On the biological response to different biomaterials under normal and irradiated conditions

Clinical and experimental studies

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To my wonderful loving parents, who have always supported me

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ABSTRACT

There are several challenges when introducing an implant into a host. Concerning soft tissues, major complications are fibrosis and capsule contracture, whereas for bone, failure of osseointegration may occur. In addition to the properties of the biomaterial, compromising conditions of the host can influence the outcome, and in the case of irradiation, the risk increases for both capsule contracture and failed osseointegration. To improve the success of implants, increased knowledge about the mechanisms of integration is needed.

In Study II, *in vitro*, isolated human mononuclear cells showed increased adhesion to ultrathin amorphous hydroxyapatite (HA)-coated titanium (Ti) and increased secretion of the proinflammatory cytokine TNF-α compared to crystalline HA. No difference between Ti and HA was observed. Study I, *in vivo*, showed no difference between the HA surfaces, but Ti provoked a more intense inflammatory response in regard to HA. The materials were the same in Studies I and II and had similar microscale topography (Study I: S_a 0.24-0.26 µm; Study II: S_a 0.23-0.3 µm), while amorphous HA was the least hydrophilic (Study II: Ti 60.9°, crystalline HA 58.2°, amorphous HA 89.0°). In Study III, the expression of inflammatory and fibrogenic molecular markers was determined after the insertion of silicone implants in irradiated and nonirradiated human soft tissue. Downregulation of IL-8 and upregulation of BCL-2 were detected in the peri-implant tissue in the irradiated side compared with the nonirradiated side. The antifibrotic transcription factor FOXO1 was downregulated in implant-adherent cells on the irradiated side. Correlation and regression analyses showed that irradiation dose and time since irradiation as well as chemotherapy and anti-neutropenic drugs influenced the gene

expression response, both in irradiated and nonirradiated sides. The results of Study IV demonstrated that pre-exposure to irradiation significantly reduced bone-implant contact and implant removal torque in the recipient bone. The irradiation-induced detrimental effects on osseointegration were associated with high expression of proinflammatory TNF-α and osteoclastic CatK and reduced expression of bone formation gene ALP in the implant-adherent cells, in parallel with high expression of the inflammatory cell recruiter MCP-1, proinflammatory TNF-α and pro-fibrotic TGF-β genes in the peri-implant soft tissue. All compartments around the implant in the irradiated site revealed reduced expression of FOXO1.

It is concluded that under normal experimental conditions, titanium and ultrathin coated HA are associated with cytocompatibility, biocompatibility and a transient inflammatory process, although differences in surface chemistry, nanotopography and hydrophilicity/hydrophobicity can alter the cellular response. In contrast, irradiation of soft and hard tissues causes dysregulation of biological activities in the different tissue compartments around the implant, of which perturbed inflammation and profibrotic propensity seem to hamper tissue healing and regeneration around implanted biomaterials.

Keywords: biomaterials, bone, fibrosis, hydroxyapatite, inflammation, irradiation, osseointegration, silicone, soft tissue, titanium

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SAMMANFATTNING PÅ SVENSKA

Biomaterial används idag i stor utsträckning bland annat som implantat och även om resultaten generellt sätt är mycket bra finns det flera utmaningar med inläkningen av implantat. När det gäller komplikationer är fibros och kapselkontraktur förekommande i mjukvävnad medan utebliven eller förlorad benförankring kan ske i ben. Förutom biomaterialets egenskaper kan mottagande vävnad påverka hur väl implantatet integreras, exempelvis innebär strålning i samband med cancerbehandling en ökad risk för både kapselkontraktur och försämrad benförankring. För att förbättra inläkningen av implantat behövs därför ökad kunskap om mekanismerna kring integration.

Studie II, *in vitro*, visade att isolerade humana mononukleära celler i högre grad adhererade till ultratunn amorf hydroxyapatit (HA)-belagd titan (Ti) och frisatte mer av den proinflammatoriska cytokinen TNF-α jämfört med kristallin HA. Ingen skillnad kunde observeras mellan Ti och HA-ytorna. Studie I, in vivo, visade ingen skillnad mellan HA ytorna medan Ti ledde till en mer uttalad inflammatoriskt reaktion i jämförelse med HA ytorna. Materialen var desamma i Studie I och II och hade liknande topografi (Studie I: S_a 0.24-0.26 µm; Studie II: S_a 0.23-0.3 µm), medan amorf HA var minst hydrofil (Studie II: Ti 60.9°, kristallin HA 58.2°, amorf HA 89.0°). I Studie III, analyserades genuttrycket av inflammatoriska och fibrotiska markörer som svar på silikon implantat i strålad och icke-strålad human mjukvävnad. En nedreglering av IL-8 och uppreglering av BCL-2 observerades i mjukvävnaden kring implantaten i strålad vävnad. Den antifibrotiska transkriptionsfaktorn FOXO1 noterades däremot vara nedreglerad i implantat-adherenta celler i strålad vävnad. Korrelations- och regressionsanalyser visade att strålningsdos och tid efter strålningen, liksom kemoterapi och behandling mot neutropeni, påverkar genexpressionen både i strålad och icke-strålad vävnad. Resultaten i Studie IV visade att tidigare strålning reducerade ben-implantat kontakten samt vridmomentet för avlägsnande av implantatet i ben. Strålningens negativa effekter på benförankringen var associerade med högt genuttryck av proinflammatoriska TNF-α och osteoklastiska CatK och reducerat uttryck av benbildande ALP i implantat-adherenta celler, parallellt med höga uttryck av en markör för cellrekrytering, MCP-1, proinflammatoriska TNF-α och pro-fibrotiska TGF-β i mjukvävnaden kring implantaten. Alla analyserade vävnadskompartment kring implantaten uppvisade reducerat uttryck av FOXO1 när strålning förekommit.

Sammanfattningsvis visar avhandlingen att under normala experimentella förhållanden så är titan och ultratunn HA associerade med cytokompatibilitet,

biokompatibilitet och en övergående inflammatorisk process medan skillnader i ytkemi, nanotopografi och hydrofili/hydrofobicitet kan påverka cellsvaret. Däremot orsakar strålning av både mjukvävnad och ben en dysreglering av biologiska processer i olika vävnadskompartment runt implantet varav störd inflammation och profibrotisk benägenhet verkar hindra läkning, benregeneration samt osseointegration av implantatet.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Rydén, L., Molnar, D., Esposito, M., Johansson , A., Suska, F., Palmquist, A., Thomsen, P. 2013. Early inflammatory response in soft tissues induced by thin calcium phosphates. Journal of Biomedical Materials Research - Part A 101 A(9), pp. 2712-2717

II. Rydén, L., Omar, O., Johansson, A., Jimbo, R., Palmquist, A., Thomsen, P. 2017 Inflammatory cell response to ultra-thin amorphous and crystalline hydroxyapatite surfaces. J Mater Sci Mater Med. Jan;28(1):9, Epub 2016 Nov 28.

III. Rydén, L., Zdolsek, J., Johansson, A., Emanuelsson, L., Omar, O., Palmquist, A., Thomsen, P. Early inflammatory reaction to clinically used silicone implant material in irradiated and non-irradiated tissue: A clinical study – Submitted

IV. Omar, O., Rydén, L., Rahman Wamied, A., Al-Otain, I., Abdullah Alhawaj, H., Abuohashish, H., Al-Qarni, F., Johansson, A., Emanuelsson, L., Palmquist, A., Thomsen, P. Impact of irradiation on molecular, histological, and biomechanical determinants of osseointegration: An experimental study - In manuscript

CONTENT

ABBREVIATIONS

ALP	alkaline phosphatase
BA	bone area
BIC	bone-implant contact
BMP-2	bone morphogenetic protein-2
BCL-2	B-cell lymphoma 2
CatK	cathepsin K
COX-2	cyclooxygenase 2
ECM	extracellular matrix
FBGC	foreign body giant cell
FGF	fibroblast growth factor
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FOXO1	forkhead box protein O1
HA	hydroxyapatite
IL - 8	interleukin 8
IL - 10	interleukin 10
LD	lactate dehydrogenase
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
OC	osteocalcin
OPG	osteoprotegerin

PAA	poly(acrylic acid)
PDGF	platelet derived growth factor
PDMS	polydimethylsiloxane
PGA	poly(ethylene glycol)
PMN	polymorphonuclear neutrophils
RANKL	receptor activator of nuclear kappa B ligand
RNA	ribonucleic acid
ROS	reactive oxygen species
Runx2	runt-related transcription factor 2
SEM	scanning electron microscopy
TGF β	transforming growth factor β
TNF- α	tumour necrosis factor α
TRAP	tartrate-resistant acid phosphatase
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 BIOMATERIALS AND THEIR PROPERTIES

There are several definitions of biomaterials, but the essence is that biomaterials are natural or synthetic materials used in direct or indirect contact with tissue. Furthermore, a requirement for all biomaterials is that they should be biocompatible. The most commonly used definition of biocompatibility is as follows:

The ability of a material to perform with an appropriate host response in a specific application (1)

A more practical approach is presented by the ISO standards on biocompatibility, which specifies tests and requirements that must be met. Among these tests are screens for genotoxicity, carcinogenicity, reproduction toxicity, cytotoxicity, irritation, sensitivity and sterilization agent residues, local effects, etc.

In addition, the local effects should be a normal foreign body reaction, which is described as the development of a thin though avascular capsule and a modest local reaction (2). The most important factor determining the local effect of a biomaterial is its surface characteristics, which include its chemistry, topography, wettability, surface energy and stiffness.

1.1.1 BIOMATERIALS

Titanium

Titanium (Ti) is a metal that comes in four different commercial pure (CP) grades as well as in different alloys, of which Ti-6Al-4V is the most widely used within medicine. Due to its moderate elastic modulus (106 GPa for CP Ti and 112 GPa for Ti-6Al-4V), stress shielding is reduced compared to other metals, such as stainless steel (193-200 GPa) and cobalt-chromium (210 GPa), which is an advantage when using this material in bone. Titanium is considered relatively inert due to its thin oxide layer; titanium dioxide TiO_2, contributes to its high corrosion resistance and biocompatibility. Although regarded as highly inert, this material has the ability to interact with bone and is regarded as osteoconductive (3). Although the oxide layer is hydrophilic, contamination from inorganic anions and organic hydrocarbons in combination with cleaning and certain sterilization methods increases the hydrophobicity of titanium (4-

6). With chemical alterations, such as fluoride modification, nitrogen treatment or oxygen plasma cleaning, surfaces can be made more hydrophilic (7, 8). Modifications of the titanium surface both in terms of its chemistry and topography have been widely studied, primarily with the purpose of optimizing osseointegration. An extensively investigated chemical modification of titanium is coating with calcium phosphate (9).

Calcium phosphate

The calcium phosphate family consists of various variations, of which the most widely used biomaterials are tricalcium phosphate (TCP), biphasic calcium phosphate (BCP) and hydroxyapatite (HA).

Calcium phosphates are classified as ceramics due to their hard and brittle nature and non-metallic origin. Being a brittle material, calcium phosphate is generally restricted to non-loadbearing applications or as a coating on implants (10-14). Since the main component in bone tissue is calcium phosphate, it is possible for this material to bond to bone and it may also be osteoinductive. Among the calcium phosphates, hydroxyapatite (HA) has the most similar composition to bone, with a Ca/P ratio of 1,67, and has been widely studied. The thickness of the HA coating has been under investigation, and thin coatings have been found to remain stable during the process of bone mineralization while thicker coatings might crack and result in alteration of the implant topography as well as release of HA particles, which could alter the biological reaction (15, 16). Furthermore, thin coatings have been shown to possess the same osteogenic capacity as thicker coatings (11).

Silicone

Silicones include a group of synthetic polymers consisting of repeating silicon and oxygen. Among the silicones, polydimethylsiloxane (PDMS) is the most widely used biomaterial, and it has methyl groups bound to silicon with the dimethylsiloxane oligomers crosslinked together. Curing will, however, not be 100%, leaving oligomers that are not crosslinked and can migrate to the surface, affecting the surface characteristics (17).

Regarding their material properties, silicones are optically transparent, viscoelastic, hydrophobic, and stable from a chemical and thermal perspective as well as having a low elastic modulus and a low surface tension.

An advantage of silicones is that they can be produced in various forms, such as fluids, emulsions, compounds, resins, and elastomers. However, the

additives and processing can affect the properties of the material (18). These treatments are, for example, plasma etching and the addition of PEG, which makes the silicones more hydrophilic to facilitate cell adhesion (19, 20).

1.1.2 SURFACE CHARACTERISTICS

The surface topography has been shown to be of importance both on the micro- and nanoscale. Microscale roughness was the first to be explored and has been shown to be able to promote bone formation in a positive way by influencing the cell shape and expression of various mediators (21, 22). Microscale topography has also been reported to reduce the incidence of capsular contracture compared to a smooth surface (23, 24). Unfortunately, some textured silicone implants have been associated with a specific type of lymphoma, (anaplastic large cell lymphoma, ALCL), indicating that the rough texture is able to influence cell changes (25). There are also reports that the micropattern is important since it has been shown to influence the phenotype of macrophages and the inflammatory response (26, 27).

Nanoscale topography has also been associated with improved bone formation through decreased osteoclast differentiation as well as better adherence and proliferation of osteoblastic cells (28, 29). Since both nano- and microscale modifications appear to influence cell behaviour, several studies describe a combination of these modifications to promote osseointegration (30, 31).

Chemistry is of major importance for the host response, as observed by several studies, which show that some materials are more prone to provoking an intense inflammatory reaction and fusion of macrophages to giant cells (32-35).

Wettability has proven to be of importance for the adherence, spreading and migration of cells (36, 37). There are also reports that more hydrophilic surfaces downregulate proinflammatory cytokines and promote bone formation (38, 39). The wettability is also correlated with the surface energy and the roughness of the surface, with roughness enhancing hydrophobicity in general (40). Higher surface energy and hydrophilicity have been shown to promote cell adhesion (41).

Another property that has been shown to be important to the cellular response is the stiffness of the biomaterial (42). Integrins, which are cell adhesion receptors, bind to the surroundings, including biomaterials, and depending on the stiffness, the cells then behave differently. There are reports of neurons forming more branches on softer material, mesenchymal stem cells producing myogenic markers when cultured on stiff material and on even stiffer material

early osteogenic markers were expressed (43, 44). The differentiation of fibroblasts into myofibroblasts has been shown to be facilitated when cultured on a stiffer material, which could partially explain some diseases (45).

1.1.3 CLINICAL APPLICATIONS OF BIOMATERIALS

The applications of biomaterials can be divided into two groups in relation to the tissue in which they will be used. A suitable grouping would be soft tissue and bone applications since the requirements regarding the material properties differ considerably between these tissues.

Regarding soft tissue applications, there are both external and internal applications. Among the external applications are wound dressings, catheters and contact lenses, while examples of internal applications are intraocular lenses, breast implants, facial implants, vascular grafts, cochlear implants, pacemakers and sutures. The most common biomaterial used for soft tissue applications is silicone (PDMS), which can be manufactured to mimic the softness of the tissue in which it is placed. Silicone (PDMS) has been shown to be successful as a breast implant, contact lens, catheter, facial implant, and wound dressing (46-50). Other common biomaterials with soft tissue applications are polyurethane for catheters, polyethylene for facial implants, lactide and glycolide copolymers as well as polyesters for sutures, polyethylene terephthalate (PET) and expanded polytetrafluoroethylene (ePTFE) for vascular grafts (see Table 1)(49, 51-53).

The most common complication with soft tissue implants is capsular contracture, the genesis of which is unknown. The problem is widely described with breast implants, and although various treatments have been suggested, none is generally accepted. Another complication is bone resorption when using chin implants, although the incidence is reduced by the supraperiosteal placement of the implant (54, 55). There is also a general risk of infection, seroma, displacement and extrusion of the implant (49).

Table 1.

Soft tissue	
Contact lenses	Poly (methyl metacrylate) (PMMA), silicone hydrogel, poly(2-hydroxyethyl metacrylate) so called poly-HEMA, polyvinyl (PVA)
Breast implants	Silicone (PDMS), polyurethane
Wound dressing	Silicone, poly (vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), methylcellulose, pectin, gelatin, polyisobutylene, polyurethane, alginate,
Vascular grafts	Polyethylene terephthalate (PET), expanded polytetrafluoroethylene (ePTFE)
Sutures	Polyesters, polydioxanone, poly (lactic-co-glycolic acid), synthetic polyester-glycolide, caprolactone, trimethylene carbonate and lactide, copolymer of glycolide and epsilon-caprolactone
Catheters	Silicone, polyurethane
Facial implants	Silicone, polyethylene
Bone	
Hip prosthesis	Titanium (Ti-6Al-4V), stainless steel, Co-Cr-alloy, polyethylene, polymethacrylic acid (PMMA), alumina, zirconia, hydroxyapatite
Knee prosthesis	Titanium (Ti-6Al-4V), Co-Cr-alloys, polyethylene, alumina, zirconia, hydroxyapatite
Cranioplasty	Titanium (Ti-6Al-4V), polyether ether ketone (PEEK), acrylics (PMMA), polyethylene, bioceramics
Dental implants	Titanium (mostly CP Ti), hydroxyapatite, zirconia

Regarding applications of biomaterials in bone, the major areas of use are within orthopaedics, such as plates to stabilize fractures, hip and knee prostheses, within dentistry as dental implants, and within neurosurgery/maxillofacial surgery as cranio- and maxillofacial implants. For hip and knee prostheses, combinations of metals, ceramics and polymers are used to take advantage of the different material properties and minimize wear debris, which otherwise can create local and systemic effects (56, 57). A significant improvement in quality of life is described with hip and knee prostheses, and the long-term results are usually very good (58, 59).

For dental implants, mostly CP titanium is used with or without HA coating. The success rate regarding dental implants, including in elderly patients and in type II diabetes patients, has been reported to be excellent (60, 61). Additionally, within cranioplasty, titanium mesh or plates are widely used, as is polyetheretherketone (PEEK), which has shown similar results (62).

The most frequent complications with bone implants are implant failure, loosening, wear debris and infection (63). With hip implants, dislocation is the far most common complication, followed by aseptic loosening and infection (64). One of the most frequent complications with dental implants is peri-implant mucositis, while bone loss, which jeopardizes the implant, has been reported to be more infrequent (61).

Compromised biology

A special challenge regarding the use of biomaterials as implants is when the tissue has undergone irradiation. Important factors related to the risk of complications due to irradiation are the radiation dose, number of fractions and time between irradiation and surgery (65-68). The type of radiation used in radiotherapy is mostly electromagnetic radiation, which includes X and gamma rays (69).

When radiation precedes or is performed postoperatively in relation to implant breast reconstruction, there is an increased risk of complications such as skin flap necrosis, infection, explant and capsular contracture compared to the absence of radiation (70). Regarding dental implants, a significantly higher rate of soft tissue complications, dehiscence of mucosa, local infection and delayed wound healing have been observed after radiation (71). Reports on the success rate of dental implants in radiated and non-irradiated head-neck cancer patients have shown the failure rate to be significantly higher among patients receiving radiation therapy (72). The failure of dental implants in radiated tissue is primarily explained by failed osseointegration and peri-implantitis (71).

Furthermore, the failure of implants in radiated tissue continues to be high even long after radiation therapy (72).

1.2 THE TISSUES OF THE HOST

1.2.1 SOFT TISSUE

Soft tissue includes a variety of tissues, such as muscles, skin, adipose tissue, vessels, nerves, and lymph vessels. The two main tissue components are cells and extracellular matrix (ECM). In each specific tissue, there are specialized cells that provide the tissue with its characteristics, such as adipocytes in adipose tissue, myocytes in muscles, and chondrocytes in cartilage. The cells are situated in the ECM, which acts as a scaffold for the cells to grow in, but it also regulates cellular processes such as growth, differentiation and migration. The main components of the ECM are water, collagen, glycoproteins (elastin, fibronectin, and laminin), proteoglycans and various mediators, with the composition depending on the specific tissue (73). For instance, in adipose tissue, the ECM consists of collagen I, IV and VI, laminin, elastins, fibronectins and glycosaminoglycans (GAGs) and growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth hormone ß (TGF-ß)(74).

Tissues undergo continuous remodeling through degradation and subsequent regeneration. The degradation is achieved by the actions of proteinases, among which metalloproteinases have a prominent role (75). Regeneration is facilitated by the actions of growth hormones bound to the ECM, such as FGFs (75). There is a balance between degradation and regeneration, which, if disturbed, could result in fibrotic diseases or cancer (76, 77).

Soft tissue response to biomaterials

Introducing a biomaterial into soft tissue will activate several biological processes. One of the first systems to be activated is the coagulation system, which limits bleeding due to surgery. Other processes, such as the complement and fibrinolytic systems, will also be activated, which are part of the immune defence and controls the dissolution of clots, respectively. The inflammatory process has a significant impact on the integration of the biomaterial. As the biomaterial is exposed to the blood, a provisional matrix will form initially as a blood clot consisting of platelets, fibrin, and fibronectin (78). Depending on the properties of the biomaterial, the proteins absorbed on its surface will vary, which in turn determines which cells will adhere to the protein layer and subsequently decide the soft tissue response.

Various modifications have been made to biomaterial surfaces to improve their integration. Coating with various proteins, such as fibronectin, collagen and albumin, has been shown to promote the differentiation of human preadipocytes to adipocytes and has been suggested as a scaffold for adipose tissue graft transplantation (79). Other coatings are, for instance, polymers such as PEG coated on PDMS, which resulted in an anti-proliferative effect on fibroblasts, while adding PDMS to vascular grafts resulted in a decrease in platelet adhesion, indicating improved haemocompatibility (80, 81). When coating hydroxyapatite on titanium, improved connective tissue attachment was observed in humans (82). Similar observations were reported for titanium, to which connective tissue attachment increased with increased surface roughness, which is supported by the finding of better adhesion of fibroblasts than epithelial cells to rougher surfaces (83-85). The growth of epithelial cells has, on the other hand, been reported to improve when cultured on PDMS with a microscale pattern (86). Furthermore, combining micro- and nanoscale patterns on PDMS has been reported to stimulate human adipose stem cells to undergo tenogenic differentiation (87). Microscale rough silicone breast implants have also been associated with a very rare lymphoma indicating the topography's influence on the immune system (88, 89).

Inflammation

Inserting a biomaterial will, like all trauma, trigger an inflammatory response in the host. During the acute inflammatory phase, chemoattractants such as platelet derived growth factor (PDGF) and TGF-β1 released from platelets within the provisional matrix will attract inflammatory cells, initially primarily neutrophils (polymorphonuclear cells, PMNs), to the site of the biomaterial (90, 91). These cells will be joined by monocytes in abundance, and they have the ability to differentiate into macrophages, which are the cell type that characterizes the chronic inflammation phase. Both neutrophils and monocytes/macrophages are able to adhere to the adsorbed protein layer on the biomaterial through adhesion receptors (92). The soft tissue inflammatory response has been shown to differ significantly between materials; for instance, both copper and gold have been shown to elicit significantly stronger inflammation than titanium (32, 93). When comparing silicone with polyurethane (PU) cultured with human monocytes, silicone reportedly induced a generally more intense inflammatory response (94). Furthermore, the addition of PEG to PU increased the anti-inflammatory response (95).

It has been proposed that the biological response caused by macrophages depends on the balance between two subtypes of macrophages, classically (M1) or alternatively activated macrophages (M2) (96). The activation of

macrophages along the classical pathway is considered to result in the release of proinflammatory cytokines, whereas activation along the alternative pathway results in the release of anti-inflammatory cytokines and is considered to facilitate wound healing and tissue integration (97). There are several reports that different topography, both the pattern and the distance between the pattern, influences the chosen pathway of the macrophages, their mediator release and their morphology (26, 27, 98). A study comparing roughness and HA coating showed that within an interval of microscale roughness (S_a=0,66-2,91 um), more implant-adherent macrophages were activated through the alternative pathway, resulting in more M2 macrophages, whereas in the presence of HA coating, a hybrid macrophage subtype was detected that had both M1 and M2 characteristics (99). Whether this difference in the macrophage phenotype has any implication for the inflammatory response is inconclusive in the literature, and there are reports of HA eliciting less inflammation than titanium (100, 101).

In addition to topography and chemistry, wettability has been shown to influence the inflammatory response, and the combination of a rough and hydrophilic surface resulted in the highest number of M2 macrophages (8).

The main role of both neutrophils and monocytes is to destroy any foreign material through phagocytosis. Free biomaterial particles can therefore be engulfed by these cells and provoke different responses. Depending on the phenotype of the macrophage, the inflammatory response will differ when exposed to microscale titanium particles, with M1 macrophages eliciting TNF-α and IL-8 (102). Microscale titanium dioxide particles were shown to induce the M1 phenotype, which was not observed for nanoscale particles (103). A similar finding was described for HA particles with a maximum TNF-α secretion observed for particle sizes of 1-2 μm (104).

Fibrosis and capsule formation

With the persistent presence of a foreign body such as a biomaterial, the macrophages that have adhered to the biomaterial surface can fuse and form foreign body giant cells (FBGCs) in a further attempt to destroy the foreign material. Factors that promote the formation of FBGCs are IL-4 and IL-13, which are also associated with the switch in phenotype to anti-inflammatory or alternative macrophages promoting fibrosis. The FBGC will secrete enzymes and create an acidic environment to degrade the material, which can result in implant failure. It has been shown to make a difference in the number of FBGCs depending on the material, topography and also that fewer FBGCs adhere to hydrophilic non-ionic surfaces (34, 105, 106). In addition, FBGCs

release mediators that are initially proinflammatory cytokines, such as IL-1beta and IL-8, but over time, they release profibrotic TGF-ß and IL -10 (34). When the foreign body persists, the process of shielding it off begins with the formation of granulation tissue, which will mature into a fibrous capsule. Fibroblasts have an important role in this process since they are one of the major cells in soft tissue and are responsible for ECM formation by secreting fibrous molecules such as collagen. Fibroblasts have been shown to be susceptible to the chemistry of the surface as well as the topography in regard to their motility and proliferation (107, 108). Depending on the biomaterial, the fibrous capsule can be more or less pronounced. For instance, in the case of copper, a thicker fibrous capsule has been described when compared to titanium (32, 33). When titanium was coated with HA, an even thinner capsule was described (109). Fibroblasts grown on silicone with different topography showed the best proliferation, attachment and reduced initial foreign body reaction on silicone modified to resemble an acellular dermal matrix (110). Under the stimuli of TGF-ß and PDGF, fibroblasts can differentiate into myofibroblasts with contractile ability. A great deal of research has focused on how to suppress capsular contracture, and there are various suggestions, such as multi-layered micropatterns with poly-L-lysine and hyaluronic acid or the addition of PAA micropatterns to silicone, which have been shown to reduce the number of fibroblasts and myofibroblasts and the capsular thickness (111, 112). In addition to fibroblasts and myofibroblasts, there have been reports that adipocytes can express profibrotic genes and ECM production in response to a stiff biomaterial since these cells are mechanosensitive and mechanoresponsive (113).

The clinical results for biomaterials are generally very good. A long-term study on titanium plates has shown them to be encapsulated more a decade after insertion and the capsule containing titanium particles did not create any inflammatory response (114). Silicone breast implants, which in the majority of cases have good long-term results, have complications such as a capsular contracture associated with a smoother implant surface and a thicker capsule (115). Although the microscale roughness of silicone breast implants has been shown to reduce the incidence of capsular contracture compared to smooth surfaces, it has also been associated with a rare type of lymphoma (BIA-ALCL) (23-25).

Radiation-induced inflammation and soft tissue response

Radiation negatively affects the tissue and gives rise to both acute and chronic effects. The acute effects regarding soft tissue are erythema, dry or moist desquamation and possibly ulcers, whereas chronic effects are changed pigmentation, fibrosis of the skin, telangiectasia, sebaceous and sweat gland

malfunction. These clinical findings correlate with the acute and chronic inflammatory responses due to the DNA damage and cell death caused by radiation. The radiation effect on macrophages has shown reduced growth but increased protein production and phagocytic ability (116, 117). Initially, proinflammatory cytokines are released due to exposure to various molecules called damage-associated molecular patterns (DAMPs) from necrotic cell death as well as due to the production of reactive oxygen species (ROS) (118, 119). Some of these inflammatory cytokines stimulate even more ROS production as a consequence of tissue damage (enzymes, proteins, DNA and cellular phospholipids). Apoptotic cells have, on the other hand, been described to stimulate macrophages to produce anti-inflammatory cytokines, such as TGF-β1, which can stimulate fibrosis through the action of FGF (120, 121). Depending on the radiation regimen (dose, number of fractions, volume of tissue, and duration of complete treatment) and concomitant treatments such as surgery and chemotherapy, fibrosis can be more or less pronounced (122-124). The clinical symptoms of radiation fibrosis can emerge from changes in vessels, skin, subcutaneous tissue, muscles, and skeleton and are known as radiation fibrosis syndrome. Studies on how to restore radiation-injured tissue have found PDGF to improve wound healing and fat transplant to normalize adipose tissue (125, 126).

Radiation-induced inflammation and soft tissue response around implants

Most studies of radiation effects on implants in soft tissue have been performed on silicone because breast implants are exposed to irradiation in the context of breast cancer treatment. An intense and prolonged inflammatory reaction has been observed around silicone implants that have been subjected to radiation (127, 128). In addition to the increased number of inflammatory cells, it has been noted that the number of myofibroblasts also increased with radiation, which could possibly be a consequence of the prolonged inflammatory response and has been suggested to increase the risk of capsular contracture (127, 129). Another observation is that with radiation, the thickness of the capsule around the silicone implant increases and continues to increase with time, unlike the capsules around nonirradiated implants (128). Increased capsule thickness has been associated with capsule contracture, which is consistent with the increased risk of capsule contracture observed with irradiation (115, 130).

Although the cause of capsular contracture due to radiation is unknown, it has been suggested that the wingless signaling system involving beta-catenin and TGF-beta is important, which is supported by the role of TGF-ß in radiation-induced fibrosis (131-133). The use of an acellular dermis has been shown to

reduce the incidence of capsular contracture, which might be explained by the decreased levels of PDGF and TGF-ß1 as well as fewer myofibroblasts with acellular dermis (134). Another treatment being investigated is the use of leukotriene inhibitors, which have an antagonistic effect on TGF-ß and have shown promising results among patients with capsular contracture (135, 136).

Regarding titanium implants on which mesenchymal stem cells subjected to radiation were grown, increased secretion of IL-6, IL-8, MCP-1 and VEGF was observed, although no difference in cell viability could be detected (137). A drawback of metal implants is the risk of backscattering and beam attenuation when radiotherapy is used, which can affect the soft tissue next to the implant (138).

1.2.2 BONE

Like soft tissue, the two main tissue components of bone are cells and ECM. The specialized cells in bone are primarily osteoblasts, osteocytes, and osteoclasts. As for soft tissue, the ECM consists of collagen and various proteins, such as proteoglycans but also bone-specific proteins, such as osteonectin, osteopontin, osteocalcin, and bone sialoprotein. In addition, the ECM of bone contains inorganic material, calcium phosphate primarily in the composition of HA ($Ca_{10}(PO_4)_6(OH)_2$), which is responsible for the compressive strength of bone, while collagen is responsible for the tensile strength. Depending on the organization of the collagen, bone is divided into lamellar or woven bone. The structure of lamellar bone is parallel collagen fibres in layers in the cortical bone that are arranged in concentric circles, while in the trabecular bone, it is arranged in plates or rods in a porous network with a lower degree of mineralization than cortical bone. In general, the inorganic and organic components make up 70% and 30% of the ECM, respectively, if the cells are disregarded. The cells responsible for mineralization are osteoblasts, through the presence of the enzyme alkaline phosphate (ALP), which hydrolyses pyrophosphate, supplying phosphate for the production of HA. Furthermore, osteoblasts produce collagen, bone-specific proteins, growth factors, and hormones, which are all important for building the ECM. Certain osteoblasts will stop collagen production and instead differentiate into osteocytes, while during this process, they will be enclosed by the ECM by neighbouring osteoblasts. Osteoclasts, on the other hand, have the function of resorbing bone through the action of proteolytic enzymes and by creating an acidic environment.

Osseointegration

Osseointegration is defined as "a direct – on the light microscopic level - contact between living bone and implant" (139). The new bone that develops at the biomaterial can either be produced by existing osteoblasts or by recruited mesenchymal cells that differentiate into preosteoblasts when exposed to inductive agents such as bone morphogenetic protein (BMP). Biomaterials that promote bone formation from mesenchymal cells and thereby are able to create bone at an ectopic site are called osteoinductive materials, and materials considered to possess this ability are calcium phosphate ceramics and bioactive glass (140-142). In attempts to promote osteoinductivity, biomaterials have been coated with BMPs, which in vivo showed increased bone-to-implant contact (143). A biomaterial's ability to support bone formation at its surface is called osteoconductivity and is a requirement to achieve osseointegration. In addition to osteoinductive biomaterials, others, such as titanium, stainless steel, and collagen type I, have shown this ability (144-146). Since titanium is primarily used in bone due to its mechanical properties, there has been extensive research on how to increase its osseointegration, and one of the most investigated modifications is coating with HA (3, 11). Several studies point to important differences in favour of HA-coated implants (147-150). However, there is also an opposition claiming no additional benefits of HA-coating (151). Other modifications of titanium to improve its osseointegration are the addition of fluoride ions and coatings with various proteins, such as bone sialoprotein, osteogenic protein 1, strontium and collagen (152-157).

As for the soft tissue response, the topography also influences osseointegration. Both micro- and nanoscale topography have been reported to be of importance, while microscale topography increases removal torque nanoscale topography increases bone-implant contact and influences cellular behaviour such as proliferation and increased ALP production (158-161). A combination of micro- and nanoscale topography was shown to be superior in regard to stimulating osteoblasts to produce osteogenic markers, and if combined with high surface energy, the response was even greater (162). The high surface energy, which correlates with hydrophilicity, has been reported to promote bone growth, although the effect with time appears to be less important (163, 164).

Structure of the material-bone interface

The material-bone interface has been shown to be an area of mineralized, partly mineralized and unmineralized zones. Depending on the topography, the interface differs. Bundles of mineralized collagen have been visualized to be in direct contact with the oxide layer of the implant, whereas areas with

nanoscale topography showed bone mineral growth into the surface of the implant (165, 166). A network of canaliculi reaching from the nearby osteocytes to the implant surface suggests that these cells could contribute to infrastructure adaptive remodeling (167). Blood vessels are also found close to the implant surface, usually in mineralized areas or in areas of remodeling (165). Although the interface appears to be similar for different implants regarding the ultrastructural morphology, the amount of bone in contact with the implant varies, and factors that can influence bone formation include the implant surface, stability, load, and geometry (168-170).

Biomechanics

The biomechanics between bone and implant are determined by several factors, such as the surgical method, bone quality, surface characteristics of the implant, and compromising treatment (such as irradiation) (171-173). The primary stability, which is the time just after implantation, is primarily determined by how well the implant was attached and the quality of the bone. If this stability is not sufficient, micromovements can occur, causing fibrous tissue to impair osseointegration, at least with movements above 150 μm (174). The secondary stability, i.e., when healing occurs is dependent on bone-implant contact to occur to an adequate degree. It has been shown that a bone-implant contact of 60% is sufficient, and no extra benefits were shown with higher bone-implant contact (175). The healing begins with the formation of immature bone, and as this bone matures, the biomechanical stability increases, and higher stresses via the implant can be tolerated. If the implant bone is subjected to excessively high stresses, bone resorption can occur (169). The bone within 100-150 μm surrounding the implant appears to withstand higher strains without any damage compared to bone further away from the implant (176). A plausible explanation is the lower degree of mineralization in the newly formed bone adjacent to the implant. In analysing the stresses in the bone around the implant, it has been shown that the highest stress occurs in the cortical bone at the neck of the implant; therefore, high bone contact between the implant and cortical bone is crucial for good stability (177). Microscale topography reportedly increases the removal torque (178).

Inflammation

Inflammatory cells, primarily macrophages, are crucial for bone formation (181). Both the M1 and M2 phenotypes of macrophages are important at different timepoints. The M1 phenotype has been shown to be dominant during the first three days, after which the M2 phenotype increased in number (179, 180). Although the M2 phenotype is associated with regenerative potential, the

suppression of M1 has been shown to reduce bone healing through reduced MSC recruitment (181). However, a prolonged inflammatory response has been reported to be negative for bone healing (182). An in vivo study in rabbits showed that 10 days after implantation, M2 macrophages were dominant around titanium implants, whereas PEEK and copper presented a mixed M1/M2 phenotype, which was suggested as an explanation for the reduced bone formation around PEEK and copper (183).

Topography is known to influence inflammatory cells, and a combination of micro- and nanoscale topography showed a weaker proinflammatory response than both nanoscale and smooth titanium *in vitro* (184). This trend could also be influenced by the micro/nanoscale surface being more hydrophilic since an increase in hydrophilicity has been shown to promote the expression of anti-inflammatory mediators (8). These *in vitro* findings correlate with the increased bone implant contact reported for micro/nanoscale implants in vivo (184).

Both titanium and HA microscale particles have been shown to trigger a proinflammatory response, and an in vivo study has shown that titanium particles promote osteoclastogenesis (185, 186).

Bone formation and bone remodeling

Bone remodeling, which is characterized by a balance between bone resorption and bone formation, occurs in response to changes in load and to repair damage to the bone. The process starts with osteoclasts resorbing existing bone and osteoblasts replacing the bone loss. The regulation occurs both at the systemic and local levels through the action of hormones and various mediators, such as parathyroid hormone, oestrogen, TGF beta, and BMP2.

Osteoblasts and osteoclasts have been shown to communicate with cell-to-cell contact as well as through mediators such as receptor activator of nuclear kappa B ligand (RANKL) and osteoprotegerin (OPG) (187). Both RANKL and OPG are regarded as coupling factors, with the main function of RANKL being to promote bone resorption, while that of OPG is to inhibit bone resorption (188). An increased ratio of RANKL/OPG and thereby bone resorption is reported for wear debris or degradable biomaterial through the inflammatory response they trigger (189-191). Other markers of bone resorption are cathepsin K (CatK) and tartrate-resistant acid phosphatase (TRAP), and their expression has been observed to be increased together with that of bone forming markers osteocalcin and runt-related transcription factor 2 (Runx2) at oxidized compared to machined titanium, indicating an upregulated bone remodeling at

the rougher oxidized surface (192). The importance of the topography over the chemistry was confirmed when titanium and calcium phosphate were compared with regard to the presence of osteoclasts and bone formation (193).

Radiation-induced inflammation and bone response

Bone is less susceptible to small fraction sizes or low total dose radiation compared to soft tissue due to the lower proliferation rate of bone (194). Although the mineral content is generally not considered to be affected by radiation, non-mineralized bone is more sensitive to radiation, resulting in hypovascular, hypocellular and hypoxic tissue (195, 196). In addition to the cells directly affected by radiation, there are theories that both nearby "bystander" and distant "abscopal" cells can be affected by radiation through processes involving inflammatory mediators such as TNF-α, IL-6, IL-1β, TGF-β, FGF-2, and reactive oxygen species (ROS) and that this effect can persist for a long time (197). Interestingly, low-dose radiation (<1 Gy) has been shown to reduce bone resorption as well as increase bone mineralization, contributing to the complexity of radiation (198).

Radiation-induced inflammation and bone response in association with implants

When irradiation precedes the insertion of an implant, the rate of complications and the failure rate increase (173). In vivo studies have verified these results by showing lower bone-to-implant contact (BIC), more pronounced bone resorption and reduced stability of the implant as a consequence of radiation (199, 200). Furthermore, the negative consequences of radiation have been shown to be dose-dependent (199, 200). With a proinflammatory response being associated with radiation, this could be a reasonable explanation for the higher rate of failure when radiation was present. This hypothesis was to some degree confirmed by an in vivo study in rats that were given a single dose of radiation of 20 Gy, which resulted in a proinflammatory response through an increased expression of TNF-α as well as a reduction in the bone formation marker ALP and removal torque (201).

2 AIM

The aim of this thesis was to investigate the early inflammatory reaction induced by the common biomaterials titanium, HA and silicone in normal and irradiated tissues. Both the magnitude of the inflammatory reaction and its potential effects on osteogenesis and fibrogenesis were investigated to improve the present understanding of the inflammatory process associated with implanted biomaterials.

The specific aims were:

Study I – To study the initial inflammatory reaction to biomaterials (titanium and HA) in a rat soft tissue model

Study II – To determine the initial human mononuclear cell response *in vitro* to biomaterials (titanium and HA)

Study III – To determine the constitutive and induced expression of inflammatory and fibrogenic molecular markers after insertion of biomaterials (silicone) in irradiated and nonirradiated human soft tissue

Study IV – To determine the morphological, biomechanical and molecular responses in irradiated and nonirradiated bone and soft tissues after insertion of biomaterials (titanium) in a rat model

3 PATIENTS AND METHODS

The present thesis is based on three experimental studies (Studies I & IV, in vivo; Study II, *in vitro*) and one human study (Study III).

Study I is an experimental biocompatibility study performed in a previously described rat dorsum soft tissue model (202, 203), providing a comparative analysis of the relatively early (24 h and 72 h) inflammatory response to biomaterials (titanium and titanium-coated HA), whereas **Study IV** is an experimental study in a rat tibia model (199, 201, 204), investigating the peri-implant cell and tissue response 6 and 28 days after titanium implant insertion in previously nonirradiated or irradiated sites.

Study II is a cytocompatibility study performed *in vitro*, determining the viability and secretory response of human peripheral blood mononuclear cells cultured for 24 h and 96 h on titanium and titanium coated with amorphous and crystalline HA, respectively.

Study III is a human study focusing on gene expression in cells in soft tissue and adherent silicone discs in women previously treated with breast cancer surgery and irradiation for unilateral breast cancer.

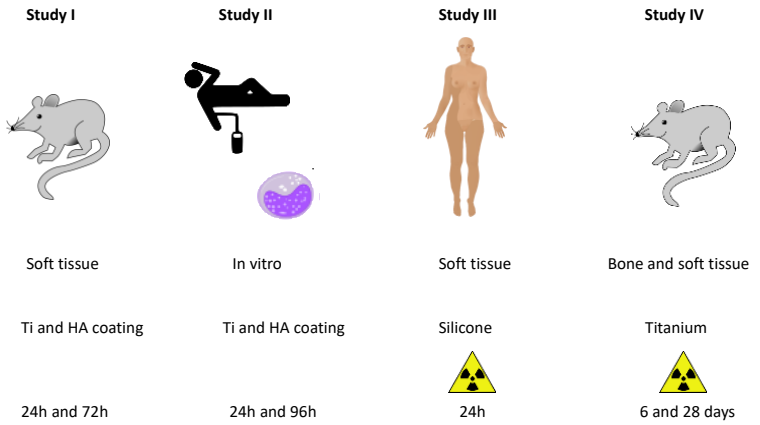

Figure 1. Overview of Study I-IV. (with permission from publicdomainvectors.org, commons.wikimedia.org, maxpixel.net)

3.1 BIOMATERIALS

Titanium discs (Study I & II)

Titanium discs were machined from commercially pure titanium grade 2 rods (Stupino Titanium Company, Russia). Two different diameters were manufactured, 10 mm (Study I) and 15 mm (Study II), with a thickness of 1 mm. The surface was left as-machined.

Cleaning and sterilization: The discs were cleaned and exposed to ultraviolet ozone for 30 min (Study I) or placed in an ultrasonic bath containing 70% ethanol for 30 sec (Study II). The discs were then sterilized by gamma radiation (1 x 25 Gy, IBA, Esbergærde, Denmark).

Hydroxyapatite (HA) coated discs (Study I & II)

Titanium discs were subjected to radio frequency magnetron sputtering with hydroxyapatite (HA). Ultrathin coatings of amorphous HA with thicknesses of 0,1 μm and 2,0 μm were produced. Some of the 0.1 μm amorphous HA coatings were crystallized by heat treatment (gas flow reactor, 600°C).

Cleaning and sterilization: The procedures were performed in a similar manner as previously described for the titanium discs.

Silicone discs (Study III)

The silicone discs originated from a commercially used silicone implant (Mentor reference number 350-1650, a gift from Promeduc Surgical AB, Knivsta, Sweden). The dimensions of the discs were 10 mm in diameter and a thickness of 1 mm.

Cleaning and sterilization: The discs were punched from sterile implants under sterile conditions and sterilized using autoclaving (120^0 ℃ for 15 min).

Titanium screws (Study IV)

Titanium screws (diameter 1.8 mm; length 2 mm) were made by machining a commercially pure titanium rod (Christers Finmekaniska AB, Skövde, Sweden). The screws were then glass bead (0.08 – 0.25 mm) blasted.

The titanium screws were initially cleaned according to the manufacturer's cleaning protocol using two different degreaser, ultrasonic bath for 15 min and

rinsing in water. At the laboratory the screws were further cleaned in an ultrasonic bath with 70% ethanol 3 times for 10 min and in distilled water once for 10 min. They were sterilized by autoclaving for 30 min at 120 °C and 100 kPa steam pressure.

3.2 SURFACE CHARACTERIZATION

Optical interferometry (Studies I, II & IV)

Surface topography was quantified by white light interferometry (MicroXAM, Phase Shift, AZ, USA). A Gaussian filter (filter size: 50 μm x 50 μm) was applied to reduce waviness and vibrations. The following parameters were measured in Study I: height descriptive parameter, S_a (arithmetic mean deviation of a surface), and in Studies II & IV: S_a, hybrid parameter, S_{dr} (the ratio between the developed surface area and a flat reference area); and functional parameter, S_{ci} (core retention fluid index). The software used to create 3D images was MountainsMap 6.2 imaging software (Digital Surf, Paris, France) in Studies I and II, while the Veeco Wyko NT9100 system and 4.1 Vision analysis software (Veeco Instruments, Inc., New York, USA) was used in Study IV.

Scanning Electron Microscopy (SEM) (Studies II, III & IV)

SEM was used to investigate the surface morphology. The apparatuses used were an Ultra 55 FEG SEM (Leo Electron microscopy, Zeiss, Oberkochen, Germany) (Studies II & IV) and a Quanta 200 environmental SEM (FEI Company, The Netherlands) with a 20 kV accelerating voltage, water vapour pressure of 0.5 Torr and no conductive coating (Study III).

Contact angle measurement (Studies II & III)

The methods used to measure the contact angle, Fibro-DAT1100 (Fibro System AB, Stockholm, Sweden) (Study II) and Attension Theta optical tensiometer (Biolin, Västra Frölunda, Sweden) (Study III), calculated the contact angle automatically and a steady-state value was chosen.

X-ray photoelectron spectroscopy (XPS) (Study II)

The surface chemistry of the HA discs used in Study II (the same manufacturing was used for the HA discs in Study I) was analysed with a Quantum 2000 (Physical Electronics, Germany) equipped with a

monochromatic Al Kα X-ray source. Prior to the spectrum analysis, the HA-coated surfaces were cleaned for five sec with Ar. The scans analysed for Ca2p, P2p and O1 s, thereby determining the Ca/P ratio.

X-ray diffraction (XRD) (Study II)

The X-ray diffraction patterns were created using a D5000 diffractometer (Siemens, Germany) with a Cu Kα anode. The patterns were collected between the angular range of 25° and 45°.

Auger Electron Spectroscopy (AES) (Study IV)

The screw-shaped titanium implants in Study IV were characterized with respect to surface chemistry using Auger electron spectroscopy (AES) (PHI 700 Scanning Auger Microprobe, Physical Electronics Inc., Chanhassen, Minnesota) operating at 3 keV.

3.3 STUDY MODELS

3.3.1 IN VIVO (STUDY I & IV)

Study I

Animals

The animals used were female Sprague–Dawley rats with a weight of approximately 250 g, n=7 (24 h) and n=8 (72 h). They were permitted to acclimatize for one w, and their food consisted of a standard pellet diet and water.

Surgical procedure

The animals were anaesthetized using isoflurane gas. The backs of the rats were prepared by shaving and subsequently cleaned with chlorohexidine. Four incisions with a length of approximately 12 mm were placed approximately 15 mm lateral to the midline on the back. For each incision, a subcutaneous pocket was created. In three of the pockets, an implant was inserted, and the fourth pocket was a sham. Each rat received one crystalline HA, one amorphous HA, and one titanium implant. Nonresorbable Suturamid VR 5-0 Fs-2 sutures

(Ethicon, Sommerville, NJ) were used to close the wound. The surgery was performed under aseptic conditions.

At retrieval after 24 h and 72 h, the rats were anaesthetized, and the skin was shaved and cleaned. In addition, they were injected intraperitoneally with an overdose of pentobarbital. Each implant was retrieved and transferred to polystyrene tissue culture wells containing 400 mL of 0.05 M sodium phosphate buffer containing 2 M NaCl and 2 mM ethylenediaminetetraacetic acid (EDTA). The culture wells with the implants were stored at -20°C. The pockets, now without the implants, were rinsed with 0.3 mL of heparinized (10 IE/mL) sterile Hank's balanced salt solution (HBSS) with Ca^2 and enzyme inhibitors (0.15 mM aprotinin and 2.2 mM leupeptin; Sigma Aldrich AB, Sweden) and 0.5 mM pefabloc (Boehringer Mannheim, Scandinavia). The exudates were kept on ice until they were centrifuged at 400 g for 5 min to separate the cells in the exudate. The cell pellets were dissolved in 0.05 M sodium phosphate buffer with 2 M NaCl and 2 mM EDTA and stored at -20°C for subsequent DNA measurement. The supernatants were used for analysis of lactate dehydrogenase (LD) and cytokines (TNF-α, IL-10, and MCP-1).

To determine the cell concentration and viability of the exudate cells, Turk's staining solution and Trypan blue staining, respectively, were used.

Study IV

Animals

The animals used were Wistar albino male white rats weighing approximately 300-350 g and 16 w old, n=40.

Radiation procedure

Prior to irradiation, the animals were anaesthetized with intraperitoneally injected ketamine (70 mg/kg) and xylazine (7 mg/kg). Each animal in the irradiation group received a single dose of irradiation of 20 Gy towards both hind legs. Irradiation was performed with 4-mV photon radiation from a linear accelerator (VARIAN Clinac 600 CDA) at the Radiation Oncology Department at King Fahad Specialist Hospital – Dammam, Saudi Arabia. The protocols of irradiation and implantation have previously been published (201, 204). To treat the animals as similarly as possible, the nonirradiated group (control) was also transported to the Radiation Oncology department. Both the irradiated and nonirradiated groups were returned to the animal house on the same day.

Surgical procedure

The surgery was performed 8 w after irradiation. All animals, both the irradiation and the nonirradiation (control) groups, were anaesthetized using sevoflurane (Sevorane, Aesica Queenborough Ltd, UK) with Sigma Delta Vapourizer (Penlon Ltd., Abingdon, UK) and received local anaesthetics (1 ml lidocaine with epinephrine). A sharp incision was made over the tibial metaphysis, a rotary round bur was placed to prepare the implantation site, and then the titanium screw implant was screwed into the bone. Surgery and implantation were performed bilaterally in the tibia with two implants per tibia. The wounds were closed with an internal 5/0 absorbable polyglycolic acid suture (Ernst Kratz GmbH, Dreieich, Germany) as well as an external 5/0 nonabsorbable silk suture (B. Braun Surgical, Rubi, Spain). The surgical protocol has previously been published (192, 201, 205).

3.3.2 IN VITRO (STUDY II)

Cells

Human peripheral blood mononuclear cells were isolated from buffy coats received from blood donors at Sahlgrenska University Hospital, Gothenburg, Sweden, n=6.

Isolation protocol

The protocol of Pertoft using a gradient, polyvinylpyrrolidone-coated silica gel (Percoll™, Amersham Pharmacia Biotech AB, Sweden) was used to isolate human peripheral blood mononuclear cells (206). In brief, a Percoll gradient with a density of 1.076 g/mL was placed in a tube, and on top of this gradient was a buffy coat gently placed before centrifugation (800 g for 30 min) to separate the mononuclear cells. The mononuclear cells were collected and subsequently washed in HBSS (without Ca^{2+} and Mg^{2+}) and centrifuged (350 g for 5 min at 4 °C). The suspension of the resulting cell pellet was placed on top of the Percoll gradient at a density of 1.064 g/mL and then centrifuged (400 g for 60 min at 4 °C). The collection of mononuclear cells and washing were repeated. In the final step, the viability was determined by Trypan blue to 97.5 ± 2.5%.

Cell Culture

The isolated human mononuclear cells were suspended in media (RPMI 1640, Gibco, with 5% foetal calf serum and 1% PEST (penicillin and streptomycin)). The cell suspension, at a concentration of 10^6 cells/mL, was seeded onto

titanium and HA discs, which were placed in 24-well plates (NUNC™, Nalge Nunc International, Denmark). In total, 1 mL cell suspension was added to each well. The addition of exogenous lipopolysaccharide (LPS) (Escherichia coli, serotype 0127:B8, Sigma) was made to half of the wells. The concentration of LPS in the wells was 10 ng/mL. The incubation conditions were 37 °C with 5% CO_2 and 95% humidity. Half of the wells, including both with and without LPS, were cultured for 24 h, and the other half were cultured for 96 h. At the end of culture process, the cell suspension was collected and centrifuged (400 g for 5 min). The supernatant was subsequently frozen at -70 °C until analysis. All samples were performed in duplicate.

3.3.3 CLINICAL (STUDY III)

Patient selection

Women with previous unilateral breast cancer treated with surgery and irradiation were included in the study. To be included, the woman should not have any disease other than the history of breast cancer, no medication that is known to affect the inflammatory response (except medication against breast cancer), known coagulation defects, allergies towards local anaesthesia, or being a smoker. The women enrolled (n=15) were either on the waiting list for surgery at the Clinic of Plastic Surgery, Region Östergotland, Linköping University Hospital, and/or previous patients at the Department of Radiation Oncology, Region Östergötland, Linköping University Hospital. Each patient received both written and oral information about the study and gave their written consent.

Surgical procedure

In the inframammary fold at the site of incision, local anaesthetic mepivacaine 10 mg/mL (Carbocain®; Aspen Nordic, Ballerup, Denmark) was subcutaneously administered. An incision of 1 cm was made bilaterally. Biopsies were collected for gene expression and histology from both the irradiated and nonirradiated adipose tissue. The silicone discs were thereafter inserted into the adipose tissue, with one disc per incision. The wound was closed with medical tape.

After 24 h, the discs were retrieved, and biopsies were taken for gene expression as well as for histology. The discs and biopsies intended for gene expression were placed in separate tubes with RNA*later* (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania) and stored at 4 °C until isolation of the RNA. The biopsies for histology were placed in 4% formaldehyde. After retrieval, the wound was closed with resorbable sutures. All surgeries were

performed under aseptic conditions with special care not to contaminate the samples collected for gene expression.

3.4 ANALYSES

Limulus quantitative chromogenic assay (Studies I, II & III)

To determine if there was any endotoxin contamination, the Limulus amebocyte lysate test was performed.

Cytotoxicity (Studies I & II)

The activity of lactate dehydrogenase (LDH) was determined as an indicator of cell death (cytolysis). LDH catalyses the bidirectional reaction, turning pyruvate to lactate by using NADH. The difference in NADH can be measured spectrophotometrically at 340 nm and is related to the activity of LDH (C-Laboratory, Sahlgrenska University Hospital, Sweden).

DNA content (Studies I & II)

Fluorescence assay (Study I)

At retrieval, the implants were placed in separate tissue culture polystyrene wells with 400 mL of buffer (0.05 M sodium phosphate buffer with 2 M NaCl and 2 mM ethylenediaminetetraacetic acid (EDTA)). The implants with the buffer were stored at -20 °C until analysis of DNA content.

The exudate from the subcutaneous pockets was centrifuged for 5 min at 400 g, and the supernatants were collected. The remaining cell pellet was stored in buffer (0.05 M sodium phosphate buffer with 2 M NaCl and 2 mM EDTA) at -20 °C.

The DNA content on the implants and in the exudate was determined after sonication of the thawed samples for 15 sec. To the samples, 2 mL of a reagent solution (1 mL H33258 in 100 mL 0.05 M sodium phosphate buffer with 2 M NaCl) was added. The samples were incubated at room temperature for 15-30 min. A luminescence spectrometer (Perkin Elmer LS 50 luminescence spectrometer) was used to measure both excitation and emission wavelengths at 360 nm and 450 nm, respectively. The reagent solution served as control. A DNA standard was used to determine the DNA content.

Nucleocounter (Study II)

The nucleocounter is an automated cell counter that measures the concentration of cells by staining the nucleus with propidium iodide (PI). For the stains to access the nucleus, lysis buffer was added to the cell suspension to break up the plasma membrane and dissolve aggregates of cells. Thereafter, a stabilization buffer is added with the main purpose of raising the pH to optimize the fluorescence of PI. The concentration of cells was determined based on known volumes of added buffer.

ELISA (Studies I & II)

Enzyme-linked immunosorbent assay (ELISA) (Quantikine®, R&D Systems, USA) was used to determine the release of specific mediators (Table 2).

Gene expression (Studies III & IV)

The tissue samples (adipose tissue in Study III; peri-implant soft tissue and bone in Study IV) were lysed and homogenized using steel beads and a TissueLyser (Qiagen Gmbh, Hilden, Germany). The implant-adherent cells were also lysed and vortexed (2x30 s in Study III; 2 min in Study IV).

RNA isolation from the tissue samples was achieved with a fatty acid RNA purification kit (Norgen, Biotek Corporation, Thorold, Canada) in Study III and with an RNeasy mini kit (Qiagen, Germany) and concentrated with an RNeasy MinElute clean up kit (Qiagen, Germany) in Study IV. RNA isolation from the implant-adherent cells in both studies was performed with an RNeasy microkit with a carrier to maximize the amount of RNA (Qiagen Gmbh, Hilden, Germany). To remove genomic DNA, all the samples were subjected to DNase treatment (Qiagen Gmbh, Hilden, Germany). The isolated RNA was frozen at -80 °C.

cDNA was produced using the TATAA GrandScript cDNA synthesis kit (TATAA Biocenter, Sweden). In the case of a high concentration, the sample was diluted to 20 ng/µl, and a spike assay (TATAA Universal RNA Spike I, TATAA Biocenter, Sweden) was added. Poled RNA within each group was used as NRT control. The cDNA was frozen at -20 °C.

Regarding the quantification, TATAA SYBR GrandMaster mix (TATAA Biocenter, Gothenburg, Sweden) was used together with primers that had been developed and validated by the same company. All samples were analysed in

duplicate. To compare the analysis of the same gene when more than one plate was needed, an interplate calibrator (TATAA Interplate Calibrator) was used.

For the reference screening, human and rat reference gene panel kits (TATAA Biocenter, Sweden) were used in Study III and Study IV, respectively. The best reference genes were calculated by GenEx software (MultiD Analyses AB, Sweden). In Study III, 18S and TBP were chosen for the tissue samples, whereas YWHAZ was selected for the implant-adherent cells. The reference genes selected in Study IV were GAPDH, ACTB and YWHAZ. The mediators analysed in the respective study are found in Table 2.

Table 2.

Study	*Mediators*
Study I	TNF-α, IL-10, and MCP-1
Study II	TNF-α, TGF-β1 and BMP-2
Study III	TNF-α, PDGF-B, MCP-1, VEGF, MMP2, FOXO1, DDIT-4, PTGS2 (COX2), IL-8, COLL1A1, SCF, CysLTR2, PAI, CTNNB1, FGF2, BCL2, AMAC-1, TGF-β1
Study IV	CXCR4, MCP-1, iNOS, MCR1, TNF-α, IL6, HIF-1α, FOXO1, DDIT4, BCL2, ALP, OC, CatK, RANKL, OPG

Removal torque

The removal torque (RTQ) required to loosen the screws in Study IV was measured using an RTQ device (Cedar® Model DIS-RL2; Electromatic Equipment Co., Inc., NY) attached to the head of the screw. The animals were meanwhile sedated on sevoflurane gas.

Histomorphometric analysis

The screws in Study IV were removed together with surrounding bone, i.e., *en bloc*, and fixated in 10% buffered formalin. The samples, i.e., the screws and bone were embedded in plastic (LR White resin, The London Resin Co, Agar Scientific Ltd, Stansted, England). Longitudinal sections of 15-20 µm thickness through the screw and bone were prepared by sawing and grinding (Exakt, Apparatebau, Norderstedt, Germany). Sections were stained with basic fuchsin and blindly evaluated under a light microscope (Nikon Eclipse E600; Nikon Ltd, Tokyo, Japan) to quantify the bone in the implant threads (bone area; BA) and bone in contact with the screw (bone-to-implant contact; BIC).

ETHICAL APPROVALS

Study I: Experiments were approved by the Local Ethics Committee for Laboratory Animals. (Dnr 81/2001)

Study III: The study received approval from the Human Ethics Committee, University Hospital, Linköping, Sweden (Dnr 2011/46-31).

Study IV: The study was approved by the Institutional Animal Care & Use Committee (IACUC) at Imam Abdulrahman Bin Faisal University (IRB 2020-02-201).

4 RESULTS

4.1 STUDY I

This work investigated the early inflammatory response to thin HA coatings compared to titanium in vivo.

Surface characteristics

Amorphous and crystalline HA-coated titanium discs showed similar topography as the titanium discs, S_a 0,24-0,26 μm.

Soft tissue response

DNA content

Higher DNA contents on the titanium discs and in the exudate around the titanium discs were observed compared to the exudate around the HA-coated discs as well as on the amorphous HA-coated discs at 24 h (Figure 2). No difference among the materials at 72 h or among the different HA surfaces was detected.

Inflammatory cells

Monocytes were dominant over the PMN on all the different surfaces. Hydroxyapatite surfaces showed the highest percentage of monocytes, but due to large variation between the animals, no statistical significance was found.

Release of mediators

None of the analysed mediators, MCP-1, TNF-α or IL-10, showed any significant difference among the surfaces. The release of MCP-1 was, however, markedly reduced for all surfaces with time.

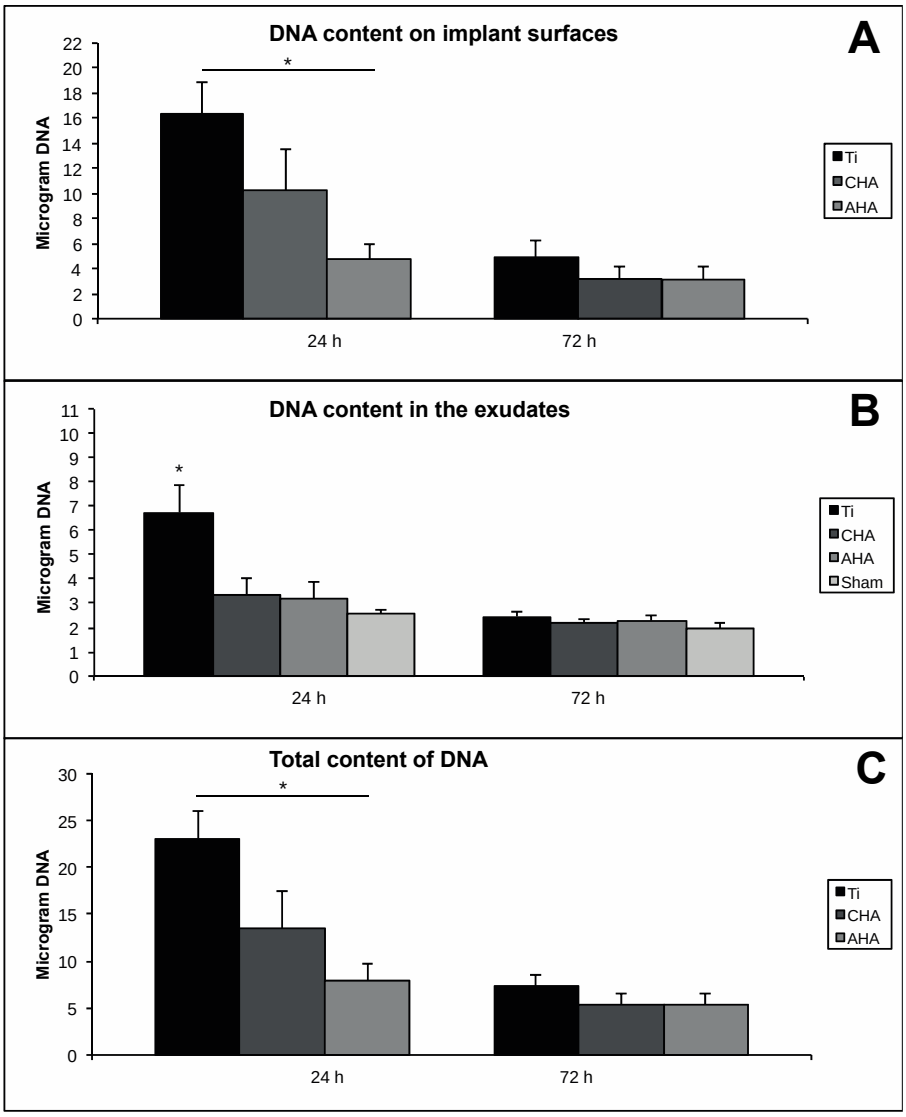

*Figure 2. DNA content at 24 hours and 72 hours. **A** At implant (disc) surface. Ti versus amorphous HA (AHA) at 24 hours * $p < 0.05$ B In the exudates. Ti versus amorphous HA (AHA), crystalline HA (CHA) and sham site at 24 hours, * $p < 0.05$ C Total DNA contant. Ti versus AHA at 24 hours, * $p < 0.05$. Data presented as mean (±SE). (Reproduced with permission from Wiley online).*

4.2 STUDY II

This work investigated the early response of human peripheral blood mononuclear cells to thin HA coatings compared to titanium *in vitro*.

Surface characteristics

The same surfaces evaluated in Study I were used. (Figure 3). The topography was similar for titanium and HA surfaces; for example, S_a varied between 0.23-0.3 μm. The Ca/P ratios were confirmed to be 1.61 ± 0.08 and 1.60 ± 0.12 for crystalline and amorphous HA, respectively. XRD confirmed a crystalline phase in the crystalline HA. Amorphous HA was the least hydrophilic surface (89.1° ± 4.9) compared to crystalline HA (58.2 ± 2) and titanium (60.9° ± 0.77) surfaces.

Human mononuclear cell response

DNA content

The lowest number of cells in the exudate was found at the amorphous HA which also showed significant higher ratio of adherent cells to cells in the exudate compared to the crystalline HA at 24 h (Figure 4A). Neither the presence of LPS nor at 96 h was no difference observed between the different surfaces.

Release of mediators

The release of the proinflammatory cytokine TNF-α was significantly higher from human mononuclear cells grown on amorphous HA compared to crystalline HA at 24 h (Figure 4B). At 96 h or in the presence of LPS, no difference between the surfaces was observed.

Regarding the anti- and proinflammatory cytokine TGF-β1, no difference between the surfaces at any of the timepoints with or without LPS was observed.

There was no detection of BMP-2 with the used method.

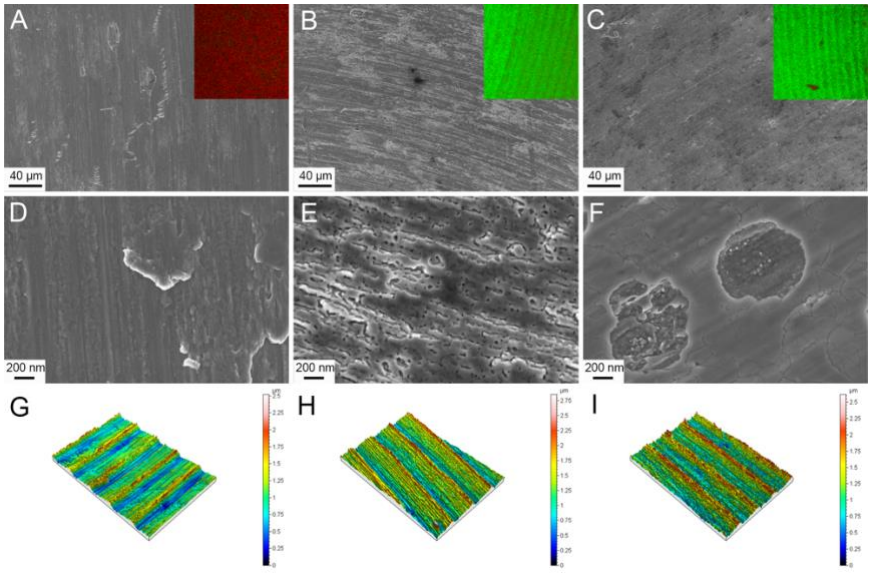

Figure 3. Surface characterization. SEM micrographs and 3D reconstructions of the interferometry analysis. Machined titanium (A, D, G), amorphous HA (B, E, H), and crystalline HA (C, F, I). In the pictures A-C the inserts show the TOF-SIMS signals for the corresponding surfaces. (Reproduced with permission from Springer).

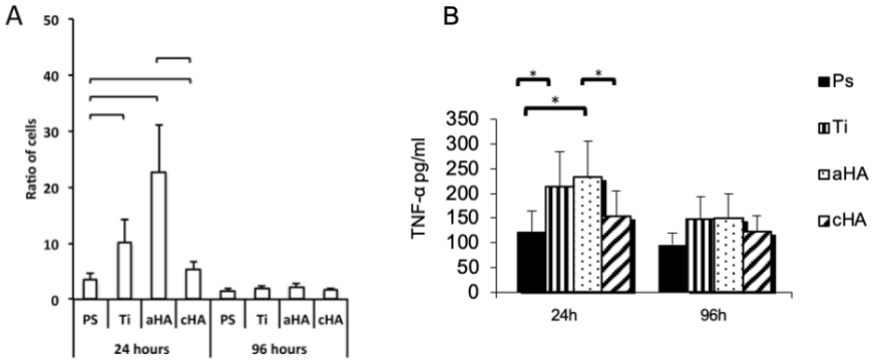

Figure 4. Ratio of implant-adherent cells in relation to cells in supernatant, at 24 and 96 hours (A) TNF-a release from mononuclear cells (B). Polystyrene (Ps), titanium (Ti), amorphous HA (aHA), crystalline HA (cHA). Statistically significances indicated with bars, $p < 0.05$. Data presented as mean (+SEM). (Reproduced with permission from Springer.)

4.3 STUDY III

This work investigated the influence of irradiation on the early inflammatory and fibrotic response in humans in the presence of silicone implants.

Surface characteristics

The round silicone discs showed a smooth surface and were hydrophobic (contact angle between 104° and 120°).

Histology

Haematoxylin-eosin staining of the adipose tissue showed inflammatory cells, mast cells, adipocytes and fibroblasts in both the irradiated and nonirradiated tissue without any structural differences.

Gene expression

Peri-implant tissue

In the irradiated tissue, there was less expression of 18S compared to nonirradiated tissue, suggesting a lower number of cells, both in the adipose tissue around the silicone discs and in the disc adherent cells (Figure 5). There was no difference in 18S expression between irradiated and nonirradiated tissue before implantation.

The difference between the irradiated and nonirradiated soft tissue before implantation was a lower expression of SCF and higher PAI in irradiated tissue

The expression of IL-8 was lower in the irradiated tissue, while PDGF-B and BCL-2 increased 24 h after the implantation of silicone discs. Unlike the nonirradiated tissue, there was no significant reduction in MMP2 in the irradiated tissue.

Disc adherent cells

The only difference in the disc adherent cells was a lower expression of FOXO1 in the irradiated group.

Correlation and Regression analysis

The IL-8 expression was shown to be related to time after irradiation in the soft tissue while this was the case for FOXO1 in the disc-adherent cells. The higher radiation dose the higher expression of COLL1A1 both in the soft tissue and disc-adherent cells, the same was observed for FGF in the soft tissue. The expression of BCL-2 was found to increase in nonirradiated tissue when G-CSF treatment been administrated.

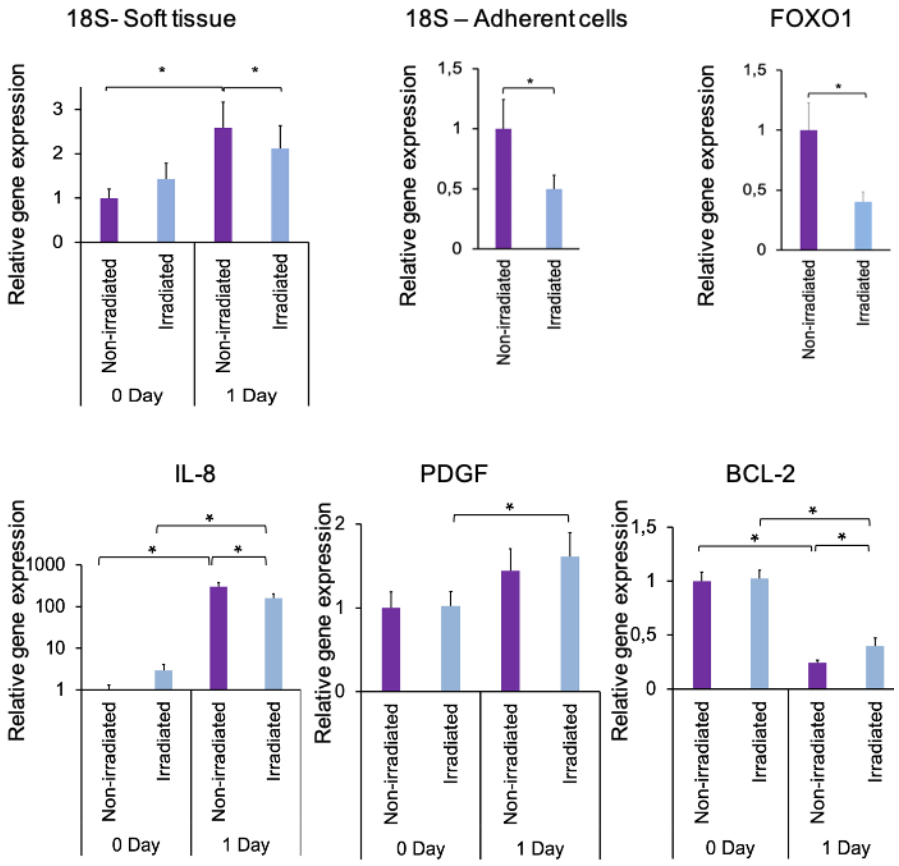

Figure 5. Gene expression in disc-adherent cells: 18S – adherent cells and FOXO1. Gene expression in soft tissue: 18S – soft tissue, IL-8, PDGF, BCL-2. Statistical significance indicated with bars, $p < 0.05$. Data presented as mean (+SEM).

4.4 STUDY IV

This work investigated the influence of irradiation on the osseointegration of a titanium implant from molecular, histological and biomechanical perspectives.

Surface characteristics

The titanium screws had a topography of S_a = 0.707 um, S_{dr} = 13.1 %, S_{ci} = 1.3. The titanium oxide layer was 7 – 10 nm. The surface chemical composition was dominated by carbon (52.7 ± 0.4 %), oxygen (27.7 ± 1.9 %) and titanium (9.2 ± 1.0 %), with minor contamination by the blasted particles silicon (3.6 ± 1.5 %) as well as Na, Ca, Cl, most likely originating from salts.

Gene expression

Peri-implant soft tissue

In the peri-implant soft tissue, the expression of iNOS, a marker of M1 macrophages, was significantly higher at 6 days with prior irradiation than in the absence of irradiation. In the irradiated group, the expression of MCP-1, TNF-α and TGF-β1 was significantly higher than in the nonirradiated group at both 6 and 28 days.

Peri-implant bone

In the peri-implant bone, the expression of MRC-1, a marker of M2 macrophages, was significantly higher when prior irradiation at the early timepoint, 6 days. This was in line with the downregulation of the proinflammatory cytokine IL-6 at this time. The irradiated group also had higher expression of the chemotactic chemokine CXCR4 and the bone formation markers ALP and OC at 28 days.

Implant adherent cells

The expression of inflammatory genes in the implant adherent cells showed a downregulation of IL-6 at 6 days and upregulation of TNF-α at 28 days in the irradiated animals compared to that of nonirradiated animals (Figure 6). At 28 days, the bone resorption gene CatK was expressed to a higher degree, while both the ALP and the anti-apoptosis gene BCL-2 were expressed to a lower degree in the irradiated animals.

For all the different tissue compartments, there was a significant reduction in the expression of the anti-fibrotic gene (FOXO-1) in the irradiated animals at 6 days after implantation.

Removal torque

The removal torque required to loosen the titanium screws was significantly lower in the irradiated bone than in the nonirradiated bone at both 6 and 28 days after implantation (Figure 7).

Histology and histomorphometry

The bone-implant contact (BIC) was significantly lower in the irradiated bone than in the nonirradiated bone at both 6 and 28 days (Figure 7). There was no significant difference in the bone area (BA) between the irradiated and nonirradiated bone.

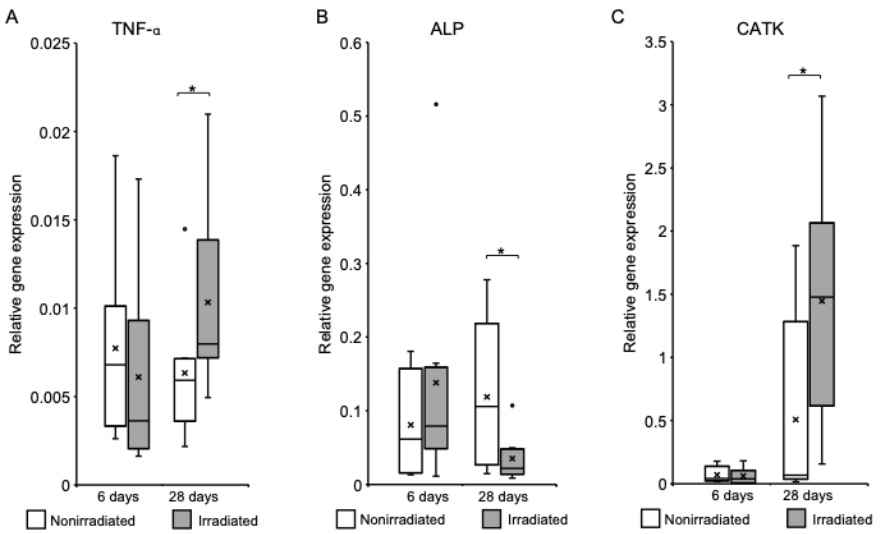

Figure 6: Boxplots showing the gene expression of selected factors in the implant-adherent cells: (A) Pro-inflammatory cytokine TNF-α, (B) Bone formation marker ALP and (C) Bone resorption marker CatK. The boxplots show the median (line), mean (multiplication sing), first and third quartiles (box), minimum and maximum (whiskers) and marked outlier (black circle). Statistically significant differences (P<0.05) between irradiated and nonirradiated groups are indicated by asterisks.

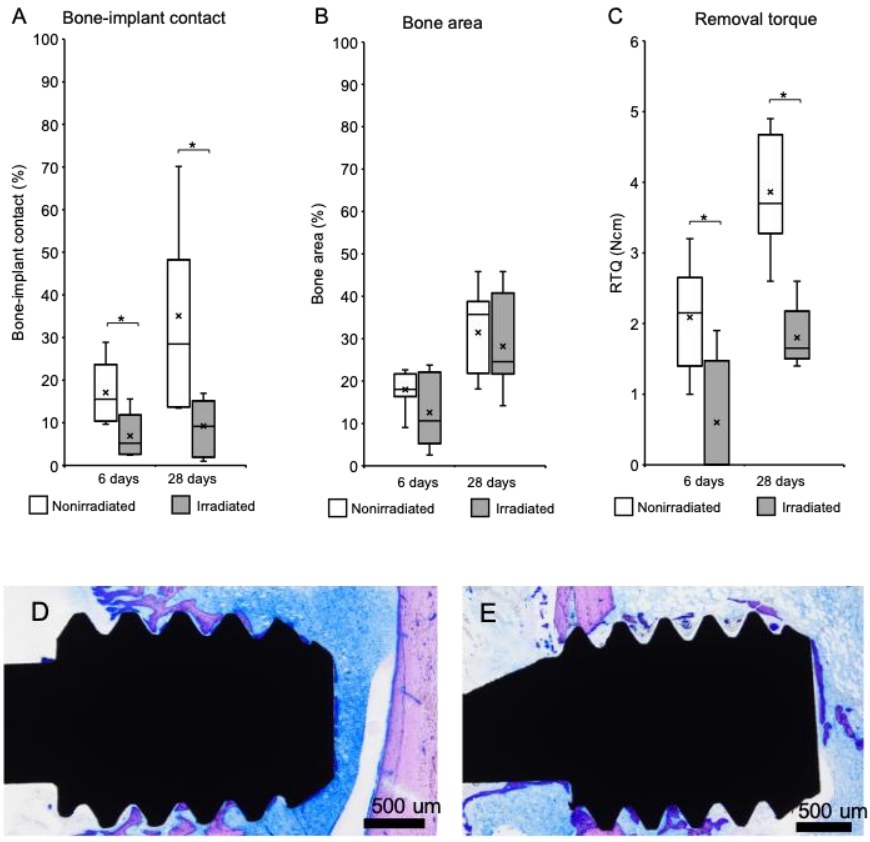

Figure 7: Histomorphometry, removal torque and histology. Graphs showing the percentage of bone-implant contact (A), the percentage of bone area (B) and the removal torque (C) for implants at nonirradiated and irradiated sites after 6 and 28 days. The boxplots show the median (line), mean (multiplication sign), first and third quartiles (box), minimum and maximum (whiskers). The histology section at 28 days for nonirradiated (D) and irradiated bone (E). Statistically significant differences ($P<0.05$) between irradiated and nonirradiated groups are indicated by asterisks.

5 DISCUSSION

5.1 METHODOLOGY

5.1.1 EXPERIMENTAL MODELS

To investigate a specific hypothesis, various experimental models may be more or less suitable. The advantages of *in vitro* studies include the ability to separate the process or reaction that is being studied from the influence of surrounding cells and the ability to control the environmental conditions. *In vitro* models are in particular used to study biological mechanisms and the influence of materials on cells and to investigate toxicity and immunogenicity (207, 208). Cells used in *in vitro* models can be primary cells, i.e., cells isolated directly from human or animal tissue, or they can be cells from cell lines, i.e., cells that have been subcultured repeatedly. With cell lines, there is a risk that the cells may lose the characteristics of the original tissue, and this could explain differences in the results obtained using cell lines and primary cells (209).

Several of the shortcomings of the *in vitro* model can be solved with the in vivo model, which offers a much more complex environment, including a systemic influence. The use of in vivo models is crucial in studying the biocompatibility of implants, as implantation triggers numerous biological processes, such as inflammation and coagulation, that interact with the implant. When choosing an animal model, however, several considerations must be taken into account. Nonhuman primates are rarely used, and large animals such as dogs and pigs are also less commonly used due to ethical issues and cost (210). Instead, the majority of animals used are small animals such as rabbits and rodents, which are also suitable for studying the bone implant interface as well as pathophysiological pathways. One of the foremost advantages of small animals is their short life cycle, which offers the advantage of accelerating the research and making it possible to obtain more information in a shorter time (210). Today, the majority of animals used in animal models are rodents. The strains of rats most often used are Sprague–Dawley and Wistar; these strains are used in Study I and Study IV, respectively.

Although *in vitro* and in vivo models are both extensively used, the optimal experimental model would in most cases be the clinical model. However, the main problem with clinical models is that ethical considerations limit their use. Other disadvantages are that they are often time-consuming and costly and that the investigator has less control of environmental factors.

As irradiation is a compromising factor in many clinical situations that involve biomaterials as part of reconstruction, it is important to understand the effects of irradiation. The most common use of radiotherapy is as a cancer treatment, and such treatment often involves high doses of radiation. For breast reconstruction patients, there is currently a trend toward performing immediate reconstruction, and data indicate a reduced risk of capsular contracture when irradiation is performed after breast reconstruction compared to placement of the implant in previously irradiated tissue (130). However, delayed exchange of the expander with the permanent implant from 3 to 6 months post-irradiation showed a reduced number of failures (211). Similar observations have been made with titanium implants in patients receiving radiation to the head and neck; in those patients, the survival of the implants increased as the time between irradiation and implant placement increased (212). The general consensus appears to be that fewer complications occur after delayed implantation; this is consistent with our finding in Study III that certain mediators (IL-8 and FOXO1) were less affected as the time from irradiation increased.

5.1.2 IMPLANT SURFACE CHARACTERIZATION

The most frequently clinically used biomaterials are titanium with and without HA for bone applications and silicone for soft tissue applications. The topography of commercially used titanium dental implants varies (213); a machined surface is used in Studies I and II, and a glass beaded blasted surface is used in Study IV. For silicone implants, both smooth and textured topographies are clinically used; the use of silicone implants with smooth topography has been shown to increase the risk of capsular contracture and is therefore studied in Study III (24).

5.1.3 ANALYTICAL TECHNIQUES

Isolation of cells can be performed using various methods such as centrifugal sedimentation, fluorescence-activated cell sorting and magnetic cell separation. Although the last two methods are associated with high purity, they are also limited to use with cells that display specific markers on the cell surface, and tagged antibodies to these markers may be expensive. The centrifugal sedimentation that we used is rapid and inexpensive but yields cell preparations of lower but acceptable purity. In a previous study, we showed that the method yielded a preparation in which 75±10% of the cells were monocytes and the remaining cells were lymphocytes (214). Regarding the analysis of mediators produced by cells, qPCR and ELISA determine the RNA expression and secreted protein, respectively. While the ELISA method may not be sensitive enough to detect small amounts of a released mediator, qPCR

is a method that is very sensitive to contamination. This difference in sensitivity might explain the contradictory results obtained in Study II, in which no BMP-2 was detected in mononuclear cells, whereas BMP-2 was detected by qPCR in a cell line; the use of different methods might explain the discrepancy (21).

5.2 SOFT TISSUE

5.2.1 INFLAMMATORY RESPONSE

The inflammatory response of soft tissue when an implant is introduced differs depending on the implant material. However, in common is an initial recruitment of PMN and, soon thereafter, recruitment of monocytes, which then dominate in number. The dominance of monocytes 24 hours after implantation was confirmed in Study I. The higher DNA content around the titanium discs was significant to both HA surfaces, while the DNA content *on* the titanium discs was only significant for the amorphous HA surface. This indicates greater proliferation or increased recruitment of cells around titanium. Since the topographies of the materials were similar, differences in topography are unlikely to be the cause of the difference. Instead, the difference might be partially explained by the greater hydrophilicity of the titanium surface, but this might not be the entire explanation, as the crystalline HA discs had wettability similar to that of the titanium discs. It is more likely that differences in the surface chemistry of the materials are the primary reason for the difference in DNA content. A study of biocompatibility in which titanium and HA were tested reported a difference in cell adherence to these two materials; however, in that study, fewer cells adhered to titanium than to HA (although the topography was not specified), and the timepoint at which adherence was measured was 14 days (215). Furthermore, cell adherence to HA surfaces has been shown to be influenced by crystallinity as well as by the crystal phase (216, 217).

When focusing on isolated human monocyte responses to titanium and HA surfaces in Study II, it was observed that there were fewer cells around the amorphous HA corresponding to Study I, but it was also shown that monocytes adhered to the amorphous HA to a higher degree than to the crystalline HA. This would not be explained by the difference in wettability but could be explained by a rougher topography, as amorphous HA has been shown to degrade (218). With dissolution of the HA coating, HA particles could be released, and this has been shown to provoke an inflammatory response that we observed, represented by an increase in TNF-α secretion (104).

5.2.2 APOPTOSIS AND REGENERATION

Depending on the chemical properties of biomaterials, apoptosis of the cells surrounding the implant can be more or less pronounced. For instance, in comparison to titanium, copper implants are associated with a significantly higher percentage of monocytes with signs of apoptosis after one hour of culture (219). In addition to their chemical properties, the size of the biomaterials appears to be of great importance since wear particles are known to increase apoptosis. Titanium particles stimulate apoptosis, which has been shown to occur through autophagy (220). A study in which nanoscale particles of HA and titanium were compared showed significantly higher apoptosis of epithelial cells at 24 hours due to the titanium particles than to the HA particles, consistent with our findings in Study I of higher LD around titanium than around amorphous HA (221). However, in Study II, in which isolated monocytes were studied, no difference between titanium and HA was observed in regard to LD expression, but an increase in LD with time was observed, as previously reported for titanium (203).

As apoptotic cells are phagocytosed, they have been reported to stimulate macrophages to produce anti-inflammatory cytokines such as TGF-β1, a mediator that is of major importance for regeneration (120). In neither Studies I nor II did we observe any difference in IL-10 or TGF-β1 release as a measurement of regeneration between the titanium and HA. There is limited research on the use of titanium and HA in soft tissue; however, one study reported that certain topographies (Sa 0.66-2.91 μm) of titanium are more prone than others to activate macrophages through the alternative pathway (99). This research group also observed higher TGF-β1 secretion on HA surfaces than on titanium surfaces, an observation that differs from ours. Possible explanations for this difference are the use of cells from different sources (primary cells versus cell lines) and materials with different types of surfaces (smooth versus rough). Improved attachment of soft tissue has been observed in vivo with HA abutments compared with titanium; however, the topography of the surfaces as well as the design of the two types of abutments differed (222).

5.2.3 EFFECT OF IRRADIATION ON THE RESPONSE OF SOFT TISSUE TO IMPLANTS

Irradiation is known to have a negative effect on tissue, and an in vivo study showed decreased cell proliferation and increased apoptosis as a consequence of high-dose irradiation (223). This could explain our finding of fewer adherent

cells on silicone discs in irradiated tissue in Study III. An alternative explanation is the lower expression of IL-8 in the irradiated group. Since IL-8 is a chemoattractant cytokine for neutrophils, which are present immediately after implantation, it is possible that a reduced number of neutrophils could be the reason for the reduced number of adherent cells. No report on the presence of neutrophils in previously irradiated tissue has been found, but neutropenia is a known side effect of irradiation; however, whether this "memory" persists after previous irradiation is still unknown. In contrast to our findings are some reported observations that IL-8 levels increase as a consequence of irradiation of endothelial cells and keratinocytes; however, this was an acute response to radiation, and the persistent response has not, to our knowledge, been investigated (224, 225). Investigations of acute and persistent changes due to irradiation show that their characteristics differ profoundly (226).

The soft tissue response 6 days after implantation of titanium implants into previously irradiated tissue (Study IV) showed a more intense inflammatory response, as indicated by increased expression of TNF-α and MCP-1, corresponding to the presence of more M1 macrophages. Irradiation has been shown to increase the M1 phenotype of macrophages as well as TNF-α levels in lung tissue in mice (227, 228). The prolonged high expression of TNF-α observed in Study IV might contribute to the development of fibrosis, as chronic inflammation is associated with fibrosis. Signs of increased fibrosis in the soft tissue of the irradiated animals in Study IV were the higher expression of TGF-β1 at both 6 and 28 days and a lower expression of FOXO1 6 days after implantation. TGF-β1 is regarded as the foremost marker of fibrosis, and FOXO1 has been reported to inhibit fibrosis in various tissues (229, 230). Although no increase in TGF-β1 was observed in cells adherent to implants of either titanium or silicone, these cells presented lower expression of FOXO1 (Studies III and IV). FOXO1 has also been shown to promote apoptosis and to protect cells from oxidative stress caused by compounds such as reactive oxygen species (ROS), by stimulating antioxidant enzymes (231). Radiation has furthermore been reported to inactivate FOXO1, and in Studies III and IV, we found that the production of FOXO1 was affected even long after exposure to radiation (232). In response to silicone disc implants, the soft tissue around the discs showed increased expression of fibrotic markers such as PDGF and BCL-2 and a trend toward an increase in FGF expression. The finding that expression of both PDGF and BCL-2 is increased is not surprising since PDGF can stimulate the JAK2 pathway to produce BCL-2 (233). PDGF has also been shown to inactivate FOXO1 by phosphorylation via the PI3K/Akt pathway; this may explain the proliferative effect of PDGF on fibrotic cells (234).

5.3 BONE

5.3.1 INFLAMMATORY RESPONSE

Since inflammatory cells have been suggested to be important in regeneration, including osteogenesis, it is of interest to study the inflammatory response to implants (8, 235). In the nonirradiated implant-adherent cells and peri-implant bone in Study IV, we observed a trend of a reduced proinflammatory response, indicated by reduced TNF-α and IL-6 levels over time; this is consistent with events that occur during the normal wound healing process. Such a reduction has also been observed in other in vivo studies of titanium implants; however, a major factor that affected this response was the topography of the surface, with significantly higher TNF-α expression around a machined surface than around a rougher oxidized surface (192). When the hydrophilicity of a rough surface was increased, an increase in the number of M2-activated macrophages was observed, an effect that is thought to be beneficial for osseointegration (8). Here, we observed a combination of markers for M1 and M2 macrophages both in implant- adherent cells and in peri-implant bone.

In addition to the importance of topography in determining the chemistry of the proinflammatory response, coatings with HA have been shown to cause expression of a stronger proinflammatory response *in vitro*. This is partially consistent with our observations of higher expression of TNF-α around amorphous HA than around crystalline HA (Study II), although this was not confirmed in vivo (Study I) (236). The comparable results for titanium and HA have also been confirmed in clinical studies (237, 238).

5.3.2 APOPTOSIS

During osseogenesis, the majority of osteoblasts undergo apoptosis, a process that is important for the development and homeostasis of bone. However, the timing of apoptosis is important since the number of osteoblasts is crucial for bone formation. It has been shown that the chemistry of the implant is of major importance for apoptosis (219, 239). The mechanisms by which osteoblasts undergo apoptosis are still not fully understood, but certain cytokines such as FOXO1 and TNF-α are known to be proapoptotic, while BCL-2 is antiapoptotic (240). We observed in Study IV that the expression of FOXO1 was initially high at 6 days under normal conditions both in implant-adherent cells and in peri-implant bone but decreased with time. This indicates an initially higher rate of apoptosis but is also associated with osteogenesis (241). A similar trend of reduced expression of TNF-α with time was also observed. An interesting finding was that an increase in BCL-2 occurred in the implant-adherent cells in noncompromised tissue. This might be explained by ongoing

bone formation at the implant surface requiring the presence and action of osteoblasts.

5.3.3 BONE FORMATION, REMODELING AND THE EFFECTS OF IRRADIATION

Bone formation and remodeling are associated with the presence of various markers such as ALP, osteocalcin, CatK, RANKL and OPG. We observed a difference between implant-adherent cells and peri-implant bone in the expression levels of several of these markers, again verifying the different behaviour of cells in different tissue compartments (Study IV). In implant-adherent cells in previously irradiated bone, relatively lower bone formation activity and higher bone resorption activity were observed. This indicated an uncoupling of bone formation and resorption, which otherwise go hand in hand. Since osteocytes play a critical role in bone remodeling through their influence on both osteoblasts and osteoclasts, it appears possible that these cells are to some degree responsible for the abnormal remodeling process observed at the implant surface in irradiated tissue. This is supported by reports of the effects of irradiation on primary mouse osteocytes; irradiated osteocytes showed a dominance of bone resorption over bone formation activity and a senescence-associated secretory phenotype (SASP) with dysfunctional secretion of inflammatory cytokines (242). It has also been shown that the canaliculi from osteocytes extend all the way to the surface of the implant, making it possible for osteocytes to have a direct effect on the implant-adherent cells (167).

Regarding the differences in the responses of irradiated and nonirradiated peri-implant bone we observed an increase in remodeling at both 6 and 28 days in the irradiated animals (Study IV). This correlates with the results of an in vivo study conducted in rabbits showing that the remodeling of irradiated bone was initially depressed but later increased to exceed the remodeling that occurred in nonirradiated bone and was at its highest between 3 and 6 months post-radiation depending on the location of the irradiated bone (243). We were unable to correlate the increase in remodeling with any significant change in bone area at the timepoints we observed. Our finding has been confirmed by others who however, observed a decrease in bone area at 2 weeks that recovered at 7 weeks (244). This indicates that irradiated bone has the ability to regenerate through remodeling and to compensate for the early radiation-induced damage to osteoblasts, osteocytes and, to some extent, osteoclasts (245).

5.3.4 OSSEOINTEGRATION AND THE EFFECTS OF IRRADIATION

We found that osseointegration was affected by irradiation prior to implantation; the effect was observed as early as 6 days and also at 28 days after implantation (Study IV). In the irradiated bone, a reduction in removal torque corresponded to the reduction in bone implant contact. Similar results showing reduced removal torque at 5 weeks after implantation with the same time between radiation and implantation as in Study IV, i.e., 8 weeks, have previously been reported (201). Overexpression of FOXO1 has been reported to promote bone formation, and without prior irradiation, we observed an initial increase in FOXO1 that could have contributed to the increase in bone implant contact at 6 days (246). Radiation induces oxidative stress, a type of stress that has been reported to shift Wnt/TCF-mediated transcription to transcription of FOXOs and, as a result, less differentiation of osteoblasts occurs (247). Considering previous reports of persistently high ROS production in mitochondria as well as in haematopoietic stem cells long after irradiation, it is plausible that an increased oxidative stress level in the irradiated tissue can explain its reduced capacity for osseointegration (248, 249). It has been shown that the stability of bone implants is dependent on the radiation dose and that delaying the implantation improves osseointegration, possibly due to a certain decrease in oxidative stress with time (199, 249, 250). Clinical reports confirm the negative effect of irradiation on osseointegration, as the success rate after prior irradiation is reduced (173). We further found a prolonged increased proinflammatory response after prior irradiation in both implant-adherent cells and peri-implant bone. In the implant-adherent cells, the increase in TNF-α corresponded to a decrease in ALP, consistent with the results of a previously mentioned study in which the tissue was examined at similar timepoints (201). We also observed an increase in CatK, a further indication of increased bone resorption. Taken together and with the knowledge that chronic inflammation is associated with increased bone resorption, the findings suggest that a prolonged proinflammatory response after irradiation could possibly be a contributing cause of failed osseointegration (251).

6 SUMMARY AND CONCLUSION

In **Study I**, which was an in vivo experimental soft tissue study in rats, the titanium implants showed significantly higher cell infiltration after 24 h, but not after 72 h, than amorphous and crystalline ultrathin HA-coated titanium implants. Furthermore, cell death was higher for the titanium implants and the sham site than with the amorphous HA-coated implants at 24 h. In contrast, at 72 h, titanium revealed the lowest cell death compared with the crystalline HA-coated implants and sham sites, which, in turn, showed the highest LD activity. Monocytes predominated in the peri-implant exudates around all surfaces and in sham sites. Moreover, no significant differences in the amounts of secreted MCP-1, TNF-α and IL-10 were detected between titanium, HA-coated surfaces and sham sites. Since the material surface roughness was similar, the material-induced differences in the inflammatory response could mainly be attributed to differences in the surface chemical properties. Under these normal conditions, a transient inflammatory response was observed in soft tissues of both titanium and ultrathin coated amorphous and crystalline HA, which was comparable with nonimplanted sham sites after 72 h. Thus, both the titanium and ultrathin HA coatings demonstrate soft tissue biocompatibility.

In **Study II**, which was a cytocompatibility study performed *in vitro*, no difference in peripheral blood mononuclear cell viability was detected between polystyrene, titanium, crystalline HA and amorphous HA after culturing for 24 h and 96 h. A main observation was a higher ratio of adherent cells to supernatant cells as well as a higher TNF-α secretion for amorphous HA in comparison with crystalline HA under normal culture conditions (i.e., without the addition of the inflammatory stimulus LPS). LPS induced 30-fold higher secretion of TNF-α compared with normal conditions, but no differences between the surfaces were detected. A similar level of secreted TGF-β1 was found in cells on all surfaces, irrespective of the presence of LPS. BMP-2 secretion was not detected. Taken together, this study revealed cytocompatible properties for all the materials evaluated and an early peak and transient course of TNF-α secretion. Furthermore, differences in CaP phase composition, nanotopography and wettability may influence the monocyte early response.

In **Study III**, the expression of inflammatory and fibrogenic molecular markers was determined after the insertion of silicone implants in irradiated and nonirradiated human soft tissue. Whereas the PAI gene expression was higher, the SCF gene expression was lower in previously irradiated tissue compared with the nonirradiated control side. After 24 h of implantation, downregulation of IL-8 and upregulation of BCL-2 were detected in the peri-implant tissue in

the irradiated side compared with the nonirradiated side. The antifibrotic transcription factor FOXO1 was significantly downregulated in implant-adherent cells on the irradiated side. Correlation and regression analyses showed that factors related to irradiation (dose and time since irradiation) and its adjunctive therapy (chemotherapy and anti-neutropenic drugs) influenced the gene expression response, both in the irradiated and nonirradiated sides. These observations suggest that previous exposure to irradiation imprints a profibrotic propensity in the irradiated tissue that is expressed rapidly after surgical implantation.

In **Study IV**, an experimental study using titanium implants in rats demonstrated that pre-exposure to irradiation significantly reduced bone-implant contact and implant removal torque in the recipient bone. The irradiation-induced detrimental effects on osseointegration were associated with high gene expression of proinflammatory TNF-α and osteoclastic CatK and reduced expression of bone formation gene ALP in the implant-adherent cells. This was in parallel with high expression of inflammatory cell recruitment MCP-1, proinflammatory TNF-α and pro-fibrotic TGF-β genes in the peri-implant soft tissue. All compartments around the implant in the irradiated site revealed reduced expression of the antifibrotic gene FOXO1.

In conclusion, under normal experimental conditions *in vitro* and in soft tissue, titanium and ultrathin coated HA are associated with cytocompatibility, biocompatibility and a transient inflammatory process, although differences in surface chemistry, nanotopography and hydrophilicity/hydrophobicity can alter the cellular response. In contrast, irradiation of soft and hard tissues causes dysregulation of biological activities in the different compartments at the implant, of which perturbed inflammation and profibrotic propensity seem to hamper tissue healing and regeneration around implanted biomaterials.

7 FUTURE PERSPECTIVES

In recent decades, biomaterials used as implants and prostheses have become increasingly important for the rehabilitation of patients after trauma and cancer treatment. Implants and prostheses are often required to restore function and aesthetics, and sustaining a good quality of life. The biomaterials mostly used for implants are titanium, HA and silicone. To improve their integration with tissue, further knowledge is required regarding the cellular and molecular mechanisms that direct the tissue response towards healing, regeneration and integration vs. protracted inflammation, fibrosis and failure to achieve integration.

It would be interesting to more closely investigate implant-adherent cells in vivo since their response differs from that of the surrounding tissue. Regarding bone, it would be of value to learn more about the role of osteocytes in the abnormal bone remodeling observed at the implant surface in the case of previous radiotherapy since osteocytes have been shown to be influenced by radiation and are in contact with the implant surface. Another relevant pursuit would be to gain more knowledge about the change in the inflammatory response at the implant surface with time from irradiation as well as after implantation. There appears to be a "memory" due to irradiation, including not only exposed cells but also neighbouring and distant cells with dysfunctional expression and secretion of inflammatory cytokines. The exploration of treatments targeting specific proinflammatory mediators in conjunction with postirradiation implant rehabilitation is of considerable importance and might be of significant clinical value. To what extent this "memory" includes altered responsiveness to oxidative stress over time would be interesting to study. FOXO1 seems to be particularly interesting in this context. Moreover, additional knowledge is needed regarding the relationship between other treatments, such as chemotherapy, and the integration of implants

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