In vitro and in vivo performance of a novel surface treatment to enhance osseointegration of endosseous implants

Roberto Chiesa, PhD,[a] Gianluca Giavaresi, MD,[b] Milena Fini, MD,[b] Enrico Sandrini, PhD,[a] Carmen Giordano, PhD,[a] Andrea Bianchi, MD,[c] Roberto Giardino, MD,[b] Milan and Bologna, Italy
POLITECNICO DI MILANO, RIZZOLI ORTHOPEDIC INSTITUTE, AND VITA-SALUTE UNIVERSITY

Objective. This article shows the in vitro and in vivo characterization of a new biomimetic treatment developed to enhance the osseointegration of titanium dental implants.
Study design. A novel biomimetic treatment of titanium was developed. Its physicochemical properties and biologic and in vivo performance were considered and studied. Mineralization capability was assessed by soaking test in simulated body fluid solution, and cytocompatibility was assessed using osteoblast-like MG63 cell culture. Histomorphometric analysis was performed at 3 time points using a sheep animal model.
Results. In vitro tests confirmed the biomimetic potential of the considered novel treatment. Histomorphometric analysis indicated its potential for rapid and good-quality osseointegration.
Conclusion. The in vitro and in vivo test results indicated that the proposed novel treatment possesses a significant potential to increase the rate of osteointegration of titanium for endosseous dental implants. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;103:745-56)**

Currently, endosseous implants have proven to be successful and predictable in the rehabilitation of patients with poor bone (i.e., osteoporosis) and those partially or completely edentulous. One of the key factors for the success of these implants is the achievement of close contact between the bone and the implant surface. Additionally, the long treatment time is primarily related to the slowness of the osseointegration process. This is perceived by some patients and clinicians as a major problem, especially compared with conventional prosthetic techniques. Different implant surface textures have been studied and introduced to solve this limitation, but restoration achieved by early or immediate loading still remains controversial compared with the reliability of endosseous implants.[1,2] The occurrence of excessive parafunctional loads, the chemical severity action of the biologic fluids on the implant materials, the possibility of infiltration, adhesion, and proliferation of bacteria from the oral cavity onto the implant surface, and contamination of the implant surface are all factors which may contribute to impairing both short- and long-term functionality of dental implants.

Besides implant design and surgical technique, surface chemical composition and topography are recognized to be key factors for achieving a fast and durable osseointegration, as well as for implant stability over time. The required surface roughness and texture is still a debated factor. Implant anchorage is obtained by different systems with varying surface roughness. Together with topography, the chemical properties of the implant surface in contact with the biologic tissues are known to play a fundamental role in the healing process.[3,4] The implant-to-bone interface should be able to promote the apposition of new bone by a proper triggering of the biochemical functions. In a similar way, the surface of the implant area in contact with the gingival tissues should enhance the apposition of soft tissues, sealing the way to the ingress, proliferation, and colonization of bacteria from the oral cavity.

Titanium (Ti) is considered to be the best material for endosseous implants. Besides its mechanical, chemical, and physical properties, Ti is known to possess excellent biocompatibility.[5] Osseointegration may be improved by modifying the surface properties with different surface treatments. An appropriately modified Ti surface might actually be the key factor in achieving a fast and stable implant fixation through optimal osseointegration. Among the several surface modification and deposition techniques developed and applied to biomaterials,[6] Ti is particularly suitable for electrochemical anodic modification techniques,[7] such as low-

[a]Department of Chemistry, Materials, and Chemical Engineering "G. Natta," Polyitecnico di Milano.
[b]Department of Experimental Surgery, Rizzoli Orthopedic Institute.
[c]Department of Dentistry, Vita Salute "S. Raffaele", University.
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voltage anodization and anodic spark deposition (ASD).[8] Anodization treatments can be effectively applied to modify the crystalline structure and chemical composition of the titanium oxide (TiO_2), and ASD can be used to increase the surface oxide thickness and to modify the morphology and roughness of the surface.

A new biomimetic treatment performed using the ASD technique was previously developed and investigated by the present authors.[9,10] This ASD technique was proven to improve titanium biocompatibility by enhancing its bioactivity and osseointegrative properties without introducing any detrimental effects on the mechanical properties of the material. The results obtained in the previous studies suggested the possibility to use such treatment to improve the osseointegration of endosseous implants. In the present study, the behavior of the newly developed biomimetic treatment, designated AAT (for anodized alkali-treated titanium), was compared with a chemical etched surface, called BioRough, and to a simply machined surface. As suggested by ISO 10993-1, "Biological evaluation of medical devices," the materials have been preliminarily analyzed in vitro to assess cytocompatibility, in particular the effect of material release and surface chemical-physical properties on cell behavior. An in vitro cellular model based on MG63 human osteosarcoma cells was selected because they show several characteristics of immature osteoblast and can be used as a model to investigate the early stages of osteoblastic response. Subsequently in vivo tests were performed on the materials.

MATERIALS AND METHODS

Samples preparation

Commercially pure grade 2 titanium (ISO 5832/2) is chosen by many dental implant producers for its good mechanical properties and superior biocompatibility. This material was selected and used to prepare samples for in vitro and in vivo tests (Loterios, Legnano-Milan, Italy). Titanium sheets were used to prepare plates of 10 x 10 x 1 mm, and Ti bars were used to prepare screw-thread fixtures, 12 mm in length and 4 mm in diameter. All samples were cleaned by ultrasonic rinsing (Branson Automatic Cleaner; Branson Ultrasonic Corp., Danbury, CT) in acetone (RPE; Carlo Erba Reagenti, Milan, Italy) for 5 minutes, then in distilled water for 5 more minutes, to degrease and clean the surface of contaminants. Two different surfaces were made on both plates and fixtures: an electrochemical surface treatment performed by ASD and followed by an alkali etching process (AAT) or an acid etching process (BioRough).[8,9]

AAT surface preparation. The AAT samples were prepared according to the patent EP 1515759. The treatment involved the application of 2 consecutive ASD processes[11,12] carried out in different electrolyte solutions at different voltage ranges, and followed by an alkali etching process.[9,13] The first ASD was performed in a solution containing phosphate anions and calcium cations, and the second ASD was performed in a solution containing only calcium cations. A variable DC power supply (BVR1200-500-1; Belotti Variatori, Milan, Italy) and 2 voltmeters were used to supply power to the circuit and monitor the voltage gap between cathode and anode, as well as between cathode and the points of the electrolytic solution in between the 2 electrodes. The solution was continuously stirred by magnetic stirring in a double-wall glass beaker. Electrolytic solution temperature was monitored and kept at 0 ± 2°C by refrigerating fluid flow through the external and the inner wall of the beaker. The Ti specimens were connected one by one to the anode. A polytetraflouroethylene sleeve was used to shield the Ti specimen surface and avoid sparking at the atmosphere–sample–electrolyte solution interface. The cathode was formed by a Ti cylinder-shaped net, whose surface was about 60 times bigger than the surface of the anodizing sample.

BioRough surface preparation. BioRough specimens were prepared by a double-step etching process. An initial etching was carried out in a solution of 1 mol/L NaOH, mixed with 2% v/v H_2O_2, kept at 80°C for 10 minutes. The second etching step consisted of an acid treatment performed at 28°C for 1 hour. After each etching step, specimens were rinsed 3 times for 5 minutes in distilled water by ultrasonic rinsing.

Physicochemical and morphologic properties

Physicochemical and structural analyses were carried out to interpret the results obtained in the biologic experiments; an extensive chemical and structural study of Ti, BioRough, and AAT surface has already been reported.[8]

Macroscopic Analysis. A digital camera (Nikon Coolpix 950, Nikon Imaging Company, Tokyo, Japan) was used for collecting magnified pictures of the whole treated implants.

Scanning electron microscopy. The plate and fixture surface before cell seeding and in vivo implantation was investigated on selected specimens by scanning electron microscopy (SEM) (Stereoscan 430, equipped with a backscattered electron detector; Leica Cambridge Instruments, Cambridge, UK) at an accelerating voltage of 20 kV. All of the samples investigated with SEM were coated with gold by the sputtering technique (Sputter Coater SC7640; Polaron, Hertfordshire, UK). SEM was carried out also on plates after the cell test to

analyze the morphology and to assess the cell adhesion at an early stage (48 h culture) on the different surfaces.

Energy dispersive spectroscopy. The plates and fixtures were investigated also by energy dispersive spectroscopy (EDS) (Link eXL analyzer, Cambridge, UK). Specimens analyzed with EDS were not sputter coated, to avoid the gold signal overlapping with other elements on the surface. Elements analyzed were calcium, phosphorus, and oxygen. The EDS allowed qualitative comparative estimation of the differences in the chemical composition of the finishing treatments.

Noncontact laser profilometry. Roughness measurement was carried out on plates and fixtures using a noncontact laser profilometer (Microfocus Compact, UBM, GmbH, Ettlingen, Germany). Roughness parameters were calculated as an average of 5 profiles 5.6 mm in length. The analysis of the results was performed considering the following parameters:

R_a: the arithmetic mean of the departures of the roughness profile from the mean line

R_{max}: the vertical distance between the maximum peak to lowest valley within a single sample length

R_t: the distance between the highest peak and the lowest valley of the whole sample

K: the distribution of the profile height around an ideal average line (kurtosis)

Sk: the symmetry of the distribution of the profile height around an ideal average line (skewness)

Although attempts were made to perform roughness analysis directly on the surface of the fixtures, the restricted area of the distal drilled channel, which was the only planer surface of the fixture available for roughness measurement, did not allow us to perform reliable profile measurement. Nevertheless, the morphology observed at high magnification with SEM showed the same topography between plates and fixtures for each considered material.

Thin-film x-ray diffraction. The superficial structure of Ti, BioRough, and AAT was investigated with thin-film x-ray diffraction (TF-XRD) (D500 Kristalloflex, Siemens, Munich, Germany) at 40 mA and 40 kV. X-ray diffraction was carried out on treated plates to analyze the crystalline structure of the differently treated surfaces, and to assess the presence of titanium oxide (TiO_2) on the surface and its structure.

In vitro cytocompatibility

Sample treatments and controls. The material plates were tested after sterilization by 70% ethanol in distilled water followed by UV irradiation (254 nm). Tissue culture plate polystyrene (TCP) (Corning-Costar, Celbio, Milan, Italy) was used as control. All assays were performed at least in triplicate.

Human osteosarcoma cell cultures. The human osteosarcoma cell line MG63 was obtained from the European Collection of Animal Cell Cultures and cultured as described in a previous study.[14] After trypsinization, cell viability was assessed using the trypan blue exclusion dye (Sigma-Aldrich, Milan, Italy). The test materials were seeded with a 50-μL drop of a 2×10^5 cells/mL suspension placed in the center of the sample, allowed to adhere in the incubator for 1 hour, flooded with 1 mL culture medium, and placed back into the incubator. After 24 hours, tissue culture plate wells containing the samples were checked with light microscopy and, if cells adhering on the TCP around the specimens were visible, the samples were transfered to a new tissue culture plate, flooded with 1 mL fresh culture medium, and incubated.

Elution study. Four samples for each material were flooded with 1 mL culture medium and incubated. At the selected time points (1, 3, 7, 14, and 21 days), the aqueous extracts were collected in sterile conditions and replaced with 1 mL fresh medium. One hundred–microliter/well cell suspensions (10^5 cell/mL) were seeded into a TCP, incubated to confluence, and then culture medium replaced with 100 μL/well aqueous extract (3 replicates for each sample). After 48 hours of incubation, the extracts were replaced with 100 μL/well 0.5 mg/mL MTT (Sigma-Aldrich) solution in culture medium, and the plates were incubated for 4 hours. Finally, the solution was replaced by 100 μL/well DMSO (Sigma-Aldrich), the plate was mixed until complete dissolution of the crystals, and the absorbance was measured using a spectrophotometric plate reader (Tecan Genius Plus, Tecan Italia, Milan, Italy) at a wavelength of 570 nm (reference wavelength 630 nm).

Cellular adhesion and morphology. Samples were prepared for SEM analysis as follows: 48 hours after seeding of sample surface (2 samples for each material), the cells were fixed with 1.5% w/w glutaraldehyde (Fluka; Sigma-Aldrich) in 0.1 mol/L sodium cacodylate (Fluka) then dehydrated through a series of ethyl alcohol solutions (Sigma-Aldrich) (from 20% to 100% v/v in distilled water), followed by a series of hexamethyldisilazane (Sigma-Aldrich) solutions (from 25% to 100% v/v in ethyl alcohol), and then air dried. The samples were sputter coated (Sputter Coater S150B, 1 min at 15-20 10^{-1} mm Hg; Edwards, Crawley, UK) before examination by SEM (accelerating voltage 10 keV).

Cellular proliferation. Cells were seeded onto the tested surfaces (4 samples for each material) as previously described. At each time point (1, 3, 7, 14, 21

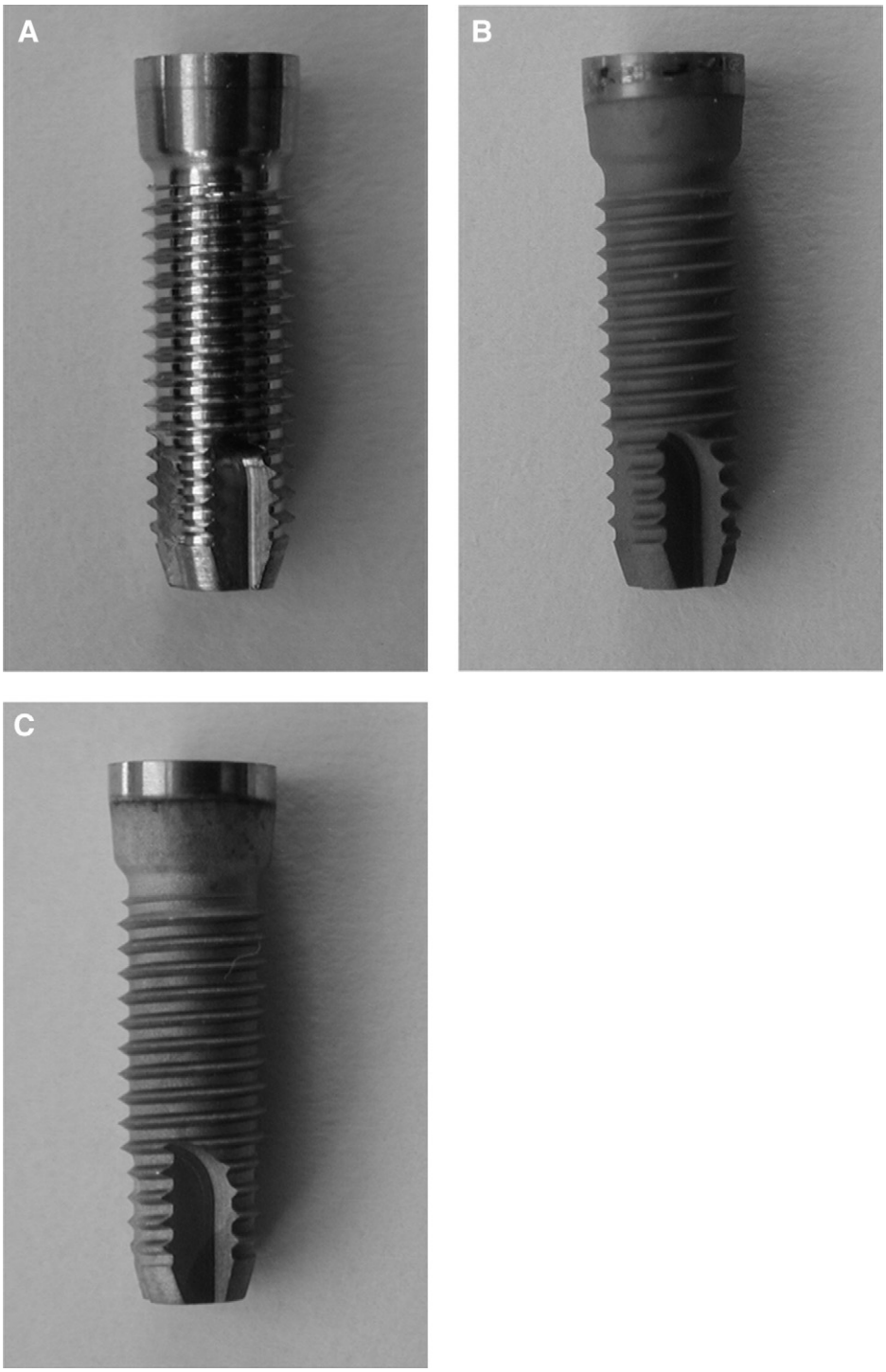

Fig. 1. Magnified pictures of in vivo–tested Ti grade 2 (ISO-5832/2) dental implants: **A**, machined Ti implant; **B**, BioRough-treated implant; and **C**, AAT-treated implant. BioRough and AAT implants were treated only on the threads of the distal part of the fixture, and the implant neck was left machined.

days), the samples were transfered to a new tissue culture plate, the culture medium was replaced with 1 mL 10% v/v Alamar Blue (Serotec; Prodotti Gianni, Milan, Italy) solution in culture medium, and the plate was incubated for 4 hours. One hundred–microliters/well Alamar Blue solution (3 replicates for each sam-

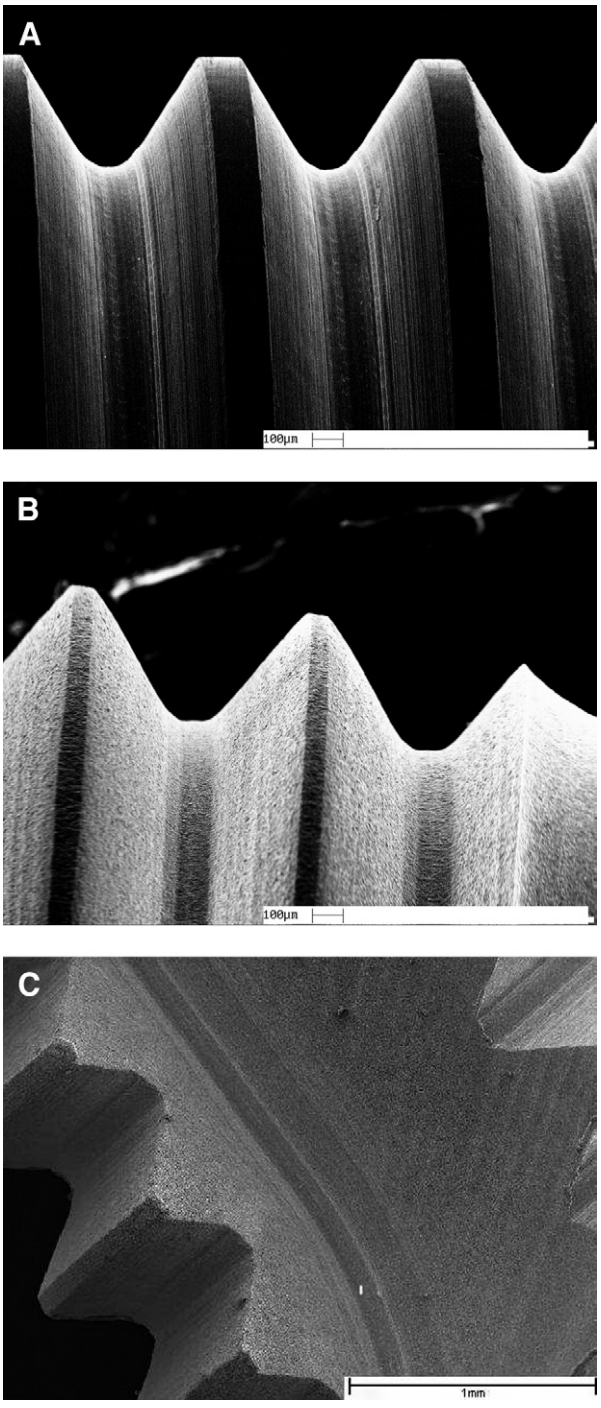

Fig. 2. Low-magnification secondary electron emission micrographs of: **A**, Ti implant surface; **B**, BioRough implant surface; and **C**, AAT implant surface. Geometry and shape of the implant were not modified by AAT and BioRough treatments, and the thread sharpness originated by the machining process was not significantly affected by the surface treatments.

ple) was removed and transfered to a new tissue culture plate, and the absorbance was measured using a spectrophotometric plate reader (Tecan Genius Plus; Tecan Italia) at a wavelength of 570 nm (reference wavelength 630 nm). The samples, rinsed with PBS and flooded with 1 mL culture medium, were returned to the incubator.

In vivo study

The study was performed according to European and Italian Law on animal experimentation, as stated in the "Guide for the Care and Use of Laboratory Animals." The animal experimental protocol was received from and approved by the Ethical Committee of Rizzoli Orthopedic Institute–Bologna and the Ministry of Health (Italy).

Implants in sheep trabecular bone. Twelve crossbred (Bergamasca-Massese) sheep, 3.0 ± 0.5 years old and 70 ± 5 kg, were subjected to bilateral fixture implantation in the femoral condyles. Animals were premedicated with an IM injection of 10 mg/kg ketamine and 0.3 mg/kg xylazine, and an SC injection of 0.0125 mg/kg atropine sulphate. General anesthesia was induced with 10 mg/kg IV sodium thiopentone (2.5% solution) and maintained with 60%/40% O_2/N_2O and 1.5%-2% fluothane. The lateral surface of the femoral condyle was exposed and 3 3.9-mm-diameter holes were drilled at low speed under sterile 0.9% NaCl. The holes were then flushed and cooled with sterile 0.9% NaCl to remove bone debris, and the fixtures were randomly placed and tightened (one fixture for each surface treatment for each femoral condyle). Postoperatively, antibiotics and analgesics (1 g/day cephalosporin for 5 days and 500 mg/day ketoprofen for 3 days) were administered.

Four animals were pharmacologically killed at 4, 8, and 12 weeks after surgery, and the femurs were excised and cleaned of soft tissue. Cubic bone segments, each one containing an implant, were obtained from the femoral condyles using an Exakt B System 300 CL (Exakt Apparatus, Norderstedt, Germany). Bone segments were fixed in 4% paraformaldehyde, and blind histomorphometric evaluation was performed.

Histomorphometry. Bone segments were dehydrated in a series of ethanol solutions and embedded in polymethylmethacrylate. Three bone-implant sections of 60 µm thickness were obtained parallel to the long axis of the fixture by means of the Leica SP 1600 diamond saw microtome (Leica, Milano, Italy) cutting system and were then further ground to 20 ± 5 µm with the Exakt 400CS Micro Grinding System (Exakt Apparatus). Two sections for each fixture were stained with basic fuchsin and fast green. Histomorphometric analyses were performed using an image analyzer system (Qwin;

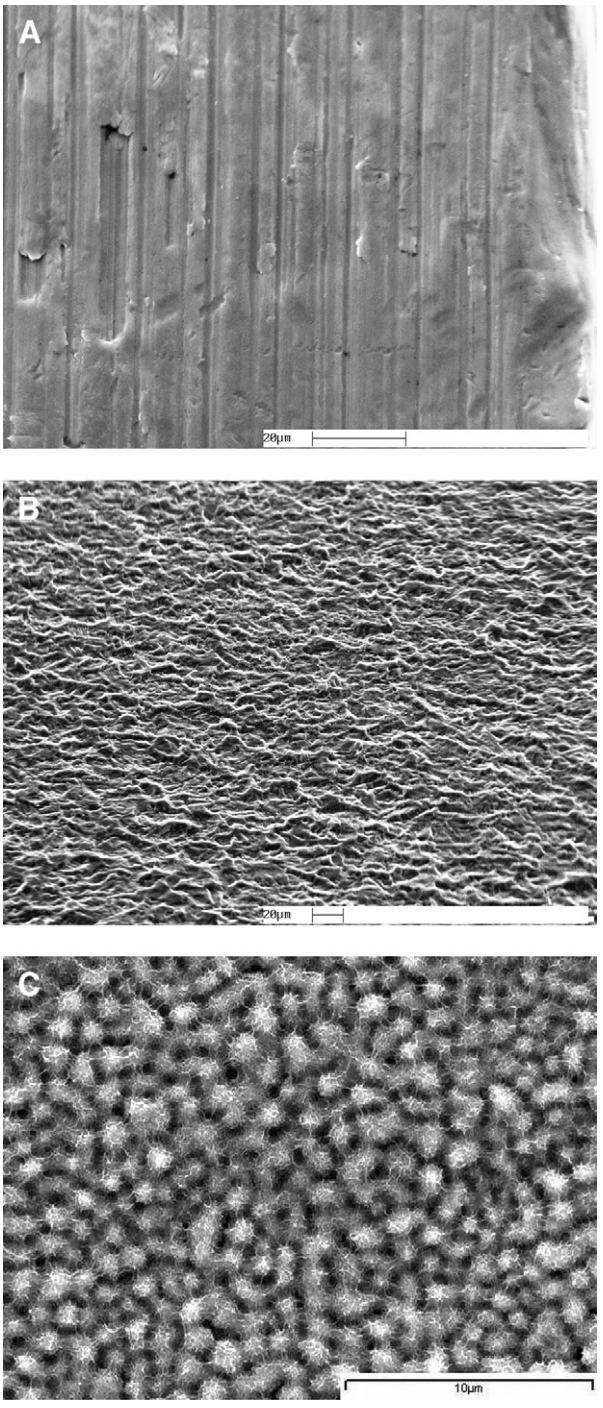

Fig. 3. High-magnification secondary electron emission micrographs of: **A**, Ti implant surface; **B**, BioRough implant surface; and **C**, AAT implant surface.

Leica Microsystems SpA, Milan, Italy) connected to an optical microscope (Olympus BX41, Olympus Italia Srl, Segrate, Italy). Each measurement was taken semiautomatically at an original magnification of 4×. The following histomorphometric parameters were measured bilaterally to the implants in a 2000 µm long and 1400 µm wide reference area calculated from the base of the threads starting from the endosteal side.

The histomorphometric parameters measured were the following:

Affinity index (AI, %): the interface contact between bone and implant calculated on the 3 consecutive best threads and considered as the length of the bone profile directly opposite the implant and beyond the length of the bone-implant interface[15-17]

Bone ingrowth (BI, %): the amount of bone growth inside the threads measured in an area located between the bottom and the top of the threads and expressed as a percentage

Bone mirror area (BMA, %): the amount of bone in mirror-image area outfolded from the thread, expressed as a percentage[18]

Statistical analysis

Statistical analysis was performed using SPSS v.12.1 software (SPSS Inc., Chicago, Illinois). After having verified the normal distribution and the homogeneity of the variance, 2-way (surface treatments and experimental times) analysis of variance (ANOVA) was used to assess significant difference among selected factors and histomorphometric data. In the case of roughness results and if no interaction was found, a 1-way ANOVA for each selected factor (if factor effect was significant), followed by Scheffé multiple comparison test was done to assess for the presence of significant differences. Data are reported as mean ± SD at a significance level of $P < .05$.

RESULTS

Physicochemical and morphologic properties

The Ti fixtures appeared as a shiny silver color as expected from untreated clean machined titanium specimens (Fig. 1, *A*). The BioRough surface (Fig. 1, *B*) exhibited a light silver color, less shiny than the smooth machined surface because of light refraction due to the roughness induced by the acid etching. The AAT (Fig. 1, *C*) showed a dark gray color.

The SEM images of the implant surfaces did not exhibit any macroscopic defects; fixtures appeared well machined, and neither chips nor overlapping of ridges resulting from the machining process were observed (Fig. 2). No appreciable differences could be observed between the textures achieved on plates and fixtures for each of the 2 surface treatments at higher magnification (Fig. 3, *A*). The BioRough texture was characterized by high and sharp edges, with deep valleys (Fig. 3, *B*),

Table I. Surface roughness for the 3 different surface treatments (mean ± SD; n = 5)

	Titanium	*BioRough*	*AAT*	*ANOVA*
R_a (m)	0.47 ± 0.01*,†	1.07 ± 0.09‡	0.29 ± 0.03	$p < .0005$
R_{max} (m)	3.54 ± 0.10*,†	8.78 ± 1.38‡	2.24 ± 0.41	$p < .0005$
R_t (m)	3.69 ± 0.21*,†	9.17 ± 0.93‡	2.31 ± 0.36	$p < .0005$
Sk	−0.13 ± 0.18	0.07 ± 0.27	0.07 ± 0.21	*ns*
K	3.06 ± 0.17	3.61 ± 0.74	3.12 ± 0.36	*ns*

Scheffé multiple comparison test ($P < .001$): * Ti versus BioRough; † Ti versus AAT; ‡ BioRough versus AAT.

whereas the AAT texture showed micropores and an overlapped nanometric net of filaments (Fig. 3, *C*).

Qualitative EDS analysis indicated that the cleaning procedure was effective in preventing machining oil contaminants from spoiling the surfaces. The smooth Ti and BioRough fixtures exposed an external layer of pure Ti, whereas the anodically-treated AAT surface exhibited high oxygen content enriched by Ca and P.

The AAT surface exhibited the lowest average roughness, R_a, whereas BioRough showed the highest values (Table I). The K and S_k parameters did not show any important differences in the peak profiles and valley distributions of treated and untreated surfaces. The R_{max} and R_t parameters followed the trend of R_a, with the BioRough surface exhibiting the highest values.

No crystalline oxides or other crystalline structures, apart from those typical of Ti, were detected by TF-XRD analysis on smooth Ti (Fig. 4, *A*) and the BioRough (Fig. 4, *B*) surface, whereas the AAT surface exhibited both Ti and TiO_2. The AAT surface was covered by a layer of crystalline anatase TiO_2 with traces of rutile (Fig. 4, *C*).

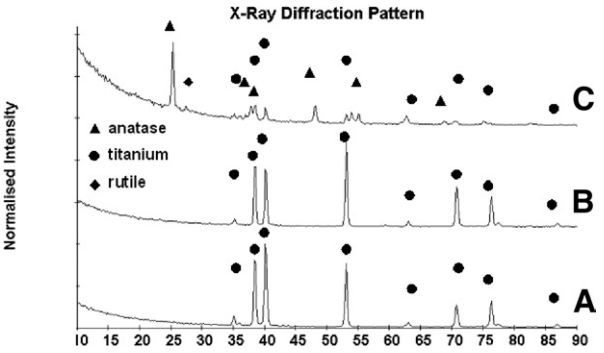

Fig. 4. Thin-film x-ray diffractometry of: **A**, Ti implant surface; **B**, BioRough implant surface; and **C**, AAT implant surface.

In vitro biologic response

In vitro cytocompatibility. After incubation with eluted media, cell viability slowly decreased from 1 to 7 days for all the tested materials, then showing an increasing trend from 14 to 21 days with values comparable with the TCP with the exception of day 7, where TCP resulted in the highest value ($P < .05$). In general, no remarkable differences in viability were observed among smooth Ti, BioRough, and AAT elution extracts at all the time points selected (Fig. 5).

A good cellular adhesion was observed on all tested surfaces 48 hours after seeding, with cells showing a mature, cuboidal, flattened shape and a high spreading level (Fig. 6). Compared with smooth Ti (Fig. 7, *A*) and BioRough (Fig. 7, *B*) samples, cells on AAT (Fig. 7, *C*) achieved an intimate bonding with the surface, characterized by high focal points with fewer and shorter filopodia. Cells cultured on BioRough (Fig. 7, *B*) were visibly anchored to the valley edges with their filopodia.

Cell proliferation showed values increasing with time, comparable from 1 to 3 days with the TCP for all the samples. On days 7 and 14, higher values were collected for cells cultured on AAT compared with TCP and in particular with smooth Ti and BioRough ($P < .05$), which showed lower values in the same time range. On day 21, the values collected for AAT were higher than TCP as well as smooth Ti and BioRough ($P < .05$), which showed decreasing values compared with the previous time point.

In vivo study

Histomorphometric results of implants in sheep trabecular bone. All animals tolerated surgery well and survived the postsurgical period without any infective complications. When dissecting the femurs, the fixture implants were checked, and neither macroscopic malpositioning nor signs of infection were observed.

The histologic findings demonstrated that all samples were correctly implanted in the trabecular bone of femoral condyles, and neither inflammatory cell infiltrate nor signs of infection were observed (Fig. 8). The best histomorphometric results in terms of AI and BI were found for AAT implants. The ANOVA test highlighted significant increases in AI results for AAT and BioRough implants compared with smooth Ti implants at each experimental time (Table II). The AI results of

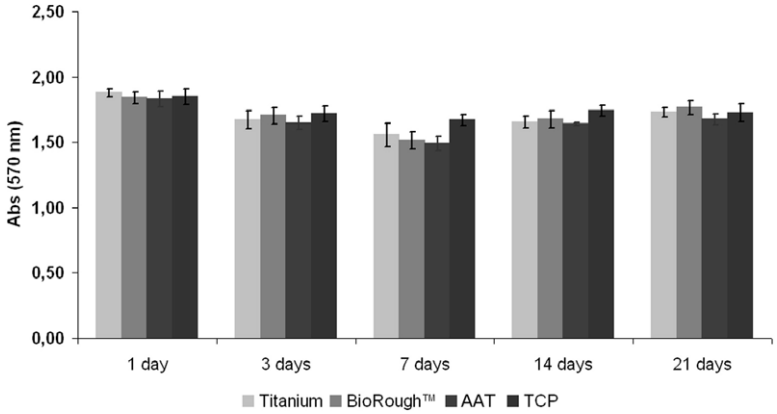

Fig. 5. MTT assay. Response of MG63 osteoblast-like cells cultured with media previously kept in contact with tested plate materials at different time points (1, 3, 7, 14, and 21 days). The effect of material ion release in the media was assessed by this method.

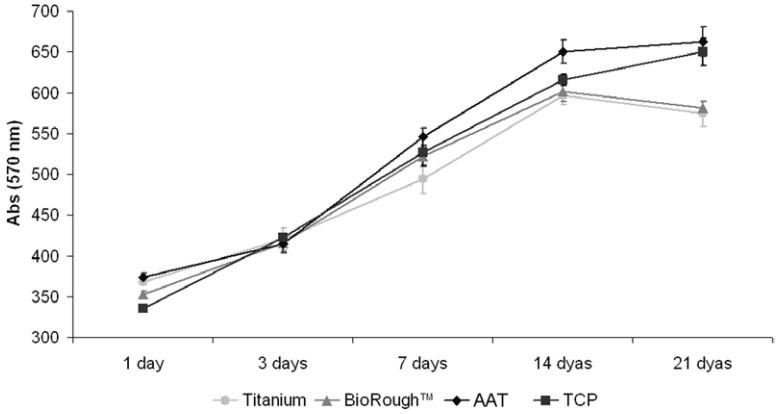

Fig. 6. Alamar blue test. Response of MG63 osteoblast-like cells to tested plate materials at different time points (1, 3, 7, 14, and 21 days). Cell behavior while cultured directly in contact with tested surfaces was assessed by this method.

AAT resulted also to be significantly higher that those of BioRough implants, and at 8 weeks the difference achieved was highly significant at about 110% ($P < .0005$). Significant increases in BI were seen between 8 and 4 weeks of implantation ($P < .001$) and between 12 and 4 weeks of implantation ($P < .005$), independent of surface treatments (Table II). When BI results were analyzed independently from experimental time, the 1-way ANOVA showed a significant increase in BI results for AAT implants compared with smooth Ti implants ($P < .05$). The comparison of bone area inside a thread (BI) with the area in the mirror-image of the same thread (BMA) showed less bone inside the threads than outside for all tested surface treatments. The BMA results of AAT implants were significantly higher than that of smooth Ti implants (16%; $P < .001$), independent of experimental times. The BI/BMA ratio, always less than 1, increased for all tested surface treatment between 4 and 8 weeks ($P < .01$), persisting almost unchanged between 8 and 12 weeks, independent of surface treatments.

DISCUSSION

The present findings on the new biomimetic AAT surface treatment extended previous data on physicochemical and morphologic properties, and the in vitro behavior of Ti, BioRough, and AAT were also reported in other papers.[8-10] Fixture and plate surfaces presented the same characteristics and properties, thus confirming the applicability of AAT treatment to differently shaped implants and prostheses. The smooth Ti surface and the finely roughened BioRough surface, both clean and decontaminated, were found to support cell adhesion and viability without enhancing proliferation.

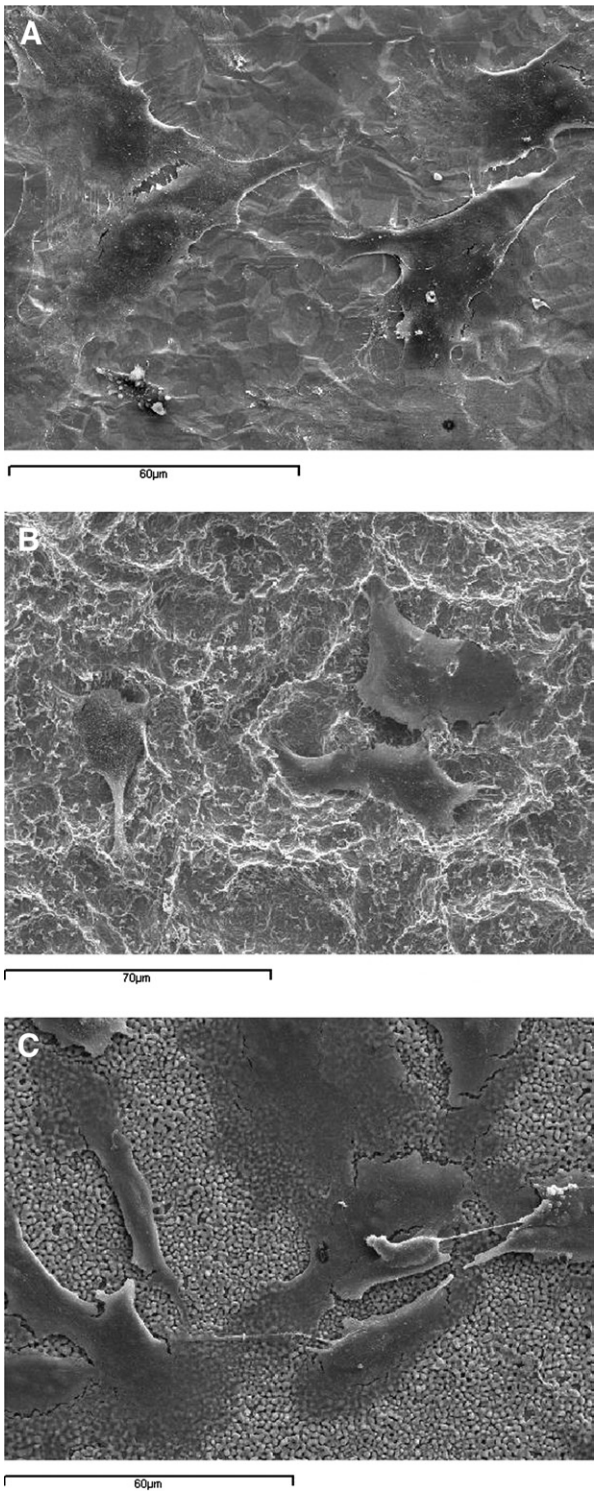

Fig. 7. Low-magnification secondary electron micrographs of MG63 osteoblast-like cells cultured for 48 hours on: **A**, Ti plates; **B**, BioRough plates; and **C**, AAT plates.

In contrast, the AAT surface exhibited peculiar properties: a bioactive microporous and nanotexture Ca and P–enriched thick TiO_2 layer, with a predominant crystalline anatase structure, whose bioactivity and catalytic properties are well known.[19] Actually, the stable and small crystalline anatase form of the TiO_2 has been recently considered for future clinical application, even though the rutile crystalline form is the most common and well characterized.[20,21] The catalytic properties of anatase play an important role in in vitro hydroxyapatite (HA) nucleation[19] and are associated with high in vivo osseointegration performances.[22,23] Because the osseointegration of Ti takes place through a process that involves the chemical modification of the outer oxide layer of titanium,[7] and the AAT treatment enhances the amount of Ca and P, this doping process may become the basis for the intimate bonding formation between bulk Ti and the bone tissue.

Regarding in vitro experiments, MG63 response indicated that all tested materials were a suitable environment for cellular adhesion and proliferation with comparable values until day 14. However, the highest values collected for AAT treated surface starting from day 14 showed features able to enhance cell proliferative activity, a phenomenon that occurs by changing surface roughness, as already observed.[24] Elution tests showed a slow decrease in cell viability from day 1 to 7 for all the tested samples except for the control, thus suggesting a release of leachables, probably due to material processing before cell seeding, which did not have a negative effect on cellular viability, as confirmed by the results and the following trend to day 21.

After 2 days of cell culturing on material sample surfaces, BioRough showed cells anchored on the valley edges covering the whole area without tightly adhering to it, resulting in a stretched but less flattened morphology than smooth Ti. Cells cultured on AAT showed a more advanced adhesion phase, with cells tightly adherent on the surface. This phenomenon could be due not only to the nanoroughened surface which offers an appropriately dimensioned nanopattern for cell mechanical attachment but also to the presence of a high amount of fibronectin, a well known vector for osteoblast adhesion, adsorbed on the surface.[10,25,26]

Previous evaluations of cell differentiation induced in osteoblast-like cells MG63 by AAT, showed an intrinsic osteogenic activity of the obtained nanostructured anatase TiO_2 coating, bringing about increases in the production of alkaline phosphatase and collagen type I synthesis.[10] These findings, together with the high Ca/P ratio typical of newly deposited mineral phase and the preferential adsorption of fibronectin observed on AAT, suggest the possibility of an improvement in osseointegration

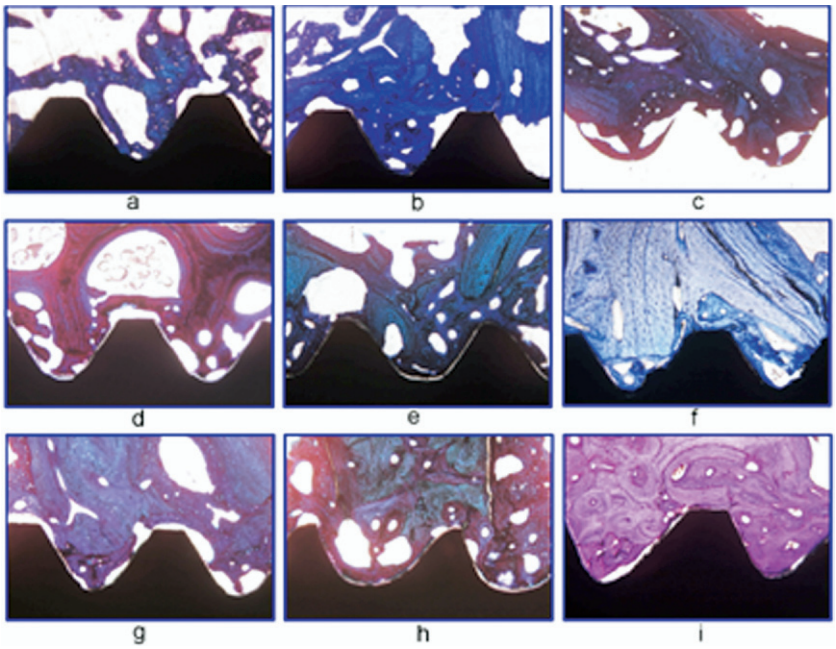

Fig. 8. Histologies of new bone in-growth on implant surface after 4 (**A, B, C**), 8 (**D, E, F**), and 12 (**G, H, I**) weeks in vivo of smooth Ti implant (**A, D, G**), BioRough implant (**B, E, H**), and AAT implant (**C, F, I**). At 4 and 8 weeks there is a lot of immature bone in the interface, reflecting ongoing bone regeneration, whereas the state after 12 weeks reflects a remodeling of tissue in good bone contact at the resolution level of the light microscopy (10×; scale bar = 100 μm).

osteogenic process performed by osteoblastic cells once in vivo. Actually, fibronectin, an extracellular matrix protein with receptor sites for the cell membrane integrins,[25,26] is known to bind to different substrates with different conformations through charged groups at the surface, and thus to be involved in the initial cell attachment and spreading by cell reorganization of actin microfilaments.[20]

Histologic observations highlighted that a direct bony contact was established with all tested surface treatments 8 weeks after surgery. The experimental times and implant sites were selected on the basis of previous studies, also taking into account that the biologic bone response to implants depends on the material properties and the trauma of surgery.[27-30] In particular, the forces acting on condylar trabecular sites, which are a combination of shear and compression forces, have been demonstrated to have a beneficial effect on the remodeling process of the bone-implant interface.[31] The implant site has in fact been reported to greatly affect the rate of both osseointegration and biodegradation of the ceramic surface treatments, with higher material degradation in trabecular than in cortical bone.[32]

From a histomorphometric point of view, AAT treatment was shown to positively affect the metabolism and healing capability of the surrounding bone at all experimental times. The AAT treatment improved the osseointegration at 4 weeks after surgery and achieved the greatest osseointegration at 8 weeks. The decrease of AI and BI values observed at 12 weeks seems to be related to the bone remodeling process around implants. In fact, the decrease of BI/BMA ratio showed exactly this phenomenon in the absence of evident adverse reactions to the implants as confirmed by the positive results in terms of AI and BI. The comparison of the obtained histomorphometric results of the AAT implants with those previously obtained from the in vitro experiments[10] further confirmed the direct relationship existing between low surface roughness, high synthesis of collagen type I, and high level of AI.[33] Finally, the BI/BMA results of AAT and BioRough implants, equal or higher than those of smooth Ti implants, indicated that no adverse effects due to implant material occurred. Nevertheless, the progressive increase in BI/BMA values of Ti implants from 4 to 12 weeks, even if less than 1, indicated a physiologic improvement of bone turnover.

CONCLUSION

The in vitro and in vivo tests of the present study indicated that all 3 surfaces analyzed could be suitable for endosseous implants. Simple mechanical finishing of the Ti fixture was successfully used and applied over

Table II. Histomorphometric results for type of implants at 4, 8, and 12 weeks (mean ± SD; n = 4)

			Surface treatments		
Time	*Parameter*	*Unit*	*Ti*	*AAT**	*BioRough‡*
4 weeks†	AI	%	35.5 ± 8.6	68.6 ± 7.4[a,c]	56.8 ± 13.7[b]
	BI	%	56.1 ± 7.3	70.7 ± 11.4	66.2 ± 11.5
	BMA	%	83.6 ± 3.5	94.9 ± 1.9	90.1 ± 7.4
	BI/BMA		0.67 ± 0.08	0.75 ± 0.12	0.77 ± 0.10
8 weeks	AI	%	29.8 ± 7.5	88.3 ± 9.6[d]	41.9 ± 7.0[e]
	BI	%	76.9 ± 6.9	90.0 ± 9.1	80.6 ± 12.7
	BMA	%	88.2 ± 7.0	96.8 ± 4.48	93.2 ± 6.7
	BI/BMA		0.88 ± 0.14	0.93 ± 0.07	0.87 ± 0.20
12 weeks	AI	%	37.7 ± 5.7	70.0 ± 9.5[f,h]	52.1 ± 8.3[g]
	BI	%	77.0 ± 8.7	86.2 ± 10.9	78.4 ± 10.6
	BMA	%	89.4 ± 5.6	96.9 ± 3.7	91.4 ± 5.5
	BI/BMA		0.86 ± 0.11	0.89 ± 0.09	0.86 ± 0.10

AI, affinity index; *BI*, bone ingrowth; *BMA*, bone mirror area.
Univariate ANOVA test for AI results between tested surface treatments for each experimental times:
4 weeks: [a]AAT versus Ti ($P < .0005$); [b] BioRough versus Ti ($p < 0.001$); [c] AAT versus BioRough ($P < .05$)
8 weeks: [d]AAT versus Ti ($P < .0005$) and BioRough ($P < .0005$); [e] BioRough versus Ti ($P < .05$)
12 weeks: [f]AAT versus Ti ($P < .0005$); [g] BioRough versus Ti ($P < .05$); [h] AAT versus BioRough ($P < .05$).
One-way ANOVA test for BI: *AAT versus Ti ($P < .05$); † 4 weeks versus 8 ($P < .001$) and 12 weeks ($P < .005$); BMA: ‡ AAT versus Ti ($P < .001$); BI/BMA: not significant.

a long time, providing good results and reliability in many different implant designs. Chemical-etched surfaces, such as BioRough, can enhance the surface morphology, bringing the roughness to optimal values[3,4] without introducing any chemical modification of the titanium surface. BioRough treatment was able to effectively decontaminate the implant surface, from contaminants such as organic oils used in the machining process, by dissolving a few micrometers of Ti at the surface. In addition, the BioRough etching treatment was found to introduce no significant changes on the sharpness of the thread ridge and implant cutting edges. The AAT treatment, however, exhibited the most promising results both in vitro and in vivo and proved to have the potential of substantial improvements in achieving fast and stable osseointegration of endosseous implants.

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Reprint requests:

Prof. Roberto Chiesa
Dipartimento di Chimica, Materiali e Ingegneria Chimica "G. Natta"
Politecnico di Milano
Via Mancinelli 7
20131 Milano
Italy
roberto.chiesa@polimi.it