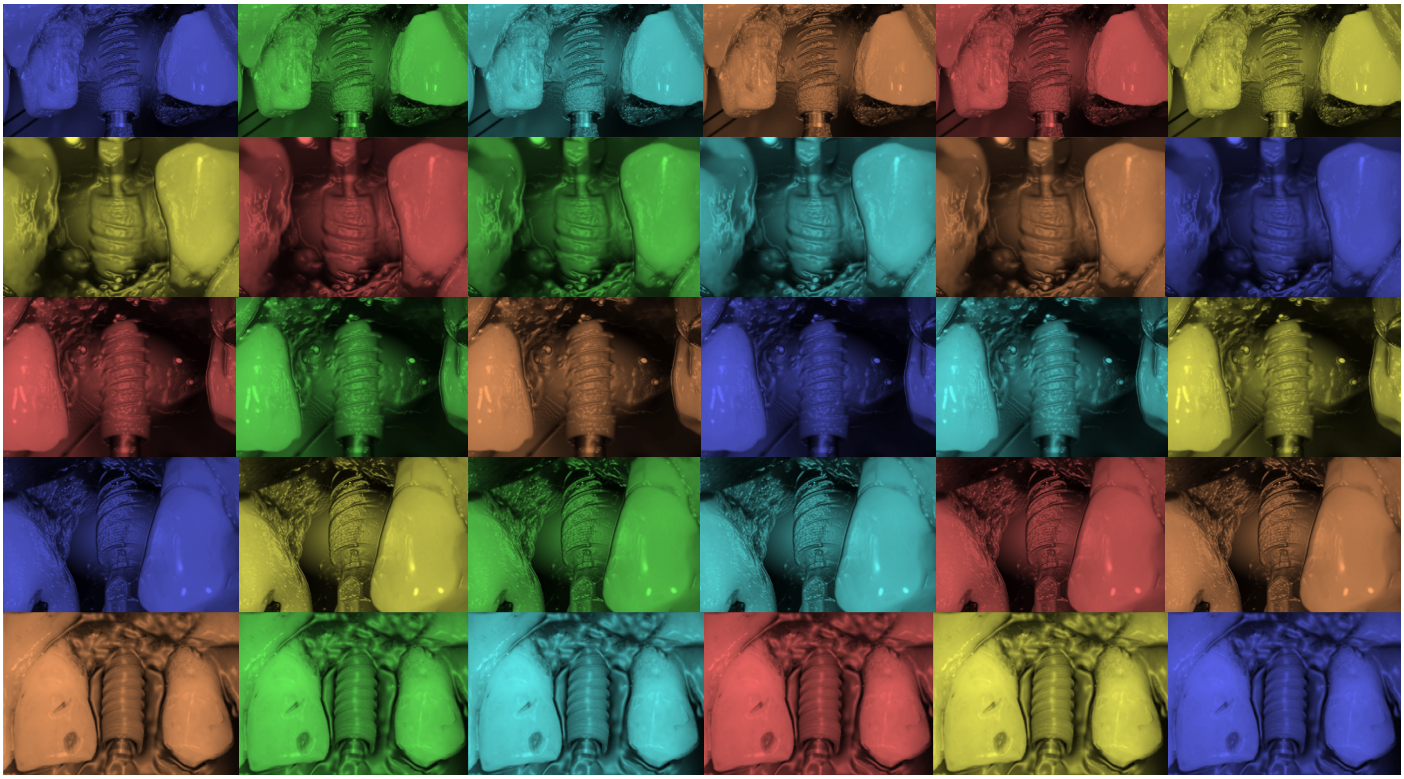
IMMEDIATE AND EARLY LOADING OF THERMO CHEMICALLY TREATED IMPLANTS WITH DEFINITIVE ABUTMENTS AT POSTERIOR AREAS OF MAXILLA AND MANDIBLE

Matteo Albertini



Immediate And Early Loading Of Thermo-chemically Treated Implants With Definitive Abutments At Posterior Areas Of Maxilla And Mandible

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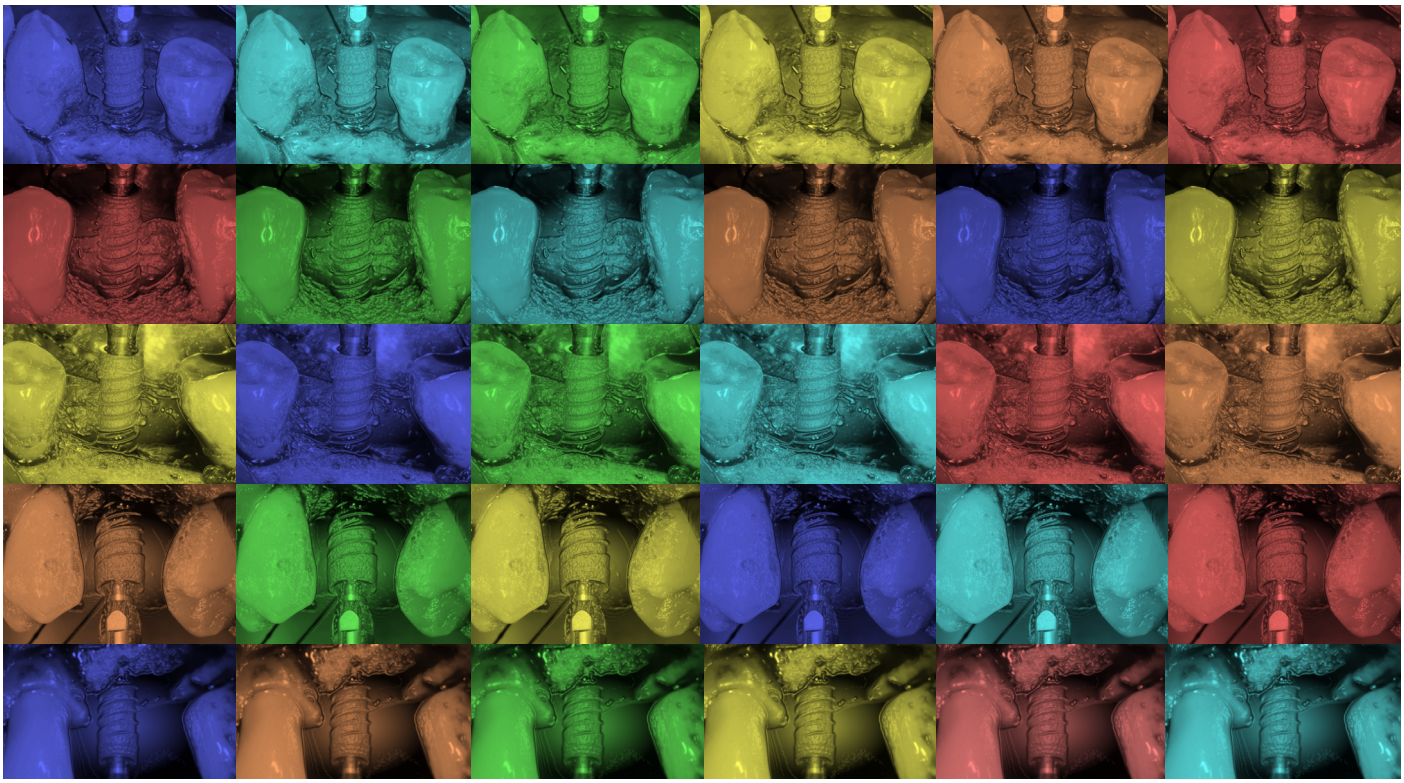

IMMEDIATE AND EARLY LOADING OF THERMO-CHEMICALLY TREATED IMPLANTS WITH DEFINITIVE ABUTMENTS AT POSTERIOR AREAS OF MAXILLA AND MANDIBLE

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Matteo Albertini

Department of Periodontology, Universitat Internacional de Catalunya

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Director: Dr. Jose Nart Molina

Co-Director: Dr. Mariano Herrero Climent

Ad Anna e alla mia famiglia.

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1. INTRODUCTION

1.1 General introduction

Implant surface characteristics have a relevant influence on the bone healing process around implants at early stages (Berglundh, et al. 2003). Recently developed surfaces ensure a more predictable application of immediate and early loading protocols (Buser, et al. 2004; Wallkamm, et al. 2015).

One of the first strategies that were developed in order to enhance the implant osseointegration was the application of hydroxyapatite coatings attained by plasma-spray technique and -although promising results have been observed in the short term (Cannizzaro, et al. 2013; Mistry, et al. 2016)- complications with some hydroxyapatite plasma-sprayed coatings have been reported in longer follow-up evaluations due to bacterial microleakage into the interface between the coating and titanium (van Oirschot, et al. 2016b).

For this reason, alternative methods have been proposed to improve the bioactive properties of the titanium implant surface by adding certain molecules which, in contact with blood and bone cells, are able to enhance the osseointegration process at early phases (Mertens & Steveling 2011; Felice, et al. 2015). In this sense, Kokubo et al. proposed a thermo-chemical treatment in which titanium was first chemically treated with alkaline solutions and then subjected to heating at high temperatures (Kokubo, et al. 1990). This method allows de novo hydroxyapatite formation through a biomimetic mechanism where hydroxyapatite is produced as a chemical reaction in which calcium and phosphate ions precipitate once the implant is in contact with the human serum without

the presence of osteoblasts (Kokubo, et al. 1996; Kokubo & Takadama 2006).

Nucleation of hydroxyapatite at implants subjected to a thermo-chemical treatment submerged in simulated body fluid has been observed by electronic microscope (Nishiguchi, et al. 2003; Kokubo, et al. 2004; Pattanayak, et al. 2011; Aparicio, et al. 2007a). The new crystalline hydroxyapatite layer is chemically adhered to titanium by a covalent union and has the potential to stimulate the osteoblasts migration to the implant surface leading to an accelerated osseointegration (Aparicio, et al. 2011a) Based on these investigations, a highly hydrophilic and osteoconductive surface has been developed (Aparicio, et al. 2002; Nogueras-Bayona, et al. 2004) and recently applied to dental implants (Albertini, et al. 2015). The substrate of the additive thermo-chemical treatment is a moderately rough surface (Albrektsson & Wennerberg 2004) with a value of 1,74 S_a attained by a grit-blasting and acid-etching procedures of grade 4 commercially pure titanium (Contac-Ti ®, Klockner Implant System, SOADCO, Andorra). *In-vitro* studies have shown new grown hydroxyapatite directly bound to this surface (Aparicio, et al. 2007a). Furthermore, *in vivo* studies observed *de novo* bone formation and an increased bone-to-implant contact at the early stages of bone healing in comparison with non-treated surfaces (Gil, et al. 2014b).

Over the years, implant surface has experienced several changes in it's chemical composition that have led to a more predictable and quicker osseointegration thereby leading to a progressive change of the standard loading protocols (Moraschini & Barboza 2016; Papaspyridakos, et al. 2014).

Immediate loading has been defined as the connection of the restoration to the implant in occlusion with the opposing dentition within the first week after implant placement, while

in early loading the connection takes place the first week and 2 months subsequent to implant placement (Weber, et al. 2009). Predictable results in terms of implant survival rates and marginal bone loss have been attained with immediate and early loading procedures in different clinical situations such as in the treatment of totally or partially edentulous patients with fixed prostheses (Esposito, et al. 2013; De Bruyn, et al. 2014). Even in posterior areas, several clinical studies have showed high implant success rates in the presence of adequate primary stability and favourable occlusal conditions (Salvi, et al. 2004; Roccuzzo, et al. 2009; Cordaro, et al. 2009). Due to the capacity of bio-active implant surfaces to accelerate osseointegration, it has been suggested that loading time could be reduced. In this regard, recent clinical investigations have shown promising results after loading these implants three to four weeks after placement (Simmons, et al. 2016; Felice, et al. 2015; Nicolau, et al. 2013).

Several experimental studies (Abrahamsson, et al. 1997; Becker, et al. 2012; Alves, et al. 2015) have suggested avoiding repeated connection/disconnection of the implant abutment due to the greater risk of crestal bone loss. Furthermore, a better maintenance of marginal bone level with the use of definitive abutments tightened at the time of surgery on platform-switched implants (‘one abutment one time’) has been demonstrated (Degidi, et al. 2011b; Grandi, et al. 2013; Degidi, et al. 2014; Molina, et al. 2017). Nevertheless, results of immediate or early loading of implants with chemical modification of their surface in association with one abutment at one time protocol are still lacking.

1.2 The evolution of dental implants and surfaces

Developments of implantology have allowed extending the reach of dental treatments providing long-term stable support for a prosthesis subjected to chewing load (Simmons, et al. 2016) and dental implants represent a valid therapeutic option for the replacement of missing teeth (Blanes, et al. 2007). The biological principles followed for implant placement have already been described by some authors and can be summarized in the concept of osseointegration, which is defined as the direct and structural connection between living and structured bone, and the surface of an implant subjected to a functional load (Brånemark, et al. 1977).

The earliest studies on this phenomenon were developed by Branemark in the 1950s, 1960s and 1970s, as well as by Schröeder (Schroeder, et al. 1981), who proved that the alveolar bone is capable of forming a direct connection with a bolt-shaped alloplastic material such as titanium after being placed on a surgically-created bed.

Since implantology's earliest stages, the growing interest of clinicians in this type of treatment has impelled research from the knowledge of the biological principles to the basis of osseointegration. A concept emerging from the studies by Johansson and Albrektsson is that osseointegration is a time-related phenomenon. Rigidity in bone-implant interface increases with time until reaching a high level 3 months after implant placement, and can increase progressively until 12 months after placement (Johansson & Albrektsson 1987).

The time necessary for implant osseointegration is variable, as it depends on a series of

factors that in turn depend, in one hand, on the bone and in other hand, on implant features. According to Branemark's (Brånemark, et al. 1977) protocol, waiting time for implant loading traditionally ranged from 3 to 6 months, depending on the implant's maxillary or jawbone position. The implants used back then were made of commercially-pure titanium obtained by bar mechanization, and their surface topography resulted from their drilling process and their subsequent electrolytic polishing, thus being known as smooth or mechanized surface.

The implant's surface features have been proven to influence the healing of the bone surrounding it (Buser, et al. 1991), and the use of rough surfaces -as proven by Beagle's histological studies on dogs- showed that osseointegration can be achieved in a 6-week period under normal conditions with rough surfaces obtained through subtraction methods (Abrahamsson, et al. 2004).

The morphology of these surfaces is involved in a series of biological events occurring after implant placement, which range from protein adhesion to periimplant bone mineralization and remodelling. These phenomena are favored by a particular surface roughness, thus allowing quicker osseointegration, which -from a clinical viewpoint- grants space for prosthesis placement within shorter time-periods. Immediate or early implant loading is a procedure that has been back in use with good medium-to-long-term results in the last years (Strub, et al. 2012). This is partly due to the use of implants with a more osteophilic surface, which allows maintaining implant stability more effectively throughout the first weeks of osseointegration. Reduction in implant primary stability due to initial bone resorption is counterbalanced by quicker bone neoformation, which leads

to increased secondary stability and more predictable osseointegration.

Implant surface treatment is aimed at providing it with some particular features involving an excellent biological response in the surrounding tissue. There are several methods for dental implant surface treatment such as mechanizing, electro-polishing, plasma-spraying coating, acid etching, surface oxidation, ionization, phosphate deposit techniques in some apathetic cases, or any combination of them (Avila, et al. 2009).

Implant surfaces can mainly be classified into three main categories according to their biological response: bioinert, osseoconductive and bioactive surfaces. The first are those around which bone healing occurs from the bone to implant surface with a slow healing process. The second are characterized by the fact that their surface morphology allows them to produce bone neoformation on implant surface and the bone starts forming from the surface to the periphery. These can present different roughness degrees and/or topographies that favor interaction with the proteins that promote migration of osteoblast precursor cells depending on their surface processing received.

Bioactive surfaces are those around which rapid bone neoformation occurs from implant surface, and are characterized by their surface showing -apart from different roughness degrees- some bioactive molecules or growth factors that induce bone formation according to different action mechanisms.

A bioactive implant surface -recently developed and based on the experimental studies by Kim et al. (Kim, et al. 1996)- can imitate osteoblast's formation of the bone mineral part in its early stages. This is possible thanks to the development of a new thermochemical

treatment of titanium that creates a calcium phosphate layer once in contact with biological fluids and prior to the arrival of osteoblastic cells. The use of implants with this type of surface would allow -from a clinical point of view- quicker and more reliable osseointegration for cases of immediate or early implant loading.

2. FUNDAMENTALS

2. Fundamentals

2.1 Osseointegration of dental implants: past and current knowledge

The bone is a mineralized connective tissue particularly structured to bear mechanical loads. Direct and structural connection between the living bone and the surface of an implant subjected to functional load was defined as osseointegration by Branemark (Brånemark, et al. 1977). This phenomenon has been described and researched since the 1950s and still generates interest in modern implantology. The most widely researched alloplastic material for dental implant manufacture is pure titanium and its alloy Ti6Al4V, always bolt-shaped. Titanium presents good biocompatibility, resistance to corrosion, and excellent mechanical properties. Implant surface osseointegration is what allows the implant to be subjected to chewing loads, which are transmitted to the bone. Osseointegration as described by Branemark is a clinical concept referred more to the stability of the implant subjected to chewing loading and in close contact with the bone rather than to the true microscopic joint of bone tissue and implant surface. This joint is the consequence of the biological events that lead to the interaction of bone cells with implant surface after surgical trauma.

The bone reacts to implant placement with a healing process that is very similar to intramembranous ossification produced after bone fracture, except that the neoformed bone is in contact with the surface of an alloplastic material -the implant.

We can mainly recognize different biological events during implant-surrounding bone healing -protein resorption, clot formation, granulation tissue formation, provisional matrix formation, interface formation, apposition and bone remodelling.

2.1.1 Protein adsorption

In a first moment after dental implant placement, the latter is blood soaked and the present proteins will subsequently be absorbed by its surface. The degree of wettability of the implant surface plays a relevant role in blood protein adsorption, since it has been proven that either excessive hydrophilia -unlike generally thought- or hydrophobia hinders protein adsorption (Pegueroles, et al. 2012). Indeed, both highly hydrophilic and extremely hydrophobic surfaces allow no formation of a liquid drop with enough volume for proteins to be absorbed by the implant surface. Once blood can ideally soak implant surface, proteins (cytokines) can be absorbed and remain on the surface to work as a signal for the migration of osteoblastic cell lines, which will form the new bone around the implant and allow implant osseointegration. Subsequently, neutrophils and macrophages question the implant and -according to the formation, orientation and type of absorbed proteins (O'Brien, et al. 2008)- macrophages interact with implant surface and segregate a particular type and number of cytokines that can either gather the osteoblastic cells in charge of bone formation in direct contact with surface implant, or the fibroblast cell line that encapsulates the biomaterial in fibrous connective tissues and results in osseointegration failure. Protein adsorption occurs instantaneously, thus inhibiting direct cell-biomaterial contact. Indeed, after exposing the surface to contact with blood, adsorption time is around 5 seconds (Nygren 1996). Implant surface's nature of one layer

of absorbed proteins constitutes the key factor of cell response, since cells have been proven to depend on specific proteins to adhere themselves (Chatakun, et al. 2014). Particularly, osteoblasts demand specific interactions to adhere, proliferate and differentiate, and these interactions are defined by the number and type of proteins adsorbed in implant surface. Implant surface's chemical and topographic nature will determine protein adsorption and conformation in its surface (Pegueroles, et al. 2010).

2.1.1.1 Types of proteins

For osteoblasts to be able to onset bone formation around the implant, they must previously adhere themselves to implant surface. In vitro studies observed that these cells' adhesion depends on some specific proteins absorbed in implant surfaces such as fibronectin, osteopontin and vitronectin (table 1). The last protein, proved in in-vitro and in-vivo studies, as the one that usually predominates in cell adhesion processes, followed by fibronectin (Rivera-Chacon, et al. 2013). However, the latter usually acquires more and more relevance once cells onset their differentiation process (Petrie, et al. 2009).

Implant surfaces play a determining role in the first stages of cell adhesion, since it is their topographic and physicochemical features that are capable of inhibiting the adsorption of the proteins that facilitate the migration of the undesired cells that provoke implant fibrointegration. TGF-α is an example of this, since it is a protein that favors fibroblastic cell line adhesion (Aliuos, et al. 2014). Pegueroles et al. (Pegueroles, et al. 2010; Pegueroles, et al. 2012) proved in an in-vitro study that surface treatment of titanium dental implants with a specific size of alumina oxide (A6) improves fibronectin adsorption compared to smooth titanium surfaces.

Table 1. Proteins involved in osseointegration and their functions.

Fibronectin	binding of cells, integrins, heparin, gelatin and collagen
Vitronectin	cell-binding protein that binds collagen, plasminogen and heparin
Albumin	Transportation of proteins, and inhibition of growth of hydroxyapatite crystals
Alkaline phosphatase	hydrolyzation of the inhibitors of mineral deposition (Ca2+ transporter)
Osteonectin	Mediation of hydroxyapatite deposition
Osteocalcin	Regulation of osteoclasts' activity

2.1.1.2 Cell-protein interaction

Cells are capable of interacting with proteins by means of cell receptors known as integrins. However, integrin-protein interactions are completed through recognition of a particular amino acid sequence within a protein by the integrin. This is the case of the RGD amino acid (Arg-Gly-Asp) sequence present in adhesive proteins such as fibronectin. Integrin-protein interaction determines the regulation of multiple cell functions such as adhesion. Ramaglia *et al*. proved that osteoblasts change integrin's expression according to implant surface's chemical composition and roughness degree, where alumina sand blasted and acid etched surfaces showed greater expression relative to smooth surfaces (Ramaglia, et al. 2011).

After this first protein adsorption stage, the arrival of polymorphonuclear neutrophils and macrophages to the implant surface occurs. These generate a cascade of intercellular signaling that shall derivate in implant acceptance or refusal according to the recruited cells.

2.1.2 Blood clot formation

Some minutes after implant insertion into the bed, a blood clot forms between the implant surface and the bone walls of the created bed. This mainly contains red blood cells, platelets and macrophages in a fibrin scaffold. During the first days a series of cytokines or growth factors (PDGF, TNFα, TGFα, TGFβ, FGF, EGF) are released to stimulate healing of the surgical wound gathering different cell lines. Two to three days after implant placement, leukocytes and macrophages complete 'cleaning' tasks through the phagocytosis process and the blood clot is simultaneously deconstructed through fibrinolysis to leave space for new blood vessels.

2.1.3 Granulation tissue formation

Four days after placement, blood vessel growth produces a granulation tissue that occupies the space between the implant and the bone. This tissue is characterized by the presence of non-differenced mesenchymal cells around vessel structures in a fibrin scaffold. Surgical bed preparation -due to tissue trauma itself, which releases specific cytokines such as BMP2 and BMP4- induces the differentiation of non-differentiated mesenchymal cells in the bone marrow and perivascular (pericytes) firstly in pre-osteoblasts and subsequently in mature osteoblasts.

2.1.4 Provisional matrix formation

Osteoblastic cells physically move in the space between the bone and the implant, and their migration is guided by the fibrin scaffold. In osseoconductive surfaces such as, for instance, those obtained by blasting and acid etching, cells adhere themselves to the

proteins absorbed in implant surface and start forming a provisional bone matrix (Davies 1998). Osteoblasts are incapable of producing matrix and move simultaneously, so they stop migrating along the fibrin scaffold once they have started to produce the bone matrix. If the fibrin scaffold is removed from the implant surface during migration, osteoblasts will not reach it directly and no bone formation will therefore take place from the implant surface (Davies 1998). However, fibrin adhesion to implant depends on the implant's type of surface. On those of smooth or mechanized titanium -due to the weak adhesion force- fibrin is detached from implant surface during osteoblast migration, while in rough surfaces -where fibrin's adhesion is stronger- cells can easely migrate through the fibrin scaffold to reach the implant.

Thus, two main types of osseointegration can be distinguished: contact osteogenesis as described by Osborn *et al*. (Osborn & Newesely 1980), in which progressive contact between the bone neoformed from the periphery to the implant bed; and the bone neoformation described by Davies *et al*. (Davies 1996), where osteoblasts that can migrate to the implant surface through the fibrin scaffold form new bone from the implant back to the bed walls.

2.1.5 Bone apposition

Bone neoformation starts in early healing stages, and after 7 days a provisional matrix rich in collagen fibers, vascular structures, osteoblasts and some neo-formed bone area (bone apposition) begin to form (Berglundh, et al. 2003). Some growth factors such as BMP 2 and 4 take part by stimulating the later migration of non-differentiated mesenchymal cells and by differentiating osteoblasts (BMP 7). After 14 days the implant-bone gap is occupied

by neoformed or woven bone, which is rich in collagen fibers, vascular structures and osteoblasts, which form a reticular structure. In this stage osteoblasts produce the interface bone and can be found, in parallel to the surface, in the osseoconductive surfaces in contact with the implant. However, bone neoformation on implant surface in early stages seems more characteristic of rough surfaces than of mechanized titanium (Johansson & Albrektsson 1987). At the centre of the neoformed bone tissue some osteocytes can be observed while osteoclasts appear on bed bone surface, thus indicating necrotic bone resorption. During the apposition process, bone structure progressively transforms from reticular to lamellar. Reticular bone is fragile and poor in calcium phosphate crystals, and transforms firstly into bone rich in parallel fibers and then into lamellar bone, which is mineralized tissue capable of withstanding mechanical loadings. The duration of the bone apposition process can vary according to implant surface type, being around 4 weeks on sand-blasted and acid-etched rough surfaces (Herrero-Climent, et al. 2013).

2.1.6 Bone Remodeling

Once formed, peri-implantary bone undergoes a remodelling process in which parallel fiber bone is mainly substituted by lamellar bone and bone architecture progressively adapts itself to its functional load (Frost 2004). In this stage osteoblasts and osteoclasts work synergically, apposing and reabsorbing bone according to functional needs. The bone-implant interface is under continuous remodelling and close contact between peri-implant bone and the implant is essential to keep it functioning in the long-term.

2.2 Chemically-bonded bone to the implant: a new concept of osseointegration

Osseointegration, as described by Branemark (Brånemark, et al. 1977) is a clinical concept referred more to the stability of the implant to occlusal forces and in close contact with the bone rather than to a true microscopic surface bond of the bone tissue to the implant surface. The bone reacts to implant placement with a healing process that is very similar to intramembranous ossification produced after bone fracture, except that the neo-formed bone is in contact with the surface of an alloplastic material -the implant.

Originally implants had a smooth or minimally rough (Sa < 0.5 µm) machined surface, with characteristic repeated irregularities, showing a clear orientation across the implant (anisotropic surface). Over the years new improved surfaces were released with greater roughness to facilitate cell adhesion and thus accelerate implant osseointegration.

Subtraction methods such as aluminum oxide (Al2O3) particle blasting and acid etching provide a surface topography characterized by concavities that form peaks and valleys that increase osteoconduction and, consequently, quicker bone growth with increased bone adhesion force (Herrero-Climent, et al. 2013).

Many studies have shown a greater ratio of bone surface in contact with the implant surface of rough implant surface compared to machined implant surface, leading to an improved and faster osseointegration (Aparicio, et al. 2011a). These results may be explained by the apparent different cell response in the early stages of osseointegration. A rough surface will enhance the wettability and the protein absorption of the implant

surface favoring a greater cell migration and adhesion (Pegueroles, et al. 2012).

Davies et al. stated that the more favorable osseointegration of rough surfaces compared to smooth is due to the greater adhesion force of the clot's fibrin scaffold (Davies 1998). This scaffold allows osteoblast migration towards the implant surface before these cells start to produce calcium phosphate crystals (hydroxyapatite). If fibrin's adhesion capacity to implant surface exceeds the threshold, it shall be enough to allow osteoblasts to migrate through the scaffold and get in contact with implant surface. However, in mechanized titanium surfaces, no sufficiently stable bond occurs between it and fibrin so as to withstand the 'weight' of osteoblasts during their migration, thus producing separation between the implant and the fibrin scaffold. In this situation osteoblasts do not reach implant surface and new nuclei of bone formation will be placed closer to implant bed and far from implant surface. On the contrary, the fibrin scaffold on rough surfaces does not set free from the implant during osteoblast migration due to its tighter surface bond, allowing osteoblasts to reach the surface and start the bone apposition process. Thus, difference can be made between bioinert surfaces (smooth surfaces) in which 'contact osseointegration' occurs (Osborn & Newesely 1980), progressive bone apposition from bed periphery to implant surface; and, on the other hand, osseoconductive (rough surfaces) surfaces, where the 'bone neoformation' can be observed, bone apposition contemporarily from implant surface and bed (Davies 1996).

A new concept of implant surface is the bioactive surface; characterized by some bioactive molecules or growth factors that induce bone formation according to different action mechanisms.

Chemically modified sand-blasted/acid etching surface, which has been developed in the last years, is one example of bioactive surface that promotes a faster bone healing (Buser, et al. 2004; Schwarz, et al. 2007). However no chemical bonding between titanium and surrounding bone has been observed due to the fact that commercially pure Titanium is a bioinert material without bone–bonding ability, thus the interaction between the metal and the hard tissue does not involve a chemical bond (Johansson & Albrektsson 1987).

The thermo-chemically-treated surface, as proven by experimental studies (Nagano, et al. 1996; Fujibayashi, et al. 2001), provides the implant with a chemically bonded hydroxyapatite layer with the purpose, as other bioactive surfaces, to accelerate bone healing during the critical period for osseointegration therefore producing better results with advanced clinical procedure as immediate or early loading. This method, as discussed above, provides the implant with chemically bonded hydroxyapatite layer, which produces a bone healing and mineralization, both from the implant surface and the bone-bed (Gil, et al. 2014b). Once osteoblasts start bone apposition and mineralization by producing and production of calcium phosphate, a chemical bonding between the hydroxyapatite layer of the implant surface and the new osteoblast-produced hydroxyapatite is produced. Several studies have confirmed high bond strength of the hydroxyapatite layer on the implant surface to the implant and also an increased resistance of the implant to the pull-out test in-vivo has been observed (Aparicio, et al. 2011a; Miyazaki, et al. 2002; Kato, et al. 2001).

Therefore, the classical concept of osseointegration described as 'intimate contact between living well-structured bone and the implant surface'(Brånemark, et al. 1977),

seems to start changing to a more biomimetic concept of a 'chemical bonding between living well-structured bone and the implant surface'.

2.3 The evolution of hydroxyapatite coatings

Osseointegration is a time related biological process that allows dental implant to be subjected to functional loading, and implant surface seems to be one of the most relevant factors to obtain a predictable bone healing (Wennerberg & Albrektsson 2009).

Recent years have witnessed a progressive development of dental implants and many resources have been invested to improve implant surfaces and improve clinical results when using immediate and early loading procedures.

The use of coatings with similar composition of the human bone is an attractive strategy in the development of bioactive surfaces, which provide an accelerated osseointegration during the earliest healing stages. Particularly, calcium phosphate apatite has the same chemical composition as the mineral bone phase, so that complete acceptance by the organism and no inflammatory reaction occurs (van Oirschot, et al. 2016b).

Many researchers have applied coatings on titanium implants by different techniques such as hydroxyapatite plasma spraying (Mertens & Steveling 2011). As demonstrated by clinical studies (Cannizzaro, et al. 2013), this treatment produced a quicker osseointegration at early stages after implant placement but an accelerated bone loss due to a bacterial micro-leakage between the hydroxyapatite layer and the titanium has been

observed in the long term (Yang, et al. 2005). Furthermore, additive techniques such as hydroxyapatite plasma spraying do not allow the formation of crystalline apatite like in human bone, but amorphous calcium phosphate due to high elaboration temperatures (Liu, et al. 2004). The properties of this layer are not considered appropriate for dental implants, since they are extremely soluble and titanium only achieves mechanical retention, not true adhesion.

2.3.1 A new method to attain bioactive titanium

Bioengineering studies have recently proven that alternatives methods to obtain phosphate calcium coating with higher homogeneity and chemical stability are possible (Kim, et al. 2007). These methods, propose apatite growth directly bound to the surface as a result of a precipitation reaction in the human body fluid, thus achieving true chemical adhesion and layer-thickness control.

Human body fluid is supersaturated in apatite even under normal conditions and the prerequisite for apatite formation on an artificial material in a living body is the presence of functional groups that could be an effective site for apatite nucleation on its surface (Kokubo, et al. 2004).

Based on this principle, Kokubo (Kokubo, et al. 1990) proposed a method to provide implants with a bioactive surface based on a thermo-chemical procedure where titanium, is first chemically treated with alkali solutions and then subjected to heating at high temperatures. The aim of this treatment is to reproduce the in vivo formation of crystalline hydroxyapatite on implant surface therefore accelerating bone healing and osseointegration.

The chemical treatment, as described by the author, consist of soaking the implant in a 5-10M NaOH aqueous solution at 60°C for 24h and then a gentle washing with distilled water. The thermal procedure consists of heating the implants in an electrical furnace to various temperatures below 800°C at a rate of 5°C·min-1, kept at the temperature for 1h and allowed to cool to room temperature in the furnace.

Titanium is generally covered with a thin TiO2 (titanium oxide) passive layer, which provides chemical stability and durability. During the soaking phase of the chemical treatment, the TiO2 layer gets in contact and reacts with the NaOH (sodium hydroxide) solution forming a hydrated TiO2 gel, which can be stabilized as an amorphous sodium titanate by a suitable heat treatment.

Sodium titanate layer is expected to form many Ti-OH- groups on its surface in the living body via the ion exchange of its Na+ ions from the surface with H3O- ions in the surrounding body fluid. These Ti-OH- groups make a highly negatively-charged surface that initially combine with positive Ca2+ ions -coming from human plasma- to form amorphous calcium titanate in the surface environment, and later the calcium titanate combines with the negative phosphate ions to form amorphous calcium phosphate, which, at the SBF-pH of 7,4 (simulated body fluid ph -7,4-), eventually transforms into bone-like apatite.

Indeed, nucleation of hydroxyapatite is the consequence of a reaction of precipitation between titanate (which contains Na+ ion) and serum which is normally saturated with Ca2+(calcium) and (PO4)3- (phosphorus) producing calcium phosphate (Ca3 (PO4)2) thus Hydroxyapatite, as showed in figure 1 and figure 2.

Simulated body fluid (SBF) has been used in in-vitro experimental studies to reproduce human plasma and ideal ions concentration has been recently described by Kokubo et al. (Kokubo, et al. 2004). Table 2 shows ions concentration in simulated body fluid and blood.

Table 2. Simulated body fluid and blood plasma ions concentration as described by Kokubo et al. (mM)

Ions	*Na+*	*K+*	*Mg2+*	*Ca2+*	*Cl–*	*HC03–*	*HPO24–*	*S024–*
SBF	142	5	1,5	2,5	147,8	4,2	1,0	0,5
BLOOD PLASMA	142	5	1,5	2,5	103	4,2	1,0	0,5

SBF: Simulated Body Fluid.

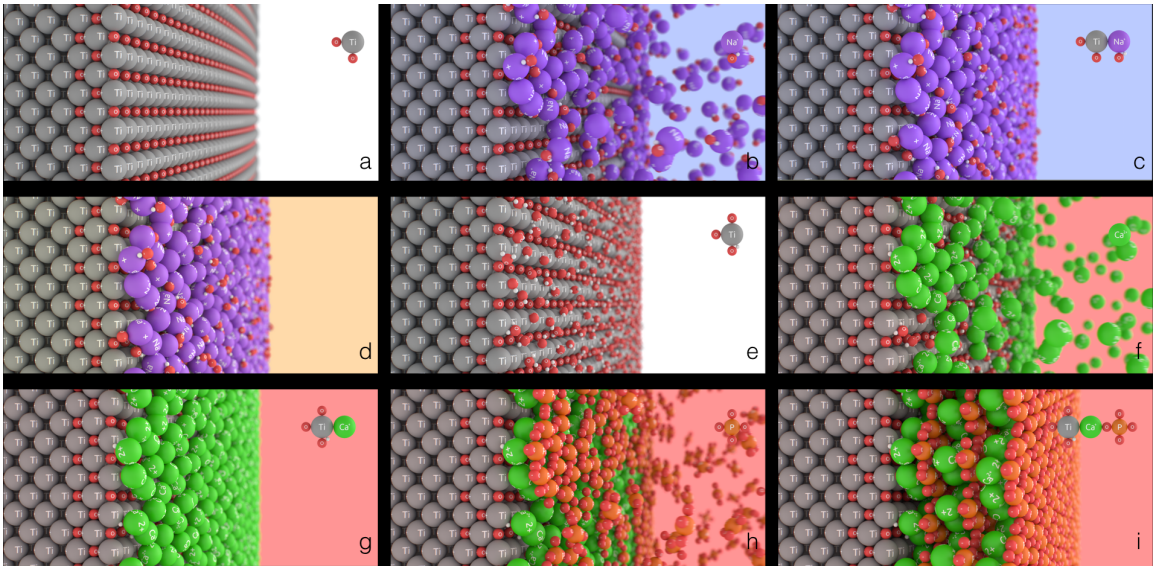
Nucleation of hydroxyapatite at implants with thermo-chemical treatment submerged in SBF has been observed by electronic microscope and x-ray diffraction by several authors (Kokubo, et al. 1990; Kim, et al. 1996; Liu, et al. 2004; Pattanayak, et al. 2011) and others have confirmed these results (Aparicio, et al. 2007b; Aparicio, et al. 2011b).

This method can be said to provide a biomimetic surface, since the implant-covering sodium titanate layer can, thanks to Na+ ion bioactivity, and once it gets in contact with biological fluids, form on its own a hydroxyapatite layer without the need of osteoblasts taking part.

Once the hydroxyapatite layer on implant surface has formed, osseointegration process continues with the selective adsorption of fibronectin from human plasma followed by migration, adhesion, proliferation and differentiation of osteoblasts, which starts bone

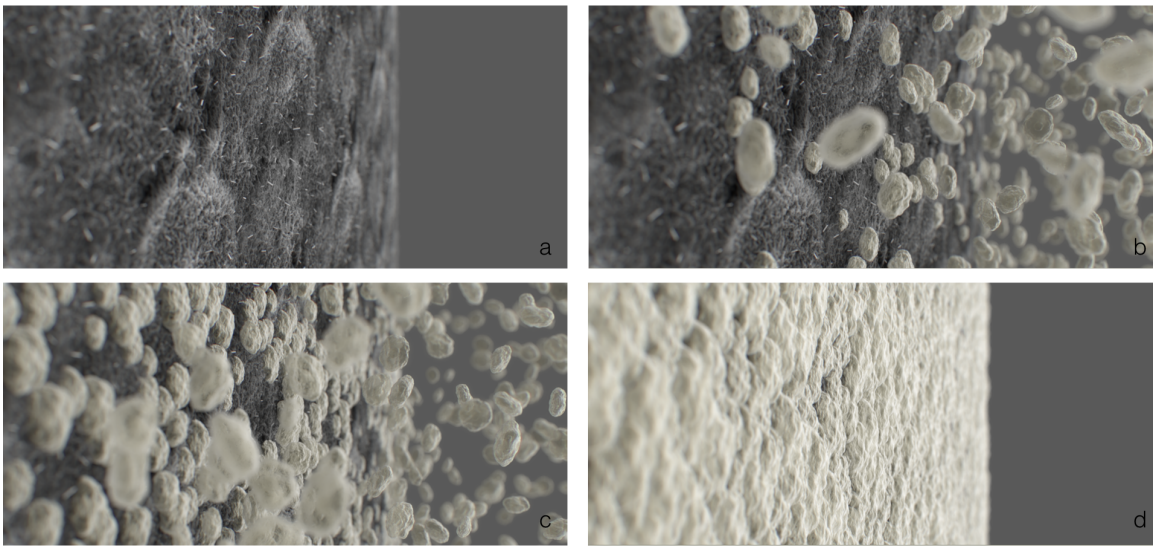
apposition on the surface.

Figure 1.



Biochemical sequence of Calcium Phosphate formation on the thermo-chemically modified surface. A) Titanium oxide, b) Soaking in NaOH solution, c) Formation of sodium titanate hydrogel, d) Heating treatment, e) Elimination of Na+ ion, f) Calcium migration from human plasma, g) Calcium adsorption, h) Phosphate migration from human plasma, i) Calcium phosphate formation on the surface.

Figure 2.



Schematic images of bone formation on the surface. A) Selective adsorption of fibronectin from human plasma, b) Migration, adhesion and proliferation of osteoblasts, c) Differentiation of osteoblasts and bone apposition on the surface.

2.3.2 The Contac-Ti Surface (2-Step Method)

Contac-Ti is the evolution of Shot Blasting surface (Klockner Implant System, SOADCO, Andorra), which was based on micro roughness obtained by grit-blasting with alumina particles and subsequent acid etching. Excellent clinical results have been demonstrated with the use of this surface by significantly increasing the BIC area as compared to an untreated surface (Herrero-Climent, et al. 2013).

It is well known that moderately rough surfaces (Sa = 1-2 μm) obtained by means of grit-blasting and acid-etching provide a better bone healing (Albertini, et al. 2015) and has also been observed that roughness can improve biological response of bioactive titanium surfaces (Aparicio, et al. 2007b).

The new surface is the result of the combination of subtraction procedures to attain a

moderately rough surface and a thermo-chemical method based on the principles described by Kokubo et al. (Kokubo, et al. 1990).

A 2-step procedure, in which first girt-blasting and acid-etching and then a thermo-chemical treatment is performed on machined titanium to obtain the Contac-Ti surface.

2.3.2.1 The first step: grit blasting/acid etching treatment

Combination of grit blasting and acid etching treatment, which consists of first bombarding a surface with a myriad of small abrasive biologically-inert ceramic particles and then soaking the implant in an corrosive-acid solution, is one of the most frequently used treatments for obtaining a rough surface of dental implants (Jones 2001).

There is a consensus in the literature about the improvement of osteoblastic response provided by grit-blasting/acid-etching treatment (Anselme 2000; Boyan, et al. 2001). Moreover, a better long-term in-vivo response is achieved when the surface roughness increases since the percentage of implant in direct contact with bone increases as well as loads and torques for extracting implant from bone (Gotfredsen, et al. 2000).

Improvements in fibronectin adsorption at implants which received grit blasting treatment with a specific size of alumina (A6) has been demonstrated by in-vitro studies (Pegueroles, et al. 2010; Pegueroles, et al. 2012) as well as a better osteoblasts response in terms of integrin expression at implants with grit-blasted/acid-etched surfaces (Ramaglia, et al. 2011).

Commercially pure grade IV titanium (according to ASTM F67) is used as substratum to obtain the Contac-Ti surface (Klockner Implant System, SOADCO, Andorra) and particles

smaller than the ones used for the Shot Blasting since lower roughness value was pursued. During the grit-blasting treatment, 300 µm Aluminium oxide particle size is used; in a second stage acid-etching procedure with HCl is performed to attain a 1,74 Ra value of the implant surface.

2.3.2.2 The second step: the thermo/chemical treatment

The second step to attain Contact-Ti is the thermo-chemical treatment for rough titanium surfaces (Aparicio, et al. 2002; Nogueras-Bayona, et al. 2004).

It consists in submerging the metal in a NaOH solution at 60º C for 24 hours, then rinsed with distilled water and dried at 40º C for 24 hours and finally it is submitted to a thermal treatment in a tubular furnace at 600ºC for an hour and finally subjected to a cooling process. After completing the surface treatments, all implants are ultrasonically cleaned in soap and distilled water for 10 min, dried with nitrogen gas, and sterilized in ethylene oxide at 37 ◦C and 760 mbar for 5 hours.

The main difference between this treatment and the one previously described (Kim, et al. 1996) is that the conditions of reagent concentrations, temperatures changes and heat treatment times have been optimized for moderately rough titanium surfaces as well as heating and cooling rates.

2.3.3 Microscopical characterization of the 2-step surface

2.3.3.1 Surface roughness

Surface characterization of Contact-Ti compared with machine titanium has been recently analyzed by our research group using an optical profiling system device (Optical Profiling System, Wyko NT9300, Veeco Instruments, EEUU) and data analysis means of Wyko Vision 232TM software (Veeco). 10 measurements have been performed and Sa, Sq, Sz and S area index topographic parameters have been used to describe surface characterization. Values of a 1,74 Sa, 2,20 Sq, 16,74 Sz and 1,03 S area index have been obtained from the analysis as shown in table 3.

Table 3. Roughness values of Contac-Ti

SURFACE	*Sa (µm)*		*Sq (µm)*		*Sz (µm)*		*S area index*	
	Mean	**S.d.**	**Mean**	**S.d.**	**Mean**	**S.d.**	**Mean**	**S.d.**
Machined	0,15	0,01	0,19	0,02	3,47	1,53	1,04	0,01
Contac-Ti	1,74	0,07	2,20	0,09	16,74	1,11	1,03	0,01

Machined: machined titanium, Contac-Ti: surface attained after the 2-step treatment, S_a: average surface roughness, S_q: quadratic mean surface roughness, S_z: maximum peak/valley surface, S area index: index between surfaces, homogeneity of the surface.S.d.: standard deviation.
S.D.: standard deviation. *Statistically significant difference (p 0.005).
from Aparicio et al., 2011.

Grit-blasting and acid-etching procedure as described above produces a moderately rough surface with good homogeneity as described by Albrektsson (Albrektsson & Wennerberg 2004) and the additional thermo-chemical treatment seems not to alter surface topography (Aparicio, et al. 2011b).

2.3.3.2 Surface Hydrophilia

Implant surface can be defined as hydrophilic when it's characterized by a high wettability which is the process by which a drop of liquid spreads over the surface as a result of the interaction of adhesive forces, between liquid and substrate, and internal cohesive forces of the liquid. "The contact angle (CA) is a technique used to determine the wettability of materials and, as the name suggests involves determining the angle between a drop of liquid in contact with the surface a solid. This value depends on the relationship between the adhesive forces between the liquid and the solid and liquid cohesive forces. When the adhesive forces with the solid surface are greater than the cohesive, the contact angle is less than 90 degrees, so that the liquid wets the surface.

Our research group performed an analysis of the wettability of Contac-Ti compared with machined and other rough surfaces by measuring the contact angles so that information above hydrophilic and hydrophobic characteristics could be obtained.

A device for contact angle (CA) measurements and drop dispenser (DATAPHYSICS OCA 15 model) has been used to obtain CA values, 5 measurements for each surface were made and a drop of 1 µl of pure water (Milli-Q, Merck Millipore) was used. A constant of 3 seconds was the time the water drop laid on the surfaces before measurements were performed.

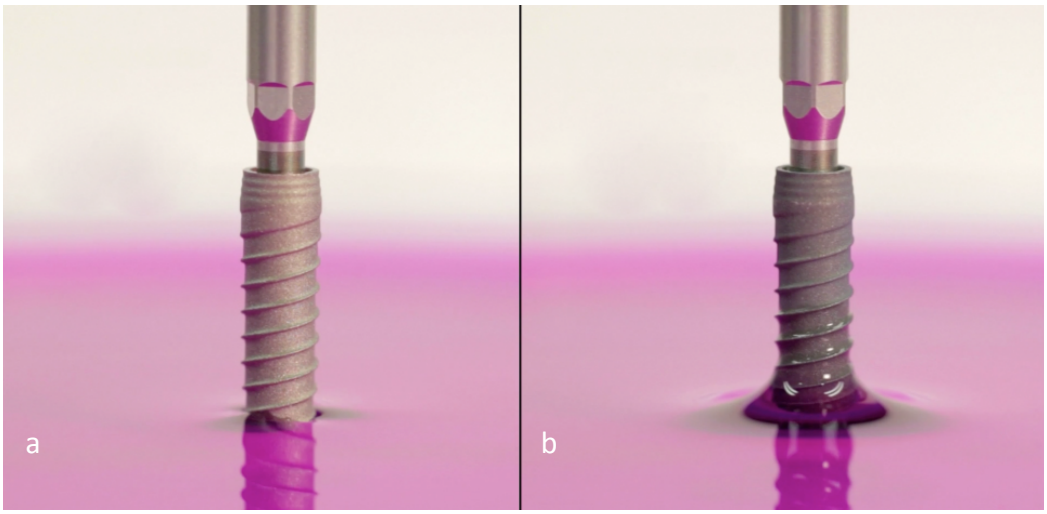
CA higher than 90 have been registered on the machined titanium showing the hydrophobic behavior of this surface, while all the other surfaces have hydrophilic characteristics (table 4). However, results show the new surface to have the lower value of contact angle, therefore the highest wettability (figure 3). These hydrophilic

characteristics are able to promote protein adsorption and cells adhesion, which contribute to accelerate osseointegration (Pegueroles, et al. 2012; Albertini, et al. 2015).

Table 4. Contact angle measurements of machined titanium, Contac-Ti surface, highly rough surface and extremely rough surface

Surface	*Machined*	*Contac-Ti Ra 1,5*	*Ra 2,5*	*Ra 3,5*
Mean	90.88	**77.70**	80.92	87.94
S.d.	5.90	3.09	0.85	1.62

Figure 3.



Implant surface Wettability. a) Grit-blasted/acid etched surface: low wettability due to the hig contact angle; b) 2 Step surface (Contac-Ti): high wettability due to the low contact angle with the liquid.

2.3.4 Influence of the 2-steps treatment on mechanical properties of titanium

The lack of osseointegration due to several factors in the early stages after implantation is the most common form of implant failure whilst peri-implantitis and implant fracture represents the most common causes of implant loss in the long term (Berglundh, et al. 2002). Therefore, fatigue is a very important aspect to be taken into account when considering the long-term behavior of dental implants. Fatigue of a material is closely related to the surface structure, meaning that all these surface modification methods conducted to promote a better osseointegration may affect the fatigue performance of the implant. Furthermore, it has to be considered that post-thermal processes may alter the microstructure of the implant material.

Gil et al. (Gil, et al. 2014a) carried out an in-vitro study where mechanical properties of 2-step-treated-implants were assessed. Fatigue test were carried out at 37 ºC on 500 dental implants, residual stresses and fatigue-crack nucleation were analyzed comparing machined, grit-blasted and 2-step surfaces. Although a minimal decrease (10%) in fatigue life of 2-step implants in comparison with grit-blasted was registered, a high fatigue limit of 315 N was registered and all of the implants showed fractures at 15 106 cycles. The slight decrease was due to the oxygen diffusion inside the titanium of the dental implant with thermo-chemical treatment, which significantly reduced the ductility of the alloy.

According to previous works that compared apatite coatings obtained by different methods like plasma spray, laser ablation, the coatings did not last longer than 106 cycles in any of the cases, being the rapid propagation of the crack either in the coatings or at the interface with the metal implant the main cause of failure (Chang, et al. 1999; Geesink,

et al. 1987).

The 2-step procedure, obtained by grit-blasting and thermo-chemical treatment reaches a 10 times higher fatigue life in comparison with classical plasma-spray apatite coating. This encouraging results, which has to be confirmed by clinical studies, make implants treated with this new technology allows a great balance in an excellent between enhanced osseointegration and long-term fatigue life.

2.3.5 Adhesive properties of the hydroxyapatite coating

Hydroxyapatite coating is a highly osteoconductive material and allows a predictable osseointegration of dental implants in a short period of time. Nevertheless one of the most critical considerations of hydroxyapatite-coated implants is the adhesion of the apatite layer to the titanium. Plasma-spray was used in the past to provide the apatite layer over the implant surface, however only a scarcely-adhered to titanium amorphous calcium phosphate was produced with this technology leading to a progressive loss of osseointegration due to a bacterial micro-leakage between titanium and apatite coating (Yang, et al. 2005).

The thermo-chemical treatment, as discussed previously, provides the implant with a chemically bonded hydroxyapatite layer by means of a chemical reaction of precipitation of calcium phosphate from ions-saturated human plasma. Adhesion force between implant titanium and the hydroxyapatite layer attained by the 2-step treatment have been investigated in the last years by several authors.

Aparicio et al. (Aparicio, et al. 2011a) assessed the adhesion strength of the apatite-coating layer attained by plasma-spray and by the 2-step procedure with different grinding agents after immersion in SBF. The adhesion strength for the plasma-sprayed apatite layers was around 170 mN with a mean thickness of 20 µm, which were statistically lower than those measured for the 2-step samples, with mean values of 470 mN and a mean thickness of the apatite layer of 15 µm.

Similar results have been attained by other authors (Nogueras-Bayona, et al. 2004; Miyazaki, et al. 2002) which demonstrate that the bonding strength of apatite layers formed after immersion in SBF of thermo-chemically-treated samples is significantly higher than those of plasma-sprayed hydroxyapatite layers (table 5). These results confirm the thermo-chemical treatment provides a chemical bonding between titanium and hydroxyapatite layer.

Table 5. Mean adhesion force values of different titanium substrates with apatite layers

Samples	***Force adhesion* ± S.D. (mN)**
Ti-2-steps	451±124
Ti-PS	160±56*
AL6-2-steps	501±90
AL6-PS	190±65*
SI6-2-steps	-
SI6-PS	178±66*

Ti-2 steps: machined titanium + thermo-chemical treatment; Ti-PS: machined (lathe cut) commercially pure titanium surface + Plasma-spray treatment; AL6-2-step: titanium grit-blasted with Al2O3 particles with a mean diameter of 425-600 µm at a pressure of 2.5 MPa + thermo-chemical treatment; AL6-PS: titanium grit-blasted with Al2O3 particles with a mean diameter of 425-600 µm at a pressure of 2.5 MPa + Plasma-spray treatment; SI6-2-step: titanium grit-blasted with SiC particles with a mean diameter of 425–600 µm at a pressure of 2.5 MPa + thermo-chemical treatment; SI6-PS: titanium grit-blasted with SiC particles with a mean diameter of 425–600 µm at a pressure of 2.5 MPa + Plasma-spray treatment.

2.3.6 Biological behavior of the thermo-chemically treated surface

2.3.6.1 Cellular response

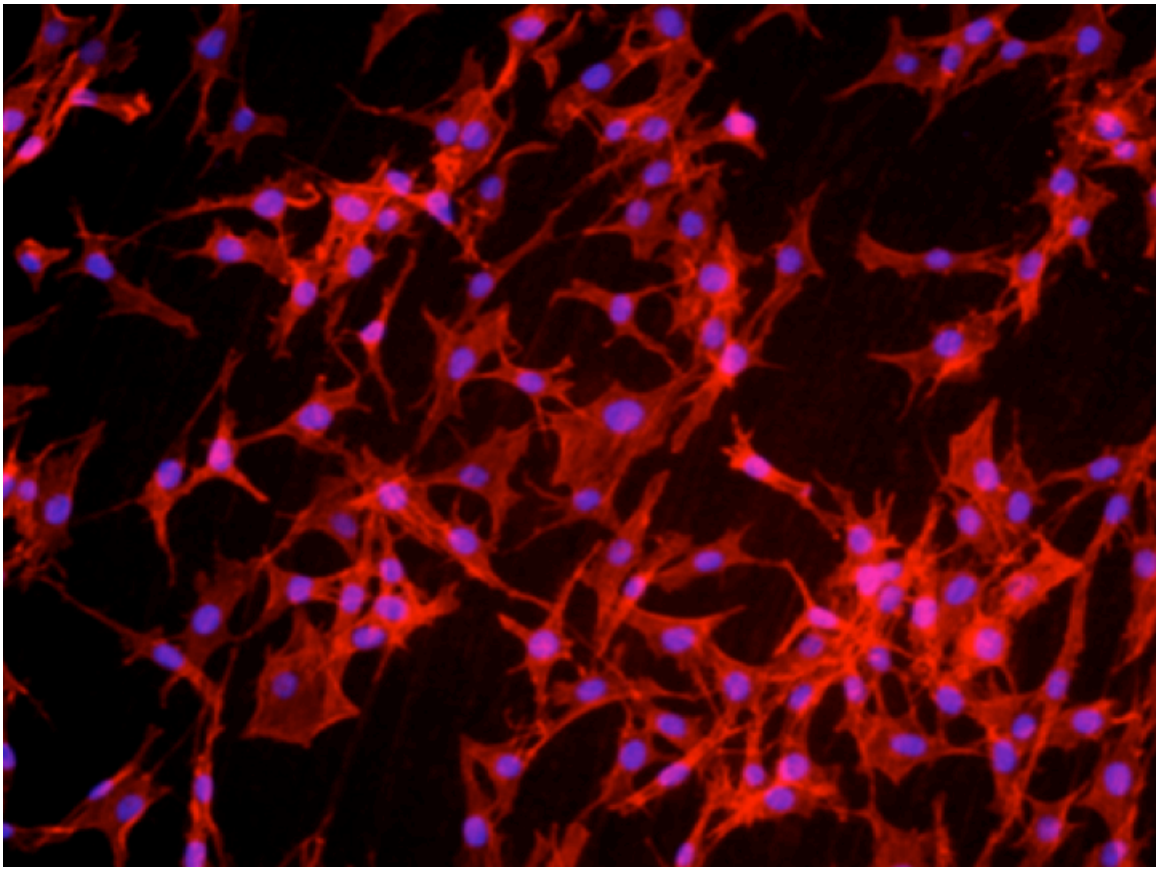
Osteblasts are the cells responsible for bone apposition and mineralization, thus the main cells implicated in the osseointegration process. The assessment of human osteoblasts response (proliferation, differentiation, and cell morphology) to implant surfaces is on one of the most used in-vitro methods to investigate the potential of osseointegration of dental implants.

Aparicio et al. in 2002 (Aparicio, et al. 2002) investigated in-vitro biological response as proliferation, differentiation -ALP (alkaline phosphatase) activity- and cell morphology by means of environmental scanning electron microscopy of human osteoblasts on machined, grit-blasted and 2-step-treatment titanium. Cells response was assessed by the cell count (proliferation), the analysis of alkaline phosphatase activity (differentiation) and the observation of cell morphology with environmental scanning electron microscopy (ESEM). An increased cell proliferation after 1 day was registered on 2-step-treated surface compared with machined and grit-blasted ones showing the bioactive surface to provide better cell adhesion probably due to an augmented initial protein adsorption. No statistically significant difference at 3 and 7 days between the samples was registered and a lower proliferation of 2-step surface was shown at 7 and 14 days confirming the good behavior and the higher differentiation of the cells, which -as described by other authors- is reciprocally related to the late proliferation process (Anselme 2000).

ALP-activity was always higher (statistically significant) in the thermo-chemical treated surfaces, indicating stimulation of human-osteoblasts differentiation because of the

bioactive surfaces and this result confirms the conclusions of other authors (Nishio, et al. 2000; Sandrini, et al. 2007) (figure 4).

Figure 4.



ESEM picture showing human osteoblasts on the thermo-chemically treated implant surface. The shape and distribution of the cells on the surface shows the good differentiation achieved.

Nisho et al. (Nishio, et al. 2000) investigated the behavior of rat bone marrow cells on commercially pure titanium (Cp Ti), thermo-chemical treatment (Tc Ti) and thermo-chemical treatment incubated in a simulated body fluid (SBF) to deposit crystalline hydroxyapatite on the surface (Tc AP Ti). The alkaline phosphatase (ALP) activity of the cells cultured on Tc AP Ti was significantly higher at day 7 and day 14 than the ALP activity

observed for the other titanium surfaces. At day 14, the ALP activity on Tc Ti was significantly increased compared with the ALP activity on Cp Ti. Northern blot analysis of alpha1(I) collagen mRNA was assessed revealing that expression of osteocalcin and alpha1(I) collagen mRNA was higher in the cells cultured on Tc AP Ti than the cells cultured on Tc Ti at day 14 and the cells cultured on Cp Ti showed the lowest mRNA levels. This study confirms that the thermo-chemical treatment provides the most favorable conditions for differentiation of bone marrow cells. The rough and bioactive surface obtained by a grit-blasting thermo-chemical treatment provided enhanced adhesion and differentiation of human osteoblast cells. This fact may play an important role in a rapid formation of the extracellular matrix and, consequently, in an accelerated short-term osseointegration.

Similar results have recently been reported by Quan et al. on bio-activated zirconia implants (Quan, et al. 2016). Zirconia implant disks were submerged in SBF for 1, 4, 7, and 14 days and statistically significant differences of ALP activity of cultured osteoblasts was observed between treated and non-treated samples at 9 days; cell attachment, proliferation, and differentiation of SBF-treated zirconia disks was superior to that of non-treated disks.

2.3.6.2 In-vivo results. Histological studies

Several animal studies which investigates bone healing around implants with the novel 2-step treatment have been carried out in the last years and the encouraging results attained by previous in-vitro studies have been confirmed.

The first histological study on implants coated with Kokubo method was conducted by

Nagano et al. in 1996 (Nagano, et al. 1996) where coated and non-coated polyethersulfone (PSE) discs were implanted in rabbit tibia. Mechanical analysis by means detachment test and histological measurements were obtained after sacrificing the animals. Differences in failure loads were statistically significant between coated samples and uncoated ones, with values at 8,16 and 30 weeks of 1.7 ± 0.35, 2.36 ± 0.53, 1.45 ± 0,48 kg in the first ones and 0.08 ± 0.06, 0.04 ± 0.03, and 0.023 ± 0.038 kg, in the second ones. Examination at SEM (scanning electron microscope) showed differences between the two groups of samples with a direct contact of bone to the plate at coated whilst areas of soft tissues were observed at uncoated. Authors claims apatite layer after 30 weeks seemed to have been incorporated to the bone after an osteoclasts-mediated resorption.

These results are in line with others from animal studies carried out by Fujibayashi (Fujibayashi, et al. 2001) and Nishiguchi et al. (Nishiguchi, et al. 2001; Nishiguchi, et al. 2003) where machined, porous and porous–apatite-coated cylinders were implanted in rabbit tibia and pull-out and histological analysis were assessed. Statistically significant differences were obtained after pull-out test between apatite-coated cylinders and machined ones; no apatite layer detachment was registered at histological examination.

In 2011 Aparicio et al. (Aparicio, et al. 2011b) conducted a study, in mini-pigs, comparing the new 2-steps treatment to a grit-blasted and acid etched surface, with a machined surface as control. Histological and histomorphometric analysis was performed at 2, 4, 6 and 10 weeks' time points, showing a new mineralized bone growth around the 2-step implants at only 2 weeks. The investigated surface reached the highest values of BIC (bone-to-implant contact) compared to the other samples, with 22% at 2 weeks, 55% at 4

weeks, 65% at 6 weeks and 52% at 10 weeks. The differences between the last three values and the first values were statically significant.

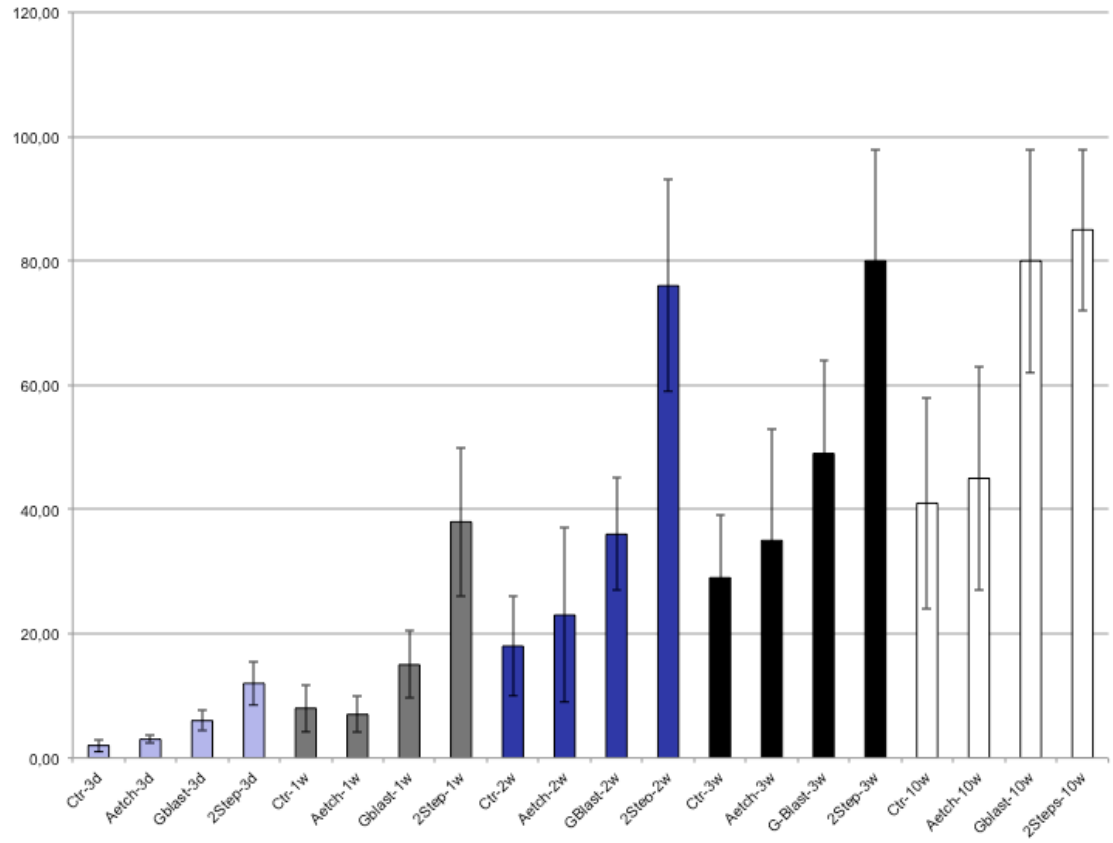
A similar study was recently undertaken by Gil et al. (Gil, et al. 2014b), in which three hundred twenty implants were used in a mini-pig model assessing the BIC %, surface composition, topography and wettability in a mini-pig animal experimental model, comparing the 4 surfaces previously described at 3 days, 1, 2, 3 and 10 weeks. Low BIC values for the acid-etched surface and the machined surface were obtained, while the results for the bioactive surface were significantly higher than all the other surfaces for all time points with exception to the alumina blasted surface at the 10 weeks' time point, where there was no statically significant difference (figure 5).

The surface presented surprisingly high osseointegration values in early healing stages after placement in this animal model, being around 75% and 80% 2 and 3 weeks, respectively, and 85% of BIC was achieved at 10 weeks. The 2-step surface was the only one that clearly showed extensive areas of bone neo-formation in direct contact with the implant after only one week after implantation (figure 6).

Van Oirschot et al. (van Oirschot, et al. 2016a) have recently investigated the influence of a bioactive hydroxyapatite and composite hydroxyapatite/bioactive glass coatings on the iliac crest of 8 goats. A total of 96 implants were placed and removal torque test and histomorphometrical evaluation were carried out after 4 weeks. Significant higher bone area attached to the implants and BIC% was registered for bioactive implants compared to grit-blasted/acid-etched ones showing the bioactive surface treatments enhanced the bone healing.

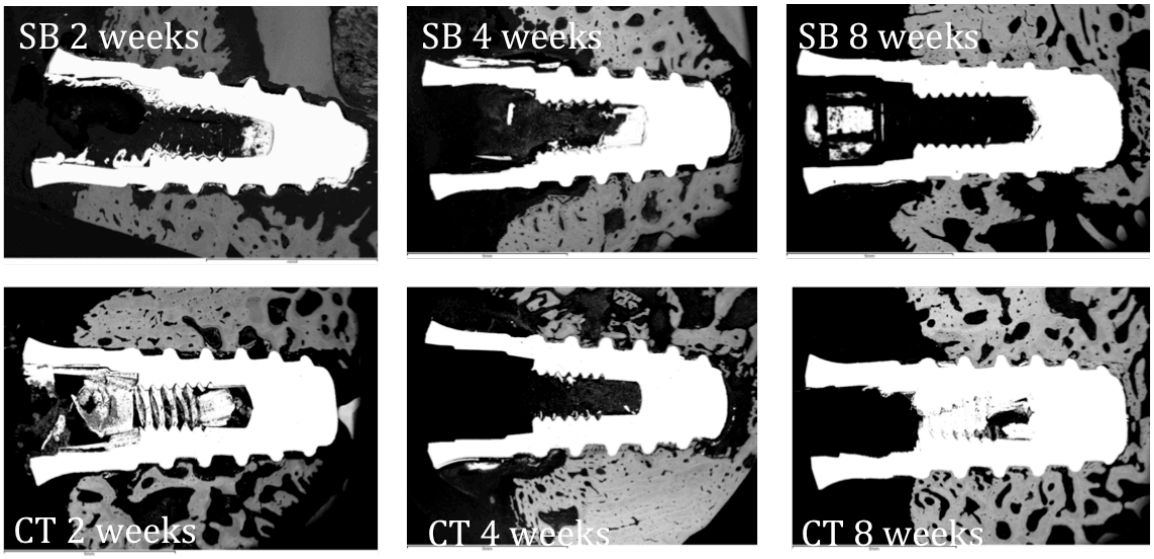
Caparrós et al. in 2016 (Caparrós, et al. 2016) also found significant differences in terms of BIC% between thermo-chemically treated and non-treated porous titanium implants. In vivo results demonstrated that the bioactive titanium achieved over 75 % tissue colonization compared to the 40 % value for the untreated titanium.

Figure 5.



Bone to implant contact (%) of the thermo-chemically modified surface and controls at 3 days and 1, 2, 3, 10 weeks in a mini-pig model. Ctr: machined surface; AEtch: acid-etched surface; Gblast: grit-blasted surface; 2-Step: grit-blasted, acid-ecthed and thermo-chemical treated surface. Significantly quicker osseointegration occurs at 2-step surface with a BIC greater than 70% at 2 weeks. At 3 weeks osseointegration is completed.

Figure 6.



Histologic samples of implants with the thermo-chemically modified surface and sand-blasted implants controls in rabbit model.
SB: Sand-blasted implants controls, CT: Contact-Ti implants (thermo-chemically modified surface).

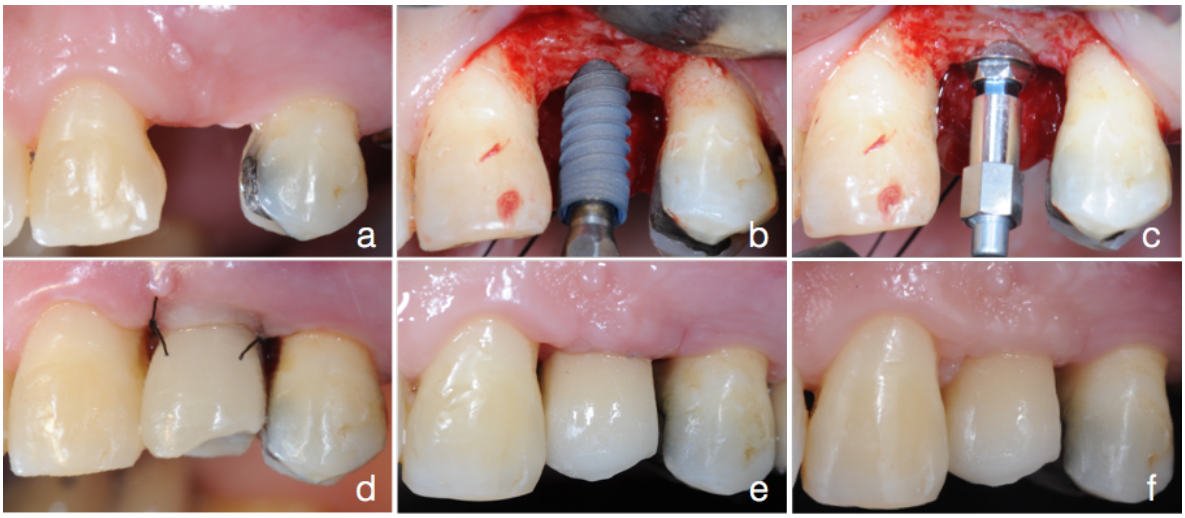
2.4 Immediate and early loading protocols at posterior areas of jaws

Advances in surfaces characteristics led to a more predictable and quicker osseointegration of dental implants, which in turn originated a progressive change of loading protocols in the last years (Albertini, et al. 2015; Esposito, et al. 2013). Immediate loading has been defined as the connection of the restoration to the implant in occlusion with the opposing dentition earlier than one week after implant placement while early loading is defined as being between 1 week and 2 months subsequent to implant placement (Weber, et al. 2009). Predictable results in terms of implant survival rate and marginal bone loss have been attained with immediate and early loading procedures in different clinical situations such as in the treatment of either totally or partially edentulous patients with fixed prostheses (Esposito, et al. 2008; De Bruyn, et al. 2014; Papaspyridakos, et al. 2014). Rehabilitation of premolar and molar areas of the maxilla and mandible with dental implants is one of the most frequent clinical situations in oral implantology and, in some cases, the placement of fixed provisional restorations at early stages can improve sensitively aesthetic and comfort of the patient. Immediately and early loaded implants and prostheses at posterior areas have been investigated in clinical studies with high success rates under favourable occlusal conditions and good primary stability of implants (Cordaro, et al. 2009).

Bio-active implant surfaces, due to their capacity of accelerate osseointegration, have been proposed to be used for immediate or early loading at 3-4 weeks of implants at

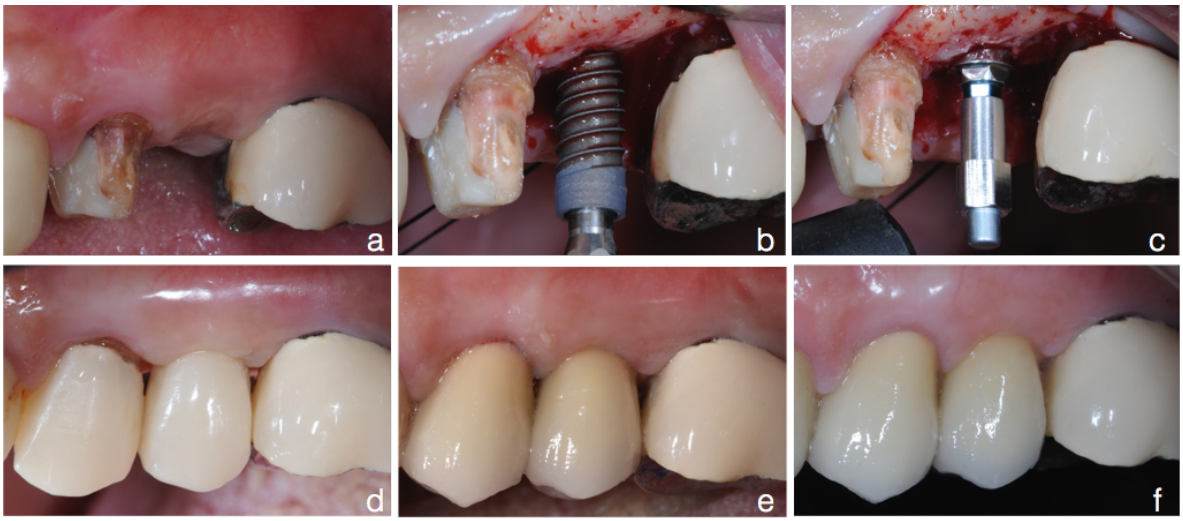
posterior sites in order to further increase predictability of non-bio-active rough surfaces (Nicolau, et al. 2013; Simmons, et al. 2016). Due to the capacity of enhancing osseointegration an early and immediate loading protocol has been proposed in this project for the clinical investigation of the thermo-chemically modified surface (figure 7, figure 8).

Figure 7.



The immediate loading procedure. a) Pre-operative situation of a patient from the clinical trial where the left first premolar is missing, b) implant placement, c) ISQ measurement at the abutment, d) installation of the provisional restoration after 24 hours, e) installation of the definitive restoration after 12 weeks, f) the restoration after 1 year.

Figure 8.



The early loading procedure. a) Pre-operative situation of a patient from the clinical trial where the left second premolar is missing, b) implant placement, c) ISQ measurement at the abutment, d) installation of the provisional restoration after 4 weeks, e) installation of the definitive restoration after 12 weeks, f) the restoration after 1 year.

2.5 Rationale for the present study

Longitudinal studies have shown that both implants and prostheses can function properly for teeth replacement for periods of 20 years or more. Currently the standard protocol for non-submerged implants requires that both implants in the maxilla and mandible remain free of charge for 6-8 weeks after placement to facilitate osseointegration in all situations, however a period without loading of 3 or 4 months in poor bone quality (type IV) is recommended.

Immediate loading has been defined as the placement of a restoration in occlusion with the opposing dentition within 7 days after implant placement while early loading is when the restoration is placed in occlusion with the opposing dentition between 7 days and 2 months after implant insertion (Weber, et al. 2009). Immediate and early loading (after 3, 4, 6 and 8 weeks) is a scientifically approved procedure (Nicolau, et al. 2013; Simmons, et al. 2016; Cochran, et al. 2002; Roccuzzo, et al. 2001; Ioannidou & Doufexi 2005; Romanos & Nentwig 2006) and the latter is now considered a standard procedure for moderately rough implant surfaces in a bone of good quality according to the ITI Consensus Conference 2009 (Gallucci, et al. 2009).

The implants used in this research have a surface obtained by a mechanical treatment of shot-blasting consisting of blasting with alumina particles, etching with hydrochloric acid and finally thermo-chemically treated (Kim, et al. 1996; Kokubo, et al. 1990), providing it with unique characteristics in current implantology. This surface is indeed capable of imitate the early stages of bone formation once it comes into contact with the plasma

without the osteoblastic cells taking part. Histological studies in animals have demonstrated (Aparicio, et al. 2011b; Gil, et al. 2014b) this bioactive surface allows an accelerated osseointegration compared to rough titanium surfaces currently in use, which would significantly benefit to patients needing missing teeth replacement with dental implants.

However, even though reliable results of implants with this type of surface in animal models have been presented, there are currently no randomized clinical trials in the literature to investigate their behaviour in humans. The use of these implants would allow connecting the prostheses immediately or within a very short time after surgery with increased reliability, which will be a great benefit for patients who need implant treatment.

3. HYPOTESIS

3. Hypothesis

Main hypothesis:

H_0: A statistically significant difference concerning the survival rate does not exist between immediate and early-loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery.

H_1: A statistically significant difference concerning the survival rate exists between immediate and early-loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery.

Secondary hypotheses:

H_0: A statistically significant difference concerning bone level changes does not exist between immediate and early-loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery.

H_1: A statistically significant difference concerning bone level changes exists between immediate and early-loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery.

H_0: A statistically significant difference concerning implant stability does not exist between immediate and early-loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery.

H_1: A statistically significant difference concerning implant stability exists between immediate and early-loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery.

4. OBJECTIVES

4. Objectives

Main objective:

To compare the survival rate of either immediate or early loaded thermo-chemically treated implants with definitive abutments placed at the time of surgery at posterior areas of the maxilla and mandible.

Secondary objective:

To compare bone level changes and implant stability of either immediate or early-loaded implants with definitive abutments placed at the time of surgery at posterior areas of the maxilla and mandible.

5. MATERIAL & METHODS

5. Material & Methods

5.1 Study design

This is part of an on going 3-year multicenter prospective randomized clinical trial carried out in 3 centres in Spain (BDI, CEROM, NCD). Interventions have been performed by 3 experienced periodontists (implant surgery) (MA, MH, JN) and prosthodontists (provisional and final restorations) of the respective centres. The study was conducted in accordance with the 'Declaration of Helsinki' (1964) and amendments (Tokyo, 1975; Venice, 1983; Hong Kong, 1989; Somerset, 1996; Edinburgh, 2000) for patients participating in clinical studies. The study protocol was reviewed and approved by the Ethics Committee for clinical studies of the Universitat Internacional de Catalunya with reference number 500077. All the patients were given an informed consent document, which had to be read and signed before entering the study.

5.2 Patients and sites

5.2.1 Subject Population

Subjects attending the 3 dental centres were screened following these study criteria:

5.2.1.1 *Inclusion criteria*

- ≥ 18 years of age;
- At least one tooth missing at posterior areas of the maxilla (FDI positions 4–7, ADA positions 2–5 and 12–15) or the mandible (FDI positions 4–7, ADA positions 18–21 and 28–31);
- Need of an implant-supported fixed restoration;

- Patients willing to participate and to attend the planned follow-up appointments.

5.2.1.2 Exclusion criteria

- Uncontrolled endocrine or immune disorders;
- Metabolic disorders such as osteoporosis;
- Severe or uncontrolled bone diseases;
- Drug abuse or alcoholism;
- Smoking > 10 cigarettes per day;
- Severe bruxism;
- Severe TMJ disorders;
- Gingival inflammation, untreated periodontitis or mucosal diseases.

Additionally, sites had to fulfil the following criteria:

- Molars or premolars areas with a sufficient space for restorations;
- Healed sites (teeth extracted or lost ≥ 4 months before implantation);
- Natural teeth or restored teeth with fixed restorations as opposite dentition;
- A minimum bone availability of 6,5mm of width and 10 mm of height;
- Presence of at least 4mm of keratinized tissues from buccal to lingual side of the edentulous ridge.

And exclusion criteria were:

- Previous bone augmentation procedures performed on the implant site;
- Local infections;
- History of implant failure;

- Lack of primary stability of the implant after placement;
- Need for augmentation procedure during implant surgery;
- Inability to place the implant according to the prosthetic requirements.

5.2.2. Implants and abutments

Vega Contac-TI® implants (Klockner Implant System, SOADCO, Andorra) of 3,5; 4,0 and 4,5mm in diameter (with a platform-switching of 0.3, 0.35 and 0.60 mm, respectively) and 8, 10 and 12mm in length were used. Permanent abutments of 2 mm of height for screw-retained restorations (Klockner Implant System, SOADCO, Andorra) were immediately placed after surgery. No cemented restorations were used in this study.

5.3 Pre-treatment procedures

Intra-oral photographs, impressions, cast models, diagnostic wax-up, radiographs and a cone beam computerized tomography were performed. All patients received cause-related periodontal therapy including oral hygiene instructions. Previously calibrated examiners registered modified Plaque Index (mPLI), (Mombelli, et al. 1987) modified Sulcus Bleeding Index (mSBI), (Mombelli, et al. 1987)clinical attachment level (CAL) and probing depth (PD) with a manual periodontal probe (PCP UNC 15-Hu-Friedy). Periodontal status was assessed according to the classification proposed by Armitage et al. (Armitage 1999).

5.4 Surgical procedure

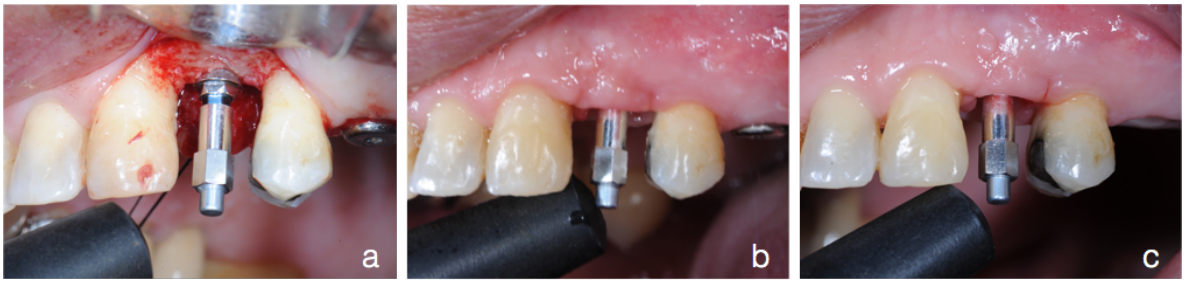
Antibiotic prophylaxis (amoxicillin 1 g or clindamycin 600 mg) was given 1 hour prior to surgery. Chlorhexidine 2.0% solution (Bohmclorh®, Bohm, Madrid, Spain) was extraorally

applied and patients were told to rinse with 0.12% clorhexidine for 1 minute (Perio Aid®, Dentaid, Spain). Every surgical procedure was performed under local anaesthesia with articaine 40mg/ml + epinephrine 0,01 mg/ml (Artinibsa®, Inibsa Dental, Barcelona, Spain). A crestal incision preserving at least of 2mm of keratinized mucosa on the buccal and lingual/palatal aspects and intra-sulcular incisions at the neighbouring teeth were made to elevate a full-thickness flaps to expose the alveolar crestal. Bone quality was registered following Lekholm & Zarb (1985) classification (D1, D2, D3 and D4) and bone width was measured with a periodontal probe (PCP-UNC 15, Hu-Friedy). The drilling protocol was performed according to the manufacturer's specifications for Klockner Vega® implants and adapted to the bone quality. Primary stability was firstly tested manually (Type A: insertion torque > 35 N/cm and the implant cannot be rotated manually; Type B: insertion torque < 35 N/cm and the implant cannot be rotated manually; Type C: the implant can be rotated manually, Type D: the implant can be rotated and moved vertically) after implant placement and, if any kind of vertical mobility of the implant was noted, the patient was excluded from the study. In these cases, a delayed loading protocol was carried out.

5.5 Implant Stability Quotient measurements

Permanent abutments for screw-retained restorations (Klockner Implant System, SOADCO, Andorra) were tightened at 20 N/cm with a dynamometric wrench (Klockner Implant System, SOADCO, Andorra) immediately after implant insertion. Smart-Pegs® (Osstell®, Gothenburg, Sweden) were manually screwed to the abutment at 5 N/cm and RFA (Resonance Frequency Analysis) measures in ISQ both buccolingually and mesiodistally were obtained by means Osstell® ISQ device (Osstell®, Gothenburg, Sweden). After the Smart-pegs® were removed, healing caps were manually screwed on to the permanent abutments and flaps were sutured with 5/0 Polyamide avoiding any tension. Antibiotics (amoxicillin 500 mg three times per day for 7 days or clindamycin 300 mg for 7 days in penicillin-allergic patients), a non-steroidal anti-inflammatory drugs (dexketoprofen 25 mg every 8 hours if needed), and a gastric protector (pantoprazole 20 mg one per day for 7 days) were prescribed. Figure 9 shows an example of a patient treated in the early loading group.

Figure 9.



ISQ measurements of an immediate loading group patient. a) Measurement immediately after implant placement, b) Measurement at 4 weeks, c) Measurement at 12 weeks, before installation of the definitive restoration.

5.6 Prosthetic procedure

Abutment-level impressions with open trays and hydrophilic vinyl polysiloxane impression material (Virtual®, Ivoclar Vivadent, Italy) were taken the same day of the surgery at permanent abutments, and cast models for provisional restorations were obtained. Screw-retained provisional restorations were prepared for all implants. Provisional prostheses were inserted and tightened at 15 N/cm within the first week after implant placement in patients of group A and after 4 weeks in patients of group B. 8 weeks after implant placement, provisional restorations were unscrewed and definitive impressions with open trays and hydrophilic vinyl polysiloxane impression material (Virtual, Ivoclar Vivadent, Italy) were taken. All definitive prostheses were delivered after 10-12 weeks; abutments were tightened at 30 N/cm and restoration screws at 25 N/cm with a dynamometric wrench. The occlusal screw access hole was filled with a composite material.

5.7 Randomization

All patients were randomly allocated to one of the following treatment groups: implants in group A were loaded with provisional restorations within the first week after surgery while group B implants were loaded at 4 weeks after placement.

The randomized allocation of subjects into the treatment groups was performed by flipping a coin where heads corresponded to group A and tails to group B. Someone who was not involved in the investigation performed the assignment of patients the day of surgery after impressions for provisional restorations had been taken.

5.8 Radiographic evaluation

Standardized periapical radiographs were taken immediately after implant placement (baseline), after the temporary restoration installation (at 1 week and 4 weeks respectively), after definitive restoration installation (at 10-12 weeks) and at 6 and 12 months. Baseline radiographs were taken with the definitive abutment and the corresponding healing cap screwed. Radiographs for assessing the impression coping fit and prosthetic components were also performed, but only for clinical purposes. Radiographs using the long-cone parallel technique were taken using customized film holders (Rinn XCP®, Dentsply Rinn) to allow standardization of radiographs at different times. The stock radiograph film holders were customized with acrylic resin recording the occlusal surfaces of the adjacent and opposing teeth (figure 10). At least the coronal two thirds of the implant had to be clearly visible on the radiograph and no distortions or artefacts had to be detected (figure 11, figure 12).

Figure 10.

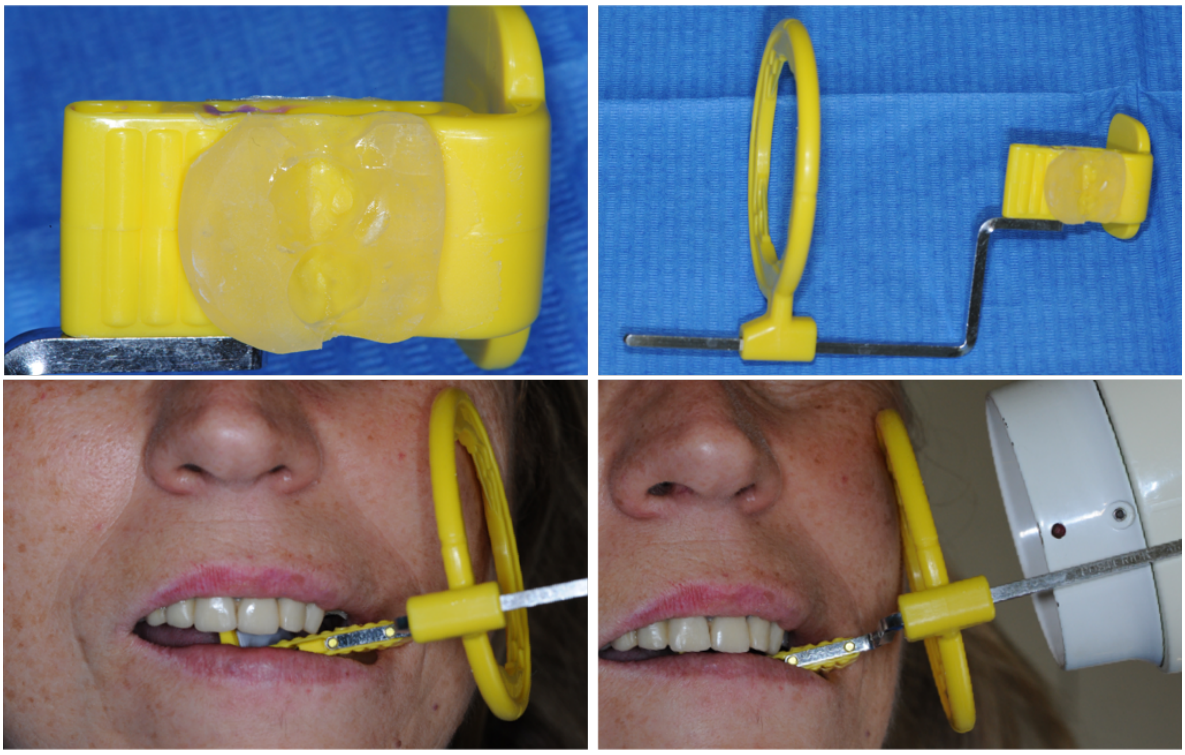
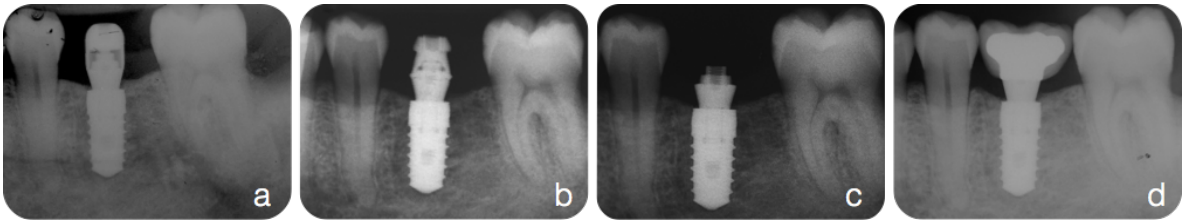
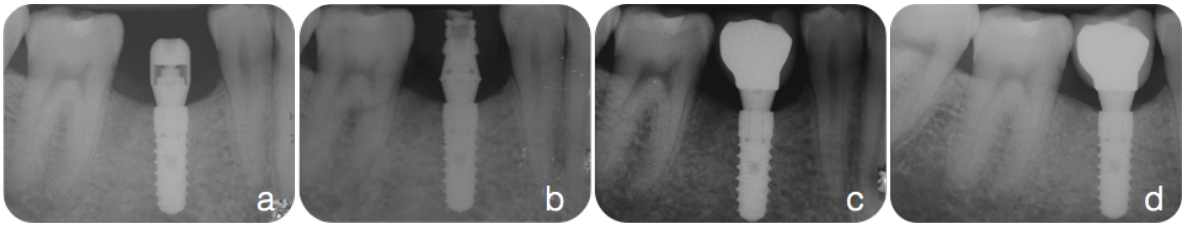


Figure 11.



An example of standardized periapical radiographs of an immediate-loaded implant. a) Radiograph immediately after implant placement, b) radiograph at 4 weeks, c) radiograph at 6 months, d) radiograph at 1 year.

Figure 12.



An example of standardized periapical radiographs of an early-loaded implant. a) Radiograph immediately after implant placement, b) radiograph at 4 weeks, c) radiograph at 6 months, d) radiograph at 1 year.

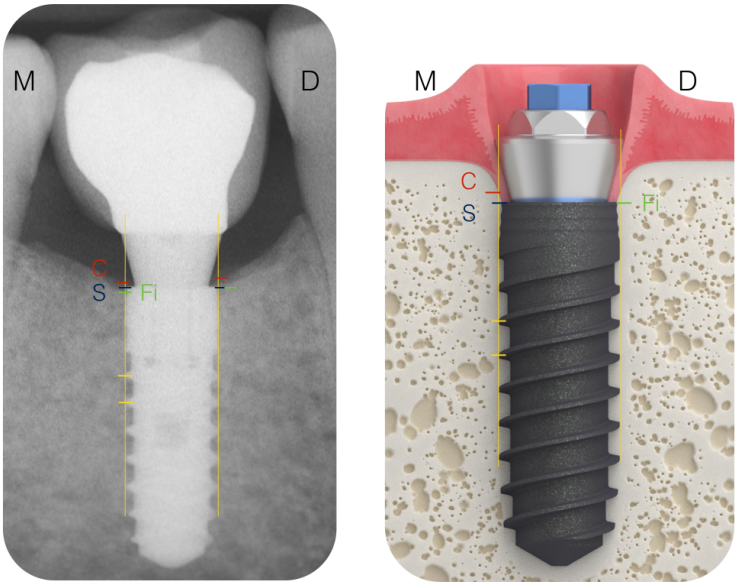
5.8.1 Radiographic measurements

Radiographs were used to measure bone level changes at periimplant bone level and at the crestal bone level with an image analysis software (ImageJ®, version 1.39F, U.S. National Institutes of Health). All measurements were determined at the mesial and distal surfaces of each implant using magnifications (×7). An independent blinded researcher performed the radiographic analysis. A previous calibration procedure was carried out as follows: the same examiner measured a subset of 10 radiographs on three separate occasions, 72 hours apart, to determine the intra-examiner reproducibility. An interclass coefficient of 0.99 ($P < .05$) was obtained.

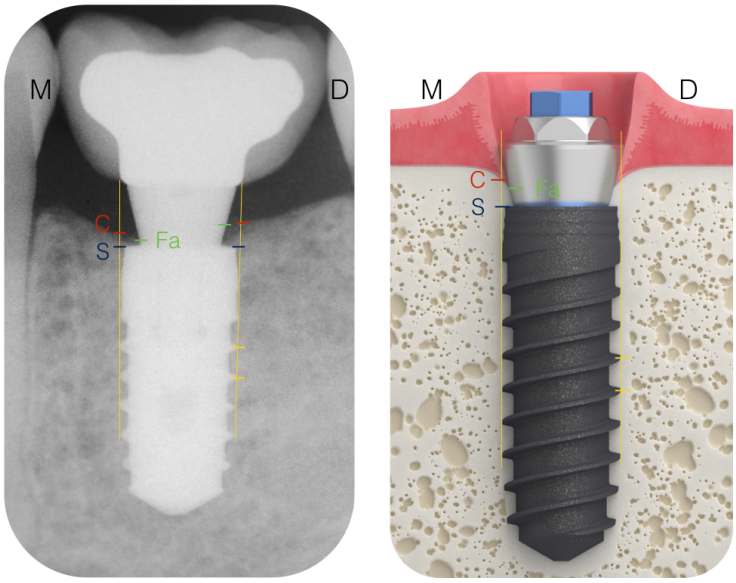
In each radiograph the most coronal part of bone crest (C), the position of implant shoulder (S) and the level of the first visible contact of the periimplant bone with the implant (Fi) or abutment (Fa) were recorded. The distance in millimetres from S to C was defined as the marginal bone level at the crest (MBLc), while the distance from S to Fi was defined as the marginal bone level at the implant (MBLi). When the first bone contact was localized on the abutment, the distance between S and Fa was registered as marginal

bone level at the abutment (MBLa). The MBLc distance was considered positive if the C mark was coronal to S, negative if C was apical to it and as zero if C was coincident with the implant shoulder. In the same manner, MBLi was considered negative if Fi was apical to S and as zero if Fi was coincident with S or bone to abutment contact was detected (figue 13). MBLc changes (CMBLc) were assessed as the differences between MBLc at 1 year, 6, 3 months, 4 weeks and baseline. MBLc, MBLi and MBLa changes (CMBLc, CMBLi, CMBLa) were defined as the differences between MBLc, MBLi or MBLa measurements at the same follow-up periods.

Figure 13.



(a) Implants with the first bone contact on the fixture. C: bone crest; S: implant shoulder; Fi: first bone to implant contact; M: mesial; D: distal; SC distance: marginal bone level at the crest (MBLc); SFi distance: marginal bone level at the implant (MBLi).



(b) Implants with the first bone contact on the abutment. C: bone crest; S: implant shoulder; Fa: first bone to abutment contact; M: mesial; D: distal; SC distance: marginal bone level at the crest (MBLc); SFa distance: marginal bone level at the abutment (MBLa).

5.9 Patient-reported outcomes (PROMS)

PROMS were assessed by means of a questionnaire regarding comfort, aesthetics of the restorations, chewing ability and overall satisfaction. A 4 items rate scale was used to assess the degree of satisfaction: excellent, good, fair, poor.

5.10 Status of provisional and definitive restorations

Post-loading status of provisional and definitive restorations was assessed at each follow-up visit. Mobility of prostheses, restoration and/or abutment screw loosening were clinically evaluated as well as the presence of porcelain chippings or fractures of the framework or abutment fractures

5.11 Adverse events

Adverse events and complications related to the implant treatment ("implant-related adverse events") like acute or chronic pain, sensorial alterations, bone fractures, osteomyelitis, loss of osseointegration of the implant, discomfort and local or systemic infections were recorded. "Non-implant associated adverse events" such as serious illnesses or any condition requiring hospitalization for more than 1 day were also reported.

5.12 Statistical analysis

Sample size was previously calculated with N Query Advisor 4.0 software (Statistical Solutions®, Cork, Ireland) using data from previous studies with a similar design (Kokovic, et al. 2014). A significance level of 5% (α=0.05) and a statistical power of 80% were used

for the analysis. Considering a mean difference of 0.25mm of radiographic bone changes as acceptable, a standard deviation of 0.21mm and dropout rate of 10%, a minimum of 20 patients had to be included in the study.

The interclass correlation coefficient (ICC) to study consistency among the different consecutive radiographic measurements provided by the same operator on the same patients were significant with a confidence level of 95% and they all were greater than 0.9, considering 1 as a perfect consistency.

Descriptive statistics, such as means, SEs, SDs, medians and range of the measurements, were calculated for ISQ, marginal bone level and the rest of clinical values. As variables appeared to be parametric up to the 3-month visit, T-student test and ANOVA to analyse parameters evolution and to compare values between groups were used. Nonparametric tests such as Wilcoxon and Mann-Whitney tests were used to compare data after 3 months. The mean differences were considered statistically significant at $P \leq 0.05$ with a confidence interval of 95%. Data analysis was completed with software package IBM SPSS 21.0® for Windows (IBM, SPSS, Chicago, IL, USA).

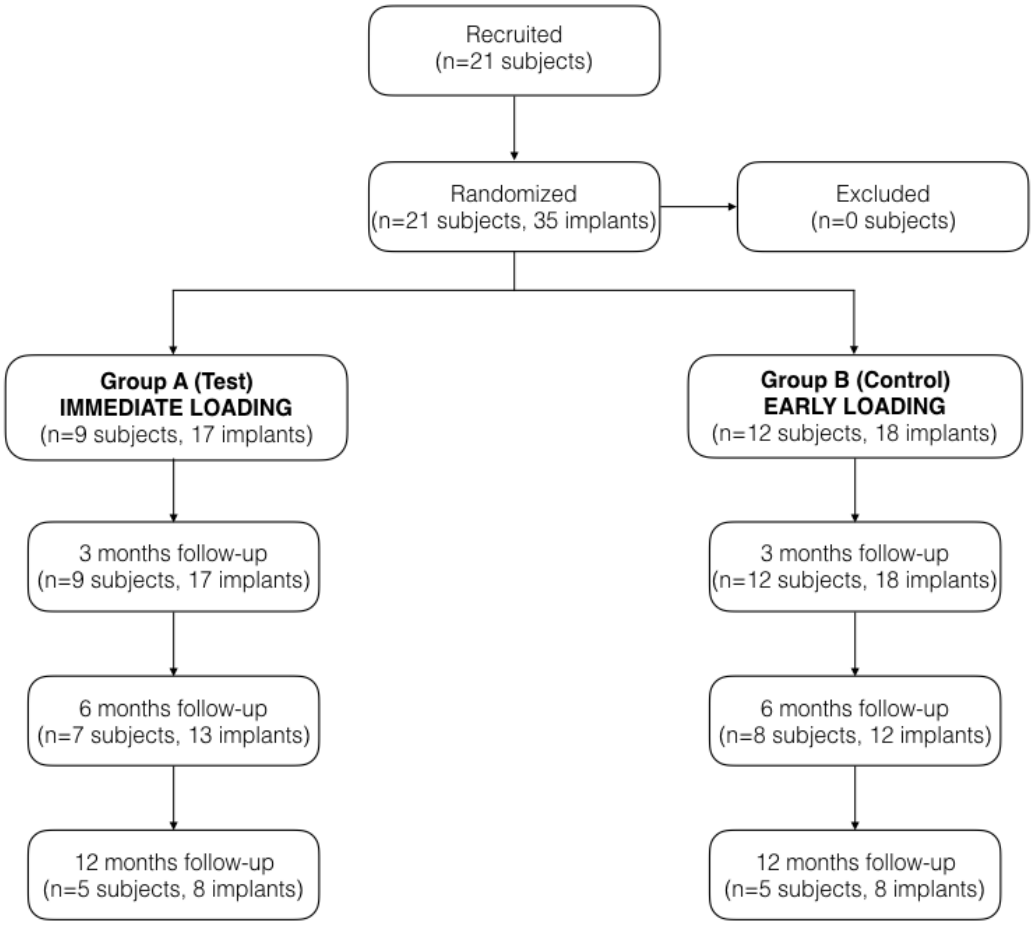
6. RESULTS

6. Results

6.1 Study population

Twenty-one patients were recruited in this randomized clinical trial from October 2015 to July 2016 and there were no dropouts. A total 35 implants were included in the study. No implants were lost. Two patients (2 implants) were not subjected to x-ray exposure at the 6-months visit as they were pregnant at that time. Figure 14 depicts the study sample flow-chart.

Figure 14.



Study sample flow-chart.

6.2 Demographic data

Table 6 depicts demographic data of the study. Of the 21 patients, 12 (57,1%) were men and 9 (42,9%) women with a mean age of 51.9 years (SD: 15.3 years). No statistically significant differences between groups were observed of these variables at baseline (P = 0.113 and P = 0.382, respectively).

Regarding the smoking habit, 4 patients (19%) were smokers and 17 not (81%). From a periodontal point of view, 4 patients were healthy (19%), 4 had a treated gingivitis (19%), 7 a treated slight chronic periodontitis (33%), 5 a treated moderate chronic periodontitis (23,8%) and 1 had a treated severe chronic periodontitis (4,8%). Again, no statistically significant differences between test and control group were observed of any of the parameters at the baseline (P = 0.414 and P= 0.586, respectively).

6.3 Interventions

Nine patients (42,8%) and 17 implants (48,6%) were assigned to group A and 12 patients (57,2%) and 18 implants (51,4%) to group B (early loading). A total of 14 implants (40%) were placed in the maxilla and 21 implants (60%) in the mandible. Twenty-four implants (68,5%) were restored with single crowns and 11 implants (31,5%) with partial fixed prostheses. 83,3% and 16,7% of the implants, respectively, were restored with single crowns and partial fixed prostheses in the group A, whereas in group B they were 52,9% and 47,1%, respectively. With regard to bone quality, the majority of the implants (20) were inserted in D2 bone (57,1%) and 15 implants in D3 (42,9%). Seven implants (20%) were inserted with a torque ranging between 15 and 20 N/cm, 16 implants (45,7%) between 20 and 35 N/cm and 12 implants (34,3%) with a torque ≥ 35 N/cm. No

statistically significant differences between groups were observed in any of these variables (P >0.05). Characteristics of interventions are shown in Table 5.

With respect to complications, in 2 patients (2 implants) of the group B minor surgical complications occurred. On the other hand, in 2 A-group patients (2 implants) and 3 B-group patients (3 implants) slight screw loosening of the definitive abutment and of the provisional restoration were observed at the 4-week and 3-month visit.

Table 6. Demographic data and interventions

PATIENTS		Total n=21		A, Immediate Loading n=9		B, Early loading n=12		P-value
		n	%	n	%	n	%	
SEX	TOTAL	21	100.00%	9	100.00%	12	100.00%	0.113
	MALE	12	57.10%	7	77.80%	5	41.70%	
	FEMALE	9	42.90%	2	22.20%	7	58.30%	
AGE	TOTAL	21		9		12		0.382
	MEAN	51.86		55.33		49.25		
	SD	15.28		15.70		15.11		
	MEDIAN	57.00		58.00		50.50		
	RANGE	24-74		30-74		24-74		
SMOKING	TOTAL	21	100.00%	9	100.00%	12	100.00%	0.414
	SMOKERS ≤10 cigs/days	4	19.00%	1	11.10%	3	25.00%	
	NON-SMOKERS	17	81.00%	8	88.90%	9	75.00%	
PERIODONTAL CONDITION	TOTAL	21	100.00%	9	100.00%	12	100.00%	0.586
	HEALTHY	4	19.00%	2	22.20%	2	16.70%	
	TREATED GINGIVITIS	4	19.00%	1	11.10%	3	25.00%	
	TREATED SLIGHT PERIODONTITIS	7	33.30%	4	44.40%	3	25.00%	
	TREATED MODERATE PERIODONTITIS	5	23.80%	1	11.10%	4	33.30%	
	TREATED SEVERE PERIODONTITIS	1	4.80%	1	11.10%	0	0.00%	
INTERVENTIONS		**Total n=35**		**A, Immediate Loading n=17**		**B, Early loading n=18**		
		n	%	n	%	n	%	
JAW TYPE	TOTAL	35	100.00%	17	100.00%	18	100.00%	0.053
	MAXILLA	14	40.00%	4	23.50%	10	55.60%	
	MANDIBLE	21	60.00%	13	76.50%	8	44.40%	
IMPLANT POSITION	TOTAL	35	100.00%	17	100.00%	18	100.00%	0.415
	14	1	2.90%	0	0.00%	1	5.60%	
	16	3	8.60%	1	5.90%	2	11.10%	
	24	2	5.70%	2	11.80%	0	0.00%	
	25	3	8.60%	1	5.90%	2	11.10%	
	26	2	5.70%	0	0.00%	2	11.10%	
	27	3	8.60%	0	0.00%	3	16.70%	
	34	1	2.90%	1	5.90%	0	0.00%	

	35	1	2.90%	1	5.90%	0	0.00%	
	36	9	25.70%	4	23.50%	5	27.80%	
	37	2	5.70%	1	5.90%	1	5.60%	
	45	1	2.90%	1	5.90%	0	0.00%	
	46	3	8.60%	2	11.80%	1	5.60%	
	47	3	8.60%	2	11.80%	1	5.60%	
	48	1	2.90%	1	5.90%	0	0.00%	
BONE QUALITY	**TOTAL**	35	100.00%	17	100.00%	18	100.00%	
	D2	20	57.10%	12	70.60%	8	44.40%	**0.118**
	D3	15	42.90%	5	29.40%	10	55.60%	
MANUAL PRIMARY STABILITY	**TOTAL**	34	100.00%	17	100.00%	17	100.00%	
	A	15	44.10%	8	47.10%	7	41.20%	**0.730**
	B	19	55.90%	9	52.90%	10	58.80%	
INSERTION TORQUE	**TOTAL**	35	100.00%	17	100.00%	18	100.00%	
	<15	2	5.70%	0	0.00%	2	11.10%	
	15	10	28.60%	6	35.30%	4	22.20%	
	20	4	11.40%	2	11.80%	2	11.10%	
	25	1	2.90%	0	0.00%	1	5.60%	**0.671**
	30	3	8.60%	1	5.90%	2	11.10%	
	35	3	8.60%	2	11.80%	1	5.60%	
	>35	12	34.30%	6	35.30%	6	33.30%	
SURGICAL COMPLICATIONS	**TOTAL**	35	100.00%	17	100.00%	18	100.00%	
	NO	33	94.30%	17	100.00%	16	88.90%	**0.257**
	YES	2	5.70%	0	0.00%	2	11.10%	
IMPLANT DIAMETER	**3,5**	5	14.30%	3	17.60%	2	11.10%	
	4	24	68.60%	11	64.70%	13	72.20%	**0.844**
	4,5	6	17.10%	3	17.60%	3	16.70%	
IMPLANT LENGTH	**8**	9	25.70%	4	23.50%	5	27.80%	
	10	22	62.90%	10	58.80%	12	66.70%	**0.531**
	12	4	11.40%	3	17.60%	1	5.60%	
TYPE OF PROSTHESES	**SINGLE**	24	68.60%	9	52.90%	15	83.30%	
	MULTIPLE	**11**	**31.40%**	**8**	**47.10%**	**3**	**16.70%**	**0.057**

6.4 Follow-up

All the patients attended the 3-month appointment, 15 patients (71.4%) and 25 implants (71.4%) at the 6-month visit; and 10 patients (47.6%) and 16 implants (45.7%) at 12-month visit (Fig. 14). Two patients did attend to the show up at 6-month recall but radiographs could not be taken, as they were pregnant at that time.

The overall survival rate at 3, 6 and 12 months was 100% in both groups.

6.5 Clinical variables

6.5.1 Modified Plaque Index (mPLI)

Mean values of mPLI are shown in Table 7. There were no significant changes of mPLI values throughout the study ($p<0.05$) without differences between groups ($p<0.05$).

6.5.2 Modified Sulcus Bleeding Index (mSBI)

No statistically significant changes of mSBI over time were registered ($p>0.05$) neither differences between tests and controls ($p>0.05$). Mean values of mSBI are depicted in Table 7.

6.5.3 Probing Depth (PD)

A significant decrease in PD ($p=0.049$) with no differences between groups ($p=0.716$) was observed throughout the study. However changes were not significant from a clinical point of view. Results of mean PD values are shown in Table 7.

Table 7. Clinical Variables

		A, test (IMMEDIATE LOADING)		B, control (EARLY LOADING)		P-values
		Mean	SD	Mean	SD	
mPLI	4 weeks	0.132	0.252	0.083	0.171	0.590
	3 month	0.368	0.587	0.181	0.224	0.807
	6 months	0.058	015	0.062	0.113	0.769
	12 months	0.031	0.088	0.156	0.229	0.382
mSBI	4 weeks	1.441	0.603	1.25	0.507	0.424
	3 month	1.309	0.348	1.167	0.284	0.219
	6 months	1.135	0.282	1.313	0.386	0.376
	12 months	1.063	0.177	1.188	0.513	0.105
PD	4 weeks	1.956	0.525	2	0.781	0.845
	3 month	2.132	0.65	1.792	0.654	0.132
	6 months	1.962	0.62	1.958	0.673	0.990
	12 months	1.531	0.86	2.156	0.352	0.078

mPLI: modified plaque index; mSBI: modified sulcus bleeding index; PD: probing depth.

6.6 Implant Stability Quotient measurements

ISQ measurements were taken at abutment (ISQa) and implant level (ISQi) with a mean of 74.2±8.3 and 69.6±7.9, respectively. This difference was statically significant (p=0.007). Mean ISQi was 77.5±6.9 in group A and 71.1±8.6 in group B with a statistically significant difference (p=0.021) while mean of ISQa was 72.0±8.0 and 67.3±7.2 for groups A and B respectively almost reaching a significant difference (p=0.074). The analysis of other variables such as manual stability (P=0.022), insertion torque (P=0.037), bone quality (P=0.003) and maxillary/mandibular location (P=0.002) evidenced an association with baseline ISQ values (ISQa and ISQi), which was independent from the type of loading. Mean ISQa at 2, 4, 8 weeks and 3, 6 and 12 months are shown in Table 8**.** ISQa values were higher at all time points in the test than in the control group (p=0.027). Regarding ISQa

values throughout the follow-up period, although no significant changes were found between baseline and 4 weeks (P=0.628), a statistically significant increase was observed between 4 and 8 weeks (P=0.000), between 8 and 12 weeks (P=0.002) and between 12 weeks to 6 months (P=0.001) From baseline to the 12-month visit ISQa increased from 70.5±8.9 to 83±10.9, which was statically significant (P=0.004). Type of loading did not influence changes in ISQa since no significant differences were detected between groups (P=0,512). Figure 15 shows the changes of ISQa throughout the study.

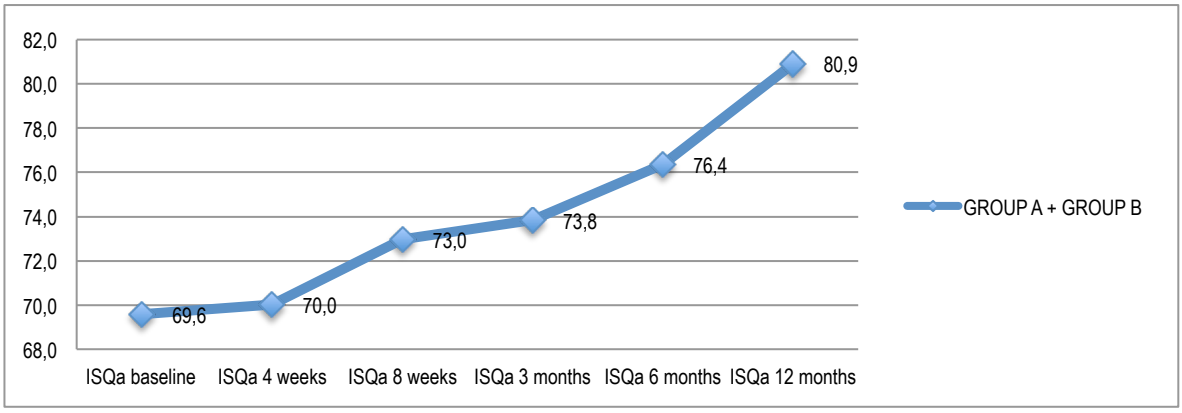
Table 8. Implants Stability

	Total				A (test, n=17 implants)				B control n=18 implants)				*P*-value
	N	Mean	SD	Median	N	Mean	SD	Median	N	Mean	SD	Median	
ISQi	35	74.2	8.3	76.0	17	77.5	6.9	79.5	18	71.1	8.6	72.0	**0.021***
ISQa baseline	35	69.6	7.9	67.0	17	72.0	8.0	70.0	18	67.3	7.2	66.0	0.074
ISQa 4 weeks	35	70.0	8.9	67.5	17	73.3	9.8	70.5	18	66.9	7.0	66.3	**0.033***
ISQa 8 weeks	35	73.0	9.1	69.5	17	76.6	9.5	74.0	18	69.6	7.5	68.8	**0.023***
ISQa 3 months	35	73.8	8.9	70.0	17	77.2	9.4	72.0	18	70.7	7.4	70.0	**0.033***
ISQa 6 months	25	76.4	10.1	72.0	13	78.6	11.0	73.5	12	74.0	8.9	70.5	0.270
ISQa 12 months	16	80.9	10.9	83.0	8	85.3	10.6	86.0	8	76.5	9.9	71.5	0.195

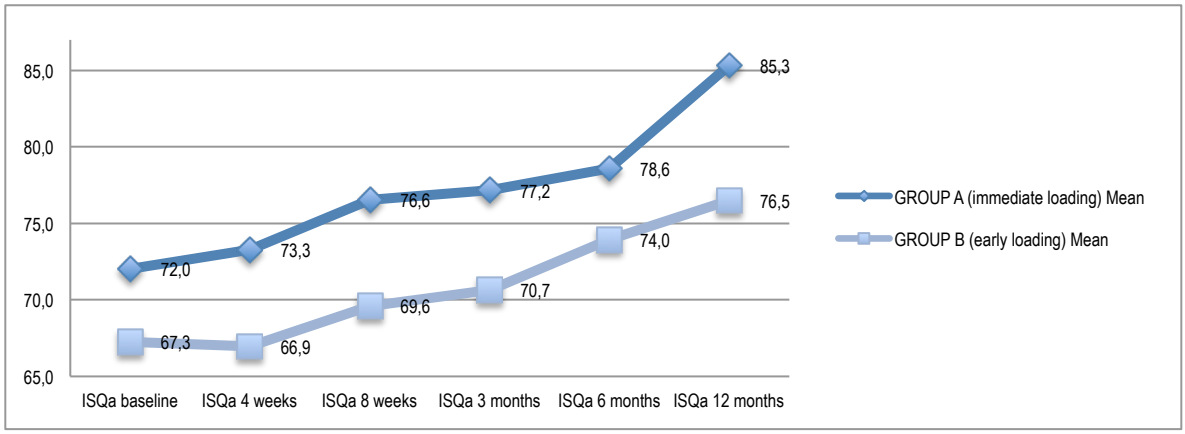
ISQi: ISQ values measured at the implant immediately after implant placement; ISQa baseline, 4, 8 weeks, 3, 6, 12 months: ISQ values measured at the abutment at baseline, 4, 8-week, 3, 6, 12-month visits.
*Statistically significant (P≤0.05).

Figure 15.

(a)



(b)



ISQa changes. (a) Implant stability quotient changes at the abutment in the overall sample. (b) Implant stability quotient changes at the abutment in group A and group B.

6.7 Changes of marginal bone level (CMBL)

Figure 16 depicts the changes of marginal bone level at the crest, the implant and the abutment over time.

6.7.1 Changes of marginal bone level at the crest (CMBLc)

Mean MBLc at the baseline, was 1.04±0.55mm in the whole sample, 0.83±0.61mm in group A and 1,25±0.39mm in group B. This difference between groups was statistically significant not only in baseline (P=0.024), but also at all follow-up visits. When comparing MBLc along the evaluation period, significant changes occurred in both groups between baseline and 4 weeks (P=0.000) and not between 4 weeks and 3 months (P=0.422). A bone loss of 0.32±0.40mm in group A and 0.41±0.51 mm in group B was registered with no statistically significant differences between them in this time lapse (P>0.05). From 3 to 6 months, no significant changes in MBLc occurred (P=0.194). However, a significant bone loss at the crest was observed between 6 months 1 year (-0.28 mm, P=0.004). Mean MBLc at 12 months was 0,31±0.51 m in the immediately loaded and 0.62±0.82mm in the early-loaded group (P=0.195). No significant differences in changes of marginal bone level at the crest (CMBLc) between the two groups were observed throughout the study (p>0.05). Table 9 shows CMBLc values for both groups during the investigation.

6.7.2 Changes of marginal bone level at the implant (CMBLi)

MBLi at baseline was 0mm in 97.1% of the implants meaning that only 2.9% of the implants (1 implant) had a supracrestal position at the day of surgery. No differences were observed between groups (P>0.05). The rate of implants with no bone loss at 4 weeks and 3, 6 and 12 months was 94.3%, 77.1%, 77.3% and 75%, respectively. Mean CMBLi at 4 weeks and 3, 6 and 12 months were 0.012mm, 0.048mm, 0.040mm and 0.068mm. These changes were not statistically significant (P>0.05) and no differences between groups were observed (P>0.05). Table 9 depicts the means of CMBLi throughout the study.

6.7.3 Changes of marginal bone level at the abutment (CMBLa)

Bone contact at the abutment was observed at 62.9% and 60% of the implants at 4 and 12 weeks, respectively. These values remained constant after 6 and 12 months with a rate of 59.1% and 62.5%, respectively (P>0.05). Table 10 depicts the incidence of bone to abutment contact over time. The mean of MBLa was 0.40±0.45mm at 4 weeks, 0.22±0.31mm at 3 months, 0.28±0.45mm at 6 months and 0.25±0.46 at 12 months. Changes of marginal bone level at the abutment (CMBLa) were statistically significant between 4 and 12 weeks (P=0.028). CMBLa at 12 months was 0.29mm in group A and -0.13mm in group B which was statistically significant (P=0.007). CMBLa values were statistically significant between 4 and 12 weeks (P=0.028). CMBLa at 12 months was 0.4 mm in the test and -0.01 in control group; the difference was a statistically significant (P=0.007). Results of MBLa are summarized in Table 10.

Table 9. Radiographic variables

		TOTAL		A, test (IMMEDIATE LOADING)		B, control (EARLY LOADING)		P-value
		Mean Bone loss (mm)	SD (mm)	Mean Bone loss (mm)	SD (mm)	Mean Bone loss (mm)	SD (mm)	
CMBLc	1 MONTH	0.309	0.262	0.26	0.231	0.355	0.287	0.291
	3 MONTHS	0.369	0.458	0.323	0.4	0.412	0.514	0.572
	6 MONTHS	0.275	0.519	0.193	0.43	0.394	0.634	0.235
	12 MONTHS	0.464	0.680	0.311	0.517	0.616	0.819	0.161
CMBLi	1 MONTH	0.012	0.113	0.014	0.158	0.011	0.046	0.807
	3 MONTHS	0.048	0.138	0.054	0.169	0.042	0.107	0.732
	6 MONTHS	0.04	0.16	0.053	0.204	0.021	0.062	0.695
	12 MONTHS	0.068	0.164	0.038	0.078	0.099	0.222	1.000
CMBLa	3 MONTHS	0.177	0.409	0.308	0.509	0.054	0.241	0.303
	6 MONTHS	0.189	0.515	0.278	0.540	0.079	0.485	0.601
	12 MONTHS	0.139	0.526	0.398	0.522	-0.013	0.519	**0.007***

CMBLc: changes of marginal bone level at the crest; CMBLi: changes of marginal bone level at the implant; CMBLa: changes of marginal bone level at the abutment; SD: standard deviation.
*Statistically significant (P≤0.05).

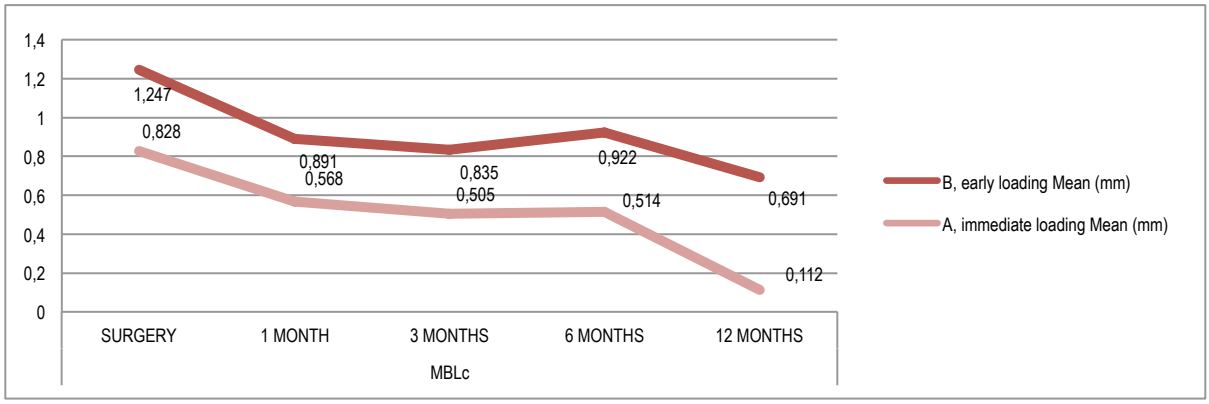
Table 10. Bone to abutment contact

		TOTAL	A, test (IMMEDIATE LOADING)	B, control (EARLY LOADING)	P-values (Chi-square)
		% > 0 (implants)	% > 0 (implants)	% > 0 (implants)	
Mbla	1 MONTH	62.9%	70.6%	55.6%	0.358
	3 MONTHS	60.0%	52.9%	66.7%	0.407
	6 MONTHS	59.1%	61.5%	55.6%	0.779
	12 MONTHS	62.5%	37.5%	87.5%	0.119

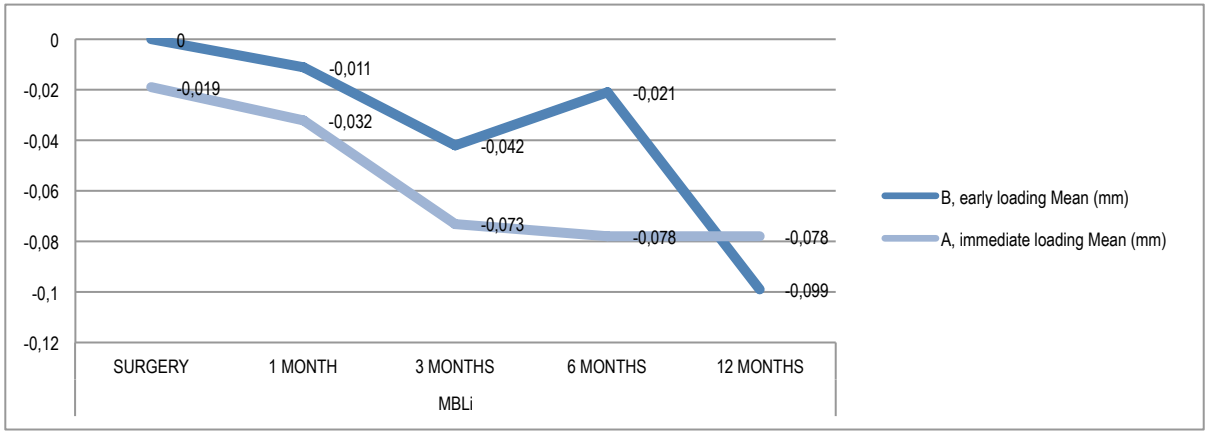
MBLa: Marginal bone level at the abutment; % > 0: percentage of implants with visible bone to abutment contact.

Figure 16.

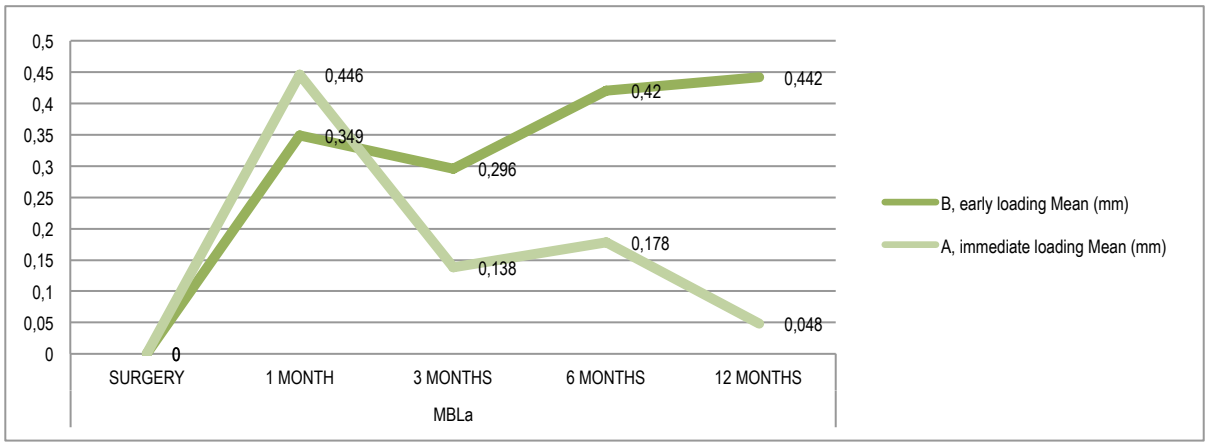
(a)



(b)



(c)

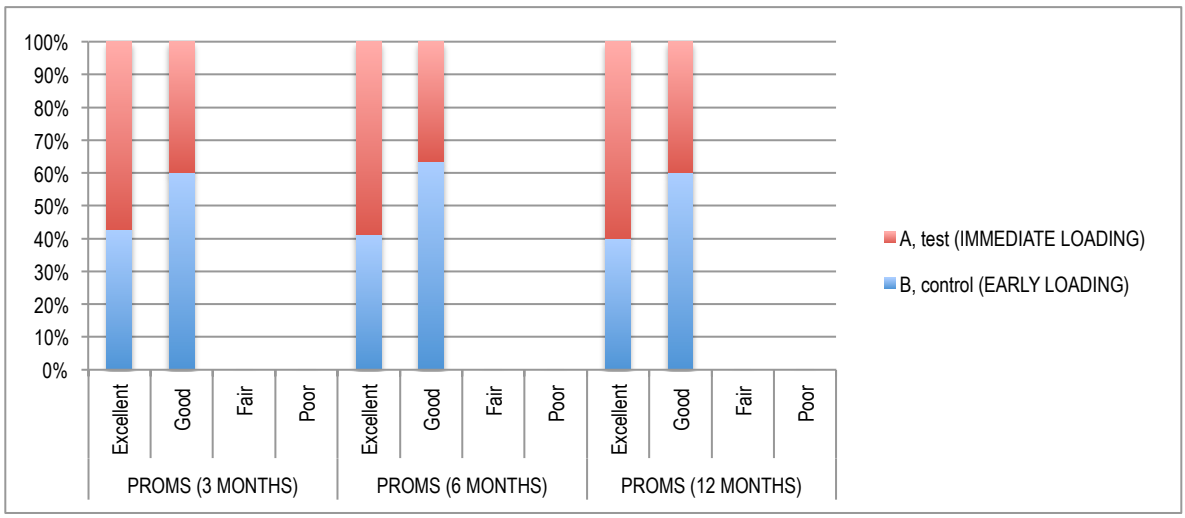


Marginal Bone Level. (a) MBLc: marginal bone level at the crest. (b) MBLi: marginal bone level at the implant. (c) MBLa: marginal bone level at the abutment.

6.8 Patients-reported outcomes (PROMS)

Reported satisfaction of the patients was high throughout the study and no statically significant differences were found between 3, 6 and 12 months between groups (figure 17).

Figure 17.



Patient-related Outcomes Measurements (PROM).

6.9 Association between variables

Association and correlation between variables are shown in Table 11.

6.9.1 Implant Stability Quotient at implant (ISQi)

A statistically significant association between ISQi and insertion torque < 30 N/cm (p=0.037), mandibular location (p=0.040), implant length of 12 mm (p=0.027) and type A manual stability (p=0.001) was found.

6.9.2 Implant Stability Quotient at the abutment (ISQa)

ISQa was associated with the following variables at baseline mandibular location (p=0.002), bone type D2 (p=0.003), type A manual stability (p=0.022), and multiple prostheses (p=0.000).

There was an inverse correlation between ISQa and MBLc at baseline (p=0.027) and at 3 months (p=0.01). At 12 months ISQa and MBLa values were also inversely correlated (p=0.01). An association between ISQa changes (from baseline to 12 months) and multiple prostheses was also found in Mann-Whitney test (p=0.042).

6.9.3 Marginal bone level at the crest (MBLc)

An association between MBLc values at baseline and the following variables were observed: maxillary location (p=0.026), single prostheses (p=0.040) and an insertion torque <25 N/cm (p=0.043). MBLc at surgery was also associated with changes of marginal bone level at the abutment (CMBLa) at 4 weeks (p=0.002) and 12 months (p=0.016).

An association between changes of marginal bone level at the crest (CMBLc) during the first 3 months and mandibular location of the implants was also found (p=0.028). A direct correlation between MLBc and MBLa was found at 12 months (p=0.037).

6.9.4 Marginal Bone Level at the implant (MBLi)

At 3 months, an inverted correlation between MBLi changes (CMBLi) and ISQa changes was observed (p=0.01), however this association at 12 months was not confirmed. None of the other variables were associated with MBLi and its changes during the study (p>0.05).

6.9.5 Marginal Bone Level at the abutment (MBLa)

Changes of marginal bone level at the abutment (CMBLa) from baseline to 12 months were associated with type B manual stability (p=0.022) and a tendency to be associated with a maxillary location was also observed (p=0.055). An association with CMBLa at 4 weeks and 12 months time points was also found for baseline MBLc values (p=0.002; p=0.016).

Table 11. Associations and correlations between variables

DEPENDANT VARIABLES	TIME POINT	PREDICTORS	ASSOCIATION	CORRELATION	STATISTICAL TEST	STANDARD COEFFICENT	P-value
ISQi	**baseline**	Insertion torque < 25 N/cm	direct		t-Student		p=0.037*
		Mandible	direct		t-Student		p=0.040*
		Implant length of 12 mm	direct		Anova		p=0.027*
		Type A manual stability	direct		t-Student		p=0.001*
ISQa	**baseline**	Mandible	direct		t-Student		p=0.002*
		Bone type D2	direct		t-Student		p=0.003*
		Type A manual stability	direct		t-Student		p=0.022*
		Multiple prostheses	direct		t-Student		p=0.000*
		MBLc		inverse	Spearman's Rho	(-) 0.373	p=0.027*
	3 months	MBLc		inverse	Spearman's Rho	(-)0.535	p=0.01*
	12 months	MBLa		inverse	Spearman's Rho	(-) 0.734	p=0.01*
	0-3 months	MBLi		direct	Pearson	(+) 0.425	p=0.011*
	0-12 months	Multiple prostheses	direct		Mann-Whitney		p=0.042*
MBLc	**baseline**	Maxilla	direct		t-Student		p=0.026*
		Single prostheses	direct		t-Student		p=0.040*
		Insertion torque < 25 N/cm	direct		t-Student		p=0.043*
		CMBLa (4 weeks)		direct	Spearman's Rho	(+) 0.511	p=0.002*
		CMBLa (12 months)		direct	Spearman's Rho	(+) 0.590	p=0.016*
	12 months	MBLa		direct	Spearman's Rho	0.524	p=0.037*
	0-3 months	Mandible	direct		Multivariate contrasts	0.179 (Pillai's Trace)	p=0.028*
						0.821 (Wilks' Lambda)	p=0.028*
						0.218 (Hotelling's Trace)	p=0.028*
						0.218 (Roy's Largest Root)	p=0.028*
MBLi	**0-3 months**	ISQa 0-3 months		direct	Pearson	(+) 0.425	p=0.011*
MBLa	**0-4 weeks**	MBLc at baseline		direct	Spearman's Rho	(+) 0.511	p=0.002*
	0-12 months	MBLc at baseline		direct	Spearman's Rho	(+) 0.590	p=0.016*
		Type B manual stability	inverse		Mann-Whitney		p=0.022*

ISQi: ISQ values measured at the implant immediately after implant placement; ISQa: ISQ values measured at the abutment; MBLc: marginal bone level at the crest; MBLi: marginal bone level at the implant; MBLa: marginal bone level at the abutment.
*Statistically significant (P≤0.05).

7. DISCUSSION

7. Discussion

The outcomes of this randomized clinical trial show no differences in the survival rate and marginal bone loss between immediate and early loading protocols of implants located in posterior areas. No implant was lost during the study and mean bone loss was 0.07±0.16 mm at the implant and 0.46±0.68 mm at the crest level. These results are in accordance with other clinical trials with a similar design in which high survival rates during the observation period were reported (Kokovic, et al. 2014; Nicolau, et al. 2013; Ganeles, et al. 2008; Zollner, et al. 2008). Kokovic et al. did not find any differences in a randomized controlled clinical trial including 72 immediately and early-loaded smooth-collar implants with a chemically modified surface placed in the posterior mandible (Kokovic, et al. 2014). At 5 years, the survival rate was 100% and the mean bone loss 0.4±0.24mm and 0.8±0.15mm in test and control groups, respectively. Similarly, a recent investigation on tissue-level implants with an SLActive® surface showed a survival rates of 97.4% and 96.7% and mean bone level changes of 0.88±0.81mm and 0.57±0.83mm for immediate and early loading groups, respectively, with no differences between them (Nicolau, et al. 2013). The thermo-chemically modified implant surface used in the present study has shown highly osseoconductive properties in *in vivo* studies with a mean bone to implant contact of 80% at 3 weeks (Aparicio, et al. 2011b; Gil, et al. 2014b; Albertini, et al. 2015). Perhaps this may have contributed to reach high survival rates of the implants under immediate and early loading conditions in posterior areas. The predictability of early-loaded platform-switched implants with a similar hydrothermally modified surface has been recently demonstrated in a 3-year randomized clinical trial in which two types of

surfaces were compared (Felice, et al. 2015). Implants were restored in function after 10-14 weeks and a 100% survival rate after 3 years was attained in both groups with no differences in marginal bone loss (1.12±0.49mm versus 1.10±0.38mm). Immediate and early loading after 3 weeks with hydrothermally treated hydroxyapatite implants with platform-matching connection has been investigated in a prospective clinical study (Simmons, et al. 2016). The survival rate was 100% and 94.7% for the immediate and early loading group, respectively and the mean bone loss was 0.75±0.50 mm with no differences between groups after 2 years of function. In a randomized clinical trial with platform-switched implants carried out by Grandi et al. in 2013 no differences in survival rates between immediate and early loading at 3 weeks after 3 years were observed (Grandi, et al. 2013). Immediately loaded implants lost 0.42±0.59mm and 0.90±1.17mm of peri-implant bone at 1 and 3 years, respectively; for early loaded implants bone loss was 0.46mm (95% CI 0.20, 0.72) and 1.10±1.39mm) at the same time points. However, a minimal bone loss of 0.07±0.16 mm at implants was observed in the present study at 1 year. The current literature has reported some differences in the maintenance of peri-implant bone with different implant designs. In fact, platform-switched implants and conical internal connections obtained better results compared to platform-machting and external connection implants (Atieh, et al. 2010; Annibali, et al. 2012; Gracis, et al. 2012; Strietzel, et al. 2015). The biological response to platform-switching implants with a conical connection has been recently investigated in a dog model by Cochrane et al. (Cochran, et al. 2013). They observed that, unlike in matching-platform implants, in platform-switched implants the connective tissue was located coronally with respect to the microgap and the epithelium was never found at implant-abutment interface. From a

biological point of view this means that the epithelial attachment can be kept far from microgap and only a minimum bone resorption may be expected after healing.

In order to minimize bone resorption, implants with a conical internal connection and a platform switching of 0.60mm, 0.35mm and 0.3mm were used in the present investigation. In addition, definitive abutments were tightened immediately after implant placement according to the 'one abutment at one time' protocol (Degidi, et al. 2011b; Grandi, et al. 2012; Luongo, et al. 2015; Molina, et al. 2017). In this sense, Molina et al. (Molina, et al. 2017) compared platform-switched implants with one-time abutment placement or implants in which repeated connections/disconnections were performed and found a statistically significant less bone resorption at 6-months post-loading between the test (0.61±40 mm) and the control group (1.24±0.79 mm).

In the present trial implants were inserted 1mm subcrestally as evidenced by the radiographic distance from the top of bone crest to the implant shoulder (mean MBLc: 1.04±0.55mm). Whether a subcrestal position of an implant can have an influence on the rate of marginal bone loss is still a matter of discussion (Cochran, et al. 2009; Fetner, et al. 2015; de Siqueira, et al. 2016). Recent reports suggest that it may lead to a better maintenance of peri-implant bone (Huang, et al. 2015; Cassetta, et al. 2016). Fetner et al. investigated the differences between subcrestal and equicrestal position of implant shoulder in an *in vivo* study (Fetner, et al. 2015). Similar rates of bone loss at the crest were showed between groups, but there was an increased bone loss at the implant in the equicrestal group. The histology showed bone contact at the abutment in the subcrestal groups while in any case this phenomenon was observed in the equicrestal. These results have been confirmed by other clinical and histological studies (Koutouzis, et al. 2014;

Degidi, et al. 2011a). A histomorphometric evaluation of retrieved human implants showed that all subcrestal implants presented bone contact at the abutment, while 0.5-1.5mm bone loss was observed at equicrestal implants (Degidi, et al. 2011a).

Within the limitations of the radiographic method used in the present work, bone contact at the abutment was observed in 62.5 % of the implants at 1 year with a significant difference (p=0.007) between groups (37.5% group A, 87.5% group B). However, this difference could be related with the different position of implant shoulder (MBLc) at baseline between the two groups (0.83±0.62mm in group A and 1.25±0.34mm in group B), confirming the results from Koutozis et al. (Koutouzis, et al. 2014) and Degidi et al. (Degidi, et al. 2011a). However, no association was found between subcrestal position of the implant and periimplant bone loss over time (CMBLi), which is in accordance with previous findings (Huang, et al. 2015; de Siqueira, et al. 2016).

Differences in bone loss at the implant were demonstrated comparing equi and subcrestal position of Ankyloss implants in another clinical trial, however a higher bone resorption at the crest for subcrestal implants was showed in radiological CBCT evaluation (Koutouzis, et al. 2014). Bone loss at the crest was 0.08±0.25 mm, 0.65±0.45mm and 0.85±0.75mm at equicrestal, 1 mm subcrestal and 2 mm subcrestal group, respectively at 1 year. These results are in line with those presented in this research were the bone loss at the crest was 0.46±0.68mm at 1 year. Degidi in 2016 with the same type of implants reported a bone loss at the crest of 0.42±0.77mm after 3 years (Degidi, et al. 2016). Although the rate of bone loss at the crest seems to be similar between subcrestal and equicrestal position of the implant a deeper position of the platform could be recommended to avoid an

exposure of the first implant threads, which could lead to more probable exposure to dental plaque in case of mucositis (Schwarz, et al. 2014).

The available literature suggests that primary stability is one relevant factor that can influence the osseointegration of immediately loaded implants (Lioubavina-Hack, et al. 2006; Strub, et al. 2012; Esposito, et al. 2007; Esposito, et al. 2008; Esposito, et al. 2013) and Resonance Frequency Analysis is considered the most reliable method to evaluate implant stability over time after implant placement (Huang, et al. 2003; Aparicio, et al. 2006; Lachmann, et al. 2006; Herrero-Climent, et al. 2012; Manzano-Moreno, et al. 2015) ISQ values at implant (ISQi) and abutment (ISQa), insertion torque and manual stability were used to assess primary stability in this study.

A significant difference between ISQi and ISQa values was found just after surgery, were mean of ISQa was 5 units lower than ISQi. This could be explained because the 2mm height abutment increases the distance between the top of the transductor and the implant shoulder and this tends to make ISQ values decrease as demonstrated by Sennerby et al. (Sennerby & Meredith 2008). Anyway, the ISQ measurement at the abutment was necessary in order not to unscrew the definitive abutment according to the protocol of 'one-abutment-one time'. The high ISQ values registered at surgery (ISQi 74.2±8.3 and ISQa 69.6±7.9) remained stable at 4-weeks. After 4 weeks ISQ values kept increasing up to the 1-year visit in a similar manner in both groups. A progressive increase in implant stability after complete osseointegration has also been observed by Kokovich et al. who compared immediate and early loading in 72 self-tapping implants with SLA® surface and observed a progressive rise of ISQ values from surgery up to 5 years.

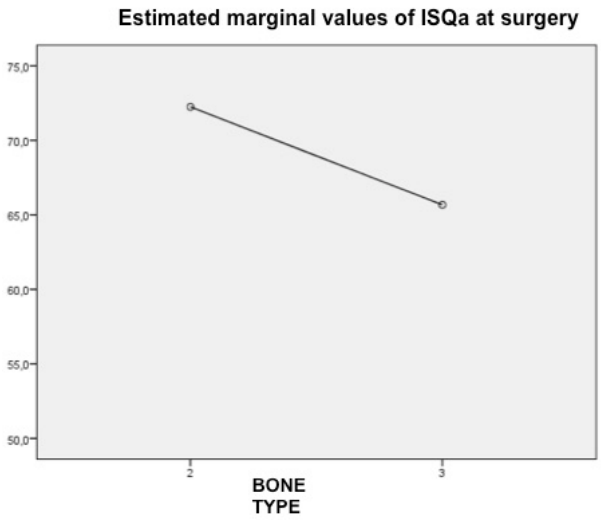
Immediate loading group showed a significant higher stability over time compared to early loading group (Kokovic, et al. 2014).

A common finding when assessing implant stability in clinical trials is the initial drop of ISQ values within the first 2-4 weeks of healing after implant placement (Nedir, et al. 2004; Han, et al. 2010; Huwiler, et al. 2007) which is caused by osteoclastic bone resorption (Bischof, et al. 2004). The chemical modification of the implant surface seems to reduce this phenomenon due to an accelerated bone formation around the implant. Nevertheless, a slight decrease of ISQ values between surgery and 4 weeks was still found in some studies were implants with a chemical modification of the surface were used, especially with high value (>70 ISQ) of primary stability (Oates, et al. 2007; Han, et al. 2010; Gómez-Polo, et al. 2016). Unfortunately, in this study ISQ values were not registered at 2 weeks and we were not able to determine whether there was a reduction in implant stability during this time.

Implant stability in the immediate loading group was significantly higher than in the early loading group at each follow-up visit. However, this difference was associated with a higher proportion of implants placed in the mandible and D2 bone quality in group A. Indeed, a direct correlation between higher ISQ values and mandibular bone ($p=0.002$) and D2 type ($p=0.003$) bone and was observed in the study (figure 18).

Figure 18.

(a)



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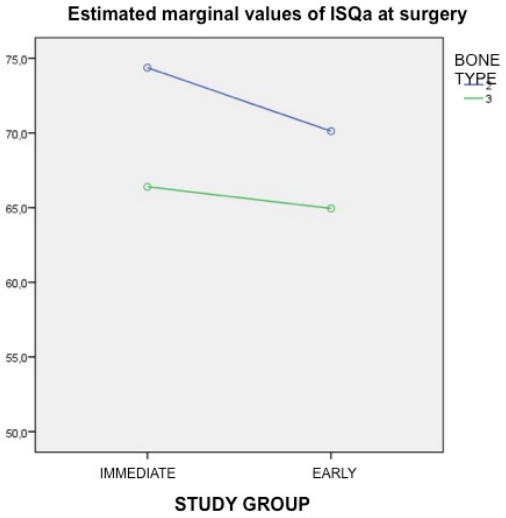
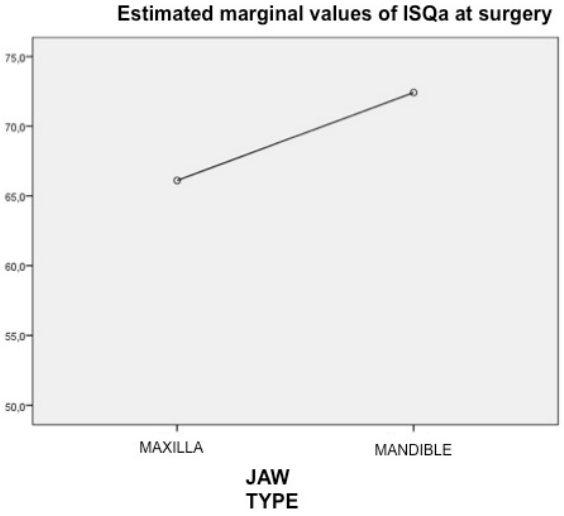
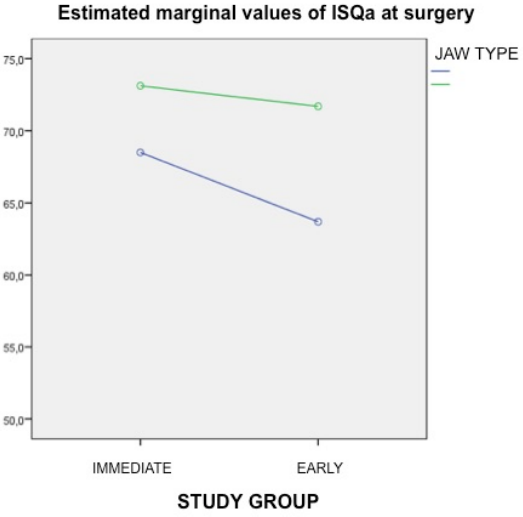


(c)



(d)



a) The graphic depicts the direct correlation between bone type and ISQa values observed in the multifactorial analysis. (b) The absence of intersections between the two lines demonstrates that the type of loading (immediate or early loading) did not influence this correlation. c) Direct correlation between the jaw type and ISQa values observed in the multifactorial analysis. (d) The absence of intersections between the two lines demonstrates that the type of loading did not influence this correlation. Jaw and Bone type variables are making a mask effect on type of loading regarding implant stability.

These results agree with an investigation by Sim et al. (Sim & Lang 2010) where higher ISQ values of implants were found at surgery, 2, 4 and 8 weeks in areas with bone type D2 compared to bone type D3 and D4 (Sim & Lang 2010). This correlation between the presence of cortical bone and a higher primary stability has been well documented in the available literature (Friberg, et al. 1999; Bischof, et al. 2004; Ito, et al. 2008; Andrés-García, et al. 2009; Merheb, et al. 2010).

Another interesting finding of the present investigation was the inverse correlation between ISQ values at surgery and the insertion torque, so that lower values of torque were associated with higher ISQ values. Whether an association between insertion torque and resonance frequency analysis measurement exists is still controversial in the literature (Brizuela-Velasco, et al. 2015; Gómez-Polo, et al. 2016; Staedt, et al. 2017; Levin 2016). One possible explanation could be the design at the neck of the implants used in this study These implants have an inverted conical shape at their coronal part leaving an horizontal space between implant shoulder and the bone that could be filled by the coagulum. Once implant shoulder has been inserted in a subcrestal position, implants tends to loose rotatory stability, especially in thin cortical bone, Thus, the insertion torque tends to decrease. However, a subcrestal position of the implant tends to increase ISQ values (Meredith, et al. 1996; Sennerby, et al. 2005; Sennerby & Meredith 2008; van Eekeren, et al. 2015) and this could explain the high ISQ values found at surgery.

Although this was not an objective of the study, from this investigation it could also be highlighted that implants with multiple splinted prostheses showed a significant higher increase in implant stability over time compared to single crowns at 1 year ($p=0.042$). This finding has not been reported in other similar trials (Ganeles, et al. 2008; Nicolau, et al.

2013; Kokovic, et al. 2014). However, significant differences in bone loss between splinted and non-splinted implant restorations have been recently demonstrated (Vigolo, et al. 2015), the association between splinting and implant stability is still unclear.

A limitation of this study was the use of standardized intraoral radiographs to assess marginal bone level, even though this is a well-validated method in the literature, (Meijer, et al. 1992) since buccal and lingual bone could not be evaluated. Furthermore, the first bone to abutment contact point of implants has been considerably challenging to be visualized and interpret with the used method. On the contrary, this reduces the radiation exposure of patients compared to Cone beam computed tomography (CBCT).

The preliminary results of this randomized clinical trial should be interpreted with caution due to the limited sample size and follow-up. In fact, this is an on-going investigation and complete results will be presented after 3 years.

8. CONCLUSIONS

8. Conclusions

Within the limitations of this study, it could be concluded that immediate and early loading of implants with thermo-chemically treated surface, with the use of definitive abutments tightened at the time of surgery, is a predictable treatment option at posterior areas of maxilla and mandible. The thermo-chemical treatment of the implant surface seems to limit the drop of implant stability in the first 4 weeks after surgery. These results have to be confirmed by further studies with a larger sample and longer follow-up periods.

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10. ANNEXES

Annex I: approval of PhD Project

Universitat Internacional de Catalunya | Escola de Doctorat

MATTEO ALBERTINI
C/ ARIBAU 213, 5º2ª
08021 BARCELONA

Estimado Sr. Albertini,

Con la presente, le comunico que la Comisión Académica del Doctorado en Ciencias de la Salud en su sesión del pasado 8 de julio, y una vez estudiada su solicitud acordó aprobar el proyecto de tesis con título "Carga inmediata y temprana de los implantes dentales con una superficie biomimética" y que sea admitido al período de investigación del Doctorado en Odontología.

Se acuerda nombrar al Dr. José Nart Molina como Director y al Dr. Mariano Herrero Climent como Codirector.

Por otro lado le hacemos saber que la normativa de la UIC establece que se debe obtener una evaluación favorable del Comité de Ética en la Investigación, antes del inicio de la Investigación. Deberá aportar este informe en cuanto lo obtenga.

Finalmente le queremos informar que para poder tramitar la matrícula se puede poner en contacto con la Sra. Sònia Soriano (ssoriano@uic.es).

Para cualquier otra cuestión que desee comentar no dude en ponerse en contacto con nosotros.

Atentamente,

Empar Lorda
Secretaria Comisión Académica
Doctorat en Odontología
Escuela de Doctorado
Universitat Internacional de Catalunya

Barcelona, 17 de julio de 2014

Annex II: approval of Ethic Comitee

CEIC Comitè Ètic d'Investigació Clínica

CUO Clínica Universitària d'Odontologia | Universitat Internacional de Catalunya

CARTA APROVACIÓ ESTUDI PEL CEIC

Número de l'estudi: IMPL-ECL-2014-01
Versió del protocol: 1.1
Data de la versió: 03.10.2015
Títol: "Carga inmediata y temprana de los implantes dentales con una superficie biomemética".

Sant Cugat del Vallès, 19 d'abril de 2016

Dr. José Nart

Referència: "Carga inmediata y temprana de los implantes dentales con una superficie biomemética".

Benvolgut Doctor,

Els membres del CEIC de la Clínica Universitària d'Odontologia, els hi agraeixen l'aportació científica en el camp de la investigació i la presentació del Protocol en aquest Comité per a la seva avaluació.

Valorades les noves aportacions realitzades a l'estudi, sol.licitades pel nostre CEIC, el passat dia 16 de març de 2016, li comuniquem que el dictamen final ha sigut FAVORABLE.

Li notifiquem que la Direcció General d'Ordenació Professional i Regulació Sanitària del Departament de Salut de la Generalitat de Catalunya ha resolt favorablement incloure dins l'àmbit d'actuació del CEIC-CUO el centre sanitari Nart Clínica Dental, i no autoritzar l'ampliació de l'àmbit d'actuació del CEIC els centres Centro Medico Cerom i Barcelona Dental Institute. (*Adjuntem resolució)

Li recordem que s'haurà de presentar al Comitè d'Ètica d'investigacions clíniques de la CUO, i a través de la Comissió Científica, un informe preliminar mensual del seguiment de l'estudi i un informe final un cop finalitzat aquest.

Quedem a la seva disposició per a qualsevol dubte o aclaració al respecte.

Atentament,

Dr. Magí Brufau
President CEIC

CEIC Comitè Ètic d'Investigació Clínica
20 OCT. 2016
Núm. de registre
S00077

Annex III: paper I

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Advances in surfaces and osseointegration in implantology. Biomimetic surfaces

Matteo Albertini [1], Marc Fernandez-Yague [2], Pedro Lázaro [3], Mariano Herrero-Climent [3], Jose-Vicente Rios-Santos [3], Pedro Bullon [3], Francisco-Javier Gil [4]

[1] Padrós Dental Institut, Marti i Julià, 6-8 bajos, 08034 Barcelona, Spain
[2] ETSEIB, Universitat Politècnica de Catalunya, Avda. Diagonal 647, 08028 Barcelona, Spain
[3] Faculty of Dentistry, Universidad de Sevilla, C/. Avicena s/n, 41004 Seville, Spain
[4] Department of Materials Science and Metallurgical Engineering (ETSEIB), Universitat Politècnica de Catalunya, Avda. Diagonal 647, 08028 Barcelona, Spain

Correspondence:
C/ Avicena s/n
41009 Sevilla, Spain
jvrios@us.es

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Abstract
The present work is a revision of the processes occurring in osseointegration of titanium dental implants according to different types of surfaces -namely, polished surfaces, rough surfaces obtained from subtraction methods, as well as the new hydroxyapatite biomimetic surfaces obtained from thermochemical processes. Hydroxyapatite's high plasma-projection temperatures have proven to prevent the formation of crystalline apatite on the titanium dental implant, but lead to the formation of amorphous calcium phosphate (i.e., with no crystal structure) instead. This layer produce some osseointegration yet the calcium phosphate layer will eventually dissolve and leave a gap between the bone and the dental implant, thus leading to osseointegration failure due to bacterial colonization. A new surface -recently obtained by thermochemical processes- produces, by crystallization, a layer of apatite with the same mineral content as human bone that is chemically bonded to the titanium surface. Osseointegration speed was tested by means of minipigs, showing bone formation after 3 to 4 weeks, with the security that a dental implant can be loaded. This surface can be an excellent candidate for immediate or early loading procedures.

Key words: *Dental implants, implants surfaces, osseointegration, biomimetics surfaces.*

Introduction
Dental implants represent a valid therapeutic option for the replacement of missing teeth (1). Developments in implantology have allowed to extend the reach of dental treatments through implant placement, since the latter provide long-term stable support for a dental prosthesis subjected to chewing load (2).

The biological principles followed for implant placement have already been described by some authors and can be summarized in the concept of osseointegration, which is defined as the direct and structural connection between living and structured bone, and the surface of an implant subjected to a functional load (3).
The earliest studies on this phenomenon were develo-

ped by Branemark in the 1950s, 1960s and 1970s, as well as by Schröeder (4), who proved that the alveolar bone is capable of forming a direct connection with a bolt-shaped alloplastic material such as titanium after being placed on a surgically-created bed.

Since implantology's earliest stages, the growing interest of clinicians in this type of treatment has impelled research from the knowledge of the biological principles to the basis of osseointegration. A concept emerging from the studies by Johansson and Albrektsson is that osseointegration is a time-related phenomenon. Rigidity in bone-implant interface increases with time until reaching a high level 3 months after implant placement, and can increase progressively until 12 months after placement (5).

The time necessary for implant osseointegration is variable, as it depends on a series of factors that in turn depend, in one hand, on the bone and in other hand, on implant features. According to Branemark's (3) protocol, waiting time for implant loading traditionally ranged from 3 to 6 months, depending on the implant's maxillary or jawbone position. The implants used back then were made of commercially-pure titanium obtained by bar mechanization, and their surface topography resulted from their drilling process and their subsequent electrolytic polishing, thus being known as smooth or mechanized surface.

The implant's surface features have been proven to influence the cicatrization of the bone surrounding it (6), and the use of rough surfaces as proven -by Beagle's histological studies on dogs- showed that osseointegration can be achieved in a 6-week period under normal conditions with rough surfaces obtained through subtraction methods (7).

The morphology of these surfaces is involved in a series of biological events occurring after implant placement, which range from protein adhesion to peri-implant bone remodeling. These phenomena are favored by a particular surface roughness, thus allowing quicker osseointegration, which -from a clinical viewpoint- grants space for prosthesis placement within shorter time-periods.

Immediate or early implant loading is a procedure that has been back in use with good medium-to-long-term results in the last years (8). This is partly due to the use of implants with a more osteophilic surface, which allows maintaining implant stability more effectively throughout the first weeks of osseointegration. Reduction in implant primary stability due to initial bone resorption is counterbalanced by quicker bone neoformation, which lead to increased secondary stability and more predictable osseointegration.

Implant surface treatment is aimed at providing it with some particular features involving an excellent biological response in the surrounding tissue. There are several methods for dental implant surface treatment such as mechanizing, electropolishing, plasma spraying, coating, acid etching, surface oxidation, ionization, phosphate deposit techniques in some apathetic cases, or any combination of them (9).

Implant surfaces can mainly be classified into three main categories according to their biological response: Bioinert, osseoconductive and bioactive surfaces. The first are those around which bone cicatrization occurs from the bone to implant surface (slow cicatrization). The second are characterized by the fact that their surface morphology allows them to produce bone neoformation on implant surface (i.e., the bone starts forming from the surface to the periphery). These can present different roughness degrees and/or topographies that favor interaction with the proteins that promote migration of osteoblast precursor cells depending on their surface processing received.

Bioactive surfaces are those around which rapid bone neoformation occurs from implant surface, and are characterized by their surface showing -apart from different roughness degrees- some bioactive molecules or growth factors that induce bone formation according to different action mechanisms.

A bioactive implant surface -recently developed and based on the experimental studies by Pattanayak *et al.* (10)- can imitate osteoblast's formation of the bone mineral part in its early stages. This is possible thanks to the development of a new thermochemical treatment of titanium that creates a calcium phosphate layer once in contact with biological fluids and prior to the arrival of osteoblastic cells. The use of implants with this type of biomimetic surface would allow quicker and more reliable osseointegration for cases of immediate or early implant loading.

The present paper is aimed at updating osseointegration mechanisms through the description of tissue response to different implant surfaces, as well as introducing the concept of the new biomimetic surface obtained by means of thermochemical methods.

Implant osseointegration. Present-day concepts

-Present-day concepts

The bone is a mineralized connective tissue particularly structured to bear mechanical loads. Direct and structural connection between the living bone and the surface of an implant subjected to functional load was defined as osseointegration by Branemark (3). This phenomenon has been described and researched since the 1950s and still generates interest in modern implantology.

The most widely-researched alloplastic material for dental implant manufacture is pure titanium and its alloy Ti6Al4V, always bolt-shaped. Titanium presents good biocompatibility, resistance to corrosion, and excellent mechanical properties. Implant surface osseointegration is what allows the implant to be subjected to

chewing loads, which are transmitted to the bone.
Osseointegration as described by Branemark (3) is a clinical concept referred more to the stability of the implant subjected to chewing loading and in close contact with the bone rather than to the true microscopic joint of bone tissue and implant surface. This joint is the consequence of the biological events that lead to the interaction of bone cells with implant surface after surgical trauma.
The bone reacts to implant placement with a cicatrization process that is very similar to intramembranous ossification produced after bone fracture, except that the neoformed bone is in contact with the surface of an alloplastic material -the implant.
We can mainly recognize different biological events during implant-surrounding bone cicatrization -protein resorption, clot formation, granulation tissue formation, provisional matrix formation, interface formation, apposition and bone remodeling.
-Protein adsorption
In a first moment after dental implant placement, the latter is blood soaked and the present proteins present will subsequently be absorbed by its surface. The degree of wetting of the implant surface plays a relevant role in blood protein adsorption, since it has been proven that both excessive hydrophilia -unlike generally thought- or hydrophobia, hinders protein adsorption (11). Indeed, both highly hydrophilic and extremely hydrophobic surfaces allow no formation of a liquid drop with enough volume for proteins to be absorbed by the implant surface. Once blood can ideally soak implant surface, proteins (cytokines) can be absorbed and remain on the surface to work as a signal for the migration of osteoblastic cell lines, which will form the new bone around the implant and allow implant osseointegration.
Subsequently, neutrophils and macrophages question the implant and -according to the formation, orientation and type of absorbed proteins (12)- macrophages interact with implant surface and segregate a particular type and number of cytokines (biological molecular messengers) that can either gather the osteoblastic cell line in charge of bone formation in direct contact with surface implant, or the fibroblast cell line that encapsulates biomaterial in fibrous connective tissues and results in osseointegration failure.
Protein adsorption occurs practically instantaneously, thus inhibiting direct cell-biomaterial contact. Indeed, after exposing the surface to contact with blood, adsorption time is around 5 seconds (13).
Implant surface's nature of one only layer of absorbed proteins constitutes the key factor of cell response, since cells have been proven to depend on specific proteins to adhere themselves (14). Particularly, osteoblasts demand specific interactions to adhere, proliferate and differentiate, and these interactions are defined by the number and type of proteins adsorbed in implant surface. Implant surface's chemical and topographic nature will determine protein adsorption and conformation in (its) surface (15).
-Types of proteins
For osteoblasts to be able to onset bone formation around the implant, they must previously adhere themselves to implant surface. In vitro studies observed that these cells' adhesion depends on some specific proteins absorbed in implant surfaces such as fibronectin, osteopontin and vitronectin. The last protein, proved in in-vitro and in-vivo studies, as the one that usually predominates in cell adhesion processes, followed by fibronectin (16) (Table 1). However, the latter usually acquires more and more relevance once cells onset their differentiation process (17).
Implant surfaces play a determining role in the first stages of cell adhesion, since it is their topographic and physicochemical features that are capable of inhibiting the adsorption of the proteins that facilitate the migration of the undesired cells that provoke implant fibrointegration. TGF-α is an example of this, since it is a protein that favors fibroblastic cell line adhesion. For instance, fibroblasts can trigger migration to the implant of undesired cells (18).
Pegueroles *et al.* (11,15) proved in an in-vitro study that surface treatment of titanium dental implants with a specific size (A6) of alumina sand improves fibronectin adsorption relative to smooth titanium surfaces.
-Cell-protein interaction
Cells are capable of interacting with proteins by means of cell receptors known as integrins. However, integrin-protein interactions are completed through recognition of a particular amino acid sequence within a protein by

Table 1. Proteins and their functions.

Fibronectin	binding of cells, integrins, heparin, gelatin and collagen
Vitronectin	cell-binding protein that binds collagen, plasminogen and heparin
Albumin	Transportation of proteins, and inhibition of growth of hydroxyapatite crystals
Alkaline phosphatase	hydrolyzation of the inhibitors of mineral deposition (Ca2+ transporter)
Osteonectin	Mediation of hydroxyapatite deposition
Osteocalcin	Regulation of osteoclasts' activity

the integrin. This is the case of the RGD amino acid (Arg-Gly-Asp) sequence present in adhesive proteins such as fibronectin. Integrin-protein interaction determines the regulation of multiple cell functions such as adhesion. Ramaglia *et al*. proved that osteoblasts change integrin's expression according to implant surface's chemical composition and roughness degree, where alumina sanded and acid etched surfaces showed greater expression relative to smooth surfaces (19).

After this first protein adsorption stage, the arrival of polymorphonuclear neutrophils and macrophages to the implant surface occurs. These generate a cascade of intercellular signaling that shall derivate in implant acceptance or refusal according to the recruited cells.

Blood clot formation

Some minutes after implant insertion into the bed, a blood clot forms between the implant surface and the bone walls of the created bed. This mainly contains red blood cells, platelets and macrophages in a fibrin scaffold. During the first days a series of cytokines or growth factors (PDGF, TNFα, TGFα, TGFβ, FGF, EGF) are released to stimulate healing of the surgical wound gathering different cell lines. Two to three days after implant placement, leukocytes and macrophages complete 'cleaning' tasks through the phagocytosis process and the blood clot is simultaneously deconstructed through fibrinolysis to leave space for new blood vessels.

-Granulation tissue formation

Four days after placement, blood vessel growth produces a granulation tissue that occupies the space between the implant and the bone. This tissue is characterized by the presence of non-differenced mesenchymal cells around vessel structures in a fibrin scaffold. Surgical bed preparation -due to tissue trauma itself, which releases specific cytokines such as BMP2 and BMP4- induces the differentiation of non-differentiated mesenchymal cells in the bone marrow and peri-vascular (pericytes) firstly in pre-osteoblasts and subsequently in mature osteoblasts.

-Provisional matrix formation

Osteoblastic cells physically move in the space between the bone and the implant, and their migration is guided by the fibrin scaffold. In osseoconductive surfaces such as, for instance, those obtained by blasting and acid etching, cells adhere themselves to the proteins absorbed in implant surface and start forming a provisional bone matrix (20).

Osteoblasts are incapable of producing matrix and move simultaneously, so they stop migrating along the fibrin scaffold once they have started to produce the bone matrix. If the fibrin scaffold is removed from the implant surface during migration, osteoblasts will not reach it directly and no bone formation will therefore take place from the implant surface (20). However, fibrin adhesion to implant depends on the implant's type of surface. On those of smooth or mechanized titanium, fibrin is removed during osteoblast migration, while in rough surfaces fibrin's adhesion force is higher and cells can migrate to reach implant surface.

Thus, two main types of osseointegration can be distinguished: contact osseogenesis as described by Osborn *et al*. (21), in which progressive contact between the bone neoformed from the periphery to the implant bed; and the bone neoformation described by Davies *et al*. (22), where osteoblasts that can migrate to the implant surface through the fibrin scaffold (,) form new bone from the implant back to the bed walls.

-Bone apposition

Bone neoformation starts in early cicatrization stages, and after 7 days a provisional matrix rich in collagen fibers, vascular structures, osteoblasts and some neoformed bone area (bone apposition) begin to form (23). Some growth factors such as BMP 2 and 4 take part by stimulating the later migration of non-differentiated mesenchymal cells and by differentiating osteoblasts (BMP 7). After 14 days the implant-bone gap is occupied by neoformed or woven bone, which is rich in collagen fibers, vascular structures and osteoblasts, which form a reticular structure. In this stage osteoblasts produce the interface bone and can be found, in parallel to the surface, in the osseoconductive surfaces in contact with the implant. Bone neoformation on implant surface in early stages seems more characteristic of rough surfaces than of mechanized titanium (23). At the centre of the neoformed bone tissue some osteocytes can be observed while osteoclasts appear on bed bone surface, thus indicating necrotic bone resorption.

During the apposition process, bone structure progressively transforms from reticular to lamellar. Reticular bone is fragile and poor in calcium phosphate crystals, and transforms firstly into bone rich in parallel fibers and then into lamellar bone, which is mineralized tissue capable of withstanding mechanical loadings. The duration of this bone apposition process can vary according to implant surface type, being around 4 weeks on blasting- and acid etching-obtained rough surfaces (24).

-Remodeling

Once formed, peri-implantary bone undergoes a remodeling process in which parallel fiber bone is mainly substituted by lamellar bone and bone architecture progressively adapts itself to its functional load (25). In this stage osteoblasts and osteoclasts work synergically, apposing and reabsorbing bone according to functional needs.

The bone-implant interface is under continuous remodeling and close contact between peri-implantary bone and the implant is essential to keep it functioning in the long-term.

Osseointegration on bioinert and osseoconductive surfaces

Recent years have witnessed a progressive development of dental implants and much resources have been invested to improve implant surfaces. The bolt-shaped implant developed by Adell *et al.* was a pioneer in implantology and its use has proven good long-term clinical results (26). This titanium implant is characterized by its smooth or minimally-rough (Sa < 0.5 μm) surface, resulting from drilling, which provides it with characteristic unevenness which are repeated showing a clear orientation across the implant (anisotropic surface).

This type of surface has been improved throughout the years with the creation of greater roughness so as to facilitate cell adhesion and thus accelerate implant osseointegration. While the first rough surfaces were obtained through additive particle processes such as those obtained by titanium plasma spraying, the most modern rough surfaces are obtained by subtraction methods. Among those most widely-used to obtain rough surfaces, aluminum oxide blasting, acid etching, surface oxidation and combinations of the aforementioned methods stand out (9).

These different procedures can produce mainly three types of implant surfaces: micro-structured rough surfaces (Sa = 0.5-1 μm), moderately rough surfaces (Sa = 1-2 μm), and highly rough surfaces (Sa > 2 μm) (27).

Results in literature confirm the greater effectiveness of rough surfaces relative to mechanized titanium ones, since a greater ratio of bone surface enters in contact with the implant (5), and they lead to improved (28) and quicker (24) osseointegration.

These results may be explained by the apparent different cell response in the earliest osseointegration stages. Firstly, surface roughness leads to significantly increased wetting and protein absorption, which in turn favor cell migration and adhesion (11).

However, Davies et al. hold that more favorable osseointegration is due to the clot's fibrin scaffold's greater adhesion force on rough vs. smooth surfaces (20). The fibrin scaffold allows osteoblast migration toward implant surface before these cells start to produce calcium phosphate crystals (hydroxyapatite). If fibrin's adhesion capacity to implant surface exceeds the threshold, it shall be enough to allow osteoblasts to migrate through the scaffold and get in contact with implant surface. However, in mechanized titanium surfaces, no sufficiently stable bond occurs between it and fibrin so as to withstand the 'weight' of osteoblasts during their migration, thus producing separation between the implant and the fibrin scaffold. In this situation osteoblasts do not reach implant surface and new nuclei of bone formation will be placed closer to implant bed and far from implant surface. On the contrary, the fibrin scaffold on rough surfaces does not set free from the implant during osteoblast migration due to its tighter surface bond, thus allowing osteoblasts to reach the surface and start the bone apposition process.

Thus, difference can be made between mechanized titanium bioinert surfaces in which 'contact osseointegration' occurs (21) -i.e., progressive bone apposition from bed periphery to implant surface; and, on the other hand, osseoconductive surfaces, where the 'bone neoformation' can be observed -i.e., bone apposition contemporarily from implant surface and bed (22).

-A new paradigm -the biometric surface

Rough surfaces obtained through subtraction methods such as aluminum (Al2O3) particle blasting and acid etching prove improvements in in-vivo response relative to smooth surfaces (28). This procedure gets a surface topography characterized by concavities that form peaks and valleys that favor increased osteoconduction and, consequently, quicker bone growth with increased bone adhesion force (24).

However, the use of these surfaces still leads to reduced implant stability for the time period between the second and fourth week after placement. This is due to resorption of the bone initially in contact with the implant and, and also, to still slow bone neoformation, which fails to confer stiffness to the bone-implant bond. Consequently, increased amount of implant micro-movements may occur. Increased implant movements have been proven to determine the formation of a fibrous connective tissue and finally lead to its failure (29). This phenomenon has been observed to occur more frequently when the implant is subjected to functional load in its cicatrization stage, such as immediate loading. In these procedures the surface's biological behavior gathers still more relevance, since the objective of obtaining an ideal surface also includes increasing implant stability during the critical stage of osseointegration.

Once the implant gets in contact with the bed after placement, osteoblasts from mesenchymal cells in the bone marrow form the first layers of calcium phosphate on the implant surface. This process, which is responsible for the formation of the first reticular bone, takes place by the process of bone resorption of the bed walls launched by osteoclastic cells.

A surface that provides quicker bone apposition in the first weeks after placement would allow lower reduction of implant stability during this critical stage and, thus, lower risk of osseointegration failure in an implant subjected to chewing load.

The use of coatings with similar composition to that of the bone are an attractive strategy to accelerate osseointegration during the earliest cicatrization stages. Particularly, calcium phosphate apatite has the same chemical composition as the mineral bone phase, so that complete acceptance by the organism and no inflammatory reaction occurs (30). Many researches have applied

coatings on titanium implants by different techniques such as hydroxyapatite plasma spraying (31).

Although literature reports good clinical results for hydroxyapatite-coated implants and results are comparable to those achieved with titanium-surface ones (32), coatings obtained with certain techniques seem to have important drawbacks such as scarce adherence between implant titanium and the hydroxyapatite layer. In fact, additive techniques such as hydroxyapatite plasma spraying doesn't allow formation of crystalline apatite but amorphous calcium phosphate due to high elaboration temperatures (33). The properties of this layer are not appropriate, since it is extremely soluble and titanium only achieves mechanical retention, no true adhesion.

Indeed, plasma spraying-obtained hydroxyapatite surfaces have proven scarce long-term clinical behavior, where -in spite of obtaining quick initial implant osseointegration- detachment of the osteophilic surface layer with time produces bacterial filtration into the interface and progressive osseointegration loss due to peri-implantitis (34).

New studies have recently proven that other methods to obtain phosphate calcium coating with higher homogeneity and chemical stability are possible (35). These new methods propose in-vitro apatite growth directly bound to the surface, thus achieving greater adherence and layer-thickness control. This can be achieved through surface, thermal and chemical treatments.

Pattanayak *et al*. completed apatite deposits based on the formation of a thick and amorphous gel of surface sodium titanate that, once immersed in ion-supersaturated serum (mainly calcium and phosphorus), can spontaneously generate a thin apatite layer that increases direct and structural connection with the structured bone (10). There are huge differences between this thermochemical treatment and those producing calcium phosphate deposits by plasma, since plasma starts from very high temperatures (6000-9000 ºC), under which the projected calcium phosphate is in plasma state and solidifies when launched to the dental implant. The first fact is that plasma solidification provides no crystalline calcium phosphate structure but an unstructured material known as calcium amorphous state, which cannot be known as apatite because it has no crystalline structure and is more similar to a frozen liquid. This is a very important aspect because in plasma-coated surfaces amorphous calcium phosphate dissolves much quicker than the crystalline phosphate. On the non-crystalline calcium phosphate layer also starts osteoblasts' bone apposition process, although this neoformed bone will not get in direct contact with the implant surface when the calcium phosphate layer has dissolved; consequently, this phenomenon delays initial stages of the osseointegration process. Besides, calcium phosphate cooled down from such high temperatures is very fragile, since ceramic materials cannot withstand volume changes caused by such sudden temperature changes. Finally, the main limitation of plasma-formed layers is that they present no titanium-layer chemical bond, and their stability is mainly due to some mechanical clamp between titanium roughness and the amorphous mass of calcium phosphate. The biological consequence of this phenomenon is a bacterial microfiltration in the interface that in turn leads to osseointegration loss due to progressive peri-implantitis (34).

Obtaining calcium phosphate on implant surface by means of thermochemical treatments involves numerous advantages. Firstly, calcium phosphate is not organized amorphously but in a crystalline way, since it is formed by precipitation. This makes its structure (measured by X ray diffractograms) be the same as the calcium phosphate that forms bone mineral content (hydroxyapatite) (36), which provides a material with lower dissolving capacity in biological fluids and allows titanium chemical covalent bonds (37). This chemical bond renders excellent long-term stability and prevents all bacterial colonization between the calcium phosphate and the titanium (38).

Another important advantage of the thermochemical treatment relative to other hydroxyapatite obtaining methods is the obtained layer's high mechanical resistance, since high temperature changes in plasma treatment are avoided (39).

This method can be said to provide a biomimetic surface, since the implant-covering sodium titanate layer can -thanks to Na^+ ion bioactivity, and once it gets in contact with biological fluids- form on its own a hydroxyapatite layer without the need of osteoblasts taking part. This phenomenon has been proven both in-vitro and in-vivo by our research group, and accelerated osseointegration has been observed relative to untreated surfaces (28,37) (see Fig. 1).

Gil et al. have proven in histological studies in minipigs that thermochemical treatment of dental implant type-3 titanium surfaces can render full implant osseointegration within 4 weeks (37).

In their most recent study Gil et al. focused on the osseointegration capacity of 320 implants in minipigs, comparing bone response to different types of surface (37). The assayed surfaces were biomimetic surfaces obtained by combined aluminum oxide blasting and acid etching plus thermochemical treatment, rough surface obtained by aluminum oxide blasting, rough surface obtained by acid etching, and smooth surface as control. The implants used in this study were characterized by their 1.5-mm polished neck, 12-mm length and 1-mm thread pitch. Implant surface roughness was characterized first through electron microscopy, measuring surfaces contact angles and then the in-vivo test was completed by placing implants into minipigs to which teeth

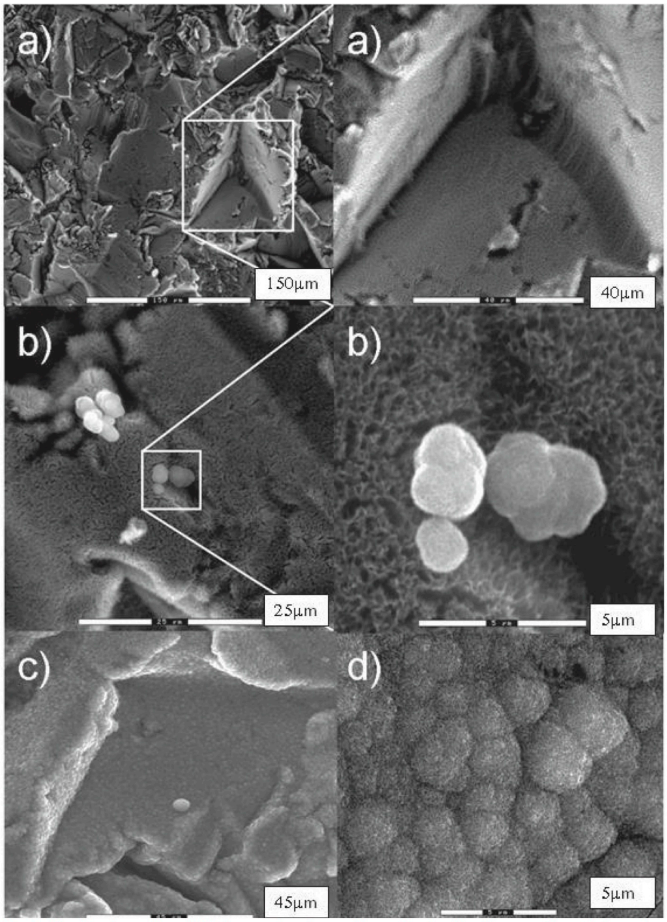

Fig. 1. Biomimetic surface. SEM images showing apatite nucleation directly on surfaces 3 days after immersion.

were extracted 4 months before. Four implants of each type were placed in each animal, which were slaughtered 3 days, and 1, 2, 3 and 10 weeks after intervention to complete histological studies. Regarding surface characterization, no significant differences were observed in roughness values (Sa and Sm) between the biomimetic surface and the blasting-obtained rough surface. However, significant differences were found between these two and the acid etching-obtained rough surface (see Table 2). The biomimetic surface proves lower contact angle relative to the blasting-obtained rough one, which shows up greater wetting and better behavior under blood contact.

Regarding bone-implant contact (i.e., the ratio of bone in contact with the implant), the biomimetic surface proves significantly higher values relative to the other surfaces 3 days, and 1, 2, 3 and 10 weeks after placement, though similar values are observed in blasting-obtained rough surface after 10 weeks (see Fig. 2).

This surface has presented surprisingly high osseointegration values in early cicatrization stages, being around 75% and 80% 2 and 3 weeks, respectively, after placement in this animal model. The biomimetic surface was the only one that clearly showed extensive areas of bone neoformation in direct contact with the implant after only one week of cicatrization (see Fig. 3). This

Table 2. Values of surface roughness in mechanized (Ctr), acid etched (AEtch), aluminum oxide blasted (GBLast) and biomimetic surfaces obtained by blasting, acid etching and surface thermochemical treatment.

SURFACE	Sa (µm) ± SD	Sm (µm) ± SD	Index Area ± SD
Ctr	0.21 ± 0.02	0.34 ± 0.02	1.09 ± 0.01
AEtch	1.59 ± 0.13	2.51 ± 0.23	1.24 ± 0.06
GBlast	3.64 ± 0.15	5.67 ± 1.07	2.56 ± 0.05
2Step	3.20 ± 0.45	5.10 ± 1.08	2.52 ± 0.20

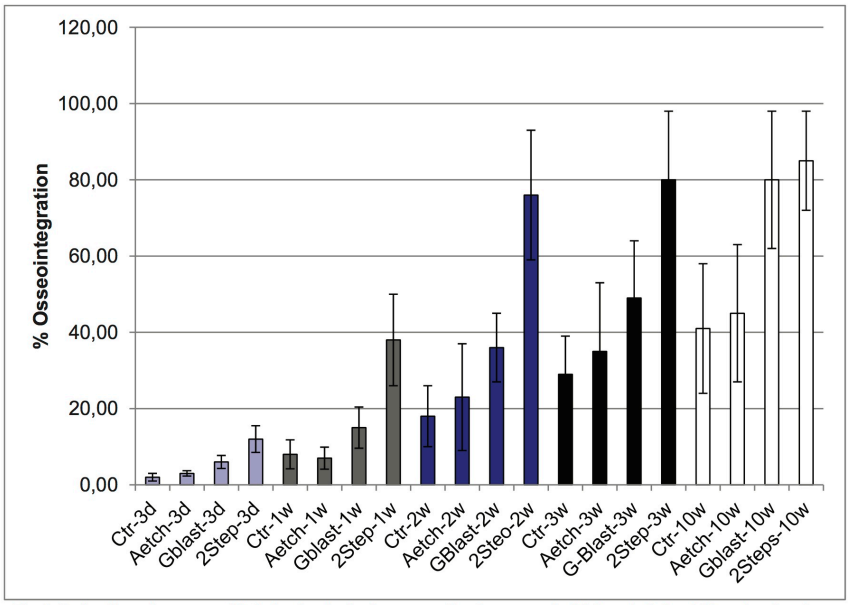

Fig. 2. Ratio of bone in contact with the implant in the four types of surface researched 3 days, 1, 2, 3 and 10 weeks after placement. Crt: mechanized surface; AEtch: acid etching-obtained rough surface; Gblast: aluminum oxide blasting-obtained rough surface; 2 Step: rough surface obtained by aluminum oxide particle-blasting, acid etching and thermochemical treatment. Bone neoformation occurs significantly more quickly on the rough surface obtained by aluminum oxide particle-blasting, acid etching and thermochemical treatment, whose bone-implant contact (BIC) is over 70% and maximum at two and three weeks after stabilization, respectively.

phenomenon can be explained by the combination of osseoconductive phenomena provided by thermochemical treatment, which in turn naturally leads to the formation of calcium phosphate crystals on the implant surface once it gets in contact with biological fluids.

These encouraging results in this new surface can contribute to great clinical benefits for the application of immediate or early implant-loading protocols, however still need to be confirmed by clinical tests on humans, which are currently in developmental stages.

Conclusions

Dental implant osseointegration is a phenomenon that has been studied for a long time. However, recent bioengineering has enabled us to understand the different biological events that characterize it —namely, protein adsorption, clot formation, granulation tissue formation, provisional matrix formation, interface formation, bone apposition and remodeling.

Protein adhesion has proven to play a key role in the earliest stages of osseointegration, where the presence

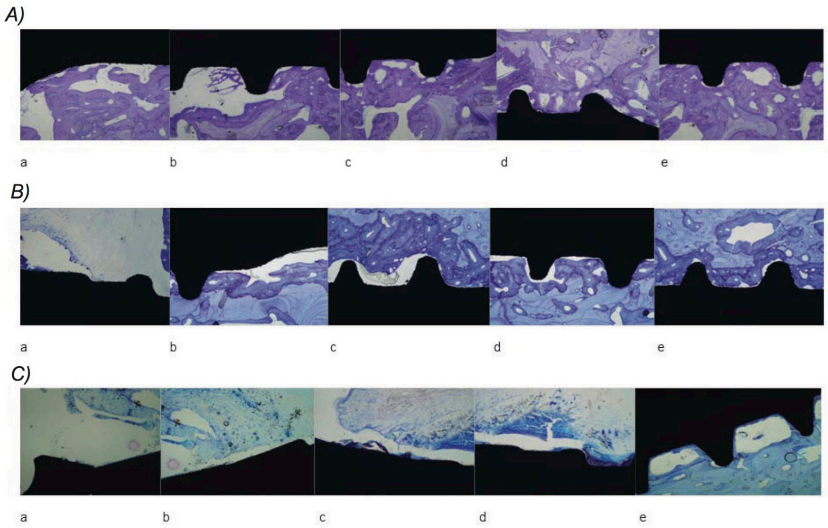

Fig. 3. A) Acid-etching rough surface implant histology 3 day s(a), 1 (b), 2 (c), 3 (d) and 10 weeks (e) after placement. B) Histology of an rough surface implant obtained by aluminum oxide particle-blasting and acid etching 3 days, 1, 2, 3 and 10 weeks after placement. Surface shows accelerated ossification relative to the treatment including only acid etching. C) Histology of a rough surface implant obtained by aluminum oxide particle-blasting, acid etching and thermochemical treatment 3 days, 1, 2, 3 and 10 weeks after placement. Surface shows accelerated ossification relative to the treatment including aluminum oxide blasting and acid etching. Note the abundant presence of neoformed mature bone in contact with the implant surface.

of fibronectin and vitronectin favor osteoblastic cell line proliferation, while proteins such as TGF-α inhibit it.

Rough implant surfaces (Sa) over 1-2 μm lead to quicker osseointegration relative to micro-rough surfaces (Sa = 0.5-1 μm) due to the phenomenon of bone neoformation, where bone starts to form from implant surface toward the periphery at greater speed.

Implants presenting hydroxyapatite in their surface lead to accelerated osseointegration due to osteoblasts' affinity to calcium phosphate. However, the surfaces produced up to date have presented long-term problems due to the bonding of this layer to the underlying titanium.

A biomimetic surface has been developed by means of thermochemical processing of titanium that allows the formation of a calcium phosphate layer in crystalline shape (hydroxyapatite), when the implant gets in contact with biological fluids. Studies in animals prove that this new surface can produce osseointegration in significantly shorter times relative to rough surfaces obtained by aluminum oxide blasting and acid etching. In-vivo studies show full implant osseointegration within 3 weeks, which would facilitate the use of immediate and early loading protocols.

These encouraging results need to be confirmed by clinical studies.

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Annex IV: paper II

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JSM Dental Surgery

Review Article

A Biomimetic Surface for Immediate and Early Loading of Dental Implants Surface Characterization and Results from Histological Studies

Albertini M[1]*, Herrero-Climent M[2], Nart J[1], Falcao C[2], and Gil FJ[3]

[1]Department of Periodontology, International University of Catalunya, Spain
[2]Private practice, Porto Dental Institute, Portugal
[3]Faculty of Dentistry, International University of Catalunya, Spain

*Corresponding author
Albertini M, Department of Periodontology, Universidad Internacional de Catalunya, Campus SantCugat, JosepTrueta, 08195 SantCugat del Vallès, Barcelona, Spain, Tel: 34670485694; Email: malbertini@uic.es

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Keywords

- Dental implants
- Immediate loading
- Early loading; Implant surfaces
- Biomimetic surfaces
- Hydroxyapatite

Abstract

The main purpose of this paper is to describe the characteristics of a biomimetic implant surface obtained by a two-step procedure, which combines grit-blasting with a thermo-chemical treatment. This method produces a crystalline hydroxyapatite with the same mineral content as human bone that is chemically bonded to the titanium surface instead of the amorphous calcium phosphate produced with a plasma-projection treatment. This surface favors an accelerated bone healing and de-novo bone formation at the early phase of healing, making it ideal for immediate or early loading. Chemical treatment, surface characterization, biological mechanism as well as histological results are discussed.

INTRODUCTION

Osseointegration is a well-known biological phenomenon that allows us to restore function and aesthetic in partially or totally edentulous patients through implant-supported prostheses [1,2]. Implant surface characteristics have been demonstrated to be one of the most important factors for early stages of bone healing to reach successful osseointegration [3].

Thanks to advances in technology and bioengineering, development of new implant surfaces has been possible in the last years, leading to a better response of bone cells during healing process, therefore a more predictable osseointegration can be achieved [4].

The development of moderately rough surfaces, through sandblasting and acid-etching procedures, has allowed a faster osseointegration making immediate and early loading more predictable [5].

Implants have recently been provided with bio-active surface treatments by introducing certain molecules which, in contact with blood and bone cells, are able to produce a further enhancement in osseointegration at early phases of bone healing as proved by experimental studies [6-8].

Hydroxyapatite coatings have been used on implant surface due to the increased affinity of the bone to calcium phosphate with good results in the short term [9-11]. However some plasma-sprayed Hydroxyapatite-coated implants have shown a significant failure rate in the long term [12]. This was due to the poor adherence of the apatite layer to the titanium surface, as a result of the high temperatures used in the formation of the amorphous calcium phosphate. This fact led to the bacterial infiltration of the interface and ultimately the progressive bone loss.

Kokubo described an alternative method for a more homogeneous and chemically stable calcium phosphate coating [13,14]. This method allows an in-vitro apatite growth directly bound to the surface, thus achieving a greater adherence and better control in the layer thickness.

Based on Kokubo's studies our research group has recently developed a novel implant surface called Contac-Ti [15, 16], which is based on the combination of a subtraction procedure and an optimized thermo-chemical treatment for moderately rough of titanium surfaces.

The aim of this paper is to describe the biological principals, characteristics and the method used to attain this new implant surface as well as to analyze and discuss the histological evidence

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Albertini et al. (2016)
Email: malbertini@uic.es

of the early phases of bone healing around dental implants provided with this surface.

The evolution of hydroxyapatite coatings

Osseointegration is a time related biological process that allows dental implant to be subjected to functional loading, and implant surface seems to be one of the most relevant factors obtain a predictable bone healing [17].

Recent years have witnessed a progressive development of dental implants and many resources have been invested to improve implant surfaces and improve clinical results when using immediate and early loading procedures.

The use of coatings with similar composition of the human bone is an attractive strategy in the development of so-called bioactive surfaces, which provide an accelerated osseointegration during the earliest healing stages. Particularly, calcium phosphate apatite has the same chemical composition as the mineral bone phase, so that complete acceptance by the organism and no inflammatory reaction occurs [9]. Many researchers have applied coatings on titanium implants by different techniques such as hydroxyapatite plasma spraying [8]. As demonstrated by clinical studies [11], this treatment produced a quicker osseointegration at early stages after implant placement but an accelerated bone loss due to a bacterial micro-leakage between the hydroxyapatite layer and the titanium has been observed in the long term [12]. Furthermore, additive techniques such as hydroxyapatite plasma spraying do not allow the formation of crystalline apatite like in human bone, but amorphous calcium phosphate due to high elaboration temperatures [18]. The properties of this layer are not considered appropriate for dental implants, since they are extremely soluble and titanium only achieves mechanical retention, not true adhesion.

Bioactivities of titanium: the thermo-chemical method

Bioengineering studies have recently proven that alternatives methods to obtain phosphate calcium coating with higher homogeneity and chemical stability are possible [19]. These methods, propose apatite growth directly bound to the surface as a result of a precipitation reaction in the human body fluid, thus achieving true chemical adhesion and layer-thickness control.

Human body fluid is supersaturated in apatite even under normal conditions and the prerequisite for apatite formation on an artificial material in a living body is the presence of functional groups that could be an effective site for apatite nucleation on its surface [20].

Based on this principle, Kokubo [14], proposed a method to provide implants with a bioactive surface based on a thermo-chemical procedure where titanium, is first chemically treated with alkali solutions and then subjected to heating at high temperatures. The aim of this treatment is to reproduce the in vivo formation of crystalline hydroxyapatite on implant surface therefore accelerating bone healing and osseointegration.

The chemical treatment, as described by the author, consist of soaking the implant in a 5-10M NaOH aqueous solution at 60°C for 24h and then a gentle washing with distilled water. The thermal procedure consists of heating the implants in an electrical furnace to various temperatures below800°C at a rate of 5°C·min-1, kept at the temperature for 1h and allowed to cool to room temperature in the furnace.

Biomimetic formation of hydroxyapatite on the implant surface

Titanium is generally covered with a thin TiO2 passive layer, which provides chemical stability and durability. During the soaking phase of the chemical treatment, the TiO2 layer gets in contact and reacts with the NaOH solution forming a hydrated TiO2 gel, which can be stabilized as an amorphous sodium titanate by a suitable heat treatment.

Sodium titanate layer is expected to form many Ti-OH- groups on its surface in the living body via the ion exchange of its Na+ ions from the surface with H3O- ions in the surrounding body fluid. These Ti-OH- groups make a highly negatively-charged surface that initially combine with positive Ca2+ ions -coming from human plasma- to form amorphous calcium titanate in the surface environment, and later the calcium titanate combines with the negative phosphate ions to form amorphous calcium phosphate, which, at the SBF-pH of 7,4 (simulated body fluid ph -7,4-), eventually transforms into bone-like apatite.

Indeed, nucleation of hydroxyapatite is the consequence of a reaction of precipitation between titanate (which contains Na+ ion) and serum which is normally saturated with Ca2+(calcium) and (PO4)3- (phosphorus) producing calcium phosphate (Ca3 (PO4)2) thus Hydroxyapatite (Figure 1).

Simulated body fluid (SBF) has been used in in-vitro experimental studies to reproduce human plasma and ideal ions concentration has been recently described by Kokubo et al. [20] (Table 1).

Nucleation of hydroxyapatite at implants with thermo-chemical treatment submerged in SBF has been observed by electronic microscope and x-ray diffraction by several authors [13,14,18,22] and our research group has confirmed these results [23,24] (Figure 2-3).

This method can be said to provide a biomimetic surface, since the implant-covering sodium titanate layer can, thanks to Na+ ion bioactivity, and once it gets in contact with biological fluids, form on its own a hydroxyapatite layer without the need of osteoblasts taking part.

Once the hydroxyapatite layer on implant surface has formed, osseointegration process continues with the selective adsorption of fibronectin from human plasma followed by migration, adhesion, proliferation and differentiation of osteoblasts, which starts bone apposition on the surface (Figure 4).

Chemically bonded bone to the implant: a new concept of osseointegration

The classic Osseointegration, as described by Branemark [1] is a clinical concept referred more to the stability of the implant toocclusal forces and in close contact with the bone rather than to a true microscopic surface bond of the bone tissue to the implant surface.

Albertini et al. (2016)
Email: malbertini@uic.es

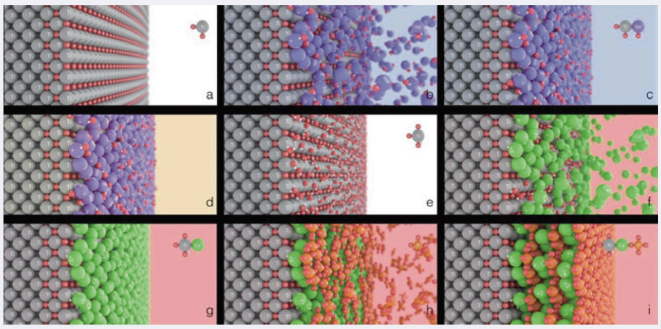

Figure 1 Biochemical sequence of Calcium Phosphate formation on Contac-Tiimplant surface.A) Titanium oxide, b) soaking in NaOH solution, c) formation of sodium titanate hydrogel, d) heating treatment, e) elimination of Na+ ion, f) calcium migration from human plasma, g) calcium adsorption, h) Phosphate migration from human plasma, i) calcium phosphate formation on the surface.

Table 1: Simulated body fluidand blood plasma ions concentration as described by Kokubo et al. (mM).

Ions	*Na+*	*K+*	*Mg2+*	*Ca2+*	*Cl–*	*HC03–*	*HPO24–*	*S024–*
SBF	142	5	1,5	2,5	147,8	4,2	1,0	0,5
BLOOD PLASMA	142	5	1,5	2,5	103	4,2	1,0	0,5
SBF: Simulated Body Fluid								

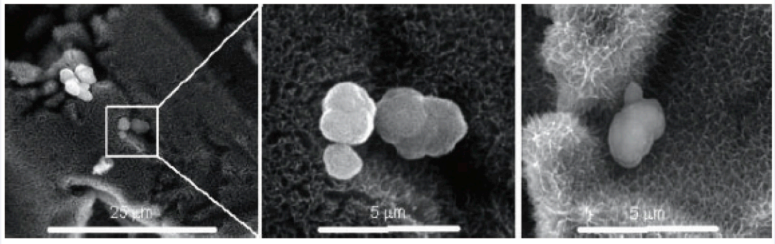

Figure 2 ESEM picture showing nucleation of hydroxyapatite on implant surface after 3 days of soaking in simulated body fluid (SBF).

The bone reacts to implant placement with a healing process that is very similar to intramembranous ossification produced after bone fracture, except that the neo-formed bone is in contact with the surface of an alloplastic material -the implant.

Originally implants had a smooth or minimally rough (Sa <0.5 µm) machined surface, with characteristic repeated irregularities, showing a clear orientation across the implant (anisotropic surface). Over the years new improved surfaces were released with greater roughness to facilitate cell adhesion and thus accelerate implant osseointegration.

Subtraction methods such as aluminum oxide (Al2O3) particle blasting and acid etching provide a surface topography characterized by concavities that form peaks and valleys that increase osteoconduction and, consequently, quicker bone growth with increased bone adhesion force [25].

Many studies have shown a greater ratio of bone surface in contact with the implant surface of rough implant surface compared to machined implant surface, leading to an improved and faster osseointegration [27].

These results may be explained by the apparent different cell response in the early stages of osseointegration. A rough surface will enhance the wet ability and the protein absorption of the implant surface favoring a greater cell migration and adhesion [28].

Davies et al. stated that the more favorable osseointegration of rough surfaces compared to smooth is due to the greater adhesion force of the clot's fibrin scaffold [29]. This scaffold allows osteoblast migration towards the implant surface before these cells start to produce calcium phosphate crystals (hydroxyapatite).

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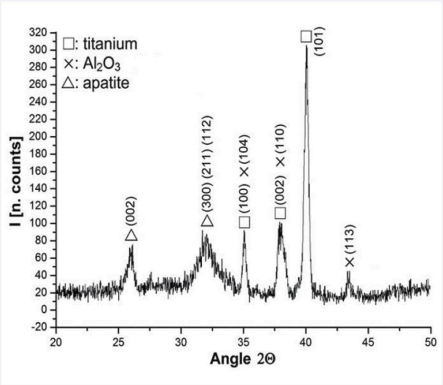

Figure 3 X-ray diffraction (GI-XRD) of2-step method treated titanium after soaking in SBF.

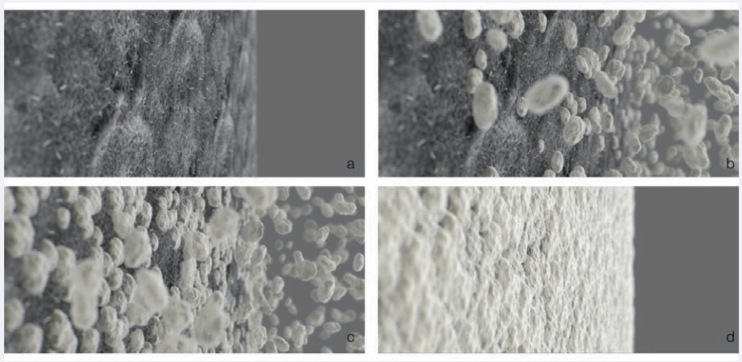

Figure 4 Schematic images of bone formation on the surface.A) Selective adsorption of fibronectin from human plasma, b) migration, adhesion and proliferation of osteoblasts, c) differentiation of osteoblasts and bone apposition on the surface.

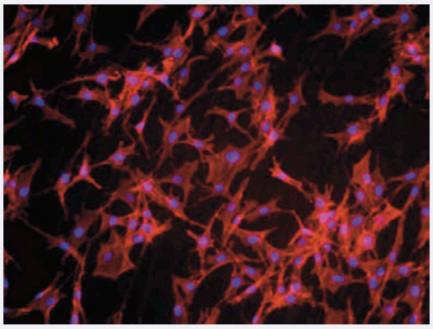

Figure 5 ESEM picture showing human osteoblasts on the thermo-chemically treated implant surface.The shape and distribution of the cells on the surface shows the good differentiation achieved.

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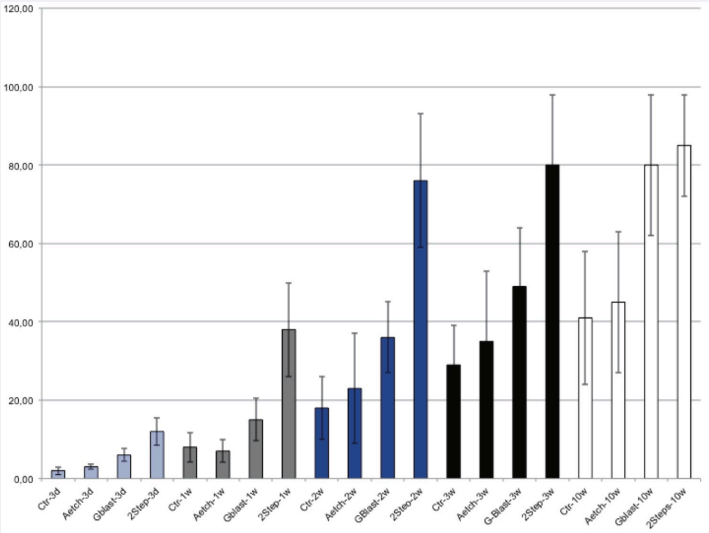

Figure 6 Bone to implant contact (%) at the new surface and controls at 3 days and 1, 2, 3, 10 weeks in a mini-pig model. Ctr: machined surface; AEtch: acid-etched surface; Gblast: grit-blasted surface; 2-Step: grit-blasted, acid-ecthed and thermo-chemical treated surface. Significantly quicker osseointegration occurs at 2-step surface with a BIC greater than 70% at 2 weeks. At 3 weeks osseointegration is completed.

If fibrin's adhesion capacity to implant surface exceeds the threshold, it shall be enough to allow osteoblasts to migrate through the scaffold and get in contact with implant surface. However, in mechanized titanium surfaces, no sufficiently stable bond occurs between it and fibrin so as to withstand the 'weight' of osteoblasts during their migration, thus producing separation between the implant and the fibrin scaffold. In this situation osteoblasts do not reach implant surface and new nuclei of bone formation will be placed closer to implant bed and far from implant surface.

On the contrary, the fibrin scaffold on rough surfaces does not set free from the implant during osteoblast migration due to its tighter surface bond, allowing osteoblasts to reach the surface and start the bone apposition process. Thus, difference can be made between bio-inert surfaces in which 'contact osseointegration' occurs [30], progressive bone apposition from bed periphery to implant surface; and, on the other hand, osseoconductive bioactive surfaces, where the 'bone neoformation' can be observed, bone apposition contemporarily from implant surface and bed [31].

A new concept of implant surface is the bioactive surface; characterized by some bioactive molecules or growth factors that induce bone formation according to different action mechanisms.

Chemically modified sand-blasted/acid etching surface is one example of bioactive surface, which promotes a faster bone healing [32,33]. However there's no chemical bonding between titanium and bone due to the fact that commercially pure Titanium is a bio-inert material without bone–bonding ability, thus the interaction between the metal and the hard tissue does not involve a chemical bond [23].

The thermo-chemically-treated surface, as proven by experimental studies [24], provides the implant with a chemically bonded hydroxyapatite layer with the purpose, as other bioactive surfaces, to accelerate bone healing during the critical period for Osseointegration therefore producing better results with advanced clinical procedure as immediate or early loading.

This method, as discussed above, provides the implant with chemically bonded hydroxyapatite layer, which produces a bone healing and mineralization, both from the implant surface and the bone-bed [24]. Once osteoblasts start bone apposition and mineralization by producing and production of calcium phosphate, a chemical bonding between the hydroxyapatite layer of the implant surface and the new osteoblast-produced hydroxyapatite is produced. Several studies have confirmed high bond strength of the hydroxyapatite layer on the implant surface to the implant and also an increased resistance of the implant to the pull-outtest *in-vivo* has been observed [27,34,35].

Therefore, the classical concept of Osseointegration described as 'intimate contact between living well-structured bone and the implant surface', as described by Branemark [1], seems to start changing to a more biomimeticconcept of a 'chemical contact between living well-structured bone and the implant surface'.

The contac-ti surface (2-stepmethod)

Contac-Ti is the evolution of Shot Blasting surface (Klockner Implant System, SOADCO, Andorra), which was based on micro

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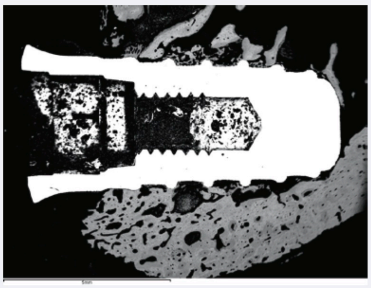

Figure 7a Histologic samples of implants with Contac-Ti surface in rabbit model. Sample at 2 weeks.

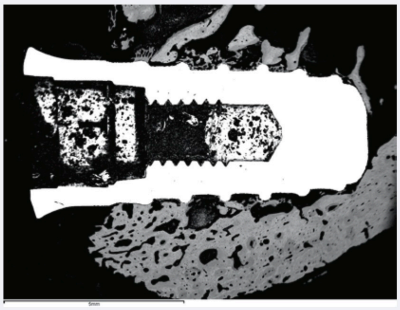

Figure 7b sample at 4 weeks.

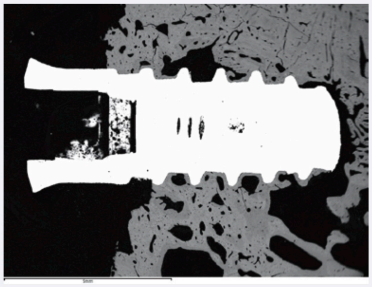

Figure 7c sample at 8 weeks.

roughness obtained by grit-blasting with alumina particles and subsequent acid etching. Excellent clinical results have been demonstrated with the use of this surface by significantly increasing the BIC area as compared to an untreated surface [43,44].

It is well known that moderately rough surfaces (Sa = 1-2 μm) obtained by means of grit-blasting and acid-etching provide a better bone healing [36] and has also been observed that roughness can improve biological response of bioactive titanium surfaces [23].

The new surface is the result of the combination of subtraction procedures to attain a moderately rough surface and a thermo-chemical method based on the principles described by Kokubo et al. [14].

A 2-step procedure, in which first girt-blasting and acid-etching and then a thermo-chemical treatment is performed on machined titanium to obtain the Contac-Ti surface.

The first step: grit blasting/acid etching treatment

Combination of grit blasting and acid etching treatment, which consists of first bombarding a surface with a myriad of small abrasive biologically-inert ceramic particles and then soaking the implant in an corrosive-acid solution, is one of the most frequently used treatments for obtaining a rough surface of dental implants [37].

There is a consensus in the literature about the improvement of osteoblastic response provided by grit-blasting/acid-etching treatment [38,39]. Moreover, a better long-term in-vivo response is achieved when the surface roughness increases since the percentage of implant in direct contact with bone increases as well as loads and torques for extracting implant from bone [40].

Improvements in fibronectin adsorption at implants which received grit blasting treatment with a specific size of alumina (A6) has been demonstrated by in-vitro studies [28,41] as well as a better osteoblasts response in terms of integrin expression at implants with grit blasted/acid-etched surfaces [42].

Commercially-pure grade IV titanium (according to ASTM F67) is used as substratum to obtain the Contac-Ti surface (Klockner Implant System, SOADCO, Andorra) and particles smaller than the ones used for the Shot Blasting since lower roughness value was pursued. During the grit-blasting treatment, 300 μm Aluminium oxide particle size is used, in a second stage acid-etching procedure with HCl is performed to attain a 1,6 Ra value of the implant surface.

The second step: thermo/chemical treatment

The second step to attain Contact-Ti is the thermo-chemical treatment for rough titanium surfaces [45,46].

It consists in submerging the metal in a NaOH solution at 60 ºC for 24 hours, then rinsed with distilled water and dried at 40 ºC for 24 hours and finally it is submitted to a thermal treatment in a tubular furnace at 600ºC for an hour and finally subjected to a cooling process. After completing the surface treatments, all implants are ultrasonically cleaned in soap and distilled water for 10 min, dried with nitrogen gas, and sterilized in ethylene oxide at 37 ºC and 760 mbar for 5 hours.

The main difference between this treatment and the one previously described [14] is that the conditions of reagent concentrations, temperatures changes and heat treatment times have been optimized for moderately rough titanium surfaces as well as heating and cooling rates.

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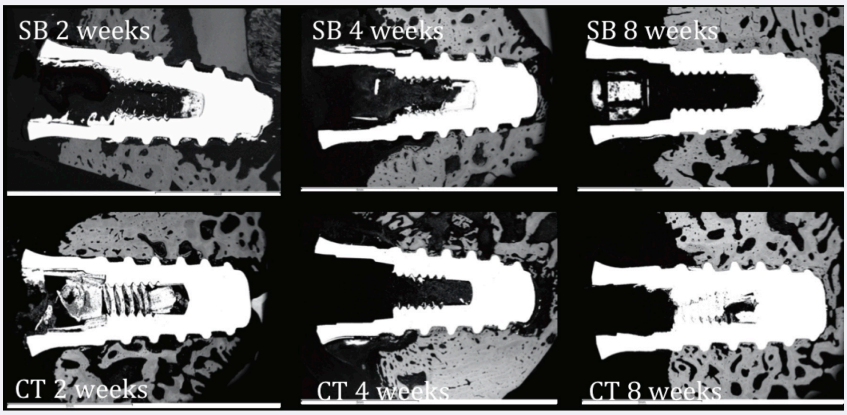

Figure 8 Histologic samples of implants with Contac-Ti surface and sand-blasted implants controls in rabbit model.
SB: Sand-blasted implants controls, CT: Contact-Ti implants.

Microscopical characterization

Surface roughness: Surface characterization of Contact-Ti compared with machine titanium has been recently analyzed by our research group using an optical profiling system device (Optical Profiling System, Wyko NT9300, Veeco Instruments, EEUU) and data analysis means of Wyko Vision 232TM software (Veeco). 10 measurements have been performed and Sa, Sq, Sz and S area index topographic parameters have been used to describe surface characterization. Values of a 1,74 Sa, 2,20 Sq, 16,74 Sz and 1,03 S area index have been obtained from the analysis as shown in Table (2).

Grit-blasting and acid-etching procedure as described above produces a moderately rough surface with good homogeneity as described by Albrektsson [3], and the additional thermo-chemical treatment seems not to alter surface topography [27].

Surface hydrophily: Implant surface can be defined as hydrophilic when it's characterized by a high wettability which is the process by which a drop of liquid spreads over the surface as a result of the interaction of adhesive forces, between liquid and substrate, and internal cohesive forces of the liquid. "The contact angle (CA) is a technique used to determine the wettability of materials and, as the name suggests involves determining the angle between a drops of liquid in contact with the surface a solid. This value depends on the relationship between the adhesive forces between the liquid and the solid and liquid cohesive forces. When the adhesive forces with the solid surface are greater than the cohesive, the contact angle is less than 90 degrees, so that the liquid wets the surface.

Our research group performed an analysis of the wettability of Contac-Ti compared with machined and other rough surfaces by measuring the contact angles so that information above hydrophilic and hydrophobic characteristics could be obtained.

A device for contact angle (CA) measurements and drop dispenser (DATAPHYSICS OCA 15 model) has been used to obtain CA values, 5 measurements for each surface were made and a drop of 1 µl of pure water (Milli-Q, Merck Millipore) was used. A constant of 3 seconds was the time the water droplaidon the surfaces before measurements were performed, results are shown in Table (3,4).

CA higher than 90 have been registered on the machined titanium showing the hydrophobic behavior of this surface, while all the other surfaces have hydrophilic characteristics. However, results show the new surface have the lower value of contact angle, therefore the highest wettability. These hydrophilic characteristics are able to promote protein adsorption and cells adhesion, which contribute to accelerate osseointegration [28,36].

DISCUSSION AND CONCLUSION

Influence of the 2-step treatment on mechanical properties of titanium

The lack of osseointegration due to several factors in the early stages after implantation is the most common form of implant failure whilst peri-implantitis and implant fracture represents the most common causes of implant loss in the long term [47]. Therefore, fatigue is a very important aspect to be taken into account when considering the long-term behavior of dental implants. Fatigue of a material is closely related to the surface structure, meaning that all these surface modification methods conducted to promote a better osseointegration may affect the fatigue performance of the implant. Furthermore, it has to be considered that post-thermal processes may alter the microstructure of the implant material.

Gil et al. [48], carried out an in-vitro study where mechanical properties of 2-step-treated-implants were assessed. Fatigue test were carried out at 37 ºC on 500 dental implants, residual stresses and fatigue-crack nucleation were analyzed comparing machined, grit-blasted and 2-step surfaces. Although a minimal

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Table 2: Roughness values of Contac-Ti.

SURFACE	*Sa (µm)*		*Sq (µm)*		*Sz (µm)*		*S area index*	
	Mean	**S.d.**	**Mean**	**S.d.**	**Mean**	**S.d.**	**Mean**	**S.d.**
Machined	0,15	0,01	0,19	0,02	3,47	1,53	1,04	0,01
Contac-Ti	1,74	0,07	2,20	0,09	16,74	1,11	1,03	0,01

Machined: machined titanium, Contac-Ti: surface attained after the 2-step treatment, S_a: average surface roughness, S_q: quadratic mean surface roughness, S_z: maximum peak/valley surface, S area index: index between surfaces, homogeneity of the surface.S.d.: standard deviation.
S.D.: standard deviation. *Statistically significant difference (p 0.005).
from Aparicio et al., 2011.

Table 3: Angle contact measurements expressed in degrees of the analysed surfaces.

	Contact angle (°)		**Contact angle (°)**
Machined	83.1	**Ra 2,5**	80.6
	86.8		80.8
	94.3		80.6
	97.8		82.4
	92.4		80.2
Contact-Ti Ra 1,5	79.0	**Ra 3,5**	86.2
	79.2		89.8
	81.3		88.4
	75.1		88.9
	73.9		86.3

Machined: machined titanium, Contac-Ti Ra 1,5: Contact-Ti surface, Ra 2,5: higly-rough surface, Ra 3,5: extremely-rough surface.

Table 4: Mean and standard deviation (S.d.) of contact angle measurements of the analysed surfaces.

Surface	*Machined*	*Contac-Ti Ra 1,5*	*Ra 2,5*	*Ra 3,5*
Mean	90.88	**77.70**	80.92	87.94
S.d.	5.90	3.09	0.85	1.62

Machined: machined titanium, Contac-Ti Ra 1,5: Contact-Ti surface, Ra 2,5: higly-rough surface, Ra 3,5: extremely-rough surface.

Table 5: Mean adhesion force values of the different samples with apatite layers†.

Samples	***Force adhesion*** ± S.D. (mN)
Ti-2-steps	451±124
Ti-PS	160±56*
AL6-2-steps	501±90
AL6-PS	190±65*
SI6-2-steps	-
SI6-PS	178±66*

Ti-PS: machined (lathe cut) commercially pure titanium surface + Plasma-spray treatment; AL6-2-step: titanium grit-blasted with Al2O3 particles with a mean diameter of 425-600 µm at a pressure of 2.5 MPa + thermo-chemical treatment; AL6-PS: titanium grit-blasted with Al2O3 particles with a mean diameter of 425-600 µm at a pressure of 2.5 MPa + Plasma-spray treatment; SI6-2-step: titanium grit-blasted with SiC particles with a mean diameter of 425–600 µm at a pressure of 2.5 MPa + thermo-chemical treatment; SI6-PS: titanium grit-blasted with SiC particles with a mean diameter of 425–600 µm at a pressure of 2.5 MPa + Plasma-spray treatment.

decrease (10%) in fatigue life of 2-step implants in comparison with grit-blasted was registered, a high fatigue limit of 315 N was registered and all of the implants showed fractures at 15 106 cycles. The slight decrease was due to the oxygen diffusion inside the titanium of the dental implant with thermo-chemical treatment, which significantly reduced the ductility of the alloy.

According to previous works that compared apatite coatings obtained by different methods like plasma spray, laser ablation, the coatings did not last longer than 106 cycles in any of the cases, being the rapid propagation of the crack either in the coatings or at the interface with the metal implant the main cause of failure [49,50].

The 2-step procedure, obtained by grit-blasting and thermo-chemical treatment reaches a 10 times higher fatigue life in comparison with classical plasma-spray apatite coating. This encouraging result, which has to be confirmed by clinical studies, make implants treated with this new technology allows a great balance in an excellent between enhanced osseointegration and long-term fatigue life.

Adhesive properties of the hydroxyapatite coating: Hydroxyapatite coating is a highly osteoconductive material and allows a predictable osseointegration of dental implants in a short period of time. Nevertheless one of the most critical considerations of hydroxyapatite-coated implants is the adhesion of the apatite layer to the titanium. Plasma-spray was used in the past to provide the apatite layer over the implant surface, however only a scarcely-adhered to titanium amorphous calcium phosphate was produced with this technology leading to a progressive loss of osseointegration due to a bacterial micro-leakage between titanium and apatite coating [12].

The thermo-chemical treatment, as discussed previously, provides the implant with a chemically bonded hydroxyapatite

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layer by means of a chemical reaction of precipitation of calcium phosphate from ions-saturated human plasma. Adhesion force between implant titanium and the hydroxyapatite layer attained by the 2-step treatment have been investigated in the last years by several authors.

Aparicio et al. [51], assessed the adhesion strength of the apatite-coating layer attained by plasma-spray and by the 2-step procedure with different grinding agents after immersion in SBF. The adhesion strength for the plasma-sprayed apatite layers was around 170 mN with a mean thickness of 20 μm, which were statistically lower than those measured for the 2-step samples, with mean values of 470 mN and a mean thickness of the apatite layer of 15 μm (Table 5).

Similar results have been attained by other authors [16,34] which demonstrate that the bonding strength of apatite layers formed after immersion in SBF of thermo-chemically-treated samples is significantly higher than those of plasma-sprayed hydroxyapatite layers. These results confirm the thermo-chemical treatment provides a chemical bonding between titanium and hydroxyapatite layer.

Biological behavior

Cellular response to the surface: Osteblasts are the cells responsible for bone apposition and mineralization, thus the main cells implicated in the osseointegration process. The assessment of human osteoblasts response (proliferation, differentiation, and cell morphology) to implant surfaces is on one of the most used in-vitro methods to investigate the potential of osseointegration of dental implants.

Aparicio et al., in 2002 [45] investigated in-vitro biological response as proliferation, differentiation -ALP (alkaline phosphatase) activity- and cell morphology by means of environmental scanning electron microscopy of human osteoblasts on machined, grit-blasted and 2-step-treatment titanium. Cells response was assessed by the cell count (proliferation), the analysis of alkaline phosphatase activity (differentiation) and the observation of cell morphology with environmental scanning electron microscopy (ESEM). An increased cell proliferation after 1 day was registered on 2-step-treated surface compared with machined and grit-blasted ones showing the new bioactive surface to provide better cell adhesion probably due to an augmented initial protein adsorption. No statistically significant difference at 3 and 7 days between the samples was registered and a lower proliferation of 2-step surface was shown at 7 and 14 days confirming the good behavior and the higher differentiation of the cells, which –as described by other authors- is reciprocally related to the late proliferation process [52].

ALP-activity was always higher (statistically significant) in the thermo-chemical treated surfaces, indicating stimulation of human-osteoblasts differentiation because of the bioactive surfaces and this result confirms the conclusions of other authors [53,54] (Figure 5).

Nisho et al. [53], investigated the behavior of rat bone marrow cells on commercially pure titanium (Cp Ti), thermo-chemical treatment (Tc Ti) and thermo-chemical treatment incubated in a simulated body fluid (SBF) to deposit crystalline hydroxyapatite on the surface (Tc AP Ti). The alkaline phosphatase (ALP) activity of the cells cultured on Tc AP Ti was significantly higher at day 7 and day 14 than the ALP activity observed for the other titanium surfaces. At day 14, the ALP activity on Tc Ti was significantly increased compared with the ALP activity on Cp Ti. Northern blot analysis of alpha1(I) collagen mRNA was assessed revealing that expression of osteocalcin and alpha1(I) collagen mRNA was higher in the cells cultured on Tc AP Ti than the cells cultured on Tc Ti at day 14 and the cells cultured on Cp Ti showed the lowest mRNA levels. This study confirms that the thermo-chemical treatment provides the most favorable conditions for differentiation of bone marrow cells. The rough and bioactive surface obtained by a grit-blasting thermo-chemical treatment provided enhanced adhesion and differentiation of human osteoblast cells. This fact may play an important role in a rapid formation of the extracellular matrix and, consequently, in an accelerated short-term osseointegration.

Similar results have recently been reported by Quan et al., on bio-activated zirconia implants [55]. Zirconia implant disks were submerged in SBF for 1, 4, 7, and 14 days and statistically significant differences of ALP activity of cultured osteoblasts was observed between treated and non-treated samples at 9 days; cell attachment, proliferation, and differentiation of SBF-treated zirconia disks was superior to that of non-treated disks.

In-vivo results histological studies

Several animal studies which investigate bone healing around implants with the novel 2-steps treatment have been carried out in the last years and the encouraging results attained by previous in-vitro studies have been confirmed.

The first histological study on implants coated with Kokubo method was conducted by Nagano et al., in 1996 [56] where coated and non-coated polyethersulfone (PSE) discs were implanted in rabbit tibia. Mechanical analysis by means detachment test and histological measurements were obtained after sacrificing the animals. Differences in failure loads were statistically significant between coated samples and uncoated ones, with values at 8,16 and 30 weeks of 1.7 ± 0.35, 2.36 ± 0.53, 1.45 ± 0,48 kg in the first ones and 0.08 ± 0.06, 0.04 ± 0.03, and 0.023 ± 0.038 kg, in the second ones. Examination at SEM (scanning electron microscope) showed differences between the two groups of samples with a direct contact of bone to the plate at coated whilst areas of soft tissues were observed at uncoated. Author's claims apatite layer after 30 weeks seemed to have been incorporated to the bone after an osteoclasts-mediated resorption.

These results are in line with others from animal studies carried out by Fujibayashi [57] and Nishiguchi et al. [58, 59], where machined, porous and porous–apatite-coated cylinders were implanted in rabbit tibia and pull-out and histological analysis were assessed. Statistically significant differences were obtained after pull-out test between apatite-coated cylinders and machined ones; no apatite layer detachment was registered at histological examination.

In 2011 Aparicio et al., [27] conducted a study, in mini-pigs, comparing the new 2-steps treatment to a grit-blasted and acid etched surface, with a machined surface as control. Histological and histomorphometric analysis was performed at 2, 4, 6 and

10 weeks' time points, showing a new mineralized bone growth around the 2-steps implants at only 2 weeks. The investigated surface reached the highest values of BIC (bone-to-implant contact) compared to the other samples, with 22% at 2 weeks, 55% at 4 weeks, 65% at 6 weeks and 52% at 10 weeks. The differences between the last three values and the first values were statically significant.

A similar study was recently undertaken by Gil et al. [23], in which three hundred twenty implants were used in a mini-pig model assessing the BIC %, surface composition, topography and wettability in a mini-pig animal experimental model, comparing the 4 surfaces previously described at 3 days, 1, 2, 3 and 10 weeks. Low BIC values for the acid-etched surface and the machined surface were obtained, while the results for the bioactive surface were significantly higher than all the other surfaces for all time points with exception to the alumina blasted surface at the 10 weeks' time point, where there was no statically significant difference (Figure 6).

The surface presented surprisingly high osseointegration values in early healing stages after placement in this animal model, being around 75% and 80% 2 and 3 weeks, respectively, and 85% of BIC was achieved at 10 weeks. The 2-steps surface was the only one that clearly showed extensive areas of bone neo-formation in direct contact with the implant after only one week after implantation (Figure 7).

Van Oirschot et al. [60], have recently investigated the influence of a bioactive hydroxyapatite and composite hydroxyapatite/bioactive glass coatings on the iliac crest of 8 goats. A total of 96 implants were placed and removal torque test and histomorphometrical evaluation were carried out after 4 weeks. Significant higher bone area attached to the implants and BIC% was registered for bioactive implants compared to grit-blasted/acid-etched ones showing the bioactive surface treatments enhanced the bone healing.

Caparrós et al., in 2016 [61] also found significant differences in terms of BIC% between thermo-chemically treated and non-treated porous titanium implants. In vivo results demonstrated that the bioactive titanium achieved over 75 % tissue colonization compared to the 40 % value for the untreated titanium.

Up to the present, very encouraging results have been attained with this surface; nevertheless, randomized-controlled clinical trials are needed in order to validate them in humans under functional loading conditions. According to the biologic bone response of the surface emerged from in-vivo studies, early and immediate loading protocols have been proposed for human-clinical trials, which are currently being carried out by our research group.

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Annex V: submission of paper III

CLINICAL ORAL IMPLANTS RESEARCH WILEY

IMMEDIATE OR EARLY LOADING OF THERMO-CHEMICALLY TREATED IMPLANTS WITH DEFINITIVE ABUTMENTS: 1-YEAR RESULTS OF A RANDOMIZED CONTROLLED CLINICAL TRIAL

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Para: malbertini@uic.es

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Dear Mr. Albertini:

Your manuscript entitled "IMMEDIATE OR EARLY LOADING OF THERMO-CHEMICALLY TREATED IMPLANTS WITH DEFINITIVE ABUTMENTS: 1-YEAR RESULTS OF A RANDOMIZED CONTROLLED CLINICAL TRIAL" has been successfully submitted online and is presently being given full consideration for publication in Clinical Oral Implants Research.

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Annex VI: summary

The objective of this PhD project is to investigate the clinical and radiological behaviour of thermo-chemically treated implants comparing immediate and early loading protocols with definitive abutments in a functionally high-demanding clinical situation such as posterior areas of maxilla and mandible.

Before starting the clinical trial a literature review on the latest scientific advances in osseointegration and physical-biochemical characteristics of the thermo-chemically treated surface used in the study has been conducted. The results of in-vitro and in-vivo studies on this surface until the present day have also been reviewed.

Currently bio-engineering has enabled us to understand the different biological events that characterize osseointegration -namely, protein adsorption, clot formation, granulation tissue formation, provisional matrix formation, interface formation, bone apposition and remodelling. Protein adhesion has proven to play a key role in the earliest stages of osseointegration, where the presence of fibronectin and vitronectin favor osteoblastic cell line proliferation, while proteins such as TGF-α inhibit it. Rough implant surfaces (Sa over 1-2 μm) lead to quicker osseointegration relative to micro-rough surfaces (Sa = 0.5-1 μm) due to the phenomenon of bone neoformation, where bone starts to form from implant surface toward the periphery at greater speed. Implants presenting hydroxyapatite in their surface lead to accelerate osseointegration due to osteoblasts' affinity to calcium phosphate. However, the surfaces produced up to date have presented long-term problems due to the bonding of this layer to the underlying

titanium.

Biomimetic behaviour of the investigated surface in this project has been demonstrated by current studies since -out of a chemical reaction of precipitation of plasma ions- is able to produce a crystalline hydroxyapatite layer chemically bonded to the titanium of the implant without osteoblasts taking part. Results from In-vitro assays have demonstrated a surface roughness (S_a) of 1,74µm, high hydrophilia with a contact angle of 77.6 degrees, an increased osteoblastic cellular activity and high mechanical resistance of thermo-chemical treated titanium. Augmented adhesion strength of titanium-bonded hydroxyapatite without detached areas has been observed in in-vivo assays and a BIC (bone-to-implant contact) of 80% at 3-weeks has been registered histologically.

Results of the 1-year randomized clinical trial suggest that there are no statistically significant differences in terms of survival, implant stability and radiographic bone loss between implants restored with immediate or early loading protocols. 100% survival rate was registered in both groups. A mean radiographic bone loss of 0.04±0.08mm at the implant and 0.3±0.5mm at the crest in the immediate-loaded group has been observed. In the early-loaded group the means of radiographic bone loss were 0.1±0.2mm at the implant and 0.6±0.8 mm at the crest. 62.5 % of the implants showed bone contact at the abutment after 1 year.

Thermo-chemically treated implants may be considered a reliable treatment option for the rehabilitation of the posterior areas of the maxilla or mandible using immediate or early loading protocols with definitive abutments placed at the time of surgery.

These results must be confirmed by studies with longer follow-up.