Title: Proteome analysis of human serum proteins adsorbed onto different titanium surfaces used in dental implants

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Abstract

Titanium dental implants are commonly used due to their biocompatibility and biochemical properties; blasted acid-etched Ti is used more frequently than smooth Ti surfaces. In this study, physicochemical characterisation revealed important differences in roughness, chemical composition and hydrophilicity, but no differences were found in cellular *in vitro* studies (proliferation and mineralization). On the other hand, the deposition of proteins onto the implant surface might affect *in vivo* osseointegration. To test that hypothesis, protein layers formed on both surface type discs after incubation with human serum were analysed. Using mass spectrometry (LC/MS/MS), 139 proteins were identified, 31 of which were associated with bone metabolism. Interestingly, Apo E, antithrombin and protein C adsorbed mostly onto blasted and acid-etched Ti, whereas the proteins of the complement system (C3) were found predominantly on smooth Ti surfaces. These results suggest that physicochemical characteristics could be responsible for the differences observed in the adsorbed protein layer.

Keywords: titanium, surface properties, human serum, apolipoprotein E, bone regeneration, proteomics

Introduction

Titanium dental implants are commonly used due to their biocompatibility and biochemical properties (Lemons & Lucas 1986; Smith 1993; Nakajima & Okabe 1996). Blood plasma is the main biological fluid interacting with these implants (Park & Davies 2000). The first event that takes place at the biomaterial–tissue interface is the interaction of water molecules and salt ions with the surface of the implant. Shortly after the formation of a hydration layer, a variety of blood proteins adsorbs onto implant surfaces. This occurs within seconds or minutes after implantation (Puleo 1999; MacDonald et al. 2002). The resulting protein film mediates all subsequent biological interactions between the material and the surrounding environment; the cells are unlikely ever to interact directly with the native material surfaces. The concentration, composition and conformation of the protein layer on a biomaterial surface may vary. These characteristics of the protein layer are important for synergistic interactions promoting either favourable or adverse cellular and tissue responses, such as attachment to material surfaces, proliferation, and phenotypic changes (Molino et al. 2012; Fernández-Montes Moraleda et al. 2013).

Rough and blasted acid-etched Ti have replaced smooth Ti after reports of a positive correlation between surface roughness and bone integration (Wennerberg & Albrektsson 2010). Moreover, rough Ti surfaces adsorb more proteins than smooth Ti due to the increased surface area (Sela et al. 2007; Rockwell et al. 2012).

Protein adsorption is a dynamic process involving non-covalent interactions such as hydrophobic interactions, electrostatic forces, hydrogen bonding and Van der Waals forces (Andrade & Hlady 1987). The non-covalent interactions are controlled by many protein parameters, such as protein size, pI and secondary and tertiary structures (Haynes & Norde 1994; Rabe et al. 2011). The specific physicochemical properties of the biomaterial surface, such as its chemistry, wettability, charge and surface morphology, also affect the protein adsorption process (Schmidt, D.R., Waldeck, H., Kao 2009).

For these reasons, the researchers have been focusing on the elucidation of the mechanisms governing protein interactions with various biomaterials including polymers, metals and ceramics (Wehmeyer et al. 2010). A number of surface-sensitive techniques have been used for the quantification of protein adsorption: surface plasmon resonance, optical waveguide lightmode spectroscopy, ellipsometry, quartz crystal microbalance with dissipation and total internal reflection fluorescence spectroscopy (Malmström et al. 2007).

Many studies evaluating the kinetics of protein adsorption onto Ti have been focused on the exposure of Ti to single protein solutions or protein mixtures (Sousa et al. 2008; Imamura et al. 2008; Pei et al. 2011; Pegueroles et al. 2012; Kohavi et al. 2013). However, the protein adsorption process is a complex phenomenon depending on many parameters, some of which

are not considered in these studies. For instance, in the multi-protein systems such as blood plasma/serum, increasing the protein concentration or/and the number of small molecules improves their diffusion and accelerates the displacement; thus, they are the first to be adsorbed onto the surface. With time, molecules with greater affinity for the surface but slower rate of diffusion (due to their low concentration or large size) replace the smaller molecules. This is known as the Vroman effect (Kay C Dee, David A. Puleo 2003; Wang et al. 2012).

A study using mass spectrometry techniques has identified fibronectin, albumin, fibrinogen, IgG and complement C3 adsorbed on a modified Ti surface incubated in human plasma for 24 h (Sela et al. 2007). The same study has shown that the adsorption of plasma proteins depends on the roughness of the surface. Recently, label-free quantitative proteomics has been used in a study of the composition and function of adsorbed protein layers (Montoya et al. 2011). Dodo et al. have characterized the proteome of the protein layer adsorbed onto a rough Ti surface, after exposure to human blood plasma. The study has shown that the layer adsorbed on this surface is composed mainly of proteins associated with cell adhesion, molecular transportation and coagulation processes. This layer creates a polar and hydrophilic interface for subsequent interactions with host cells (Dodo et al. 2013).

At present, biological evaluation of medical devices includes a battery of standardized tests, as defined in ISO 10993, highly accepted in the biomaterials research field. Typical tests for biocompatibility of biomaterials involve cytotoxicity, cells attachment, cells proliferation and mineralization assays. However, a lack of correlation between *in vitro* and *in vivo* results is observed in many occasions. Since the first step before cells attachment on the materials surface is protein adsorption, we propose using proteomic to progress in knowledge of materials biocompatibility.

Thus the aim of our study was to compare the protein layers adsorbed onto two types of Ti surfaces, smooth Ti and blasted acid-etched Ti, after incubation in the serum. To achieve this goal, we employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Furthermore, a comparison of the more relevant results with cells *in vitro* tests outcomes was performed.

Therefore, this article shows our interest in testing surfaces, such as those currently used in commercial dental implants, in order to establish a correlation between protein deposition and *in vitro* outcomes.

Materials and methods

Surface disc preparation

Ti discs (12 mm in diameter, 1-mm thick) were fabricated from a bar of commercially available, pure, grade-4 Ti (Ilerimplant SL). Some of the discs sandblasted-acid-etched (SAE) Ti were abraded with 4-μm aluminium oxide particles and acid-etched by submersion in sulfuric acid for 1 h to obtain a moderately rough implant surface. All discs were then washed in acetone, ethanol and 18.2Ω purified water (for 20 min in each liquid) in an ultrasonic bath and dried under vacuum. Finally, all Ti discs were sterilised using UV radiation.

Physicochemical characterisation of titanium discs

The surface topography of titanium discs was characterized using atomic force microscopy (AFM, Newport Multimode) under dry conditions. Images were taken at different amplitudes. Measurements at scan size of 60 and 1 μm, with a scan rate of 1 and 0.3 Hz, respectively, were carried out (n = 3). The results were analysed using the NanoScope Analysis software. The scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX, Leica-Zeiss LEO) was used to study these surfaces under vacuum. Platinum sputtering was employed to make the samples more conductive for the SEM examination. SEM micrographs were analysed by image processing with Image J programme.

The roughness of the samples was determined using a mechanical Dektak 6M profilometer (Veeco). Two samples of each material were tested, with three measurements for each sample to obtain the average values of the parameters R_a and R_t.

The wettability was evaluated by measuring the contact angle using an automatic contact-angle meter (DataPhysics OCA 20), after depositing 10 μL of ultrapure water W04 on the titanium surface at room temperature. The drops were formed at dosing rate of 27.5μL s^{-1} and the angles were determined using SCA 20 software. Five discs of each material were studied after depositing two drops on each sample.

Cell culture

MC3T3-E1 (mouse calvaria osteosarcoma cell line) cells were cultured in DMEM with phenol red (Gibco–Life Technologies, NY, USA), supplemented with 1% of (100×) penicillin/streptomycin (Biowest Inc., USA) and 10% of FBS (Gibco–Life Technologies) for the first 24 h. Then, the medium was replaced with differentiation medium: DMEM with phenol red (1×) containing 1% of penicillin/streptomycin, 1% of ascorbic acid (5 mg mL^{-1}) and 0.21% of β-glycerol phosphate.

Cells were cultured (at a concentration of 1×10^4 cells/well) with the titanium discs in 24-well culture plates (Thermo Scientific®) at 37 ºC in a humidified (95%) atmosphere of 5% CO_2.

Titanium discs were not exposed to blood serum before cell culture. The culture medium was changed every forty-eight hours. In each plate, wells with the same concentration of cells, but no Ti discs, were used as a control of culture conditions.

Cell proliferation

For measuring cell proliferation, the commercial cell viability assay alamarBlue® (Invitrogen) was used. This kit measures the cell viability based on a redox reaction with resazurin. The cells were cultured in wells with the discs (3 replicates per treatment) and examined following the manufacturer's protocol after 24 h, 72 h and 120 h. The percentage of reduced resazurin was used to evaluate cell proliferation.

ALP activity

ALP activity was assayed by measuring the conversion from p-nitrophenyl phosphate (p-NPP) to p-nitrophenol, and the specific activity of the enzyme was calculated.

Aliquots (0.1 mL) of the solution used for measuring the protein content were assayed for ALP activity. To each aliquot, 100 µL of p-NPP (1 mg mL^{-1}) in substrate buffer (50 mM glycine, 1 mM $MgCl_2$, pH 10.5) was added. After two hours of incubation in the dark (37 ºC, 5% CO_2), absorbance was spectrophotometrically measured at 405 nm using a microplate reader. ALP activity was acquired from a standard curve obtained using various concentrations of p-nitrophenol in 0.02 mM sodium hydroxide. Results were calculated in mmols of p-nitrophenol/h (mM PNP h^{-1}), and data were expressed as ALP activity normalized to the total protein content after 14 and 21 days.

Statistical analysis

Data were submitted for analysis of variance (ANOVA) and a Newman–Keuls multiple comparison test, when appropriate. Differences at $p \leq 0.05$ were considered statistically significant.

Total protein

Total protein content was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) for colorimetric protein quantitation based on copper reduction. The culture medium was removed from the wells, the wells were washed 3 times with 1 × DPBS, and 100 µL of lysis buffer (0. 2% Triton X-100, 10 mM Tris-HCl pH 7.2) were added to each. After 10 min, the lysate was sonicated and centrifuged for 7 min at 13300 rpm and 4 ºC. 20 µL of the supernatant were used for colorimetric measurement of BCA at 570 nm on a microplate reader Multiskan FC® (Thermo Scientific®). Total protein content was calculated from a standard curve

for bovine albumin and expressed as μg μL^{-1}. These data were used to normalize the alkaline phosphatase (ALP) activity after 14 and 21 days.

Formation of the protein layer

Each disc (12 mm Ø) was incubated in a well of a 24-well plate (Thermo Scientific®) with 2 mL of human blood serum from male AB plasma (Sigma®) for 180 min (37 ºC, 5% CO_2). The use of blood serum tries to deplete some very high abundant proteins, such as fibrin-related proteins. Then, the serum was removed, and the discs were put through five consecutive washes with 200μL of double-distilled water and a final wash with 100 mM NaCl in 50 mM Tris-HCl, at pH 7.1, to remove unadsorbed proteins. The final eluate was obtained by submerging the discs in a solution containing 4% SDS, 100 mM DTT and 0.5 M TEAB. This method was based on previous studies (Kaneko et al. 2011). Three elutions were performed for each surface treatment; each eluate was obtained from 4 distinct discs. Total protein of the serum was quantified before the assay, using the method described above (Pierce™ BCA Protein Assay Kit), yielding a concentration of 51 mg mL^{-1}.

Proteomic analysis

Eluted protein sample was resolved in 10% polyacrylamide gels, using a Mini-Protean II electrophoresis cell (Bio-Rad). A constant voltage of 150 V was applied for 45 min. The gel was then stained using SYPRO Ruby stain (Bio-Rad) following the manufacturer's instructions. The gel was then washed, and each lane was cut into 4 slices. Each of these slices was digested with trypsin following a standard protocol (Anitua et al. 2015).

The resulting peptides were resuspended in 0.1% formic acid, separated using online NanoLC and analysed using electrospray tandem mass spectrometry. Peptide separation was performed on a nanoACQUITY UPLC system (Waters) connected to a SYNAPT G2-Si spectrometer (Waters). Samples were loaded onto a Symmetry 300 C18 UPLC Trap column (5 μm, 180 μm × 20 mm, Waters) connected to a BEH130 C18 column (1.7 μm, 75 μm × 200 mm, Waters). The column was equilibrated in 3% acetonitrile and 0.1% FA. Peptides were eluted at 300 nL/min using a 60-min linear gradient of 3%−50% acetonitrile.

A SYNAPT G2-Si ESI Q-Mobility-TOF spectrometer (Waters) equipped with an ion mobility chamber (T-Wave-IMS) for high definition data acquisition analyses was used for the analysis of the peptides. All analyses were performed using electrospray ionization (ESI) in a positive ion mode. Data were post-acquisition lock-mass corrected using the double charged monoisotopic ion of [Glu^1]-fibrinopeptide B. Accurate LC-MS data were collected in HDDA mode, which enhances signal intensities using the ion mobility separation. Searches were performed using Mascot search engine (Matrix Science) in Proteome Discoverer v.1.4 software (Thermo). Mascot generic files (MGF) files were generated from the original SYNAPT RAW

files using ProteinLynx Global Server 3.0.2 (PLGS, Waters) and further processed using the Proteome Discoverer. Peptide mass tolerance of 10 ppm and 0.2-Da fragment mass tolerance were used for the searches. Carbamidomethylation of cysteines was selected as the fixed modification and oxidation of methionine as a variable modification for tryptic peptides. Proteins identified with at least one peptide with an FDR < 1% were kept for further examination.

Progenesis LC-MS software (Nonlinear Dynamics) was used for differential protein expression analysis. Raw files were imported into the programme, and one of the samples was selected for a reference run to which the precursor masses in all the other samples were aligned. Abundance ratio between the run to be aligned and the reference run were calculated for all features at given retention times. These values were then logarithmized and the programme, based on the analysis of the distribution of all ratios, automatically calculated a global scaling factor. Once normalized, the samples were grouped into the appropriate experimental categories and compared. Differences between groups were only considered for peptide abundances with an ANOVA p-value < 0.05 and a ratio > 1.5 in either direction. A peak list containing the differing peptides was generated for each comparison and searched against a Swiss Prot database using the Mascot Search engine (www.matrixscience.com). Proteins with ANOVA $p < 0.05$ and a ratio higher than 1.3 in either direction were considered different.

Results

Physicochemical characterisation of Ti discs

Figure 1 shows SEM images of smooth Ti and sandblasted acid-etched Ti surfaces. The different topographies can be clearly seen. The particles on the titanium surface (Figure 1b) are visible in the image. EDX results demonstrated that these were alumina (Al_2O_3) particles that could have been encrusted in the material after the sandblasting process (Figure 2). The area of the disc covered by alumina particles reaches a 13.84% of the disc surface area. AFM images in Figure 3, with a scan size of 60 μm, were analysed and an increase on the surface area was detected after aluminium oxide blasting acid-etching treatment. The untreated discs showed a specific surface area of 0.69 ± 0.16%, while that of the blasted acid-etched discs was of 19.97 ± 1.40%.

Untreated titanium discs, with smoother topography (Figures 3a and 3c), showed series of grooves due to the machining process. The change in the topography of Ti after the surface blasting and acid-etching treatment is clearly visible in Figures 3b and 3d. Machining grooves disappeared as a result of sandblasting, and the roughness increased significantly ($p<0.05$) when the surface was marked by alumina powder. As it can be seen in Figures 3c and 3d, the blasting

and acid-etching resulted in larger irregularities, but the surface was smoother in comparison with the untreated Ti. This can be attributed to the acid-etching treatment.

The mechanical profilometer revealed that for the smooth Ti surface, R_a and R_t parameters were 0.14 ± 0.04 and 1.28 ± 0.40 μm, respectively. After blasting and acid-etching, R_a and R_t were 0.93 ± 0.06 and 8.38 ± 0.99 μm, respectively. Thus, the surface roughness of the treated discs was significantly higher than the roughness of the untreated samples.

Contact angle measurements were carried out to determine the wettability of the surface. Significantly ($p<0.05$) lower contact angles were observed for blasted acid-etched Ti surfaces than for the untreated discs, namely, 85.70 ± 2.83° and 94.53 ± 2.59°, respectively. Thus, the treated discs showed greater hydrophilicity.

In vitro cultures

Analysis of cell proliferation (Figure 4) clearly showed that disc treatment had no significant effect on the cellular growth. Cells proliferated equally on both types of discs during the 5-day protocol. A threefold increase in cell numbers was observed between 24 h and 3 days in culture. Proliferation slowed down between 3 and 5 days of incubation, showing a plateau and a reduction in proliferation.

ALP enzyme activity (Figure 5) was not affected by disks topography after 14 and 21 days (ANOVA, $p > 0.05$). Moreover, between these time points, there was a slight decrease in the ALP activity, as expected. These *in vitro* data indicate that the disc topographies examined in our study do not affect the metabolic and division processes of MC3T3-E1 cells, related to mineralization.

Proteomic analysis

Identification of proteins adsorbed onto the blasted acid-etched Ti and smooth Ti

The LC-MS/MS analysis of the protein layers adsorbed to both Ti surfaces resulted in the identification of 218 different proteins. The identified proteins are presented in Table 1. Serum proteins involved in cell adhesion and extracellular matrix, important for implant integration, were also found: vitronectin (Salasznyk et al. 2004; Kundu & Putnam 2006; Di Benedetto et al. 2015) and proteoglycan 4 (Novince et al. 2012). Intriguingly, we found cellular/cytoplasmic components of cell adhesion and cell junction adsorbed to the Ti surfaces, integrin alpha-V (Kumar 2003; Roux 2010; Kaneko et al. 2014), junction plakoglobin (D'Alimonte et al. 2013), gelsolin (Kwiatkowski et al. 1989; Thouverey et al. 2011; Kim et al. 2013) and actin cytoplasmic 1 (Sen et al. 2015). LCMS/MS analysis also revealed the cellular and secreted proteins associated with bone homeostasis, such as peptidyl-prolyl cis-trans isomerase B (Pyott et al. 2011) and lysozyme C (Siebert et al. 1978; Briggs & Arinzeh 2014). We also found serum

proteins involved in bone formation to a certain degree, serum paraoxonase/arylesterase 1 (Dowling et al. 2014), vitamin D binding protein (Benis & Schneider 1996; Swamy et al. 2001; Schneider G.B., Grecco K.J., Safadi F.F. 2003) and pigment epithelium-derived factor (Li et al. 2013; Li et al. 2015).

Gene ontology analysis of the identified proteins

The proteomic analysis led to the identification of 181 and 162 proteins on smooth Ti and blasted acid-etched surfaces, respectively. Adsorbed proteins were classified using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system (Figure 6). The results of protein classification according to *biological processes* were almost identical for the two types of surfaces (Figure 6a and 6b). However, classification of proteins according to the *pathways* in which they are involved revealed differences between the two types of surfaces (Figure 6c and 6d). Interestingly, we observed that smooth Ti-adsorbed proteins intervene in a wider range of pathways than those found on the blasted acid-etched Ti. Blood coagulation (43.35%), inflammation mediated by cytokines (17.34%) and integrin signalling (13.29%) were the three major process-classified protein categories found on the treated (blasted and acid-etched) Ti. For smooth Ti, blood coagulation (28.52%) and inflammation (11.91%) were the most significant categories. However, a major group of proteins related to glycolysis (11.91%) was adsorbed on smooth Ti, which is absent on sand-blasted acid-etched surfaces. Integrin signalling was only represented by a relatively minor proportion of proteins on the smooth Ti (4.69%) in comparison with the treated Ti surfaces. Proteins related to diseases such as Parkinson's and Alzheimer's and proteins related to CCKR signalling pathways were found on both disc types (a very small proportion). In addition to these categories, smooth Ti surfaces adsorbed a small percentage of proteins involved in apoptotic and plasminogen signalling pathways.

Specifically enriched proteins

To find the specifically enriched proteins adsorbed onto the two surface types that might reflect their different osteoinduction capabilities, a differential analysis was performed (in triplicate) using the Progenesis QI software. This method identified 9 proteins differentially enriched/associated with each surface (Table 2).

Proteins enriched on the blasted acid-etched Ti were apolipoproteins ApoA-I, ApoE, ApoA-IV, plectin, antithrombin III and Vitamin K-dependent protein. The largest difference between the two surface types was found for ApoA-IV and plectin. We also found that complement C3 and some immunoglobulins (Ig gamma and lambda chains) were significantly enriched on the smooth Ti but not on the blasted and acid-etched Ti discs.

Discussion

The main part of our study characterises the protein layer adsorbed onto titanium discs with two different surface types: a sandblasted, acid-etched Ti and an untreated, smooth Ti. It is reasonable to assume that the different surface characteristics will affect the adsorption of proteins.

Roughness is a key parameter in the assessment of the osseointegrative properties of material (Buser et al. 1991). The two surface types studied in this work have different topography, i.e. sand-blasted acid-etched Ti is rougher than the untreated Ti surface. These results are consistent with previous studies (Grassi et al. 2006). Moreover, the presence of alumina is also associated with a good bone response (Wennerberg et al. 1995) and a change in hydrophilicity affecting both chemical and physical composition of the surface. All these physicochemical features will affect the affinity of the protein layer formed on the material.

Ti surfaces are widely used in implants; techniques advancing the osteogenesis are needed to improve the quality of health care and patient recovery. The surface types described here have been extensively used in orthopaedic implants with overall similar outcomes (Schwartz et al. 2008).

Our *in vitro* experiments, using an osteosarcoma cell line, showed no differences between both samples either in proliferation or mineralization. Both surfaces showed very similar proliferation results evolution with time, increasing gradually in all the test period. Mineralization in cells, measured by ALP activity, an enzyme that becomes very active during osteoblast differentiation, decreased on both Ti surfaces with time, while differences founded had not statistical meaning. In similar studies, no significant differences in neither proliferation nor mineralization were found (Yoshida et al. 2012). These results are supported by the proteomic analysis of proteins adsorbed onto the different discs since the vast majority of proteins attach on a similar way to both surfaces. The extensive list of adsorbed proteins shows that at least 30 of these proteins are involved in bone homeostasis in a direct or indirect way (Table 1).

However, the blasted and acid-etched Ti surfaces and smooth Ti surfaces show different osteogenic properties in *in vivo* models (Wennerberg & Albrektsson 2009). Furthermore, Aparicio *et al*, showed that high R_a values favour osseointegration of the dental implant in comparison with smoother surfaces (Aparicio et al. 2011). This effect is attributed to the higher implant-bone contact interface as a consequence of greater roughness. Nevertheless, in this study we find also chemical differences between treatments. To test this premise we performed a detailed analysis of the proteins adsorbed to the two surface types to find statistically significant differences between them. In order to isolate and identify these surface adsorbed proteins, we established a protocol where, following serum incubation, discs were washed and

final proteins elution was obtained with an SDS-containing buffer. This approach permitted us to wash the surfaces thoroughly and to get a good protein yield for the characterization of the differences between both surfaces. The procedure shows that under the same regime of washes and the same elution strength a number of different proteins bind more consistently to any of the surfaces in a statistically significant way, revealing differences in the surface-protein interactions. Although other approaches cannot be discarded, our method has proved to be useful for our intended purpose. On the other hand, the use of harsher buffer could release proteins that might have remained attached after SDS wash, however, we believe that although the total list of proteins could increase, it should not affect the differential analysis. We get an average of 114 proteins identified in the smooth Ti surface discs, and 108 proteins in the blasted and acid-etched Ti surface. This suggests that the differences observed between surfaces arise from differential binding of certain proteins and not from the total amount of protein.

The proteomics differential quantification analysis performed by Progenesis found some significant differences for plectin, antithrombin-III and several other apolipoproteins. Plectin is a cytoskeleton protein that links intermediate filaments to other cytoskeletal systems and anchors them to the membrane junction sites. It binds mostly to vimentin and is very important for preserving the mechanical integrity of the tissue (Burgstaller et al. 2010). Plectin is not a typical serum protein; therefore, its presence in the protein layer formed by incubation of Ti discs with the serum was unexpected. Antithrombin (AT) is a glycoprotein that inactivates several enzymes of the coagulation system. Specifically, AT-III inactivates thrombin, which catalyses the formation of fibrin from fibrinogen. Fibrin architecture at the clot affects bone healing (Shiu et al., 2014). However, apolipoproteins are important serum proteins involved in lipid transport; different isoforms have different properties and activities. Apolipoprotein A-IV has antioxidant-like activity and is involved in the inhibition of lipid oxidation (Spaulding et al. 2006). It has been reported that patients with osteonecrosis, a skeletal pathology with intense bone degeneration, have lower levels of ApoA-IV in comparison with healthy individuals (Wu et al. 2008). Lipid metabolism and oxidative injury are important processes in the pathophysiology of the disease. Apo A-IV mutations are linked to corticosterone-induced osteonecrosis in patients with renal transplants (Hirata et al. 2007). In our study, Apo A-IV level was significantly higher on the blasted acid-etched Ti than on the smooth Ti. This observation might account for a favourable osseointegration environment created by the treated discs as the protein acts as an antioxidant. Another important apolipoprotein, ApoA1, was found adsorbed to the treated Ti in larger amounts than to smooth Ti. ApoA1 is the main component of the high-density cholesterol complex, but it has not been associated with bone formation or resorption. Interestingly, ApoE, which is involved in the regulation of bone metabolism, was also adsorbed to the blast acid-etched Ti in larger amounts than to smooth Ti. Although the data interpretation is still controversial, ApoE has been extensively reported to be involved in bone

homeostasis (Niemeier et al. 2012), possibly via promotion of vitamin K uptake into the osteoblasts (Newman et al. 2002). However, various ApoE alleles behave very differently in this process. ApoƐ2 is the allele with the lowest involvement in the transport of vitamin K (Saupe et al. 1993). The ApoƐ4 allele has been associated with a low bone mass in several studies in postmenopausal women (Shiraki et al. 1997; Sanada et al. 1998). More recently, epidemiological studies have confirmed that ApoƐ2 represents an increased risk for trabecular bone fracture (Dieckmann et al. 2013). The most frequent ApoE allele is ApoƐ3, found with a frequency of 79%. ApoƐ2 is present in approximately 7% of the population and ApoƐ4, in 14%. ApoƐ3 is also called the *neutral allele* because it is not associated with any of the human diseases. Apoe2 and 4 have been associated with increased probability of developing arthrosclerosis and Alzheimer's disease (Eisenberg et al. 2010).

The method that we used to characterise the protein layer on Ti surfaces did not allow the determination of the type of ApoE allele adsorbed. Moreover, is not clear whether physicochemical properties of the surface discriminate between the allele types. It is tempting to hypothesize that blasted acid-etched Ti has the ability to enrich the microenvironment of the implant with ApoE. However, this could only improve the osseointegration outcome if the patient carried the ApoƐ3 alleles. Following this line of thought might help to determine the mechanisms of the variability in the outcomes of the same implant type in different patients.

Kaneko and colleagues have published a similar study using different surfaces, octacalcium phosphate (OCP) and hydroxyapatite crystals (HA). They have found that ApoE and complement component 3 (C3) were among the proteins differentially associated with these surfaces. They have observed that HA adsorbed more C3 than OCP, whereas OCP adsorbed more APoE (Kaneko et al. 2011).

Interestingly, in our study, C3 was enriched on smooth Ti discs. C3 belongs to a family of proteins involved in immune and inflammatory responses (Sahu & Lambris 2001). Osteoclasts are bone macrophages derived from the myeloid lineage that requires complement C3 and C5 for optimal differentiation (Tu et al. 2010). Osteoclasts are necessary for bone resorption and the optimal balance between osteoblast and osteoclast differentiation must be reached to achieve healthy bone formation. It is not clear whether the increased C3 adsorption onto smooth Ti surfaces alters this balance.

To summarise, two types of surfaces, smooth and SAE, were studied by physicochemical, *in vitro* and proteomic analysis. Al_2O_3 was found in the SAE surface while only Titanium in smooth sample. Roughness and hydrophilicity were increased by SAE treatment. Bibliography showed differences in *in vivo* experiments giving more osseointegration SAE surfaces. In our study and, in accordance with literature, no differences in *in vitro* tests (proliferation and mineralization) were found. Proteome analysis of the proteins adsorbed onto both surfaces

showed the presence of proteins related to bone generation. Proteins enriched on the SAE Ti were apolipoproteins ApoA-I, ApoE, ApoA-I, plectin, antithrombin III and Vitamin K-dependent protein. The largest difference between the two surface types was found for ApoA-IV and plectin. We also found that complement C3 and some immunoglobulins (Ig gamma and lambda chains) were significantly enriched on the smooth Ti but not on the blasted and acid-etched Ti discs.

Although significant physicochemical differences are found between samples (chemical composition, roughness and hydrophilicity), *in vitro* test did not show any. Further work is needed to demonstrate that proteomic analysis is able to explain *in vivo* behaviour.

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Figure captions:

Figure 1: SEM images of disc surface: (a) smooth-Ti and (b) SAE-Ti (x1000).

Figure 2: SEM/EDX images of titanium sandblasted and acid-etched disc for Al_2O_3 particles identification.

Figure 3: AFM images at scan size 60 μm: (a) untreated titanium and (b) SAE treated titanium; and 1 μm: (c) untreated titanium and (d) SAE treated titanium. The z-axis could not be normalized to the same scale due to the height difference between treatments.

Figure 4: MC3T3-E1 cells proliferation on different treated discs: Smooth-Ti (White circle), SAE-Ti (black semi-square with dotted line). Cells, on an empty well, without disc was used as a control (white circle). No statistically significant differences were found between treatments.

Figure 5: MC3T3-E1 cells ALP activity normalized to the total protein (BCA) levels (mM PNP/h) / (μg/μl) on different treated discs at 14 (a) and 21 (b) days.; Smooth-Ti (White column); SAE-Ti (sqared/dotted column). Cells, on an empty well, without disc was used as a control (Black column). No statistically significant differences was found between treatments

Figure 6: PieCharts pathways, of the biological processes, of the proteins adhered to (a) SAE-Ti and (b) Smooth-Ti.

Figure 7: PieCharts pathways of the proteins adhered to (c) SAE-Ti and (d) Smooth-Ti.

Table titles

Table 1: Plasma proteins adsorbed on SAE Ti and Smooth Ti as identified by LC-MS/MS. Spectral counts indicates number of MS/MS spectra obtained for each protein

Table 2: Specific Proteins (Progenesis method)