On Biological Response and Wear Particles around Oral Implants and Implant Components

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Cover illustration: Titanium particle in mucosa taken by Julia Olander with a Scanning Electron microscope.

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To my family with love, Petrus, Levi, and Harriet

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ABSTRACT

Wear particles released from implant components are gaining interest in the dental literature. In orthopedic medicine, wear particles are known to cause bone loss around medical implants in an aseptic manner and several in vitro studies have shown proinflammatory responses to titanium particles. In dentistry and medicine, several materials are used for implant constructions, with a variation in material properties such as hardness and surface roughness. Theoretically, dissimilarity in material combination may cause aggravation as the materials wear. Due to aesthetical advantages, ceramic abutments made from zirconia are increasingly used in dentistry. Zirconia is harder than titanium, which could lead to more wear and particle release. It is unknow whether wear particles in tissues around dental implants cause peri-implant bone loss.

This thesis investigates whether the combination of materials in dental implant constructions alters biological responses and release of wear particles. Specifically, the studies included in this thesis compare single implant crowns manufactured with two abutment materials regarding clinical outcomes (Study I), in vitro wear on two implant materials (Study II), inflammatory cell responses (Study III), gene expression and presence of particles in soft tissues (Study IV).

In Study I, we retrospectively analyzed data from patients treated at a specialist clinic with implant-supported crowns manufactured with two abutment types – zirconia and titanium. These analyses focused on clinical outcome variables with respect to function up to five years after prosthetic placement. In Study II, we evaluated experimental dynamic loading to compare wear, corrosion, and wear particle generation when

these two abutment materials were used to connect to two types of implant materials. In Study III, we investigated the proinflammatory response to human peripheral blood mononuclear cells (PBMCS) when exposed to two types of disc materials combined with two particle materials**.** In Study IV, we evaluated mucosa biopsies from patients with single implant supported crowns manufactured on two abutment materials, focusing on gene expression and presence of wear particles.

The following conclusions were drawn from the thesis. Study I show that abutment material type was statistically significantly associated with amount of yearly bone loss and accumulated five-year bone loss in this cohort but did not affect occurrence of technical complications. Implants with zirconia abutment showed an increase in bone loss but a decrease in technical complications compared to titanium. However, limitations in this finding include small sample size and only slight differences in bone loss values, which may not be clinically relevant. Study II shows that all implants had signs of wear irrespective of abutment material. No clear difference was seen comparing material combinations. Particles were released from the implant-abutment junction and the internal connections harbored wear particles inside the implants. More particles were released when using zirconia abutments. Study III shows that titanium particles and discs generated a higher proinflammatory response compared to zirconia. Neutrophils reacted to zirconia particles by releasing neutrophilic extracellular traps (NETs), which was not seen when exposed to titanium particles. In Study IV, we found titanium wear particles in soft tissue samples and zirconia particles on implant heads. More particles were found in mucosa around zirconia abutments. Gene expression showed upregulation of several proinflammatory genes when using zirconia abutment compared to titanium abutment. Wear particles may trigger pro-inflammatory reactions in the peri-implant mucosa.

Keywords: Dental implants, zirconia, titanium, wear particles, gene expression
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SAMMANFATTNING PÅ SVENSKA

Partikelbildning vid belastning av dentala implantat är ett nytt fokusområde inom odontologisk forskning. I medicinsk litteratur har det visats att partiklar kan orsaka bakteriefri benförlust runt medicinska implantat. Cellstudier har visat utsöndring av pro-inflammatoriska cytokiner när immunceller växer i närvaro av titanpartiklar. Runt dentala implantat har titan partiklar hittats i mjukvävnad och ben. De material som används i dentala implantat stödda konstruktioner har olika egenskaper, och varierar i hårdhet, kemi och yt-råhet. Kombination av materialtyper påverkar grad av slitage. Det är dock oklart i nuläget om frisättning av partiklar runt dentala implantat kan vara en bidragande orsak till benförlust.

Avhandlingen syftar till att jämföra biologisk respons och slitagepartikelbildning mellan olika biomaterialkombinationer i dentala implantat. Avhandlingens olika delstudier syftar till att jämföra det kliniska utfallet av singelimplantatkonstruktioner med två olika distansmaterial (studie I), jämföra slitage mellan olika materialkombinationer (studie II), utvärdera inflammationssvaret hos PBMC celler växandes i närvaro av två olika material och vid tillsättning av partiklar (studie III), och genuttryck samt partikelförekomst i mucosa runt dentala implantatkonstruktioner (studie IV).

I studie I, granskades patientjournaler avseende patienter behandlade med singelimplantat och två distansmaterial typer fem år tidigare. Utfallet av benförluster och tekniska komplikationer bedömdes. I studie II jämfördes fyra materialkombinationer med avseende på slitage, korrosion och bildning av slitagepartiklar i ett dynamiskt belastningstest. I studie III mättes frisättning av pro-inflammatoriska cytokiner från immunceller (PBMCs) växandes på olika materialytor och vid tillsättning av olika typer av partiklar. I studie IV undersöktes patienter med singelimplantatkonstruktioner och två olika materialkombinationer kliniskt, samt genom analys av vävnadsprover där genuttryck och förekomst av slitagepartiklar undersöktes.

Följande slutsatser kan dras av avhandlingens resultat:
Studie I: Användning av zirkonia distans var statistiskt signifikant associerat till högre grad av benförlust mellan årskontrollerna och

ackumulerat efter fem år i funktion. Implantat med titandistans hade fler fall av teknisk komplikation, men denna skillnad nådde inte statistisk signifikans.
Studie II: Synligt slitage på både implantat och distanser förkom hos alla testade materialkombinationer där ingen skillnad uppmättes dem emellan. Fler partiklar syntes frisättas från de implantat med zirconia distans. Partikel frisättning kunde mätas men en del större partiklar var lokaliserade innanför den interna kopplingen på implantatet.
Studie III: Celler som odlats tillsammans med metallpartiklar uppvisade högre grad av pro-inflammatoriska cytokiner jämfört med de som odlats tillsammans med keramiska partiklar.
Studie IV: I biopsier identifierades partiklar av varierande storlek och material. Fler partiklar fanns i vävnaden runt zirkoniadistanser. Ovanpå implantathuvudet detekterades keramiska partiklar hos de prover där implantatet var kopplat till keramisk distans. Genuttrycket skiljde sig mellan mucosan runt keramiska- respektive metalldistanser, där ett flertal proinflammatoriska gener uttrycktes i högre nivåer runt de keramiska distanserna.

LIST OF PAPERS

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

I. J. Olander, A. Wennerberg and V. F. Stenport. Implant-Supported Single Crowns with Titanium or Zirconia Abutments: A Retrospective Up-to–5-year Follow-up Study. The International journal of prosthodontics 2022 vol:35 iss:4, 387–395

II. J. Olander, A. Ruud, A. Wennerberg, and V. F. Stenport. Wear particle release at the interface of dental implant components: Effects of different material combinations. An in vitro study. Dental Materials 2022 Vol. 38 Issue 3, 508–516

III. J. Olander, AK. Östberg, K. Christenson, P. H. Johansson, A. Wennerberg, V. F. Stenport. Inflammatory response to wear particles: comparisons between zirconia and titanium in vitro. *In manuscript*

IV. Olander J, Barkarmo S, Hammarström Johansson P, Wennerberg A, Stenport VF. Inflammatory Gene Profile and Particle Presence in Peri-Implant Mucosa: a Pilot Study on 9 Patients. J Oral Maxillofac Res 2023;14(3):e2

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ABBREVIATIONS

3Y-TZP	3 mol% yttria stabilized tetragonal zirconia polycrystal
ARG1	Arginase -1
ASTM	American Society of Testing and Materials
BoP	Bleeding on probing
CAD/CAM	Computer aided design /computer aided manufacturing
CP	commercially pure
DAPI	4',6-diamidino-2-phenylindole
DAMPS	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
EDX	Energy-dispersive X-ray spectroscopy
EFSA	European food safety authority
EU	European union
FGF- basic /FGF2	Basic fibroblast growth factor
G-CSF/CSF3	Granulocyte colony stimulating factor
GM-CSF/CSF2	Granulocyte-macrophage colony-stimulating factor
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma atomic emission spectroscopy
IFN-γ	Interferon gamma
IL10	Interleukin 10
IL-17	Interleukin 17
IL-1β	interleukin 1 beta
IL-1ra	Interleukin 1 receptor antagonist
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-9	Interleukin 9
IFN-γ	Interferon gamma
IP-10 /CXCL10	Interferon gamma-induced protein 10
ISO	International Organization for Standardization
MCP-1 /CCL2	Monocyte chemoattractant protein 1
MIP-1α/CCL3	Macrophage Inflammatory Protein-1 Alpha
MIP-1β/CCL4	Macrophage Inflammatory Protein-1 beta
MMP8	matrix metalloproteinase 8

MMP9	Matrix metallopeptidase 9
N	Newton
NE	Neutrophil elastase
NETs	Neutrophil extracellular traps
NIOM	Nordic institute of dental materials
OPG	Osteoprotegerin
OR	Odds ratio
PBMCs	Human peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PEEK	Polyether ether ketone
pH	Potential of hydrogen
PMMA	Poly methyl methacrylate
PPIA	Peptidylprolyl isomerase A
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES /CCL5	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
Sa	Surface roughness
Sdr	Developed interfacial area ratio
Sds	Density of summits
SEM	Scanning electron microscope
Ti	Titanium
Ti-6Al-4V	Titanium-aluminum-vanadium
Ti-6Al-4V ELI	Titanium-aluminum-vanadium extra low interstitial
TiO_2	Titanium dioxide
Ti-Zr	Titanium-zirconium alloy
TNF-α	Tumor necrosis factor
TRAP	Tartrate resistant acid phosphatase
TREM1	Triggering receptor expressed on myeloid cells 1

1 INTRODUCTION

1.1 BIOMATERIALS

Biomaterials used for rehabilitative purposes exist in both medical and dental implantology. The literature on biomaterials has introduced several definitions. For example, in 1991, a conference in the United Kingdom defined a biomaterial as follows:

"Any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual. [1]"

However, this definition does not consider the structure of the material or the biological response when implanted. Moreover, biomaterials can be classified based on their chemical structure, tissue interaction capacity, or origin of the material [1].

Biomaterials can be used to deliver drugs, act as immunotherapy, and replace tissues such as in the form of medical and dental implants [2]. For example, biomaterials used in dental rehabilitation are exposed to various oral environments, such as occlusal forces from the dentition and acidic elements derived from oral bacteria and diet. The materials need to be biocompatible and nonallergic or noncytotoxic to the nearby tissues. Finally, aesthetics is a concern when selecting replacements for teeth and surrounding tissue.

1.2 DENTAL IMPLANTS

Since it was first discovered that titanium can osseointegrate with bone, dental implants have been developed to rehabilitate partial and complete edentulism. The implants are anchored through osseointegration in bone to support a crown or bridge construction via the transmucosal abutment. In Sweden, approximately 100,000 dental implants are placed every year according to statistics from the Swedish Social Insurance Agency [3]. After ten years of function, the survival rate of implant

constructions is above 90% [4, 5], indicating a safe and functional therapy for rehabilitating of lost teeth. However, survival does not mean without complications as will be discussed further in this thesis.

1.2.1 SURGICAL PROCEDURE

In dental implantology, the first step of successful implant treatment is careful planning of surgical and prosthodontic protocols. Several surgical approaches exist. However, the implant placement starts with local surgery where a series of drills are used to create a bone cavity matched for the specific dental implant [6]. After placing the implant, post-surgical healing varies depending on the surgical method used. In the two-step approach, the implant is completely covered by gingiva and left for undisturbed submerged healing for 3–6 months. The one-step approach includes immediate placement of healing abutments protruding the mucosa with either immediate or delayed loading by a prosthetic construction [7]. Another approach is to place implants immediately in fresh extraction sockets. Surgical method and healing time affects the outcome results. Systematic reviews have found more marginal bone loss and early implant loss for immediately placed implants in extraction sockets compared to when allowing some healing time [8, 9]. A recent systematic meta-analysis found the two-step method to be associated with fewer implant failures (i.e., loss of implant) compared to the one-step procedure but found no difference in marginal bone loss or post-operative infections between the two techniques [7].

SURGICAL DEBRIS AND BIOLOGICAL RESPONSES

Several sources of material debris that release into nearby tissues during surgical treatment have been proposed. Surgical drills used for bone preparation undergo surface deformation and increased surface roughness over time [10], which could result in metal debris in the alveolar bone. Post-surgical healing includes covering the implant with cover screws or healing abutments of various materials, such as titanium, zirconia, and the polymers polyetereterketone (PEEK) and polymethyl methacrylate (PMMA) [11]. When soft tissue is in direct contact with cover screws, metal particles such as titanium, vanadium, and aluminum are sometimes found [12]. Self-cured PMMA materials used as healing abutments increase the risk of monomer residuals

leaking into the mucosal tissues during polymerization [11]. A clinical study on 22 implants found that PEEK, presently used in healing abutments, induces a higher degree of inflammation compared to titanium [13]. Furthermore, remnants of surgical sutures used to secure the wound can result in pro-inflammatory reactions. Dapunt et al. found proinflammatory response to suture both in vitro and in bone tissue samples from retrieved knee implants [14]. The size of the particles may alter the response. For example, Lovric et al. found that micrometer suture debris compared to larger debris created a higher subcutaneous inflammatory response in rats [15].

1.2.2 SOFT TISSUE AROUND DENTAL IMPLANTS

The soft tissue around dental implants is referred to as mucosa. This terminology reflects the difference in tissue structure compared to gingival tissue around natural teeth. Although the mucosa has sulcular epithelium and junctional epithelium, no root cement exists, which causes a difference in the orientation of collagen network fibers [16]. After surgical implantation, the mucosa starts a healing process, which is completed approximately 6–8 weeks post-surgery [17]. The process of soft tissue healing has been described in animal and human sample models. Animal models reveal several important steps during healing, beginning with an initial coagulum formed between the implant and mucosa. This is followed by early infiltration of neutrophils and clustering of leukocytes in a fibrin network. After one week of healing, a collagen structure and fibroblast can be seen and by week two connective tissues contain vascular structures adhering to the implant. After 4 weeks, the barrier epithelium has formed [18]. A similar process in human mucosal samples has been found: an early healing process of connective tissues occupied with inflammatory cells and a later healing process (after 12 weeks) of well-organized and the well-defined connective tissues and barrier epithelium [19]. These results demonstrate the activity of the inflammatory system during mucosal healing. Similarly, Tomasi et al. found that human mucosa samples contained macrophages and polymorphonuclear cells in the connective tissues during the first 12 weeks of healing [20].

BONE FORMATION AROUND DENTAL IMPLANTS

The bone healing around dental implants involves a process called osseointegration. An osseointegrated implant is defined as "direct contact between implants and bone at the resolution level of the light microscope" [21]. Several cells participate in bone formation and remodeling around an implant. The osteoblasts, the main bone forming cell, cooperates with osteoclasts responsible for bone resorption [22]. The most common cell type in bone are osteocytes, which act as a mechanosensory inside the bone [23]. These cell types interact during bone formation and bone resorption by signaling and receiving molecules. Osteocytes cause differentiation of unmature osteoclast by releasing the Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) protein leading to a stimulation of bone resorption. Both osteocytes and osteoblast can also release osteoprotegerin (OPG), a decoy receptor for the RANKL protein, which inhibits the osteoclast to mature [23]. The immune system is also involved in the bone formation and bone resorption process, including cytokine release from macrophages and lymphocytes [23]. Bone formation around dental implants takes several steps: the initial blood clot formation around the surgically-placed implant; the subsequent replacement by formation of woven bone (a less structured bone); and the formation of lamellar bone around the implant [24]. Bone remodeling (i.e., adaptation of bone mass) continues as a reaction of mechanosensory stimulus [25].

BONE AUGMENTATION

Sometimes bone augmentation is necessary because the amount of bone is not sufficient for implant insertion. Several possible techniques exist, such as using autogenous bone and autologous bone graft materials [26]. However, bone augmentation therapy can be unsuccessful, especially in medically compromised patients. Several complications have been shown, such as infections, compromised hard and soft tissue healing, and a fibrous tissue layer forming between the graft and bone [27]. There are some studies on implant treatment in augmented bone. A systematic review published in 2019 found that implants with simultaneously placed lateral bone augmentation showed no difference in marginal bone loss compared to native bone, although the authors note a lack of studies on this subject [28]. Another systematic review looked at short dental

implants (<10 mm) and standard length implants (≤ 10 mm) placed in augmented bone and found both groups have high survival rates (96.7% and 97.3%, respectively) with no significant difference in marginal bone loss, although the follow-up was limited to one year [29]. In contrast, Jemt et al. found an enhanced risk of peri-implantitis and late implant loss in patients with bone graft with autologous bone in a large retrospective study following 1017 patients for up to 10 years [30].

1.2.3 IMPLANT MATERIALS

TITANIUM

Titanium is an element found in rocks in several regions such as North America and Scandinavia [31]. Since the discovery of titanium's ability to fixate in bone around the 1960s, it has been used as a bone anchor in medical and dental implant constructions [32]. In dentistry, titanium is compiled of 99,5 % titanium and other elements such as carbon, oxygen, iron, nitrogen, and hydrogen, which make up the residual 0,5 % [31]. The American Society of Testing and Materials (ASTM) classifies titanium (grades 1–4) based on purity measured by the amount of oxygen involved. That is, titanium becomes increasingly harder as the amount of oxygen and iron increases, so titanium grade 4 is harder than grade 1. This standard also includes alloys (Ti-6Al-4V and Ti-6Al-4V ELI) used as dental biomaterials [33]. The diverse types of titanium used in dentistry all have unique properties and hardness. Moreover, incorporation of other metals such as vanadium and aluminum creates an alloy that is both harder and more fatigue resistant [33]. As titanium has low density, constructions can be light [34]. The material has several other characteristics such as the ability to withstand corrosion, mechanical strength, and chemical stability, characteristics that further add to its biocompatibility in the oral tissues [35]. When a titanium surface is exposed to oxygen, an immediate reaction occurs, resulting in an oxide layer; this process is called passivation. This oxide layer contributes to the capacity to withstand corrosion [35].

New theories on osseointegration challenge previous beliefs on titanium's biocompatibility. These theories posit titanium as the cause of a low degree of continuous inflammation, which results in

encapsulation of bone as a result of a foreign body reaction [36]. This has been disputed in a recent review, which claims that the dental implant exists in a homeostatic or steady state under healthy conditions [37].

TITANIUM ALLOYS

Titanium can be strengthened by alloying it with other metals. Several of these titanium alloy materials are used in implant dentistry, such as Ti-6Al-4V (also called grade 5) [38] and titanium-zirconium, although commercially pure (CP) titanium still remains the most common implant material in dentistry [39]. Ti-6Al-4V is more resistant to fatigue than CP grade 1–4 titanium, but it runs the risk of releasing aluminum and vanadium ions, substances that are harmful to surrounding tissues [40]. The Ti-6AL-4V alloy, also called Ti grade 5, is 40% stronger than CP-Ti grade 4 (480/850 mPa yield strength) and therefore is widely used in orthopedic medicine [38]. However, there is little clinical evidence on the survival and success rates for dental implants made from this alloy. One review claimed success rates similar to CP Ti after ten years [38], although this review is lacking in both systemization and number of included articles.

Implants made from titanium-zirconium (Ti-Zr) alloys have also demonstrated 40% higher strength compared to CP 4 titanium in vitro [39]. This improved strength could support the use of this alloy for narrow diameter implants, where a lack of bone width can limit the possibility for implant placement. In vivo studies have shown bone-implant contact or percentage of bone formation on the implant surface to be similar to those for titanium implants [41]. The clinical performance of Ti-Zr implants, according to one systematic review of nine studies, has a 97.3% success rate after two years [42]. However, the need to change Ti-Zr implants in narrow diameters is uncertain. A systematic review with a one-year follow-up did not find any differences in implant failures or marginal bone loss comparing Ti-Zr implants ($\leq$ 3.5 mm) with narrow titanium implants [43].

CERAMIC IMPLANTS

Ceramic alternatives have gradually been introduced to the dental implant market during the past 20 years. Initially, ceramic implants were made of aluminum oxide; however, due to uncertain long term results, the implants were withdrawn from the market in the 1990s [44]. The first abutment made of zirconia was made by a Swiss company in 1997 [45], and the first dental zirconia implant system was developed in 1987 [46]. Zirconia is a polymorphous material that transforms upon thermal changes. The monoclinic phase in zirconia is the largest and its volume shrinks about 4% when reaching the tetragonal phase (at about 1170 ºC). The cubic phase, also the smallest volume, is reached at about 2370 ºC [47]. A stabilizer is added as changes in volume can produce cracks in the material, and zirconia can revert to monoclinic phase at room temperature. Usually, the stabilizing material is made of yttria but ceria and alumina has also been used in dental products [47]. The main zirconia used in dentistry is stabilized with 3 mol% yttria [48]. Crack formation in the material can be counteracted with some minor phase changes. When a micro crack starts progressing in the material, stresses on the grains in the ceramic material results in a volume expanding phase shift nearby, where the tetragonal structure turns monoclinic. This expansion leads to a suppression of the crack and minimizes crack propagation [44].

Initially, the implant system was based on the one-piece system, a combined implant and abutment construction. During the last years, new zirconia implants with a removable abutment have been introduced; this is referred to as a two-piece system. Clinical results on zirconia implants are scarce as compared to titanium. A systematic review from 2020 showed one-year survival rates for zirconia implants (one and two piece) above 90% [49]. In a study published in 2021, Borganovo et al. followed 26 one-piece zirconia implants over ten years and found 100% survival rate [50]. However, a recent study by Kohal et al. found only a 78.2% survival rate following one-piece zirconia implants for five years (n = 66 implants) [51]. Regarding two-piece implants, Lorentz et al. followed 19 patients for approximately 15 months and found no implant failures [52]. Brunello et al. followed 30 patients with two-piece zirconia implants over nine years and found only one implant failure [53]. The

results indicate acceptable results for ceramic implants although only on small sample studies.

One important factor regarding implant success is biological response to the biomaterial. Bienz et al. compared 42 patients with titanium or zirconia implants without supraconstructions in an experimental mucositis test (i.e., no brushing of implant for 3 weeks). The author found lower plaque values and bleeding on probing in the zirconia implant group. However, no histological difference was seen when examining inflammatory cells in the soft tissue biopsies [54].

SURFACE TREATMENTS AND SURFACE ROUGHNESS

Initial stability and rapid osseointegration of dental implants might be improved when the surface of implants is treated [55]. Various rough implants have been used in dentistry such as the originally machined surface with a surface roughness (Sa) value of around 0.5 μm and below, a moderately rough surface with a Sa value between 1–2 μm, and rougher implants with Sa value above 2 μm [56].

Surface modifications of dental implants include subtractive and additive methods [57]. Subtractive methods involving sand blasting and etching with acids on the implant surface allow for a higher bone-to-implant contact percentage compared to smoother machined surfaces in vivo [58]. Anodization or anodic oxidation is a electrochemical method using voltage to increase the oxide layer from its natural thickness of 2–5 nm up to hundreds of micrometers, a technique that creates a porous surface [59]. In vitro testing has shown a rougher surface when anodized titanium discs are used; however, this did not affect cell proliferation or viability compared to regular titanium and zirconia discs [60]. Coating implants with a plasma spray of titanium or hydroxyapatite also increases the surface roughness of the implants [57]. These surface modifications are shown to affect the survival of the implant. A meta-analysis has shown that anodized surface-treated implants are less likely to fail relative to turned implant surfaces, although no difference was reported on marginal bone loss values [61]. Furthermore, a systematic review has shown that implants with a moderately rough surface have higher survival rates after 10 years compared to turned and more rough implant surfaces. However, lower marginal bone loss values were seen

for the turned surfaces [62]. Furthermore, in a large retrospective analysis following patients from 1986 to 2013, one specialist clinic found a clear trend of less early implant failure when switching from machined to moderately rough implants [63].

CONNECTION DESIGN

There are mainly two types of implant head macro designs: external platforms, where the hexagon is placed externally, and platforms that are connected internally using anchorage inside the implant either by an internal hexagon or a conical taper. The influence of the platform design on the biological short-term and long-term outcomes is not clear. A systematic review found lower marginal bone loss values for internally-connected implants compared to externally-connected implants [64], but another systematic review found no difference in marginal bone loss [65]. Nonetheless, technical complications appear to be affected by type of connection. External connections suffer from more abutment screw loosening, and internal connections have a higher frequency of reported porcelain chipping of the crown structure [65]. During load of a dental implant, the implant components experience stress. A finite element analysis model showed a variation in stress levels in experimental bone between two implant systems (internal and external hex) and found the highest stress to be located around the abutment-implant connection, with higher stress in 30 degree loading situations compared to zero degree loading situations [66].

1.2.4 ABUTMENT MATERIALS

The abutment is the transmucosal part of the implant-supported construction. In single implant crowns, the abutment can be covered by porcelain fused on the core material [67] or it can designed as two separate parts where a crown is cemented on the abutment [68]. Cemented implant crowns entail the risk of excess cement leading to inflammatory reactions in the peri-implant tissues [69]. The abutment is screwed on to the implant and connected via an external or an internal connection as previously mentioned. However, one-piece implants, where the implant and abutment are made as one part, are available. In these cases, a separate crown is cemented directly on top of the implant without any screw joint [6]. Abutments can be prefabricated or

individually designed using CAD/CAM techniques. A recent systematic review comparing the clinical outcome between these manufacturing techniques found no statistically significant differences, although the review included only short follow-ups (1–3 years) [70].

Several abutment materials are available in dentistry such as titanium, titanium alloys, gold alloys [65], cobalt chromium [71], and ceramics such as alumina and zirconia [65]. In Sweden, there is no official data registry for abutment material choice in dental clinics. In the dental literature regarding abutment materials on single implant constructions, the focus has primarily been on zirconia, titanium, and its alloys.

TITANIUM ABUTMENTS

Titanium has been the preferred abutment material for decades [72]. Dental implants with metal abutments including titanium exhibit very high survival rates–close to 97.5% in one systematic review [73]. A major issue with titanium is the risk of the metal producing a darker shading that is visible through the thin gingiva, not an aesthetically desirable outcome, especially for the front teeth. However, surface alterations of titanium abutments can alter their appearance, creating colors ranging from gold-yellow to pink to enhance aesthetics. One technique alters the surface by anodization, causing thicker oxide layers on the surface, creating a new exterior color [74]. Alternatively, titanium abutments can be coated with a thin titanium-nitride layer, creating harder and a more wear resistant surface, which appears yellow [75]. Little evidence on surface alterations of titanium abutments and clinical success is available. One systematic review found that abutments coated with titanium nitride show promising results, although there are few studies on the type of abutments [75]. In addition, only a few clinical studies on anodized surfaces exist. A study found no difference in peri implant health compared to regular titanium, although the same study found no aesthetic enhancement [76]. Another study found the color difference in mucosa around anodized titanium abutments compared to natural teeth to be smaller compared to regular titanium [77].

CERAMIC ABUTMENTS

The introduction of ceramic abutment materials offers the clinician and patient a metal-free option. A more tooth-like appearance can be achieved, particularly in the frontal region, using white abutments, which do not create a dark metal shading in the thin mucosa covering, a finding that has created a market for zirconia implant abutments [72]. In addition, these abutments have been proposed to allow for better soft tissue aesthetics compared to metal abutments [78]. However, a limit of clinical evidence exists. Zembic et al found 96. 3 % of the 27 implants with zirconia abutments to be in function after 11 years [79]. Another study followed 23 patients for 10–11 years and found a high survival rate for implants with zirconia abutments [80]. Additionally, a systematic review of nine studies comparing zirconia and titanium abutments on single implants measuring marginal bone loss values showed no statistical significant differences [72]. Another systematic review including 53 studies looked at biological complications related to implants. The study defines biological complications as "soft tissue complications, soft tissue recessions, and substantial (>2 mm) marginal bone loss" [65]. This review found that internally-connected single implants with ceramic (zirconia and alumina) abutments had significantly higher frequency of biological complications compared to the metal counterparts [65]. In contrast, another systematic review concluded that implants connected to titanium abutments were associated with more bleeding on probing and plaque accumulation compared to zirconia abutments [81]. Similarly, another study found more soft tissue inflammation (BoP) around implants with titanium abutments than around implants with zirconia abutments [82].

However, a major issue is fractures. Because zirconia abutments are brittle, especially when used in internal connections, they tend to fracture more often than metal abutments [83, 84]. The issue of brittle zirconia internal hexes has been addressed by adding a titanium base that is either screwed together with the zirconia abutment or cemented in the laboratory or clinic. These two-piece abutments have shown good fractural strength in laboratory studies: zirconia abutments with a titanium base showed more resistance to loading than conventional zirconia abutments, although not as high as titanium abutments [85].

Basically, this is a return to a metal abutment in some sense. Clinical studies on titanium-based abutments or hybrid abutments, as they are also called in the dental literature, are few and little long-term follow-up data exists [86, 87]. Concerns have been raised on loss of cement retention between the titanium base and ceramic component. Various treatment modalities have been introduced to reduce this complication, including chemical and mechanical surface treatments such as sandblasting the mating surfaces and using resin cements [87].

1.2.5 IMPLANT ABUTMENT JUNCTION AND MICROMOVEMENT

The implant abutment junction (IAJ) is the interface created between the implant and the abutment [88]. A tight seal is preferable as micro gaps could result in microbial leakage [89]. Measured micro gaps in the dental literature vary from a tight fit of 1–2 μm [88] up to a wider fit of 49 μm [90]. Furthermore, larger micro gaps between implant and abutments may cause misfits resulting in an increased movement between the implant and abutment, defined as micromovement [91]. In experiments, the micromovements in the IAJ during chewing have been measured to range up to 94 μm [92]. Furthermore, micromotion could cause fretting wear on the components, leading to an enlargement of the micro gap [93].

1.3 COMPLICATIONS RELATED TO ORAL IMPLANT SUPPORTED PROTHESES

Complications following implant treatment can be of a biological or a technical nature. Technical complications include implant fracture, bridge or abutment fracture, screw loosening, screw fractures and chipping of porcelain veneer. Biological complications include soft tissue recession, marginal bone loss, peri-implant mucositis, peri-implantitis, and implants loss. Occurrence of complications varies in the available literature. In one recent study of 2,666 patients with single, partial, or full jaw implant supported constructions, 42% of the implants over 9 years had technical and/or biological complications and most complications were biological [94]. However, a meta-analysis published

in 2014 on single, partial, and full jaw implant supported constructions showed most of the complications to be technical (16-53%) and fewer to be biological (6%) during a five-year period [95].

1.3.1 BIOLOGICAL COMPLICATIONS

FAILURE OF IMPLANT TO OSSEOINTEGRATE

After implant insertion, the tissues surrounding the implant start the delicate process of healing. Disturbance in this process can cause failure of the osseointegration, resulting in early implant loss. Early failures of implants to osseointegrate are most commonly seen during the first year of function according to a large retrospective analysis [96]. There are several proposed explanations for why the implants lose bone retention during the early healing phase: poor primary stability, over loading, lack of initial bone and/or other implant, and patient-related factors [97, 98]. A recent large retrospective analysis found that the shift from low surface roughness to moderate surface roughness in implant dentistry resulted in a lowered occurrence of early implant loss in the maxilla [63].

MARGINAL BONE LOSS

A normal process of bone remodeling during healing is a common observation during the first year of function [99]. Several definitions exist for what constitutes acceptable marginal bone loss values. Kathic et al. propose a definition of success to be > 0.2 mm of marginal bone loss annually following the first year of function [100]. The International Congress of Oral Implantologist Pisa Consensus Conference in 2008 concluded that “the bone loss measurement should be related to the original marginal bone level at implant insertion, rather than to a previous measurement (e.g., 1 year prior)” but added that the most important criteria to assess implant health is absence of pain or mobility [101]. The reason for marginal bone loss around dental implants is disputed. A consensus report from 2008 stated that marginal bone loss with signs of mucosal inflammation is a result of bacterial infections– i.e., peri-implantitis [102]. The prevalence of peri-implantitis varies greatly in the dental literature. One systematic review found that the prevalence ranged between 1% and 47% [103]. Bacteria have been

found in the mucosal pockets around implants. Persson et al. found several bacteria species associated with periodontitis in mucosal pockets around healthy and peri-implantitis diseased implants [104].

In contrast, Albrektsson et al. argues that merely by placing a dental implant (i.e., a foreign body of some sort) activates the immune system, which could result in marginal bone loss [105]. Furthermore, titanium particles from wear on the implant head have been proposed as another possible cause of inflammation in peri-implant tissues, although there is no evidence of a causal relationship in the current dental literature [37, 106]. Titanium particles have been found in tissues around peri-implantitis diseased implants [107-109]. Safioti et al. found more titanium ions in plaque from implants diagnosed with peri-implantitis than from healthy implants [110]. Moreover, Pettersson et al. found higher amounts of titanium ions and particles in the soft tissue around implants with peri-implantitis than in samples from teeth with periodontitis [111].

INFLAMMATION OF THE PERIIMPLANT MUCOSA

Peri-implant mucositis is defined as an inflammation of the mucosa surrounding the dental implant without any signs of contemporary marginal bone loss [112]. Some authors consider plaque accumulation around the dental implant as the key reason for developing mucosal inflammation [112, 113]. However, other researchers regard placement of a dental implant as a trigger of a chronic inflammatory response much like that of a foreign body response [114]. According to a systematic review, prevalence values of peri-implant mucositis varies greatly, ranging between 19% and 65% [103], possibly due to difference in disease characterization and variations in the patient group.

SOFT TISSUE COMPLICATIONS

Using a removable prosthesis may cause mucosal wounds superior to the submerged implants due to compression and movement of the prosthesis [115]. This complication affects 4–13.7% of the implants [116]. However, this complication may not cause severe implant failure if corrected by surgical closure and prothesis adjustment to secure reduced loading of the area [117].

Buccal mucosal recession, where the mucosa partially does not cover the implant, can lead to dissatisfying aesthetics if it is visible in the aesthetic zone [118]. The reason for recession of the mucosa is not clear, but several reasons have been proposed, such as mucosal thickness, malposition of implants, implant angulation, thickness of covering bone, and amount of keratinized mucosa [118].

1.3.2 TECHNICAL COMPLICATIONS

Several types of technical complications are reported in the dental literature with various prevalence rates. In two recent studies, technical complications occurred in 9% of the single implants during a five-year follow-up [119] and in 25% of the patients receiving single tooth or partial or full jaw restoration after 9 years [120]. A retrospective study of patients receiving dental implants (single tooth or partial or full jaw restoration) at a specialist clinic in Sweden found that 32% of the patients had experienced a technical complication after up to 15 years of function [121].

FRACTURES OF IMPLANTS AND COMPONENTS

Fractures of dental implants are a rare but severe complication. In one narrative review, the occurrence of implant fractures ranged between 0.2% and 1.4% with a 5–10-year follow-up [122]. One large retrospective study found that the factors associated with implant fractures were implant width and length, bruxism, and implant material; the prevalence of implant fracture was evident in 0.44% of the implants (external and internal platforms and single, partial, and full jaw implant constructions) [123]. However, in a systematic review focusing on anteriorly placed implants, no evidence was found for higher numbers of abutment or implant neck fractures on narrow diameter implants after a five-year follow-up [124]. In another systematic review, the prevalence of fractures of the abutment was estimated to be around 0.5% for single implants over five years, and these fractures occurred more frequently for ceramic abutments (regardless of connection type) than for metal abutments [65]. A large retrospective study of single tooth and partial or full jaw restoration reported that fractures of abutment screws were a more common complication, occurring in 10% of the patients up to 15 years of function [121]. In yet another systematic review, it was

concluded that the 10-year cumulative complication rate was 20.8% for abutment screw fractures on full jaw prostheses (metal-acryl and metal-porcelain) [125].

DETACHMENT OF IMPLANT CROWNS/BRIDGES

A common technical complication is the loss of crown retention, either through loosening of the abutment screws or debonding of the implant crown cement. In a recent study following patients up to 15 years (single implants and partial or full jaw restorations), abutment screw loosening was reported in 28% of the patients and debonding or loss of retention in 22% of the patients [121]. In another study, 2.8% of the single implants with external connection were reported with abutment screw loosening after up to five years of function [119]. A systematic review focusing on fixed complete dental prosthesis reported a cumulative 10-year abutment screw loosening rate of 18.5% [125].

FRACTURES OF PORCELAIN AND ACRYLIC

Other common complications include fractures of the acrylic and porcelain veneering on dental implant crowns and bridges. A meta-analysis reported a 10-year cumulative complication rate of 66.7% for full jaw prostheses [125]. In a recent large retrospective study (single, partial, and full jaw replacement), fractures of porcelain were reported in 16% of the patients and acrylic fractures in 6% of the patients. Severe fractures of the implant crown–i.e., constructions that could not be polished in the clinic–were found in 4% of the patients [121].

In conclusion, technical complications are common, mainly affecting the supraconstruction rather than the implant itself, allowing for chair-side repair or adjustment at the dental technician.

1.4 PARTICLES AROUND DENTAL IMPLANTS

In recent years, researchers have questioned the biocompatibility of titanium, with one potential factor being very important: particle release from the implant during function [106]. Some propose that the particles are derived from the initial insertion of the implant or from wear at the implant abutment interface during function and loading [126]. A systematic review concluded that there is evidence in several studies of titanium remnants in peri-implant tissues after implant insertion [89]. However, Sridhrar et al., in an experiment that tested metal release in simulated bone with different density, found no metal particles after insertion irrespective of bone density [127]. In contrast to this, Pettersson et al found metal content after implant placement in pig jaw bones, where rougher surfaced implants displayed more metal shedding in to bone compared to machined surfaces [128]

In orthopedic research, many articles report on aseptic loosening–i.e., implant loss caused by inflammation in the absence of bacteria. One of the most discussed reasons for aseptic loosening is released metal and plastic particles due to wear [129]. According to the Swedish Arthroplasty Register the most common reason for revision of hip implants in Sweden is aseptic loosening [130]. A recent review concluded that choice of mating surface materials can affect the overall survival of hip implants, where ceramic on polyethylene or ceramic on ceramic had the best results and metal on metal the worst [131]. In orthopedic medicine, ceramic on metal is a rather uncommon joint replacement combination in the UK [132], the US [133], and Sweden [130]. In Sweden, the most common material combination in joint replacements is metal on highly cross-linked polyethylene (HXLPE) [130]. In a recent randomized controlled trial comparing material couplings in total hip replacements, researchers found metal on metal (cobalt chromium) to initially produce more metal ions measured in blood compared to ceramics (zirconia toughened alumina) on metal; however, after three years, the ceramic group had increased blood ion levels, and during revision surgery of two patients, extensive wear debris on the ceramic femoral head was found [134]. However, the difference in loading conditions compared to dental implants is obvious. Hip implant construction parts are supposed to withstand high loading conditions while moving against each other during activities such as

walking and running [135]. In addition, increased physical activity and load leads to more wear on the hip implants [136].

Oliveira and co-workers [137] concluded that metal particles and ions stimulated inflammatory response and activated osteoclasts in peri-implant tissues and degenerative changes in macrophages and neutrophils after phagocytizing of titanium particles. Furthermore, Pettersson et al. [138] demonstrated that in vitro titanium particles stimulated a proinflammatory reaction. Titanium ions did not promote inflammation but had a cytotoxic effect. Another study concluded that the reaction with particles depends on the metal particle size, shape, concentration, and chemical composition [139]. In vitro studies have also demonstrated that cleaning or scaling implants as a part of peri-implantitis treatment could further result in titanium debris [89]. The oral cavity is also exposed to corrosive substances from diet or bacteria acids, which can affect the titanium surface chemically [140].

MATERIAL EFFECT ON WEAR

When two materials with different hardness are connected, there is always a risk of wear on the softer material. Two recent studies have shown that zirconia abutments produce more fretting wear on the titanium implant [141, 142]; this finding is expected given that zirconia is five times harder than titanium (1200 HV and 220 HV, respectively) [143] [144]. However, in contrast to these findings, a laboratory study test designed to measure both wear and corrosion found five times more wear on a titanium-titanium interface compared to a zirconia-titanium interface [145]. A recent systematic review containing nine studies concluded that more wear was evident on titanium implants connected to a zirconia abutment than a titanium abutment. Moreover, the authors found an increased misfit or presence of a micro gap between the abutment and implant for zirconia abutments compared to titanium counterparts after loading in an experimental test set up [146]. Micro gaps between abutments and implant could lead to accumulation of bacteria in this region [147].

SIZE AND TYPE OF PARTICLES

Experimental studies focusing on wear between implant components have found particles ranging from nano sizes to micrometer sizes (up to ≈50–90 μm) [71, 148]. Although smaller particles (>10 μm) can be phagocytized by human immune cells [137], larger particles (<15–20 μm) or non-biodegradable fibers (e.g., asbestos) cannot be phagocytized and could lead to chronic inflammatory reactions and tissue damage [149]. Additionally, the shape of the particle (e.g., round or spherical) can affect phagocytosis: spherical particles of ~3 μm in diameter are regarded as ideal for phagocytizing [149]. The shape of particle affects the reaction of the immune system. Spiky particles with a rough surface can cause inflammasome activation (a large macromolecular complex) and cell death [149]. Nanoparticles, on the other hand (e.g., TiO_2 particles), can cause inflammatory reactions in surrounding tissues and can travel through lymph vessels to the draining lymph nodes [150]. Nanoparticles can agglomerate to larger complexes, and larger agglomerates of titanium dioxide nanoparticles have been shown to produce a stronger pro-inflammatory response compared to smaller agglomerates in vivo [151].

BIOLOGICAL RESPONSE TO PARTICLES – MACROPHAGES AND NEUTROPHILS

Neutrophils and macrophages can phagocytize particles [149]. Macrophages and neutrophils are first responders to the injury as the result of implantation [152]. Macrophages residing in bone tissue (osteomacs) play a key role in bone remodeling and formation [153]. Macrophages fuse into multi-nucleated giant cells to form three subtypes: osteoclasts, foreign body giant cells, and Langhans giant cells [154]. Neutrophils can release neutrophil extracellular traps (NETS) when encountering particles and pathogens. NETS, released immune cell DNA, is seen when large pathogens are encountered [155]. Macrophages, on the other hand, produce several signaling molecules in contact with particles–e.g., TNF-α, IL-1β, and M-CSF–and can polarize to the proinflammatory M1 type or anti-inflammatory/tissue regenerating M2 type [156]. Titanium particles added to cell media containing the M1/M2 macrophage types enhance the M1 proinflammatory type [156]. In an in vivo test (on rats), a stimulation of

M1 types of macrophages was observed when in contact with added titanium particles by measuring specific macrophage markers using an immunofluorescent microscope [157]. In a small clinical study, an increased number of M1 type macrophages was observed in mucosa biopsies affected by peri-implantitis compared to biopsies affected by periodontitis [158]. However, titanium elements, analyzed using inductively coupled plasma mass spectrometry (ICP-MS), has been found in bone samples from humans without dental implants, although titanium particles were only found in bone samples with dental implants [159]. Titanium dioxide is a common food coloring used in various products such as candy, chewing gum, and toothpastes and therefore might end up in the biologic context [160]. However, recently the European Food Safety Authority (EFSA) has judged titanium dioxides as unsafe as a food additive:

"Considering all available scientific studies and data, the Panel concluded that titanium dioxide can no longer be considered safe as a food additive. A critical element in reaching this conclusion is that we could not exclude genotoxicity concerns after consumption of titanium dioxide particles. After oral ingestion, the absorption of titanium dioxide particles is low, however they can accumulate in the body [161]"

During the year 2022 the Swedish Food Agency (Livsmedelsverket) stated on its website that The European Union (EU) has banned the use of titanium dioxide in food products [162].

1.4.1 ION LEAKAGE AND CORROSION

Titanium and zirconium are highly reactive and form oxide layers outside of the metal bulk when in contact with fluid or air [24]. This passive layer on the dental titanium implants helps resist corrosion [163]. However, this protective layer can be disrupted in the oral environment by corrosive substances and mechanical wear. Recently, the term "tribocorrosion" has been introduced in the dental literature to describe "material deterioration or transformation resulting from simultaneous action of wear and corrosion" [71]. Research has found corrosion products such as metal ions in vitro when loading dental implant constructions in an acidic environment [71, 148]. Bacteria can also release acids that could cause corrosion on dental implants and

implant components. In vitro tests have shown surface corrosion signs on dental implants immersed in *Streptococcus mutans* medium as early as after two days [164]. Rodrigues et al. analyzed five retrieved implants with peri-implant disease and found severe corrosion on the implant surface [165]. Furthermore, inflammation can be associated with an acid environment due to the release of chemical agents by the immune cells involved [89]. An acidic environment was found around failed hip implants due to aseptic loosening [166]. For dental implants, a small sample study (n = 17) found a pH above 7 around implants with radiographic signs of bone loss, although two patients had implants that they characterized with acute peri-implantitis and a pH below 7 [167].

Several factors could improve implant corrosion resistance, such as alloys with other metals to create more stable oxide layers, fewer pores or irregularities on the surfaces by surface alterations, or creating thicker oxide layers through anodization [168]. However, in vitro and in vivo tests have not found any correlation between surface roughness on titanium implants and a higher degree of ion release [169].

In contrast to orthopedic medicine, the dental literature lacks clear guidance about whether titanium particles can generate a proinflammatory response resulting in bone loss.

2 AIM

This thesis investigates the effect of different biomaterials on biological responses and particle generation during function.

Study I compare the clinical outcome of single implant crowns with zirconia or titanium abutments after up to five years of function. We hypothesized that no difference between the two abutment materials would be seen.

Study II compares wear and wear particle release between two implant materials connected to zirconia or titanium abutments in an experimental set up. We hypothesized that no difference would be seen between the different abutment-implant combinations.

Study III compares the immune cell response to particles of titanium or zirconia growing on different growth materials. We hypothesized that no difference in cellular response would be seen with respect to different particle and disc groups.

Study IV compares gene expression in mucosa around single implants connected to zirconia or titanium abutments and investigates wear particle release in soft tissues after five years of function. We hypothesized that no difference in particle presence and gene expression would be seen.

3 PATIENTS

The patients included in this thesis were retrospectively included from data records (Study I) or recruited at the 5-year recall (Study IV) in the specialist clinic in oral prosthodontics Brånemark, Västra Götalandsregionen, Göteborg, Sweden. Blood samples were collected from anonymous donors at Sahlgrenska University Hospital (Study III).

3.1 ETHICAL CONSIDERATIONS

The data from patient records in Study I and IV were registered with care to maintain the patient's integrity and anonymity. All data concerning patients in Study I and IV were anonymized to prevent identification. Blood donor samples in Study III was anonymous, so no ethical approval was needed. Tissue sample retrieval in Study IV was carefully harvested from the mucosal tissues on the palatal/lingual side of the implant to avoid endangering the survival of implant constructions.

4 METHODS

4.1 STUDY I

This retrospective analysis was conducted by gathering information from patient records at the specialist clinic in Prosthodontics, Brånemark Clinic, Folktandvården, the Region of Västra Götaland, Gothenburg, Sweden.

Dental records from patients receiving single implant surgeries between 2011 and 2013 were analyzed and the following inclusion and exclusion criteria were used:

- Inclusion: Patients with a single implant supported crown with external platform, surgery, and prosthetic treatment at the clinic, a zirconia or titanium abutment, and a screw retention of crown.

- Exclusion: Patients with severe illnesses (e.g., ongoing cancer treatment and mental disabilities) or syndromes or defects involving the implant surgery area (i.e., cleft palate, radiation therapy, and fractures).

The following data were collected from the dental records:

- Patients: age at surgery, gender, previous orthodontic treatment, reason for needing implant treatment, smoking, and number of received dental implants.

- Implants: type, length, diameter, and placement in the dental arches.

- Abutment material and type.

- Dentist performing surgical placement of implant and prosthetic therapy.

- Control visits: number and time since prosthetic treatment.

Furthermore, information on biological and technical outcome during the follow-up period was collected. The baseline was set at time of prosthetic placement. Marginal bone loss was calculated by analyzing marginal bone level at each year by counting threads not covered in bone as visualized on the radiographic images, which were converted to millimeters using the manufacturer's given reference point. Information about bleeding on probing and oral hygiene during each visit was collected from the digital dental records. Technical complications–e.g., fracture of abutment, chipping of surface porcelain, abutment screw loosening, and repair or replacement of the implant crown–were included in the data collection.

4.2 STUDY II

This experimental study was conducted in collaboration with the Nordic Institute of Dental Materials (NIOM) in Oslo, Norway. Two implant materials, titanium-zirconium alloy (Straumann Roxolid bone level, Basel, Schweiz) and Titanium grade 4 (Straumann SLA, Basel, Schweiz) and two types of abutment materials–zirconia and titanium (Straumann CARES, Basel, Schweiz) –were connected according to the manufacturer's instructions. The complexes were divided into four groups: titanium implant-titanium abutment (Ti/Ti); titanium implant-zirconia abutment (Ti-Zr); titanium-zirconium implant-titanium abutment (Ti-Zr/Ti); and titanium-zirconium implant-zirconia abutment (Ti-Zr/Zr). From each group, one sample was randomly selected and analyzed in a scanning electron microscope before testing (Figure 1).

All samples were placed in a customized holder filled with epoxy in accordance with ISO-14801 (Figure 2). Furthermore, an immersion liquid was added in the plastic containers. One sample from each group had distilled water to allow for analyzing particle production and two from each group immersed with lactic acid (pH: 2.3), to allow for testing of corrosion and ion leakage (Figure 1).

A steel cap was added on top of the abutment to prevent fractures during loading force. The holders were placed in a dynamical loading machine

(BOSE Electroforce 3330, TA instruments, Detroit, USA) and a cyclic load ranging from 10 to 100 N was applied on top of the complex for 240,000 cycles at 2 Hz. After loading, the corrosion group was placed in 37 ºC heating cabinet for 7 days (ISO 10271). All sample liquids from both trial groups were separately collected from the chambers and placed in plastic tubes. The tubes were centrifuged for 15 minutes to allow particle debris to gather at the bottom. For the corrosion group, the supernatant was collected in new tubes and sent to Sheffield Analytical Services (Sheffield, United Kingdom) for analysis using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES). The residual liquid at the bottom of the tubes was collected on aluminum foil and dried in a heating cabinet. The inside of all implants was brushed gently with a micro brush (Dab Dental, Gothenburg, Sweden) and pressed on carbon tape to collect the particle debris. Scanning electron analysis (TM4000, Hitachi High-tech Corp. Tokyo, Japan) was conducted post loading on all implants, abutments, carbon tape, and liquid residuals.

SUPPLEMENTARY TESTING

Two additional tests were conducted on two samples. One titanium implant previously connected to a zirconia abutment was sectioned in half to allow for inspection of wear inside the implant. One Ti-Zr/Zr complex was tested for an additional 5 million cycles after rinsing the plastic chamber with distilled water. The implant-abutment sample was placed in the loading machine, at 10–100 N cyclic load at 2 Hz for another 5 million cycles. The sample was placed in a heating cabinet at the same degree for 7 days, and the liquid collected and sent for ICP-OES analysis.

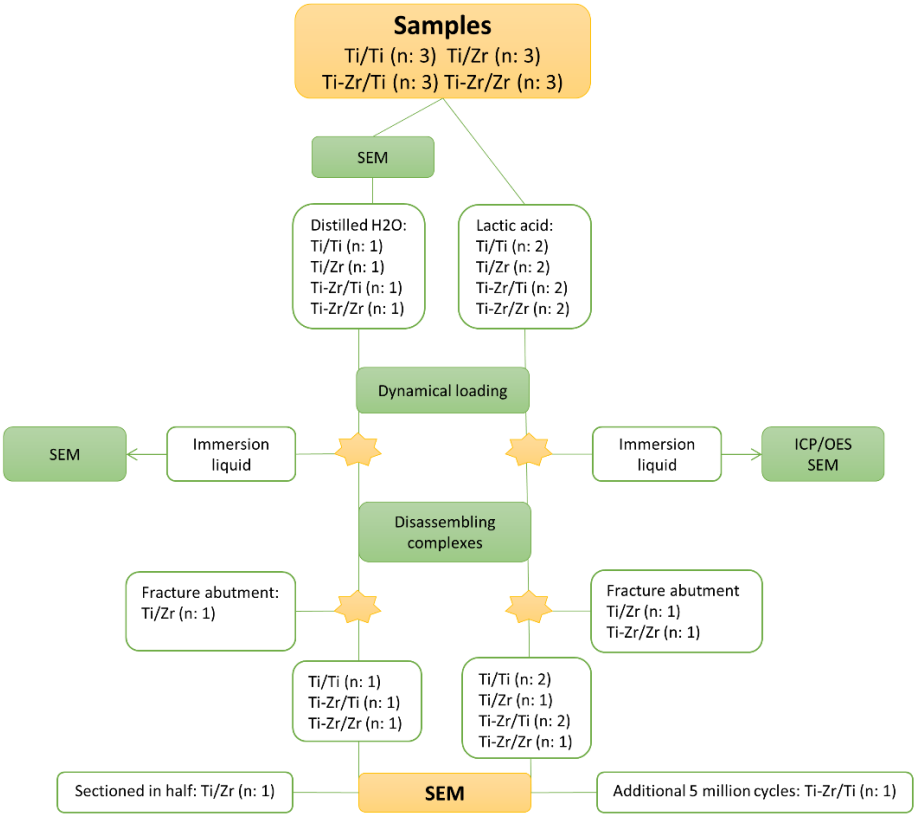

Figure 1. Flowchart samples in Study 2. *Ti: Titanium, Zr: Zirconia, Ti-Zr: Titanium-zirconium alloy. SEM: Scanning electron microscope, ICP/OES: Inductively Coupled Plasma Atomic Emission Spectroscopy.*

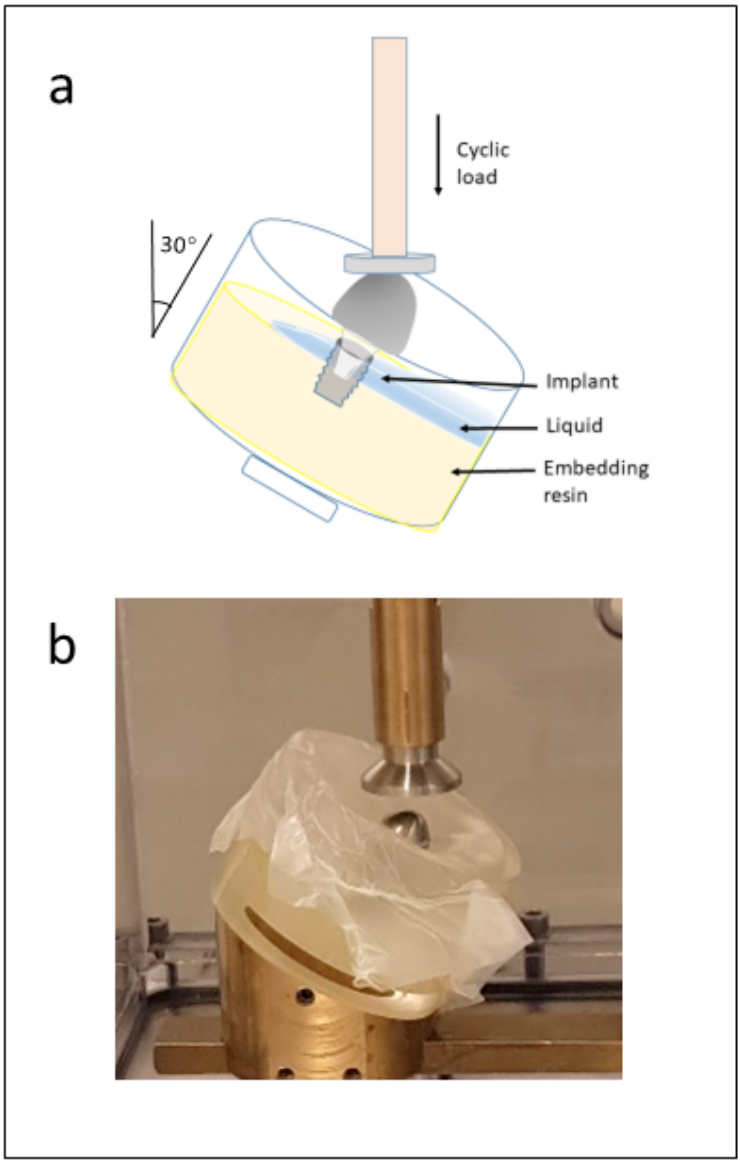

Figure 2. Test set up of dynamical loading machine. a) Schematic image of testing set up of dynamical loading machine. b) Photograph of actual set up of dynamical loading machine. The customized brass holder set at 30° relative to sample.

4.3 STUDY III

This in vitro study was conducted by allowing human peripheral blood mononuclear cells (PBMCs) to grow on titanium or zirconia coins to analyze inflammatory responses. The coins were 2 mm in thickness and 5 mm in diameter. Before cell growth, the roughness of three coins from each group were analyzed with an interferometer.

PBMCs

For this study, PBMCs from 10 healthy anonymous donors from Sahlgrenska Hospital were used. The cells were filtered from plasma using centrifugation (Ficoll-Paque, Plus Density Gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells were washed in PBS twice and suspended in a medium (Dulbecco's modified eagle medium + GlutaMAX-1™, Gibco). Next, 5% heat inactivated human serum type AB (Sigma-Aldrich), penicillin (100 Units/mL), and streptomycin (100 μg/mL) (Sigma-Aldrich) were added. The cells were counted using a hematocytometer, and viability was verified using a trypan blue exclusion assay (Trypan Blue 0.4%, Sigma-Aldrich Sweden AB, Stockholm, Sweden).

DISCS

All discs (2-mm thick and 5-mm diameter) were bought from one manufacturer (Kullberg's Mikroteknik AB, Lycke, Sweden). The discs were made of CP grade 4 titanium (Zapp, GmbH, Schwerte, Germany) and zirconia blanks (Z-cad HTL, Metoxit AG, Switzerland). The zirconia discs were received presintered and were fully sintered on site using Vario S400, Zubler, (USA) according to the manufacturer's instructions.

PARTICLES

The titanium particles and zirconia particles were bought from one manufacturer (Goodfellow, London, England). The titanium particles (99.5% titanium) were up to 70 μm and zirconia particles ranged from 0.1 to 2 μm. Both discs and particles were cleaned before testing according to a standardized protocol [170]. The samples were immersed in 1% Extran Ma 01 (Merck, Darmstadt, Germany) and 70 °C distilled water for 15 minutes, rinsed twice in pure grade 2 water (Elix Advantage System, Merck, Germany), and treated ultrasonically for 10 minutes.

Next, the discs were immersed in absolute ethanol (Histolab, Gothenburg, Sweden) and placed in sterilization pouches. The titanium particles were filtered through a 20-µm mesh.

SURFACE ANALYSIS

Three discs from each material were analyzed in an optical interferometer (Smart WLi extended, Gesellschaft für Bild- und Signalverarbeitung (GBS), Ilmenau, Germany). Each disc was analyzed on three sites with a 50X magnification objective lens. Surface characteristics were calculated with MountainsMAP Premium ver. 7 software (Digital Surf, Besancon, France). The following parameters for these discs were compared to a flat surface: mean values of surface roughness (Sa), difference in height of each point compared to the mean plane, the density of summits (Sds), number of peaks per unit, and the developed interfacial area ratio (Sdr), the percentage of added surface by surface roughness.

CELL CULTURE

The cells were placed in a 96-well plate (Nunc) in triplicates from each donor sample. The cell count of each well was 2 x 10^5 cells. The samples were cultured with a titanium or a zirconia disc only or with the addition of titanium-zirconia mix of particles, creating different test groups. Particle concentration was set at 0.05 mg/mL. Cells without a disc or particles were used as controls. The samples were cultured in 37 ºC in a humidified atmosphere (5% CO_2) for 3 days. The supernatants were collected and used for cytokine and cell death analysis.

CELL COUNTING

One disc from each triplicate sample donor was prepared for immunofluorescence. The samples were fixated in 4% paraformaldehyde for 15 minutes to attach to the disc surfaces. Next, the fixative was removed, and the samples were washed in phosphate-buffered saline buffer and permeabilization of the cells with 1% Triton X-100. After a second washing, the cells were immunofluorescent stained with 2 µg/mL of HCS CellMask™ Stain (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The samples were protected from light for 30 minutes. After one last washing, the samples were mounted using ProLong Antifade mounting medium with DAPI from Molecular Probes (Eugene, OR, USA) onto #1.5H glass-bottom plates

(Cellvis, Sunnyvale, CA, USA). The samples were visualized using a AxioObserver Z.1 microscope (Zeiss, Oberkochen, Germany). A 20X LD objective (LD Plan-Neofluar 20x/0.4 Ph2 Korr) was used to receive a nine-tile image at the center of each of the disc. Attached cells were calculated using the cell counting tool in Image J (Wayne Rasband, NIH, USA).

PARTICLE VISUALIZATION

One sample from each group was prepared for the SEM by washing it in 0.1 M pipes buffer (EMS, Hatfield, PA, USA) six times and incubating this in a mixture of 1% osmium tetra oxide (EMS, Hatfield, PA, USA) and 0.1 M pipes for 30–60 minutes at room temperature in a dark environment. Next, the samples were washed with distilled water 5 times and dehydrated in ascending concentrations of ethanol (Fischer Chem., New Jersey, USA). Finally, the samples were incubated in hexamethyldisilane (Merck KGaA, Darmstadt, Germany) solution for 2–3 minutes and left to dry. The samples were placed on stub holders in Gemini SEM 450 (Zeiss, Oberkochen, Germany) using the backscatter (BSD1) and secondary electron (SE) detector.

CYTOKINE ANALYSIS

The supernatant was analyzed using multiplex panel Bio-Plex Pro™ Human Cytokine 27-plex (Bio-Rad Laboratories, Hemmel Hempstead, UK) in accordance with manufacturer's instructions. Cytokine concentrations data were collected using a Bio Plex 200 instrument and the Bio Manager analysis software (Bio-Rad). See Table 1 for selected cytokines.

CELL DEATH

The Cytotoxicity Detection Kit (LDH, Roche Diagnostics) was used to calculate cell death in the samples preparing the supernatant according to the manufacturer's instructions. Positive control samples were prepared by lysing all cells with 1% Triton X-100 and negative controls by using cell free supernatant. The relative cell death in each sample was calculated as percentage compared to maximal LDH release–i.e., Triton X-100-lysed cells.

RELEASE OF NETS FROM NEUTROPHILS

Calculation of NET release was performed by using a Sytox green assay (Molecular Probes, Eugene, OR, USA). Neutrophils were added in a RPMI medium with 1.25 µM Sytox green DNA stain and plated in a 96-well plate with 5 x 10^4 cells per well. The plate was incubated for 5 minutes in 37 ºC with 5% CO_2. A reference value was determined by measuring the wells in a ClarioStar plate reader (BMG Labtech, Ortenberg, Germany). Next, titanium and zirconia particles were added in a mixture or separately in three different concentration levels: 0.025 mg/mL, 0.05 mg/mL, or 0.1 mg/mL. The fluorescence values were measured repeatedly at the stated times.

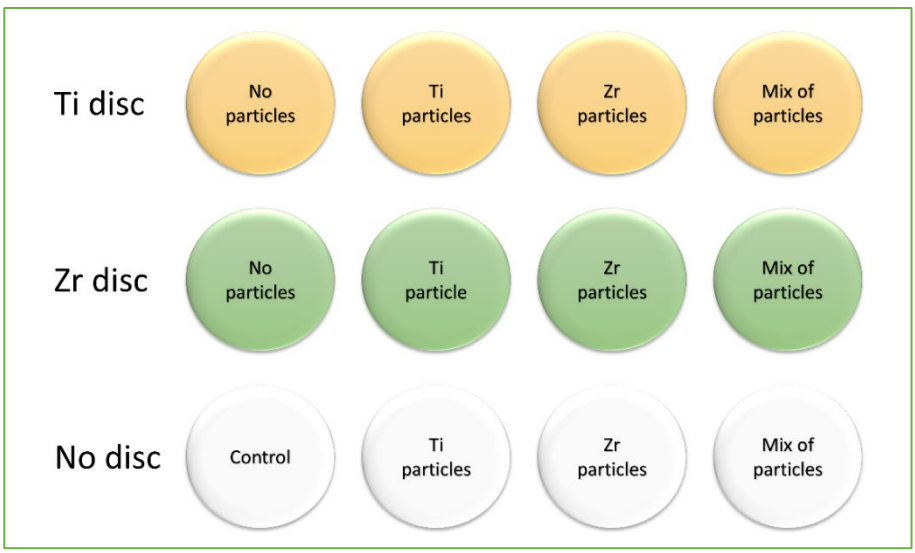

Figure 3. Group division of discs and particles. Ti: Titanium, Zr: Zirconia.

Table 1. Cytokines selected for statistical tests

Cytokine	Name	Function
IL-1β	Interleukin 1 beta	Pro-inflammatory [171, 172]
IL-1ra	Interleukin 1 receptor antagonist	Anti-inflammatory [171]
IL-2	Interleukin 2	Proliferation of T-cells and B-cells [173]
IL-4	Interleukin 4	Regulates Th cell differentiation [174]
IL-6	Interleukin 6	Pro inflammatory [175]
IL-9	Interleukin 9	T cell growth factor [176]
IL-17	Interleukin 17	Pro-inflammatory [177]
FGF- basic (FGF2)	Basic fibroblast growth factor	Bone homeostasis and skeletal development [178]
G-CSF/CSF3	Granulocyte colony stimulating factor	Neutrophil development [179]
GM-CSF/CSF2	Granulocyte-macrophage colony-stimulating factor	Proinflammatory, macrophage development [180]
IFN-γ	Interferon gamma	Proinflammatory, effector of cell mediated immunity [181]
IP-10/CXCL10	Interferon gamma-induced protein 10	Proinflammatory, attracting of immune cells [182]
MCP-1 /CCL2	Monocyte chemoattractant protein 1	Proinflammatory, monocyte recruitment [183]
MIP-1α/CCL3	Macrophage Inflammatory Protein-1 Alpha	Proinflammatory, cell recruitment and osteoclast genesis [184]
MIP-1β/CCL4	Macrophage Inflammatory Protein-1 beta	Proinflammatory, cell recruitment and preosteoclast migration [185]
RANTES /CCL5	Regulated on activation, normal T cell expressed and secreted	Chemoattractant for T-cells and monocytes [186]
TNF-α	Tumor necrosis factor alpha	Proinflammatory, osteoclast differentiation [187]

4.4 STUDY IV

The patients in this study were recruited at the five-year follow-up at the Brånemark Specialist Clinic, a public dental health clinic in the Västra Götaland Region (Göteborg, Sweden).

The following inclusion and exclusion criteria were used:

- Inclusion: Healthy (no signs of periimplantitis) single implants with external platform, a zirconia or titanium abutment, screw retention of crown.

- Exclusion: Patients with severe bone defect around the implant construction.

The patients were asked the following questions about their experience of the implant treatment with the response options Yes, No, or Don't know:

- Are you generally satisfied with the implant crown?

- Are you satisfied with your chewing ability?

- Are you satisfied with the shape and form of implant crown?

The following clinical examinations were conducted at the clinical examination:

- Health of surrounding mucosa, pocket depth, and mucosal discoloration.

- Aesthetic appearance of crown and inspection of construction with regards to chip-off of the veneering porcelain and abutment.

The implant crown was removed and a sample from the top of the implant and the surrounding mucosa were collected on pre-treated glass plates. The glass plates were fixed directly in glutaraldehyde until

further testing. A small biopsy was taken from the mucosa with a 2-mm stent and cut into pieces. One piece was directly put in RNA (Thermo Fisher, Waltham, MA, USA) and stored at −80 ºC and the other piece was fixed in glutaraldehyde until further preparation.

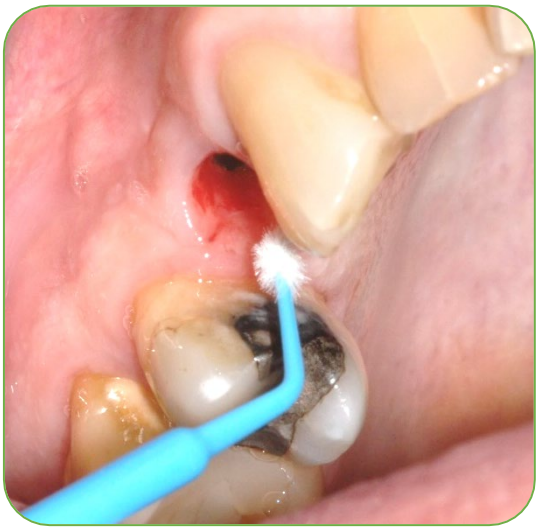

Figure 4. Image of sampling from dental mucosa with micro brush

GLASS SLIDES:

Pretreatment of the glass slides (Thermo Menzel Glas, Braunschweig, Germany) were made by cleaning with soap and water and incubating with 0.5% gelatin (Sigma-Aldrich, Missouri, USA) and 0.05% chromium potassium sulphate (Sigma-Aldrich, Missouri, USA). Glow discharge treatment was prepared using GLOQUBE plus (Quorum, Darmstadt, Germany) to improve adhesion of cells and better visualize in the SEM but still allowing for the use of a light microscope. The post-treatment swabs were rinsed with 0.1M cacodylate buffer and the cells were fixated in 1% osmium tetroxide (EMS, Pennsylvania, USA) to allow for visualization in the SEM.

ICP-MS ANALYSIS

Swab samples from the mucosa/implant head after implant crown removal was used for ICP-MS analysis. A region of interest was chosen in a Raman spectrometry (Horiba LabRam HR Evolution Raman

spectrometer) where samples had particle like material in and around the cells. Spectra were compared to the Horiba/Wiley internal database (KnowItAll software package, Wiley Science Solutions, New Jersey, USA). Analyses were performed using a Laser Ablation (LA) Inductively Coupled Plasma (ICP) Mass Spectrometry (MS) system New Wave NWR 213 laser ablation system coupled to an Agilent 8800 mass spectrometer (Agilent Technologies, California, USA)

Analyses were run during three measurement sessions with a 20-µm round laser beam at a 1-µm/s scan speed and set to 5 Hz and a fluence of 3 J/cm2.
The reference glass NIST SRM 610 was used to tune and to document signal stability along a line scan.

BIOPSIES
Biopsies for PCR analysis was sent to Tataa Laboratory (Gothenburg, Sweden) in freezing bags after being kept at −80 ºC. At the laboratory, the samples were prepared for quantitative Polymerase Chain Reaction (qPCR) analysis. The genes were selected using previous assays from our research group (not published) and adding genes representing the inflammatory cytokines in Study III to allow for comparison. Biopsy samples used for SEM/EDX analysis were prepared by fixating in 1% osmium tetroxide (EMS, Pennsylvania, USA), dehydrating, and embedding in resin (Durcupan, Sigma-Aldrich, Missouri, USA). The samples were left to polymerize for 48 hours until sectioning with a diamond knife.

GENE EXPRESSION ANALYSIS
The collected samples from the freezer were transported to Tataa Biocentre for PCR analysis and stored at the lab in −80 ºC until starting analysis.
Extraction of the tissue samples (n: 9) was made using the extraction kit Total RNA Purification Kit (Cat.17200, Norgen Biotek, Ontario, Canada). RNA quality control was conducted by using a spectrophotometer (Lunatic, Unchained Labs, California, USA) and with capillary gel electrophoresis (Fragment Analyzer, Agilent Technologies Inc., California, USA) using the SS Total RNA 15nt Kit (Cat No. DNF-471-33, Agilent Technologies Inc., California, USA). All

samples except samples 6 and 8 was normalized prior to reverse transcription. These samples had instead the maximum input volume.
The TATAA GrandScript cDNA Synthesis Kit (Cat. No. A103, TATAA Biocentre AB) was used for reversed transcription into cDNA. The reference gene was selected using the built-in function in the CFX maestro software.

SCANNING ELECTRON MICROSCOPY/ELEMENT ANALYSIS:
Biopsies were placed on metal stubs and glass slides on holders in a SEM (Gemini 450, Oberkochen, Germany) and analyzed with EDX (Bruker, Massachusetts USA) on findings of radiolucent areas, which could indicate metal particles.

4.5 STATISTICS

Study I
The statistical tests of marginal bone level and loss were made using Repeated measures mixed models with annual data (T=5). A multivariate logistic regression model was used for presence of bone loss at year five, chipping, bleeding on probing, remaking the crown, and screw loosening. Wilcoxon rank sum test was performed for the bone loss outcome for patient's first implant at year five. All statistics was performed in Stata (STATAcorp, College Station, Texas, USA).

Study II
Mixed-effects regression analysis for displacement and particle sizes were performed using Stata (STATAcorp, College Station, Texas, USA).

Study III
The analyses were performed using a mixed effects regression for topographical measurements and cell count in Stata (STATAcorp, USA). The Wilcoxon matched-pairs signed-rank test was used for cytokine and LDH assays, and the Friedman test corrected by Dunn's test was used for multiple comparison of NET formation using GraphPad Prism 9.0. software (La Jolla, CA, USA).

Study IV
Differences in gene expression analysis was performed using the Student's t test in CFX Maestro (Bio-Rad, California, USA) to compare the two groups.

In general, statistical significance level was set at a *P*-value below 0.05.

4.6 ETHICAL APPROVAL

For Study I, ethical approval was given by the regional ethical review board (Dnr: 2019-00830/1205-18). For Study IV, ethical approval was given the Swedish Ethical Review Authority (Dnr: 2019-01899). For Study III, no ethical approval was needed for these samples as these blood samples were anonymously provided and cannot be traced (Swedish legislation section code 4§ 3p SFS2003:460).

5 RESULTS

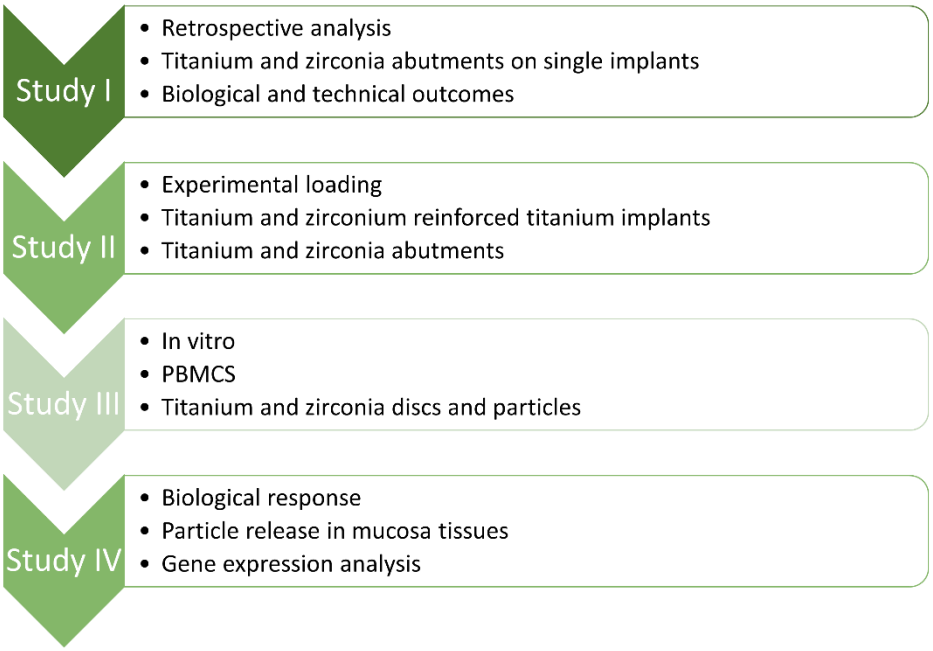

Figure 5. Flowchart of included studies in thesis.

5.1 STUDY I

In this retrospective study, 132 patients and 174 implants were included. The mean age of patients was 37 years and a most were men (52%).

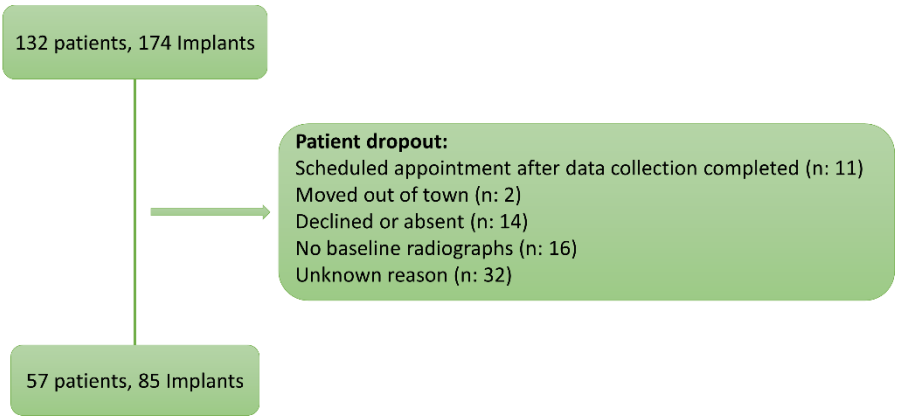

Figure 6. Drop-out tree from baseline to five-year appointment.

TECHNICAL COMPLICATIONS

In this patient cohort, 9% of the implant-supported crowns had a technical complication. Most of these occurred during the first year of function. The titanium abutments showed higher percentage of technical complications compared to zirconium abutments, and the anterior region of the dentition showed a higher percentage of technical complications compared to the posterior region. One zirconia abutment fractured the day after placement of the implant crown. Chipping of porcelain was the most common complication, occurring in 5.7% of the implant-supported crowns, followed by loss of retention in abutment screw (2.8%). None of these complications was affected by the abutment material ($p = 0.247$); however, the age of the patient was statistically significantly related to the occurrence of complication. Patients between 50 and 70 years old had an OR of 4.74 for technical complications as compared to the other groups.

M*ARGINAL BONE LOSS*

Marginal bone loss was measured as annual change as well as calculated as an accumulated bone loss at year five, compared to baseline (when receiving implant crown). Annual marginal bone loss ranged between 0.05 and 2.15 mm. The group of implants with zirconia abutments showed a higher mean annual marginal bone loss compared to implants with titanium abutments (0.32 mm and 0. 21 mm, respectively), and this difference was statistically significant ($p = 0.05$) when controlled for dentist placing the implant crown. At year five, 18 of 174 implants displayed 1-mm bone loss or more.

At year five, 85 implants (48%) had both baseline values and a five-year control evaluation to allow for comparison. 48 implants with a titanium abutment and 37 implants with a zirconia abutment. In this group, the mean age was slightly lower (32 years) than the whole group although still a majority were men ($n = 44$ and $n = 41$, respectively). For 35 implants (41%), no bone loss was evident, but the residual 50 implants showed bone loss between 0.05 mm and 4.25 mm. In total, 27 (71%) of the implants connected to zirconia abutments and 24 (51%) of the of the implants connected to titanium displayed bone loss. A difference in bone loss values was observed between the groups. The implants with zirconia abutment had higher mean bone loss values compared to implants with titanium abutment (0.56 mm and 0.38 mm, respectively). A difference which was statistically significant ($p = 0.034$).

5.2 STUDY II

All samples were loaded for 240,000 cycles without receiving any fractures during the experiment. Three zirconia abutments were fractured during disassembling and could not be used for post loading SEM analysis.

DISPLACEMENT OR BENDING ON LOAD

The displacement values during loading were measured during the experiment. A difference between the combinations of abutment and implants was found. Zirconia abutments provided stiffness to the construction ($p = 0.032$) regardless of implant material.

MECHANICAL WEAR

SEM visualization revealed signs of wear on the implant heads, abutment bases, and inside the upper parts of the implant. These areas had grooves in the metal, abraded metal flakes, and loose particles. One implant was cut in half and the inside showed signs of metal wear in the connecting parts, where the abutment and implant had met during loading. All samples showed signs of wear in the connected parts, and no differences were found between different groups of material combinations.

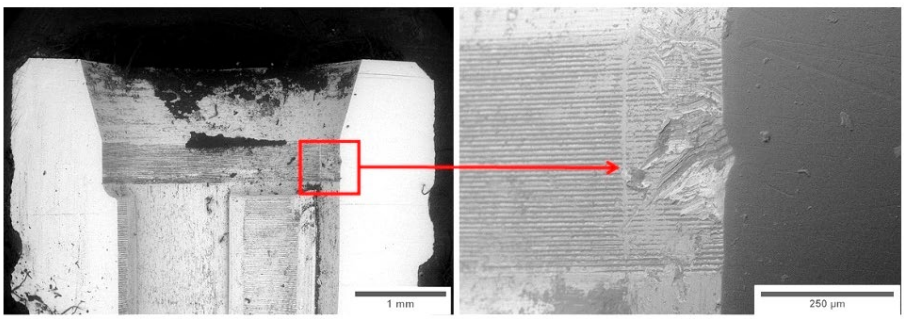

Figure 7. *Internal view of implant wear. Sectioned implant visualized in SEM–25 x zooms 5kV (left) and zoomed in on wear scar, 120 x zoom 5kV (right).*

GENERATED PARTICLES

Particles were found where the implants and abutments were connected, inside the implants, and in the container liquids. In the EDX analysis the

metal zirconium together with a peak of oxygen was regarded to origin from zirconia abutments. Peaks of both titanium and zirconium was regarded to originate from the zirconium reinforced titanium implant. More particles were found in the immersion liquids for zirconia abutment samples (regardless of implant type) compared to the titanium abutment samples (n = 14 and n = 6, respectively). The implant and abutment material type that showed most particles release was titanium implant and zirconia abutment (n = 9), and the fewest were found on titanium-zirconium alloy titanium abutment group (n = 1). Most of the particles were found in the corrosive immersion liquid and not inside the implants. In the liquids, the particles ranged from 253 nm to 1.7 μm. Titanium particles in the immersion liquids were slightly smaller compared to zirconia particles (mean = 535 nm and mean = 633 nm, respectively).

Inside the implants the particles were found by gently swiping with a micro brush; here the particles were larger, ranging from 3 to 95 μm. We found only titanium and titanium-zirconium particles inside the implants. The particles from inside the titanium-zirconium alloy were larger than the particles from titanium (mean = 12.84 μm and mean = 25.33 μm, respectively). Table 2 describes material type, location, and particle sizes.

Table 2. Particle size in Study III

Particle type	*Mean (μm)*	*Min*	*Max*	*Location*
Zirconia	0.63	0.33	1.21	Immersion liquids
Titanium	7.83	0.25	56.90	Immersion liquids, inside implant
Titanium-zirconium	25.33	4.40	95.30	Inside implant

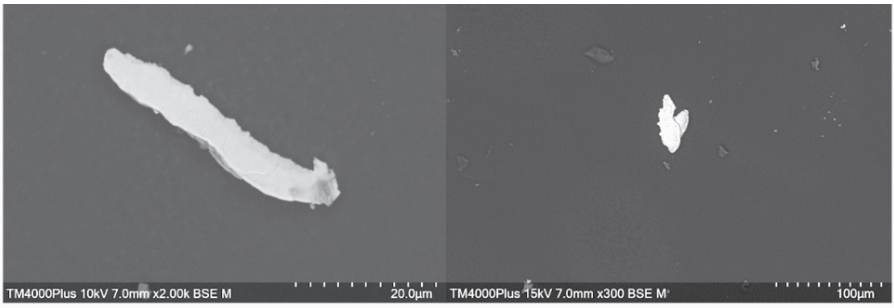

Figure 8. Titanium particles found inside implant.

CORROSION

The lactic acid immersion was collected and sent for analysis with ICP-OES to detect titanium and zirconium ions. All samples had levels below the detection limit of 0.1 mg/L. One sample was analyzed for further 5 million cycles; in the corrosive liquid of this sample, there were 0.29 mg/L titanium ions. Thus, corrosion might be related to time in lactic acid.

5.3 STUDY III

SURFACE ANALYSIS

The surface roughness (Sa) on the zirconia discs was higher compared to the titanium discs, and Sdr (percentage of additional surface area) was ten times higher for zirconia discs (Table 3).

Table 3. Surface analysis

Material	Sa (μm)	Sdr (%)	Sds (1/μm2)
Titanium	0.197 (SD = 0.036)	1.365 (SD = 0.280)	0.206 (SD = 0.018)
Zirconia	0.493 (SD = 0.113)	10.254 (SD = 1.461)	0.288 (SD = 0.009)

CELL COUNTING

On the 80 discs imaged using a widefield microscope, the attached cell count ranged between 48 and 2620. Two samples had disturbances and were discarded from the analysis. The mean number of attached cells was 976. The highest mean number was found on discs without particles, where zirconia discs displayed the highest attached cell count and titanium discs the lowest. The addition of particles resulted in an increase of attached cells on the titanium discs but a reduced number of cells on the zirconia discs. This difference was not statistically significant ($p = 0.832$). Large individual differences were observed between the samples from different blood sample donors (Figure 9).

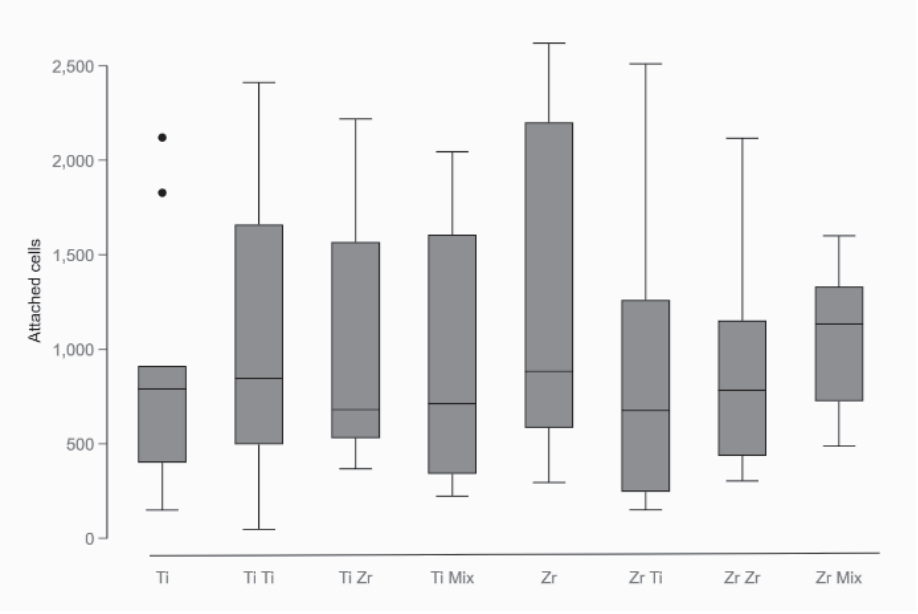

Figure 9. Graph of attached cells on the surface groups, Ti: Titanium, Zr: Zirconia.

PARTICLE VISUALIZATION

A SEM was used to study the particle and cell interaction. Several cells were found near or on top of the particles. Zirconia particles were clustered, creating large aggregates with cells positioned between the voids (Figure 10).

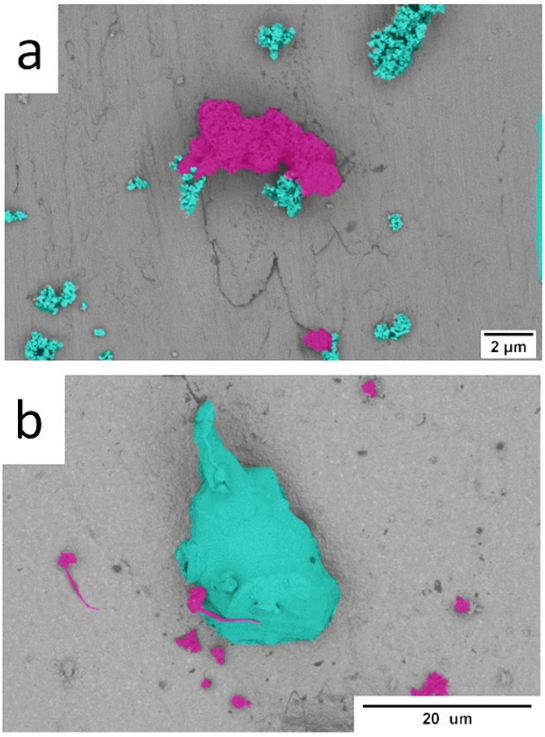

Figure 10 SEM images particles and cells on discs. a) Titanium disc with zirconia particles, 28.8kX zoom, BSD1 detector. Particle and cell interaction are shown; particles show a granular appearance and aggregated clusters measuring around 1–2 µm. b) c) Zirconia disc with titanium particle, 4.98kX zoom, BSD1 detector. Titanium particle around 20 µm with cells attached to the surface. The zirconia disc has a granular appearance.

CYTOKINE ANALYSIS

Several cytokines were measured using the multiplex panel Bio-Plex Pro™ Human Cytokine 27-plex. In general, most of the cytokines (76%) followed a similar pattern where the titanium discs displayed higher concentrations of proinflammatory cytokines than the polystyrene control surfaces. The cytokine levels were further raised after titanium particles were added. This did not occur when measuring cytokine levels released by cells growing on zirconia discs after adding zirconia particles.

Statistical analyses of 10 selected cytokines displayed the following significant differences. Ti particles in absence of discs had elevated IL-1b, IL-6, MCP-1/CCL2, TNF-α, IL-1ra, and IL-9 levels compared to control samples. Zirconia particles, on the other hand, had reduced TNF-α and RANTES/CCL5 and elevated MCP-1/CCL2 levels compared to control samples. Titanium particles compared to zirconia particles generated higher levels of IL-1b, IL-6, IL-2, TNF-α, IL-1ra, IL-9, and RANTES/CCL5. The cells on titanium discs displayed higher levels of IL-1b, IL-2, IL-4, TNF-α, IFN-γ, IL-1ra, and IL-9 than the zirconia discs. When adding particles to the disc surface, a combination effect of the disc and the particles was observed for a few cytokines. Titanium disc and particles displayed higher TNF-α values. When adding zirconia particles to the zirconia disc, a lowered expression of TNF-α and RANTES/CCL5 and an increased expression of IFN-γ and MCP-1/CCL2 were observed.

CELL DEATH

Cell death was affected by disc and particle material. After three days in culture, the zirconia particles in polystyrene control samples had lower cell death values compared to titanium and a mixture of particles. After adding zirconia particles onto titanium discs and zirconia discs, a lower cell death was observed.

RELEASE OF NETS FROM NEUTROPHILS

Levels of NET formation were measured at two time points and at three concentrations (0.025, 0.05, and 0.1 mg/mL). The release of NETS from neutrophils in the wells was increased with addition of zirconia particles compared to the addition of titanium particles as well as compared to unexposed control cells. This effect was observed in particle concentration levels of 0.05 m /mL and 0.1 mg/mL (Figure 11)

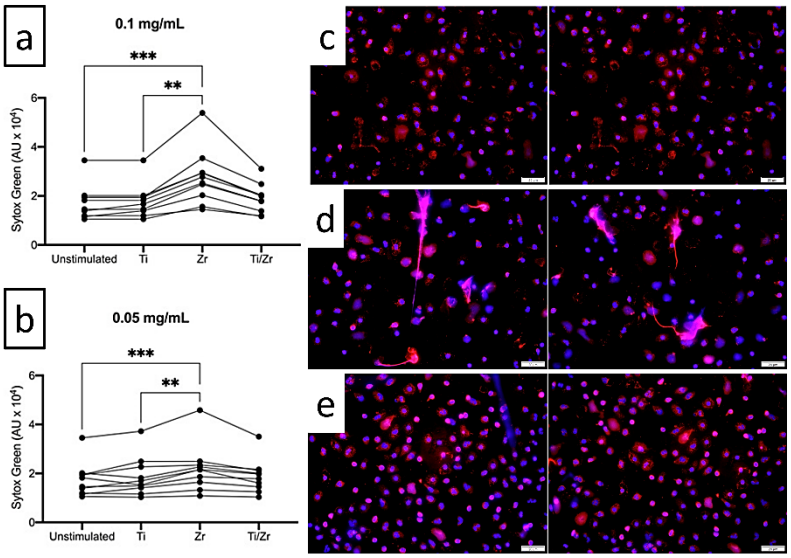

Figure 10. Formation of NETS by neutrophils after 3 h. Left images show NETS release from neutrophils at concentration levels of 0.01 mg/mL (upper) and 0.05mg/mL (lower) after 3 h of culture. Right images (c, d, and e) show two microscopy images of NETS from the same group. Neutrophils were cultured on #1.5H glass-bottom plates in the presence of 0.1 mg/mL of Titanium particles (c), Zirconia particles (d), or left untreated (e).

5.4 STUDY IV

Ten patients were included in this study: five with titanium implants connected to titanium abutments and five with titanium implants connected to zirconia abutments. One patient with zirconia was unable to attend an examination visit during the inclusion time due to illness. Six patients were female and three were male. Mean age of patients at examination was 42 years old, with a range of 25–67 years old. Table 4 lists age, gender, abutment material, and implant position for patients included in the study.

Table 4. Description of patients in Study IV

Patient id	*Gender*	*Age at examination*	*Implant position*	*Abutment material*
1	F	41	24	Ti
2	F	49	25	Ti
3	M	67	14	Ti
4	M	49	36	Ti
5	F	25	35	Ti
6	F	53	21	Zr
7	F	36	21	Zr
8	F	26	23	Zr
9	M	28	12	Zr

CLINICAL EXAMINATION

All patients (n = 9) were satisfied with their implant construction regarding chewing ability, color, and shape of the implant crown. Two patients had signs of mucositis, which was found at the clinical examination (one titanium and one zirconia abutment). All implant crowns except one had good aesthetics according to the two operating dentists.

PARTICLE PRESENCE IN TISSUES AND GLASS SLIDES

Particles were found on the glass slides of sample 1 and 4; however, due to poor conductivity, the element type was not able to be detected by EDX and further testing with this method was discarded in favor of ICP-MS analysis.

Samples 1, 2, and 4 (titanium abutments) and 6, 8, and 9 (zirconia abutments) were analyzed with SEM. The particles, detected in all the six biopsies, were titanium and iron. Particles embedded in the mucosa tissue ranged from around 20 nm to 2 μm, with a mean value of 480 nm (Figure 12). Some particles were clustered into larger aggregates (Figure 12a), some with round edges (Figure 12c), and some with sharper edges (Figure 12d), which in general was the most common appearance. More particles were observed in the mucosal samples with zirconia abutments than with the titanium abutments (17 and 12, respectively).

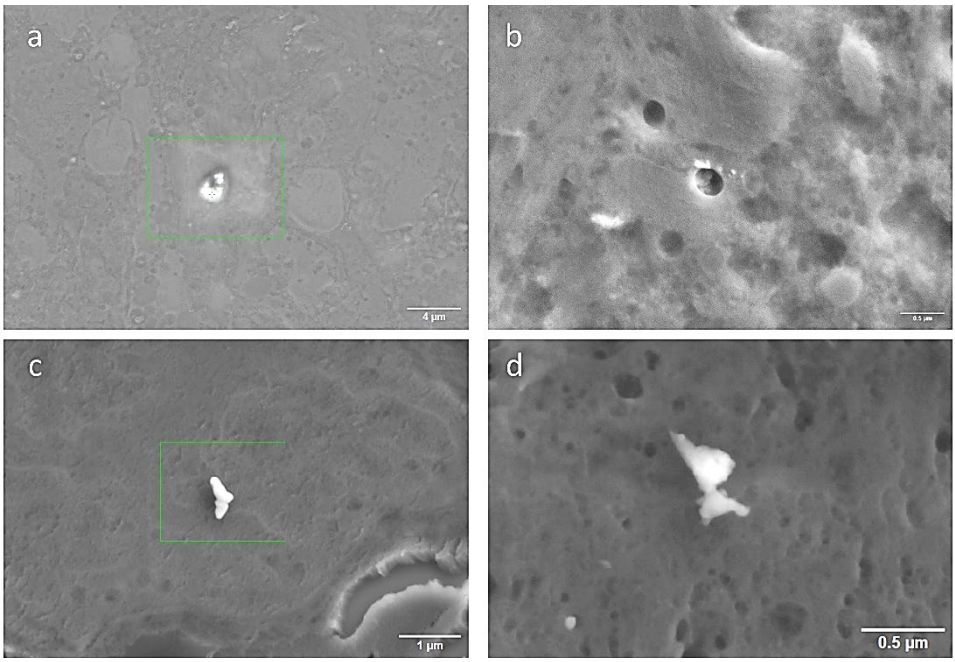

Figure 11. Nano-sized titanium particle in mucosal tissues. a) Titanium particles in one large cluster around 2 μm across, which were found in mucosa around titanium abutment. b) Titanium particles at the edge of a circular shaped structure, which were found in patient with titanium abutment. c) Titanium particles found in patient with zirconia abutment: rounded edges and half-moon shape. d) Sharp-edged titanium particle found in patient with zirconia abutment.

ICP ANALYSIS

Swab samples on glass plates from all patients were analyzed using ICP-MS (Agilent technologies, California, USA). Particles were only identified on samples from the zirconia abutment group. Most of the particles were zirconia (zirconium) (figure 13), however, a few titanium particles were found. This method did not allow for size measurement of the particles.

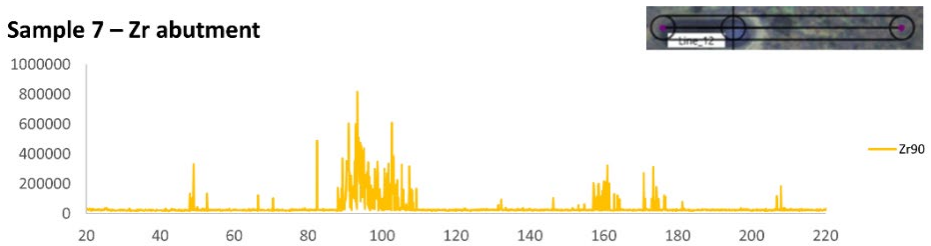

Figure 12. ICP analysis. Graph of ICP-MS analysis of glass slides from samples from zirconia abutment group. Peaks indicate when the laser hit particles in the samples as seen in the inserted image up in the right corner.

GENE EXPRESSION

Reference genes were selected by using the CFX maestro program. The gene PPIA was found to be the most appropriate. Gene expression analysis using quantitative PCR (qPCR) displayed several differences between mucosal samples from patients with the two abutment types. Compared to titanium samples, the zirconia samples had a two-fold upregulation for the following genes: MCP-1/CCL2, RANTES/CCL5, IP-10/CXCL10, FGF2, IL-2, IFN-γ, NE, and RANKL. The differences for IL-2, IP-10/CXCL10, and RANTES/CCL5 were statistically significant. The genes TREM1 and G-CSF/CSF3 had a two-fold downregulation in the zirconia group compared to titanium. The difference between the groups regarding the gene TREM1 was statistically significant (Figure 14).

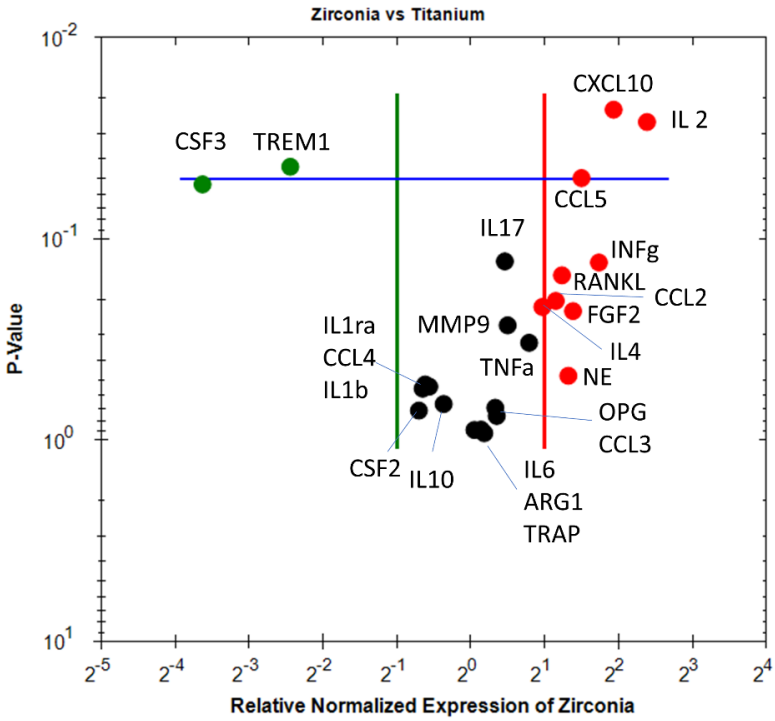

Figure 13. . Volcano plot PCR analysis displaying mean values. Volcano plot of gene expression in mucosa comparing zirconia (treated) with titanium (control). Blue horizontal line represents a P-value of 0.05. Green vertical line represents two-fold downregulation and red vertical line two-fold upregulation. CXCL10(IP-10), IL2, and CCL5 (RANTES) were upregulated and CSF3 (G-CSF) and TREM1 were downregulated for zirconia.

Table 5. Gene expression for zirconia group compared to titanium group

Gene target	*Fold Change*	*P-Value*	*Significant P-Value*
ARG1	1.1	0.88	No
MCP1/CCL2	2.22	0.20	No
MIP-1α/CCL3	1.27	0.76	No
MIP-1β/CCL4	-1.54	0.53	No
RANTES/CCL5	2.82	0.05	Yes
GM-CSF/CSF2	-1.63	0.71	No
G-CSF/CSF3	-12.4	0.05	No
IP-10/CXCL10	3.82	0.02	Yes
FGF2	2.61	0.23	No
IL10	-1.29	0.66	No
IL17	1.38	0.13	No
IL1β	-1.48	0.54	No
IL1ra	-1.57	0.55	No
IL2	5.22	0.03	Yes
IL4	1.96	0.22	No
IL6	1.13	0.92	No
IFN-γ	3,32	0.13	No
MMP9	1.41	0.27	No
NE	2.49	0.48	No
OPG	1.26	0.69	No
RANKL	2.35	0.15	No
TNFα	1.73	0.33	No
TRAP	1.03	0.89	No
TREM1	-5.44	0.04	Yes

6 DISCUSSION

6.1 STUDY I

The results from Study I reveal a difference in annual marginal bone loss and total marginal bone loss after five years when comparing the two abutment material groups. Technical complications were associated with the age of the patient but not the abutment material. The patients in this study represent a cohort that received specialist treatment in both surgical and prosthodontic aspect, which could be described as ideal conditions (efficacy) compared to "real world conditions" (effectiveness) [188], which could limit the generalizability. The experience and skill of the dentist placing the implants could influence the outcomes. We found the dentist to be linked with amount of marginal bone loss. The dentist involved in the prosthodontic rehabilitation had varying experience, from general dentist to specialist dentist. In line with our results, previous research has shown prosthetic treatment by less experienced dentists to increase the odds of moderate or severe peri-implantitis [189].

One possible confounding factor is that 18% of the implant sites had been treated with bone volume adjustment and were unevenly distributed on 22 implants with zirconia abutment and nine with a titanium abutment. Furthermore, the patients were not randomly assigned an abutment material and the choice of abutment type could have been affected by amount of buccal bone and mucosal type. As stated above, several biological complications may occur to implants placed in augmented bone, such as infection, formation of fibrous tissue around the graft, and suppressed healing. However, a systematic review on the subject found no difference in marginal bone loss between augmented bone (immediately placed) and natural bone [28]. This possible confounder could be solved using data from a larger sample group. Previous studies comparing single implants with zirconia abutments to titanium abutments have not found a difference between the abutment types on marginal bone loss. However, the studies are based on small samples or with cemented restorations, which could affect the results as excess cement can cause inflammation in the mucosa [190]. Zembic et al. followed 18 patients for up to five years after crown placement with a blend of cemented and screw retained

crowns in the posterior region [191]. Lops et al. followed 81 patients who had crowns cemented on the abutments for five years [192]. Hosseini et al. followed 59 patients who had gold, titanium, or zirconia abutments and cemented crowns for three years [193].

In this study, we acquired the reference position from the dental implant manufacturer and compared baseline and yearly bone level values to this position to retain marginal bone loss values. A Pisa consensus report from 2008 concluded that "clinical observations obtained by probing or radiographic measurements of 0.1 mm for bone loss are operator sensitive and are not reliable" [101]. Using the manufacturer's reference on distance between reference point and threads, we found that the smallest value measured in the radiographs could be half a thread–i.e., 0.25 mm.

Technical complications were not associated with abutment type but age of patient when receiving implant surgery. Patients between 50 and 70 years old had a odds ratio of 4.74. This finding contrasts with findings from a systematic review on implant-abutment connections where ceramic abutments had more fractures than metal abutments. However, the studies included in that review used several ceramic and metal material types besides titanium and zirconia [65]. Furthermore, unlike our findings, age of patients when receiving implant treatment and technical complications was not statistically significant in a recent large retrospective study on single, partial, and full jaw treatments [121]. The study design in that study included full, partial, and single implant construction types, which could explain the different outcomes.

LIMITATIONS

For all retrospective studies, there is a risk of missing information in patient records, which could affect the data analysis. Furthermore, the data records lacked standardization as the study period covered several years and various examiners. Further studies should use a large multicenter approach to generate a larger sample size.

6.2 STUDY II

In this experimental in vitro study, we found wear particles in the immersion liquid, on the surfaces of the connecting parts of the constructions, and inside the screw channel of the implants. Both titanium and zirconia particles were found ranging from nanometer to micrometer sizes. One purpose of the study was to quantify differences in wear between various implant and abutment materials. We did not find differences in wear, although the number of particles observed in the immersion liquids was different between the groups; the highest amount was shown in the samples with zirconia abutments. In a systematic review, zirconia abutments have been found to allow for more wear and greater misfit compared to titanium abutments [146], which could explain why more particles were found around zirconia abutments. However, finding internally captured particles inside the implants has not, to the author's knowledge, been found in previous studies. Alrabeah et al., comparing externally-connected implants focusing on wear scarring, found titanium and zirconia particles up to 50 micrometers released in the immersion liquids [71]. Klotz et al. simply stated that titanium residue was found on zirconia abutments and did not specify the sizes of the wear particles [141]. Capturing particles inside the internally-connected implants is interesting as a recent systematic review and meta-analysis concluded internally-connected implants to maintain bone levels better compared to externally-connected implants [64]. Considering that wear particles and ions can stimulate an inflammatory response in vitro [106, 137, 194], this finding could have clinical relevance. The size of the particles is also important, as shown in previous vitro studies, where smaller particles are more proinflammatory than larger particles [195, 196]. In our study, we found small nano-sized particles of up to a micrometer, which could contribute to inflammatory responses if released into the peri implant tissues.

Displacement or bending during loading were also measured during this test and found to be less prominent in the zirconia abutments than the titanium abutments regardless of implant system. In orthopedic medicine, stiffness of implants is important. An implant that is too stiff when bending or loading leads to bone degradation as the implant absorbs to much stress and the bone is subjected to less stress (i.e., stress shielding) [197].

LIMITATIONS

Experiments like this generate results in specific conditions. ISO 14801: 2016 is a standard for measuring single endousseous implants, under "worst case" applications [198], and has been used in similar in vitro tests [71, 199, 200]. This test allows for comparison using a structured testing protocol but might not be regarded as clinically representative in general. In the oral cavity one could speculate that several conditions could affect the results, such as diet, muscular strength, implant position, and opposing teeth.

The ICP-OES technique only shows differences between the samples with respect to being below the detection limit (except one sample used for supplementary loading). The ICP-MS, with a higher detection limit [201] could have been used for better comparison to be able to find differences below the threshold value.

The SEM proved very effective for the qualitative analysis as it allowed for the visualization of wear scars on the implant components. However, the possibility of quantitative analysis was influenced by the imaging technique. Other techniques have been used in previous research such as Micro Computer Tomography [142] and optical scanners [202], which could be used in future studies to compare surface morphological changes in a quantitative approach.

Presence of particles in the immersion liquids differed some between the groups; however, a larger sample size would allow for better quantification on abutment choice and particle generation.

6.3 STUDY III

In Study III, we found higher levels of inflammatory markers for titanium both as particles and disc compared to zirconia counterparts. This finding agrees with previous research that found zirconia particles to be less proinflammatory than titanium particles [194, 203]. The addition of titanium and zirconia particles in the cell medium growing on titanium or zirconia discs only altered the levels of a few cytokines: MCP-1/CCL2, TNF-α, and IFN-γ. MCP-1/CCL2 regulates migration and infiltration of macrophages [204] and IFN-γ activates macrophages [181]. TNF-α is released by activated macrophages and T-lymphocytes [205], and both TNF-α and IFN-γ are thought to be associated with proinflammatory M1 macrophages [206]. M1 macrophages are shown to be enhanced in soft tissue samples from implants with peri-implantitis compared to tissue samples with periodontitis [158]. Furthermore, in vivo tests have shown an activation of M1 macrophages in contact with titanium particles in peri-implant tissues [157].

The release of NETs when encountering zirconia particles is interesting. Previous research has found released NETs to be a non-specific response to microbes and cause tissue destruction and an increase in the proinflammatory response [207]. The release of NETs is also seen in autoimmune diseases such as rheumatoid arthritis and psoriasis [208]. Release of NETs is one possible reaction by the neutrophils as a response to pathogens and particles in the body in an attempt to shield it from the surrounding cells [209]. NET formation has been reported in vitro when encountering particles such as asbestos [210], a fibrous mineral used in the past in building materials and a cause of lung cancer in exposed humans [211]. Previous research, which has found a neutrophilic response to titanium and zirconia materials, concluded a rougher titanium surface increases the response of NETs formation, but no difference was found in neutrophil reactions to titanium alloy and zirconia toughened alumina surfaces in vitro [212]. However, the authors stated that a lack of available evidence on neutrophilic reaction towards titanium and zirconia hinders the possibility to draw any conclusion.

The surface roughness of the discs was measured using an optical interferometer and showed higher Sa and Sdr values for zirconia discs

than titanium discs. Zirconia surface roughness has been measured on discs in previous in vitro studies, which found Sa mean values ranging from 0.6 [213] to 1.6 μm [214], slightly above our results. Surface roughness has been shown to affect adhesion of cells; one in vitro study comparing different surface roughness of titanium discs showed higher adherence of monocytes to rougher surfaces than to turned surfaces [215]. Barkarmo et al. also reported increased cell attachment on rougher PEEK discs compared to smoother titanium alloy discs [216]. In our in vitro design, no difference in cell adhesion was found comparing rougher zirconia discs to smoother titanium ones; this could be because cell clustering occurred on most of the samples, which hindered the possibility to calculate each cell individually.

LIMITATIONS

The discs and particles in this study were acquired to try to represent a clinical situation. However, differences in particles derived from dental implants might occur as the particles are produced in laboratory settings. One positive aspect is that the particle size and material are specified and manufactured for research. However, future studies could try to generate particles from real implants and abutments.

6.4 STUDY IV

In Study IV, we found titanium particles in the biopsy samples and on top of the implant head. Zirconia particles were observed in samples from patients with zirconia abutments. Gene expression differed between the patients receiving different abutment materials: the group with zirconia showed upregulation of several pro-inflammatory genes. For one patient with zirconia abutment, the metal shine trough was evident through the mucosa. Several studies have shown a greater discoloring compared to natural teeth for titanium abutment compared to zirconia using a spectrophotometer [217-219]. However, other authors found no differences between the two abutment types regarding mucosa color upon clinically examination [220] and high resolution image examination [221], findings in line with the majority of patient samples in our small pilot study. We argue that the metal shine might represent the implant rather than the abutment seen in one case in this study.

The particles found in our study ranged between 20 nm and 2 μm, slightly smaller compared to findings in peri-implant bone (ranging between 0.5 and 40 μm) [159]. However, a review from 2018 concluded that metal particles found in soft and hard tissues around dental implants ranged between 100 nm and 54 μm [126], displaying the great variation also seen in our results. Particle size matters. Small particles, below 10 μm, can be phagocytized by human immune cells [137] and nano-sized particles have the ability to travel through lymph vessels to the draining lymph nodes [150]. In our study, the shape of the particles varied greatly–from round to needle shaped to sharp edged; however, most were rough edged. Phagocytosis of particles with these shapes could cause inflammasome activation and cell death [149]. Researchers have previously found titanium particles in samples from implants diagnosed with peri-implantitis [108] and more titanium particles and ions present in peri-implantitis samples than in healthy samples [126] [110]. The patients in our study did not show signs of peri-implantitis, and future studies should compare the results from this study to mucosa samples from around diseased implants.

Gene expression analysis differed between sample groups. The zirconia abutment group showed a two-fold upregulation of several

proinflammatory genes: CCL5 /RANTES, a chemoattractant for T-cells and monocytes [186], and CXCL10/IP-10 expressed by T-cells, which attract other immune cells to sites of inflammation [182, 186]. IL-2 from T-cells and NK cells causes proliferation of other T-cells, NK cells, but also B-cells [173]. CCL2, also known as MCP-1, is a chemokine expressed in tissue during inflammation, bone formation, and resorption [183] and is released from PBMCS in the presence of titanium and zirconia particles (Study III). Moreover, IFN-γ, an activator of macrophages [181], was seen upregulated in samples around zirconia abutments and on cells cultured on zirconia discs with zirconia particles. A possible explanation for this could be the presence of zirconia particles found near the implant-abutment junction in the ICP-MS analysis. In addition, an upregulation was seen for neutrophil elastase (NE), which is released during formation of NETs [222]. NETs were found to be released from neutrophils challenged by zirconia particles but not titanium particles (Study III), which could indicate that the presence of zirconia particles in peri-implant mucosa could cause formation of NETs, a process that causes tissue destruction [223]. Furthermore, upregulation was seen for RANKL around zirconia abutment. RANKL is released by osteocytes to cause differentiation of unmature osteoclasts [23], which indicates bone degeneration. However, contrary to this, upregulation of FGF2 (FGF basic) was seen, which is a growth factor related to bone homeostasis, skeletal development [178], and angiogenesis [224], indicating a simultaneous tissue healing process. Downregulation was seen for CSF-3/G-CSF, a growth factor triggering development of neutrophils in the bone marrow [179], and TREM1, a receptor reacting to infections and damage associated molecular patterns (DAMPs) causing and strengthening inflammatory responses [225] [226]. This indicates that the tissues around the implants with titanium abutments might also display some sort of inflammatory process.

Several proinflammatory genes did not show any differences in the sample groups–e.g., Il-6, which is secreted by macrophages in the early stages of inflammation [227]. This finding is contrary to the results in Study III, which showed an enhanced release for PBMCS grown with titanium particles. A possible explanation could be that the mucosa around the implants is in a state of chronic rather than acute inflammation. A theory that could further explain why no upregulation

was seen in the gene expression of TNF-α and IL1β, pro-inflammatory cytokines involved in the acute inflammatory phase [228]. Similar results were found for tartrate-resistant acid phosphatase (TRAP) secreted by osteoclasts and macrophages and acts as a marker of bone resorption and inflammation [229].

Several anti-inflammatory genes did not differ between the sample groups, such as IL-1ra and IL-10, anti-inflammatory cytokines produced by leukocytes [230]. Furthermore, no significant upregulation or downregulation was seen for Arg-1, expressed by M2 macrophages (anti-inflammatory) [231], or MMP-9, an enzyme that cleaves extracellular matrix and several cytokines, expressed by neutrophils, macrophages, and fibroblasts [232].

Studies on gene expression in dental mucosa are scarce in the dental literature. However, Slotte et al. found a correlation between early gene expression of TNF-α in crevicular fluid around dental implants and latter clinical complications (e.g., implant removal or loose implant) [233].

In this study, we selected genes based on results from cytokine release in Study III and previous research from our research group. However, selecting other genes for further analysis on samples from similar test groups could focus on the M1 and M2 macrophage relation as M1 macrophages have been found to be increased in peri-implantitis tissues compared to periodontitis samples [158], and titanium particles have shown to increase M1 macrophage phenotype in an in vivo study on rats [157].

LIMITATIONS

Retrieving samples from the mucosa had to be done after detaching the implant crown. This technique could cause particle release and, in the worst case, generate cracks or fractures when reattaching the abutment screw. This could generate particles in the tissue samples from the detaching itself and not due to wear, which could confound the results. However, detachment of implant crowns occurs in the clinical milieu as well in case of overload or when repair or a new crown is needed. Furthermore, retrieving similar sized tissue samples was challenging. This was performed using a regular 2-mm punch. Future studies should

include specialized punches that generate precisely sized biopsies. Another limit of this study is the small sample size, a result of a low interest in being a part of the study. Future studies should include a multicenter approach to gain a larger study sample. Furthermore, in this study, we used mucosa around titanium abutments as control in the gene expression analysis to test whether these tissues displayed similar expression patterns. However, to find differences compared to untreated gingiva, future studies should consider using a control sample from gingiva around teeth.

6.5 IN GENERAL

This thesis shows differences in biological response towards zirconia and titanium in a clinical view and in vitro. Both titanium and zirconia have been proposed to be biocompatible [65, 72, 234]. The definition proposed by a consensus conference in UK in 1991 states that a biocompatible material "augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual" [1]. This definition may be too broad, as it does not consider the possible long-term effects, such as biological response with time and biomaterial changes during function. In 1987, Williams argued that biocompatibility is "the ability of a material to perform with an appropriate host response in a specific application" [235]. This definition includes the biological response and suggests possible difference in responses in different situations. In another article, Williams suggests that a biocompatibility biomaterial has two pathways when implanting: an initial host response after surgical trauma and implantation with a resolution of the response and tolerated by the patient or a continued host response that is not tolerable by the patient [236]. Hence one could argue that biomaterials might function well in certain situations and patients but might not be tolerated in other situations.

7 CONCLUSION

- Abutment material was associated with marginal bone loss both yearly and at five-year accumulation in the study cohort. Around zirconia abutments, more marginal bone loss was seen compared to around titanium abutments.

- Particle generation is possible when subjecting implant-abutment to dynamical loading. Large particles seem to be trapped inside internal connected implants. More particles were released from zirconia abutment connected implants than titanium abutment connected implants.

- Cells challenged by titanium particles generate a greater proinflammatory responses compared to zirconia particles. Neutrophils release NETS when in contact with zirconia particles.

- Particles were present in soft tissues around single implants after five years of function. More particles were found in samples with a zirconia abutment. More proinflammatory genes were upregulated around zirconia abutments than around titanium abutments.

In this thesis, we found indications of more wear particle release for zirconia abutments than for titanium abutments both in vitro and in vivo. We further found an upregulated proinflammatory response around zirconia abutments. Susceptible patients with a zirconia abutment might exhibit marginal bone loss due to increased wear particles.

8 FUTURE PERSPECTIVES

- Future studies concerning marginal bone loss and particles should use retrieved implants to compare tissue response, findings of particles, and inflammatory cells.

- Future studies should include comparisons of the results seen from single implants in this thesis to full jaw restorations regarding metal particle presence in mucosa and inflammatory responses.

- Future large retrospective studies should compare clinical outcomes of different combinations of implant and abutment materials.

- Future studies should compare wear particle release when subjecting externally-connected implant types to these internally-connected implants to analyze amount, position, and type of particles generated.

- Future studies should focus on gene expression in cells challenged with zirconia and titanium particles on specific M1/M2 polarization.

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