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# Surface modification of ultrafine-grained titanium: influence on mechanical properties, cytocompatibility and osseointegration potential

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**Running title: Surface modification of ultrafine-grained titanium**

**Abstract**

Objective: The main objective of this study was to demonstrate that dental implants made from ultrafine-grain titanium (UFG-Ti) can be created that replicate state of the art surfaces of standard coarse-grain titanium (Ti), showing excellent cytocompatibility and osseointegration potential while also providing improved mechanical properties.

Material and methods: UFG-Ti was prepared by continuous equal channel angular processing (ECAP) and surfaces were treated by sand-blasting and acid-etching. Mechanical properties (tensile and fatigue strength), wettability and roughness parameters were evaluated. Human trabecular bone-derived osteoblast precursor cells (HBCs) were cultured on all samples to examine cytocompatibility and mineralization after 4 and 28 days, respectively. Biomechanical pull-out measurements were performed in a rabbit *in vivo* model 4 weeks after implantation.

Results: Both yield and tensile strength as well as fatigue endurance were higher for UFG-Ti compared to Ti by 40%, 45% and 34%, respectively. Fatigue endurance was slightly reduced following surface treatment. Existing surface treatment protocols could be applied to UFG-Ti and resulted in similar roughness and wettability as for standard Ti. Cell attachment and spreading was comparable on all samples, but mineralization was higher for the surfaces with hydrophilic treatment with no significant difference between UFG-Ti and Ti. Pull-out tests revealed that osseointegration of surface treated UFG-Ti was found to be similar to that of surface treated Ti.

Conclusion: It could be demonstrated that existing surface treatments for Ti can be translated to UFG-Ti and furthermore, that dental implants made from surface-treated UFG-Ti exhibit superior mechanical properties while maintaining cytocompatibility and osseointegration potential.

Keywords:

Ultrafine-grain titanium, yield strength, tensile strength, fatigue endurance, cytocompatibility, mineralization, osseointegration

**Introduction**

The development of novel dental materials, especially for bone applications, is shifting in emphasis from achieving a bioinert material that avoids a negative tissue response to instead stimulating specific cellular responses at the molecular level (Lu et al. 2012). Titanium, being the staple material for dental implants for decades, is used particularly for its corrosion resistance when in contact with body fluids, demonstrated biocompatibility in numerous applications and its high

strength to weight ratio (Liu et al. 2004). However, with the increasing life expectancy of patients, the active implant lifespan is also assumed to increase and new, more mechanically strenuous materials are desired for implants. Thus, it has been the focus of continuing research to increase the strength and fatigue resistance of titanium. The most promising routes for this are twofold: 1) alloying titanium to other elements/materials or, 2) secondary processing techniques (Niinomi 1998). While certain titanium alloys have indeed been shown to drastically increase the overall material strength, it has been reported that metal ion release from commonly used titanium metal alloy implants can have adverse biological effects or elicit allergic reactions (Okazaki et al. 1998a b; Köster et al. 2000; Li et al. 2010). A particularly promising secondary processing technique that has been developed, called equal channel angular processing (ECAP), has been shown to reduce the material grain size to the nanometer range (grain size ranges: coarse-grained Ti = approx. 20μm; ultrafine-grain Ti = approx. 150nm) (Valiev et al. 2008). Considering a material's strength is inversely proportional to the square root of the material's grain size (Hall 1951; Petch NJ 1953), reduction of grain size into the nanometer range results in a disproportionate increase in strength as compared to micrometer shifts. ECAP of titanium results in a commercially pure material with increased tensile and fatigue strengths, comparable to the mechanical properties of high performance titanium alloys (Valiev et al. 2006; Semenova et al. 2008). Termed ultrafine-grain titanium (UFG-Ti), this material has recently been considered as appropriate candidate material for implants destined to bear heavy loads (Latysh et al. 2006; Valiev et al. 2008; Bernhard N, Berner S, de Wild M 2009; Elias et al. 2013).

Like titanium, the basic crystal structure of UFG-Ti is hexagonal close-packed (referred to as α-phase), in contrast to certain titanium alloys that, because of non-miscibility between the different metal components, have an additional body-centered cubic phase (referred to as β-phase). A homogenously phased material harbors important potential for biomaterial translation, particularly in terms of surface chemistry and structure (Variola et al. 2008). Indeed, recent work has begun to concentrate on applying topographically-patterned surface features to medical grade UFG-Ti in order to translate this technology to the medical device field (Latysh et al. 2006; Valiev et al. 2008).

Controlling the surface of implant materials destined for bone applications is critical to the overall clinical success of the device (Shalabi et al. 2006). While surface modification achieved by many different methods has been shown to have a positive effect on bone-to-implant interaction, many strategies involve the alteration of surface physicochemical and biochemical properties to obtain the desirable tissue/material interface (Puleo 1999; Nayab et al. 2005; Das et al. 2007). Promising for their medical application, these strategies present developmental complications in terms of obtaining a final product of industrial interest, either in terms of scaling-up procedures or

standardization. Other studies have demonstrated that morphological changes of a material surface can drive a more desirable biological response, even rivaling that of biochemical supplements (Yim et al. 2010; Murphy et al. 2014; Faia-Torres et al. 2015).

Surface topography can be modified in the micro- and/or nano-scale. Sandblasting followed by acid etching results in a micro-textured surface commonly used on dental implants (SLA®) (Cochran 1999). Extended immersion of the SLA surface in a salt solution not only imparts a hydrophilic surface chemistry, but also leads to the spontaneous formation of nanocrystals of titanium oxide on top of the micro structures, resulting in a randomly-distributed nanotopography that grows from the bulk material (SLActive®) (Wennerberg et al. 2013). Complex micro-topographies have been shown to not only induce osteoblastic differentiation and proliferation *in vitro*, but also to result in a better osseointegration of the material *in vivo*, as demonstrated in histological and mechanical assessments (Zhao et al. 2005; Ismail et al. 2007). On the nanoscale, studies have shown that surface features can be used to either influence the osteoblastic differentiation of resident cells at an implant site or, in direct contrast to this, maintain these same cells in a niche state, underlining the importance of nanoscale features on the surrounding environment (Dalby et al. 2007; McMurray et al. 2011). Applying these concepts to dental materials, crystalline nanostructures of titanium oxide can spontaneously form on the surface of medical grade IV titanium upon sand-blasting/acid-etching surface treatment, in addition to the microtopography, if undergoing oxidation (Wennerberg et al. 2013). Subsequent work demonstrated that such nanostructures enhance protein adsorption and deposition of blood components *in vitro* and that they directly contribute to a better material osseointegration *in vivo* (Wennerberg et al. 2014; Kopf et al. 2015a). The ability of micro-and nanostructured surfaces to influence cell activity combined with materials having high strength and fatigue resistance properties offer the potential to develop a clinically relevant generation of implantable biomaterials that can improve interactions with the host tissue.

While surface treated coarse-grained titanium implants have been widely studied and represent the current gold standard for metal dental implants, only a few studies have addressed the short- to mid-term cytocompatibility of surface-structured UFG-Ti (Valiev et al. 2008; Estrin et al. 2009; Park et al. 2009), but have used only smooth or micro-structured surfaces. While these studies reported positive outcomes in terms of cytocompatibility, early osteoblast attachment and spreading, it remains unknown what role a reliable and controlled surface topography and additional nanostructure formation might play on material strength and biological response *in vivo*. The limited amount of information regarding the applicability of this material to the medical device domain

warrants a concerted analysis of a medical grade UFG-Ti presenting an existing, industry standard, surface modification.

The main objective of the present study was: 1) to evaluate if a homogenous, microstructured surface topography can be applied to commercially pure, medical grade UFG-Ti, 2) to characterize this surface-treated UFG-Ti in terms of cytocompatibility and osseointegration potential *in vitro,* 3) to determine the surface-treated material's fatigue and tensile properties and 4) evaluate its *in vivo* osseointegration capacity. The presented results show that it is possible to apply a homogenous microstructure topography onto the surface of UFG-Ti that is cytocompatible and imparts an osseointegration potential similar to coarse titanium. Furthermore, the overall resistance to fatigue and increase in ultimate strength of the surface-treated material remains within acceptable limits for an implantable device destined to be used in high load applications, e.g. dental implants. The ability of this material to quickly osseointegrate and to resist fracture, in a manner similar to coarse-grained titanium, demonstrates its suitability to be developed as an implantable medical material.

## Material and Methods

### Material preparation and surface treatments

UFG-Ti was obtained from grade IV titanium (Ti) bars processed through ECAP, resulting in Ø5mm UFG-Ti bars (Yuntian T. Zhu, Terry C. Lowe, Ruslan Z. Valiev 2006; Valiev & Langdon 2006). For mechanical testing, samples were cut along the longitudinal axis of the bars. For *in vitro* and *in vivo* tests, cross sectional discs of Ø5mm and 1mm thickness were cut from the bars. The cut disc surface is referred to untreated surface in the remainder of this study. A portion of the untreated discs were then further processed for various surface treatments. Surface treatments were performed to render the materials either hydrophobic (SLA®) or hydrophilic (SLActive®), respectively, as previously described (Buser et al. 1991, 2004; Wennerberg et al. 2014). For hydrophobic SLA surfaces, the substrates were sand blasted with corundum (particle size 250-500 µm), acid-etched in a boiling mixture of HCl and $H_2SO_4$, followed by cleaning in $HNO_3$ and rinsing in deionised water, according to a proprietary process by Straumann. Discs were then air dried and packaged in aluminum foil. For the SLActive surfaces, the surfaces were prepared by employing the same sand-blasting and acid-etching process as for SLA, however, further treatment took place under nitrogen to prevent exposure to air, rendering the surfaces hydrophilic. The samples were rinsed in 0.9% NaCl and finally stored in 0.9% NaCl solution at pH 5. All proprietary surface treatments were performed at Straumann (Basel, Switzerland) and resulted in material surfaces with opposing wettability. Coarse-grained/standard Ti SLA and Ti SLActive samples were produced as controls for all experiments.

**Cell isolation, culture and seeding**

Bone marrow samples were obtained from patients undergoing surgical hip replacement after informed consent (with approval from the ethics committee of the canton St.Gallen, CH, EKSG 08/14) and human trabecular bone-derived osteoblast precursor cells (HBCs) were isolated as described previously (Born et al. 2009). After isolation, HBCs were expanded in culture flasks (TPP) until use for cell culture experiments on test samples. All experiments were performed with HBCs of passage 2 with a cell-seeding density of 20'000 cells per $cm^2$ ($c/cm^2$). Before cell seeding, the different Ti samples were placed into wells of a 96-well plate (TPP) and covered with 200µl proliferation medium (α-MEM (D-5796, Sigma), 10% fetal calf serum (FCS, Lonza), 1% PSN (penicillin (5 mg/l), streptomycin (5 mg/l), neomycin (10 mg/l; all from ThermoFisher), 1 mg/l basic fibroblast growth factor (FGF-2, F0291, Sigma)) for 30 minutes. Subsequently, the medium was removed and 200µl of the HBC suspension in differentiation medium (α-MEM, 10% FCS, 1% PSN, 10 nM 1.25 dihydroxy-vitamine D3 (D-1530, Sigma), 50 µM ascorbic acid phosphate (A-8960, Sigma), 2 mM β-glycerophosphate (G-9891, Sigma), 10 nM dexamethasone (D-4902, Sigma)) was applied to each Ti sample and to cells seeded into a well (tissue culture polystyrene, TCP) of a 96-well plate as a control. After 24h the samples were transferred into wells of a new 96-well plate containing 200 µl of fresh differentiation medium; cells on TCP were also supplemented with fresh medium. Surface parameters, especially surface topography, are known to be crucial for implant osseointegration. Smooth (machined) surfaces are clinically not relevant for osseointegration and were therefore not included in biological evaluations.

**Analysis of blood coagulation on Ti substrates**

Blood coagulation on surfaces was studied as previously described (Kopf et al. 2015b). Whole human blood from healthy volunteers was obtained with approval from the ethics committee of the canton St.Gallen, CH (BASEC Nr. PB_2016-00816). The blood samples were used within 1 hour after withdrawal. Two samples per material type were placed into a custom made device, made out of polytetrafluorethylen (PTFE, Teflon), and each mold was filled with blood. The device was closed with a PTFE lid, sealed with parafilm and incubated on a wave shaker (Polymax, Heidolph®, Germany) at 10 rpm and 37 °C for 10 min. After incubation, blood was removed carefully and Ti samples were gently washed with pre-warmed PBS. Finally the samples were transferred into a new 96-well plate, cells fixed in modified Karnovsky solution for 1 h at room temperature and then prepared for SEM analysis as described below.

***In vivo* experimentation**

The present study has been approved by the Ethical Committee of the University of Lund (Sweden) (N° M-138-14), and is reported according to the ARRIVE guidelines regarding all relevant items (Berglundh & Stavropoulos 2012). Twelve adult Swedish looped ear rabbits of both sexes with a weight of 3.3–4.3 kg were used. Animals were housed in standard cages and fed with laboratory animal diet pellets. The animals had free access to tap water. General anesthesia and surgery were performed as previously described (Wennerberg 2014). Briefly, the tibia was exposed through a skin incision and gently accessed through the aponeurosis tissue. Four guide holes were made with a 1.0-mm-diameter twist drill (Medartis) using a drill guide to ensure standardized and correct positioning. A custom-made bur was used in order to form a platform on the bone for the disc implants. Each rabbit received 1 disc of each Ti SLActive and UFG-Ti SLActive, which were covered with polytetrafluoroethylene (PTFE) caps to prevent the samples from bone attachment to the wall and backside of the implant disc. All animals were sacrificed after a period of 4 weeks healing time. After soft tissue reflection, the exposed tibia was fixed in a specially designed stabilization device for the biomechanical pull-out measurements, as described in 2.4 analytical methods. As the overall aim was to demonstrate the feasibility to achieve a state of the art surface on UFG-Ti, only SLActive was used to demonstrate the osseointegration potential in the in vivo model.

**Analytical methods**

**Mechanical properties testing**

Mechanical properties of UFG-Ti material was compared to standard cold-worked Ti grade IV samples. The effect of surface treatment was investigated by comparing SLA samples to untreated samples. Sandblasting and acid etching (SLA treatment) are the only surface processing steps that have an influence on the bulk material's mechanical parameters. Since SLActive samples undergo an identical sandblasting and acid etching as SLA, they were not separately studied.

Tensile tests were performed according to standard test methods [ASTM E8 (Standard Test Methods for Tension Testing of Metallic Materials) and ISO 6892-1 (Metallic material – Tensile testing Part 1: Method of test at room temperature)]. Tests were performed on bars with ø5 mm and an initial testing length of 200 mm. Cross head velocity was 0.005mm/min in the elastic range till yielding and was then increased to 0.5mm/min till fracture. Tests were performed with a Zwick Roell machine (Zwick RetroLine UPM 200 kN) equipped with a strain gauge.

The fatigue tests were performed according to ISO 1143 (ISO 1143:2010-11 (E): Metallic materials - Rotating bar bending fatigue testing) in air at room temperature by a rotating bending fatigue test. At each load level, five specimens were tested until either failure or ultimate number of cycles ($10^7$) without fracture was reached. Using the raw data, the S/N-curves were calculated using the arc sin √P –method (Dengel 1975). For each load level within the region of finite life fatigue strength, the finite number of cycles was calculated for a failure probability of 50%. A partial regression line was plotted through the calculated points. The fatigue endurance limit (50% failure probability) was calculated using the raw data of load levels which contained specimens with and without fracture.

**Contact angle measurements**

The wettability of the different samples was examined by water contact-angle measurements as previously described (Kopf et al. 2015a). Briefly, water droplet sizes of 0.3 µL for the samples stored dry and 0.1 µL for the samples stored in liquid were used. The measurements were performed using a sessile-drop test with ultrapure water (EasyDrop DSA20E, Krüss GmbH). Three samples were analysed for each type of sample.

**Roughness measurements**

The surface roughness at the micrometer scale was measured with a confocal microscope (µsurf explorer, NanoFocus AG). Three samples of each group were selected at random and each sample was assessed at three random positions. 3D images were obtained with the confocal microscope equipped with a 20x lens on a measurement area of 798 × 798 $\mu m^2$ with a lateral resolution of 1.56 µm (512x 512 pixels). The 3D roughness parameters were calculated with the software µsoft Analysis XT (NanoFocus AG) by applying a Gaussian filter with a cut-off wavelength of 50 × 50 $\mu m^2$. Five different parameters were selected to characterize the surface topography:

- $S_a$ = average height deviation from the mean plane, measured in µm and represents a pure height descriptive parameter.
- $S_z$ = maximum height of the roughness image, represents the distance between the highest peak and the deepest valley.
- $S_{sk}$ = skewness of the height distribution, a parameter used to distinguish whether the height deviation is mainly due to dominating valleys or peaks. A positive value indicates distinct peaks, a negative value indicates distinct valleys.

- $S_{dr}$ = developed surface area, measured in % enlargement compared to a totally smooth reference area (equal to the measured area).
- $S_{pd}$ = density of peaks, measured in number of peaks per square mm and represents a pure spatial parameter.

**Immunofluorescence analysis of cell morphology**

Immunofluorescence (IF) analysis was performed to monitor the actin cytoskeleton and nuclei of HBCs cultivated on Ti samples for 4 days as described previously with minor modifications (Kopf et al. 2015a). In brief, HBCs were cultivated on Ti substrates and on TCP controls in differentiation medium. On day 4, cells were fixed in 4% paraformaldehyde (Sigma) supplemented with 0.2% Triton-X (Sigma) for 10 min. Actin filaments and nuclei were stained for 1 h with Alexa Fluor 488-labelled phalloidin (1:40; A12379, Invitrogen) and DAPI (1:1000; 4'-6-Diamidino-2-phenylindole, 10 µg/ml, D9542, Sigma) in phosphate-buffered saline (PBS, Sigma), respectively. Cells on substrates were imaged using a fluorescence microscope (Axio Imager.M1, Carl Zeiss).

**Imaging of cellular mineralization and quantification of calcium**

For imaging of cellular mineralization and quantification of calcium, HBCs were cultivated in differentiation medium on Ti samples and on TCP controls for 28 days. For imaging of mineralization, Xylenol Orange (XO; 20 mM in distilled water, 227854, Sigma) was added directly to the cell culture medium to a final concentration of 20 µM on day 27 and incubated overnight. Before microscopic examination, XO-containing medium was replaced by fresh medium without XO. Two samples per Ti substrate type were imaged using a fluorescence microscope (Axio Imager.M1, Carl Zeiss). Cells cultivated in proliferation and differentiation medium on TCP served as negative and positive control, respectively.

To quantify mineralization, cell number and $Ca^{2+}$ content of HBCs on the respective surfaces were assessed as described previously (Kopf et al. 2015b). Briefly, the number of HBCs was determined using the alamar blue (AB) assay (alamarBlue® Cell Viability Reagent; DAL1025, Invitrogen), according to the manufacturer's protocol, interpolating fluorescence readings from a 6-point standard curve (measured from HBCs on TCP after 1 day in culture). Afterwards, the $Ca^{2+}$ content of the same samples was assessed using a Quanti Chrom™ Calcium Assay Kit (Gentaur), according to the manufacturer's protocol. The $Ca^{2+}$ concentration and number of metabolically active cells was assessed for each sample in triplicate.

**Surface characterization and characterization of cell/blood interaction with surfaces by scanning electron microscopy**

For surface characterization experiments, the surface morphology of the different materials and different surface modifications was investigated by scanning electron microscopy **(**SEM). The measurements were performed on three different samples selected at random for each surface type. The samples were investigated with a Zeiss Supra 55 SEM (Carl Zeiss AG) equipped with a field-emission electron source, an Everhart-Thornley secondary electron detector and an in-lens secondary detector. The overview SEM images were acquired with an acceleration voltage of 15 kV using the Everhart-Thornley detector and the high-resolution images with an acceleration voltage of 5 kV using the in-lens detector.

For the *in vitro* experiments in which SEM was used, HBCs were cultivated for 4 days on the different Ti materials in differentiation medium before fixation. SEM imaging was performed as previously described (Kopf et al. 2015b). Briefly, samples were fixed in modified Karnovsky's solution, dehydrated in a gradient series of ethanol, dried in hexamethyldisilazane (HMDS, Sigma) and sputter-coated with 10 nm gold–platinum (Leica EM ACE 600) before acquiring the images using a Hitachi S-4800 scanning electron microscope (Hitachi High-Technologies Corporation) at accelerating voltage of 5 kV and 10 µA current.

**Biomechanical pull-out test**

Pull-out tests were performed as previously described (Rønold et al. 2003). Stabilized tibias were adjusted to be in line and perpendicular with the load cell using a level tube, as previously described (Wennerberg et al. 2014). The tensile test was performed with a Zwick PL 5kN dos 700030 testing machine (Zwick, Germany) fitted with a calibrated load cell of 200 N (Xforce P load cell, Zwick). Cross-head speed range was set to 1.0 mm/min.

**Statistical analysis**

For the statistical analysis of the contact angle measurements, it was assumed that all groups were sampled from Gaussian populations, but that the standard deviations would be different, due to the intrinsic differences in the base materials. Therefore, an unequal variance t-test (Welch t-test) was used in this case (GraphPad Prism, USA). For the *in vivo* results, the pull-out force measurement for one disc type was paired to the other disc implanted within each animal and the Wilcoxon signed

rank test was used for analysis of data (results not shown). Comparisons were adjusted, using regression mixed models, for animal effect (as a random effect), side, position and distance of the implanted disk to the knee joint (as fix effects). The Dunnett-Hsu adjustment was used to adjust the p-values in the case of multiple comparisons. Non-inferiority of a certain disc type to another was tested using regression mixed models and adjusted for the same factors as above. The average effect and its two-tailed 90% confidence interval (equivalent to a one tailed 95% CI, to test for non-inferiority) were calculated in order to obtain a measure of how much different (a tolerance range) the pull out force for the 'test' disc could be, while still being considered as good as the comparison treatment. Conventional coarse-grained Ti SLActive was used as the reference. SAS release 9.4 (SAS institute Inc, 2002-2012, Cary, NC, USA) was used for the statistical analysis. The level of significance was set at an alpha level of <0.05.

## Results

### Physical properties of bulk and surface treated UFG-Ti

The yield strength of UFG-Ti versus Ti was ~40% higher, reaching significantly higher values of ~1010 MPa versus ~722 MPa. Correspondingly, the ultimate tensile strength was also significantly higher for UFG-Ti versus Ti by ~45%, reaching an ultimate strength higher than 1250 MPa (Table 1). The effect of the SLA surface treatment on the strength of the material was insignificant when compared to untreated surfaces, i.e. the surface roughness had no negative effect on the tensile properties of the material ($p$ = 0.741). All $p$ values were < 0.0001 (comparison of UFG-Ti sample types to their respective Ti counterparts).

The surface roughness treatment had a negative effect on the fatigue endurance limit of both material types (Figure 1). For both UFG-Ti and Ti, the SLA surface treatment resulted in ~12% and ~17% reduction in the fatigue endurance limit compared with their untreated counterparts, respectively. However, for both surface conditions, UFG-Ti had a considerably higher fatigue endurance as compared to Ti, being 34% higher for the untreated and 26% higher for the SLA treated surface (Table 2).

**Surface treatment characterization of UFG-Ti**

SLActive surface treatment resulted in super-hydrophilic surfaces for both materials, with contact angles (C.A.) measured as 0° in all cases. C.A.s for SLA surface-treated samples were comparable for the two materials (Ti SLA: 115.5° ± 26.8°; UFG-Ti SLA: 131.1° ± 2.2°). While the standard deviation for Ti proved to be higher than that of UFG-Ti, no significant difference was found ($p$=0.4206; Unpaired, 2-way t-test with Welch's correction) (Table 3).

SEM analysis demonstrated that stable nanostructures formed on the Ti and UFG-Ti materials after 1 month in liquid storage (Figure 2). Furthermore, these structures remained stable over time, with no observable differences up to the 9 months analyzed in this study. No differences were visible between the different time points, i.e. the nanostructures were equally evolved after 1 month compared to the samples stored for 9 months (Figure 2 (month 1 & 9 time-points for UFG-Ti shown; 2, 3, 4, 5, 6, 7 & 8 months were comparable to the 1 and 9 month timepoints (data not shown); Ti SLActive surface topography evolution published previously)) (Wennerberg et al. 2013). For SLA treated surfaces, SEM imaging demonstrated a comparable morphology for all surfaces with clear macro- and micro-structures typical to sand-blasting and acid-etching processing.

Further analysis of surface roughness corroborated the results generated from SEM analysis. Overall, all surfaces were found to be moderately rough ($S_a$ 1-2 µm), as per the definition established by Wennerberg & Albrektsson (Wennerberg & Albrektsson 2009). Within the four groups, UFG-Ti SLActive and Ti SLA tended to have comparable roughness values while Ti SLActive and UFG-Ti SLA were more comparable to one another. Generally, higher roughness was observed for UFG-Ti SLA and Ti SLActive as compared to the other two groups (Table 4).

**Cytocompatibility, cell morphology and cell differentiation potential on surface-treated materials**

To evaluate the effect that standardized surface treatments might have on the cytocompatibility and the osseointegration potential of UFG-Ti, qualitative assessment of HBC cell proliferation and morphology as well as qualitative and quantitative evaluation of calcium production upon osteoblast mineralization were performed. Furthermore, blood coagulation on standard Ti and UFG-Ti substrates was evaluated.

HBCs cultivated on standard Ti and UFG-Ti substrates in differentiation media for 4 days showed a comparable cell density on all materials and surface treatments (Figure 3a). Evaluation of cell attachment and cell morphology by SEM imaging by staining for actin and nuclei showed

comparable cell attachment on the different surfaces and a similar degree of spreading of HBCs on all substrates after 4 days of cultivation (Figure 3). When assessing the mineralization of HBCs on standard Ti versus UFG-Ti surfaces, mineral nodule formation was observed on all material- and surface-types (Figure 4a). Higher levels of mineralization was found on both SLActive materials compared to SLA surfaces. HBC number and calcium content was quantified on the respective surfaces. Cells on Ti SLActive and UFG-Ti SLActive displayed much higher $Ca^{2+}$ levels compared to Ti SLA and UFG-Ti SLA, respectively (Figure 4b). However, no difference between Ti and UFG-Ti substrates could be observed.

Faster and enhanced blood coagulation was observed on Ti SLActive and UFG-Ti SLActive when compared to Ti SLA and UFG-Ti SLA (Figure 5). While homogenously distributed fibrin networks were seen on SLActive surfaces (Ti and UFG-Ti), only aggregated platelets with some spots of fibrin network formation could be found on Ti SLA and UFG-Ti SLA after incubation in blood for 10 minutes.

***In vivo* performance of surface-treated materials**

To determine the *in vivo* osseointegrative performance of the UFG-Ti material, SLActive surface-treated samples of Ti and UFG-Ti were implanted into tibial defects in rabbits for 4 weeks. In parallel, and on the day of implantation, matching-lot discs were analyzed via SEM to verify that the specific surface topography was indeed present.

Surface features common to both SLActive-treated Ti materials were found to be present and homogenously distributed across the surface. Both materials were found to have comparable surface characteristics at the time of implantation (Figure 6A).

Osseointegration, indirectly measured by pull-out force measurements, proved to be similar between the two material types. Table 5 shows the descriptive statistics of the outcome for the examined materials. UFG-Ti had a slightly lower average pull-out value than Ti (UFG-Ti: 55.28 N versus Ti: 63.37 N), but not statistically significant ($p = 0.3104$) (Table 6 & Figure 6B). Further statistical analysis for non-inferiority determined that UFG-Ti would need to have a weaker pull-out force by at least 14.26 N to be considered inferior (Table 6).

**Discussion**

Current trends in implant materials research are directed towards both strengthening of the base material and/or augmenting the tissue integration properties. This study analyzed the various factors associated with the successful development of a novel surface-modified, UFG-Ti material and its applicability as an implant material for bone tissues by developing a strategy to transfer a market-standard surface technology to an medical grade UFG-Ti and demonstrates a novel approach to creating a standardized, bioactive and chemically pure titanium-based implant material with increased mechanical properties when compared to traditional coarse-grained titanium. While UFG-Ti has been purported to be an applicable implant material for over a decade, no clinical translation has yet been demonstrated (Latysh et al. 2006). Employing a concerted analytical workflow, this study takes into account the parameters influencing the success of the eventual implant (mechanical constraints, cytocompatibility and *in vitro* mineralization as well as *in vivo* performance).

While titanium represents the material of choice for modern, bone-specific implants, limitations exist in which a stronger material is necessary. In this study, surface-modified UFG-Ti was found to have an ultimate tensile strength of roughly 1.5 times that of Ti-Grade 4. Interestingly, the strength values presented for this particular surface-modified UFG-Ti outperform the strength values of the most commonly used titanium alloys for implant materials: Ti-6Al-4V and Ti-6Al-7Nb (International Organization for Standardization 1996). Most importantly, this UFG-Ti material presents a chemically pure implant material and does not contain any contaminating alloy elements that have been previously shown to potentially be detrimental to the cytocompatibility and/or long term success of the implant (Perl & Moalem 2006; Percy et al. 2011).

UFG-Ti has been an increasingly important topic in translational research and other studies have indeed demonstrated a capacity to modify UFG-Ti surfaces. While a previously published study did show the cytocompatibility of a lab-produced surface-treated UFG-Ti, information on the grain size was not provided and the post-production annealing process involved heating of the material to up to 300°C (Zheng et al. 2011). However, it has been demonstrated that the original UFG-Ti material structure cannot be retained at these kinds of temperatures (Aliofkhazraei 2015). Indeed, the mechanical properties of this surface-modified UFG-Ti were not provided in this aforementioned study.

In the present study, a state of the art surface technology (SLActive) was adapted to a UFG-Ti material. To generate an SLActive-modified surface, the base material is first sand-blasted and acid etched to create an SLA surface. The modified material is then immersed in a saline solution to

impart a hydrophilic component. During the storage process, titanium dioxide nano-structures grow on the surface in a randomized, needle-like configuration (Wennerberg et al. 2013). The mechanical properties of the bulk material are only negatively affected by the SLA process and not by any effect of the treatment rendering the surface hydrophilic. Therefore, only SLA-modified (and not SLActive-modified) UFG-Ti was evaluated for its mechanical strength and fatigue profiles in this study. Overall, the presented results demonstrate that the tensile properties of the UFG-Ti and Ti show no difference arising from the surface treatment, whereas the fatigue properties are reduced by the surface treatment. A roughened surface has predominantly more sites for increased stress concentrations near the surface. Those stress concentrations are potential sites for crack initiation (Ritchie 1988). A material such as UFG-Ti that has been subjected to a high deformation prior to the fatigue testing and has limited ability to accommodate further stress concentrations and to transform them into plastic strain. As a consequence, if the local sites reach localized stresses beyond a critical level, crack initiation and propagation is accelerated (Ritchie 1988). However, as the critical stress level lies at a significantly higher level for UFG-Ti with SLA surface than that of Ti with SLA surface, a far higher fatigue endurance level can be reached. In consequence, the fatigue performance of UFG-Ti, with or without a modification of its surface, can be considered superior to that of Ti.

Considering the characterization of the surface modification on UFG-Ti, this study raises several interesting points. The wettability is found to be the same on both UFG-Ti and Ti having undergone the same surface modifications and is, therefore, only a factor of the surface modification and does not appear to be influenced by the bulk material properties. The presented results on the surface modification on Ti were also in line with a previous study in which similar surface modifications were performed on Ti, thereby suggesting that SLActive increases the surface free energy of the UFG-Ti material (Rupp et al. 2006). The standard deviation present in the wettability measurements can perhaps be explained by the local variations in roughness due to batch to batch or local differences in the sand blasting process. Overall, the wettability and roughness measurements are both similar for Ti and UFG-Ti materials according to a previously defined acceptable range of values (Wennerberg et al. 2013). Indeed, it has been suggested that only a very specific surface microtopography with an $S_a$ value between 1 and 2 µm provides an optimal surface for bone integration (Wennerberg & Albrektsson 2009). Interestingly, the SLActive-treated UFG-Ti developed in this study was found to have an $S_a$ value of approximately 1.4, thus fulfilling the criteria for a moderately rough surface optimal for bone integration applications. Furthermore, all four groups of materials tested in this study fell within an $S_a$ range of 1-2µm, thus suggesting they should be equally efficient in terms of osseointegration. Finally, this observation that the surface modification and not

the bulk material appears to be the critical parameter in determining material properties is supported by the SEM results. The nanostructures produced on UFG-Ti were found to be comparable to those produced on Ti. The formation of nanostructures seems to be independent of differing grain sizes of the base materials, an effect which was not predictable.

It has been shown that blood coagulation and blood protein adsorption depend on microtopography and chemistry of Ti surfaces (Kopf et al. 2015a). On coarse-grained titanium, an increased blood protein adsorption of the proteins fibrinogen and fibronectin, and increased blood coagulation on Ti SLActive compared to Ti SLA, was previously demonstrated (Kopf et al. 2015a). The same process has now been demonstrated to occur on SLA- and SLActive-modified UFG-Ti (Figure 5). Not only the initial blood-material interaction, but also cell attachment and cell spreading was found to be similar on surface treated UFG-Ti and Ti (Figure 3). It has previously been shown that the nanostructures formed during SLActive surface modification on Ti have a direct impact on osseointegration (Wennerberg & Albrektsson 2009; Wennerberg et al. 2014). In the present study, surface modification of UFG-Ti also demonstrated the same trend towards an increased osseointegration potential and *in vivo* performance of SLActive over SLA surfaces. Importantly, no differences could be observed between UFG-Ti and Ti materials. In line with these *in vitro* observations, *in vivo* performance of UFG-Ti versus Ti demonstrated statistically equivalent osseointegration of the two materials. It is interesting to note that the pull-out measurements obtained in this study for Ti-SLActive were almost identical to those reported previously (Wennerberg et al. 2014).

The *in vivo* data further suggest that the nanocrystalline structure of the bulk material does not have a direct effect on the osseointegration of the material and that the SLActive surface modification can be transferred to this new material type. While the measured differences in pull out force between the two materials was not statistically significant, a non-statistical p-value is generally not enough to state that UFG-Ti is non-inferior to Ti (Walker & Nowacki 2011). Therefore, a non-inferiority test was performed which showed that the mean pull-out force of UFG-Ti must be at least 14.26N less than Ti for it to be considered inferior. The tolerance range was given by the lower 90% confidence interval for the difference between the discs. This study demonstrated a pull-out force difference of only 8.08N and therefore, this material can be considered as being non-inferior to a gold standard titanium material commonly used in the clinic.

Bone-specific implants are required to meet the ever increasing specifications regarding cytocompatibility and osseointegration, as well as mechanical resistance to fatigue and fracture. The UFG-Ti material presented in this study fulfills these specifications by offering a material that

conserves its increased resistance to fracture and fatigue profile while at the same time presenting a similar osseointegration potential as Ti after state of the art surface treatment. This study demonstrates the feasibility of applying an industry standard, micro-structured surface topography to a scale-up processed UFG-Ti bulk material that results in a consistent micro-topography, similar to that produced on coarse-grained titanium. The novel surface modified UFG-Ti implant material was also found to support an equivalent biological response, in terms of *in vitro* cell response, mineralization and osseointegration, when compared to surface modified Ti implants. Surface modified UFG-Ti therefore shows great potential to specifically address the clinical need for bone implant devices strong enough to provide support to the damaged tissue but also resist in terms of fatigue over the life of the patient.

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**Disclosures**

Dr. Rottmar, Dr. Kopf and Dr. Maniura-Weber declare no conflict of interest. Dr. Stübinger reports personal fees from Straumann AG during the conduct of the study and outside of this work. Dr. Pippenger, Dr. Berner and Dr. Dalla Torre are currently employees of Straumann and participated in the study as contributing scientists. The study was funded by CTI (Grant No: 13747.1 PFFLE-LS). Institut Straumann AG (Basel, Switzerland) solely provided sample materials for the study and had no role in study design.

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**Tables**

*Table 1: Yield and ultimate tensile strengths of standard Ti (Ti) and UFG-Ti with untreated and SLA-treated surfaces. "Relative strength" columns represent percentage increase of UFG-Ti when taking Ti as the reference material.*

| n = 8 | Yield strength (MPa) | *Relative strength (%)* | Ultimate strength (MPa) | *Relative strength (%)* |
|---|---|---|---|---|
| Materials | mean (SD) | *Ti as ref. Material* | mean (SD) | *Ti is ref. Material* |
| **Ti** | 722 (8) | 100 | 864 (10) | 100 |
| **Ti SLA** | 712 (9) | 100 | 851 (6) | 100 |
| **UFG-Ti** | 1010 (18) | 140 | 1251 (12) | 145 |
| **UFG-Ti SLA** | 1019 (21) | 143 | 1254 (4) | 147 |

*Table 2: Calculated fatigue endurance strength of untreated and surface-treated UFG-Ti compared to standard Ti.*

| Material | Calculated Endurance limit (MPa) | Change (normalized to reference) (%) |
|---|---|---|
| **Ti (Ref)** | 430 | - |
| **Ti SLA** | 380 | 88 |
| **UFG-Ti** | 577 | 134 |
| **UFG-Ti SLA** | 480 | 126 |

*Table 3: Contact angles of UFG-Ti and standard Ti materials with both SLA and SLActive surface treatments. SD= standard deviation.*

| Sample | Sample 1 | Sample 2 | Sample 3 | Mean | SD |
|---|---|---|---|---|---|
| | C.A. [°] | C.A. [°] | C.A. [°] | C.A. [°] | C.A. [°] |
| **Ti SLA** | 129.3 | 132.6 | 84.6 | 115.5 | 26.8 |
| **Ti SLActive** | 0.0 | 0.0 | 0.0 | 0.0 | - |
| **UFG-Ti SLA** | 133.5 | 129.3 | 130.4 | 131.1 | 2.2 |
| **UFG-Ti SLActive** | 0.0 | 0.0 | 0.0 | 0.0 | - |

*Table 4: Comparison of 3D roughness parameters for standard Ti versus UFG-Ti subject to SLA and SLActive surface treatments.*

| Sample n= 3 | $S_a$ [µm] (SD) | $S_z$ [µm] (SD) | $S_{sk}$ (SD) | $S_{dr}$ [%] (SD) | $S_{pd}$ [1/mm$^2$] (SD) |
|---|---|---|---|---|---|
| **Ti SLA** | 1.456 (0.080) | 20.77 (2.63) | 0.161 (0.072) | 16.86 (1.37) | 1590 (170) |
| **Ti SLActive** | 1.759 (0.067) | 25.95 (2.45) | 0.082 (0.062) | 24.56 (1.83) | 1709 (102) |
| **UFG-Ti SLA** | 1.719 (0.075) | 24.35 (2.51) | 0.052 (0.123) | 23.53 (1.27) | 1833 (169) |
| **UFG-Ti SLActive** | 1.439 (0.027) | 21.30 (0.98) | 0.049 (0.069) | 16.71 (0.50) | 1513 (69) |

*Table 5. Pull-out force descriptive statistics for different disc surface treatments*

| Disc Surface Treatment | Paramenter | Pull-Out Force [N] |
|---|---|---|
| UFG-Ti SLActive | n<br>Mean ± SD<br>Median (Q3 –Q1)§ | 12<br>54.6 ± 25.3<br>54.7 (34.7 to 70.0) |
| Ti SLActive | n<br>Mean ± SD<br>Median (Q3 –Q1)§ | 12<br>64.1 ± 23.2<br>67.5 (46.2 to 78.9) |

§ (Q3 – Q1) = interquartile range

SD = standard deviation

*Table 6. Adjusted§ association between pull-out force [N] and disk surface treatment*

| Factor | Value | Regression parameters | | Adjusted parameters | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Estimate | SE | Adjusted[§] mean | 95% CI for the adjusted mean | p | Average effect of the factor | (90% CI) |
| Disc type | UFG- Ti SLActive | -5.25 | 4.85 | 56.70 | 39.76 to 73.65 | 0.3104 | -5.25 | -14.26 to 3.76 |
| | Ti SLActive (Ref.) | 0 | | 61.95 | 45.01 to 78.89 | | | |

[§]The factor animal was introduced in the model as a random effect and the factors side, position and distance to knee joint as fix effects. The Dunnett-Hsu method was used as adjustment due to multiple comparisons.

**Figure captions**

**Figure 1**: Fatigue strength of UFG-Ti vs. Ti for untreated and SLA treated surfaces. Small symbols represent single datapoints (equivalent to a single specimen measured). Large symbols represent the calculated values according to the arcsin arcsin√P method.

**Figure 2**: SEM images of UFG-Ti and Ti SLActive samples after different storage times in saline solution (1 and 9 months). Scale bar = 100 nm.

**Figure 3**: HBC attachment and spreading on Ti and UFG-Ti surfaces. Cells stained for actin filaments (green) and nuclei (blue) showing HBC adhesion and spreading on UFG-Ti and Ti are shown on a) Mosaic overview images (Scale bar: 500µm) and b) representative close up images (Scale bar: 50µm). c) Representative SEM images showing cells spreading on the materials' surface (Scale bar: 50µm).

**Figure 4**: Mineralization of HBC on Ti and UFG-Ti surfaces. a) Xylenol Orange (XO) staining of mineral nodules showing mineralization of HBC on UFG-Ti and Ti. Scale bar: 100µm. b) Quantification of $Ca^{2+}$ concentration [ng] and number of metabolically active cells after 28 days. Data is presented as mean ± SEM; n=3.

**Figure 5**: SEM images of fibrin network formation on Ti SLA, UFG-Ti SLA, Ti SLActive and UFG-Ti SLActive showing enhanced blood coagulation on UFG-Ti SLActive and Ti SLActive (Scale bar= 20µm).

**Figure 6**: Relationship between disc material and pull-out force in the rabbit bone model. a) SEM images for quality control of the implanted samples demonstrated the presence of micro- and nano-surface topographies at the time of implantation. (micrometer images scale bar = 10 µm; nanometer scale images scale bar = 200nm). b) Average pull-out forces in Newtons of Ti and UFG Ti SLActive disc materials, n=24. (Values represent adjusted means for animal effect, side, position and distance of disc to knee joint, and whiskers are the 95% confidence intervals for the mean).