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GINGIVAL CELL ATTACHMENT AND CELL ADHESION COMPLEX FORMATION ON TiO_2-COATED ZIRCONIA AND TITANIUM

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The originality of this publication has been checked in accordance with the University of Turku quality assurance system using the Turnitin Originality Check service.

ISBN 978-951-29-9552-3 (PRINT)
ISBN 978-951-29-9553-0 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama, Turku Finland, 2023

To my family

UNIVERSITY OF TURKU
Faculty of Medicine
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SINI RIIVARI: Gingival Cell Attachment and Cell Adhesion Complex Formation on TiO_2 Coated Zirconia and Titanium.
Doctoral Dissertation, 119 pp.
Finnish Doctoral Programme for Oral Sciences (FINDOS-Turku)
November 2023

ABSTRACT

Weak gingival attachment to implant surface exposes to easier bacterial invasion to peri-implant tissues, which increases the risk for peri-implant infections. In the worst case, peri-implantitis can lead to the loss of an implant. Therefore, firm peri-implant tissue integration is of a paramount importance.

The aim of the thesis is to find out, if nanoporous, bioactive TiO_2-coating is able to enhance gingival cell adhesion and growth on titanium and zirconia, which are commonly used dental implant or abutment materials.

In the study, half of the zirconia and titanium samples were coated with sol gel derived TiO_2-coating. In addition, hydrothermal treatment of titanium was used in the third study. Surface properties were measured with contact angle and surface free energy measurements. Further, the scanning electron microscope imaging was accomplished to detect the nanotopography of the coated surfaces. In the study, the adhesion and growth of epithelial cells and fibroblasts were studied on TiO_2-coated and non-coated surfaces. The expression of adhesion complexes was studied by western blotting and confocal microscopy. In addition, the effects of saliva exposure to surface properties and cell adhesion were studied.

The results of the thesis demonstrated nanoporous surface and increased hydrophilicity on TiO_2-coated zirconia and titanium. Epithelial cell adhesion and proliferation were faster on coated surfaces. Moreover, expression of adhesion proteins was enhanced on coated surfaces. Saliva exposure increased surface hydrophilicity, but decreased cell attachment on titanium surface. To conclude, bioactive TiO_2-coating is able to enhance cell adhesion to zirconia and titanium in vitro. However, saliva exposure weakens the positive effects of bioactive surface.

KEY WORDS: Epithelium, Fibroblasts, Cell adhesion, Zirconia, Titanium

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Hammaslääketieteen laitos
Protetiikan ja purentafysiologian oppiaine
SINI RIIVARI: Iensolujen kiinnittyminen ja adheesiokompleksien esiintyminen TiO_2-pinnoitetun zirkonian ja titaanin pinnalla
Väitöskirja, 119 s.
Kansallinen suun terveystieteiden tohtoriohjelma (FINDOS-Turku)
Marraskuu 2023

TIIVISTELMÄ

Pehmytkudosten heikko kiinnittyminen implantin pintaan mahdollistaa mikrobien helpomman kulkeutumisen implanttia ympäröiviin kudoksiin ja altistaa näiden tulehduksille. Pahimmassa tapauksessa peri-implantiitti voi johtaa implantin menetykseen, minkä takia peri-implanttikudosten hyvä kiinnittyminen on tärkeää.

Tämän väitöskirjatutkimuksen tarkoituksena on selvittää, pystytäänkö nanohuokoisella, bioaktiivisella TiO_2-pinnoitteella parantamaan iensolujen kiinnittymistä ja kasvua implanttimateriaaleina käytettävien titaanin ja zirkonian pinnoilla.

Puolet zirkonia ja titaaninäytteistä pinnoitettiin soolissa TiO_2-pinnoitteella. Lisäksi kolmannessa osatyössä osa näytteistä käsiteltiin hydrotermaalisesti. Pinnan ominaisuuksia tutkittiin määrittämällä nesteen kontaktikulma pinnalla ja pinnan vapaa energia. Lisäksi pintojen topografiaa tutkittiin pyyhkäisyelektronimikroskoopilla. Väitöskirjassa tutkittiin sekä ikenen epiteelisolujen (I, II, IV), että sidekudoksen fibroblastien (III) kiinnittymistä ja kasvua pinnoitettujen ja pinnoittamattomien näytteiden pinnalle. Solukiinnityksen laadun mittaamisessa käytettiin western blot -menetelmää ja konfokaalimikroskopiaa, joilla tutkittiin adheesiokompleksien muodostumista näytteiden pinnalla. Lisäksi tutkittiin syljen vaikutusta pinnan ominaisuuksiin ja solukiinnitykseen.

Väitöskirjatutkimus osoitti nanohuokoisen pinnan sekä lisääntyneen hydrofiilisyyden TiO_2-pinnoitetulla zirkonialla ja titaanilla. Epiteelisolujen kiinnittyminen ja kasvu olivat nopeampaa pinnoitettujen zirkonian ja titaanin pinnoilla. Lisäksi adheesioproteiinien ilmeneminen oli intensiivisempää pinnoitetuilla pinnoilla. Syljelle altistuminen lisäsi pinnan hydrofiilisyyttä, mutta heikensi solukiinnitystä pinnoitetun titaanin pinnalle. Yhteenvetona voidaan todeta, että bioaktiivinen TiO_2-pinnoite kykenee parantamaan solujen kiinnittymistä zirkonian ja titaanin pinnalle laboratorio-olosuhteissa. Syljen vaikutus taas heikentää pinnoitteen positiivista vaikutusta.

AVAINSANAT: Epiteeli, fibroblasti, solujen kiinnittyminen, zirkonia, titaani

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Abbreviations

ANOVA	Analyses of Variance
AO	Anodic oxidation
cpTi	Commercially pure titanium
ECM	Extracellular matrix
FA	Focal adhesion
FAK	Focal adhesion kinase
HD	Hemidesmosome
HGF	Human gingival fibroblasts
HGK	Human gingival keratinocytes
HT	Hydrothermal coating
JE	Junctional epithelium
mQ	milli-Q (ultrapure water)
min	Minute
OW	Owens-Wendt approach
PBS	Phosphate buffered saline
SE	Sulcular epithelium
SEM	Scanning electron microscope
SFE	Surface free energy
SOL	Coating made in sol
SRC	Steroid receptor coactivator
TBST	Tris buffered saline with Tween
VO	Van Oss approach
WB	Western Blotting

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Riivari S, Shahramian K, Kangasniemi I, Willberg J, Närhi TO. TiO_2-Modified Zirconia Surface Improves Epithelial Cell Attachment. Int J Oral Maxillofac Implants. 2019 Mar/Apr;34(2):313-319.

II Riivari S, Närvä E, Kangasniemi I, Willberg J, Närhi T. Epithelial cell attachment and adhesion protein expression on novel in sol TiO_2 coated zirconia and titanium alloy surfaces. J Biomed Mater Res B Appl Biomater. 2022 Jun 22.

III Riivari S, Närvä E, Kangasniemi I, Willberg J, Närhi T. Focal adhesion formation of primary human gingival fibroblast on hydrothermally and in-sol-made TiO_2 -coated titanium. Clin Implant Dent Relat Res. 2023;1-9.

IV Riivari S, Areid N, Närvä E, Willberg J, Närhi T. Saliva exposure reduces gingival keratinocyte growth on TiO_2-coated titanium. Manuscript.

1 Introduction

Nowadays, the implant osseointegration can be accomplished with relatively high success rates. However, there are several difficulties in soft tissue attachment to implant abutment which exposes to peri-implant infections in long-run. Gingival fibers in connective tissue are not able to attach directly to implant surface as they do to tooth surface, but the fibers are oriented parallery, which makes it easier for oral microbes to access peri-implant space (Berglundh et al. 1991, Ivanovski et al. 2018, Tetè et al. 2009). In the worst case, peri-implant infections can lead to the loss of an implant and the importance of prevention cannot be highlighted too much.

Currently, both titanium and zirconia are commonly used in implant prosthetics due to their optimal properties such as good biocompatibility, beneficial osseointegration, strength and mechanical properties (Bosshardt et al. 2000, Linkevicius et al. 2015). Zirconia has a benefit in esthetic areas, as its light color mirrors the natural tooth shade performing a more aesthetic treatment result compared to dark titanium (Sivaraman et al. 2018).

Gingival attachment to implant surface can be enhanced by changing the surface properties for instance by modifying the surface topography, wettability or bioactivity. Bioactive, nanoporous TiO_2-coating has shown beneficial properties in soft tissue cell response on implant material surfaces (Areva et al. 2004, Meretoja et al. 2010, Paldan et al. 2008, Rossi et al. 2008, Shahramian et al. 2017). Earlier, TiO_2-coating was produced in dip-coating method, which has its limitation when coating more complicated structures. The dip-coating method is also used in the first paper of this thesis. However, a novel method to produce nanoporous TiO_2-coating has been developed and it has been studied in the studies II, III and IV of this thesis. The novel polycondensation method makes it easier to coat more complicated structures directly in sol with routine laboratory circumstances. In addition, hydrothermal treatment of titanium surface is able to produce a bioactive nanostructure and further modify the soft tissue cell adherence on abutment surface (Areid et al. 2018).

The aim of this thesis is to find out, if TiO_2-coatings produced in sol or hydrothermally (HT), are able to enhance human gingival keratinocyte (HGK) and fibroblast (HGF) adhesion and growth on titanium and zirconia surfaces.

2 Review of the Literature

2.1 Peri-implant tissue attachment

Peri-implant tissue consists of three tissue types, bone, connective tissue and epithelium on the top. To accomplish a desirable treatment result, a proper attachment to all these tissues is crucial. Epithelium protects the underneath tissues and forms the first barrier against microbes. Connective tissue prevents bacterial invasion into deeper tissues, helps to maintain appropriate gingival anatomy around oral implants and prevents gingival recession. Meanwhile, osseointegration stabilizes the implant and is a requirement for successful implantation (Albrektsson et al. 2019).

2.1.1 Osseointegration

Implant research has previously focused mostly on developing firm osseointegration to bone (Brånemark 1969, 1983). The implant integration to bone is rigid without flexibility, while around natural teeth the bone attachment occurs via periodontal ligaments which enables a mild movement in occlusion. Most modern implants have some kind of surface treatment to improve osseointegration. These include mechanical (blasting, grinding, machining), chemical (acid treatment, anodization, sol gel treatment) and physical (plasma spraying, ion deposition) modifications of implant surfaces (Barfeie et al. 2015). Due to surface treatments, the osseointegration is rather comparable between different implant materials. It seems, that surface morphology plays more crucial role to osseointegration than material composition (Hanawa 2018). Nano- and microtopography have been evidenced to have favorable effects on osteoblast proliferation and may promote osseointegration (Albrektsson et al. 2019, Cooper et al. 2022, Inzunza et al. 2014). Today, the success rate of osseointegration is relatively high, over 95 % (Simão et al. 2022).

2.1.2 Gingival attachment

2.1.2.1 Gingival structure and function

Oral cavity is covered by three kinds of mucosa: specialized mucosa covering the tongue, lining mucosa covering soft parts of oral cavity such as cheek and lips and masticatory mucosa on alveolar ridges and hard palate (Squier et al. 2001). Oral mucosa forms from two main layers: oral epithelium and underneath the connective tissue called lamina propria. In the junction of epithelium and connective tissue, connective tissue papillae and epithelial ridges are overlapping (Squier et al. 2011). Histologically, a thin, non-cellular basement membrane is found in the epithelium-connective tissue interface. Basement membrane connects epithelium to lamina propria and supports epithelium (Brizuela et al. 2022). Depending on the mucosa type, there can be bone or submucosa consisting of muscle and elastic fibers underneath the connective tissue. When it comes to masticatory mucosa, the connective tissue attaches to periosteum of alveolar bone.

These two mucosal layers have their own typical characteristics and important functions. Firstly, the gingival attachment forms a strong mechanical protection against shearing, compression and abrasion due to biting forces. What is more, it makes barrier against oral microbes. Furthermore, the epithelium does not only make a physical barrier against microbes but also participate in immunoprotective system of mucosa when secreting antimicrobial factors, defensins named as one (Squier et al. 2011). Gingival fluid, which rinses the gingival pocket all the time and removes biofilm from gingival sulcus, is an important factor in cleaning the periodontal area. The gingival fluid also has some anti-microbial agents that help to disinfect sulcus area. Leukocyte and T-lymphocyte migration from junctional epithelium (JE) to sulcular plaque in case of an infection is thought to be another way of gingival defense (Berglundh et al. 1992, Bullon et al. 2004, Kawahara et al. 1998).

After implantation and several weeks of the healing process, a biological width is established around implants. After six weeks of implant placement, the maturation of connective tissue and barrier epithelium accomplish its final characteristics (Berglundh et al. 2007). This biological width consists of the area from highest part of peri-implant mucosa to bone contact of implant screw including sulcular epithelium (SE), JE and fibrous connective tissue. The height of this structure is evaluated to be approximately 3-4 mm (Zheng et al. 2021). The mucosa needs a certain minimum biological width, and in case the width is too low, bone resorption occurs (Berglundh et al. 1996). The dimensions and relationships of SE, JE and connective tissues are comparable between dento-gingival and implant-mucosal tissues (Cochran et al. 1997).

2.1.2.2 Epithelial structure and attachment

The oral epithelium consists of organized squamous stratified epithelium. There is variation on thickness of keratinization depending on the area in oral cavity. Non-keratinized epithelium is found in lining, moveable mucosa. Masticatory mucosa that is found on alveolar ridges is covered with keratinized or para-keratinized epithelium. Keratinization protects the epithelium against the chewing forces. The keratinized epithelium consists of four layers, which are named as stratum basale, stratum spinosum, stratum granulosum and stratum corneum. On the bottom, the columnar basal cells attach to basal lamina. The cells flatten when moving upwards and the highest layer, stratum corneum, consists of flat, keratinized cells. The HGKs attach to each other via desmosomes (Brizuela et al. 2022).

Gingival epithelium around teeth can be divided into three parts, which are gingival oral epithelium characterized as keratinized epithelium, SE and JE. SE lines the gingival sulcus around teeth. The length of sulcus area is from the highest part of gingival papillae to the bottom of sulcus, where the epithelium attaches to tooth surface and JE begins.

JE forms as a results of tooth eruption. Following the ameloblast shortening, the enamel epithelium reduces to lower layer of cuboidal cells covering the whole enamel. When the tooth erupts, this reduced enamel epithelium and oral epithelium fuse forming the JE (Shimono et al. 2003). Length of the JE is approximately 1.4-2.9 mm (Glauser et al. 2005). The JE differs from oral epithelium with wider intercellular spaces and fewer desmosomes between cells (Hashimoto et al. 1984, 1986). In these spaces, leukocyte and neutrophilic granulocyte infiltration can be seen near the sulcus area, where they are capable to phagocyte bacteria trying to access periodontal area (Schroeder et al. 1973, 2003).

The main function of the JE is to connect gingiva to tooth surface. This occurs via hemidesmosomes (HDs) and epithelium-derived extracellular matrix (ECM) facing tooth surface, which is called internal basal lamina. HDs are commonly located on the basal part of epithelial cells and connect the epithelial tissue to basal lamina. This structure is frequent in junctions where epithelium attaches to connective tissue (external basal lamina), but also in epithelial attachment to tooth surface (internal basal lamina). There is a difference in components of these two basal laminas. The JE is rich in integrin α6β4 and laminin 332, whereas most of the common basement membrane components are missing, such as collagen type IV and VII, laminin 511 and basement membrane proteoglygan perlecan (Hormia et al. 1998, 2001).

The epithelium has been proven to be able to attach to implant surface in quite similar ways as it does to a natural tooth via HD-like structures and basal lamina (Abrahamsson et al. 1996, Arvidson et al. 1996, Gould et al. 1981, Kawawara et al. 1998, Nakamura et al. 2018, Pöllänen et al. 2003). However, the attachment is

weaker with slower cell proliferation on implant when compared to tooth surface (Fujiseki et al. 2004, Ikeda et al. 2000). Even though JE around implant can be longer than around natural tooth, the internal basal lamina is often found only on cervical third of JE (Ikeda et al. 2000, Atsuta et al. 2005). Weaker attachment exposes to easier bacterial invasion to peri-implant space, which may increase the risk of peri-implant infections.

2.1.2.3 Connective tissue structure and attachment

Clinically, there are minor visual differences between healthy periodontal and peri-implant gingiva. The interdental papillae can be lower on peri-implant tissues compared to natural tooth and deeper probing depths can also be found around implants (Berglundh 2018).

However, there are many crucial differences, when soft tissue attachment to natural teeth and oral implants is compared on histological level. Connective tissue consists mostly of collagen fibers, ECM, blood vessels and human gingival fibroblasts (HGFs). Periodontal connective tissue includes two zones. HGFs are dominant on the inner zone, whereas blood vessels and collagen fibers rule the lateral zone (Moon et al. 1999). The collagen fibers consist mainly of collagen type I (Chavrier et al. 1999). Around natural tooth, the connective tissue fibers can attach directly to tooth surface. When it comes to connective tissue attachment to implant surface, the tissue resembles to scar being in close contact to implant or abutment but not attaching to it (Buser et al. 1992, Moon et al. 1999). The thickness of peri-implant connective tissue with apical extension is around 0.7 -2.6 mm (Glauser et al. 2005). Furthermore, the peri-implant connective tissue has fewer blood vessels. Besides, collagen fibers in connective tissue seem to round implant abutment circularly, not attaching it directly. This forms a capsule-like structure which enables a straight access for bacteria to the deeper tissues (Atsuta et al. 2016, Ruggeri et al. 1992, Scierano et al. 2002). To prevent the bacterial invasion into peri-implant space and decrease the risk for peri-implant infection, there is a clear need to improve the peri-implant gingival tissue attachment to implant surface.

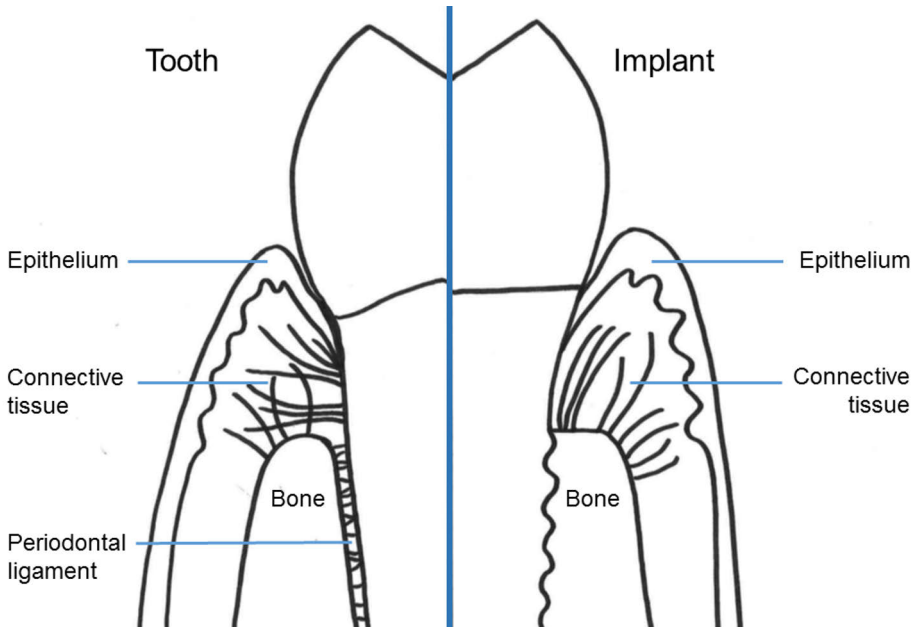

Figure 1. Gingival structure around tooth and implant. The gingival fibers in connective tissue are able to adhere directly to tooth surface, meanwhile they run parallel around implant abutment. In addition, periodontal ligaments attach root surface to bone, meanwhile bone is in immediate contact with implant surface.

2.1.2.4 Adhesion molecules on hemidesmosomes and focal adhesions

There are different ways for cells to attach to ECM, focal adhesions (FAs), focal complexes, HDs and fibrillar adhesion are ones to name (Gladkikh et al. 2018). The main function of these adhesion mechanisms is to bind intracellular parts of the cell to ECM, but also to regulate cell functions.

The HDs are precisely structured complexes, which main function is to connect cytoplasmic plaque to basal lamina via intermediate filaments forming a hemidesmosomal adhesion complex. Besides the cell adhesion, the HDs have a role in cell signal transduction (Jones et al. 1998). HDs consist of three molecular classes. First class is formed from cytoplasmic plaque proteins which form a link with the cytoskeletal parts of cell. The second group forms from transmembrane proteins that connect cell interior to ECM. Lastly, the third group consists of ECM basement membrane molecules. (Borradori et al. 1999).

The extracellular basal lamina is rich of laminins, which consist of α, β and γ subunits. The most important extracellular molecule attending epithelial attachment is laminin 332 consisting of α3, β3 and γ2 chains, earlier known as laminin-5 (Hormia et al. 2001). Laminin 332 has relatively high molecular weight 460 kDa (Marinkovich et al. 1992). The main role of laminin is to bind to integrins, which

penetrate through the cell membrane. (Shimono et al. 2003). Via integrin binding, laminins are capable to affect cell adhesion, migration, spreading, growth and differentiation. Especially, laminin 332 plays an important role in integrin α6β4 binding (Borradori et al. 1999).

Integrins are transmembrane protein family that consist of heterodimetic combinations of α and β subunits. Integrins act as adhesion receptors for ECM proteins such as laminin, collagen and fibronectin (Shimono et al. 2003). The integrin binding promotes gene expression, cell adhesion, cell spreading, cell migration and cell shape changes (Hemler 1993, Larjava et al. 2011).

Intracellularly the integrins can bind to adapter protein called plectin, which again are integrated to cytosolic keratin filaments. Plectin is a large phosphoprotein, with a size of 500 kDa. It has role in linking hemidesmosomal subunits to each other, but also in linking intercellular filaments to plasma membrane (Borradori et al. 1999). As the intracellular cytoskeleton is bonded to extracellular basal lamina, this structure will strengthen the resilience of epithelial tissue overall (Freeman, 1999).

FAs again connect the intracellular actin cytoskeleton to the extracellular fibers of fibronectin. FAs play important role in cell attachment but also in cell signaling. FAs are complex structures as over 50 different proteins have been found to be associated with focal contacts (Zamir et al. 2001). Integrins, vinculin, paxillin, focal adhesion kinase (FAK) and steroid receptor coactivator (SRC) are some important FA molecules to name.

As in HDs, intregrins also play crucial role in FAs as they pierce the cell membrane and act as receptors for ECM proteins. Most remarkable integrin subunits in FAs of HGF are α2, α4, α5, β1 and β3 (Hormia et al. 1994, Oates et al. 2005).

Vinculin and paxillin are common cytoplasmic FA molecules on the side of cytoplasm. Vinculin has a role in binding integrins to cytoskeleton. The molecular weight of vinculin is 117 kDa. The structure of vinculin consists of head and tail domains. The head domain fastens to actin-binding proteins such as talin to name one, while the tail part attaches to F-actin and paxillin (Ziegler et al. 2006). It is also thought that vinculin is able to stabilize FAs and, in this way, can enhance cell spreading (Ezzell et al. 2008). Paxillin also works between cytoplasm and nucleus, but most importantly it plays role as a FA mediator and regulates kinase signaling. In FA complexes paxillin plays role in connecting integrins to FAK. Paxillin consists of four LIM (Lin11, Isl- 1, and Mec-3) domains on carboxyl terminal that all contain two zinc-fingers divided by amino acids and 5 leucine-rich LD domains on amino-terminus. The molecular weight of paxillin is 68 kDa (Ma et al. 2018, Schaller 2001, Tumbarello et al. 2002).

FAK (120 kDa) and SRC form a dual kinase complex, which can phosphorylate other adaptor proteins such as paxillin. FAK-SRC complex is usually activated by

integrin regulated signaling. The activated complex can affect cell survival, cell cycle and cell motility (Le CoQ et al. 2022, Mitra et al. 2006).

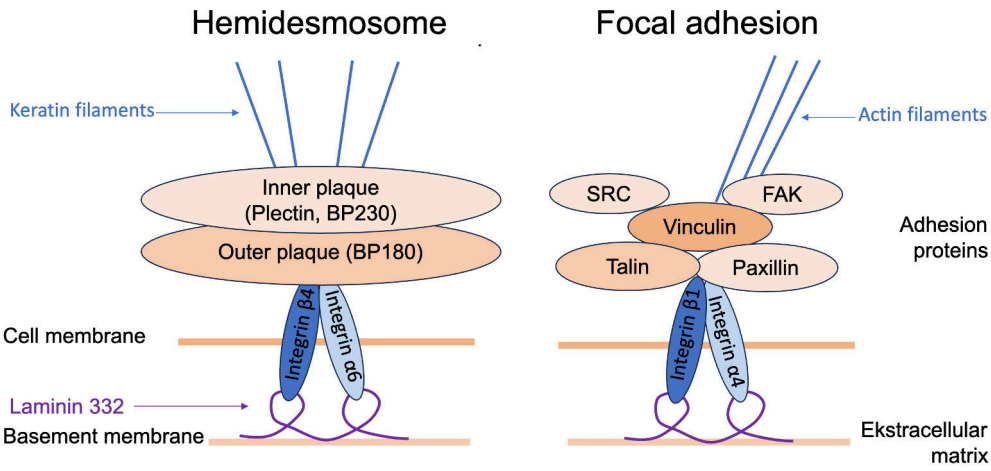

Figure 2. A simplified structure of HDs and FAs.

2.1.3 Saliva exposure

In oral conditions, the exposure to saliva can change the bioactivity of implant surfaces by forming a thin protein containing layer on implant surface. A major part of saliva is water, but there are also minerals, enzymes and over 1400 different salivary proteins. Most common salivary proteins are amylase, mucins, statherins, cystatins, histatins and proline-rich proteins (Kunrath et al. 2022, Fisher et al. 2021, Carpenter et al. 2013, Scarano et al. 2010). These salivary proteins can bind to dental material surfaces, which can either enhance or decrease their biocompatibility. Higher amounts of adherent salivary proteins can be found on rough and hydrophobic surfaces (Kunrath et al. 2021, 2022).

2.1.4 Peri-implant infections

Both periodontal and peri-implant infections are plaque associated diseases (Berglundh et al. 1992) and tissue response against biofilm formation is shown to be rather similar around dental implants and natural teeth (Berglundh et al. 2005, Ericsson 1992, Pontoriero et al. 1994, Zhang et al. 2023).

Peri-implant infections are relatively common diseases. When a reversible inflammatory reaction is found only in peri-implant soft tissues, the status is called peri-implant mucositis, which is comparable to gingivitis around natural tooth. As the infection penetrates to deeper tissues and causes loss of supporting bone, it is called

peri-implantitis (Albrektsson et al. 1994, Zitzmann et al. 2008), which is comparable to periodontitis around natural teeth. The prevalence of peri-implant mucositis has been reported to range from 50 to over 90%. Whereas the prevalence of peri-implantits is from 12 to 43% (Roos-Jansåker et al. 2006, Fransson et al. 2008). Many studies have indicated peri-implantitis to be a reason for implant loss in 10-50% of implant loss cases (Esposito 1998, Zitzmann et al. 2008). Differences between success rates of different implant types can be due to surface structure of implants or patient selection. Factors that expose to peri-implantitis are periodontitis background, poor oral hygiene and plaque accumulation, excess cement, smoking and diabetes (Renvert et al. 2015, Annibali et al. 2016). Also, lack of collagen fiber attachment, poor epithelial attachment and deeper probing depths around implants are thought to expose to microbial penetration and peri-implant infections (Schupbach et al. 2007).

Clinical diagnose of peri-implant disease require periodontal probing. Peri-implant mucositis is identified with clinical signs of inflammation, erythema, swelling, suppuration or bleeding on probing (Zitzmann et al. 2008). Increased probing depths may also occur in case of peri-implant mucositis due to swelling or decreased probing resistance of gingiva (Berglundh 2018). To diagnose bone loss around implant supporting bone, intraoral radiograph is needed.

Histological characteristic of peri-implant mucositis is a lateral inflammatory lesion to JE. The lesion consists of vascular structures, plasma cells and lymphocytes. While in peri-implantitis the inflammatory lesion with plasma cells, more lymphocytes, neutrophils, macrophages and elastase-positive cells extend to apical direction (Ericsson et al. 1992, Gualini et al. 2003, Berglundh et al. 2018, Esposito et al. 1997).

The crucial factor in preventing and treating peri-implant mucositis, is maintaining proper oral hygiene and supportive periodontal therapy (Pontorero et al. 1994). If peri-implant mucositis is not properly treated, it can develop to peri-implantitis in some cases (Jepsen et al. 2015). When it comes to treatment of peri-implantitis, besides nonsurgical therapy including scaling, often surgical treatment is needed (Renvert et al. 2017).

2.2 Oral implant abutment materials

2.2.1 Titanium

Titanium has been used in dental implantology for decades (Jorge et al. 2013). Titanium alloys (e.g. Ti-6Al-4V) are commonly used material in many implants and their components. Titanium's mechanical properties such as fatigue strength, formability and corrosion resistance are excellent. Furthermore, titanium has a desirable biocompatibility. Good biocompatibility is thought to be due to thin

titanium dioxide (TiO_2) layer that forms on titanium surface when in contact with air (Hanawa 2020, Liu et al. 2004). The disadvantage of titanium is the dark color. The dark color of implant abutment can lead to unpleasant esthetic outcome, if gingival recession occurs or patient has thin gingiva phenotype. It has been reported that in some cases titanium may cause hypersensitivity reactions (Poli et al. 2021).

2.2.2 Zirconia

As stated earlier, even though titanium has many great advantages as an implant material, there are some unfavorable properties concerning titanium abutments. Nowadays, esthetic demands are getting higher, especially when replacing teeth in the esthetic zone. Thus, a need for more esthetic abutment materials is increasing. Yttria stabilized zirconia has a light color and natural light transmission, that resembles natural teeth. By using zirconia structures, an unpleasant dark color shining under thin mucosa around implant abutment, can be avoided. Also, if gingival recession occurs, the visible zirconia abutment with light color is more esthetic compared to dark titanium. Zirconia can be found in three forms depending on the processing temperature: monoclinic, cubic and tetragonal. When zirconia transforms from one phase to another, a change in volume occurs which enhances fracture resistance and strength. Normally, tetragonal form of zirconia demands high temperatures, but it can be formed at room temperature by using yttria as a stabilizer. Thus, under mechanical pressure a beginning of a fracture can be fixed by volume transformation as tetragonal zirconia transforms into monoclinic (Piconi et al. 1999). What is more, zirconia has shown excellent biocompatibility in oral circumstances including desired osseointegration and peri-implant tissue response along with good mechanical strength. Also, desirable cell adhesion properties have been found on zirconia and titanium surface (Furuhashi et al. 2021). However, bone loss around zirconia abutments has been found to be higher than around titanium abutments (Olander et al. 2022). In addition, hyper-sensitivity to zirconia has not been reported. However, most of the studies concerning zirconia implants are short-term and longer-term clinical studies are needed to be certain of survival rates on long-term (Apratim et al. 2015, Siddiqi et al. 2017, Sivaraman et al. 2018, Özkurt et al. 2011). All in all, zirconia is suggested to be appropriate for abutment material.

2.3 Implant surface modifications

2.3.1 Surface characteristics and properties

Important things, that influence cell attachment to implant surface, are the wettability of the surface, surface roughness, porosity and nanotexture (de Santana et al. 2010,

Hicklin et al. 2015). The surface properties of titanium can be enhanced with surface topography modifications or removing contaminations by grinding, machining, polishing, acid-etching and blasting particles with high velocity (Liu et al. 2004).

Earlier studies concerning osseointegration have evidenced improved osseointegration on surfaces with roughness of Sa 1-2 µm compared to smoother surfaces (Rupp et al. 2018). Many studies have indicated that a nanoporous surface is optimal for soft tissue cell attachment. Important thing affecting the cell adhesion is the nanoparticle size. Nanoparticles with a diameter of 20 nm on titanium surface seem to improve cell adhesion compared to machined, sandblasted and acid-etched titanium surfaces (Vignesh et al. 2015). Ferrà-Cañellas et al. (2018) demonstrated that TiO_2 nanopores created with electrochemical oxidation with the diameter of ~74 nm had higher surface area and number of peaks compared to non-coated titanium. Also, cell adherence was significantly higher on nanoporous surface. Kubo et al. (2009) demonstrated that the particle size of 100, 300 and 500 nm do not increase HGF attachment or strength of adhesion on titanium surface. Thus, smaller than 100 nm nodules seem to be more favorable for cell adherence.

Kim et al. (2015) demonstrated that certain surface properties correlate with HGF attachment. These are water contact angle (WCA), developed interfacial area ratio and surface slope. They evaluated six different implant surfaces and the study showed best cell adhesion on smooth and hydrophilic surface with smallest possible WCA and surface roughness with S_a of 0.2 um or less.

2.3.2 Bioactivity and biocompatibility

The chemical composition has an influence on biocompatibility of implant materials. Titanium, zirconia and aluminum oxide have shown admissible biocompatibility concerning soft tissue cell growth on surfaces. Meanwhile lower biocompatibility was found on gold and porcelain abutments (Rompen et al. 2006).

A thin TiO_2-layer forms on titanium surface when in contact with air. The natural TiO_2-layer is rather weak, and this is why novel methods to improve bioactivity, biocompatibility, wear resistance and to create a more stable TiO_2-layer have been developed (Liu et al. 2004). When it comes to zirconia, surface modifications have been shown to enhance osseointegration and biocompatibility also on zirconia surface (Schünemann 2019).

There are many ways to enhance implant surface properties in addition to enhancing surface roughness or topography. The surface can be modified with methods, where chemical or electrochemical reactions take place while treating titanium surface. These chemical methods consist of sol-gel coating, electrochemical treatment (anodic oxidation, AO), chemical vapor deposition and biochemical modification. Besides chemical methods, also physical methods, such as thermal

spraying, can be used in surface modification. Concerning physical modification, the modification occurs via thermal or kinetic energy instead of chemical reaction (Liu et al. 2004). All in all, different coating methods have their pros and cons, and development of various techniques is going on.

The sol-gel derived TiO_2-coating is one option to modify implant surface. The deposition of TiO_2-nanoparticles on titanium surface has earlier been made with dip-coating method (Areva et al. 2004, Jokinen et al. 1998, Meretoja et al. 2010, Peltola et al. 2000, Rossi et al. 2008). The same coating method can be produced on zirconia surface (Shahramian et al. 2017, 2020). The benefits of sol-gel coating are that it can be produced as thin layer, it fastens tightly to substrate and no delamination has been found. The sol can be modified, and the aging time of sol seems to affect height of peaks and valley on the surface. Also, when more coating layers are added, the peak distances get closer, which is optimal for calcium phosphate formation (Peltola et al. 2000). In this thesis, a novel method to produce a sol gel coating has been studied (II, III, IV). The novel coating is made directly in sol and no dip-coating mechanism is needed. This makes the coating process simpler, when only basic laboratory equipment is needed. In addition, objects with more complicated shapes can be easier to coat. Furthermore, this coating method is suitable for both titanium and zirconia.

Another coating method used in this thesis, is the hydrothermal treatment (HT) of titanium. This is a chemical method, where TiO_2-coating is produced on titanium under high pressure. HT method produces a crystalline nanostructure and composes OH^- groups on titanium surface, which enhances the bioactivity of surface (Ueda et al. 2008). The HT coating has shown potential on changing surface hydrophilicity, HGF attachment and tissue response on titanium surface (Areid et al. 2018, 2021, Shi et al. 2015, Zuldesmi et al. 2015).

When studying cell attachment to implant surface, often the bacterial adhesion and growth is also in scope of interest, as peri-implantitis is a biofilm caused disease. As the nanoporous surface is optimal for cell adhesion, there is a risk, that bacterial adhesion would also be increased. Besides the improved cell attachment, it is important that the implant surface does not increase bacterial colonization. When it comes to nanoporous TiO_2-coated surfaces, the surface does not seem to increase bacterial growth on implant surface. Närhi et al. (2011) demonstrated, that sol-gel derived TiO_2-coating does not increase Aggregatibacter actinomycetemcomitans or Fusobacterium nucleatum colonization on titanium surface. Also, Areid et al. (2018) evidenced similar results concerning HT surface in vivo. Furthermore, some studies have shown lower bacterial adhesion on anodized titanium surface (Bierbaum et al. 2018, Del Curto et al. 2005, Dorkhan et al. 2014).

3 Aims

The objective of this study was to find out, whether the nanoporous, bioactive TiO_2 –coating has ability to enhance the peri-implant soft tissue cell attachment to implant material surface. The focus of the study was to examine the attachment mechanisms of the oral HGKs and HGFs to zirconia and titanium surfaces. Special emphasis was paid to examine adhesion protein expression and FA localization of HGKs and HGFs. The study was based on working hypothesis that nanoporous bioactive TiO_2 coating enhance cell attachment. Following specific aims were set to test the working hypotheses:

1. To estimate the effects of TiO_2 -coating to the HGK attachment and growth on coated and non-coated zirconia surface.
2. To compare cell attachment properties of in sol TiO_2-coated and non-coated titanium and zirconia and to study the adhesion protein expression on coated and non-coated surfaces.
3. To examine the expression of adhesion molecules, focal contact areas and cell spreading of HGFs on TiO_2-coated and non-coated titanium surfaces.
4. To study, whether saliva exposure changes surface properties and cell response on TiO_2-coated and non-coated titanium surface.

4 Materials and Methods

4.1 Material preparations (I, II, III, IV)

When cell adhesion to material surface is studied in vitro, implant surface can be mimicked with different kind of discs cut from the chosen material. In the first study (I), only zirconia was used as a test material. Half of the zirconia discs were coated with a sol gel derived TiO_2-coating made with a dip-coating method. The second study (II) extended the studied materials to zirconia and titanium. In the second study, coating made in sol technique was used instead of dip coating. In addition, zirconia and titanium discs were compared to each other. The third and fourth study focused on titanium substrates. In the third study (III), two different coating methods, in sol coated and HT titanium, were used. Meanwhile, the fourth study (IV) compared in sol coated titanium with and without saliva treatment.

In the first two experiments, 10×10 mm square shaped disc were produced from zirconia (ZrO_2 +HfO_2 + Y_2O_3 99,5% and other oxides 0.5%, Z-CAD, Metoxit, Switzerland) and titanium (grade 5, titanium 90%, vanadium 6%, aluminum 4%). The discs were produced by cutting from blocks with histological saw (Struers Secotom-50, Copenhagen, Denmark). In the third and fourth study, factory-made round (diameter 10 mm) titanium discs (grade 5) were used. To ensure homogenic surface between all the samples, the discs were polished with sandpaper (grit 1200). After polishing, the zirconia samples were sintered at 1400°C for one hour. Finally, all the samples were cleaned ultrasonically in acetone and ethanol (5'+5') and left to dry properly in the air.

4.2 Coating methods (I, II, III, IV)

4.2.1 Dip coating (I)

At first, the preparation of sol was accomplished. The same protocol was used for sol used in dip coating method and in coating-made-in-sol. To start with, tetraisopropylorthotitanate (28.40 g) [Ti(OCH(CH3)2)4] was dissolved in ethanol (95%, 21.20 g), covered with parafilm and mixed effectively with magnetic stirrer (60 s). Meanwhile another solution was prepared. Ethanol (95%, 16.7 g) was mixed

with ethylene glycol monoethyl ether (4.5 g) and HCl (1 M, 1.8 g). Again, the solution was effectively mixed. The second solution was added to the first solution and carefully blended for three minutes (min). After this, the sol was left to age for one day at 0°C.

The first study consisted of two experimental groups. Half of the zirconia discs (n=28) were coated with TiO_2 –coating, while the rest of the samples were non-coated and formed a control group. The coatings were made with a dip coating –method. The discs were dipped into sol-gel solution at a speed of 47.7 mm/min. The TiO_2 -coatings (MetAlive™, ID Creations) were finished by sintering the samples in ceramic oven at 500°C for one hour. The discs had a 2 mm tail, where the holder was placed and after the dipping and sintering, the extra part was cut away. This was followed by ultrasonic cleansing with acetone and ethanol (5' + 5').

4.2.2 Coating made in sol (II, III, IV)

In the studies II, III and IV, coating-made-in-sol –method was used. Altogether, 56 zirconia and 56 titanium discs were used in the second and 90 titanium discs in the third study. Again, half of the zirconia and titanium discs were coated for the second study and the other half formed the control group. In the third study, only one-third of the samples were coated with this method, as the study consisted of three groups – coating made in sol, HT and control group. In-sol-made coating process varied depending on used substrate. Concerning titanium samples, the coating procedure was quite simple. The washed and dried discs were covered with TiO_2 -sol, which was produced as earlier described in dip-coating section. The samples were kept in sol in a freezer (-18°C) for two hours. At the same time, zirconia samples were first treated with NaOH (0.5 mol/l, 2%, 3 '). This was followed by rinsing the samples with ethanol (95%), drying them and setting to TiO_2 -sol solution. The samples were kept in sol at glycol bath at 0°C for two hours. After coating, the samples were washed three times with ethanol and placed in a ceramic oven at 500°C to fasten the coating (10 min). In the end, the discs were again cleaned with acetone and ethanol (5' + 5').

4.2.3 Hydrothermal coating (III)

In the third study, the in sol coated and HT coated titanium were compared to each other and with the control group. Only titanium substrates were used. Titanium oxide powder (3.2 g) was mixed to water (160 g) and combined to tetramethylammonium hydroxide diluted in water (1:10). The solution was mixed for 5 min. The washed and dried titanium samples and the prepared HT suspension was put into a Teflon vessel located inside a hydrothermal reactor. The cylinder was properly tightened

and located in an oven (150°C, 48 h). During the process, the cylinder was spinning slowly. After 48 hours, the cylinder was cooled down and the coated samples were washed again with acetone and ethanol (5' + 5') in ultrasonic bath.

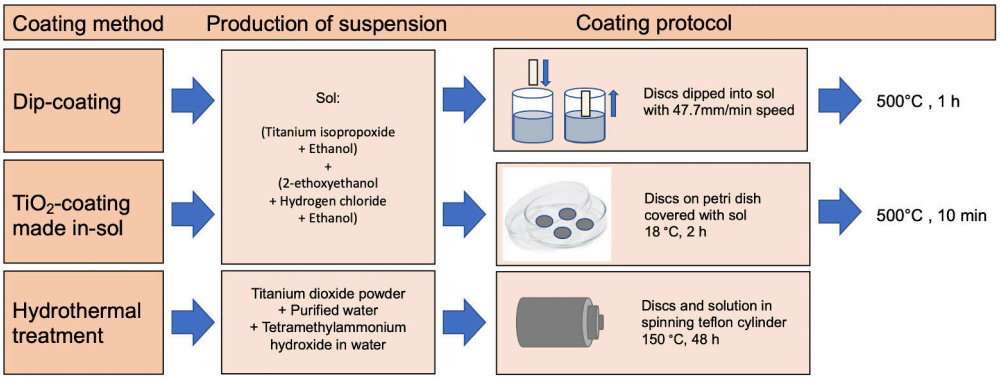

Figure 3. A simplified chart of coating protocols.

4.3 Scanning electron microscopy (II, III)

The surface topography of coated and non-coated zirconia and titanium specimens were imaged with 2 kV scanning electron microscope (SEM) (Apreo S field-emission SEM, Thermo Scientific, Netherlands) equipped with an Ultim Max energy dispersive x-ray spectrometer (EDS; Oxford Instruments, UK). To prove the presence of titanium particles on coated zirconia surface, EDS-analyze was accomplished.

4.4 Surface wettability

4.4.1 Contact angle measurements (I, IV)

Contact angles were measured with a sessile drop method (Contact angle meter Attension Theta, Biolin Scientific). Ten drops of diiodomethane, formamide and distilled water were used as a probe on coated and non-coated zirconia (I). Only water was used on contact angle measurements on coated and non-coated titanium with and without saliva treatment (IV). The contact angle meter's video camera recorded the shape of a drop on zirconia surface and analyzed the contact angles from both sides and the mean value of the drop with the Young-Laplace equation.

4.4.2 Surface free energy (I)

Surface free energy (SFE) were determined with the Owens-Wendt (OW) and Van-Oss (VO) models. Long-range dispersion (Lifshitz-van der Waals) (γ_d) and the short-range polar (hydrogen bonding) (γ_p) components of SFE are studied with OW. Meanwhile, VO approach express the dispersive (γLW) and the polar acid-base (γab) components, consisted of two parts, acidic ($\gamma+$) and basic ($\gamma-$).

4.5 Saliva treatment and salivary protein adsorption (IV)

Paraffin-wax stimulated whole saliva was collected from seven healthy, non-smoking adult volunteers. Saliva was collected into chilled test tubes for 10 min. The collected saliva was centrifuged at 9500 rpm for 40 min and pasteurized at 60°C for 30 min to eliminate bacterial growth. This followed a new centrifugation. The saliva samples were stored at -70°C. The saliva treatment of the discs was accomplished by pipetting 1 ml mixture of saliva and phosphate buffered saline (PBS, 1:1) on titanium discs. After 30 min of incubation at room temperature, the discs were washed three times with PBS.

Total protein amounts were measured before and after saliva treatment on TiO_2-coated and non-coated discs. For protein measurement heated (95°C) 2% SDS buffer was pipetted on titanium discs. After 5 min, the buffer was collected, boiled at 95°C (7 min) and diluted in PBS (1:20). A mixture of 150 µl solution and 150 µl of Micro BCA™ Protein Assay Kit (Thermo Scientific™) were set into 96-well plate and incubated for 2 hours. Protein amounts were detected with Multiskan FC reader (562 nm, Thermo Scientific).

4.6 Cell cultures (I, II, III, IV)

4.6.1 Epithelial cell cultures (I, II, IV)

In the studies I, II and IV, spontaneously immortalized HGKs were used. These cells were previously taken from a human gingival biopsy sample (Mäkelä et al. 1998). Before cell cultures, the zirconia and titanium samples were sterilized in an autoclave.

4.6.1.1 Cell adhesion

The first study explored the influence of TiO_2 -coating on adhesion and proliferation of HGKs on zirconia surface. Whereas the second study compared the effects of

coatings on both titanium and zirconia surfaces. The fourth study evaluated the effects of saliva exposure on cell adhesion on titanium surface. In these studies (I, II, IV), the HGKs were cultured in keratinocyte-serum-free medium (Gibco®, Thermo Fisher, USA). To define the levels of cell adhesion, the HGKs were cultured on the samples for 1, 3, 6 and 24 hours at 37°C at a density of 25 000 cells/ cm^2 (I, II). After the wanted adhesion period, the samples were rinsed with phosphate-buffered saline (PBS), treated with TE-buffer (10 mmol/L Tris, 1 mmol/l EDTA) and thereafter stored frozen (-70°C) while waiting further experiments. The melted samples were sonicated (30 s) to release the genomic DNA. The samples were stained with fluorescent nucleic acid stain (Pico-Green dsDNA, Molecular Probes Europe) followed by a measurement of fluorescent values with wavelengths of 490 nm and 535 nm (BioTek synergy HT). The fluorescence values were compared to standard curve to define the total amount of DNA.

4.6.1.2 Cell adhesion strength (IV)

To define the adhesion strength, the HGKs were incubated at density of 12 500 cells/cm^2 on non-coated and TiO_2-coated discs before and after saliva exposure for 2 and 6 hours (IV). Cumulative serial trypsinization method was used to determine resistance to enzymatic detachment. After wanted time period, the samples were washed with PBS followed by coverage with diluted trypsin ((0.005% trypsin (Gibco, Invitrogen) in PBS (1:5)). The trypsin was changed, and the samples were collected after 1, 5, 10 and 20 min. After 20 min, the remaining cells were collected with undiluted trypsin at 37°C (5 min). Then 500 µl of TE-Triton X-100 was pipetted on Eppendorf tubes, which were frozen at -70°C. Next day, the number of detached cells was measured using PicoGreen dsDNA-kit (Molecular Probes Europe, Netherlands). Fluorescence values were detected with wavelengths of 490 and 535 nm.

4.6.1.3 Cell proliferation (I, II, IV)

The long-term cell growth was studied by growing the HGKs on the samples for 1, 3 and 7 days (I, II, IV). This was followed by Alamar Blue treatment (Thermo Fischer, USA). After three hours of incubation in a CO_2-incubator (+37°C), the cell amounts of the samples was measured by adding 200 µl of the solution from the sample on a microtiter plate and the absorbance of the solutions was measured with a wavelength of 569 and 594 nm (Multiskan FC, Thermo Scientific). These values were again compared to standard curve to define the total amounts of attached HGKs (I, IV). In the second study (II), number of attached cells on coated discs were compared to non-coated ones and relative cell attachment was counted.

4.6.2 Cell culture of gingival fibroblasts (III)

For the third study, primary HGFs were collected. Gingival biopsies were taken from healthy adult patients during the third molar surgical extraction (Oral Health Care, City of Turku, Finland). After the extraction, the biopsies were set in cell medium [Gibco Dulbecco's Modified Eagle Medium (DMEM), Thermo Fisher Scientific] mixed with antibiotics (Pen Strep, Penicillin 10.000 units/ml, Streptomycin 10.000 µg/ml, Gibco) and transported to cell laboratory. The biopsies were set on a petri dish, cut to smaller pieces and covered with DMEM. The gingival samples were cultivated in a CO_2-incubator (+37°C) for three days before changing the media for the first time, followed by two weeks of culture. During the cell culture, media was changed three times in a week. Ethical committee permission for the collection of gingival biopsies was obtained from the Hospital District of Southwest Finland.

To determine the initial cell adhesion and cell spreading, the HGFs were cultured on titanium discs at density of 30 000 cells/cm^2 for 2 and 24 hours (DMEM, Gibco, 37°C, humidified atmosphere). For Western blotting (WB), HGFs were incubated in media for 3 days.

4.7 Western Blotting (II, III)

To define, whether there is difference in adhesion protein expression on coated versus uncoated surfaces, WB was accomplished. HGFs were cultivated on coated and uncoated discs for three days. Thereafter, the discs were washed with PBS and treated with TXLB-buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-X, 0.5% glycerol, 1% SDS, Complete protease inhibitor (Sigma- Aldrich), and phos-stop tablet (Sigma-Aldrich)] warmed to 95°C. The solution was collected in Eppendorf tubes and warmed in 95°C for 10 min. The solution was stored at -20°C. The protein amounts were defined with Protein Assay Reagent (Bio-Rad) and equal protein amounts were used. The solutions from each sample were mixed with 8x sample buffer and placed on Mini Protean TGX Precast SDS-PAGE Gels (Bio-Rad), following solution transfer to membrane (Trans-Blot Turbo Transfer System, Bio-Rad). The membranes were washed twice with mQ (milliQ, ultrapure water), once with TBST (Tris buffered saline with Tween) and blocked in milk mixed in TBST (5%, 1 hour). The membranes were stained with primary antibodies Laminin γ2 ((C-20):sc-7652, Santa-Cruz Biotechnology, Inc. 1:100), Integrin α6 (HPA12696, Sigma-Aldrich, 1:500), Integrin β4 (Ab182120, Abcam, 1:200), (vinculin V9131, Sigma-Aldrich, 1:1000), paxillin (612405BD, BD Biosciences, 1:5000), FAK (610088, BD Biosciences, 1:5000) and GAPDH (5G4MaB6C5, Hytest, 1:20 000) in 5% milk overnight at + 4℃. Thereafter, the membranes were washed three times with TBST and treated with secondary antibodies for one hour (Anti-Mouse 926-68072, LI-COR Biosciences, 1:5000, Anti-Rabbit 926-32213, LI-COR Biosciences,

1:5000, Anti-Goat 926-32214, LI-COR Biosciences, 1:5000). After this, washing three times with TBST was repeated. Finally, the membranes were imaged with Li-Cor, Infrared Imager, Odyssey. Altogether, three biological replicates were used.

4.8 Microscope analyses (I, II, III, IV)

4.8.1 Light microscopy (I)

To examine cell amount and uniformity of the cell layers, the discs were fixed and imaged microscopically. After cell culture and proliferation measurements, the zirconia discs were washed with PBS and fixed in glutaraldehyde mixed with PBS for 5 min. This followed again washing twice with PBS and serial dehydration in ethanol. After fixation, the discs were cut (Exakt 300 Diamond Band Saw, EXAKT Technologies) and cell morphology was evaluated with a light microscope (Leitz Aristoplan, Leica Microsystems, Wetzlar, Germany).

4.8.2 Confocal microscopy (II, III, IV)

To analyze the cell morphology, spreading and FA formation, the samples were fixated for confocal microscope analyze after 2 (III) and 24 (II, III, IV) hours of cultivation. The samples were treated with formaldehyde (4%, 15 min), washed with PBS and kept at 4°C while waiting further treatments. Thereafter, the samples were handled with 300 µl 0.5% TRITON-X-100 in PBS (15 min). In the studies, laminin y2 (II, IV) ((C-20):sc7652, Santa-Cruz Biotechnology, Inc., 1:100), integrin α6 (II) (MCA699, Bio-Rad, 1:100), integrin β4 (II, IV) (Ab110167, Abcam, 1:200), vinculin (II, III) (V9131, Sigma-Aldrich, 1:100), paxillin (II, III) (ab32084, Abcam, 1:500), DAPI (II, III, IV) (nucleus) and F-actin (II, III, IV) (Phalloidin Atto, 65906, Sigma-Aldrich, 1:200) were used as primary antibodies. The antibodies were mixed with 30% horse –antibody in PBS and 100 µl of mixed solution was left on the samples for 24 hours. Next day, the stained samples were washed (3 x PBS) and handed with secondary antibodies (Anti-Rat, A11077, ThermoFisher Scientific, 1:400, Anti-Mouse, A21202, ThermoFisher Scientific, 1:400, Anti-Rabbit, A21206, ThermoFisher Scientific, 1:400) for one hour under aluminum foil. The discs were washed again with PBS. A drop of glue was pipetted on a microscope glass. One sample at a time was dried and flipped on a glass upside down and left to dry in the dark for one day. A high-resolution confocal microscope (63x Zeiss Plan-Apochromat, Hamamatsu sCMOS Orca Flash4.0, 3i CSU-W1 Spinning Disk) was used in imaging. Cell spreading, signal intensities of integrin α6, β4, laminin γ2 (II, IV), vinculin and paxillin as well as FA amount and size (III) were measured from confocal images and analyzed with ImageJ, Fiji program.

4.9 Statistical analyses (I, II, III, IV)

In the first study, JMP Pro 12 statistical software (SAS Institute, USA) was used in statistical analyses. The cell and DNA amounts formed a non-continuous distribution and the Mann-Whitney U-test was used.

ImageJ, Fiji-program was used in WB and confocal microscope image analyses (II, III, IV). The data from II, III and IV studies were analyzed as well as the graphs were made with GraphPad Prism-program (GraphPad Software, USA). The statistical significance among different groups was determined with unpaired T-test, Mann-Whitney U-test and one-way ANOVA with Tukey's multiple comparisons test.

5 Results

5.1 Surface characteristics (II, III)

The surface topography of in-sol-coated zirconia and titanium, HT titanium and non-coated titanium and zirconia samples were studied with SEM using 8 000 and 50 000 × magnification. With higher magnification, the difference in surface nanostructure was revealed. All the TiO_2-modified discs showed nanosized particles on titanium and zirconia samples. With HT treated titanium, the nanostructure was more intense. To confirm, that zirconia samples certainly were covered with titanium particles, EDS-analyze was performed. EDS-analyze demonstrated a clear induction of titanium on coated zirconia meanwhile non-coated samples were titanium free (Fig. 4).

Non-coated polished titanium has a color of grey/silver. Meanwhile, the color of HT titanium's is more like bronze and the color of in sol coated titanium resembles gold (Fig. 5).

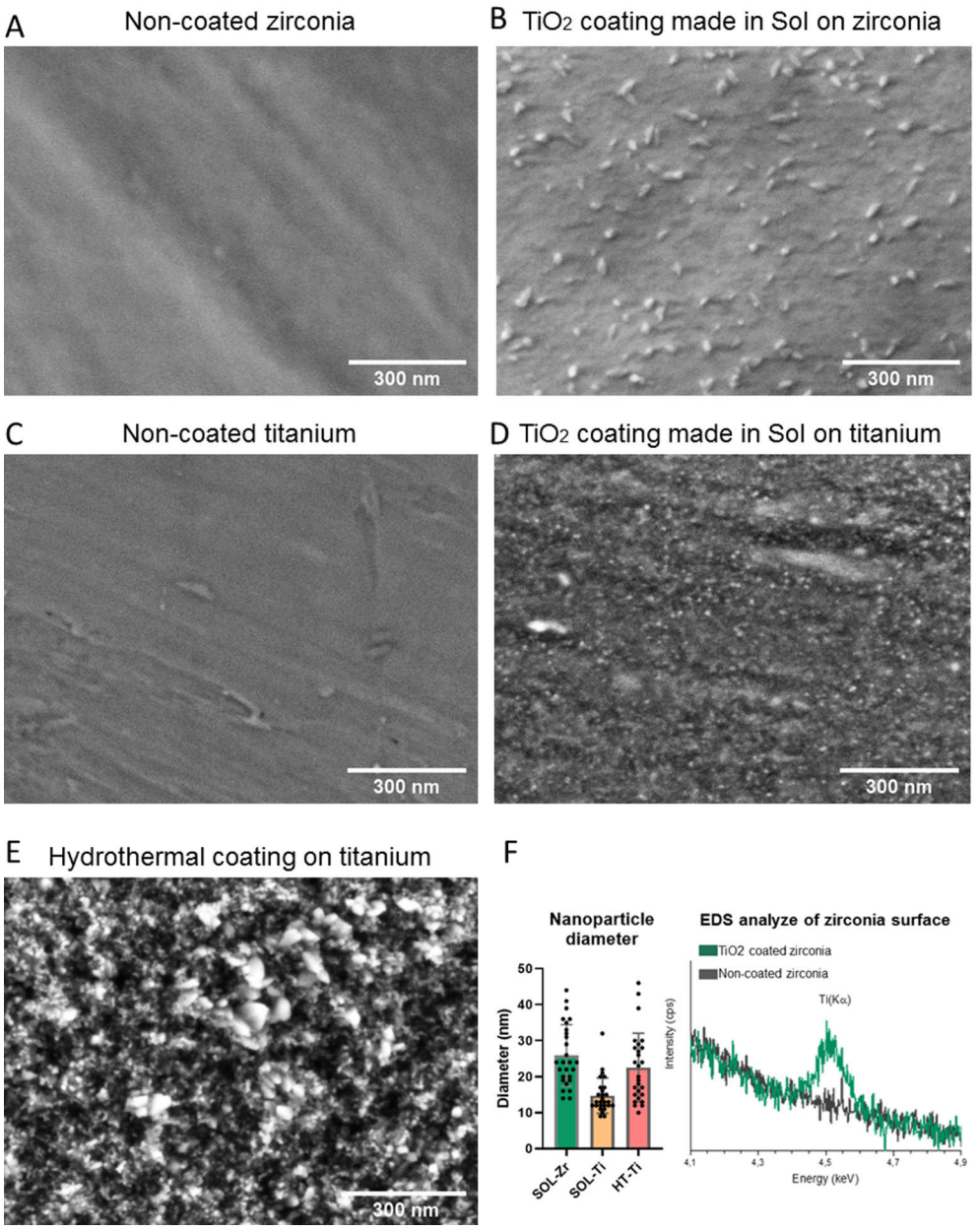

Figure 4. Surface topography of **A.**) non-coated and **B.**) in sol TiO_2 coated zirconia, **C.**) non-coated, **D.**) in sol coated and **E.**) HT titanium imaged with SEM. Representative images of each surface topography imaged with 50 000 x magnification. **F.**) Nanoparticle diameters on in sol coated zirconia (Sol-Zr) and titanium (Sol-Ti), and HT coated titanium (HT-Ti). Mean ± SD + individual values, ANOVA.

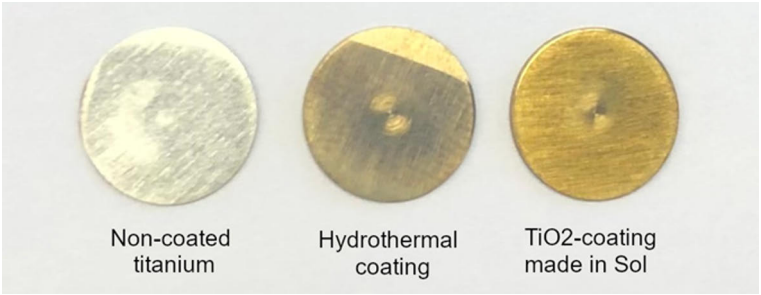

Figure 5. The color of TiO_2-coated titanium resembles bronze (HT) and gold (In sol coated).

5.2 Surface wettability (I)

To determine the surface wettability of coated and non-coated zirconia and titanium substrates, contact angles and SFE were measured.

5.2.1 Hydrophilicity of TiO_2-coated surfaces

The TiO_2-coated zirconia samples revealed significantly lower di-iodomethane and water contact angles when compared to non-coated zirconia. Water contact angle on TiO_2-coated titanium was also significantly lower compared to non-coated titanium. However, contact angles decreased significantly after saliva treatment on both non-coated and TiO_2-coated titanium surfaces. No significant difference was found among saliva coated groups. (Fig. 6)

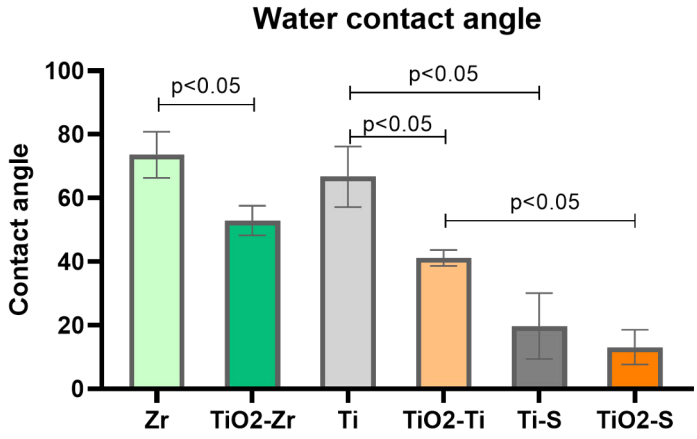

Figure 6. Water contact angle values on non-coated zirconia (Zr), dip-coated zirconia (TiO_2-Zr), non-coated titanium (Ti), in-sol coated titanium (TiO_2-Ti), saliva treated non-coated titanium (Ti-S) and saliva treated in-sol coated titanium (TiO_2-S). Shown are means ± SD. Significant p-values ($p < 0.05$) are marked in the figures.

5.2.2 Surface free energy (I)

SFE of non-coated and coated zirconia surface was measured with two methods, VO and OW approaches. With OW approach, polar SFE and consequently total SFE were significantly higher on coated zirconia compared to non-coated zirconia. With VO approach, no significant difference was found. (Fig. 7)

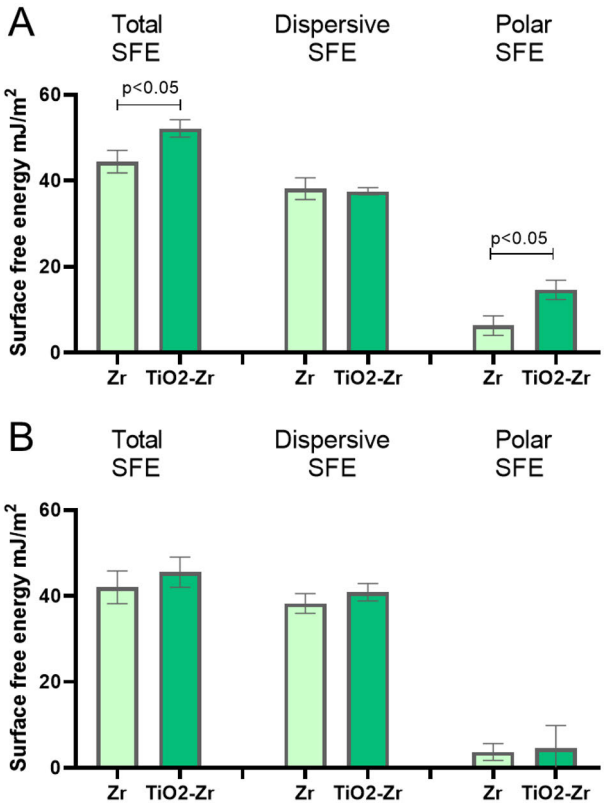

Figure 7. Total, Dispersive and polar components of SFE on non-coated and TiO_2-coated zirconia calculated with **A.**) Owens-Wendt approach and **B.**) Van Oss approach. Shown are means ± SD, ANOVA. Significant p-values ($p < 0.05$) are marked in the figures.

5.3 Cell attachment (I, II, III, IV)

5.3.1 Enhanced epithelial cell adhesion on TiO_2-coated zirconia

In studies I, II and IV the HGKs adhesion and proliferation was studied. In the first study (I), HGKs were cultured on dip-coated and non-coated zirconia surface. TiO_2-coating had favorable effects on cell adhesion. Significantly more attached HGKs

were detected on coated zirconia surface after 24 hours compared to non-coated zirconia. (Fig. 8 A)

The second study (II) measured, whether the in-sol made TiO_2-coating is able to enhance HGK attachment on zirconia and titanium samples. The HGK adhesion was determined by measuring the DNA amount from adherent cells. The amount of DNA was significantly higher after 24 hours on coated zirconia compared to non-coated zirconia. When comparing coated zirconia and coated titanium, the adhesion seemed to be significantly faster on zirconia surface during the first 6 hours. After this, the amount of DNA became quite even. Concerning cell adhesion on coated and non-coated titanium, no significant difference was found. (Fig. 8 B, C)

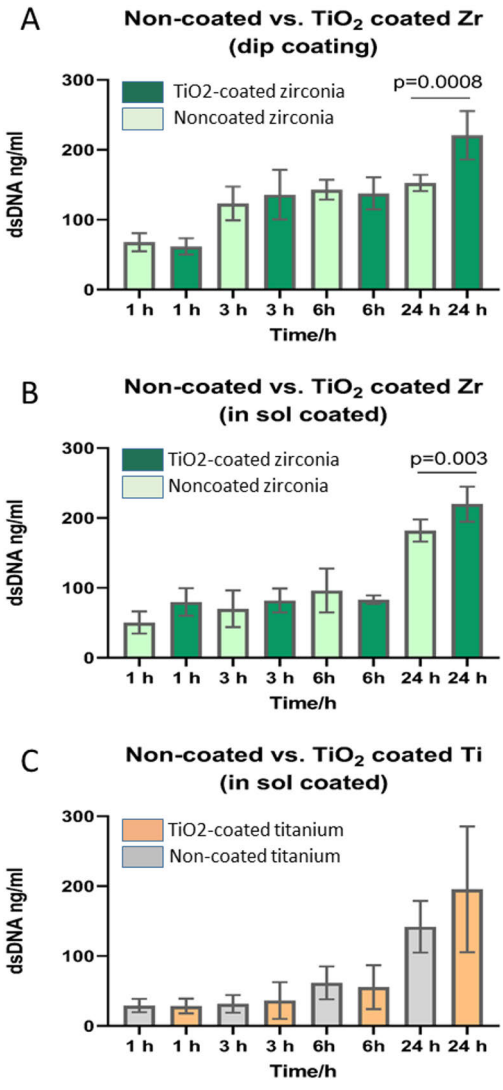

Figure 8. Adhesion of HGKs on non-coated and **A.**) dip-coated TiO_2-coated zirconia, **B.**) in sol TiO_2-coated zirconia and on **C.**) TiO_2-coated titanium after 1, 3, 6 and 24 hours. Mean ± SD, ANOVA.

5.3.2 Equal salivary protein adsorption on non-coated and TiO_2-coated titanium

Total amounts of adsorbed salivary proteins were similar on non-coated and TiO_2-coated titanium with no significant differences between the substrates (Fig. 9)

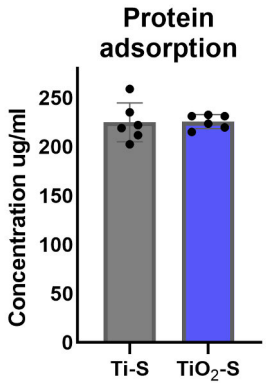

Figure 9. Salivary protein adsorption on non-coated (Ti-S) and TiO_2-coated (TiO_2-S) titanium after 30 min of saliva exposure. Mean ± SD + individual values, ANOVA.

5.3.3 Saliva exposure weakens the epithelial cell adhesion strength

HGK adhesion strength against enzymatic detachment was measured with cumulative series of trypsinization. Cell adhesion strength was weaker after saliva exposure showing increased detachment on both saliva treated non-coated and TiO_2-coated titanium after 2 and 6 hours of cultivation. The non-coated titanium with saliva exposure had significantly higher detachment levels after 1 min of trypsinization compared to all other groups indicating weaker cell adhesion on saliva treated non-coated titanium. After 10 min of trypsinization, detachment levels became relatively even. (Fig. 10)

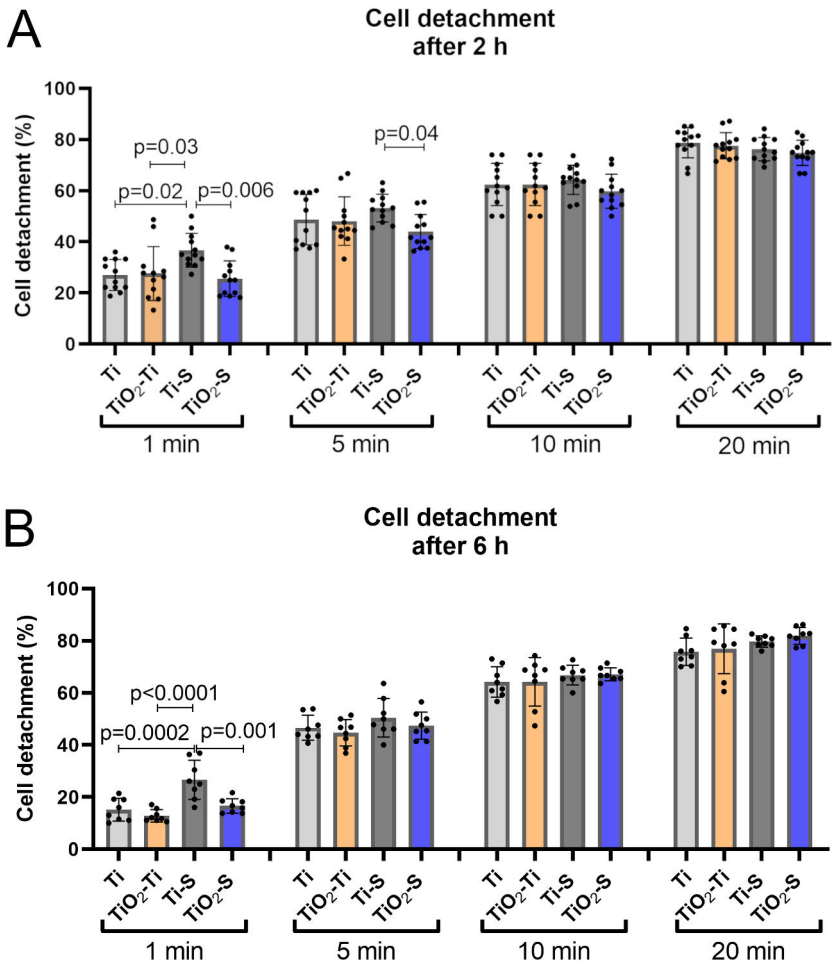

Figure 10. Detachment of HKGs after **A.**) 2 and **B.**) 6 hours. Ti= non-coated titanium, TiO_2-Ti= TiO_2-coated titanium, Ti-S=non-coated titanium with saliva treatment, TiO_2-S= coated titanium with saliva treatment. Mean ± SD + individual values, ANOVA.

5.3.4 Increased epithelial proliferation levels on TiO_2-coated surfaces

To define, if more rapid cell adhesion would correlate in enhanced cell proliferation, long term cell cultures from 1 to 7 days were accomplished. Cell proliferation was higher on TiO_2-coated zirconia after 3 and 7 days of cell culture (I). Relative cell attachment was significantly higher on TiO_2 coated titanium after 3 and 7 days, while on coated zirconia higher cell attachment was noted already after 1 day of cell culture (II). Cell proliferation was significantly higher on TiO_2-coated titanium after 1 and 7 days compared to non-coated titanium. Again, saliva exposure decreased cell

proliferation on TiO_2-coated titanium, whereas similar effects were not found between non-coated titanium with or without saliva treatment (IV). (Fig. 11)

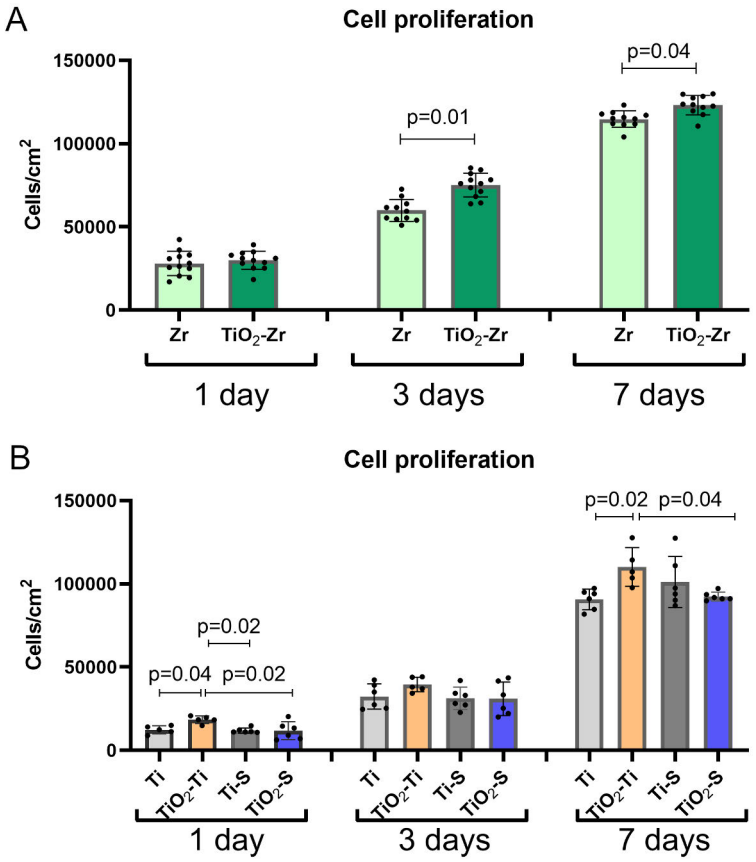

Figure 11. A.) Proliferation of HGKs on non-coated (Zr) and TiO_2-coated zirconia (TiO_2-Zr). **B.**) Proliferation levels on non-coated (Ti) and TiO_2-coated titanium (TiO_2-Ti) before and after saliva exposure (Ti-S, TiO_2-S). Mean ± SD + individual values, ANOVA.

5.3.5 Light-microscope evaluation

To see, whether the HGKs actually attach to zirconia surface, light microscope analyze was performed. Images revealed more uniform cell layer with more adherent HGKs on TiO_2-coated zirconia surface. A gap between cells and zirconia surface can be seen, but it is considered to be due to cutting protocol (Fig. 12).

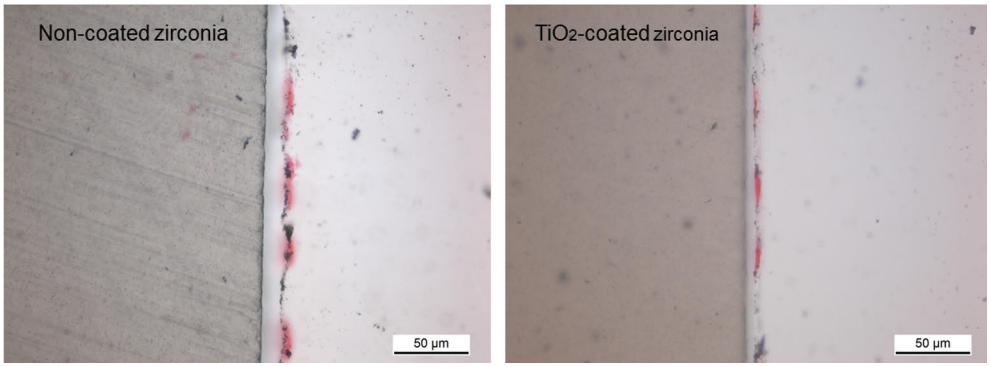

Figure 12. Light microscope images of epithelial cell layer on non-coated and TiO_2-coated zirconia.

5.4 Adhesion protein expression of gingival keratinocytes and fibroblasts (II, III)

To define, whether the increased cell amounts would stand for more decent cell adhesion, total adhesion protein levels were measured. When it comes to HGKs, WB identified significant induction of expression of laminin y2 and integrin α6 on the coated zirconia and titanium samples. Also, the expression of integrin β4 was significantly higher on coated titanium surface when compared to control titanium. Meanwhile significantly higher levels of vinculin and paxillin were found on coated zirconia surface compared to non-coated zirconia, whereas no significant difference was found between titanium samples in this respect (Fig. 13).

Concerning the expression of adhesion protein of HGFs, the levels of paxillin, vinculin and FAK appeared to be higher on in-sol and HT coated discs compared to non-coated titanium. The expression of paxillin was significantly more pronounced on in-sol coated discs (Fig. 14).

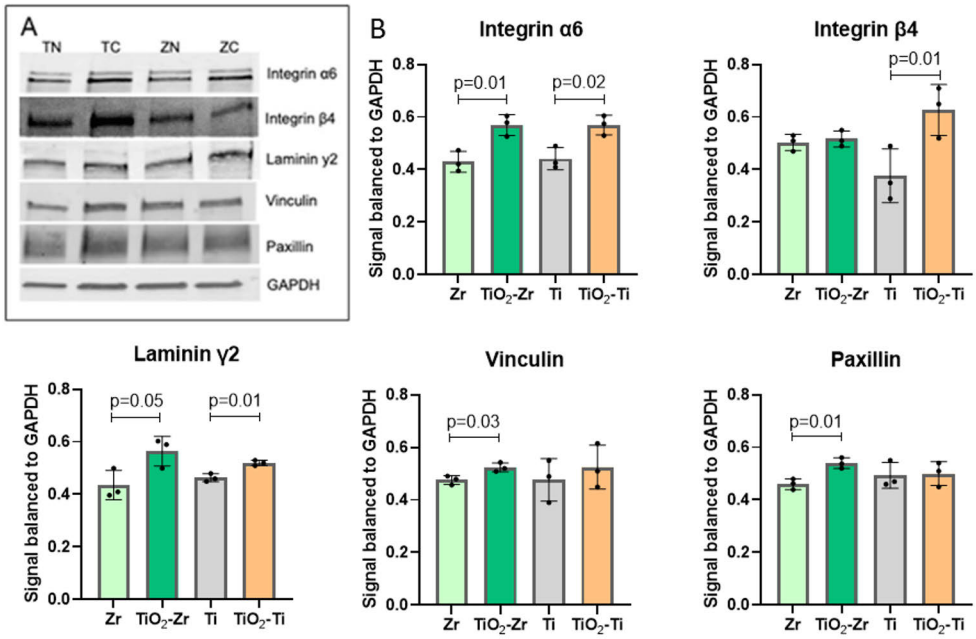

Figure 13. The expression of cell adhesion proteins on non-coated and TiO_2-coated zirconia and titanium. **A.**) WB and **B.**) quantifications of protein levels of integrin α6, β4, laminin y2, vinculin and paxillin after three days of cell culture. Mean ± SD + individual values, ANOVA.

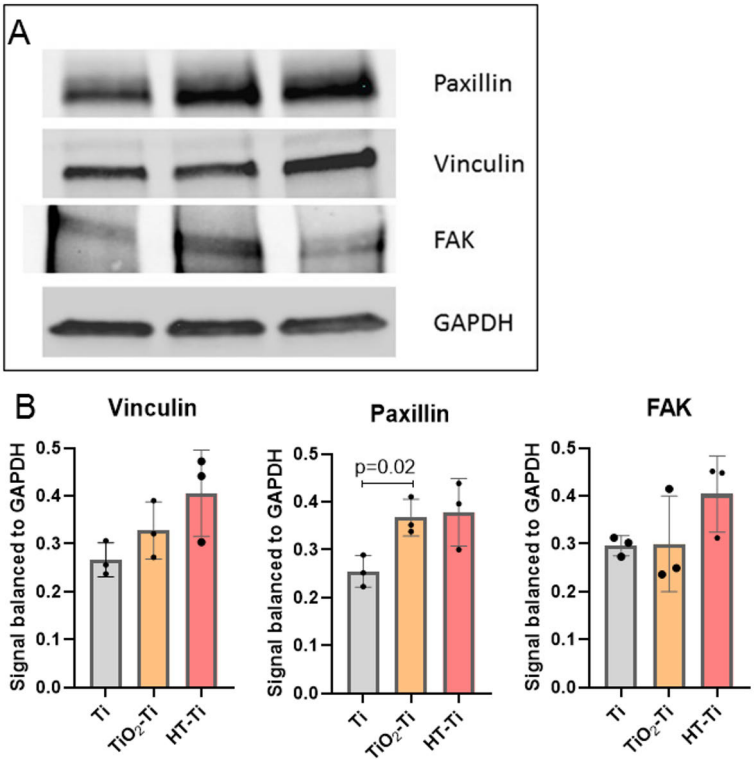

Figure 14. Expression of FA molecules paxillin, vinculin and FAK of HGFs on in sol coated (TiO_2-Ti), HT (HT-Ti) and non-coated (Ti) titanium. **A.**) WB and **B.**) quantifications. Mean ± SD + individual values, ANOVA.

5.5 Confocal microscope analyses (II, III, IV)

5.5.1 Cell morphology (II, III, IV)

In confocal microscope images, mostly well spread and elongated cells with peripheral adhesion protein expression were seen. Wider cells with higher adhesion complex expression were found on TiO_2-coated surfaces (Fig. 15, 16, 17). Meanwhile, saliva exposure seems to decrease cell adhesion to titanium surface (Fig. 18).

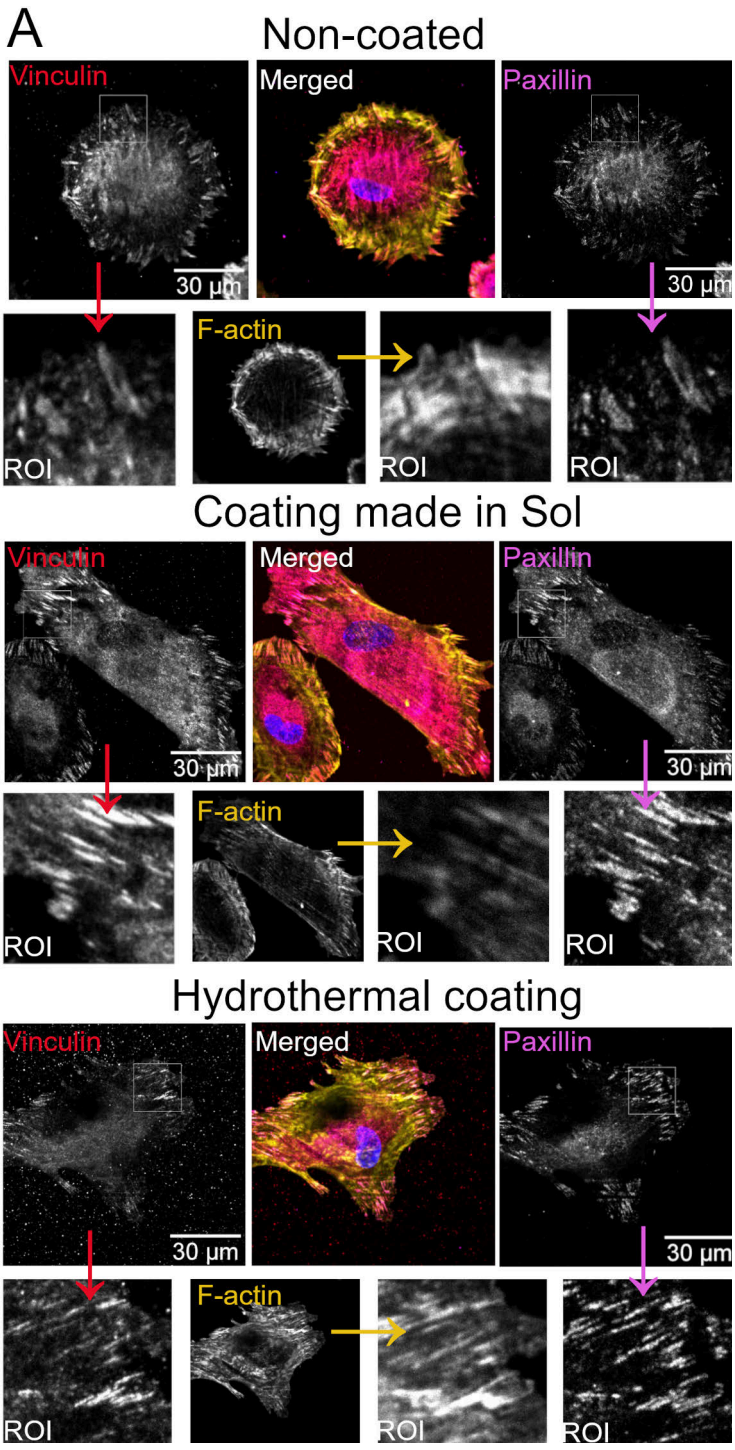

Figure 15. Faster HGF adhesion on in-sol-coated and HT titanium compared to non-coated. Cells are more spread on coated surfaces with more FAs. Representative confocal microscopy images after 2 h of cell culture and the expression of vinculin, paxillin, F-Actin. Nucleus stained as blue. ROI, region of interest (imaged with 3i CSU-W1 Spinning Disk with 63 Zeiss Plan-Apochromat objective and Hamamatsu sCMOS Orca Flash4.0 camera).

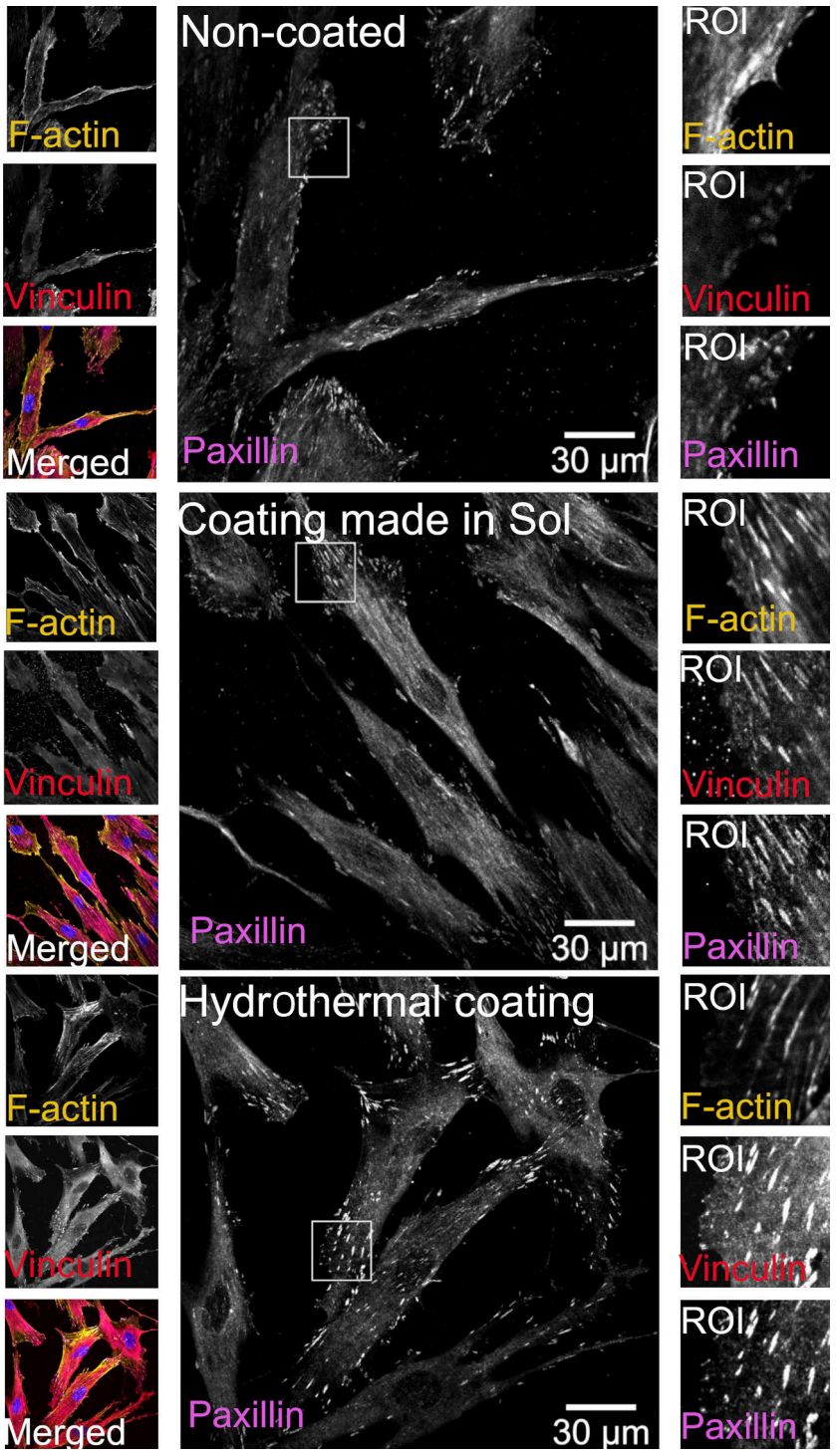

Figure 16. Higher intensities of FAs on TiO_2-coated titanium. Presentable confocal microscope images of HGFs after 24 h of cell culture.

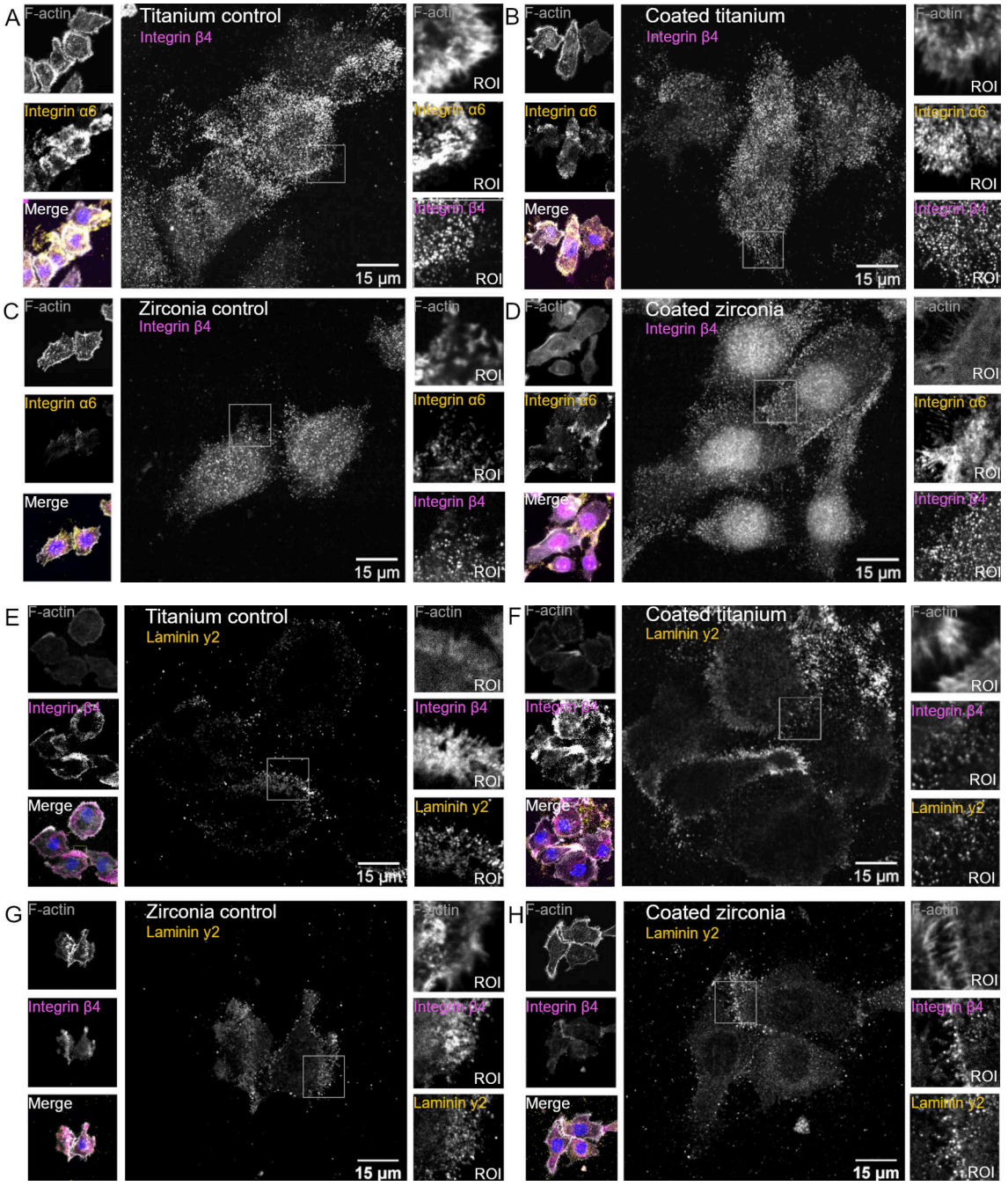

Figure 17. Adherence of HGKs on non-coated and TiO_2-coated zirconia and titanium. Representative confocal microscopy images from the cell bottom layer. The expression of integrin α6 (**A**–**D**), β4 (**A**–**H**) and laminin γ2 (**E**–**H**) stained together with F-actin and DAPI as nucleus. ROI, region of interest. Imaged with 3i CSU-W1 Spinning Disk with 63 Zeiss Plan-Apochromat objective and Hamamatsu sCMOS Orca Flash4.0 camera.

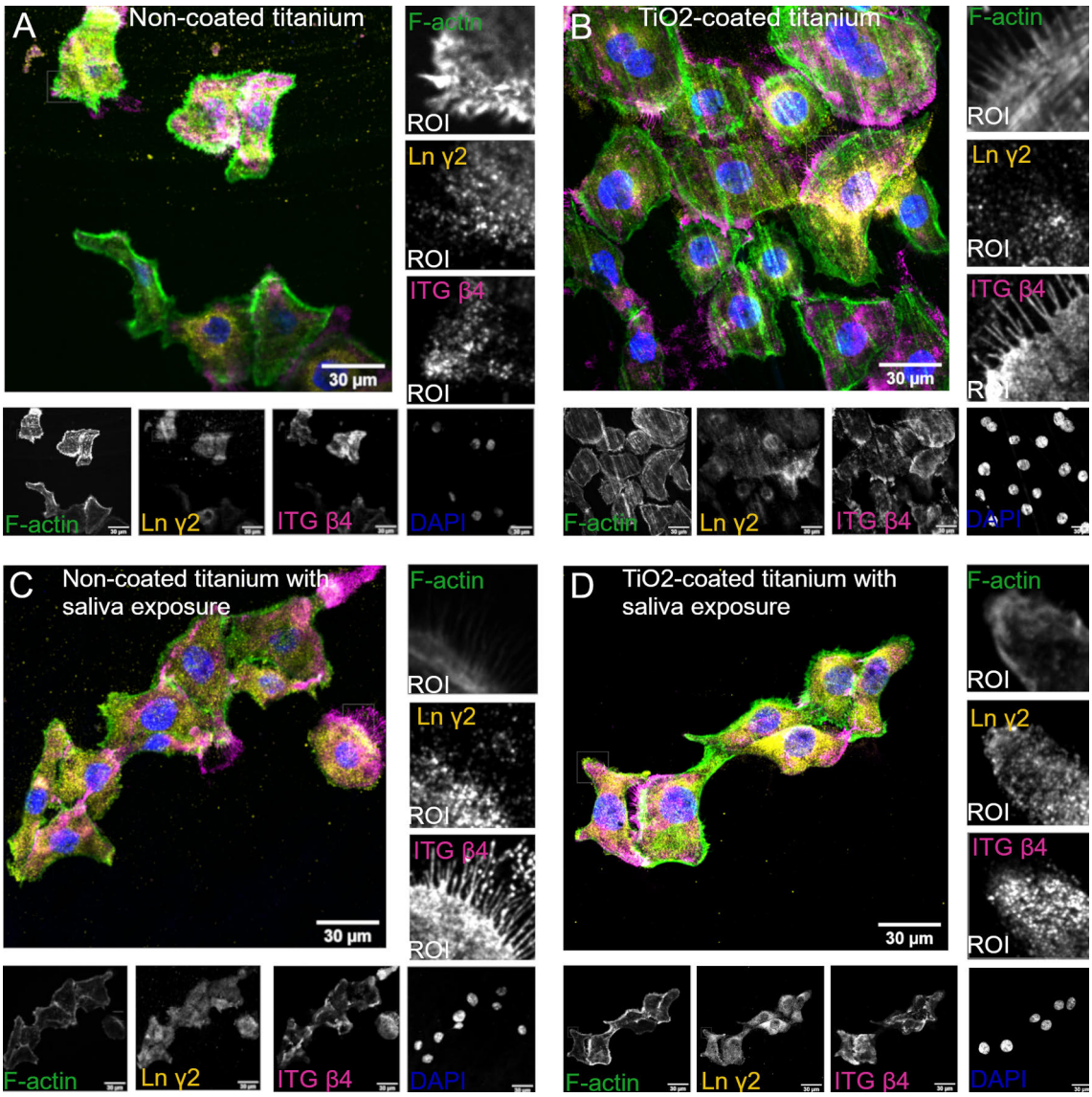

Figure 18. Saliva exposure reduces cell adhesion on titanium surface. Representative confocal microscopy images from the HGKs indicating the expression of integrin β4, laminin γ2 and F-actin on **A.**) non-coated titanium, **B.**) TiO_2-coated titanium and **C**, **D.**) previous after saliva exposure. DAPI represents nucleus. ROI, region of interest.

5.5.2 Cell spreading (II, III, IV)

To reveal, whether increased cell amounts correlated with cell size, cell spreading was measured with actin staining by confocal microscope analyze. Concerning HGKs, the cell spreading was significantly larger on coated zirconia and titanium compared to non-coated discs (II) after 24 hours of cell culture (Fig. 19 A, C). Meanwhile, the saliva exposure significantly reduced HGK spreading when compared to TiO_2-coated titanium without saliva exposure (IV), (Fig 19 B).

When it comes to HGF spreading, HGF areas were significantly wider after 2 and 24 hours on in sol coated titanium compared to non-coated titanium. The cell area appeared also higher on HT coated discs compared to non-coated titanium, but the difference was not significant (Fig. 19 D-G).

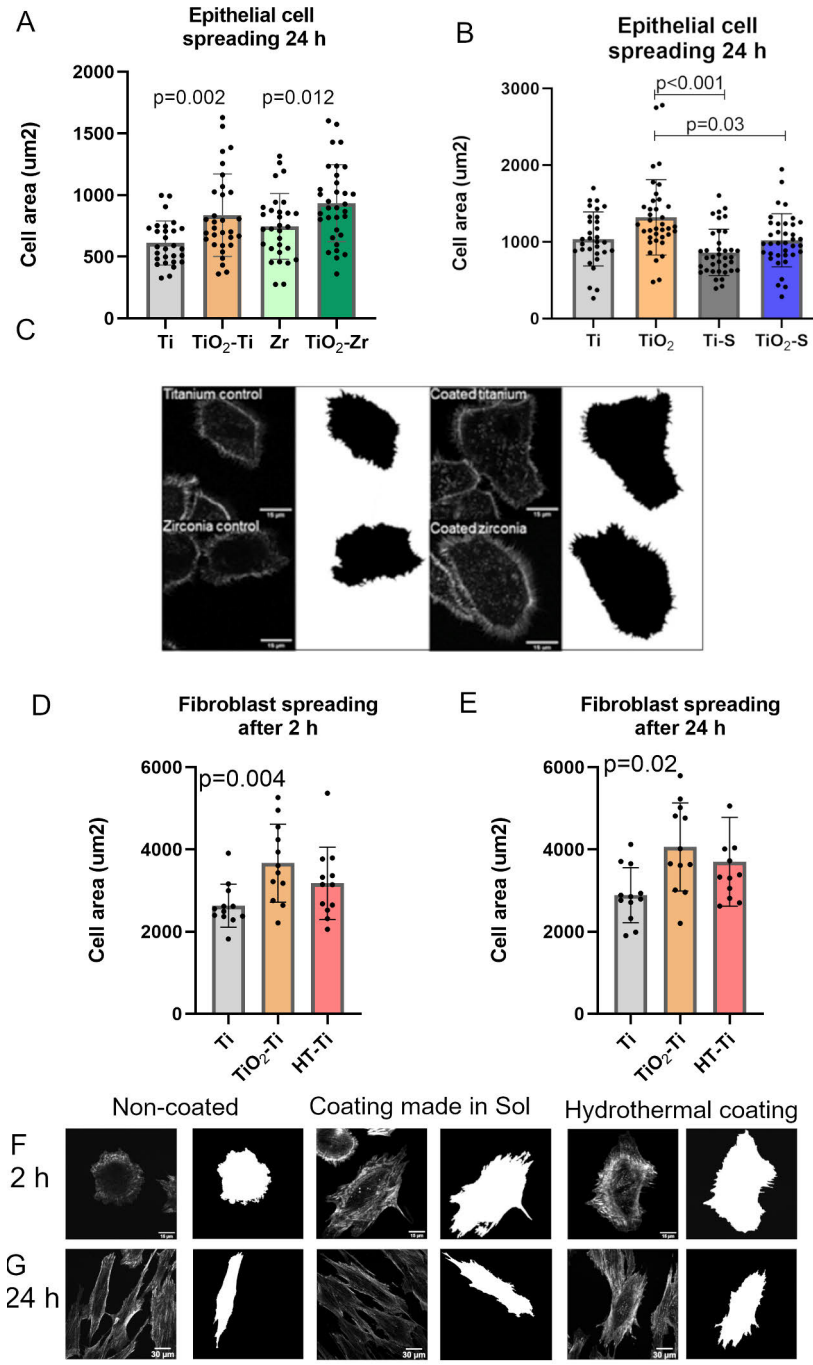

Figure 19. Spreading of HGKS on the titanium and zirconia surface with or without the TiO_2-coating (**A**) and effects of saliva exposure to cell spreading (**B**) after 24 h of cell culture. HGF spreading on in sol coated and HT titanium after 2 (**D**) and 24 hours (**E**). Shown are quantifications (**A**, **B**, **D**, **E**) and representative images of cell areas and cells stained with F-actin (**C**, **F**, **G**).

5.5.3 Signal of adhesion proteins (II, III, IV)

As earlier stated, laminin γ2, integrin α6 and β4 were located at the periphery of HGKs in HD structures, while vinculin and paxillin located more diffusely in cytoplasm. The orthogonal view of HGKs support the finding, as laminin γ2, integrin α6 and β4 have higher signal in the bottom layer of cells, meanwhile vinculin and paxillin does not have similar view (Fig. 20 F). However, when it comes to HGF, vinculin and paxillin are located punctually in focal contact areas.

To study, if the expression of adhesion proteins reflected adhesion protein signals in the bottom layer of cells, signal intensities of laminin γ2, integrin α6, β4 (II, IV), vinculin and paxillin (II, III) were measured from confocal microscope images. Altogether, the levels of adhesion protein signals of HGKs were higher on coated zirconia and titanium surfaces compared to control samples. The difference was statistically significant between zirconia samples concerning laminin γ2 and integrin β4 and between titanium samples concerning integrin α6 (II) (Fig. 20 A, B, C). After saliva exposure, the levels of adhesion protein signals significantly decreased on TiO_2-coated titanium compared to surfaces without saliva exposure (Fig. 20 D, E).

The signals of vinculin and paxillin of HGF on differently treated titanium were measured after 2 and 24 hours. Expression of vinculin and paxillin were found peripheral at the FA contacts. Level of paxillin was significantly higher after 2 and 24 hours on both in sol coated and HT surface compared to non-coated surface. Whereas signal of vinculin was significantly higher after 2 hours on in sol coated surface and after 24 hours on HT surface compared to non-coated titanium (Fig. 21).

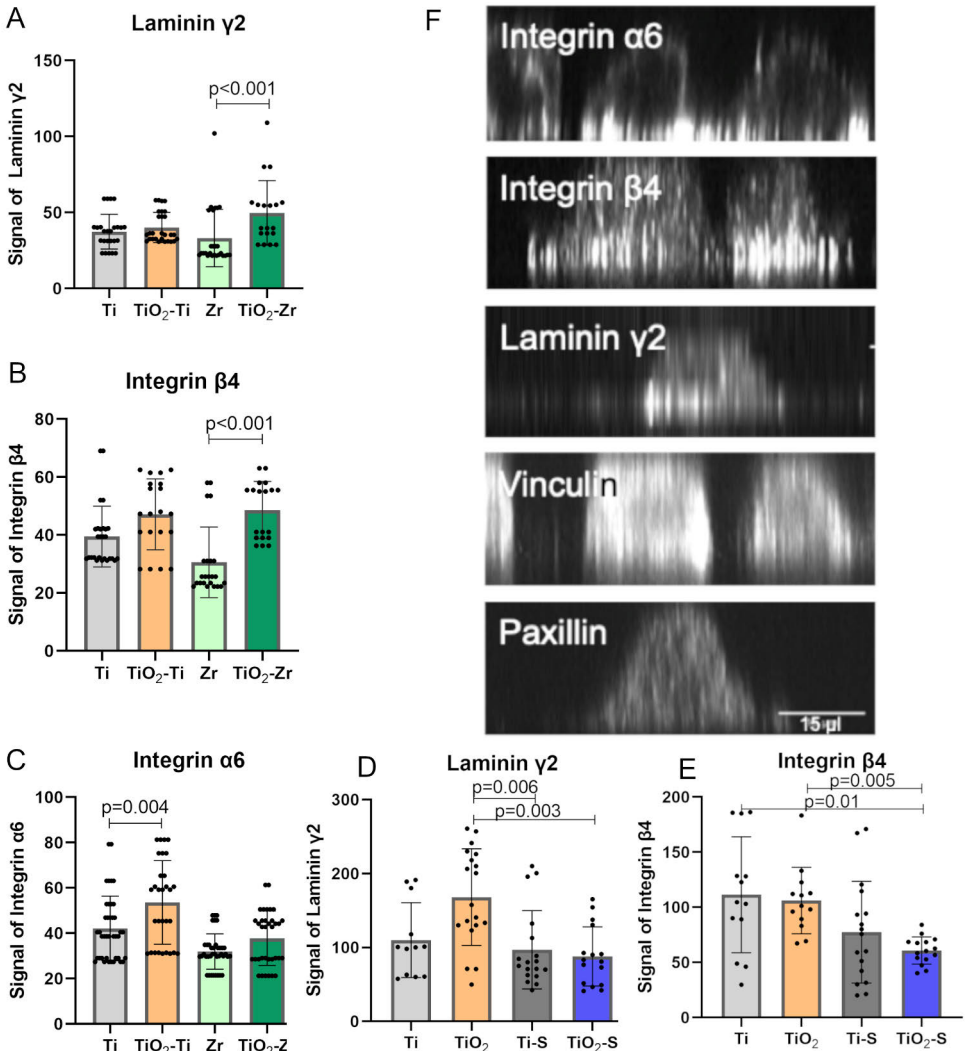

Figure 20. The levels of HD molecule signals on bottom of HGKs on non-coated titanium (Ti), TiO_2-coated titanium (TiO_2-Ti), zirconia (Zr) and TiO_2-coated zirconia (TiO_2-Zr) (**A**, **B**, **C.**) and after saliva treatment on titanium (Ti-S) and TiO_2-coated titanium (TiO_2-S). **F.**) (**D** & **E.**) Intensities of adhesion proteins in the bottom layer of cells. Mean ± SD + individual values, ANOVA.

5.5.4 Focal adhesion formation (III)

To determine, if increased adhesion protein signals are in line with FA formation in HGF, the FA size and amount per area were detected. The size of FAs were significantly larger on TiO_2-coated compared to non-coated titanium after 2 and 24 hours of cell culture. Also, there were significantly more FAs per area on TiO_2-coated compared to non-coated surfaces after both 2 and 24 hours.

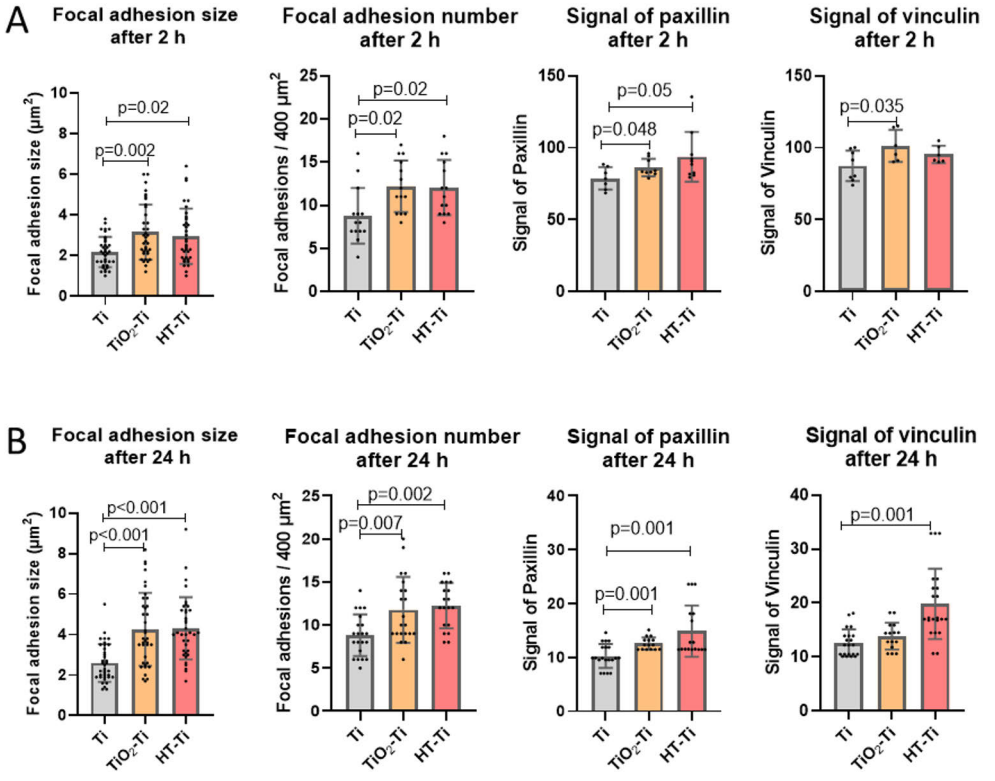

Figure 21. FA size and number and signal of vinculin and paxillin in the cell bottom after **A.**) 2 hours and **B.**) 24 hours of cell culture. Mean ± SD + individual values, ANOVA.

6 Discussion

6.1 General discussion

Bioactive TiO_2-coatings have been developed to enhance tissue integration on implant surfaces. In this study, TiO_2 -coatings produced with three different methods were evaluated in terms of adhesion of HGK and HGF. All four studies of this thesis revealed enhanced gingival cell attachment on differently produced TiO_2-coated surfaces. The studies I, II and IV showed increased wettability and adhesion, spreading and proliferation of HGKs on coated zirconia (I, II) and titanium (II, IV). In addition, more rapid FA formation of HGF was found on in sol made and HT coated titanium (III). The sol gel derived TiO_2 coatings are thin and nonresorbable (Areva et al. 2004). Instead of earlier used sol gel derived TiO_2-surface produced by dip-coating, a novel method to produce nanoporous TiO_2-surface was used (II, III, IV). The coating was induced directly in sol by deposition. This novel method enables coating of more complicated structures. What is more, in sol gelation is faster compared to dip-coating, since more samples can be coated at the same time. Furthermore, the coating can be produced with ordinary laboratory equipment without need of specific dip-coating machines. The sol gel coating seems to have favorable soft tissue cell response on both titanium and zirconia.

The benefits of this study included high quality imaging of attached gingival cells, which enabled an accurate analysis of adhesion complex formation and cell spreading indicating higher quality of cell attachment. If only cell amounts are measured, for example lack of space and energy can affect the results. More rapid cell adhesion and growth decreases space and wear out nutrition from cell culture medium faster in cell culture environment, which can lead to increased cell apoptosis. Thus, long term proliferation levels may not be the best way to study material effects on cell response.

Even though, this study evidenced enhanced cell attachment on TiO_2-coated surface, there are some limitations that ought to be discussed. Immortalized HGKs were used in epithelial cell cultures (I, II, IV). The chromosome number of the used cells has been shown to be in the hypertriploid range (70-76) (Mäkelä et al. 1999). It is not clear, if the immortalization of the cells affects the reliability of the results. Immortalized cells were used, as the survival of primary HGKs collected from

gingival biopsies is rather unsure. Also, there were some problems with WB analysis since the solutions were somewhat diluted and only faint results were available from some staining e.g., detection of phosphoproteins. The stainings with faint results were left out of analysis. Reason for faint appearance may be due to small size of samples. The titanium and zirconia discs had a size of 1 cm^2, whereas 6-microtiterplates with larger surface area are usually used in WB. In the future, samples with larger surface area could be used in WB, if wider scale of adhesion proteins is wanted to study.

6.2 Surface characteristics (I, II, III)

The SEM evaluation demonstrated small TiO_2 nanoparticles on all differently coated surfaces evidenced. HT surface has the highest peak of valley and more intense surface topography. Meanwhile in sol made surface has more porous surface with sporadic particles (II, III). The nanoparticle size on in sol derived surface is approximately 15 nm. However, dispersion on particle size is wider on HT surface varying from 10 to almost 50 nm. These particle sizes seemed to enhance cell adhesion on coated surfaces. Also, Vignesh et al. (2015) demonstrated improved cell response on titanium surface with 20 nm nanoparticles. Meanwhile Ferrà-Cañellas et al. (2018) demonstrated greater cell adhesion on nanoporous TiO_2-surface with diameter of 70-80 nm.

Measuring the contact angle of the surface is used to analyze surface wettability which affects cell adhesion (Mekayarajjananonth et al. 1999). Low contact angles indicate desirable wettability, meanwhile higher contact angles demonstrate lower hydrophilicity or hydrophobicity. Many studies have indicated lower contact angles on nanoporous TiO_2-treated surfaces. The present study evidenced significantly lower contact angles on sol gel derived TiO_2-coated zirconia compared to non-coated zirconia (I). The result is in same line with Patel et al. (2017) were zirconia had lower contact angels after 10, 20 and 30 min of anodization. In addition, significantly lower water contact angle on in sol coated titanium was shown in study IV. Also, Wang et al. (2019) and Dorkhan et al. (2014) demonstrated lower contact angles on titanium alloy treated with AO compared to non-treated titanium. Besides, contact angles of sol-gel derived dip-coated titanium and HT titanium have been lower compared to non-coated titanium (Areid et al. 2018).

SFE of the surface and especially its polar component has been shown to be important parameter, when cellular adhesion is studied (Hallab et al. 2001, Redey et al. 2000, Schakenraad et al. 1988). The present study revealed significantly higher total and polar SFE on TiO_2-coated zirconia measured with OW-approach. Higher SFE together with enhanced wettability support the results of present cell culture results.

The color of implant abutment plays important role, when aesthetic outcome is demanded. As surface modifications are able to change the surface color, not only the thickness of attached gingiva, but also the abutment color effects the aesthetic outcome. Wang et al. (2019) studied, how the anodization voltages changed the color of implant abutment from dark to yellow and pink and affected the color changes of gingiva. On yellow and pink titanium surface the color change of gingiva around implant was lower compared to dark titanium. In this study, both in sol and HT derived TiO_2 surfaces had the color of gold and bronze. As yellow seemed to be more invisible under gingiva than natural titanium (Wang et al. 2019), it can be expected that gold and bronze-colored abutments may have better aesthetic outcome than non-coated titanium. However, in Wang's study, the color change was low on zirconia surface. As the color of zirconia resembles to natural teeth, it is the safest choice when aesthetic outcome is wanted.

6.3 Epithelial attachment to coated titanium and zirconia (I, II, IV)

This study demonstrated enhanced attachment of HGKs on differently produced TiO_2-coated titanium and zirconia surfaces in vitro (I, II, IV). The results are promising, as the epithelial attachment is the first protection against oral microbes and a decent epithelial attachment could prevent microbial invasion into peri-implant space.

In the first study of this thesis (I), higher amount of attached HGKs were found on sol gel derived zirconia surface after 24 hours, 3 and 7 days compared to non-treated zirconia. The second study (II) revealed similar results concerning zirconia surface. Also, cell adhesion to coated zirconia was significantly faster during first six hours when compared to coated titanium. When it comes to cell proliferation on titanium, the cell proliferation was significantly enhanced on coated surfaces after 3 and 7 days. According to this study, there were no significant difference in adhesion strength against enzymatic detachment between TiO_2-coated and non-coated titanium after 2 or 6 hours of cell culture (IV). The larger cell spreading on both coated zirconia and titanium after 24 hours of cell culture can be due to induced wettability of TiO_2-coatings.

The HGKs attach to tooth surface via HDs. Hence, in this thesis it was decided to study if increased cell amounts would stand for higher quality in cell adhesion on molecular level. The adhesion protein expression of integrin α6, β4 and laminin γ2 were higher on coated zirconia and titanium compared to non-coated surfaces when measured with WB or signal levels from bottom plane of the cells. These molecules also located peripheral at HDs according to confocal microscope analyses. Meanwhile vinculin and paxillin were mostly cytoplasmic in HGKs. Integrin α6, β4

and laminin γ2 are important molecules in hemidesmosomal attachment and higher induction of these proteins imply enhanced cell adhesion (Kinumatsu et al. 2009). Sakamoto et al. (2019) studied the attachment of mouse derived gingival epithelial cells on HT titanium. They showed higher cell adhesion strength after 1 day and induced laminin 332 absorbance on HT surface compared to non-coated titanium. Also, they reported about higher peripheral signal of integrin β4 on epithelial cells on HT surfaces. However, the cell proliferation was lower on HT samples after 1 and 3 days. Li et al. (2023) studied HGK adhesion on anodized titanium nanotubes and found out increased cell attachment on anodized surface after 0,5 and 2 hours of cell culture. Concerning longer-term cell proliferation, no significant difference was found.

In this thesis, no significant difference in adhesion protein expression between zirconia and titanium was found. Lee et al. (2019) analyzed functional and biological properties of zirconia and titanium. They evidenced higher cell proliferation, adhesion molecule expression and more favorable biological width around zirconia surface compared to titanium. However, earlier Atsuta et al. (2019) found out lower plectin and integrin β4 expression on zirconia compared to titanium. In their study adhesion of HGKs was reduced on zirconia surface.

However, there are also studies, where no difference concerning adhesion of HGK between nanoporous TiO_2-treated surface and non-treated surface has been found. Masa et al. (2018) studied attachment of primary HGK on polished titanium and on TiO_2 copolymer films. The study revealed larger cell spreading on polished titanium compared to coated titanium and no difference was found on cell adhesion or proliferation. Also, Xu et al. (2018) evidenced lower HGK proliferation on titanium treated with AO compared to polished titanium. In addition, expression on integrin α3, α6, β3, β4 and laminin γ2 were significantly lower on AO surface after 7 days of cell culture. However, in their study the results concerning HGFs were controversial.

6.4 Fibroblast adhesion to coated titanium (III)

When it comes to HGFs, this study focused on to study initial cell attachment, cell spreading and adhesion protein expressions (III). The study revealed larger cell spreading after 2 and 24 hours on in sol made TiO_2-coatings, compared to non-coated titanium. What is more, the FA formation was faster on both in sol and HT surfaces after 2 and 24 hours. The FA size and cell spreading has been shown to correlate together, which supports findings of this study (Kim et al. 2013). In addition, signal of important FA proteins vinculin and paxillin in the bottom layer of cells were higher on in sol and HT surfaces compared to non-coated titanium. This correlates with results from WB, where signal of adhesion proteins was also higher on coated

surfaces. The difference was significant concerning expression of paxillin on in sol surface.

HGF adhesion and proliferation on implant surfaces has been studied widely. Many studies have indicated, that bioactive TiO_2-surface might improve the soft tissue attachment supporting the results of this thesis. Meretoja et al. (2010) found out, that sol gel derived TiO_2-coating on titanium surface is able to increase cell adhesion during first 24 hours, cell activity during ten days of culture and make the attachment stronger. SEM evaluation evidenced poor cell attachment with round shape and fewer cells on non-coated titanium, while the cells were more spread with extracellular fibrils on TiO_2-treated titanium. However, confocal microscope images of HGFs after 3 days of cultivation revealed more apparent focal contacts on non-coated titanium. This was explained with high background signal on coated surfaces. Wang et al. (2019) demonstrated a multidirectional network of HGF filopodia on oxidized titanium surface and on zirconia, meanwhile cells located more parallel following the surface characteristics on non-coated surfaces. The proliferation activity was higher on zirconia surface compared to treated or non-treated titanium. Wang et al. (2021) also found out improved HGF proliferation and more elongated cell morphology with numerous adhesion complexes on anodized titanium nanotube surface, meanwhile cells with round shape and lower proliferation were found on commercially pure titanium (cpTi). Moreover, Teng et al. (2014) reported about higher concentration of HGF with AO titanium compared to cpTi. In addition, Patel et al. (2017) cultured human mesenchymal stem cells on anodized zirconia. Well elongated and spread cells were seen on the anodized surface as well as significant increase of cell viability after 6 days on cell culture. Also improved HGF proliferation and more elongated cells were found on titanium treated with AO by Xu et al. (2018). Furthermore, Hoshi et al. (2010) cultured periodontal ligament fibroblasts on chemically treated and non-treated titanium revealing enhanced proliferation levels after 12 hours and 3 days on TiO_2-coated titanium. Besides, Vignesh et al. (2015) studied murine fibroblast adhesion on machined, sand blasted, acid etched, laser treated, and nanoparticle coated titanium with 20 nm diameter nanoparticles. SEM evaluation revealed an intact cell layer with cell extensions to nanocavities on titanium surfaces indicating improved HGF attachment to titanium surface treated with nanoparticles. Also, Shahramian et al. (2017) showed improved HGF proliferation on sol gel coated zirconia after first 4 days of cell culture. Besides, Xu et al. (2018) demonstrated significantly higher vinculin, integrin α3, β1 and fibronectin expression on HGFs on titanium with AO compared to mechanically polished titanium after 7 days.

However, there are also studies that have not found any benefits on TiO_2-coated surfaces when it comes to soft tissue cell attachment, or the effect was opposite to our findings. Dorkhan et al. (2014) studied TiO_2-coating produced by AO. In their

study, the HGK and HGF response was not improved comparing to cpTi. Visa versa, after 24 hours of cultivation, there seemed to less adhered cells on oxidized surfaces. Kim et al. (2015) compared differently treated implant surfaces, and TiO_2-surface produced with AO were least favorable, when cell attachment was compared. Their study reported a surface with good wettability, to be the best surface for cells to attach. Also, Kubo et al. (2009) found out enhanced osteoblast attachment, spread and differentiation on deposited TiO_2-surface with nanonodules of 300 nm. However, no effect on HGF function on TiO_2-treated titanium were detected. The differences between results can be due to different production methods of TiO_2-coating. The in sol made TiO_2-coating can be optimal for cell adhesion due to its wettability and optimal nanoporocity, where distances between peaks are favorable for cell attachment.

When it comes to tissue attachment to TiO_2-treated surfaces, Shahramian et al. (2020) studied soft tissue-implant interface on TiO_2-coated zirconia after one and two weeks of tissue culture. Immunohistological analyze showed expression of laminin γ2 around TiO_2-coated zirconia indicating formation of basal lamina after 7 days. Similar laminin expression was not found between epithelium and non-coated zirconia. Tissue culture was also accomplished by Areid et al. (2021) on HT coated titanium. Histological analyses revealed, that both epithelium and connective tissue were attached on non-coated and TiO_2-coated surface. More closely attached epithelium were found around HT surfaces after two weeks of tissue culture. Rossi et al. (2008) examined sol-gel derived TiO_2-coated titanium implants on beagle dogs. They found out immediate contact between soft tissue and coated implant abutments and less detachment on TiO_2-treated implants. Also, Paldan et al. (2007) and Areva et al. (2005) accomplished animal experiments by studying soft tissue integration on sol gel derived TiO_2-coated implants in rats. The studies evidenced firmly attached connective tissue and thinner connective tissue capsule formation around TiO_2-coated cylinders. Furthermore, Paldan et al. demonstrated higher rupture forces concerning coated titanium cylinders which indicates enhances tissue adherence around TiO_2-implants. In addition, Wennerberg et al. (2011) accomplished a clinical trial, where they compared soft tissue adherence, bone resorption and inflammation around TiO_2-coated and non-coated titanium abutments. The results revealed enhanced contact between peri-implant mucosa and abutment, lower bone resorption and decreased inflammation levels around TiO_2-coated abutments. However, these in vivo studies were accomplished with dip-coated surfaces, which is difficult to expand into industrial scale, because of its limitations when coating objects with more complicated shapes.

When implant esthetic treatment results are evaluated, gingival recession, thickness of attached gingiva and the color under the transparent gingiva are usually estimated (Totou et al. 2021). Good epithelial and connective tissue attachment may

prevent gingival regression. Also, a thick and well attached connective tissue maintains proper form of gingiva resulting a pleased esthetic result. Better soft tissue adhesion seems to reduce bone resorption, which again may reduce gingival recession (Rossi et al. 2008). Based on higher HGK and HGF attachment on nanoporous TiO_2-surfaces shown in the present study suggest that good esthetic outcome could possibly be reached on TiO_2-treated titanium and zirconia.

6.5 Saliva exposure influences cell adhesion (IV)

The fourth study (IV) of this thesis studied effects of saliva exposure on HGKs attachment. The saliva exposure significantly decreased contact angle values on non-coated and in sol made TiO_2 surfaces, which might be due to adhered water molecules on titanium surface. The result is in the same line with earlier studies, where induced hydrophilicity has been found on titanium surface after saliva exposure (Schweikl et al. 2013). Salivary protein adsorption was similar between coated and non-coated surfaces. Earlier, adsorption of plasma proteins has been shown to be similar between sol gel coated, HT and non-coated titanium (Areid et al. 2017), and TiO_2-coated and non-coated zirconia (Shahramian et al. 201). However, the serum albumin adsorption has been reported to be higher on anodized titanium (Teng et al. 2014).

When it comes to cell attachment, saliva exposure significantly decreased HGKs proliferation and adhesion protein expression on in sol made TiO_2-surface. Meanwhile cell amounts were not different on non-coated titanium before and after saliva exposure. However, the adhesion strength was lower on non-coated titanium even after saliva exposure. It appears that saliva exposure is able to neutralize the bioactive effects of TiO_2-surface and increased hydrophilicity after saliva exposure does not enhance cell spreading. Earlier studies accomplished with HGF, have also evidenced lower attachment levels after saliva exposure (Heaney et al. 1990, Zhou et al. 2019, Zöller et al. 1996). In addition, saliva exposure seems to reduce osteoblast adhesion and activity on titanium surface (Hirota et al. 2019, Kunrath et al. 2021). All in all, the results indicate decrease in cell adherence to implant surface after saliva exposure. These findings highlight the importance of good saliva control when connecting coated abutments in oral environment.

6.6 Future perspectives

In this thesis, sol gel derived TiO_2 and HT coatings were used with one coating layer. The properties of TiO_2-surface could be enhanced further. One option to increase surface hydrophilicity is ultraviolet treatment of surface. Areid et al. (2018) demonstrated how the UV treatment increases the surface wettability and lowers

contact angles on both TiO_2-coated and non-coated titanium surface. Kobayashi et al. (2022) had same kind of results and suggested lower bacterial adhesion and biofilm formation on UV treated surfaces. Also, Areid et al. (2018) evidenced lower biofilm formation and HGF adhesion on UV treated titanium alloy. In the future, the effects of UV treatment of in sol coated titanium and zirconia on soft tissue integration could be studied in more detail. Also, especially the in sol coating technique makes it possible to develop coating properties by modifying the sol. The surface characteristics can be changed with varying the aging time of sol, which changes the height of peaks, or by adding more coating layers, which increases the number of peaks (Peltola et al. 2000). Also, the sol could be developed to have anti-microbial properties for example by adding anti-microbial ions, such as zinc or strontium, in the sol. Mixture of coatings opens interesting area for next generation implant surfaces with tailored properties for various clinical and tissue conditions.

This thesis consisted of in vitro studies. However, it is not possible to mimic the real oral environment in laboratory conditions. Even though the results of this thesis are promising, clinical studies are needed in the future. Consequently, a clinical trial would be a natural continuation to this series of studies.

7 Summary/Conclusions

This thesis evidenced enhanced gingival cell attachment on different TiO_2-modified surfaces, sol gel derived dip-coated zirconia, in sol coated zirconia and titanium, and HT titanium compared to non-coated zirconia and titanium. The study contained quantitative and qualitative analyses of cell adhesion, which both revealed favorable properties of TiO_2-surfaces. Number of cells and adhesion complexes were higher on coated zirconia and titanium, which was supported by enhanced expression of important adhesion molecules of HGKs and HGFs. The results give further evidence that nanoporous TiO_2-surface seems to be ideal for promoting gingival cell adherence to implant surface. Enhanced gingival attachment may decrease the risk of peri-implant infections and improve long-term prognosis of implant treatments in general. However, saliva exposure weakens the cell adhesion on TiO_2-coated titanium surface and a proper saliva control is necessary while placing coated components in oral environment.

Acknowledgements

Firstly, I would like to thank my both supervisors, Prof. Timo Närhi, DDS, PhD and Dr. Jaana Willberg, DDS, PhD. You have both supported and guided me through this project in an enthusiastic and supportive way. You have both been an inspiration for me. I also want to thank Timo for letting me work as a half-time teacher during this project in your group in Department of Prosthodontic Dentistry and Stomatognatic Physiology. I have had an exceptional opportunity to explore both sides of academic world, thanks to you.

I want to thank Adjunct Prof. Taina Tervahartiala for accepting the invitation to be my opponent at my dissertation. Also, I would like to impress my gratitude to reviewers of this thesis, Jonathan Massera and Victoria Franke Stenport. Thank you for the reviews and comments. As well I would like thank my steering group member docent Vuokko Loimaranta.

This thesis was performed at Institute of Dentistry, University of Turku. I want to thank the dean of the Institute of Dentistry, Prof. Pekka Vallittu for creating an inspirative research environment in Dentalia. This study was accomplished in collaboration with Turku Clinical Biomaterials Centre and Johanna Ivaska group at Biocity. I want to thank Lippo Lassila, the Head of TCBC, and Prof. Johanna Ivaska for collaboration and opportunity to work in your laboratories. The study was supported by personnel grants from the Finnish Dental Society Apollonia and Finnish Women Dentists' Association. Also, a travel grant from Finnish Doctoral Program in Oral Sciences (FINDOS-Turku) was received. I am thankful for the financial support for this thesis.

I have had opportunity to collaborate with great researchers during this project. First, I want to thank docent Ilkka Kangasniemi for your help, support and expertise with coatings. Next, I would like to thank docent Elisa Närvä for all the expertise and help, you have given me. You have taught me a great deal of cell biology, methodology and how to write scientifically, thank you. Also, I would like to thank my two other co-writers DDS, PhD Khalil Shahramian and DDS, PhD Nagat Areid. You have both been very helpful and supportive during this project.

I also want to thank the laboratory personnel at Dentalia, TCBC and Biocity for help and support. Special thanks to senior laboratory technicians Katja Sampalahti and Oona Hällfors for all the help.

During this PhD project, I have had an opportunity to work part timely as a teacher at Institute of Dentistry, University of Turku. Thank you for all the colleagues at the Institute of Dentistry and especially my teaching colleagues at Department of Prosthodontic Dentistry and Stomatognathic Physiology, Ami Le Bell-Rönnlöf, Jasmina Bijelic-Donova, Inkeri Nieminen, Jaana Malmberg and Marjo-Riitta Liljeström for the support and being great colleques. Special thanks to my roommate Ami for being an inspiration and cheering me in this project.

In addition, I have many great friends, who have made my PhD journey special. Firstly, would I like to thank my dear study friends and best conference travel fellows PhD Erika Mäkinen, PhD Johanna Laakso and PhD Leeni Misin. Also, I would like to thank my dear friends from childhood, the Donna girls, who have taken care, that my life has also other entertainments besides dentistry. In addition, I would like to thank my clinical co-workers at Parainen for friendship and support in this project.

What is more, I would like to impress my gratitude towards my parents, Heidi Lähteenmäki-Riivari and Topi Riivari. You have always encouraged me to reach for my dreams and taught how hard work is worthwhile. Lastly, but most importantly, I would like to thank my dear husband, Otto Alimattila. You have had limitless amounts of patience when listening and supporting me in this project. Being my security man and baggage carrier at conference trip to Bogota was also a memorable effort. Thank you for everything.

Turku, November 2023
Sini Riivari

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TURUN
YLIOPISTO
UNIVERSITY
OF TURKU

Painosalama, Turku, Finland 2023

ISBN 978-951-29-9552-3 (PRINT)
ISBN 978-951-29-9553-0 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)