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

Francisco Romero-Gavilán, N. C. Gomes, Joaquin Ródenas, Ana Sánchez, Mikel Azkargorta, Ibon Iloro, Félix Elortza, Iñaki García Arnáez, Mariló Gurruchaga, Isabel Goñi and Julio Suaya

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, physico-chemical characterisation revealed important differences in roughness, chemical composition and hydrophilicity, but no differences were found in cellular in vitro studies (proliferation and mineralization). However, the deposition of proteins onto the implant surface might affect in vivo osseointegration. To test that hypothesis, protein layers formed on discs of both surface type after incubation with human serum were analysed. Using mass spectrometry (LC/MS/MS), 218 proteins were identified, 30 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 physico-chemical characteristics could be responsible for the differences observed in the adsorbed protein layer.

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). 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 et al. 2009).

For these reasons, researchers have focused 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, viz. surface plasmon...
Many studies evaluating the kinetics of protein adsorption onto Ti have 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 multi-protein systems such as blood plasma/serum, increasing the protein concentration and/or the number of small molecules improves their diffusion and accelerates displacement; thus, they are the first to be adsorbed onto the surface. With time, molecules with greater affinity for the surface but slower rates of diffusion (due to their low concentration or large size) replace the smaller molecules. This is known as the Vroman effect (Dee et al. 2003; Wang et al. 2012).

A study using mass spectrometric techniques 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 showed 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. (2013) characterised 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, the biological evaluation of medical devices includes a battery of standardised tests, as defined in ISO 10993, highly accepted in the biomaterials research field. Typical tests for biocompatibility of biomaterials involve cytotoxicity, cell attachment, cell proliferation and mineralization assays. However, a lack of correlation between in vitro and in vivo results has been observed in many instances. Since the first step before cell attachment on the material surface is protein adsorption, the use of proteomics is proposed to further the understanding of material biocompatibility.

Thus the aim of this 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, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was employed. Furthermore, a comparison of the more relevant results with in vitro test outcomes was performed. Therefore, this work aimed to establish a correlation between protein deposition and in vitro outcomes when testing surfaces, such as those currently used in commercial dental implants.

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, Lleida, Spain). Some of the discs, sand-blasted 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 18.2 Ω 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.

Physico-chemical characterisation of Ti discs
The surface topography of the Ti discs was characterised using atomic force microscopy (AFM, Bruker Multimode, Billerica, MA, USA) under dry conditions. Images were taken at different amplitudes. Measurements at scan sizes 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 (http://nanoscaleworld.bruker-axs.com/nanoscaleworld/media/p/775.aspx). Scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX, Leica-Zeiss LEO, Wetzlar, Germany) was used to study these surfaces under vacuum. Platinum sputtering was employed to make the samples more conductive for SEM examination. SEM micrographs were analysed by image processing using Image J software (https://image.nih.gov/ij/).

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

Wettability was evaluated by measuring the contact angle using an automatic contact-angle meter (DataPhysics OCA 20, DataPhysics Instruments GmbH, Filderstadt, Germany) after depositing 10 μl of ultrapure water W04 on the Ti surface at room temperature. The drops were formed at the dosing rate of 27.5 μl s⁻¹ and the angles were determined using SCA 20 software (http://www.dataphysics.de/startseite/produkte/software-module/). 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, Waltham, MA, USA), supplemented with 1% of (100 × ) penicillin/streptomycin (Biowest Inc., Riverside, KS, 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% penicillin/streptomycin, 1% ascorbic acid (5 mg ml^{-1}) and 0.21% β-glycerol phosphate.

Cells were cultured (at a concentration of 1 × 10^4 cells well^{-1}) with the Ti discs in 24-well culture plates (Thermo Scientific®, Waltham, MA, USA) at 37°C in a humidified (95%) atmosphere of 5% CO_2. The Ti discs were not exposed to blood serum before cell culture. The culture medium was changed every 48 h. 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 cell viability based on a redox reaction with resazurin. The cells were cultured in wells with the discs (three replicates per treatment) and examined following the manufacturer’s protocol after 24, 72 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) were added. After incubation for 2 h in the dark (37°C, 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. The results were calculated in mmols of p-nitrophenol h^{-1} (mM PNP h^{-1}) and the data were expressed as ALP activity normalised to the total protein content after 14 and 21 days.

**Total protein**

Total protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) for colorimetric protein quantitation based on copper reduction. The culture medium was removed from the wells, the wells were washed three 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 13,300 rpm and 4°C. Twenty μl of the supernatant were used for colorimetric measurement of BCA at 570 nm on a microplate reader Multiskan FC® (Thermo Scientific®). The total protein content was calculated from a standard curve for bovine albumin and expressed as μg μl^{-1}. These data were used to normalise the alkaline phosphatase (ALP) activity 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 ≤ 0.05 were considered statistically significant.

**Total protein**

Total protein content was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) for colorimetric protein quantitation based on copper reduction. The culture medium was removed from the wells, the wells were washed three 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 13,300 rpm and 4°C. Twenty μl of the supernatant were used for colorimetric measurement of BCA at 570 nm on a microplate reader Multiskan FC® (Thermo Scientific®). The total protein content was calculated from a standard curve for bovine albumin and expressed as μg μl^{-1}. These data were used to normalise the alkaline phosphatase (ALP) activity after 14 and 21 days.

**Proteomic analysis**

Each eluted protein sample was resolved in 10% polyacrylamide gels, using a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). 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 four slices. Each of these slices was digested with trypsin following a standard protocol (Anitua et al. 2015).
Progenesis LC-MS software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) 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. The abundance ratios between the run to be aligned and the reference run were calculated for all features at given retention times. These values were then logarithmised; the program, based on the analysis of the distribution of all ratios, automatically calculated a global scaling factor. Once normalised, 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 data-base 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

Physico-chemical characterisation of Ti discs

Figure 1 shows SEM images of smooth Ti and SAE Ti surfaces. The different topographies can be clearly seen. The particles on the titanium surface (Figure 1b) are visible in the image. EDX results indicated that these were alumina (Al₂O₃) particles that may have been encrusted in the material after the sandblasting process (Figure 2). The area of the disc covered by alumina particles comprises ~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
Figure 2. SEM/EDX images of titanium sandblasted and acid-etched disc for $\text{Al}_2\text{O}_3$ particle identification.

Figure 3. AFM images at a scan size of 60 μm of (a) untreated titanium and (b) SAE treated titanium; and (c) untreated Ti and (d) SAE treated Ti at a scan size of 1 μm. The z-axis could not be normalised to the same scale due to the height difference between treatments.
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 cellular growth. Cells proliferated at equal rates on both types of discs during the five-day protocol. A threefold increase in cell numbers was observed between 24 h and three days in culture. Proliferation slowed down between three and five days incubation, showing a plateau and a reduction in proliferation.

**Proteomic analysis**

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

LC-MS/MS analysis of the protein layers adsorbed to both Ti surfaces resulted in the identification of 218 different proteins, 30 of which related with bone metabolism (Table 1). Serum proteins involved in cell adhesion and
Paraoxonase/arylesterase 1 (Dowling et al. 2014), vitamin D binding protein (Benis & Schneider 1996; Swamy et al. 2001; Schneider et al. 2003) and pigment epithelium-derived factor (Li et al. 2013, 2015).

Gene ontology analysis of the identified proteins

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 (Figures 6 and 7). The results of protein classification according to biological processes were almost identical for the two types of surfaces (Figure 6a and b). However, classification of proteins according to the pathways in which they are involved 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, cellular/cytoplasmic components of cell adhesion and cell junction adsorbed to the Ti surfaces were found: 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). LC-MS/MS analysis also revealed cellular and secreted proteins associated with bone homeostasis: peptidyl-prolyl cis-trans isomerase B (Pyott et al. 2011) and lysozyme C (Siebert et al. 1978; Briggs & Arinzeh 2014). Serum proteins involved in bone formation were also found to a certain degree: serum paraoxonase/arylesterase 1 (Dowling et al. 2014), vitamin D binding protein (Benis & Schneider 1996; Swamy et al. 2001; Schneider et al. 2003) and pigment epithelium-derived factor (Li et al. 2013, 2015).

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

| Description                                      | Accession          | Smooth            | SAE-treated       | Ref. related to bone |
|--------------------------------------------------|--------------------|-------------------|-------------------|---------------------|
| Beta-2-glycoprotein 1                            | APOH_HUMAN         | 5                 | 2                 | (Adachi et al. 2002) |
| Complement component C8 beta chain               | COB8_HUMAN         | 2                 | 0                 | (Andrades et al. 1996) |
| Metal transporter CNNM2                          | CNNM2_HUMAN        | 1                 | 0                 | (Baaij et al. 2015) |
| Junction plakoglobin                             | PLAK_HUMAN         | 1                 | 0                 | (D’Alimonte et al. 2013) |
| Vitronectin                                      | VTNC_HUMAN         | 14                | 3                 | (y Salasznyk et al. 2004; Kundu & Putnam 2006; Di Benedetto et al. 2015) |
| Serum paraoxonase/arylesterase 1                 | PON1_HUMAN         | 2                 | 0                 | (Dowling et al. 2014) |
| Plasminogen                                      | PLMN_HUMAN         | 7                 | 0                 | (Engelholm et al. 2001; Daci et al. 2003; Mao et al. 2014) |
| Coagulation factor XII                           | FA12_HUMAN         | 3                 | 0                 | (Hong et al. 1999) |
| Gelsolin                                         | GELS_HUMAN         | 12                | 2                 | (y Kwiatkowski et al. 1989; Thouverey et al. 2011) |
| Integrin alpha-V                                 | ITAV_HUMAN         | 0                 | 2                 | (Kumar 2003; Roux 2010; Kaneko et al. 2014) |
| Alpha-2-macroglobulin                            | A2MG_HUMAN         | 15                | 0                 | (y Matsuoka et al. 2014; Kuo et al. 2015) |
| Complement C3                                    | CO3_HUMAN          | 135               | 31                | (Wever 1994; Driller et al. 2007; Larsen et al. 2010) |
| Tetractin                                        | TETN_HUMAN         | 2                 | 2                 | (y Matsuoka et al. 2014; Kuo et al. 2015) |
| Pigment epithelium-derived factor                | PEDF_HUMAN         | 5                 | 0                 | (Li et al. 2013; Li et al. 2015) |
| Complement C4-A                                  | CO4A_HUMAN         | 54                | 14                | (Majek et al. 2011) |
| Alpha-1-acid glycoprotein 2                      | A1AG2_HUMAN        | 1                 | 0                 | (Moussa et al. 2014) |
| Apolipoprotein E                                 | APOE_HUMAN         | 57                | 20                | (Shiraki et al. 1997; Newman et al. 2002; Kodama et al. 2007; Niemeier et al. 2012; Rodrigues et al. 2012; Dieckmann et al. 2013) |
| Proteoglycan 4                                   | PRG4_HUMAN         | 1                 | 0                 | (Novince et al. 2012) |
| Alpha-1-acid glycoprotein 1                      | A1AG1_HUMAN        | 0                 | 0                 | (Puel et al. 2004) |
| Peptidyl-prolyl cis-trans isomerase B             | PPIB_HUMAN         | 0                 | 0                 | (Pyott et al. 2011) |
| Complement component C6                          | CO6_HUMAN          | 0                 | 0                 | (Raisz et al. 1974; Sandberg et al. 1982) |
| Haptoglobin-related protein                      | HPTR_HUMAN         | 3                 | 0                 | (Rusinka et al. 2011) |
| Vitamin D-binding protein                         | VTD8_HUMAN         | 2                 | 0                 | (Benis & Schneider 1996; Swamy et al. 2001; Schneider et al. 2003) |
| Actin, cytoplasmic 1                              | ACTB_HUMAN         | 10                | 5                 | (Sen et al. 2015) |
| Lysozyme C                                       | LYS_C_HUMAN        | 0                 | 0                 | (Siebert et al. 1978; Briggs & Arinzeh 2014) |
| Haemoglobin subunit beta                         | HBB_HUMAN          | 1                 | 0                 | (Thongchote et al. 2011) |
| Apolipoprotein C-I                               | AP0C1_HUMAN        | 1                 | 0                 | (Wang et al. 2015) |
| Kininogen-1                                       | KNG1_HUMAN         | 4                 | 0                 | (Yamamura et al. 2006) |
| Serotransferrin                                  | TRFE_HUMAN         | 15                | 4                 | (y Gentili et al. 1994; Carlevaro et al. 1997; Yang et al. 2011) |
| Ceruloplasmin                                    | CERU_HUMAN         | 3                 | 0                 | (Kawada et al. 2006; Zarjou et al. 2010) |
revealed differences between the two types of surfaces (Figure 7a and b). Interestingly, smooth Ti-adsorbed proteins were observed to participate 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 SAE 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 of the total proteins). 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. This analysis revealed differences between the two types of surfaces (Figure 7a and b). Interestingly, smooth Ti-adsorbed proteins were observed to participate 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 SAE 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 of the total proteins). In addition to these categories, smooth Ti surfaces adsorbed a small percentage of proteins involved in apoptotic and plasminogen signalling pathways.
performed (in triplicate) using the Progenesis QI software. This method identified nine 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. It was 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 this study characterised the protein layer adsorbed onto Ti discs with two different surface types: a SAE Ti and an untreated, smooth Ti. It is reasonable to assume that 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, ie SAE 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 physico-chemical features affect the affinity of the protein layer formed on the material.

Ti surfaces are widely used in implants; techniques advancing 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).

The *in vitro* experiments, using an osteosarcoma cell line, showed no differences between both samples either in proliferation or mineralization. Both surfaces showed very similar cell proliferation results with time, increasing gradually throughout 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 with no statistically significant differences. In similar studies, no significant differences in either proliferation or mineralization were found (Yoshida et al. 2012). These results are supported by proteomic analysis of proteins adsorbed onto the different discs since the majority of proteins attach in 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 showed different osteogenic properties in *in vivo* models (Wennerberg & Albrektsson 2009). Furthermore, Aparicio et al. (2011) showed that high $R_a$ values favour osseointegration of dental implants in comparison with smoother surfaces. This effect is attributed to a higher implant–bone contact interface as a consequence of increased roughness. Nevertheless, in this study chemical differences between treatments were also found. To test this premise a detailed analysis of the proteins adsorbed to the two surface types was performed. In order to isolate and identify these surface adsorbed proteins, a protocol was established where, following serum incubation, discs were washed and final protein elution was obtained with an SDS-containing buffer. This approach permitted washing of the surfaces thoroughly and getting a good protein yield for the characterisation of the differences between both surfaces. The procedure indicates that under the same regime of washes and the same elution strength, a number of different proteins bound more consistently to each of the surfaces at statistically significant different concentrations, revealing differences in surface–protein interactions. Although other approaches cannot be discarded, the present method has been shown to be useful for the intended purpose. On the other hand, the use of a harsher buffer could release proteins that might have remained attached after the SDS wash. However, it is believed that although the total list of proteins could increase, it should not affect the differential analysis results. There was an average of 181 proteins identified on the smooth Ti surface discs, and 162 proteins on the blasted and acid-etched Ti surface. This suggests that the differences observed

### Table 2. Specific proteins (progenesis method).

| Accession  | Description         | Confidence score | ANOVA (p)   | Average SAE | Average smooth | Ratio SAE/smooth |
|------------|---------------------|------------------|-------------|-------------|----------------|------------------|
| PLEC_HUMAN | Plectin             | 56.31            | 2.76E-03    | 871.54      | 3.02           | 288.22           |
| AN1T_HUMAN | Antithrombin-III    | 75.68            | 5.92E-04    | 2,613.27    | 13.25          | 197.22           |
| PROC_HUMAN | Vitamin K-dependent protein C | 87.11 | 1.39E-02    | 3,106.36    | 158.53         | 19.59            |
| APO4_HUMAN | Apolipoprotein A-IV | 247.55           | 4.48E-03    | 1,326.02    | 499.46         | 2.65             |
| APOA1_HUMAN | Apolipoprotein A-I | 197.75           | 3.27E-02    | 21,111.07   | 12,019.28      | 1.76             |
| APOE_HUMAN | Apolipoprotein E    | 438.34           | 1.97E-02    | 7,706.64    | 4,798.73       | 1.61             |
| LVD301_HUMAN | Ig lambda chain V-III region SH | 37.01 | 1.21E-02    | 1,040.84    | 1,522.40       | 0.68             |
| CD3_HUMAN | Complement C3       | 205.15           | 1.23E-02    | 2,030.88    | 4,398.04       | 0.46             |
| IGHG1_HUMAN | Ig gamma-1 chain C region | 49.04 | 2.20E-02    | 506.02      | 2,040.67       | 0.25             |
between the surfaces is a result of 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 (Burgtstaller et al. 2010).

ApoE is not a typical serum protein; therefore, its presence in the protein layer formed during incubation of Ti discs with 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 the present study, Apo A-IV level was significantly higher on blasted acid-etched Ti than on 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, adsorbed to treated Ti at higher concentrations 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 at higher concentrations than to smooth Ti. Although still somewhat controversial, ApoE has been extensively reported to be involved in bone homeostasis (Niemeier et al. 2012), possibly via the promotion of vitamin K uptake into the osteoblasts (Newman et al. 2002).

However, various ApoE alleles behave very differently in this process. ApoE2 is the allele with the least involvement in the transport of vitamin K (Saupe et al. 1993). The ApoE4 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 ApoE2 represents an increased risk for trabecular bone fracture (Dieckmann et al. 2013).

The most frequent ApoE allele is ApoE3, found with a frequency of 79%. ApoE2 is present in ~7% of the population, and ApoE4 in 14%. ApoE3 is also called the neutral allele because it is not associated with any of the human diseases. ApoE2 and 4 have been associated with an increased probability of developing arthrosclerosis and Alzheimer’s disease (Eisenberg et al. 2010).

The method used to characterise the protein layer on Ti surfaces did not allow for the determination of the type of ApoE allele adsorbed. Moreover, it is not clear whether physico-chemical properties of the surface discriminate between the allele types. It is tempting to hypothesis 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 ApoE3 alleles. Following this line of thought might help to determine mechanisms of the variability in the outcomes of the same implant type in different patients.

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

Interestingly, in the present 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 increased C3 adsorption on smooth Ti surfaces alters this balance.

To summarise, two types of surfaces, smooth and SAE, were studied by physico-chemical, in vitro and proteomic analysis. Al₂O₃ was found in the SAE surface and only Ti in the smooth sample. Roughness and hydrophilicity were increased by SAE treatment. In this study, in accordance with published literature, no differences in in vitro tests (proliferation and mineralization) were found. Proteomic 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. It was also found that complement C3 and some immunoglobulins (Ig gamma and lambda chains) were significantly enriched on smooth Ti but not on the blasted and acid-etched Ti discs.
Although significant physico-chemical differences were found between samples (chemical composition, roughness and hydrophilicity), in vitro tests did not show any differences. Further work is needed to demonstrate that proteomic analysis can correlate with in vivo behaviour.

Acknowledgements

The authors would like to thank Antonio Coso and Jaime Franco (GMI-Ilerimplant) for their inestimable contribution to this study, and Iraida Escobes (CIC bioGUNE) for her valuable technical assistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by Ministerio de Economía y Competitividad (MINECO) [MAT-2014-51918-C2-2-R], Universidad de Castellón [P11B2014-19], Plan de Promoción de la Investigación de la Universidad Jaume I under grant [Predoc/2014/25] and Generalitat Valenciana under grant [Grisolia/2014/016].

References

Adachi Y, Sugimoto K, Sato AK, Mori KJ. 2002. Identification of negative regulator of interleukin-3 (NIL-3) in bone marrow. Cell Struct Funct. 27:81–89.

Andrade JD, Hlday V. 1987. Protein adsorption and materials biocompatibility: a tutorial review and suggested hypotheses. Berlin: Springer.

Andrades JA, Nimni ME, Becerra J, Eisenstein R, Davis M, Sorgente N. 1996. Complement proteins are present in developing endochondral bone and may mediate cartilage cell death and vascularization. Exp Cell Res. 227:208–213.

Anitua E, Prado R, Azkargorta M, Rodriguez-Suárez E, Iloro I, Casado-Vela J, Elortza F, Orive G. 2015. High-throughput proteomic characterization of plasma rich in growth factors (PRGF-Endoret)-derived fibrin clot interactome. J Tissue Eng Regen Med. 9:E1–E12. doi:http://dx.doi.org/10.1002/term.v9.11

Aparicio C, Padrós A, Gil FJ. 2011. In vivo evaluation of micro-rough and bioactive titanium dental implants using histometry and pull-out tests. J Mech Behav Biomed Mater. 4:1672–1682. doi:http://dx.doi.org/10.1016/j.jmbbm.2011.05.005

Benis KA, Schneider GB. 1996. The effects of vitamin D binding protein-macrophage activating factor and colony-stimulating factor-1 on hematopoietic cells in normal and osteopetrotic rats. Blood. 88:2898–2905.

Briggs T, Arinzeh TL. 2014. Examining the formulation of emulsion electrosprinning for improving the release of bioactive proteins from electrospun fibers. J Biomed Mater Res A. 102:674–684. doi:http://dx.doi.org/10.1002/jbm.a.v102.3

Burgstaller G, Gregor M, Winter L, Wiche G. 2010. Keeping the vimentin network under control: cell-matrix adhesion-associated plectin 1f affects cell shape and polarity of fibroblasts. Mol Biol Cell. 21:3362–3375. doi:http://dx.doi.org/10.1091/mbc.E10-02-0094

Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. 1991. Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. J Biomed Mater Res. 25:889–902. doi:http://dx.doi.org/10.1002/(ISSN)1097-4636

Carlevaro MF, Albini A, Ribatti D, Gentili C, Benelli R, Cermelli S, Cancenda R, Cancenda FD. 1997. Transferrin promotes endothelial cell migration and invasion: implication in cartilage Neo-vascularization. J Cell Biol. 136:1375–1384.

D’Alimonte I, Lannutti A, Pipino C, Di Tomo P, Pierdomenico L, Cianci E, Antonucci I, Marchisio M, Romano M, Stuppia L, et al. 2013. Wnt signaling behaves as a “master regulator” in the osteogenic and adipogenic commitment of human amniotic fluid mesenchymal stem cells. Stem Cell Rev. 9:642–654. doi:http://dx.doi.org/10.1007/s12015-013-9436-5

Daci E, Everts V, Torrekens S, Van Herck E, Tigchelaar-Gutt G, Wouillon R, Carmeliet G. 2003. Increased bone formation in mice lacking plasminogen activators. J Bone Miner Res. 18:1167–1176.

de Baaij JHF, Hoenderop JGJ, Bindels RJM. 2015. Magnesium in man: implications for health and disease. Physiol Rev. 95:1–46.

Dee KC, David A Puleo RB. 2003. An introduction to tissue-biomaterial interactions. New York: Wiley.

Di Benedetto A, Brunetti G, Posa F, Ballini A, Grassi FR, Colaianni G, Colucci S, Rossi E, Cavalcanti-Adam EA, Lo Muzio L, et al. 2015. Osteogenic differentiation of mesenchymal stem cells from dental bud: role of integrins and cadherins. Stem Cell Res. 15:618–628. doi:http://dx.doi.org/10.1016/j.scr.2015.09.011

Dieckmann M, Beil FT, Mueller B, Bartelt A, Marshall RP, Koehne T, Amling M, Ruether W, Cooper JA, Humphries SE, et al. 2013. Human apolipoprotein E isoforms differentially affect bone mass and turnover in vivo. J Bone Miner Res. 28:236–245. doi:http://dx.doi.org/10.1002/jbmr.1757

Dodo CG, Senna PM, Custodio W, Paes Leme AF, Del Bel Cury AA. 2013. Proteome analysis of the plasma protein layer adsorbed to a rough titanium surface. Biofouling. 29:549–557. doi:http://dx.doi.org/10.1080/08927014.2013.787416

Downing P, Hayes C, Ting KR, Hameed A, Meiller J, Mitsiadis C, Anderson KC, Clynes M, Clarke C, Richardson P, O’Gorman P. 2014. Identification of proteins found to be significantly altered when comparing the serum proteome from multiple myeloma patients with varying degrees of bone disease. BMC Genomics. 15:904. doi:http://dx.doi.org/10.1186/1471-2164-15-904

Driller K, Pagenstecher A, Uhl M, Omran H, Berlis A, Gründer A, Sippel AE. 2007. Nuclear factor I X deficiency causes brain malformation and severe skeletal defects. Mol Cell Biol. 27:3855–3867.

Eisenberg DTA, Kuzawa CW, Hayes MG. 2010. Worldwide allele frequencies of the human apolipoprotein E gene: climate, local adaptations, and evolutionary history. Am J Phys Anthropol. 143:100–111. doi:http://dx.doi.org/10.1002/ajpa.v143:1
Engelholm LH, Nielsen BS, Netzel-Arnett S, Solberg H, Chen XD, Lopez Garcia JM, Lopez-Otin C, Young MF, Birkedal-Hansen H, Danø K, et al. 2001. The urokinase plasminogen activator receptor – associated protein/Endo180 is coexpressed with its interaction partners urokinase plasminogen activator receptor and matrix metalloprotease-13 during osteogenesis. Lab Invest. 81:1403–1414.

Fernández-Montes Moraleda B, Román J, Rodriguez-Lorenzo LM. 2013. Influence of surface features of hydroxyapatite on the adsorption of proteins relevant to bone regeneration. J Biomed Mater Res A. 101A:2332–2339. doi:http://dx.doi.org/10.1002/jbm.a.v101a.8

Gentili C, Doliana R, Bet P, Campanile G, Colombatti A, Cancetta FD, Cancetta R. 1994. Orovotransferrin and ovotransferrin receptor expression during chondrogenesis and endochondral bone formation in developing chick embryo. J Cell Biol. 124:579–588.

Grassi S, Piattelli A, de Figueiredo LC, Feres M, de Melo L, Iezzi G, Alba RC, Shibli JA. 2006. Histologic evaluation of early human bone response to different implant surfaces. J Periodontol. 77:1736–1743. doi:http://dx.doi.org/10.1902/jop.2006.050325

Haynes CA, Norde W. 1994. Globular proteins at solid/liquid interfaces. Colloids Surf B Biointerfaces. 2:517–566. doi:http://dx.doi.org/10.1016/0927-7765(94)80066-9

Hirata T, Fujioka M, Takahashi KA, Takeda A, Ishida M, Akioka K, Okamoto M, Yoshimura N, Satomi Y, Nishino H, et al. 2007. Low molecular weight phenotype of apolipoprotein(a) is a risk factor of corticosteroid-induced osteonecrosis of the femoral head after renal transplant. J Rheumatol. 34:516–522.

Hong J, Andersson J, Ekdahl KN, Elgue G, Axén N, Larsson R, Nilsson B. 1999. Titanium is a highly thrombogenic biomaterial: Possible implications for osteogenesis. Thromb Haemost. 82:58–64.

Imamura K, Shimomura M, Nagai S, Akamatsu M, Nakanishi K. 2008. Adsorption characteristics of various proteins to a titanium surface. J Biosci Bioeng. 106:273–278. doi:http://dx.doi.org/10.1263/jbb.106.273

Kaneko H, Kamiiie J, Kawakami H, Anada T, Honda Y, Shiraishi N, Kamakura S, Terasaki T, Shimauchi H, Suzuki O. 2011. Proteome analysis of rat serum proteins adsorbed onto synthetic octacalcium phosphate crystals. Anal Biochem. 418:276–285. doi:http://dx.doi.org/10.1016/j.ab.2011.07.022

Kaneko K, Ito M, Naoe Y, Lacy-Hulbert A, Ikeda K. 2014. Integrin αv in the mechanical response of osteoblast lineage cells. Biochem Biophys Res Commun. 447:352–357. doi:http://dx.doi.org/10.1016/j.bbrc.2014.04.006

Kawada E, Moridaira K, Itoh K, Hoshino A, Tamura J, Morita T. 2006. In long-term bedridden elderly patients with dietary copper deficiency, biochemical markers of bone resorption are increased with copper supplementation during 12 weeks. Ann Nutr Metab. 50:420–424.

Kerachian MA, Cournoyer D, Harvey EJ, Chow TY, Bégin LR, Nahal A, Séguin C. 2010. New insights into the pathogenesis of glucocorticoid-induced avascular necrosis: microarray analysis of gene expression in a rat model. Arthritis Res Ther. 12:R124.

Kim J-M, Kim J, Kim Y-H, Kim K-T, Ryu SH, Lee TG, Suh P-G. 2013. Comparative secretome analysis of human bone marrow-derived mesenchymal stem cells during osteogenesis. J Cell Physiol. 228:216–224. doi:http://dx.doi.org/10.1002/jcp.24123

Kodama T, Goto T, Ishibe T, Kobayashi S, Takahashi T. 2007. Apolipoprotein E stimulates bone formation on titanium in vitro. Asian J Oral Maxillofac Surg. 19:96–100.

Kohavi D, Badihi Hauslich L, Rosen G, Steinberg D, Sela MN. 2013. Wettability versus electrostatic forces in fibronectin and albumin adsorption to titanium surfaces. Clin Oral Implants Res. 24:1002–1008.

Kumar C. 2003. Integrin αvβ3 as a therapeutic target for blocking tumor-induced angiogenesis. Curr Drug Targets. 4:123–131. doi:http://dx.doi.org/10.2174/1389450033346830

Kundu AK, Putnam AJ. 2006. Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells. Biochem Biophys Res Commun. 347:347–357. doi:http://dx.doi.org/10.1016/j.bbrc.2006.06.110

Kuo S-J, Wang F-S, Sheen J-M, Yu H-R, Wu S-L, Ko J-Y. 2015. Complement component C3: Serologic signature for osteogenesis imperfecta. Analysis of a comparative proteomic study. J Formos Med Assoc. 114:943–949.

Kwiatkowski DJ, Janmey PA, Yin HL. 1989. Identification of critical functional and regulatory domains in gelsolin. J Cell Biol. 108:1717–1726. doi:http://dx.doi.org/10.1083/jcb.108.5.1717

Larsen KH, Frederiksen CM, Burns JS, Abdallah BM, Kassem M. 2010. Identifying a molecular phenotype for bone marrow stromal cells with in vivo bone-forming capacity. J Bone Miner Res. 25:796–808.

Lemons JE, Lucas LC. 1986. Properties of biomaterials. J Arthroplasty. 1:143–147. doi:http://dx.doi.org/10.1016/S0883-5403(86)80053-5

Li F, Song N, Tombran-Tink J, Niyibizi C. 2013. Pigment epithelium-derived factor enhances differentiation and mineral deposition of human mesenchymal stem cells. Stem Cells. 31:2714–2723. doi:http://dx.doi.org/10.1002/stem.1505

Li F, Song N, Tombran-Tink J, Niyibizi C. 2015. Pigment epithelium derived factor suppresses expression of sst/sclerostin by osteocytes: implication for its role in bone matrix mineralization. J Cell Physiol. 230:1243–1249. doi:http://dx.doi.org/10.1002/jcp.24859

MacDonald D, Deo N, Markovic B, Stranick M, Somasundaran P. 2002. Adsorption and dissolution behavior of human plasma fibronectin on thermally and chemically modified titanium dioxide particles. Biomaterials. 23:1269–1279. doi:http://dx.doi.org/10.1016/S0142-9612(01)00317-9

Májek P, Reicheltová Z, Suttnar J, Cermák J, Dyr JE. 2011. WOMAC cytokine and endochondral bone formation in developing chick embryo. J Cell Biol. 124:579–588.

Matsuo O, Kaji H. 2014. Plasminogen activator inhibitor-1 la701233y

Matsuoka K, Park K-A, Ito M, Ikeda K, Takeshita S. 2014. Bone marrow-derived mesenchymal stem cells during osteogenesis. J Cell Physiol. 228:216–224. doi:http://dx.doi.org/10.1002/jcp.24123

Nahal A, Séguin C. 2010. New insights into the pathogenesis of glucocorticoid-induced avascular necrosis: microarray analysis of gene expression in a rat model. Arthritis Res. 12:R124.

Nakahori K, Tanaka H, Kokubun F, Kuroda Y, Ohashi K, Tanaka H, Kudo M. 2014. Osteoclast-derived complement component 3a stimulates osteoblast differentiation. J Bone Miner Res. 29:1522–1530.
Molino PJ, Higgins MJ, Innis PC, Kapsa RMI, Wallace GG. 2012. Fibronectin and bovine serum albumin adsorption and conformational dynamics on inherently conducting polymers: a QCM-D study. Langmuir. 28:8433–8445. doi:http://dx.doi.org/10.1021/la300692y

Montoya A, Beltran L, Casado P, Rodriguez-Prados J-C, Cutillas PR. 2011. Characterization of a TiO2 enrichment method for label-free quantitative phosphoproteomics. Methods. 54:370–378. doi:http://dx.doi.org/10.1016/j.ymeth.2011.02.004

Moussa FM, Hisijara IA, Sondag GR, Scott EM, Frara N, Abdelmagid SM, Safadi FF. 2014. Osteoactivin promotes osteoblast adhesion through HSPG and avß1 integrin. J Cell Biochem. 115:1243–1253.

Nakajima H, Okabe T. 1996. Titanium in dentistry: development and research in the U.S.A. Dent Mater J. 15:77–90. doi:http://dx.doi.org/10.4012/dmj.15.77

Newman P, Bonello F, Wierzbicki AS, Lumb P, Savidge GF, Shearer MJ. 2002. The uptake of lipoprotein-borne phylloquinone (vitamin K1) by osteoblasts and osteoblast- like cells: role of heparan sulfate proteoglycans and apolipoprotein E. J Bone Miner Res. 17:426–433. doi:http://dx.doi.org/10.1034/j.1600-0501.2000.011006530.x

Niemeier A, Schinke T, Heeren J, Amling M. 2012. Understanding protein expression ratio is associated with hip fragility fractures. Bone. 51:981–989.

Roux S. 2010. New treatment targets in osteoporosis. Joint Bone Spine. 77:222–228. doi:http://dx.doi.org/10.1016/j.jbspin.2010.02.004

Rusińska A, Światkowska M, Koziolkiewicz W, Skurzyński S, Golec J, Chlebna-Sokół D. 2011. Proteomic analysis of plasma profiles in children with recurrent bone fractures. Acta Biochim Pol. 58:553–561.

Salas-Znzyk RM, Williams WA, Boskey A, Batorsky A, Plopper GE. 2004. Adhesion to vitronectin and collagen I promotes osteogenic differentiation of human mesenchymal stem cells. J Biomed Biotechnol. 2004:24–34. doi:http://dx.doi.org/10.1155/S1110724304306017

Sanada M, Nakagawa H, Kodama I, Sakasita T, Ohama K. 1998. Apolipoprotein E phenotype associations with plasma lipoproteins and bone mass in postmenopausal women. Climacteric. 1:188–195. doi:http://dx.doi.org/10.3109/13697139809085540

Sandberg AL, Raisz LG, Wahl LM, Simmons HA. 1982. Enhancement of complement-mediated prostaglandin synthesis and bone resorption by arachidonic acid and inhibition by cortisol. Prostaglandins Leukot Med. 8:419–427.

Saxe J, Shearer MJ, Kohlmeier M. 1993. Phylloquinone transport and its influence on γ-carboxylglutamate residues of osteocalcin in patients on maintenance hemodialysis. Am J Clin Nutr. 58:204–208.

Schwartz Z, Raz P, Zhao G, Barak Y, Tauber M, Yao H, Boyan BD. 2008. Effect of micrometer-scale roughness of the surface of Ti6Al4V pedicle screws in vitro and in vivo. J Bone Joint Surg Am. 90:2485–2498. doi:http://dx.doi.org/10.1016/j.jbjs.2008.02.007

Sen B, Xie Z, Uzer G, Thompson WR, Stynier M, Wu X, Rubin J. 2015. Intracellular actin regulates osteogenesis. Stem Cells. 33:3065–3076. doi:http://dx.doi.org/10.1002/stem.2090
Shiraki M, Shiraki Y, Aoki C, Hosoi T, Inoue S, Kaneki M, Ouchi Y. 1997. Association of bone mineral density with apolipoprotein E phenotype. J Bone Miner Res. 12:1438–1445. doi:http://dx.doi.org/10.1002/jbmr.1997.12.9.1438

Shiu HT, Goss B, Lutton C, Crawford R, Xiao Y. 2014. Formation of blood clot on biomaterial implants influences bone healing. Tis. Eng. – Part B Rev. 20:697–712.

Siebert H, Treber N, Konold P, Pannike A. 1978. Zur Rolle des Lysozyms bei der Mineralisation von Callusgewebe [To the role of the lysozyme in the mineralization of callus tissue]. Langenbecks Arch für Chir. 346:193–199. doi:http://dx.doi.org/10.1007/BF01261242

Smith DC. 1993. Dental implants: materials and design considerations. Int J Prosthodont. 6:106–117.

Sousa SR, Lamghari M, Sampaio P, Moradas-Ferreira P, Barbosa MA. 2008. Osteoblast adhesion and morphology on TiO₂ depends on the competitive preadsorption of albumin and fibronectin. J Biomed Mater Res A. 84A:281–290. doi:http://dx.doi.org/10.1002/jbm.a.31201

Spaulding HL, Sajio F, Turnage RH, Alexander JS, Aw TY, Kalogeris Tj. 2006. Apolipoprotein A-IV attenuates oxidant-induced apoptosis in mitotic competent, undifferentiated cells by modulating intracellular glutathione redox balance. Am J Physiol Cell Physiol. 290:C95–C103.

Swamy N, Ghosh S, Schneider GB, Ray R. 2001. Baculovirus-expressed vitamin D-binding protein-macrophage activating factor (DBP-maf) activates osteoclasts and binding of 25-hydroxyvitamin D(3) does not influence this activity. J Cell Biochem. 81:535–546. doi:http://dx.doi.org/10.1002/(ISSN)1097-4644

Thongchote K, Svasti S, Sa-aridrit M, Krishnamra N, Fucharoen S, Charoenphandhu N. 2011. Impaired bone formation and osteopenia in heterozygous β(IVSII-654) knockin thalassemic mice. Histochem Cell Biol. 136:47–56.

Thouverey C, Malinowska A, Balcerzak M, Strzelecka-Kiliszek A, Buchet R, Dadlez M, Pikula S. 2011. Proteomic characterization of biogenesis and functions of matrix vesicles released from mineralizing human osteoblast-like cells. J Proteomics. 74:1123–1134. doi:http://dx.doi.org/10.1016/j.jprot.2011.04.005

Tu Z, Bu H, Dennis JE, Lin F. 2010. Efficient osteoclast differentiation requires local complement activation. Blood. 116:4456–4463. doi:http://dx.doi.org/10.1182/blood-2010-01-263590

Wang K, Zhou C, Hong Y, Zhang X. 2012. A review of protein adsorption on bioceramics. Interface Focus. 2:259–277. doi:http://dx.doi.org/10.1098/rsfs.2012.0012

Wang Y, Li W-H, Li Z, Liu W, Zhou L, Gui J-F. 2015. BMP and RA signaling cooperate to regulate Apolipoprotein C1 expression during embryonic development. Gene. 554:196–204.

Wehmeyer JL, Synowicki R, Bizios R, García CD. 2010. Dynamic adsorption of albumin on nanostructured TiO(2) thin films. Mater Sci Eng C Mater Biol Appl. 30:277–282. doi:http://dx.doi.org/10.1016/j.msec.2009.11.002

Wennerberg A, Albrektsson T. 2009. Effects of titanium surface topography on bone integration: a systematic review. Clin Oral Implants Res. 20:172–184. doi:http://dx.doi.org/10.1111/clr.2009.20.issue-s4

Wennerberg A, Albrektsson T. 2010. On implant surfaces: a review of current knowledge and opinions. Int J Oral Maxillofac Implants. 25:63–74.

Wennerberg A, Albrektsson T, Andersson B, Krol JJ. 1995. A histomorphometric and removal torque study of screw-shaped titanium implants with three different surface topographies. Clin Oral Implants Res. 6:24–30. doi:http://dx.doi.org/10.1034/j.1600-0501.1995.060103.x

Wewer UM. 1994. A potential role for tetranectin in mineralization during osteogenesis. J Cell Biol. 127:1767–1775.

Wu R-W, Wang F-S, Ko J-Y, Wang C-J, Wu S-L. 2008. Comparative serum proteome expression of osteonecrosis of the femoral head in adults. Bone. 43:561–566. doi:http://dx.doi.org/10.1016/j.bone.2008.04.019

Yamamura J, Morita Y, Takada Y, Kawakami H. 2006. The fragments of bovine high molecular weight kininogen promote osteoblast proliferation in vitro. J Biochem. 140:825–830.

Yang Q, Jian J, Abramson SB, Huang X. 2011. Inhibitory effects of iron on bone morphogenetic protein 2-induced osteoblastogenesis. J Bone Miner Res. 26:825–830.

Yang Q, Jian J, Abramson SB, Huang X. 2011. Inhibitory effects of iron on bone morphogenetic protein 2-induced osteoblastogenesis. J Bone Miner Res. 26:1188–1196.

Yoshida E, Yoshimura Y, Uo M, Hayakawa T. 2012. Influence of nanometer smoothness and fibronectin immobilization of titanium surface on MC3T3-E1 cell behavior. J Biomed Mater Res – Part A. 100A:1556–1564. doi:http://dx.doi.org/10.1002/jbm.a.v100a.6

Zarjou A, Jeney V, Arosio P, Poli M, Zavaczki E, Balla G, Balla J. 2010. Ferritin ferroxidase activity: a potent inhibitor of osteogenesis. J Bone Miner Res. 25:164–172.