in regard to the representation of the gap. The systematic non-uniform sampling of the gap caused the negligible bias of the volume density estimates [146].
Deviation from the cylindrical model: In the two studies where the stereological model of a cylinder was applied, the structure of the implant was assumed to be a perfect, smooth geometric cylinder. This was not completely the case, but the implant with rough textured coatings was considered a good approximation for the model because the surface of the implant still curved like a cylinder. Furthermore, the implant did not approach a shape that resembles any other geometrical shape. Hence, the discrepancy between the actual implant structure and a perfect cylinder is small. Any potential bias inflicted by this discrepancy is therefore considered small as well.
The second assumption was that the sections were sampled horizontally so that the implant could be represented as an isotropic circle on the sections. When the circle grid for drawing the ROI was applied it was clearly noticed that the implant was a circle and not an ellipsoid. Therefore the assumption of horizontal sampling orientation was considered valid.

Representation of the biology
As stated earlier, the histomorphometrical analysis should provide unbiased estimates. The estimates provided are only estimates of the actual values, which cannot be known. However, it is also important for the estimates to be of highest possible precision [153]. It is relevant to take into account the biological variation or heterogeneity within the tissue. If biological variation is not uniformly sampled for each specimen then the variation of data between specimens is likely to increase and subsequently lower the precision of the estimates.
This issue is dealt with by the stereological methods as long as the design method are followed and all assumptions are met. The issue arises when the stereological methods are modified, e.g. for practical reasons.

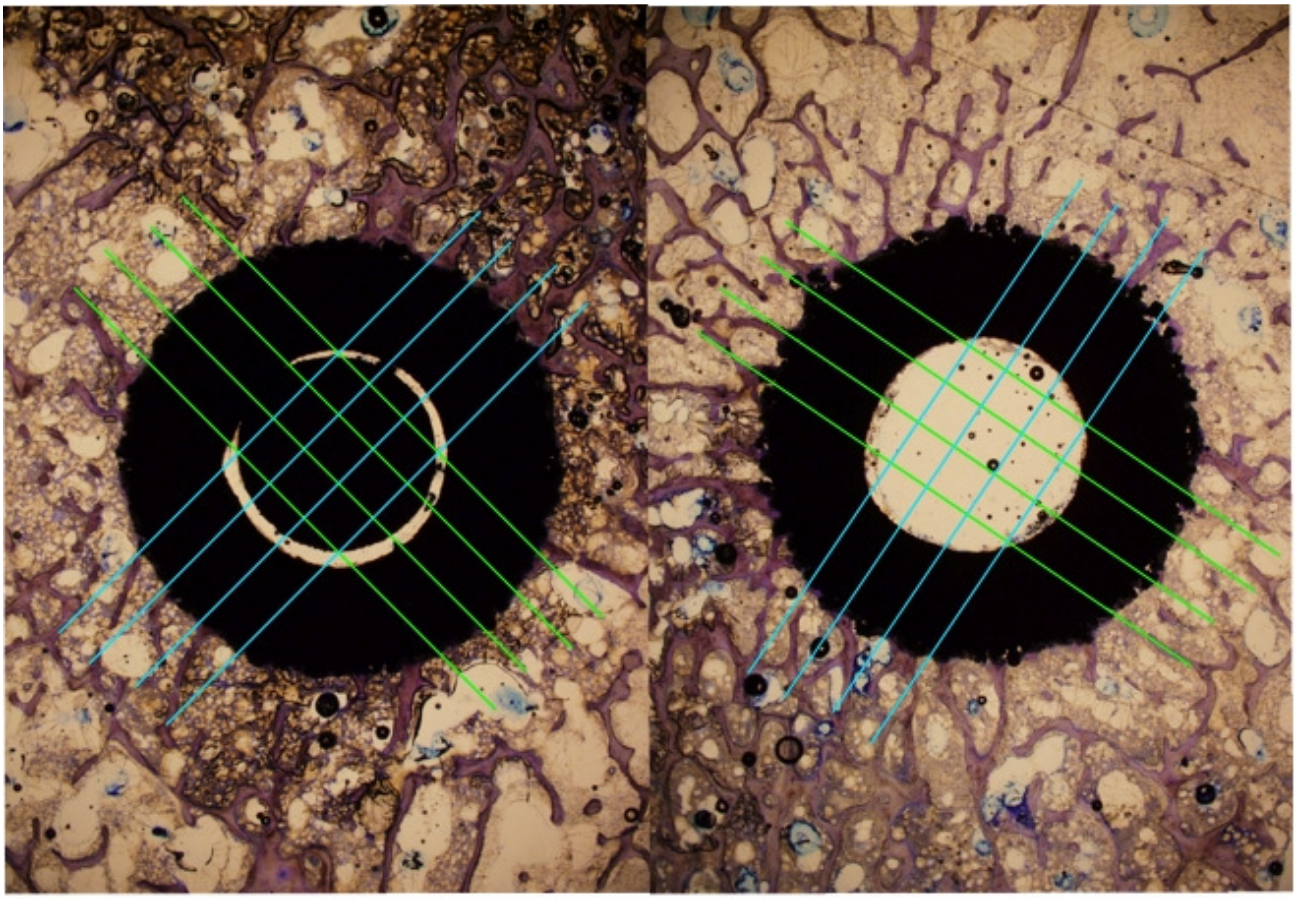

Figure 17
Inhomegeneity of the bone density can be seen in the ROI. If the sections of the central sectioned VUR method were placed as the green lines then the estimates of the histomorphometrical analysis would be low compared to sections placed as the blue lines. The estimates would not be biased but the variation of the data would increase

For the implant model used for investigating strontium in the bone-implant interface, heterogeneity of the amount of new bone formed within the ROI of the specimen was observed. The density of newly formed bone seems to be influenced by the anisotropy of the trabeculae or location of the implant e.g. in relation to the cortex. For an implant placed in proximity to the cortex, the amount of new-formed bone was more abundant in the ROI nearest the cortex (Fig. 17). With this heterogeneity in mind, it seemed likely that the estimates of volume and surface area densities from the central sectioned VUR method would be influenced. Within the same treatment group, some specimens would be sampled form an area of high bone density, which would then give relatively high estimates of bone densities. Other specimens would be sampled from an area of low bone density, which would give relatively high estimates of bone densities. In consequence the density estimates would be increasingly imprecise because variation in the data would increase, making it more difficult to detect small differences between treatment arms of the study. The estimates were not biased because the increase in variation was not systematic for a given tissue type or position. The issue arose because the sections were not uniformly sampled within the ROI and heterogeneity was considerable around the circumference of the implant. When sampling was not uniformly distributed throughout the total ROI, the representation of the biological variation decreased. Estimating the representation of the full volume of the ROI can be difficult to grasp. Therefore, for standardizing the discussion and making the presentation more understandable, total ROI will be presented as the implant surface area.

Representation of the ROI can be estimated by calculating the total area of the implant surface that was subject to the uniform random sampling. Secondly, the surface length that was subject to analysis can be calculated to see if the analyzed length is comparable to that found as a minimum surface to be estimated by Overgaard when he optimized the sampling method [152].

In the cylindrical stereological model the area subject to uniform random sampling was the whole 6 mm high implant with a standardized radius of 3 mm because the specimen block for histomorphometrical analysis was cut exhaustively throughout the implant height (Fig. 18). The surface area of a perfect cylinder is:

Equation 8

$$A = 2\pi rh$$

For each specimen the total area subject to sampling by the cylindrical model was 113mm2.

Secondly, 5 sections were subject to surface area density estimation, each with a length equal to the circumference of the circular implant:

Equation 9

$$\text{circumference} = 2\pi r$$

Equation 10

$$\text{circumference} = 19 \text{ mm}$$

The total implant surface length subject to estimation was in 94 mm.
By using the central sectioned VUR the area subject to the uniform random sampling is more difficult to calculate because 4 sections were cut from the central part of the implant. Based on the block advance evaluation, for each section cut 493µm is removed so in total the central 1972 µm of the implant was cut into 4 sections (Fig. 18). As a first step, the arch length on both sides of the cylindrical implant must be calculated and applied to equation 1, in order to calculate the surface area of the cylinder subject to the uniform random sampling.
Together with the radius of the implant of 3 mm an isosceles triangle was made from which the angle A could be calculated (Fig. 18):

Equation 11

$$\sin A/a = \sin B/b$$

Equation 12

$$\sin A = (\sin 90° \cdot (1.972\text{mm}/2))/ 3\text{mm}$$

Equation 13

$$A = 19.2°$$

and since A is half of the total angle, then 38° is equivalent to the total length of the arch and entered into the equation for the length of the arch on one side. The length of the arch is:

Equation 14

$$L\_arch = (\text{circumference} \cdot (38 \cdot \pi/180°))/2\pi$$

where the circumference is 19 mm (equation 7), so the length of arch on one side is:

Equation 15

$$L\_arch = 2.01 \text{ mm}$$

The arches on both sides were together 4.02 mm long, from which the 4 sections were cut. The total area subject to uniform random sampling was then 4.02 mm·6 mm = 24 mm^2 (according to equation 1).
When Overgaard optimized the stereological method, he found that 80 µm (the height of the implant 10 mm · 2 sides · 4 sections) implant surface length was enough for estimating densities for various tissue types [152]. Of course, the estimate of sufficient implant surface length subject to analysis is dependent on the extent of the given tissue type of interest. The tissue type of interest should be represented by approximately 100-200 counts per specimen [150]. Therefore, the estimates should be extrapolated between studies with care.
The ratio between the two methods of the area subject to sampling was 113 mm^2 / 24 mm^2 = 4.7. In conclusion, the implant surface was 4.7 times more represented by the stereological cylindrical model than by the central sectioned stereological design. The cylindrical model sampled a total length of implant surface of 94 mm, which should be sufficient compared to the length found by Overgaard of 80 mm for providing estimates with only limited variation of data due to sampling method. On the other hand, the 48 µm implant surface length subject to analysis by the central sectioned VUR method may be too low, which may have impaired the quality of the data by increasing variation. However, this possible increase in variation did not prevent large differences from being identified as statistically significant in the study of the strontium-substituted bioactive glass coatings (III).

Conclusions of the stereology

- The VUR is difficult to apply to this specific implant model.
- For the central sectioned VUR method, a negligible bias is introduced concerning the volume densities.
- By use of the cylindrical model based method, a small estimated bias was introduced.
- The representation of the biological variation is 4.7 times higher for the cylindrical model based method than the central sectioned VUR method.
- For the cylindrical model based method, the gain in biological representation seems much greater than the relatively small bias introduced.
- For the cylindrical model based method, the implant surface length subject to estimation is found comparable to the recommendation.
- Overall the applied stereological methods seem valid for use in these studies.

STATISTICAL ANALYSIS
Statistical analysis was performed using Intercooled STATA 10.0 software (StataCorp LP, College Station, TX, USA).
Study I and II: In these paired studies, the histological and me-

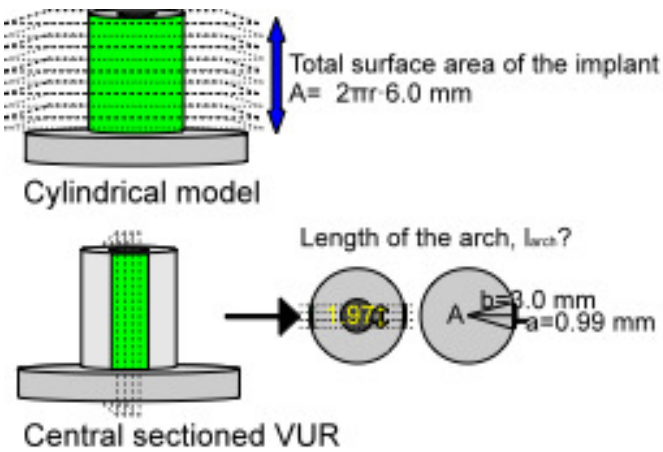

Figure 18
Size of the surface area, green, subject to sampling in the histomorphometrical analysis. For the central sectioned VUR technique, the length of the green arch must be calculated and then multiplied with the height of the cylinder.

chanical variable data were normally distributed. But the data of differences between treatment arms were not normally distributed for all variables. The histogram of the differences was skewed to the left and the effect measures show multiplicative behavior. Therefore, data were transformed by natural logarithm (ln) and found normally distributed on the natural logarithm scale. The differences between treatment arms were tested by student's t-test as the ratio of the paired data. Means and 95% CI of the t-test were transformed back by exponential function to medians and 95% CI, which are presented. Calculations of CV% for each variable was made via calculations of transformed mean and transformed standard deviation (sd) [154]:

Equation 16

$$\text{Mean} = \exp(\ln \text{mean} + (0.5 \cdot (\ln \text{sd})2))$$

Equation 17

$$\text{sd} = \text{mean} \cdot (\sqrt{} (\exp(\ln \text{sd})2 - 1)$$

Equation 18

$$\text{CV\%} = (\text{mean}/\text{sd}) \cdot 100\%$$

A P value less than 0.05 was considered statistically significant.
Study III: In this paired 4-arms study, histological and biomechanical data did not fulfil the assumptions for one-way repeated measurement ANOVA. Therefore, they were analyzed with a Friedman Repeated Measures Analyses of Variance on Ranks. When a statistically significant difference within the groups was detected, Wilcoxon signed rank test was used to identify the specific differences between two groups. The data were presented as medians with 75% and 25% interquartile ranges and p-values less than 0.05 were considered statistically significant.
The expected CVdiff % was set at 30 % and based on this expectation 10 animals were included in the studies. Unfortunately, in the strontium-doped bone graft extender study (II), the CVdiff % was often higher than 30%. Therefore, a true 30% improvement by strontium may not have been detected within the limitations of study II, a possible type 2 error.

RESULTS

STUDY I

Hypothesis: SrHA, strontiumhydroxyapatite, coating on Ti implants will enhance implant fixation both at 4 weeks and 12 weeks.
Hypothesis disproved: Yes
Comments: The surface area density fraction of ongrowth of bone

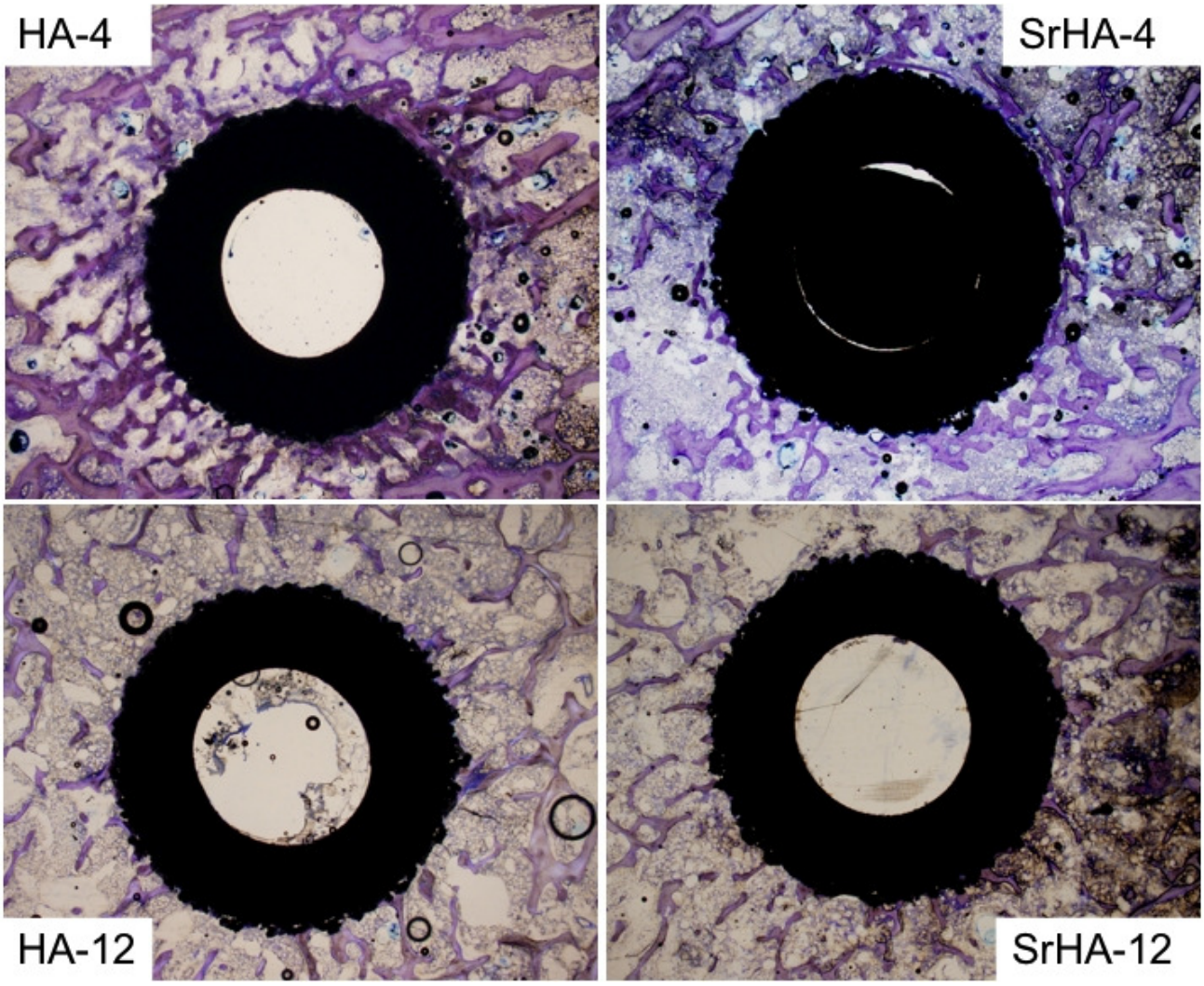

Figure 19
Gap healing and ongrowth of study I. These implants are representative of the medians for each group, but not from the same dog.

was as high as approximately 75% regardless of the strontium substitution of HA coatings. This fraction of ongrowth seemed even higher than observed in previous studies of HA coated implants in this model (Table 11 and Fig. 19). Scanning Electron Microscopy images of two pushed-out implants show bone on the HA coated implant but almost no bone on the SrHA coated implant.
In total, strontium substitution of the HA coating showed neither improvement nor impairment of any of the parameters for implant fixation or osseointegration.

Table 11
Results of study I presented as relative change to control. "0" no change.

	Coating treatment groups			
	HA 4 weeks	SrHA 4 weeks	HA 12 weeks	SrHA 12 weeks
Gap healing	0	0	0	0
Ongrowth	0	0	0	0
Implant fixation	0	0	0	0

STUDY II
Hypotheses: Strontium-doped HA as a bone graft extender mixed with allograft will enhance implant fixation.
Hypothesis disproved: Yes
Comments: Strontium doping increased the volume of new bone formed by 21%, which increased gap healing (Fig. 20). Strontium doping of the bone graft extender also preserved 18% more allograft in the gap (Fig. 21). Additionally, 39% more new bone was in contact with the strontium-doped BGE. However, the increased new bone formation had not yet reached the surface of the implant so ongrowth onto the implant was not increased (Table 9). Perhaps the implant fixation was not improved due to the lack of improved ongrowth onto the implant.

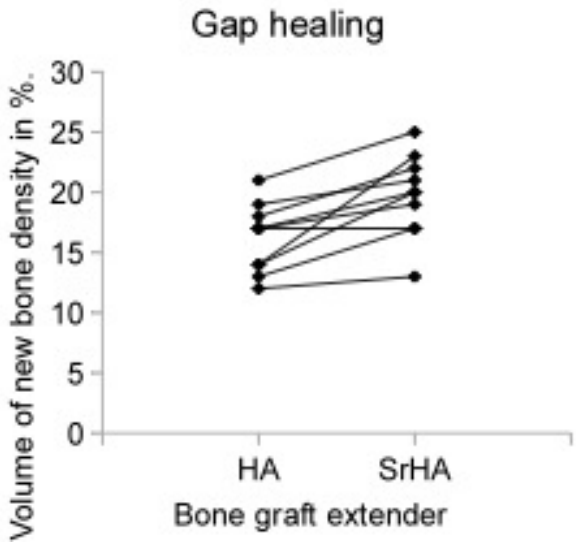

Figure 20
Fractions of new bone in gap of interconnected pairs.

Table 12
Results are presented as change relative to control. "0" no change. "+" improvement.

	Bone graft extender treatment groups	
	HA	SrHA
Gap healing	0	+
Preserved allograft	0	+
Ongowth	0	0
Implant fixation	0	0

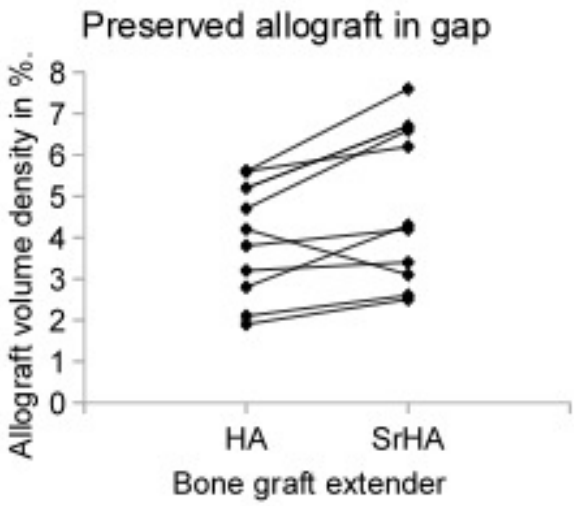

Figure 21
Fraction of allograft in the gap after 4 weeks of interconnected pairs.

STUDY III
Hypothesis 1: Bioactive glass coating of Ti implants will enhance implant fixation compared to HA coating.
Hypothesis 1 disproved: Yes
Hypothesis 2: Strontium-substitution of the bioactive glass coating on Ti implants will further enhance implant fixation compared to bioactive glass coating without strontium.
Hypothesis 2 disproved: Yes
Comments: The bioactive glass coating failed in achieving osseointegration and subsequent implant fixation (Table 13). Results of RAMAN spectroscopy suggest that the glass had become contaminated with aluminum. Therefore, the effects of strontium substitution of the glass on implant osseointegration and subsequent fixation could not be assessed. However interesting observations were made in the gap surrounding the implant and a desricptive study of the findings were performed. A homogeneous substance was observed, which could be divided into three sub-groups and the third group of the substance was mineralizes (Fig. 22).

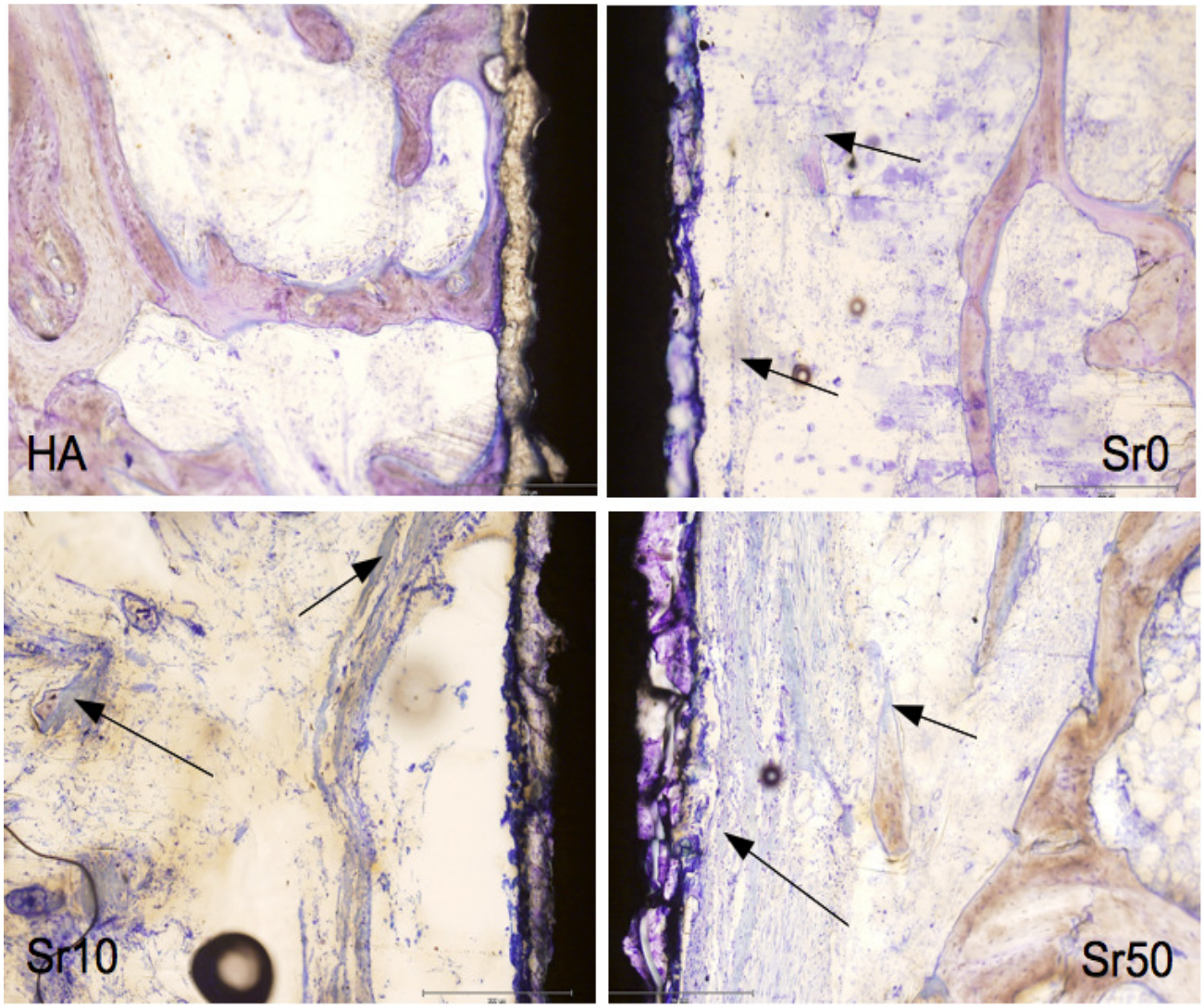

Figure 22
The HA coated implant was osseointegrated, but the implants with bioactive glass coatings were not (Sr0: bioactive glass without strontium; Sr10: 10% of the CaO2 is substituted by SrO2; and Sr50: 50% of the CaO2 is substituted by SrO2). In approximately half of the interfaces of each of the bioactive glass coated implants a homogeneous substance (arrows) was observed. The implants are representative of mean of each group but not from the same dog.

Table 13
Results of study III presented as change relative to control. "0" no change.

	Coating treatment groups			
	HA	**Sr0**	**Sr10**	**Sr50**
Gap healing	0	-	-	-
Ongrowth	0	-	-	-
Implant fixation	0	-	-	-

DISCUSSION

The purpose of the three studies performed in this dissertation was to investigate the effect of strontium topically present at the bone-implant interface. The two main questions asked were: first, what is the optimal delivery method of strontium to the bone-implant interface? Second, can strontium exercise a dual action in the bone-implant interface?

To elucidate answers to these questions, three studies were conducted where strontium was delivered to the interface by three different methods. In these three studies, different model setups were used to investigate both a possible anabolic effect and anti-catabolic effect of strontium. The methods used in the three studies were overall found to be valid for testing the hypothesis stated for each study. Therefore, the results of these studies are valid for interpretation and subsequently the stated hypotheses can be verified or disproven.

Strontium delivered to the interface by doping a HA bone graft extender produced a histological anabolic and anti-catabolic effect. However, mechanical implant fixation was not improved. These histological findings involving strontium are in agreement with the literature [98-100, 137]. The strontium-doped HA bone graft extender was of high crystallinity and of high HA purity. Therefore, only a small fraction of the bone graft extender material could have been of ß-TCP or amorphous calcium strontium phosphate compounds, which can more easily dissolve and release strontium ions into the bone-implant interface [37, 44]. Strontium must have been released into the interface milieu because more allograft was preserved when mixed with the

strontium-doped bone graft extender. The higher crystallinity and HA purity of the material the more osteoconductive it is [45]. Based on this, the non-doped HA bone graft extender was presumably a potent osteoconductive material. Still, strontium doping of the HA increased the bone ongrowth onto the granules. The increased ongrowth onto the SrHA may indicate an increase in the osteoconductive property of the HA by strontium doping. The increase in osteoconductive property of the SrHA may explain the increase in gap healing (increased volume of new bone).
Yet, when strontium was delivered as 5% strontium substituted HA by vacuum plasma sprayed coating, neither an anabolic nor an anti-catabolic effect was detected after 4 and 12 weeks. This delivery method may not be optimal because the highly crystalline and HA pure coatings are not easily dissolved. Therfore, strontium would not have been delivered to the interface but would have stayed within the coating. Interestingly, the SrHA used as a bone graft extender did exhibit the anabolic effect and also should be expected to become evident in the study using the SrHA coatings. The difference between these two studies is that the SrHA as a bone graft extender had a much larger surface area so even small amounts of released strontium may have added up to a significant total amount. Based on the SEM images of the implants after push-out, however, (Appendics, paper 1, Fig. 4 and 5) almost no bone was seen on the SrHA coating but plenty was left on the HA coating.
Therefore, I speculate that in the bone-implant interface of SrHA coated implants, a failure occured within the coating. For the HA coated implants, the failure happened in the surrounding bone. The tensile strength of the SrHA coating was not tested before implantation. The mechanical strength of the strontium-substituted coating could have been lower than the pure HA coating especially after 12 weeks in vivo because of a destabilized lattice structure [56, 57]. The destabilized strontium substituted HA more easily transformed into ß-TCP (ß-TCSrP) during the vacuum plasma spraying, was less crystalline and dissolved more easily [35, 58]. The possible tristrontiumcalciumphosphate or even amorphous strontium calcium phosphate compounds are likely to have formed a network of soluble material during the vacuum plasma spray coating. The soluble network would subsequently cause the coating to delaminate after 12 weeks in vivo when loaded during the push-out test. [155]. Li et al., enhanced the fixation of HA-coated Ti screws in osteoporotic rats by administrating systemic strontium [156]. Accordingly, perhaps it would be more optimal to deliver the strontium to the bone-implant interface by systemic treatment in large animals and humans. However, in many respects a topical delivery is optimal, since the most frequently reported side effects of systemic strontium administration by mouth are nausea and diarrhea, which could be avoided by topical treatment.
The third strontium delivery method to the bone-implant interface failed so its effects at the interface could not be investigated. Presumeably, the strontium substituted bioactive glass coating had become contaminated with aluminum during grit-blasting of the implant cores. The unintentionally present aluminum presumably changed the chemical properties of the glass, resulting in reduced, unexpected degradability [157]. Boyd and Towler successfully composed bioactive glass particles containing various amounts of strontium, which performs well in healthy and osteoporotic rats [115, 116]. Unfortunately, Lopez-Sastre et al. also unexpectedly found inferior osseointegration and mechanical fixation of bioglass coated implants compared to Apatite-Wollastonite-glass ceramic and TCP coated implants. So, it may not be very difficult to manufacture strontium containing bioactive glass but it is a great challenge to coat metallic implants with bioactive glass. Perhaps this is due to the glass being very chemical reactive and therefore difficult to control during the sintering process.
There are many delivery methods of strontium. Based on thermodynamic calculations, it has been postulated that strontium phosphate compounds, like strontium hydroxyapatite, are very readily formed and virtually non-dissolvable. Based on this, perhaps strontium in the form of strontiumacetate is a better delivery method due to this compound's increased solubility.. A study by Gentleman et al. rejects the hypothesis that strontium phosphate compounds are virtually non-dissolvable since they found strontium ions released into a media with high phosphorus concentration [158]. Clearly, further investigation into the most optimal method of strontium delivery is needed.

CONCLUSION

Strontium showed potential to work as a dual acting agent in the bone-implant interface. The dual acting effect of strontium became evident in my study using a strontium-doped bone graft extender. Strontium doping of the bone graft extender increased the volume of new bone as well as the volume of the remaining allograft compared to the control without strontium doping. However, strontium doping did not improve mechanical implant fixation.
The dual acting effect of strontium did not become evident in the two studies using strontium-substituted coatings. Strontium substituted HA VPS coating neither showed improvement nor impairment of implant fixation. No difference between the strontium substituted coating and the control HA VPS coating was detected for any of the sub-hypotheses at any of the time points. In the third study testing strontium-substituted bioactive glass coating, deterioration in implant fixation was observed for all glass coatings regardless of the doses of strontium substitution. It was presumed that the glass had been contaminated with aluminum during the coating procedure.
These studies show that strontium can work as a dual acting agent in the bone-implant interface yet the delivery of strontium to the interface is still a challenge.

PERSPECTIVE AND FUTURE RESEARCH

Despite the interesting results of these studies, there are still many uncertainties in regard to the effect of strontium in the bone-implant interface and the optimal method of strontium delivery to the interface. Since strontium showed both an anabolic and anti-catabolic effect in the interface of an allograft impacted implant, it is encouraging to pursuit the idea of adding strontium to the interface.
Clearly, further studies must be conducted to determine how strontium works in the interface to influence the osseointegration of the implant.
Most importantly, the delivery methods of strontium need study and attention. The mechanical strength of strontium-substituted HA VPS coatings must be determined before this delivery method can be considered for future in vivo investigations and at different strontium substitution doses. Moreover, SrHA may not be the optimal means of strontium delivery since SrHA does not dissolve very well, consequently, only sparse amounts of strontium would be delivered to the interface.
On the other hand, delivery of strontium to the interface by a strontium-substituted bioactive glass coating could still have potential for success, since the presumed aluminum contamina-

tion in our study likely led to the decrease in degradability and the osteoproductive property of the glass. Thus, future studies of this bioactive glass should: first, investigate the in vitro and in vivo effects of not grit blasting before coating, second, use silica or other aluminum-free particles rather than alumina for grit blasting, or third, clean the surface after grit blasting using hydrofluoric acid, for example, prior to glass coating of the implants. Additionally, it should be tested whether strontium in the bone-implant interface only benefits a sub-group of all patients, such as osteoporotic patients receiving a joint replacement.

PREFACE

The three studies of this PhD dissertation were conducted at the Orthopedic Research Laboratory, Department of Orthopedics, Aarhus University Hospital, Denmark and at the Orthopedic Biomechanics Laboratory, Excelen Center for Bone and Joint Research and Education, Minneapolis Medical Research Foundation, Minneapolis, MN, USA.

The Danish Rheumatism Association, P. Carl Petersens Foundation, and Aarhus University financed my research fellowship.

The experimental surgeries and animal care were carried out at the Orthopedic Biomechanics Laboratory, Minneapolis. Specimens were then shipped to the Orthopedic Research Lab at Aarhus University, where all analyses were performed.

Prof. Kjeld Søballe introduced me to research in year 2000, where I conducted a Pre-graduate research diploma on studies initiated by him. I am very thankful to Prof. Kjeld Søballe for all the support and possibilities he has afforded me. He also introduced me to the late Prof. Flemming Melsen, who taught me bone biology, and Assoc. Prof. Joan E. Bechtold. Assoc. Prof. Joan E. Bechtold has been very valuable to me as both a collaborator and friend and has made it possible for us to perform the experimental surgeries in Minneapolis. I am in great debt to my co-supervisors, Ellen-Margrethe Hauge and Jørgen Baas, who have willingly held endless discussions with me on various academic subjects but also in matters of my career and general life.

ACKNOWLEDGEMENTS

These studies were supported financially by the following non-commercial institutions and funds:
Danish Rheumatism Association
P. Carl Petersens Fond
Aarhus University, Denmark
NIH AR42051
The A.P. Moeller Foundation for Advancement of Medical Science
The studies have benefitted from an institutional research grant from Bioceramic Therapeutics Ltd, which holds a patent for the bioactive glass coating.
The following companies supported the studies with materials:
Osteologix Aps: SrHA and HA powder, study I and II.
Medicoat AG: VPS coating of SrHA and HA, study I.
Bioceramic Therapeutics Ltd: Bioactive glass coating and HA coating, study III.
DePuy Inc: Porous coating, study II.
During the course of this PhD study I have been involved in research, which has received institutional support from the following companies with commercial interest in orthopedics:
DePuy Inc.

SUMMARY

Total hip replacement surgery is being performed on an increasingly large part of the population and at increasingly younger age. Because we live and stay physically active longer, and since hip replacement surgery has become quite successful, the treatment is being offered to progressively more patients.

Unfortunately, about 17% of hip replacement surgeries currently involve revisions. Consequently, the longevity of both the primary and revision implant is an issue and warrants further investigation.

Implants undergoing early instability or even subsidence correlate with an increased risk of aseptic loosening, subsequently requiring revision. Thus, the goal is early fixation by osseointegration of the implant. For revision implants, this is an even greater challenge since an allograft is often needed during surgery to obtain immediate stability of the implant. Bone grafts are rapidly resorbed. Thus, instability of the prosthesis may develop before new bone formation is well established and can mechanically secure the prosthesis.

Strontium is a dual action drug; being both bone anabolic and anti-catabolic. In the form of strontiumranelate, it is used in the treatment of osteoporosis. Strontium may potentially improve the early osseointegration and fixation of implants.

This dissertation consists of three studies investigating the effect of strontium at the bone-implant interface. The questions were firstly, what is the optimal delivery method for strontium to the interface, and secondly, can strontium exercise its dual action at the interface? The studies were performed in a cementless, experimental gap model in canine. The effects of strontium were evaluated by histomorphometrical analysis of the osseointegration and mechanical push-out test of implant fixation. Different stereological methods were used for the histomorphometrical analysis of each study. The methods used were reviewed critically and found valid.

Study I compared a 5% strontium-substituted hydroxyapatite (HA) coating with an HA coating after 4 weeks and 12 weeks observation time. We examined whether fixation of the implant was improved by the strontium substitution. It was found that fixation of the implant was not improved by the strontium substituted HA coating at any of the two time points.

Study II compared a 5% strontium-doped HA bone graft extender with a HA bone graft extender. The bone graft extender was mixed with allograft and impacted around a titanium implant. The objective of this study was to determine whether strontium doping of the bone graft extender could protect the allograft from fast resorption and increase gap healing, leading to the improved fixation of the implant. We found that the strontium doping increased gap healing and protected the allograft, however, results of the mechanical test were inconclusive. The reason might have been that the increased gap healing had not yet reached the implant during the 4 weeks observation time, so ongrowth onto the implant was not improved.

Study III investigated the effects of bioactive glass coating with a 0%, 10% or 50% strontium-substitution versus HA coating of grit-blasted titanium alloy implants. The goal was to determine whether fixation of the implant would be improved by the bioactive glass coating, and then further improved by the strontium-substitution of the coating in a dose-dependent manner. Unfortunately, the bioactive glass coating failed, presumably due to aluminum contamination originating from the grit-blasting powder. The HA coated implants were superior in all parameters of osseointegration and the mechanical fixation of the implants.

These studies show the importance of performing further experimental investigation. Even when investigating a known agent for use in a new application. Strontium delivered as doping of a HA bone graft extender showed potential as a dual acting agent in

the interface. However, delivery methods of strontium to the bone-implant interface clearly need further investigation.

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