

“G. d’Annunzio”
UNIVERSITY OF CHIETI-PESCARA

School of Advanced Studies “G. d’Annunzio”

Ph.D. Course in Innovative Technologies in Clinical Medicine & Dentistry

CYCLE: XXXVII

Ph.D. Thesis

*Titanium Implant Surface Topography:
Cellular and Molecular Responses*

Department of: Innovative Technologies in Clinical Medicine & Dentistry

Scientific Disciplinary Sector: MED/38

Coordinator:

Prof. Sergio Caputi

Tutor:

Prof. Maurizio Piattelli

Ph.D. Student:

Dr Stefano Oliva

Academic Years 2021/2024

TABLE OF CONTENTS

ABSTRACT	6
1. INTRODUCTION	8
<i>1.1 Osseointegration</i>	9
1.1.1 Definition	9
1.1.2 Histology	10
<i>1.2 ECM</i>	11
1.2.1 Definition	11
1.2.2 Cell adhesion	12
<i>1.3 Implant survival rate and failure</i>	12
1.3.1 Implant success	12
1.3.2 Peri-implant pathogens	13
1.3.3 Implant failure	14
1.3.4 Peri-implant diseases	14
1.3.5 Inflammatory pathway	15
<i>1.4 Dental implant materials</i>	17
<i>1.5 Implant surface treatments</i>	18
1.5.1 Macro-level Modifications	19
1.5.2 Micro-level Modifications	19
1.5.3 Nano-level Modifications	20
1.5.4 Biofilm	20
1.5.5 Implant Surface Topography	21
1.5.6 Sandblasting	22
1.5.7 Acid Etching	22
1.5.8 Laser Ablation	22
1.5.9 Fluoride Treatment	22
1.5.10 Hydroxyapatite (HA)	23
1.5.11 UV Treatment	23
1.5.12 Anodic Spark Deposition	23

1.5.13 Plasma-spraying	24
1.5.14 Titanium Nitride (NiTi) Coatings	24
2. OBJECTIVES	25
3. STUDY I. <i>Antibacterial Activity of Titanium Nitride Coating: a Systematic Review</i>	26
Abstract	26
3.1 Introduction	26
3.2 Materials and Methods	28
3.2.1 Search Strategy	28
3.2.2 Inclusion and exclusion criteria	29
3.2.3 Paper selection	30
3.2.4 Data extraction	30
3.2.5 Quality and risk of bias assessment	30
3.3 Results	30
3.4 Discussion	33
3.5 Conclusions	35
4. STUDY II. <i>Enhanced Extracellular Matrix Deposition on Titanium Implant Surfaces: Cellular and Molecular Evidence</i>	36
Abstract	36
4.1 Introduction	36
4.2 Materials and Methods	38
4.2.1 Ethic Statement	38
4.2.2 Cell Culture Establishment	39
4.2.3 Cell Immunophenotyping	39
4.2.4 Mesengenic Differentiation and Histochemical Analysis	39
4.2.5 Dental Implants	40
4.2.6 Exposure to the Titanium Surfaces	40
4.2.7 Cell Viability Assay	40
4.2.8 Scanning Electron Microscopy (SEM) Analysis	41
4.2.9. Confocal Laser Scanning Microscopy (CLSM) Analysis	41
4.2.10 RNA Isolation and Real-Time RT-PCR Analysis	42
4.2.11. Protein Expression	43
4.3 Results	44

4.3.1 Cell Characterization	44
4.3.2 Cell Viability Assay and Morphological Features of hPDLSCs Cultured on CTRL and TEST Titanium Surfaces	45
4.3.3 hPDLSCs Adhesion	45
4.3.4 Implant Titanium Disks Affect Protein Expression at CLSM	46
4.3.5 Genes and Proteins Expression	48
4.4 Discussion	49
4.5 Conclusions	52
 5. STUDY III. <i>Anti-TLR4 Biological Response to Titanium Nitride-Coated Dental Implants: Anti-Inflammatory Response and Extracellular Matrix Synthesis</i>	53
Abstract	53
5.1 Introduction	53
5.2 Materials and methods	55
5.2.1 Titanium disk	55
5.2.2 Ethics statement	55
5.2.3 Cell culture	55
5.2.4 Experimental study design	56
5.2.5 Confocal laser scanning microscopy	56
5.2.6 Western blotting analysis	57
5.2.7 RNA isolation and real-time-PCR analysis	57
5.2.8 Statistical analysis	58
5.3 Results	58
5.3.1 Morphological features of the titanium disk surface	58
5.3.2 Machined and TiN-coated disk implants influence the inflammatory pathway and the extracellular matrix deposition.	59
5.3.3 Gene expression	68
5.4 Discussion (STUDY III)	69
 6. STUDY IV. <i>A Prospective, Blinded, Randomized Controlled Study on Antibacterial Activity of Dental Implants with Coronal Portion Treated With Anodic Spark Deposition.</i>	74
6.1 Introduction	74
6.2 Materials and methods	76
6.2.1 Surface Preparation by Manufacturer	76
6.2.2 Ethics statement	76

6.2.3 Study design	76
6.2.4 Inclusion criteria	77
6.2.5 Exclusion criteria	77
6.2.6 Randomization, Allocation Concealment and Blinding	77
6.2.7 Clinical assessment	77
6.2.8 Data Handling and Radiographic Analysis of Marginal Bone Level Changes	78
6.2.9 Soft tissues evaluation	79
6.2.10 Statistical analysis	79
6.3 Preliminary results	79
6.4 Discussion	80
 7. CONCLUSIONS	 81
 8. REFERENCES	 83

Abstract

The aim of this research project is to evaluate the effectiveness of different dental implant surface treatments in promoting osteointegration, extracellular matrix deposition, and modulating inflammatory responses, while also testing the antibacterial activity of these treatments. The research was divided into three phases, each addressing a distinct aspect of implant surface behavior and its biological implications. In the first phase, a systematic review of the literature was conducted to assess the antibacterial effectiveness of titanium nitride (TiN)-coated implants and abutments. The findings suggest that TiN coatings can significantly reduce bacterial adhesion, particularly in the context of mucositis development, thereby improving the long-term stability of dental implants. This reduction in biofilm formation is attributed to the bio-corrosion properties of TiN, which enhances the implant surface's resistance to bacterial colonization and may contribute to better clinical outcomes. However, the review's major limitation was the inclusion of only one human study, highlighting the need for further clinical trials to confirm these findings. The second phase involved two *in vitro* studies. The first study compared two different titanium implant surface topographies—sandblasted (CTRL) and dual acid etched (TEST). The aim of this study was to assess the cytotoxicity, cellular adhesion, and the enhancement in extracellular matrix (ECM) component release. Both surfaces exhibited low cytotoxicity, with no significant adverse effects on cell viability. Notably, the TEST surface promoted better cellular adhesion, which was associated with the upregulation of osteoblastic-related genes and enhanced ECM component release, suggesting a favorable environment for osseointegration. These results indicate that surface topography plays a crucial role in modulating cellular responses, potentially improving the initial steps of bone integration and long-term implant stability. The second *in vitro* study focused on evaluating the biological effects of machined titanium and TiN-coated titanium implants on inflammation levels using an *in vitro* model of human periodontal ligament stem cells (hPDLSCs). The study explored the modulation of the TLR4/MyD88/NF- κ B p65/NLRP3 signaling pathway, which is triggered by *Porphyromonas gingivalis* lipopolysaccharide (LPS), a major factor in inflammation associated with implant failure. The results showed that TiN-coated titanium implants effectively modulated the inflammatory response by suppressing the activation of the TLR4/MyD88/NF- κ B p65/NLRP3 pathway, thereby reducing inflammatory signaling. Additionally, TiN-coated surfaces promoted enhanced cell adhesion and ECM deposition, further supporting their potential to improve the early stages of osteointegration. Finally, based on the literature review and *in vitro* studies, an *in vivo* clinical study was conducted to evaluate

the performance of titanium implants with different surface treatments, specifically anodic spark deposition-treated (TEST) and standard (CTRL) implants. The primary objective was to determine whether the TEST implants offered advantages in terms of reducing peri-implant bone resorption and improving soft tissue indices. Currently, the study is ongoing, and only preliminary results are available from 6 patients, with a follow-up period of 9 months. Overall, the results of this research highlight the significant role of implant surface topography and coatings in enhancing osteointegration, modulating inflammation, and improving the long-term stability of dental implants. These findings pave the way for further clinical investigations to validate the efficacy of advanced implant surface treatments in clinical practice.

1. Introduction

The increase in life expectancy has led to an inevitable aging of the population, which presents numerous complex challenges. Scientific and cultural advancements have extended the average lifespan, yet this also brings about a higher incidence of chronic degenerative diseases. Among these, conditions affecting the stomatognathic system—comprising the jaw, teeth, and associated structures—are particularly significant. When the stomatognathic system is compromised, there is often a notable deterioration in quality of life. The loss of vital functions such as chewing and speaking not only affects physical well-being but can also have a profound impact on social relationships. In elderly populations, the diminished ability to perform these functions can lead to isolation, poor nutrition, and a reduced overall sense of independence and self-esteem. This highlights the urgent need for effective treatments and interventions that can improve both the physical and psychosocial aspects of health for older adults. A new perspective for patients requiring rehabilitations after neoplastic treatments, trauma, or bone atrophies linked to the physiological aging process is regenerative medicine. This approach, through multidisciplinary methods, represents an innovative platform for restoring functions and promoting healing processes as well as the repair of injuries. Today, scientific research is focused on the study of devices with specific chemical, physical, and geometric characteristics that stimulate tissue healing/regeneration. Many studies have shown that the long-term stability of a regenerative procedure depends on the presence of connective tissue with a well-represented myofibroblastic component. It is hypothesized that this cell type can determine the three-dimensional geometry of soft tissues, becoming activated differentially during healing. At the same time, the shape of materials and the surface characteristics with which myofibroblasts and other cellular elements interact may influence their recruitment and specific functions. Tissue regeneration and healing rely on various interdisciplinary approaches, ranging from computational biology to materials science, with innovative supports that recreate the tissue microenvironment to find therapeutic solutions that harmonize cells, biological factors, and artificial devices. The increase in world population and life expectancy, along with the growing concern about physical appearance, have significantly heightened the relevance of dental implantology in recent decades.¹The use of implant supported prosthesis to replace missing teeth is currently regarded as the gold standard in dental rehabilitation.² Dental implants are typically expected to have a success rate of approximately 90% over a period of 10 to 15 years following their placement.

1.1 Osseointegration

1.1.1 Definition

The clinical success of titanium dental implants is closely linked to the osseointegration of the implant and the bone-to-implant contact (BIC) percentage, which is influenced by the surface properties of the implants.³ Osseointegration of dental implants was previously described as the establishment of both a structural and functional bond between the newly formed bone and the implant's surface.⁴

In 2012, Zarb and Koka defined osseointegration as “a time-dependent healing process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained in bone during functional loading.”⁵

Histomorphometry, used to measure the percentage of bone-to-implant contact (BIC), is a well-established method for assessing the degree of osseointegration and the healing progress of dental implants.⁶

Albrektsson et al. have described in detail, bone behavior in contact with titanium dental implants. They have described three different terms, which are closely related to each other; *osteinduction, osteoconduction and osteointegration*.⁷

- Osteoinduction refers to the stimulation of pluripotent cells to grow into bone-forming cells. When provided with the proper stimulus (the inductive agent), an undifferentiated mesenchymal cell can be converted into a preosteoblast, a process referred to as bone induction. Osteoinduction is the process by which immature cells are recruited and stimulated to differentiate into preosteoblasts. This basic biological mechanism occurs regularly, for example, during fracture healing and the integration of implants.⁸
- Osteoconduction refers to the process where bone grows along a surface. An osteoconductive surface allows bone to grow on its surface or into pores, channels, or openings. Wilson-Hench proposed that osteoconduction is the process by which bone growth is guided to conform to the surface of a material.⁹ Bone growth on an implant surface depends on the activity of differentiated bone cells. These cells can originate either from pre-existing preosteoblasts/osteoblasts activated by trauma or from primitive mesenchymal cells recruited through osteoinduction. Bone conduction in implants depends on both bone regeneration and the biomaterial's reactions.

- Osseointegration is not an isolated phenomenon, but depends on previous osteoinduction and osteoconduction. Although bone induction and conduction have a role in initial osseointegration, the term indicates long-term bone anchoring.⁷

1.1.2 Histology

Osseointegration can be described as the final step in a cascade of processes involved in bone healing around implants.

Histological and histomorphometric parameters showed nonosseointegrated woven bone around the implant at 6 days postimplantation, increasing bone volume 7-10 days postimplantation, and lamellar bone formation on the implant surface 12-13 days postimplantation, eventually replacing the woven bone by day 14. Additional bone growth was observed after 60 days.¹⁰

The osseointegration process after dental implant insertion is strictly related to the immunoinflammatory reactions. The proinflammatory wound-healing phase precedes a regenerative phase in which down-regulation of inflammation and up-regulation of osteogenesis related genes occur during the early osseointegration process, in fact, multinucleated giant cells appear to be an integral part of the normal osseointegration event.

Upon implantation, a protein layer rapidly forms on the surface of the implant, facilitating initial tissue-implant interactions. Platelets are activated and adhere to the surface, triggering the coagulation cascade and promoting the adsorption of adhesive proteins, while also activating the complement system. Neutrophils play a key role in the acute inflammatory response by migrating to the interface and working to degrade the foreign body. Monocytes are then recruited to the implant-tissue interface, where they differentiate into macrophages, which contribute to the removal of the foreign material, marking the transition to a chronic inflammatory response.¹¹

The adsorption of plasma proteins not only facilitates the recruitment of host cells through cell-matrix interactions, accelerating bone remodeling, but also promotes bacterial adhesion and colonization. As a result, host tissue cells and bacteria compete for surface domains in what is referred to as a "race for the surface". If host cells win the race, stable bone-implant integration can be achieved, and the surface is less susceptible to bacterial colonization. However, if bacteria bind to the surface first, they will proliferate rapidly and secrete an extracellular matrix, leading to biofilm formation, which is a key factor in the development and persistence of infection.¹²

Osseointegration involves a series of intricate physiological processes that are similar to direct fracture healing. One factor that influences the integration of the implant in bone is the trauma caused during the preparation of the implant socket and the insertion of the implant.

The stages of osseointegration are divided into three overlapping steps: early immune-inflammatory response, angiogenesis, and osteogenesis.

- Early immune-inflammatory response (0-14 days): It could be shown that within a few hours of fracture (socket preparation), the extravascular blood cells form a blood clot in an early phase of osseointegration. Fibrin forms and serves as a scaffold for ingrowing capillaries, collagen fibers, mesenchymal stem cells (MSC) and pre-osteoblasts.
- Angiogenesis (14 days): During the organization of the blood clot, MSC and pre-osteoblasts begin to differentiate into osteoblasts, which start to form bone on the implant surface.
- Osteogenesis (after 28 days) After 8 to 12 weeks, the peri-implant interface is completely replaced by mature lamellar bone in direct contact with the implant surface, thus completing the initial phase of osseointegration.

Systemic factors could influence bone reparative process. It has been demonstrated that diabetes and anemia can lead to a delay in the osseointegration process.¹³

Renou et al. described the effect of total body irradiation on peri-implant tissues, demonstrating an impaired osteogenesis and absence of osseointegration.¹⁴

1.2 ECM

1.2.1 Definition

The secretion of ECM components, including fibronectin, laminin, actin, and vimentin, is a critical stage in osteogenesis and osseointegration.¹⁵ The extracellular matrix (ECM) is an essential component of tissues and organs, providing both structural and non-structural support that promotes bone osseointegration. The release of ECM components by cells is the first step that leads to the early stage of bone formation.

The extracellular matrix of bone is composed of 90% collagenic proteins (type I collagen 97% and type V collagen 3%) and of 10% non-collagenic proteins (NCP) (osteocalcin 20%, osteonectin 20%, bone sialoproteins 12%, proteoglycans 10%, osteopontin, "fibronectin").

ECM components form a dynamic and complex network structure with distinct physiological and metabolic characteristics. ECM proteins control the adhesion, migration, proliferation, and osteogenic differentiation of stem cells during bone regeneration.^{16 17}

1.2.2 Cell adhesion

Cell adhesion plays a crucial role in cell development, proliferation, and differentiation. During the adhesion process, various biological components, including the extracellular matrix (ECM), cell membrane, and cytoskeleton proteins, work together to facilitate communication between the cell and its environment.

During adhesion, cells bind to specific ECM components (such as integrins), triggering intracellular signaling pathways that regulate cell behavior. These signals can influence cell migration, survival, proliferation, and differentiation.

Cell adhesion is a crucial biological process involved in the shaping of cells and tissues. It is controlled by cell adhesion molecules, which are transmembrane receptors connected to the cytoskeleton. These molecules enable cells to organize into three-dimensional structures and interact with their surrounding environment.¹⁸

Multiple studies have demonstrated how the metabolism of osteoblasts is controlled by signaling pathways that govern cytoskeletal rearrangement.¹⁹ The initial phase of cell-material interactions, which plays a crucial role in the successful integration of dental implants into host tissue, involves the establishment of proper cellular contact with the biomaterial surface, followed by adhesion and diffusion. Extracellular matrix (ECM) proteins are key players in these cell-material interaction processes. Specifically, the glycoprotein fibronectin interacts with integrins on the cell surface, facilitating mechanical anchoring and the formation of focal adhesion points between cells and materials. In this way, cell adhesion molecules promote osteogenic responses and contribute to the healing process of the dental implant.^{20 21}

Thus, cell adhesion molecules support osteogenic cellular responses and the consequent healing of the dental implant.¹⁵

Marconi et al. have shown that modifications on titanium implants surfaces, in particular increasing surface roughness by sandblasting and double acid attack, could influence cells adhesion capacity and the ECM components release. Gene expression level of ECM components such as VIM, FN1, CDH2, LAMB1, FAK and ITGB1 showed an increased expression in roughness implant surface.¹⁵

1.3 Implant survival rate and failure

1.3.1 Implant success

Nowadays, dental implants are a reliable therapeutic option for partially or completely edentulous patients in order to secure various types of prostheses.³

Moraschini et al. in a recent review of literature, revealed dental implants mean survival rate of 94.5%, with a follow-up of 10 or more years. The studies that conducted the longest period of follow-up, 20 years presented a mean survival rate of 91.2%.²²

Criteria for long-term implant success have been proposed by several authors:

- Implant should have mobility less than 1 mm.
- Absent of continuous radiolucency around the implant.
- Vertical bone loss has to be less than 0.2mm annually after first year.
- Absence of signs and symptoms of infections, pains, neuropathies or paresthesia.
- No pocket probing depth >5mm.
- No bleeding on probing;^{23 24 25}

Clinical indices must be evaluated to establish a health-disease implant quality scale related to implant therapy.

Absence of pain, after implant healing period, is a primary subjection criterion. Pain during function from an implant body is a subjective indicator that indicates implant failure.²⁶

Absence of clinical movement does not necessarily indicate a complete lack of mobility. A healthy implant may exhibit movement of less than 75 µm, yet still appear to have no clinical mobility. A clinically mobile implant indicates the presence of connective tissue between the implant and bone and suggests clinical failure for an endosteal root-form implant.²⁶

The Pisa Consensus recommends that clinical assessments of each implant monitor marginal bone loss in 1.0 mm increments. The measurement of bone loss should be based on the original marginal bone level at the time of implant insertion, rather than on any previous measurements.

1.3.2 Peri-implant pathogens

Installing oral implants in a partially edentulous dentition can affect biofilm production differently than in a completely edentulous patient. Residual periodontal pockets may act as infection sources for nearby implants. Periodontal therapy for residual teeth is crucial before placing osseointegrated dental implants.²⁷ One year after implant placement, there was a significant correlation between individual periodontal conditions and clinical conditions around implants. This suggests that infection may spread from incompletely treated periodontal sites to peri-implant sulci.²⁸

Periodontopathogenic bacteria (e.g. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*) were also detected in implant sites and implicated in peri-implant infections.²⁹

The microbiota in healthy peri-implant tissue is similar to the healthy periodontium. However, periodontopathogenic microorganisms involved in periodontitis around natural teeth differ from those associated with diseased implant sites. *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, and uncultivable asaccharolytic anaerobic bacteria (both Gram-positive and Gram-negative rods) are commonly found in peri-implantitis sites, while opportunistic microorganisms are less frequently identified.³⁰

In comparison to peri-implant health, certain bacterial species (such as *Campylobacter rectus*, *Campylobacter gracilis*, *Treponema denticola*, *Tannerella forsythia*, *Treponema socranskii*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Staphylococcus aureus*) were strongly linked to peri-implant lesions.³¹

1.3.3 Implant failure

Implant failure is most likely the result of multiple factors. Dental implant failure is classified as early and late implant failure. Early implant failure refers to an implant demonstrating clinical movement before to the installation of a final prosthesis. This is mainly due to biological issues when the body rejects the implant. It is known as "rejection" of the dental implant. Early implant failure may be due to immunological, genetic, or immunological factors.³²

Late implant failure occurs within 1-3 years after the placement of the implant.

The failure of the long-term stability of the dental implant occurs because of biological (20% because of peri-implantitis, from microbial plaque or bacterial infections) and mechanical causes, (stress shielding causing osteopenia and clenching–bruxism overloading interfacial bone).

1.3.4 Peri-implant diseases

The most frequent and avoidable cause of dental implant failure is infection. Peri-implant diseases are caused by bacterial colonization of implant surfaces, causing an imbalance between the bacterial challenge and the host-response.

Derks et al. demonstrated that the prevalence of peri-implant mucositis ranged from 19% to 65%, while the prevalence of peri-implantitis varied between 1% and 47%.³³

Peri-implant diseases may affect the peri-implant mucosa only (peri-implant mucositis) or also involve the supporting bone (peri-implantitis).³⁴

Healthy peri-implant soft tissues are achieved when an implant is placed correctly, surrounded by sufficient bone volume, and when it is restored with a well-designed and constructed

prosthesis in a patient who maintains good plaque control and oral health. Healthy peri-implant mucosa should appear similar to healthy gingiva. There should be no visible signs of inflammation, such as redness (erythema) or swelling (edema), and when the peri-implant sulcus is probed circumferentially (at four to six sites) with light pressure (approximately 0.2 N), there should be no bleeding.

After implant placement and during the healing period, physiologic bone remodeling occurs up to 1.5mm.

The primary criteria for diagnosing peri-implant mucositis include the presence of inflammation in the peri-implant mucosa and the absence of ongoing marginal bone loss around the implant. The key clinical sign of inflammation is bleeding upon gentle probing (0.2 N), though other symptoms may include redness, swelling, and the discharge of pus. In cases of peri-implant mucositis, the probing depths around the implant may also be deeper than the baseline measurements taken after the prosthesis was placed. When these clinical signs are observed, a radiograph showing no marginal bone loss can confirm the diagnosis of peri-implant mucositis. An intra-oral radiograph to identify progressive marginal bone loss is required to confirm a diagnosis of peri-implantitis.³⁴

Peri-implantitis is a plaque-associated pathological disease that occurs in the tissues surrounding dental implants characterized by inflammation in the peri-implant mucosa and consequent gradual bone loss.

1.3.5 Inflammatory pathway

Bacterial colonization of implant surface is essential for triggering the inflammation and release of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF).

It is known that LPSs, through the activation of the Toll-like receptor 4 (TLR4), lead to the production of nuclear factor kappa B (NF- κ B) and, therefore, proinflammatory cytokines.³⁵

In recent years, human mesenchymal stem cells (hMSCs) have played a key part in the development of regenerative medicine. Human MSCs are distinguished by two characteristics: self-renewal ability and multipotent differentiation potential. Furthermore, hMSCs can develop and expand into mature cells such as osteoblasts, adipocytes, and chondroblasts, maintaining the stem cell population.³⁶

These multipotent cells are in different sites of the body. Bone marrow is the gold standard tissue for isolating hMSCs, but they can also be found in adipose and dental tissues, peripheral blood, umbilical cord blood, and amniotic membrane. Several studies have looked at alternative

tissue sources for adult mesenchymal stem cells, including the dental pulp, dental follicle, apical papilla, periodontal ligament, and palatine tonsil. In particular, hMSCs originating from oral tissues, such as dental pulp and periodontal ligament, can regulate tooth homeostasis and aid in tissue repair.^{37 38}

Stem cells generated from the human periodontal ligament (hPDLSCs) shown the ability to develop into mesengenic lineages. One of the most important characteristics of hPDLSCs is their ability to protect against infectious illnesses due to their immunomodulatory activities.³⁹ Nowadays, a large number of studies are being conducted on the MSC response to inflammatory events in combination with antioxidant compounds. Gram-negative bacteria revealed on the outside of the cell membrane a component termed lipopolysaccharide (LPS), which is regarded a crucial connection for virulence in periodontitis.⁴⁰

Lipopolysaccharide (LPS), a component of the cell wall in gram-negative bacteria, is primarily detected by the host's toll-like receptor 4 (TLR4). This bimolecular substance infiltrates periodontal tissue and is regarded as a key factor in the virulence of periodontitis.

Porphyromonas gingivalis lipopolysaccharide (LPS-G) plays a crucial role in initiating inflammation and the release of proinflammatory cytokines, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF). It is well established that LPS molecules, by activating Toll-like receptor 4 (TLR4), trigger the production of nuclear factor kappa B (NF- κ B), which subsequently leads to the release of proinflammatory cytokines.³⁵

Nuclear factor- κ B (NF- κ B) is a family of inducible transcription factors that control the expression of a wide range of genes involved in various aspects of immune and inflammatory responses.(Liu et al., 2017)

The NF- κ B pathway is activated by a variety of stimuli, including ligands binding to different cytokine receptors, pattern-recognition receptors (PRRs), members of the TNF receptor (TNFR) superfamily, as well as the T-cell receptor (TCR) and B-cell receptor.⁴¹

The pro-inflammatory role of NF- κ B has been widely studied in macrophages, a diverse group of innate immune cells found in various tissues, which play a key role in the initial immune response to infections.

Under various pathophysiological conditions, activated macrophages can differentiate into distinct phenotypic states, including classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages are marked by the production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-12, TNF- α , and chemokines, which play a role in different inflammatory processes. These M1 macrophages also promote the differentiation of inflammatory T cells, such as Th1 and Th17 cells, which further drive inflammation. In contrast, M2 macrophages

produce anti-inflammatory cytokines like IL-10 and IL-13, playing a critical role in resolving inflammation and facilitating wound healing.^{42 43}

TLR signals play a crucial role in regulating macrophage polarization. Specifically, the TLR4 ligand lipopolysaccharide (LPS) promotes the differentiation of macrophages toward the M1 phenotype. LPS activates macrophage signaling through two distinct TLR adapters, MyD88 and TRIF.

TLR4 is part of the TLR receptor family, which, upon pathogen interaction, triggers a proinflammatory response. One of the key activators of TLR4 is lipopolysaccharide (LPS, endotoxin) from Gram-negative bacteria, which activates two distinct signaling cascades. The first cascade occurs at the plasma membrane and involves the adaptor proteins TIRAP and myeloid differentiation primary response gene 88 (MyD88).

1.4 Dental implant materials

Titanium and its alloys were considered the metal of choice for dental implants, due to their biocompatibility and affordable cost.

Titanium is a bioinert substance that has minimal or no negative effects on the surrounding tissue.

Titanium's biocompatibility is due to the formation of a stable, self-limiting oxide layer on its surface, which prevents further oxidation and corrosion. This corrosion resistance helps maintain the material's mechanical integrity while ensuring the health of the surrounding tissue, thereby enhancing its biocompatibility.⁴⁴

The following grades of alloys are most commonly used in commercialized dental implants:

- Grade 2 (cp-Ti), which is commercially pure titanium containing up to 0.3% iron.
- Grade 4 (cp-Ti), containing approximately 99% titanium and up to 0.5% iron.
- Grade 5 (Ti-6Al-4V alloy), an alloy composed of 88% titanium, up to 6.75% aluminum, and up to 4.5% vanadium.

The chemical and mechanical properties of these alloys satisfy the requirements for materials used in dental implants. They are characterized by high strength at low density, high elasticity (five times greater than the elasticity of bone), and bioinertness. However, not all titanium alloys are utilized in dentistry. Despite these positive attributes, titanium and its alloys exhibit low resistance to shear and wear, particularly under conditions involving friction.⁴⁵

A suitable material for dental implants should be biocompatible with sufficient strength, toughness, corrosion resistance, and fracture resistance. These characteristics are generally associated with the oxygen content in the metal. Grade IV CpTi, with its highest oxygen

concentration (0.4%), offers exceptional mechanical strength, making it the most widely used form of titanium for dental implants.

Because of the various qualities discovered in titanium forms, titanium oxide (TiO₂) has been identified as the most commonly reported in dental implant studies.

TiO₂ is formed when titanium metal reacts with air, generating hydroxyl and hydroxide groups, giving it great corrosion resistance. This oxide layer provides titanium and its biocompatibility. In ambient settings, TiO₂ exists in three crystalline forms: anatase, brookite, and rutile.⁴⁷

The difference between the rutile and anatase phases is caused by distortions in the octahedral TiO₆ structure. Anatase is the most used in dentistry, due to its better results in osseointegration.⁴⁸

1.5 Implant surface treatments

Nowadays, there are many surface treatments for titanium implants used in dentistry and orthopedics. There has been a growing interest in utilizing advanced technologies to modify the surface properties of titanium and other metals, aiming to improve their biocompatibility and osseointegration, while also reducing the risk of material corrosion and preventing the formation of harmful bacterial biofilms.

Corrosion occurs when a metal deteriorates due to electrochemical action, releasing ions into the surrounding media.⁴⁹ Corrosion can lead to implant failure after initial success, resulting in loss of osseointegration (tight contact between bone tissue and implant). Metal particles in tissues around an implant can be caused by electrochemical corrosion, mechanical wear, or a combination of both.

The surface roughness of titanium implants affects the rate of osseointegration and biomechanical fixation.

Implant surface topography plays an important role in the osseointegration of titanium implants and includes macroscopic, microscopic and nanometric characteristics of the implant surface.⁵⁰

Over the last few decades, significant progress has been made in understanding the role of the implant material and its surface during osseous integration. This new level of biological understanding will continue to drive advancements in implant surface design, but it will also result in the development of new materials and cell-based therapies to promote overall peri-implant healing. One of the primary reasons for changing implant surfaces is to minimize the time of osseointegration, which is beneficial to both the clinician and, most importantly, the patient. Surface treatment looks to be another step toward reducing the healing period before

implant restoration, in line with the progress of implant macrodesign. One of the challenges of modern implantology is also to use implant surface treatments that can reduce bacterial adhesion on the implant surface itself.³

Physical modifications can be categorized at the macro, micro, and nano levels.

Macro-level modifications refer to visible geometric changes on a millimeter scale. Characteristics like the shape of the implant body and various thread patterns can enhance initial fixation and contribute to the long-term stability of the implant.⁵¹

Micro-level modifications can expand the implant's surface area on a micrometer scale, promoting the formation of a fibrin matrix that serves as an osteoconductive scaffold for osteogenic cells and supports bone matrix deposition.⁵²

Nano-level modifications can enhance the implant's surface roughness, wettability, and surface free energy, all of which promote improved cell growth and foster osteoblastic differentiation.⁵³

1.5.1 Macro-level Modifications

The macro-level design of dental implant takes shape (tapered or parallel), diameter and length, thread pattern. The visible characteristics of an implant are referred to as fixture design, which largely dictates the accessible surface area of the implant. This design plays a key role in achieving primary stability at the time of implant placement by facilitating mechanical interlocking between the implant and the bone. Additionally, it governs the spaces available for bone in-growth.⁵⁴ The threading on dental implants is designed to maximize primary contact, enhance primary stability, and assist in distributing stress across the bone. Since bone is more responsive to compressive forces than shear forces, thread designs should be optimized to minimize shear forces during placement.⁵⁵ V-shape threads achieve the most stability and least amount of stress.

1.5.2 Micro-level Modifications

Physical modifications to implant surfaces at the micro scale, typically ranging from 1 to 100 μm , enhance secondary integration. These micromechanical alterations promote bone growth, turnover, remodeling, and overall interlocking of the bone at the implant interface.

Surface roughness of above 10 μm is associated with implant geometry, including threaded screw and macroporous surface treatments. Numerous research have indicated that both the early attachment and long-term mechanical stability of the prosthesis can be improved by a high roughness profile when compared to the smooth surface.^{56 57 58}

Dental implants have a surface roughness of 1-10 μm , according on their microtopographic profile. This roughness range maximizes the interlocking of mineralized bone and the surface of the implant.^{58 59}

1.5.3 Nano-level Modifications

Surface profiles in the nanoscale range (1-100 nm) play a crucial influence in protein adsorption, osteoblastic cell adhesion, and thus the rate of osseointegration.⁶⁰

Surface characteristics like topography, wettability, and coatings contribute to the biological processes during osseointegration by mediating the direct interaction to host osteoblasts in bone formation.

1.5.4 Biofilm

Dental implant failure is often associated with infections, which are a frequent postoperative complication after restoration. Bacterial colonization is accompanied by biofilm formation, which shields microorganisms on the implant surface.

Biofilm is described as a complex and often undefined microbial community that adheres to tooth surfaces or any hard, non-shedding material.⁶¹

Biofilms are composed of one or more microbial communities that are non-randomly organized within a glycocalyx. This structure enables microorganisms to adhere to surfaces and proliferate. The interactions between the different bacterial species within the biofilm occur through metabolic exchanges, physical contact, genetic material transfer, and communication via signaling molecules.⁶²

Biofilms that develop on the tooth surface are known as dental plaque. The bacteria within dental plaque are the main contributors to various dental problems, including caries, gingivitis, periodontitis, and periimplantitis. Microbial infection is often cited as the primary cause of dental implant failure.⁶³ The acquired pellicle, a thin film that covers the tooth, is made up of salivary proteins and forms on the enamel within seconds after brushing. These proteins and glycoproteins adhere to the tooth surface, implants, restorations, and other materials, promoting the adhesion and coaggregation of oral bacteria. Bacterial adhesion to the pellicle is facilitated by special surface molecules, known as adhesins, with lectins being the primary type found on the bacterial cell surface. The initial colonizers are primarily Streptococci species, including *S. viridans*, *S. mitis*, and *S. oralis*. Planktonic bacteria that cannot directly adhere to the tooth surface use their receptors to bind to the cell surfaces of the initial colonizing bacteria, ultimately attaching to the tooth surface. This cell-to-cell interaction, or coaggregation, is a

crucial mechanism in the process of bacterial colonization and the formation of dental biofilms. Secondary colonizers bind to the bacteria predominantly comprising of the *Actinomyces* species, *S. mutans*, *S. sobrinus*. Mature plaque develops an inherent "circulatory system," allowing it to function as a complex microorganism. Metabolic byproducts and released cell wall components, such as lipopolysaccharides and vesicles, trigger the host's immune response.⁶⁴ Biofilm formation on dental implants follows a similar pattern of microbial colonization as that on teeth.⁶⁵ The pellicle starts to form on the implant surface within 30 minutes of its exposure to the oral cavity.⁶⁶ Due to their lower albumin absorption capacity, dental implants form a less extensive acquired pellicle, leading to reduced plaque formation around the implants. The early colonizers are primarily Gram-positive cocci, rods, and *Actinomyces* species.⁶⁷ Creating an ideal implant surface involves balancing antimicrobial effectiveness with the required osteoconductive properties. This balance is challenging because although higher surface roughness improves bone attachment, it also increases bacterial retention, which could encourage biofilm development over time.⁶⁸

To develop effective infection control strategies, new coatings have been investigated, that disrupt the two-stage process of bacterial adhesion, thereby preventing biofilm formation. Once a biofilm forms, eliminating the infection becomes difficult because the protective exopolysaccharide matrix hinders both antibiotic penetration and blood flow to the affected area.

Engineering techniques for manufacturing dental implants have had a significant impact on device design, surface topographies, biocompatibility, and cost. Increasing the healing time before implant repair.

1.5.5 Implant Surface Topography

The most important properties of an implant biomaterial are: the modulus of elasticity, tensile, compressive, and shear strength; yield and fatigue strength; ductility; hardness and toughness; surface tension and energy; and finally, surface roughness.⁶⁹

Surface topography plays a crucial role in the design and production of dental implants, as it significantly affects their bioactivity. This includes factors that impact the implant's survival, such as osseointegration and the risk of bacterial infections. Common engineering techniques employed to create bioactive surface modifications on implants include sandblasting, acid-etching, anodization, plasma spraying, and laser radiation.

These techniques alter the surface energy, chemical composition, and roughness, potentially improving osseointegration. Rough surfaces with a hydrophilic composition facilitate cellular

movement and serve as a foundation for cell adhesion, promoting osteoblast proliferation. However, excessive surface roughness may increase the risk of peri-implantitis due to bacterial growth.^{70 71}

1.5.6 Sandblasting

Sandblasting the implant surface involves using particles of different diameters and is one of the most widely used commercial methods for surface modification, offering benefits in both topography and wettability. Sandblasting is regarded as a crucial method for modifying implant surfaces to promote osseointegration and enhance contact angles, thereby improving hydrophilic properties for osteoblast adhesion. However, the use of abrasive materials in sandblasting can also lead to microbial contamination, which may ultimately result in implant failure.^{72 73}

1.5.7 Acid Etching

Acid etching creates micro-pitted surfaces by immersing the metallic core in corrosive acids like HCl, H₂SO₄, HNO₃, and HF. Typically, acid etching follows sandblasting, and the combined process is known as sandblasting and large grit acid etching (SLA), which is considered a standard surface treatment. Titanium surfaces treated with the acid-etching process have shown favorable outcomes, including increased roughness and enhanced osteogenic response, driven by the proliferation, adhesion, and differentiation of osteogenic cells.^{74 75}

1.5.8 Laser Ablation

Laser ablation is a technique used to create nano-scale channels around the implant collar area. This method offers advantages over other surface modification techniques, as it can produce a complex and highly precise surface geometry with excellent resolution, all while being clean and efficient.⁷⁶

Laser-ablated implants result in significantly greater bone-to-implant contact and higher torque removal values compared to machined surface implants.⁷⁷

1.5.9 Fluoride Treatment

Fluoride stimulates the proliferation and differentiation of undifferentiated osteoblasts and osteoprogenitor cells, enhancing their activity and increasing alkaline phosphatase levels.⁷⁸ The process of depositing fluoride onto the implant surface involves placing the titanium implant in

a hydrofluoric acid solution within an electrochemical cell. In this redox system, the titanium implant serves as the cathode, while the fluoride ions donate electrons to the titanium ions, resulting in the reduction of titanium.⁷⁹

1.5.10 Hydroxyapatite (HA)

Nowadays, Nano-HA (Nano-hydroxyapatite) is one of the most commonly used materials for coating titanium implants. HA has osteoconductive properties. The most common method to achieve this modification is plasma spraying, where a plasma flame containing HA particles is heated to around 15,000 Kelvin and then sprayed onto the titanium implant surface.⁸⁰

Clinical studies have previously shown that HA-coated implants are beneficial in situations requiring quick bone-to-implant contact, such as in grafted bone or type IV bone (which is characterized by thin cortical bone and weak, low-density trabecular bone). Interestingly, the electrochemical deposition of fluoridated hydroxyapatite has also been found to exhibit antibacterial effects, especially against *S. aureus* and *P. gingivalis*.⁸¹

1.5.11 UV Treatment

UV treatment, also known as photofunctionalization, improves the osteoconductivity of titanium implant surfaces by modifying the hydrophilicity of the TiO₂ layer. Specifically, UVA (320–400 nm) and UVC (200–280 nm) wavelengths increase the hydrophilicity of the titanium surface, which in turn enhances osteogenic cell attachment, proliferation, and plasma protein adsorption.⁷⁶

1.5.12 Anodic Spark Deposition

Anodic spark deposition is a plasma-chemical-electrochemical method for surface treatment of implants, allowing the formation of oxides on anodic metals. Depending on the voltage and duration of anodization, anodization of pure titanium produces implants with varying oxide layer thickness. The formation of an oxide layer on the implant surface can positively influence cell growth by enhancing cell adhesion and osseointegration. Additionally, the anodized surface shows mechanical advantages, such as increased corrosion resistance. Recent studies have also demonstrated that the formation of surface oxides and anatase exhibits excellent antibacterial properties.^{82 83,84}

Ueda et al. found an excellent antibacterial activity (killing ~99% bacteria) on the TiO₂ layer must contain carbon and be anatase-rich to exhibit excellent antibacterial properties.⁸⁵ Over the last decades, anodic oxidation has become popular in the surface modification of titanium for

biomedical applications due to its significant advantages, simple processing method and cost-effectiveness capability to improve coating adhesion, interfacial bonding and corrosion resistance with easily tuneable properties as well as enhanced mechanical compatibility of the coating via the creation of a porous structure with excellent potential for cell colonization.

1.5.13 Plasma-spraying

The plasma-spraying process can be combined with SLA to enhance biocompatibility and protein absorption due to the extra layer of OH groups. Specifically, the implant surface can be altered by spraying titanium particles into a plasma torch at elevated temperatures, resulting in a 30 µm thick film made up of Ti-OH residues.⁵⁰

The plasma-spraying procedure involves a thick layer of deposition. The most typical materials employed are hydroxyapatite and titanium, which are thermally heated and then sprayed on the implant surf region. The combination of hydroxyapatite coating on titanium alloys has been considered particularly fascinating because to its high biocompatibility and mechanical qualities⁸⁶. Furthermore, many techniques have been investigated in order to achieve the adherence of hydroxyapatite to titanium implants, but only plasma spraying has been employed in the market.⁸⁷

1.5.14 Titanium Nitride (TiN) Coatings

The biocompatibility, osseointegration, and therapeutic/antibacterial properties of the implant covering have been identified as the most critical elements influencing the long-term durability of dental implants. Numerous bioactive materials and local medication delivery methods have been investigated.

PVD coating of titanium alloy implants with titanium nitride creates a stable layer on the implant surface, changing its chemical makeup. In dentistry, TiN coating was first used in the 1980s. Since then, TiN coatings have been employed on dental materials and devices in implant dentistry, orthodontics, endodontics, periodontics, and prosthodontics. The coating's purpose is to increase surface hardness, abrasion/wear resistance, and corrosion resistance, as well as to reduce friction and improve interaction with neighboring biological and material substrates.^{88,89}

2. Objectives

The aim of this research project is to evaluate the effectiveness of different implant surface treatments in terms of promoting osteointegration and extracellular matrix deposition. Additionally, the actual antibacterial activity of these surface treatments has been tested.

The research project has been divided into three distinct phases:

- In the first phase, a systematic review of the literature was conducted on implant surface treatment with titanium nitride coatings to evaluate the antibacterial effectiveness of the coating.
- The second phase involved two *in vitro* studies in which two different implant surface treatments were compared, to evaluate their antibacterial and anti-inflammatory activity, as well as the differing response of the two treatments in terms of extracellular matrix component deposition.
- The third phase involved a randomized, controlled, blinded *in vivo* study, in which two types of dental implants with different surface characteristics were compared to assess their behavior in terms of peri-implant bone resorption and the differing clinical index outcomes.

3. Study I. *Antibacterial activity of titanium nitride coating: a systematic review*

Abstract

In recent years, titanium nitride (TiN) coating has been used as an adjunctive surface treatment to improve the physico-mechanical and aesthetic properties of dental implants. Several studies have investigated the antibacterial activity of TiN coatings on dental implants, demonstrating their potential to mitigate bacterial colonization and biofilm formation. The aim of this systematic review was to evaluate the actual efficacy of TiN-coated implants on antibacterial activity. Data was collected following the identification of PICO. The review was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A search was performed on PubMed-Medline, Embase, Web of Knowledge, and Google Scholar for studies published until February 2023, including the following keywords: “titanium nitride, dental implant coating, antibacterial activity, biofilm formation, plaque formation, modified implant surfaces, implant abutments”. After evaluating and scanning the articles that met the study inclusion criteria, it can be stated that the TiN implant coating can inhibit the initial biofilm formation involved in mucositis by reducing the progression into peri-implantitis related to the bio-corrosion activity of the TiN of the implant surface. In the light of these findings, more clinical studies need to be performed.

KEYWORDS: *systematic review, titanium nitride, TiN, antibacterial activity, dental implant coating, peri-implantitis, bacterial adhesion*

3.1 *Introduction*

In recent decades, the development of dental implants to restore masticatory function and dental aesthetics compromised by partial or complete edentulism has been a turning point in dentistry^{90,91}. Every year, a significant number of dental implants are placed, and a substantial proportion of these will experience biological issues, such as inflammation and the deterioration of peri-implant bone loss. When implants are affected by peri-implant mucositis, they display clinical indicators of inflammation. These signs include reddened soft tissue surrounding the implant, bleeding, and/or pus formation when probed^{30,92,93}. If left untreated, peri-implant mucositis progresses to a permanent condition known as peri-implantitis.

In peri-implantitis, the soft tissue around the implant becomes inflamed, leading to bleeding and/or pus formation upon probing ^{33,94}. Additionally, there is an increase in probing depth, which can extend to 6 mm or more ⁹⁵. Studies suggest that the prevalence of peri-implant mucositis and peri-implantitis is approximately 43% in Europe and 22% in South and North America. Treatment outcomes for this condition have been less than satisfactory in the long term, with 75% of cases remaining unresolved or recurring after 5 years. Therefore, it is advisable to prevent peri-implantitis at all stages.⁹⁶ Currently, there is a consensus among scientists that bacterial plaque is the primary cause of peri-implant disease, and its removal is crucial for restoring healthy peri-implant tissue. In fact, peri-implant infection can occur at any time from the moment implants are placed. However, the initial four weeks following implantation are considered the most susceptible period for infection.⁹⁷ This is because the implant and the bone interface have not yet fully integrated, resulting in a relatively weak anti-infective capability. Consequently, there is a risk of early implant failure before the crown is placed. Additionally, it is crucial to have a long-lasting antibacterial effect on the implant surface to prevent infection until the complete formation of the integrated interface.^{98,99,100} Recent advancements have been made in modifying the surface of implant materials to reduce bacterial adhesion and hinder biofilm formation, aiming to prevent infection during the early stages after implantation.¹⁰¹

Nevertheless, coatings with antibacterial properties applied to titanium surfaces using non-covalent adsorption methods (such as hydrogen bonding and electrostatic interactions) are less durable, as the antibacterial components can quickly detach from the surface. To overcome these problems, titanium nitride (TiN) coating has recently been introduced in titanium dental implants. TiN is commonly used to coat numerous metal instruments, including surgical ones, to improve their surface properties and aesthetics (due to its golden color). ^{102,103}

TiN-coated dental implants have higher physical-mechanical properties and allow for better camouflage under the gum tissue compared to traditional grey titanium implants. Physical Vapour Deposition (PVD) is the most common technique for coating pure titanium with TiN. It is generated in the vapor phase by interacting pure titanium with gaseous nitrogen prior to deposition. Thin coatings of TiN and TiO₂ are applied with PVD techniques, altering the surface's chemical composition and crystal structure while maintaining the implant surface's micro-thinness. This process results in an oxide layer on the implant surface that alters its chemical composition. ^{104–106}

The aim of this systematic review was to study the antibacterial properties of the TiN coating of the dental implant on microbial biofilm adhesion and suppression of bacterial growth.

3.2 Materials and Methods

3.2.1 Search Strategy

This systematic review adhered to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) guidelines and utilized the PICO(S) approach (Patient or Population, Intervention, Control or Comparison, Outcome, and Study types) ¹⁰⁷, as illustrated in Fig. 1. Thorough research was conducted on relevant literature and papers exploring the antibacterial properties of TiN-coated dental implants. Databases such as PubMed (Medline), Scopus, Web of Knowledge, and Cochrane were extensively searched.

Various combinations of keywords were employed, including Titanium nitride, dental implant coating, antibacterial activity, biofilm formation, plaque formation, modified implant surfaces, and implant abutments. Only articles in the English language were considered, and to eliminate duplicates, the references from identified records were uploaded as Research Information Systems files into Zotero (RRCHNM, Fairfax, Virginia). The PICO question was formulated as follows: P-population: *in vitro* studies and patients with dental implants; I-intervention: titanium disks with TiN coating or abutment; C-control: conventionally produced/restored teeth and implants; O-outcome: assessment of antibacterial activity of TiN coating; S-study designs: *in vivo* and *in vitro* studies, systematic and narrative reviews. The search strategy aligned with the focused PICOS question is presented in Figure 2. The review was registered in Prospero under ID CRD42023467563.

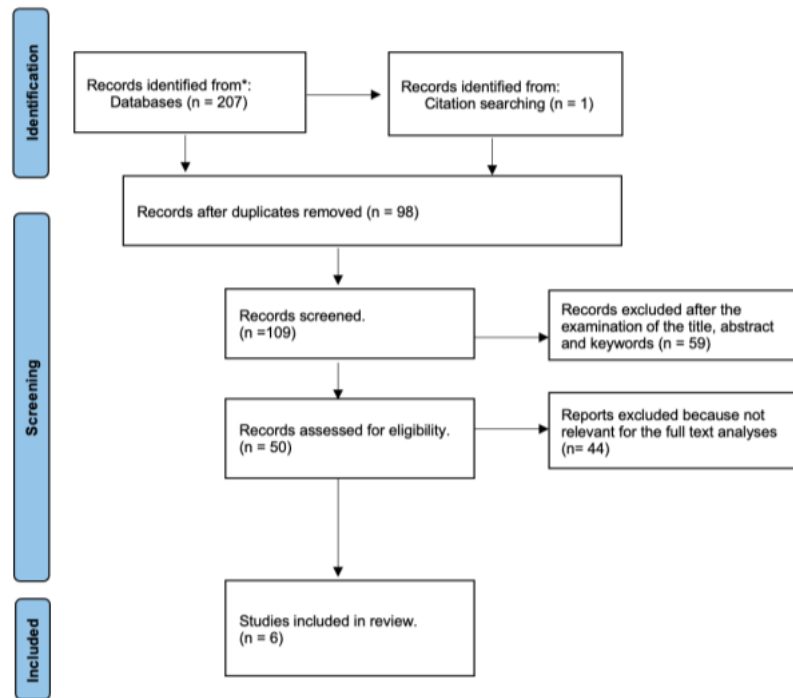


Figure 1. Search flowchart as described in the PRISMA guidelines. Caption: (n = number of records).

Focused Question (PICO)	Do TiN-coated implants exhibit antibacterial properties according to <i>in vivo</i> and <i>in vitro</i> studies?
Population	<i>in vitro</i> studies and patients with dental implants;
Intervention	titanium disks with TiN coating or abutment;
Comparison	conventionally produced/manufactured restorations (natural teeth and implants);
Outcome	evaluation of the antibacterial activity of titanium nitride coating.

Table 1. Search strategy according to the focused question (PICO).

3.2.2 Inclusion and exclusion criteria

Articles were considered appropriate when satisfying the following inclusion criteria:

1. Articles addressing at least one of the following topics regarding dental implants TiN coatings: antibacterial activity, bacterial growth, and proliferation-related to TiN coating disks compared to titanium alone or other coatings;
2. Studies performed *in vitro* or *in vivo*;
3. Systematic and narrative reviews.

Articles that did not have the above information were excluded from the review.

3.2.3 Paper selection

Clinical and *in vitro* studies were selected according to title and abstract. The participants, intervention, comparison, and outcomes (PICO) were determined to formulate a specific question: What is the efficacy of TiN on antibacterial activity?

3.2.4 Data extraction

Authors independently gathered the following information from the included studies: study design, coating type, control group, test procedure, and the effect of abutment coating on bacteria. Evaluations were conducted separately by the authors and subsequently verified through comparison.

3.2.5 Quality and risk of bias assessment

The clinical study was evaluated using the revised RoB tool based on the Cochrane RoB assessment method. For *in vitro* studies; QUIN Tool was used, which was formulated to assess the quality and measure the risk of bias in *in vitro* studies. This Tool present 12 points. The QUIN final assessment ranked each study aspect at a “low”, “medium” or “high” risk of bias.^{108,109}

3.3 Results

The preliminary search included 207 articles. It was decreased to 204 after duplicates were removed. After the title and abstract screening, 12 articles were read in full text. Six articles were chosen after a final examination included in the systematic review. Only one human study was included in the review; the other 5 were *in vitro*. It was described in Figure 1; moreover, *In vitro* studies included in the review were assessed using the Quality Assessment Tool For *In vitro* Studies (QUIN Tool) (Table 2, III). A low risk of bias was found in three studies.^{89,110,111} The other three studies exhibit a medium risk of bias.

Author	Clearly Stated Aims/Objectives	Detailed Explanation of Sample Size Calculation	Detailed Explanation of Sampling Technique	Details of Comparison Group	Detailed Explanation of Methodology	Operator Details	Randomization	Method of Measurement of Outcome	Outcome Assessment or Details
Großner-Schreiber et al.	2	2	2	2	2	2	1	2	2
Zhang et al.	2	1	2	2	2	2	1	1	1
Ji et al.	2		2	2	2		1	2	
Brunello et al.	2	2	2	2	2	2	1	2	2
Camargo et al.	2	1		2	2		1	2	1

Table 2. *The QUIN Tool assessment.*

Author	Blinding	Statistical Analysis	Presentation of Results	Total Score	Risk of Bias
Großner-Schreiber et al.	1	2	2	2	L
Zhang et al.	0	1	1	16	M
Ji et al.	0	2	2	18	L
Brunello et al.	0	1	1	19	L
Camargo et al.	0	2	1	16	M

Table 3. *The QUIN Tool assessment.*

Author	Bias due to confounding	Bias in the selection of participants for the study	Bias in classifying interventions	Bias due to deviations from intended interventions	Bias due to missing data	Bias to measuring outcomes	Bias in selecting reported results	The overall risk of biased judgment
Scarano et al. (2003)	Low	Low	Low	Moderate	Low	Low	Low	Low

Table 4. *Risk of bias of the included studies, according to the ROBINS-I tool.*

The Cochrane Collaboration tool was used to assess the risk of bias for the only *in vivo* human study that showed a low risk of bias (Table 4). The extracted data of the included studies were synthesized in Table 5.

Author (Year)	Study design	Coating	Control group	Bacteria used	Test procedure	Influence of coating on bacteria
Scarano et al. (2003)	<i>In vivo</i>	TiN coated implants	Uncoated grade 4 titanium implants	Percentage of surface covered by bacteria	Light microscope connected to a high-resolution video camera	The extent of bacterial coverage on test implants was significantly reduced compared to control implants ($P<0.0001$).
Großner-Schreiber et al. (2000)	<i>In vitro</i>	TiN	Pure titanium discs grade 2	S. Sanguis S. Mutans	Fluorescence microscope	The significantly lower number of bacteria on TiN-coated discs ($p=0.0036$)
Zhang et al. (2015)	<i>In vitro</i>	TiN	Commercial pure Ti grade 2	S. Mutans A. Viscosus P. Gingivalis	Scanning electron microscope (SEM), white light interferometer (WLI), and confocal laser scanning microscope (CLSM)	No difference in biofilm formation
Ji et al. (2015)	<i>In vitro</i>	TiN	Commercial pure Ti grade 2	S. Mutans P. Gingivalis	SEM	The number of <i>S. mutans</i> colonies on TiN decreased significantly ($p<0.05$)
Brunello et al. (2018)	<i>In vitro</i>	TiN	TiAl6V4, grade 5 disks	S. Sanguis S. Salivarius S. Mutans S. Sobrinus S. Oralis	Flow cytometry	The proportion of deceased bacteria was elevated in the biofilms cultivated on the disks coated with TiN.
Camargo et al. (2020)	<i>In vitro</i>	TiN	High pure titanium	P. Gingivalis	Fluorescence and SEM	Biofilm coverage was lower in TiN (24.22%) compared to uncoated samp

Table 5. Extracted data from included studies.

3.4 Discussion

TiN is a ceramic material with significant chemical and physical characteristics. These include a high level of hardness (measuring 2000 kg/mm²), an elevated decomposition temperature (2949 °C), modified stoichiometry, chemical stability at room temperature, excellent conductivity, and a distinctive golden-yellow color. Primarily employed as a coating, TiN enhances the properties of other materials. It contributes to near-zero hemolysis rates and exhibits exceptional biological traits, such as minimizing the release of cobalt-chromium-molybdenum ions (CoCrMo).

Previous research has revealed that TiN coatings effectively hinder the initial colonization of bacteria and the formation of biofilms. Significantly, this inhibition does not compromise the adhesion, proliferation, and differentiation of bone marrow mesenchymal cells, thereby preserving the material's biocompatibility. Studies have demonstrated that compared to polished titanium, the deposition of TiN coatings through Physical Vapor Deposition results in increased fibroblast numbers on zirconium nitride and TiN surfaces. It is worth noting that surface roughness can facilitate bacterial adhesion, and to prevent plaque formation, the roughness average (RA) value should not exceed 0.2 µm to maintain a stable soft tissue seal around the abutment.^{112,113} In light of this, the TiN coating is generally below this threshold, with documented Ra values of 0.159 ±0.040 µm and 0.217 ±0.025 µm assessed by confocal microscopy and white light interferometry.

TiN/ZrN film coatings have also been investigated for their potential to improve the surface properties of pure titanium, increasing wear resistance and abrasive hardness. Thus, the primary objective of this review was to establish the state of the art in the literature regarding the antibacterial activity and suppression of bacterial growth by TiN hard coating materials. However, the number of clinical trials published in peer-reviewed journals using TiN as a coating for implant abutments is still limited, preventing a comprehensive systematic review of clinical studies.^{76,114,115}

Therefore, our review primarily focused on five *in vitro* studies and only one human trial. This limited scope should be considered a limitation of this systematic review. Nevertheless, the antibacterial effect of TiN is summarized in Table 4. All included studies compared the antibacterial activity of TiN coating versus pure titanium using different bacteria and laboratory methods.^{116,117}

The only *in vivo* study included in this review, conducted by Scarano et al., examined early bacterial colonization of TiN-coated implant abutments. After 24 h, the test group (TiN-coated implants) showed no bacterial or salivary proteins in widely distributed zones, with only small

colonies of a few cocci found in other areas. The bacterial coverage on TiN-coated implants was found to be $13.1\% \pm 2.01\%$. In contrast, the control group, consisting of uncoated implants, displayed plaque containing a small number of cocci and a higher proportion of rod-shaped and filamentous-shaped bacteria. Additionally, a significant portion of the surface was covered by salivary proteins. The bacterial coverage on the control implants was measured to be $21.3\% \pm 1.9\%$. The authors concluded that the extent of bacterial coverage on TiN-coated implants was significantly lower compared to the control implants ($P=0.0001$). It is important to note that these findings were attributed to the distinct chemical and physical composition of the implant surface rather than differences in surface roughness, as the roughness was similar in both the test and control implants.

In their study, Grossner-Schreiber et al. investigated the effects of TiN coatings on bacterial adhesion in comparison to control surfaces with equivalent roughness. They specifically examined the adhesion of *Streptococcus mutans* and *Streptococcus Sanguis*. The findings revealed a significant decrease in bacterial colonies on TiN-coated surfaces.

Our study used hard coatings (TiTiN and Ti-ZrN) to examine their impact on plaque accumulation. These coatings effectively masked the highly reactive titanium surface underneath, regardless of the surface roughness. Previous research has suggested that physical modifications like hard coatings could influence bacterial adherence. Coating the metal components of partial dentures with TiN was found to reduce plaque formation. Notably, there was a greater reduction in *S. sanguis* than *S. mutans* due to the former's more hydrophobic surface, which exhibited a stronger affinity for the TiN coating film.

TiN coating for the implant portion in contact with soft tissue can potentially reduce plaque formation and mucosal inflammation.^{117,118} Zhang et al. found no difference in biofilm formation between TiN-coated and uncoated disks after 14 days of incubation when examining the effects of *S. mutans*, *A. viscosus*, and *P. gingivalis*. Ji et al., evaluated bacterial adhesion and osteoblast-like cell viability, focusing on *S. mutans*, which is correlated with bacterial adhesion to implant surfaces in the human oral cavity, and *P. Gingivalis*, which stimulates toxin production and inflammation associated with peri-implantitis. They reported a significant reduction in the number of *S. mutans* colonies on TiN-coated surfaces compared to polished titanium ($P<0.05$). However, no difference in bacterial colony counts was observed between the two surfaces for *P. gingivalis*.

Brunello et al. (36), investigated the antibacterial activity of TiN-coated machined titanium compared to uncoated titanium surfaces against five different bacterial strains (*Streptococcus salivarius*, *S. sanguinis*, *S. mutans*, *S. sobrinus*, and *S. oralis* (35). The percentage of dead

bacteria was calculated, and they found a higher percentage of dead bacteria on TiN-coated disks compared to uncoated samples. For example, the percentage of dead *S. oralis* on the TiN-treated disks was $51.84\% \pm 3.73\%$ ($P < 0.001$). Similar results were found for the other bacterial strains. The authors concluded that TiN-coated titanium exhibited antibacterial activity against several species associated with peri-implant infections.

In another study, Camargo et al.⁸⁸, tested the antibacterial activity of TiN-coated titanium disks compared to uncoated titanium against *P. gingivalis*. They observed a significant reduction in the number of *P. gingivalis* on TiN-coated disks (24.22%) compared to uncoated samples (85.2%, $p < 0.05$). Moreover, it has recently been reported that *Streptococcus gordonii* is an early bacterial colonizer and is essential for the initial colonization of abutments and tooth surfaces by other bacterial species. Its growth *in vitro* is significantly reduced on untreated titanium NiTi surfaces.

To summarize, the findings of this systematic review suggest that TiN-coated implants and abutments can effectively reduce mucositis and peri-implant inflammation. The TiN coating demonstrates a remarkable decrease in bacterial adherence, reducing plaque formation and potentially improving the long-term stability of the implant. However, it is important to note that further clinical trials are needed to validate these findings and explore the clinical effectiveness of TiN-coated implants.

3.5 Conclusions

To the best of the authors' knowledge, the first review on TiN coatings in clinical dentistry was published in 1992 based on their biocompatibility, mechanical properties, and corrosion. This systematic review demonstrated how TiN-coated implants and abutments could effectively reduce the initial biofilm formation involved in mucositis, improving implant long-term stability due to the strong influence related to the TiN bio-corrosion activity of dental implant surface.

The main limitation of this study is that only one human study was included in this systematic review; further clinical investigations need to be conducted.

4. Study II. *Enhanced Extracellular Matrix Deposition on Titanium Implant Surfaces: Cellular and Molecular Evidence*

Abstract

The surface structure of the titanium dental implants can modulate the activity of mesenchymal stem cells in order to promote the upregulation of osteoblastic related genes and the release of extracellular matrix (ECM) components. The present work was focused on the *in vitro* evaluation of the interaction of human periodontal ligament stem cells (hPDLSCs) and two different implant titanium surfaces topography (CTRL and TEST). This study was aimed at analyzing the cytotoxicity of the dental implant surfaces, the cellular adhesion capacity, and the improvement in the release of ECM molecules in an *in vitro* model. These parameters were carried out by means of the microscopic evaluation, viability assays, immunofluorescence, Western blot and RT-PCR investigations. The knowledge of the cell/implant interaction is essential for implant healing in order to obtain a more performing surfaces that promote the ECM release and provide the starting point to initiate the osseointegration process.

Keywords: osseointegration; osteogenesis; cellular adhesion; gene expression; extracellular matrix (ECM)

4.1 Introduction

Mesenchymal Stem Cells (MSCs) are well recognized in the regenerative and tissue engineering field for the long-lasting proliferation ability, the capability to differentiate in different lineages, immunomodulation feature and the easy accessibility^{119,120,121}. Due to their valuable characteristics and their good ability in tissue and organ regeneration, they have been utilized for clinical studies¹²². The use of periodontal ligament stem cells (PDLSCs) opens a promising possibility for development of tissue engineering technology-based therapeutics approaches for the destroyed periodontium.

The periodontium represents the supporting tissues of the teeth which consist of the attachment apparatus and the dento-gingival unit.^{123,124} The human PDLSCs (hPDLSCs) are derived from periodontal ligament, which is characterized by the presence of different cell populations, such as osteoblasts, fibroblasts, epithelial cells, endothelial cells and stem cells. They are considered as a valuable stem cell population for regenerative therapy in periodontium, and they are the widely used MSCs population in the dentistry field.^{125,126}

The possibility to obtain the ligament-anchored implants or implants surrounded by periodontal tissue is the gold standard in the dental surgery. The study in the dental implant field has led to a change from smooth machined surfaces to roughened surfaces in order to improve osseointegration thanks to the osteoconductive and osteoinductive properties¹²⁷. The characteristics of the implant surfaces have different implications in the integration that it will be possible to achieve with both hard and soft tissues during rehabilitation. A rough implant allows for a better osseointegration process than a smooth surface.

Previous studies have reported that the cellular interactions with implant surface may promote osseointegration at clinical level.^{76,128,129} In particular, the modifications of micro-topography contributed to increase surface area and increased *in vivo* Bone-to-Implant Contact (BIC) levels. Treatment of Ti surface topography altered the growth, metabolism, and migration of osteogenic cells.^{130–133} The micro-topography alteration methods, such as sandblasted or acid-etched procedures of the implant surface, have been proposed to increase the osseointegration process at cellular and protein level. At the microscopic level, the biomechanical interlocking between implant and bone can be influenced by the topography of an implant surface.^{134,135} The features of titanium surface may influence the differentiation and morphology of MSCs, the secretion of extracellular matrix (ECM) factors and the osteoblastic gene expressions.

Different findings have demonstrated that a rough surface in comparison with a smooth one promotes a better biomolecular adsorption, augments ECM production and stimulates the differentiation of the MSCs towards an osteoblastic phenotype. Different mechanisms are able to regulate the cell adhesion to a biomaterial. These mechanisms consist of definite interactions between cell surface receptors and specific ligand molecules, which are adsorbed to, placed on, or released over the biomaterial. Adhesion can be establish between adjacent cells (cell-cell adhesion) as well as between cells and the ECM (cell-matrix adhesion). The study of cell adhesion has gained a lot of interest and has been largely investigated in the cellular biology, biomedical and engineering fields.^{19,136,137} Cell adhesion is involved in inducing signals that modulate cell differentiation, cell cycle, cell migration, and cell survival. It is a fundamental biological event for establishing cell and tissue morphogenesis. This event is modulated by cell adhesion molecules, which are transmembrane receptors connected to the cytoskeleton that permit the assemblage of cells in three-dimensional tissues and their interaction with the surrounding environment.¹³⁸ The affinity of cells to substrate is a key factor in biomaterial design and development. The ECM molecules are implicated in the cell-materials interaction events of the implant.

The ECM components in most tissue are categorized into proteoglycans and fibrous proteins such as Fibronectin and Laminin. These molecules give structural support and facilitate cellular communication. Integrins, the transmembrane proteins on the cell surface, connect the cytoskeleton of cells to the ECM, activate signaling pathways that modulate cell proliferation, morphology, adhesion and cell death.

For instance, integrin binding to Fibronectin includes a conformational change in the receptor that results in mechanical coupling to the ligand.¹³⁹ Fibronectin glycoprotein interplays with integrins on the cell surface, arbitrates mechanical anchoring and establishes the focal cell–cell formation and cell–material adhesion contacts. The extracellular signals are translated into cellular responses to the areas of cell material adhesion.

In the present work, the expression levels of typical ECM markers, such as Fibronectin, Laminin, Vimentin and N-cadherin were evaluated in an *in vitro* model of hPDLSCs seeded on two different titanium implant surfaces, CTRL and TEST.

The evaluation of these essential factors may offer an important platform for assessing the biological outcomes of the surface characteristics and the basis for optimizing dental implant surfaces to assist the clinicians when choosing implant systems. Even if the limitation of an *in vitro* model, the current evidence indicates that SEM and microscopic observations may be useful in providing insight on the role of implant surface topography and the gene expression tests are useful for assessment the cellular and physio-biochemical properties.¹⁴⁰ The findings obtained by the *in vitro* tests may be correlated to the *in vivo* studies to better evaluate the surface performances. Thus, changes in micro-topography reflect its effects at a biological level, resulting in the increase of cell adhesion, gene and protein expression related to the formation of ECM, that represent a fundamental step to initiate the osseointegration process.

4.2 Materials and Methods

4.2.1 Ethic Statement

The consent form has been signed by patients enrolled in the present study. The study design has been previously approved by Medical Ethics Committee at the Medical School, “G. d’Annunzio” University, Chieti, Italy (N° 266/17.04.2014). The Department of Innovative Technologies in Medicine and Dentistry and the Laboratory of Stem Cells and Regenerative Medicine are certified according to the quality standard ISO 9001:2008 (N° 32031/15/S).

4.2.2 Cell Culture Establishment

Tissue biopsies were scraped from the horizontal fibers of the periodontal ligament of human premolar teeth. The enrolled patients were in general good health conditions and exempt from oral diseases. The phosphate buffered saline (PBS, Lonza, Basel, Switzerland) solution was used for tissue washing, then the samples were placed in culture with the specific medium for the growth of MSCs, Thera PEAK™MSCGM-CD™ Bullet Kit- chemically defined (MSCGM-CD, Lonza).¹⁴¹ Cell culture medium was refresh every 2 or three days. After two weeks of culture, cells spontaneously migrated from the tissue explants and then were sub-cultured till passage 2.

4.2.3 Cell Immunophenotyping

Human PDLSCs at the second passage were collected; 5×10^5 cells were incubated with 1 µg of the specific antibody, conjugated with fluorescein isothiocyanate, phycoerythrin (PE), for 30 min at 4 °C in the dark. Human PDLSCs were stained using the following antibodies: anti-CD14 PE-conjugated (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD34 PE-conjugated (Beckman Coulter, Fullerton, CA, USA), anti-CD73 PE-conjugated, anti-CD90 FITC-conjugated (Becton Dickinson, San Jose, CA, USA) and anti-CD105 (Ancell, Stillwater, MN, USA). After incubation, cells were acquired with a flow cytometer (FACS Calibur, BD Bioscience, Franklin Lakes, NJ, USA). Data were analyzed by the FlowJo software (v8.8.6, TreeStar, Ashland, OR, USA).

4.2.4 Mesengenic Differentiation and Histochemical Analysis

To evaluate the capacity of hPDLSCs to differentiate into adipogenic and osteogenic lineages, cells at passage 2 were used. For adipogenic differentiation expanded cultured with MSCGM-CD, were seeded into dishes at a density of 2×10^4 cells/cm². At 100% confluence, three cycles of induction/maintenance stimulated adipogenic differentiation. Each cycle consisted of feeding the hPDLSCs with supplemented adipogenesis induction medium (Lonza) for 3 days, followed by 3 days in supplemented adipogenic maintenance medium (Lonza). After three complete cycles of induction/maintenance, the culture was incubated for 7 days in supplemented adipogenic maintenance medium, replacing the medium every 2–3 days. The cells were fixed in 10% formalin for 15 min and washed with dH₂O. Subsequently, the cells were stained with Oil Red O (ORO) working solution (300 mg of Oil red O/100 mL of isopropanol) for 5 min and counterstained with hematoxylin. For osteogenic induction, the

primary cells at the second passage were seeded at 4×10^3 cells/cm² in MSCGM-CD culture medium and maintained in culture at 37 °C, in a humidified 5% CO₂ atmosphere. At sub-confluence, cells were incubated with MSCGM-CD medium with the addition of osteogenic supplements, as 100 nM dexamethasone (Applichem, Darmstadt, Germany), 10 nM β -glycerol-phosphate (Applichem), and 0.05 mM 2-phosphatascorbic acid (Sigma-Aldrich, Milan, Italy). Visualization of calcium deposition and ECM mineralization was obtained by Alizarin Red S (ARS) staining assay performed after 21 days of culture. According to Gregory et al., 32 cells were washed with PBS, fixed in 10% (v/v) formaldehyde (Sigma-Aldrich) for 30 min, and washed twice with abundant dH₂O before addition of 0.5% Alizarin red S in H₂O, pH 4.0, for 1 h at RT. After cell incubation under gentle shaking, cells were washed with dH₂O four times for 5 min and observed under light microscopy.

4.2.5 Dental Implants

In the present work, two titanium disks surfaces (Resista, Omegna, VB, Italy) have been used: sandblasted (CTRL) and dual acid etched (TEST). The disks were made of grade 4 titanium.¹⁴² In particular, the TEST surface was obtained through the non-contaminant micro-subtraction process, Double Acid Etching (DAE), changes the implant micro roughness and the surface texture maximizing the wettability and biomimetic properties.

4.2.6 Exposure to the Titanium Surfaces

Human PDLSCs were seeded on titanium disks, CTRL and TEST, with MSCGM-CD (Lonza). The culture medium was changed twice a week. Cultures were maintained for 8 weeks, then were processed as described below for further examination. The inverted light microscopy DMIL (Leica microsystem, Milan, Italy) was used to carry out the microphotographs in order to evaluate the morphological arrangement of hPDLSCs and titanium disks.

4.2.7 Cell Viability Assay

The cell metabolic activity of hPDLSCs seeded with or without CTRL and TEST samples was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). Briefly, the cells were seeded into 96-well plates with MSCBM-CD medium (Lonza) at 2400 cells/well for 24, 48 and 72 h and 1 week at 37 °C. After the different time points 15 μ L/well of MTS dye solution was added to culture medium, and

cells were incubated for 3 h at 37 °C. The quantity of formazan product, directly proportional to the number of living cells in culture, was detected by absorbance measurements at 490 nM wavelength utilizing the Synergy™ HT Multi-detection microplate reader (Biotech, Winooski, VT, USA). The MTS assay was executed in three independent experiments.

4.2.8 Scanning Electron Microscopy (SEM) Analysis

SEM analyses were then performed to evaluate the relationship between hPDLSCs and the titanium disc surfaces.

The samples for SEM analysis were fixed for 4 h at 4 °C in 4% Glutaraldehyde in 0.05 M PBS (pH 7.4) and then dehydrated in increased range of ethanol concentrations and dried out. Specimens were mounted on aluminum stubs and gold-coated in an Emitech K550 sputter-coater (Emitech Ltd., Ashford, UK). SEM EVO 50 (Zeiss, Jena, Germany) was used for analysis.

4.2.9. Confocal Laser Scanning Microscopy (CLSM) Analysis

The hPDLSCs were seeded on CTRL and TEST titanium disks surfaces for 8 weeks, the medium was replaced every 2 days, and then were fixed for 10 min at room temperature (RT) with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). After washing procedure samples were processed for the immunofluorescence staining. The specimens were permeabilized with 0.5% Triton X-100 in PBS, followed by blocking with 5% skimmed milk in PBS. Primary monoclonal antibodies to anti-human Fibronectin (antibody dilution 1:200) (sc-8422, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Laminin (antibody dilution 1:200) (sc-17810, Santa Cruz Biotechnology), N-cadherin (sc-59987, Santa Cruz Biotechnology) and RUNX2 (sc-399351, Santa Cruz Biotechnology) were used, followed by Alexa Fluor 488 green fluorescence conjugated goat anti-mouse as secondary antibodies (cat. n. A 11029, Molecular Probes, Eugene, OR, USA). Then, Alexa Fluor 594 phalloidin red fluorescence conjugate (antibody dilution 1:200) (cat. n. A12381, Molecular Probes) was used to highlight the cytoskeleton actin and TOPRO (dye dilution 1:200) (cat. n. T3606, Molecular Probes) was used to stain the cell nuclei.¹⁴³ LSM800 META (Zeiss) confocal system was used to evaluate the specific markers expression. The confocal system was connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (40×/1.3 NA, Zeiss).

4.2.10 RNA Isolation and Real-Time RT-PCR Analysis

RUNX2, VIM, FN1, CDH2, LAMB1, ITGA5 and ITGB1 mRNA expression were analyzed by real-time PCR. Total RNA was extracted using PureLink RNA Mini Kit (Ambion, Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's instructions.

Three independent biological replicates were analyzed for each sample. Two micrograms of total RNA were retrotranscribed using the High Capacity cDNA Reverse Transcription kit catalog number 4,368,814 (Applied Biosystems, Waltham, MA, USA) to synthesize cDNA for 10 min at 25 °C, 10 min at 37 °C and 5 min at 85 °C according to the manufacturer instructions. Real-Time PCR was performed with Mastercycler ep real plex real-time PCR system (Eppendorf, Hamburg, Germany). The levels of mRNA expression of RUNX2, FN1, CDH2, LAMB1, ITGA5, ITGB1 and Beta-2 microglobulin (B2M) (endogenous marker) (Table 1) were evaluated in hPDLSCs cells cultured on CTRL compared to the mRNA expression levels of hPDLSCs cultured on TEST titanium disk. Commercially available TaqMan Gene Expression Assays (RUNX2 Hs00231692_m1; VIM Hs.PT.58.38906895; FN1 Hs.PT.58.40005963; CDH2 Hs.PT.58.26024443; LAMB1 Hs.PT.58.3739165; ITGA5 Hs.PT.58.4796384 and ITGB1 Hs.PT.58.39883300) and the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) were utilized according to standard protocols. Beta-2 microglobulin (B2M Hs99999907_m1) was utilized for template normalization.¹⁴⁴ The amplification program consisted of a preincubation step for cDNA denaturation (3 min 95 °C), followed by 40 cycles consisting of a denaturation step (15s 95 °C), and an annealing step (1 min 60 °C). At the end of each run, melting curve was performed in the temperature range of 60 to 95 °C. Expression levels for each gene were performed according to the $2^{-\Delta\Delta C_t}$ method. RT-PCR was performed in three independent experiments; duplicate determinations were performed for each specimen.

Gene	Forward Primer Sequence (5-3)	Reverse Primer Sequence (5-3)
RUNX2	CTTCACAAATCCTCCCCAAGT	AGGCGGTCAGAGAACAAC
VIM	CAAGACCTGCTCAATGTTAAGATG	GTGAATCCAGATTAGTTCCCTCA
FN1	CGTCCTAAAGACTCCATGATCTG	ACCAATCTTGTAGGACTGACC
CDH2	GTTTGCCAGTGTGACTCCA	CATACCACAAACATCAGCACAAG
LAMB1	TTG GAG CAA ATG TAG TGA CCA	CTA CTG TAT CGT CAG CCA CTT G
ITGA5	ACCAACAAGAGAGCCAAAGTC	TTGTACACAGCCTCACACTG
ITGB1	GTAGCAAAGGAACAGCAGAGA	GGTCAATGGGATAGTCTTCAGC

Table 6. *Primer sequences used for real-time PCR reactions.*

4.2.11. Protein Expression

The cell lysates (50 µg) underwent electrophoresis and were transferred to the polivinilidenfluoruro (PVDF) membrane. The latter were blocked in 5% of non-fat milk in PBS 0.1% Tween-20, then membranes were incubated with primary antibodies anti-human Fibronectin (antibody dilution 1:750) (sc-8422, Santa Cruz Biotechnology), anti-Laminin (antibody dilution 1:750) (sc-17810, Santa Cruz Biotechnology), N-cadherin (antibody dilution 1:750) (sc-59987, Santa Cruz Biotechnology), anti-RUNX2 (antibody dilution 1:500) (sc-390351, Santa Cruz Biotechnology), anti-OPN (antibody dilution 1:350) (sc-21742, Santa Cruz Biotechnology), anti-SPARC (antibody dilution 1:350) (sc-25574, Santa Cruz Biotechnology) and anti-beta-actin (antibody dilution 1:750) (sc-69879, Santa Cruz Biotechnology). After five washes in PBS (Lonza) containing 0.1% Tween-20, membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibody anti-mouse (A90-116P Goat anti-mouse, Abcam, Milan, Italy) 1:2000 diluted in 1X PBS (Lonza), 3% milk, and 0.1% Tween-20.¹⁴⁵ Protein expression was visualized using the enhanced chemiluminescence detection method (ECL) (Amersham Pharmacia Biotech, Milan, Italy) with photo documenter Alliance 2.7 (Uvitec, Cambridge, UK). Signals were analyzed by ECL enhancing and evaluated through UVIband-1D gel analysis (v2.7, 1D Max software, Uvitec). Data were normalized with densitometric values derived from β -actin loading control.

2.12. Data and Statistical Analysis

The Statistical Package for Social Science (SPSS Inc., v.21.0, Chicago, IL, USA) was used for data analysis. Data were expressed as means and standard deviation of the recorded values. The differences among the levels of the factors under investigation were analyzed performing three

distinct two-way-ANOVA tests, one for each experiment. Tukey tests were applied for pairwise comparisons. A value of $p < 0.05$ was considered statistically significant in all tests. Regarding gene expression analysis, the comparative $2^{-\Delta\Delta C_t}$ method was used to quantify the relative abundance of mRNA and then to determine the relative changes in individual gene expression (relative quantification).¹⁴⁶

4.3 Results

4.3.1 Cell Characterization

Cytofluorimetric experiments showed the expression of surface molecules as CD73, CD90 and CD105. The hPDLSCs were negative for the subsequent markers CD14, CD34 (Figure 1A). To evaluate the morphological features of hPDLSCs the primary cultures were observed by means of inverted light microscopy. Adherent cells on glass cover slips showed a spindle-shaped morphology with elongated cytoplasmic processes (Figure 1B1). Alizarin Red S staining was performed to visualize the calcium deposits. To evaluate the adipogenic differentiation of hPDLSCs the cellular monolayer was stained with Oil Red O and observed by light microscopy. Adipogenic-induced cells showed the well evident intracellular lipid droplets as single or grouped (Figure 1B2). The hPDLSCs showed appositive staining for Alizarin Red S solution and that several areas with high levels of mineralization related to bone nodule were evident (Figure 1B3).

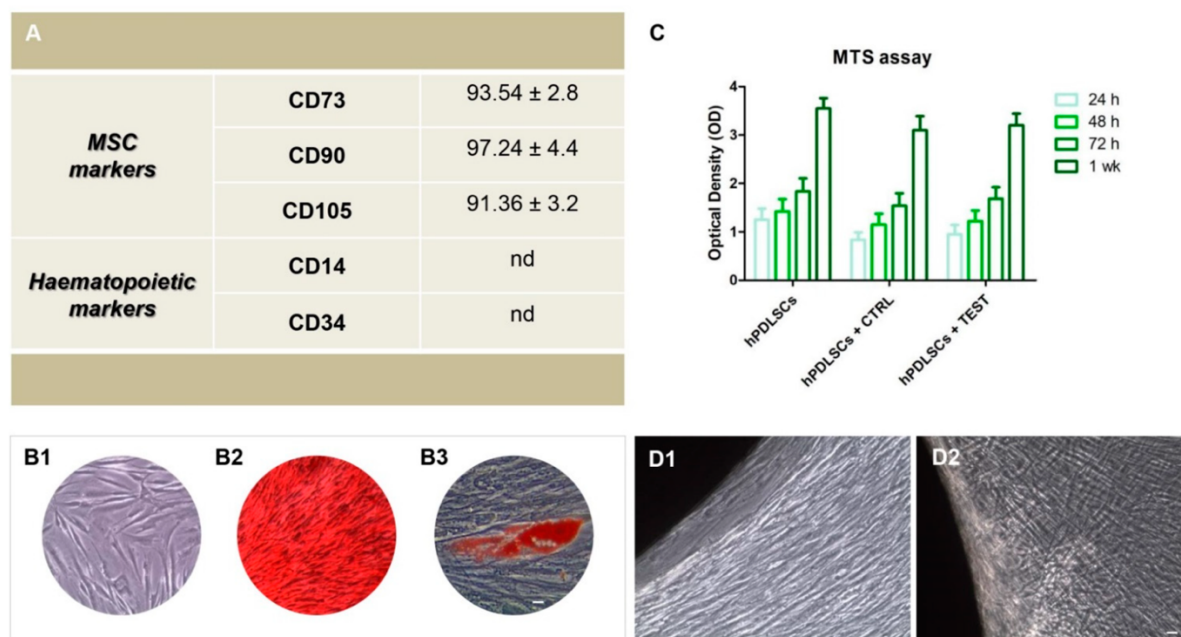


Figure 1. (A) Cytofluorimetric analysis of hPDLSCs primary culture at second passage. The values are expressed as mean fluorescence ratio (MFI) obtained by dividing the MFI of positive events by the MFI of

negative events. **(B1)** Plastic adherent primary cultures of hPDLSCs observed at light microscopy showed a spindle-shaped appearance with long cytoplasmatic processes. **(B2)** The adipogenic potential of hPDLSCs grown was analyzed through the evaluation of intracellular lipid droplets by light microscopy using Oil Red O staining after adipogenic induction. **(B3)** The osteogenic differentiation was evaluated by staining with Alizarin red S (ARS) that demonstrated the calcium deposition. **(C)** Cell viability assay at 24, 48, 72 and 1 week of culture. **(D1)** hPDLSCs cultured with CTRL surface. **(D2)** hPDLSCs cultured with TEST surface. Scale bar: 20 μ m.

4.3.2 Cell Viability Assay and Morphological Features of hPDLSCs Cultured on CTRL and TEST Titanium Surfaces

MTS assay was performed at 24, 48, 72 h, and 1 week on hPDLSCs alone and seeded on CTRL and TEST titanium disk surfaces. The hPDLSCs cultured on the CTRL and TEST disk exhibited a similar viability rate compared to the hPDLSCs at different considered time points. The cell viability rate follows a logarithmic trend (Figure 1C). Microphotographs obtained from inverted light microscopy showed the cell adhesion to both type of titanium disks, CTRL and TEST; in particular, on TEST surface cells showed a better arrangement and adherence capacity around the disk surface (Figure 1D1,D2).

4.3.3 hPDLSCs Adhesion

SEM observations evidenced the surface morphology of two different surfaces, CTRL and TEST without cells (Figure 2A,D). Human PDLSCs seeded on TEST surface for 24 h showed a better ability of adhesion compared to CTRL surface (Figure 2B,E). To better evaluate the adhesion capacity and cytoskeleton arrangement at intracellular level, the immunofluorescence experiments have been performed. Cells cultured on CTRL and TEST surfaces showed the presence of evident actin fibers stained in red (Figure 2C1–C4). On TEST surface cells were distributed in a multilayer way evidencing a better performance of the treated surface (Figure 2F1–F4).

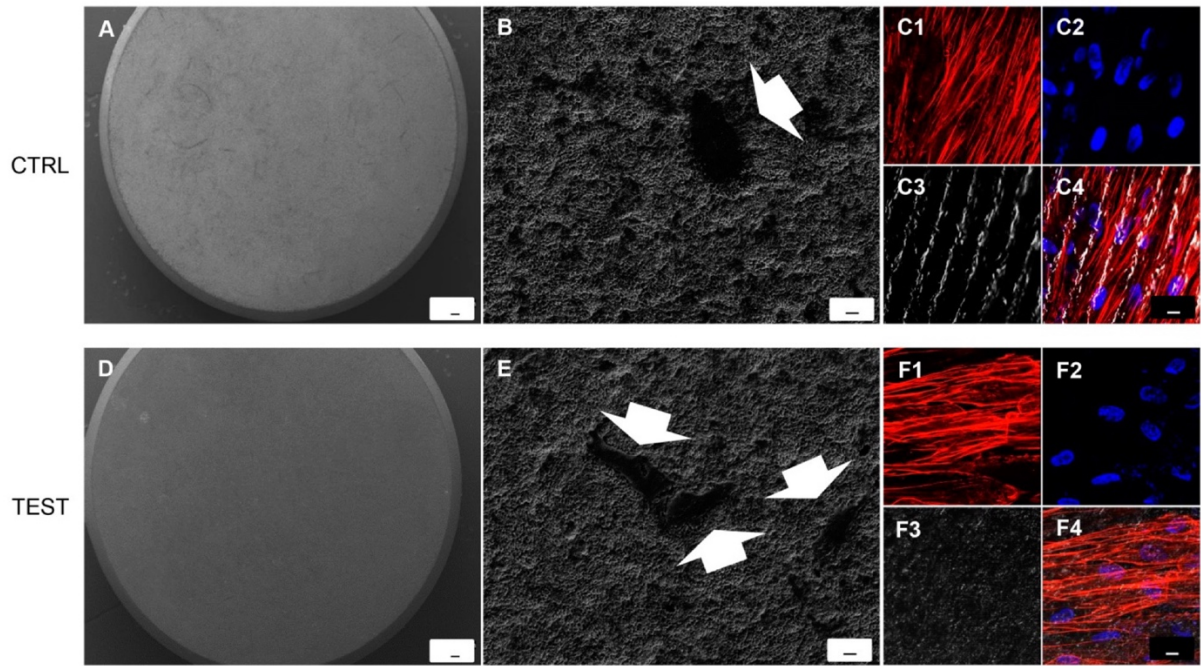


Figure 2. SEM analysis showed the hPDLSCs adhesion capacity to the titanium implant surfaces. (A) CTRL surface without cells. (B) hPDLSCs adhered on CTRL surface. (D) TEST surface without cells. (E) hPDLSCs adhered on TEST surface. Arrows indicate the cellular profile. Mag: (A,D) 50 $\times$; (B,E) 1000 $\times$. Scale bars: (A,D) 100 μ m; (B,E) 10 μ m. (C1–C4) Immunofluorescence images of hPDLSCs cultured on CTRL titanium implant surface. (F1–F4) Immunofluorescence images of hPDLSCs cultured on TEST titanium implant surface. Mag: 40 $\times$. Scale bar: 10 μ m.

4.3.4 Implant Titanium Disks Affect Protein Expression at CLSM

Figure 3 and Figure 4 showed fluorescence images of the cytoskeleton actin (phalloidin, green) and the nuclei (TOPRO, blue) of hPDLSCs seeded on CTRL and TEST specimens captured after 8 weeks of culture. The cells adhered and spread well with a spindle fibroblast-like shape on both samples which revealed that the different surface treatment did not affect the adhesion capability. The CLSM observation showed a higher RUNX2 expression level for hPDLSCs seeded on TEST compared to CTRL, suggesting a better capability of hPDLSCs seeded on TEST surface to differentiate towards the osteogenic lineage after 8 weeks of culture (Figure 3 and Figure 4). Furthermore, hPDLSCs cultured on TEST implant surface evidenced a higher expression of Fibronectin, Laminin and N-cadherin compared to cells seeded on CTRL after 8 weeks (Figure 3 and Figure 4).

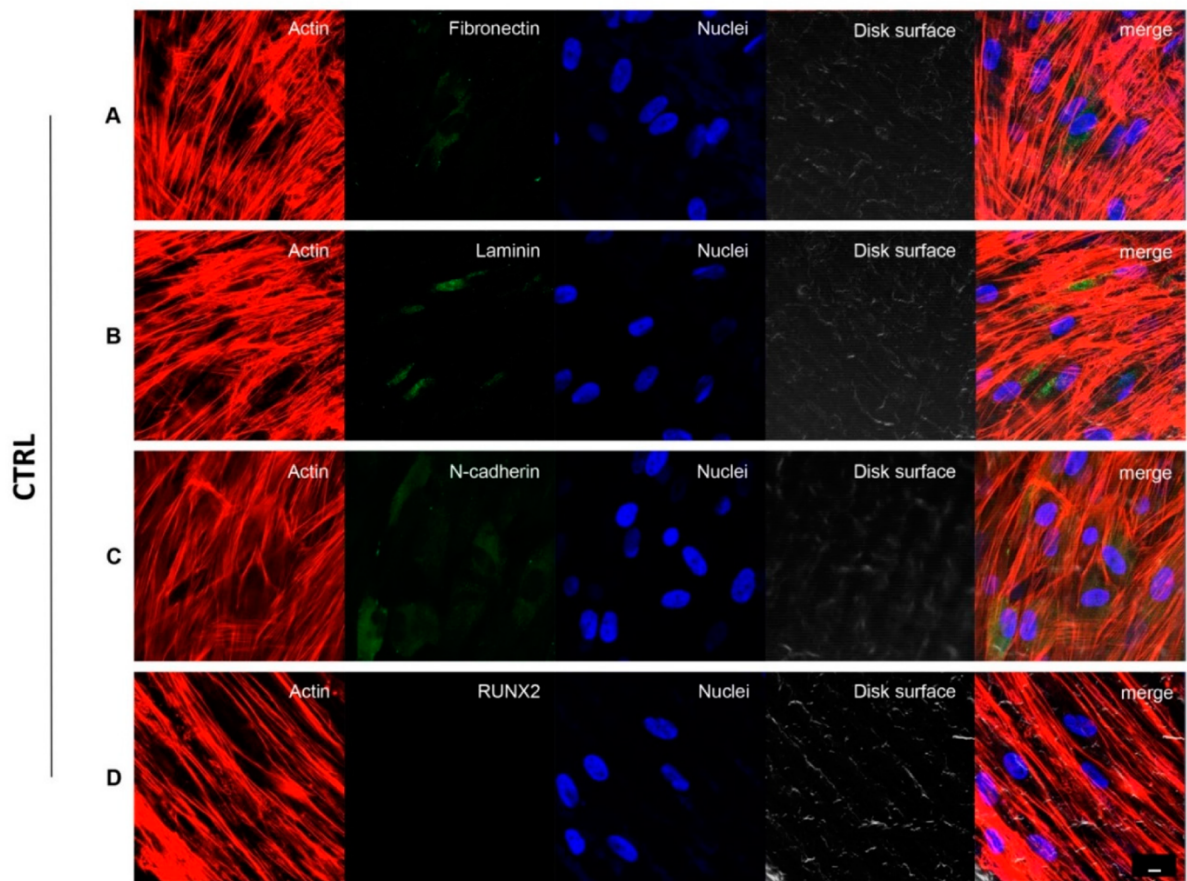


Figure 3. *hPDLSCs cultured on CTRL titanium implant surface observed after 8 weeks. Cytoskeleton actin was stained in red fluorescence; specific markers: Fibronectin (A), Laminin (B), N-cadherin (C) and RUNX2 (D) were stained in green fluorescence; nuclei were stained in blue fluorescence. Scale bar: 10 μ m.*

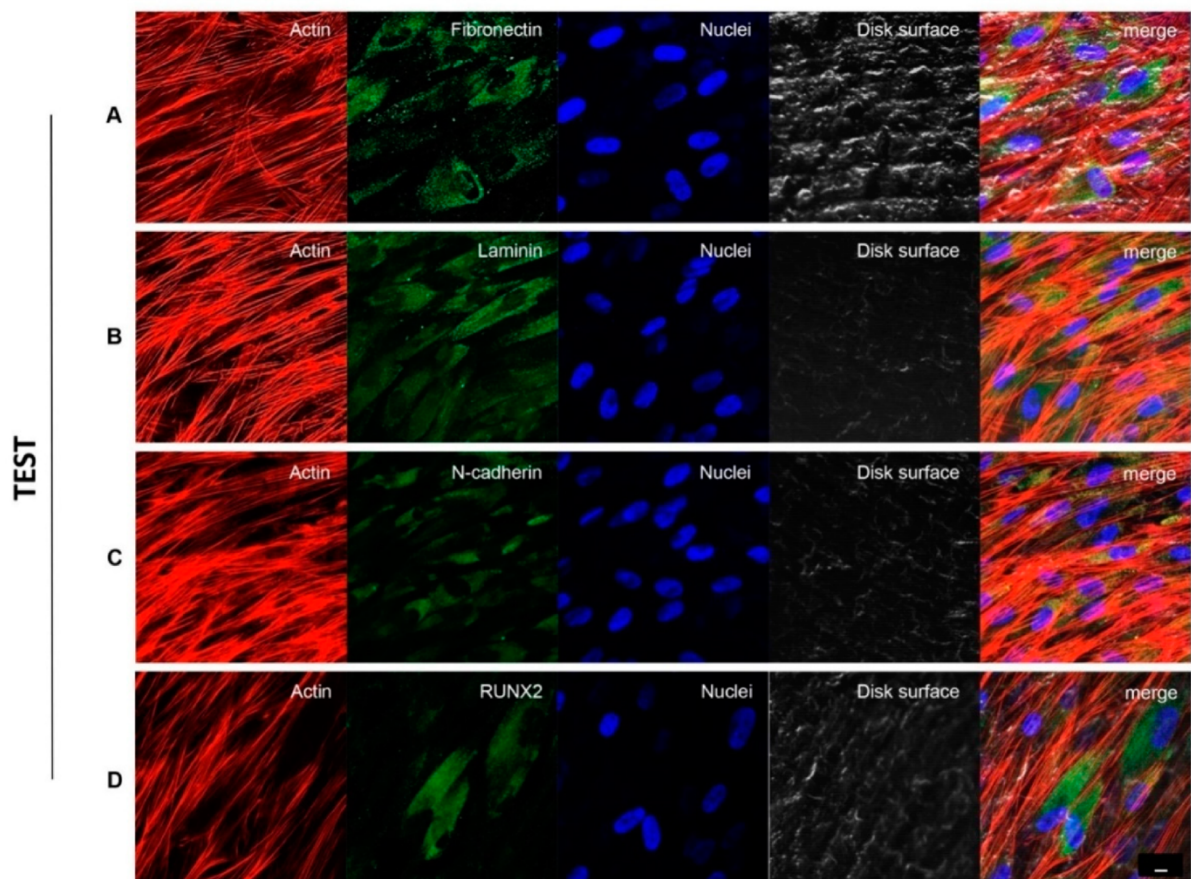


Figure 4. *hPDLSCs cultured on TEST titanium implant surface observed after 8 weeks. Cytoskeleton actin was stained in red fluorescence; specific markers: Fibronectin (A), Laminin (B), N-cadherin (C) and RUNX2 (D) were stained in green fluorescence; nuclei were stained in blue fluorescence. Scale bar: 10 μ m.*

4.3.5 Genes and Proteins Expression

The histogram indicated the gene expression of RUNX2, VIM, FN1, CDH2, LAMB1, ITGA5 and ITGB1 evaluated by RT-PCR (Figure 5A). The hPDLSCs seeded on TEST reported a significant higher expression of RUNX2 in comparison with hPDLSCs seeded on CTRL disk surface. Moreover, hPDLSCs cultured on TEST surface evidenced a significant higher gene expression of VIM, FN1 and LAMB1 compared to the CTRL surface, and a slight increase in relative gene expression level of CDH2 and ITGA5 in TEST disk in comparison with CTRL surface. Conversely, no significant difference was noticed in the gene expression levels of ITGB1 in hPDLSCs on CTRL and TEST titanium disk. Gene expression confirmed the qualitative results obtained by CLSM observations. Densitometric analysis of the relative protein expression of RUNX2, N-cadherin, Fibronectin, Laminin, OPN and SPARC showed an over expression in hPDLSCs cultured on TEST compared to the cells seeded on CTRL surface (Figure 5B). Moreover, the densitometric analysis of protein levels showed a high expression of ECM proteins which validated the immunofluorescence results (Figure 5B).

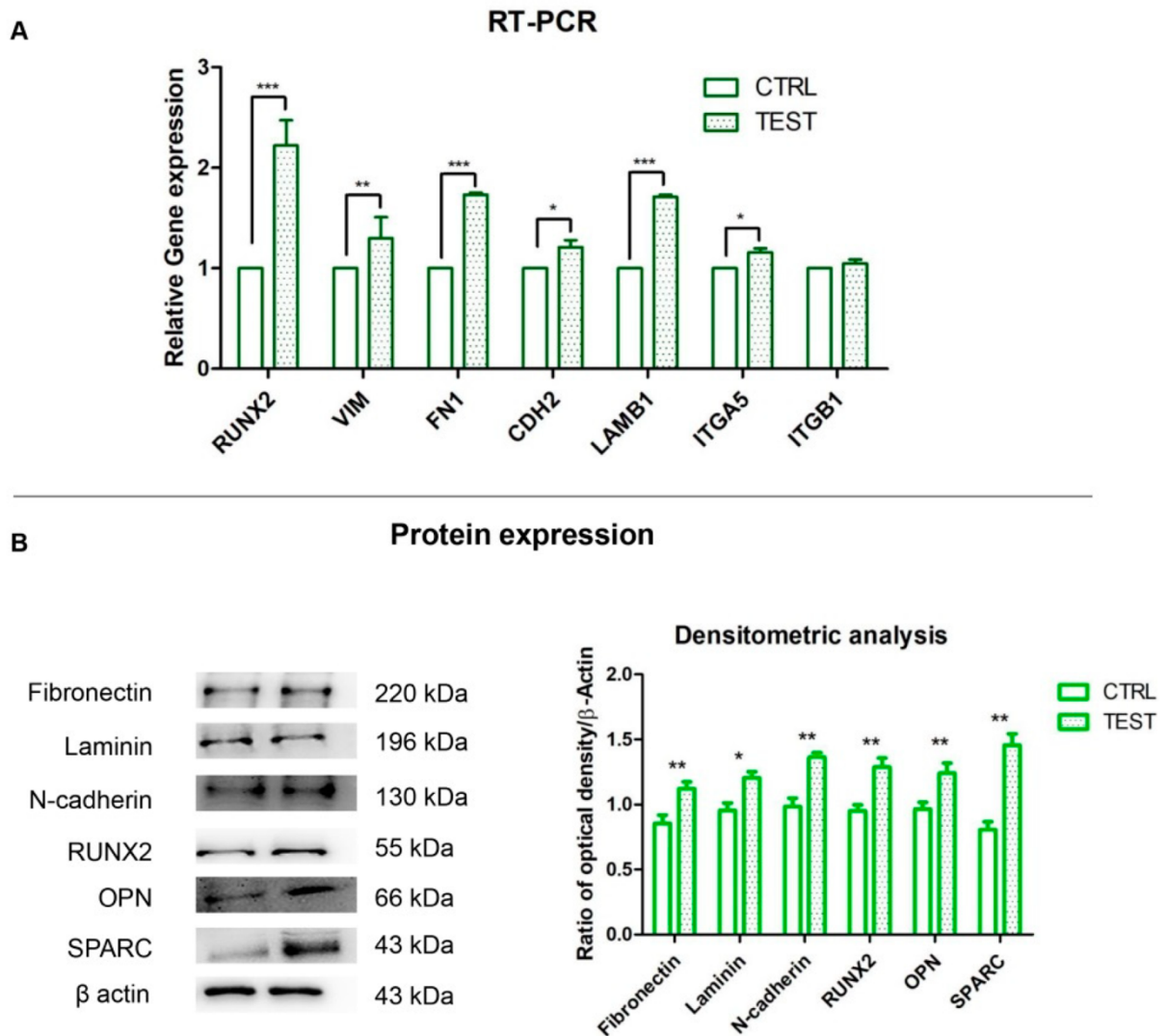


Figure 5. (A) Histograms of RT-PCR showed the mRNA levels of RUNX2, VIM, FN1, CDH2, LAMB1, ITGA5 and ITGB1 in hPDLSCs cultured on CTRL and TEST surface * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (B) Protein expression of Fibronectin, Laminin, N-cadherin, RUNX2, OPN and SPARC in hPDLSCs cultured on CTRL and TEST surface. Histograms represent densitometric measurements of proteins bands expressed as integrated optical intensity with the mean of three separate experiments. The error bars showed standard deviation ($\pm$ SD). Densitometric values analyzed by ANOVA showed significant differences. * $p < 0.05$; ** $p < 0.01$.

4.4 Discussion

Nowadays, re-establishing the oral health using dental implants is considered the gold standard treatment in oral rehabilitation of partially or fully edentulous patients. The success of dental implant engraftment is to develop a structural and functional connection between newly formed bone and the implant surface in order to obtain the biomechanical stability.⁴ The osseointegration process is a complex physiological mechanism that starts from ECM deposition leading to bone tissue formation through distinct phases of implant site healing.

Lately, a lot of scientists put their efforts on the development of biomaterials with specific characteristics which enable them to circumvent the failures of dental implant. The early step of osseointegration starts with cell adhesion and differentiation and the dental implant surface topography represents a key factor to the initial phase as well as in long-term bone remodeling.^{50,147}

Based on the literature, several treatment methods have been performed on Ti implant to promote the optimal interaction between the bone and the implant surface to ensure faster rehabilitation of the patients.¹⁴⁸ Several methods are available to modify the implant surface, such as physicochemical, morphological, and biochemical techniques.¹⁴⁹ The treatment methods, namely sandblasting and acid etching, are considered to be able to obtain high performing surfaces. Sandblasting is the bombardment of titanium surface with granules of variable diameter of oxides (titanium dioxide, aluminum oxide, zirconium dioxide and silicon carbide). Instead, acid etching method is performed with sulfuric, hydrofluoric or hydrochloric acid as stated by different procedures.¹⁵⁰ Surface features can play an important role in the biomolecular adsorption and cell adhesion to the implant surface as well as in osteoblast cell maturation.^{74,151}

MSCs can be simply isolated from craniofacial tissues during dentistry procedures. These cells exemplify as appropriate cell populations to study cell-biomaterial interaction in the craniofacial field, including osteointegrative events. MSCs have the capacity of self-renewal and differentiation into many cell lineages that comprise osteoblasts, chondrocytes, adipocytes, neuroblasts and myoblasts. In our *in vitro* cellular model, we use hPDLSCs, which are autologous and easily obtainable cell populations, showing the essential features for human clinical use.^{40,152,153} In particular, hPDLSCs represent a suitable model for the study of bone differentiation, thanks to their osteogenic capacity, compared to other types of MSCs.

In the present study we evaluated the secretion of ECM components necessary for osseointegration and osteogenesis using an *in vitro* model of hPDLSCs cultured on two different Ti surfaces, CTRL and TEST. The morphology and the cell adhesion capability of hPDLSCs on CTRL and TEST titanium surfaces were analyzed by microscopic analysis. The hPDLSCs evidenced similar morphology and adhesion capability on both CTRL and TEST Ti implant surfaces. In our study, the expression levels of principal and typical markers of ECM Fibronectin and Laminin, cytoskeleton markers actin and Vimentin, N-cadherin, a key factor in cell-cell interaction, and RUNX2, an early osteogenic marker were analyzed by Confocal laser scanning microscopy (CLSM) and RT-PCR. The results obtained from CLSM observation and RT-PCR revealed a significant upregulation of Fibronectin, Laminin, N-cadherin and RUNX2

in hPDLSCs seeded on TEST compared to CTRL surface. These results highlighted that the TEST surface induced the release of ECM matrix components fibronectin and Laminin which may promote hPDLSCs to undergo osteogenesis and implant osseointegration as evidenced from the higher expression of RUNX2 in TEST with respect to CTRL sample.

Furthermore, the slight increase of the expression level of N-cadherin could contribute to the enhancement of hPDLSCs osteogenesis, thus lead to more bone matrix deposition on the Ti implant. In parallel, these results were confirmed by the data obtained by Western Blot, which showed the quantitative level of protein expression of Fibronectin, Laminin, N-cadherin, RUNX2, OPN and SPARC. It is well known that ECM regulates cell adhesion. Cells adhere to the ECM via an integrin-mediated adhesion that connects ECM to the cytoskeleton, and integrins serve as receptors for ECM proteins. In order to ameliorate the regeneration, it is essential to evaluate the cell binding strength to the implant material surface.^{154,155} In particular, some expressed proteins are vital for the osteogenesis process, RUNX2 is essential and required for physiologic bone formation, osteopontin is a protein involved in normal calcifications and SPARC is one of the most abundant non-collagenous proteins expressed in the mineralized tissues. The evaluation of molecules such as integrins in the process of cellular attachment is needed to develop a Ti surface with optimum adherence properties for periodontal ligament. On this basis, in our study the gene expression level of RUNX2, VIM, CDH2, LAMB1, ITGA5 and ITGB1 was investigated. ITGA5 plays a fundamental role in osteogenic commitment as it is expressed by osteoblasts and osteoprogenitors and stimulates cell survival and matrix mineralization. ITGB1 is stably expressed by osteoblasts during different stages of osteogenesis, and it also mediates cell attachment to fibronectin as well as fibronectin assembly. Integrin-fibronectin interactions are vital for important cellular behaviors including embryogenesis, wound healing and cell migration.^{156–158}

In the current work, the authors demonstrated how rough titanium surfaces, obtained by double acid attack, improves the ECM components production and the expression of osteoblastic cellular profile, in order to promote the bone generation, anchorage, and osseointegration of titanium-made dental implants.

Surface modifications on the implant surfaces have been designed to improve the performance of the dental implant, in particular the roughness is considered a key factor for primary and long-term implant stability. This study was conducted to test the ECM molecules production. The ECM formation represents the early stage in the osteogenesis process, which encourages the cell migration, proliferation and differentiation, thus reducing the surgical time and morbidity of implant site via enhancing the bone tissue formation.

4.5 Conclusions

To conclude, the present results have shown a higher-level expression of typical markers of ECM Laminin and Fibronectin and its receptor Integrin α -5, and an upregulation of in cell-cell interaction marker N-cadherin, suggesting a better performance of TEST surface compared to CTRL in terms of osteogenesis and implant osseointegration as evidenced from the higher expression of RUNX2 in TEST respect to CTRL sample. Future research should examine the advancements in dental implant surface design, which are crucial to improve the healing process and to enhance the bone formation in order to permit the use of early loading protocols and to guarantee the implant success in patients with compromised bone tissue.

5. Study III. *Anti-TLR4 biological response to titanium nitride-coated dental implants: anti-inflammatory response and extracellular matrix synthesis*

Abstract

Osteointegration is a key process during dental implant placement and is related to titanium surface topography. Implant coating and surface modification methods ameliorate the bone production and the osteogenic process. The current work aimed at evaluating the biological outcomes of two different surfaces of dental implants, machined and titanium nitride (TiN) coated, at an inflammation level using an *in vitro* model of human periodontal ligament stem cells. The TLR4/MyD88/NF- κ B p65/NLRP3 pathway induced by the *Porphyromonas gingivalis* lipopolysaccharide was studied by means of gene- and protein-level expression. Moreover, the expression of vimentin, vinculin, and fibronectin was evaluated to investigate their effects on the cell adhesion and extracellular matrix deposition. The results of the present study suggest that TiN-coated titanium disks may modulate inflammation by the suppression of the TLR4/MyD88/NF- κ B p65/NLRP3 pathway and accelerate extracellular matrix apposition.

Keywords: oral stem cells, extracellular matrix, dental implants, inflammation, surface topography, titanium

5.1 Introduction

Titanium implants have demonstrated a considerable clinical success as they allow high integration between the implant surface and the host bone tissue, i.e., osteointegration. The structural properties and constitution of the extracellular matrix (ECM) are related to the differentiation capacity of cell survival and cell activity. Any alteration in the ECM may significantly modify the homeostasis of the tissues, which may have a strong impact during osteointegration. Several surface modification treatments have been recommended in order to improve osteointegration. A change in the shape and activity of mesenchymal stem cells (MSCs) can be induced through surface nanotopography and microtopography. This determines a greater induction of osteogenic differentiation of these cells through an overexpression of osteoblastic genes.^{133,159}

Dental implant surfaces with a wide variety of surface roughness values can be modified either with smooth machined surfaces or roughened with coatings, abrasion or sandblasting, acid

etching, or a combination of multiple procedures.^{117,142} Titanium nitride (TiN) has attracted significant interest due to its mechanical characteristics, including corrosion resistance, hardness, and shear strength, and biological characteristics, such as biocompatibility and hemocompatibility.¹⁶⁰ Thin coatings of TiN and TiO₂ are applied using physical vapor deposition (PVD) techniques that alter the surface chemical makeup and crystalline structure while retaining the surface microroughness of the implant.¹⁶¹ In the atomistic deposition PVD method, the material is vaporized by a solid or liquid source, passed as a vapor through a vacuum or low-pressure gaseous (or plasma) environment, and transferred on the substrate where it condenses.¹⁰³ The pathogenesis of most bacterial illnesses starts with the adhesion of bacteria to the implant surface. Accordingly, preventing bacterial infection in intraoral hard or soft tissue is essential. Surface modification treatments have been reported to reduce bacterial adhesion to the implant surface¹⁶². Grossner-Schreiber et al.¹¹¹ showed that TiN film-coated titanium displayed fewer bacterial colonies when compared to a machined uncoated titanium surface. Brunello et al.¹⁶³ found a higher percentage of dead bacteria in TiN-coated titanium disks compared to pure titanium. One of the most prevalent bacteria in the oral cavity is *Porphyromonas gingivalis*, which is involved in plaque biofilm formation. Virulence of this Gram-negative bacterium is often caused by the presence of lipopolysaccharides (LPSs)⁴⁰ *Porphyromonas gingivalis* lipopolysaccharide (LPS-G) is essential for triggering the inflammation and release of proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor (TNF)³⁵ It is known that LPSs, through the activation of the Toll-like receptor 4 (TLR4), lead to the production of nuclear factor kappa B (NF-κB) and, therefore, proinflammatory cytokines.¹⁶⁴

TLR4 belongs to the family of TLR receptors which, upon interaction with pathogens, induce a proinflammatory response. One of the activators of TLR4 is the lipopolysaccharide (LPS, endotoxin) from Gram-negative bacteria, leading to the activation of two signaling cascades. The first is stimulated in the plasma membrane and involves the adaptor proteins TIRAP and myeloid differentiation primary response gene 88 (MyD88). The second, through the adaptor proteins TRAM and TRIF, starts in the early endosomes after receptor endocytosis.¹⁶⁵ Titanium dental implants placed in the bone tissue start a series of molecular and cellular processes that guide the formation of the ECM and the apposition of newly formed bone. One of the fundamental steps for osteogenesis and for osseointegration to take place is the secretion of the components of the ECM, of which fibronectin, laminin, actin, and vimentin are fundamental markers for initiating and improving osteointegration. The ECM molecules, being involved in the cell–material interaction, regulate the cell adhesion to the biomaterial.

The present work aims to study, using an *in vitro* model of human periodontal ligament stem cells (hPDLSCs), the biological outcomes of two different surfaces of dental implants, machined and coated with TiN in the inflammatory pathway TLR4/MyD88/NF- κ B p65/NLRP3 activated by LPS-G.

5.2 Materials and methods

5.2.1 Titanium disk

In this study, 50 machined (control) and 50 TiN-coated (test) titanium disks from Resista (Omegna, VB, Italy) were used. The disks were made of grade IV commercially pure titanium and had a circumference of 5 mm and thickness of 2 mm. The disks were automatically cleaned in a cavitation washer using detergents designed to eliminate processing grease and finish purification washes. After the decontamination process in a cold argon plasma reactor in a clean room, the disks were packaged in thermally sealed autoclaving trade bags. Gammatom S.r.l. (Italy) externally sterilized the object using 35 KGy of gamma radiation. PVD coating was used to coat 50 Ti disks with TiN.

The coating had a mean thickness of $1.6 \pm 0.2 \mu\text{m}$. The coating did not affect the inherent texture of the Ti surface. The surface morphologies of the disks were characterized using a scanning electron microscope (SEM) (Jeol, JSM 5410, Tokyo, Japan), at a voltage of 10 kV.

5.2.2 Ethics statement

The Medical Ethics Committee at the Medical School, “G. d’Annunzio” University, Chieti, Italy (n° 266/17.04.14), approved the procedure and obtained informed consent from human periodontal ligament biopsy donors. Before any subjects’ samples were collected, all subjects formally consented to the experiment. The Laboratory of Stem Cells and Regenerative Medicine and the Department of Medical, Oral, and Biotechnological Sciences are certified in accordance with the quality standard ISO 9001:2008 (certificate no. 32031/15/S).

5.2.3 Cell culture

Five human premolar teeth were obtained from generally healthy subjects (between the ages of 18 and 25 years) for human periodontal ligament biopsy by root scaling using Gracey’s curettes. The tissues were cleaned five times with Dulbecco’s phosphate buffered saline (DPBS) without calcium and magnesium (Euroclone, Cat# ECB4004L) and then cultured using a TheraPEAK™

MSCGM™ (Mesenchymal Stem Cell Growth Medium) BulletKit™ (Lonza, Cat# BEBP18-936), which contains the serum-free TheraPEAK™ MSCGM™ Mesenchymal Stem Cell Growth Medium and TheraPEAK™ MSCGM Supplement. After 15 days, the tissues began to release cells after the media had been replaced twice a week. Cells were trypsinized once they reached 80% confluency to obtain subcultures up to passage 4 (P4).⁴⁰

5.2.4 Experimental study design

The hPDLSCs were used to conduct the three experimental points stated in the following research design. LPS prepared from *P. gingivalis* was applied as a stimulant to the cells (InvivoGen, Cat# tlrl-pgglps).

- hPDLSCs cultured with a machined disk for 24 h
- hPDLSCs cultured with a machined disk and stimulated with 5 µg/mL LPS-G
- hPDLSCs cultured with a machined and TiN-coated disk for 24 h
- hPDLSCs cultured with a machined and TiN-coated disk and stimulated with 5 µg/mL LPS-G

5.2.5 Confocal laser scanning microscopy

Eight-well culture glass slides (Corning, Glendale, Arizona, United States) with Ti disks were used to cultivate hPDLSCs for 24 h at a density of 6.4×10^4 /well. Then, the cells were fixed with 4% paraformaldehyde (ImmunoFix) (Bio-Optica, Cat# 05-K01015) in 0.1 M DPBS for 1 h at RT, washed three times with DPBS, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Corporation, Cat# T8787) in DPBS for 6 min, blocked for 1 h at RT with 5% of non-fat milk (Euroclone, Cat# EMR100500) in DPBS, and incubated with primary antibodies overnight at 4°C. The primary antibodies used were TLR4 (Santa Cruz Biotechnology, Cat# sc-293072, RRID: AB_10611320), anti-MyD88 (Santa Cruz Biotechnology, Cat# sc-74532, RRID: AB_1126429), anti-NF-κB p65 (Santa Cruz Biotechnology, Cat# sc-8008, RRID: AB_628017), anti-NLRP3 (Santa Cruz Biotechnology, Cat# sc-134306, RRID: AB_2152550), anti-fibronectin (Santa Cruz Biotechnology, Cat# sc-8422, RRID: AB_627598), anti-vimentin (Santa Cruz Biotechnology, Cat# sc-6260, RRID: AB_628437), and anti-vinculin (Santa Cruz Biotechnology, Cat# sc-55465, RRID: AB_630433) at a dilution of 1:200.

The cytoskeleton actin and the nuclei were marked with 1:200 Alexa Fluor 488-phalloidin green fluorescent conjugate (Invitrogen, Cat# A12379) and 1:200 DAPI (Invitrogen, Cat# D3571), respectively, for 1 h at 37°C, followed by incubation with secondary antibody Alexa Fluor 568 red fluorescence-conjugated goat anti-mouse antibody (Invitrogen, Cat# A11031, RRID:

AB_144696). LSM800 confocal equipment (Carl Zeiss, RRID: SCR_015963) was used to capture the pictures (RRID: SCR_015963). Images were collected using an argon laser beam with excitation lines at 488 nm and a helium–neon source at 543 and 633 nm. The acquisition settings were kept constant between specimens. ImageJ software was used to analyze the captured images. The quantification was based on 10 images randomly collected.

5.2.6 Western blotting analysis

The hPDLSC protein lysates were used for the Western blot analysis. After electrophoresis, 50 µg of the lysate was transferred to a PVDF membrane using a semi-dry blotting device (Bio-Rad Laboratories Srl, Cat# 1703940, RRID: SCR_019036). The membranes were first saturated with 5% non-fat milk in DPBS and 0.1% Tween-20 (Sigma-Aldrich, Cat# P1379) and then incubated overnight 4°C with the following primary antibodies: anti-TLR4 (Santa Cruz Biotechnology, Cat# sc-293072), anti-MyD88 (Santa Cruz Biotechnology, Cat# sc-74532), anti-NF-κB p65 (Santa Cruz Biotechnology, Cat# sc-8008), anti-NLRP3 (Santa Cruz, Biotechnology, Cat# sc-134306) anti-fibronectin (Santa Cruz Biotechnology, Cat# sc-8422), anti-vimentin (Santa Cruz Biotechnology, Cat# sc-6260), and anti-vinculin (Santa Cruz Biotechnology, Cat# sc-55465), all at a dilution of 1:500, and β-actin (Santa Cruz Biotechnology, Cat# sc-47778, RRID: AB_2714189) was used as a loading control at a dilution of 1:750. The membranes were then treated for 1 h at RT with the goat anti-mouse peroxidase-conjugated secondary antibody (Bethyl Laboratories, Cat# A90-116P, RRID: AB_67183). Immobilon Crescendo Western HRP substrate (Merck Millipore, Cat# WBLUR0100), the enhanced chemiluminescence exposure (ECL) method, and image documenter Alliance 2.7 (Uvitec, Cat# XD-79.LS/20M) were used to identify the expressed proteins. ECL development and evaluation were performed using UVIband-1D gel investigation (Uvitec) to evaluate the detected signals. The acquired values were adjusted using β-actin.¹⁶⁶ Three separate experiments were conducted to perform the Western blotting analysis.

5.2.7 RNA isolation and real-time-PCR analysis

Real-time PCR was used to examine the gene expression of TLR4, MyD88, NF-κB p65, NLRP3, fibronectin, vimentin, and vinculin in three separate biological replicates. The total RNA was extracted using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Cat# 12183018A). According to the technical bulletin, M-MLV Reverse Transcriptase (Sigma-Aldrich, Cat# M1302) was used to reverse-transcribe the total RNA into cDNA for 10 min at 70°C, 50 min at 37°C, and 10 min at 90°C. Real-time PCR was performed using the

Mastercycler ep realplex real-time PCR system (Eppendorf, Cat#5345) to determine the mRNA expression levels of TLR4, MyD88, RELA, NLRP3, FN1, VIM, VCL, and beta-2 microglobulin (B2M) (endogenous marker) in hPDLSCs grown on machined disks, hPDLSCs grown on machined disks with LPS-G stimulation, hPDLSCs grown on machined TiN-coated disks, and hPDLSCs grown on machined TiN-coated disks with LPS-G induction. Specifically, PrimeTime™ Predesigned qPCR Assays for TLR4 (Hs.PT.58.38700156.g, Tema Ricerca Srl), RELA (Hs.PT.58.22880470, Tema Ricerca Srl), MyD88 (Hs.PT.58.40428647.g, Tema Ricerca Srl), NLRP3 (Hs.PT.58.39303321, Tema Ricerca Srl), FN1 (Hs.PT.58.40005963, Tema Ricerca Srl), VIM (Hs.PT.58.38906895; Tema Ricerca Srl), VCL (Hs.PT.58.2753988, Tema Ricerca Srl), and the PrimeTime™ Gene Expression Master Mix (Cat# 1055772, Tema Ricerca Srl) were employed in accordance with standard protocols. The beta-2 microglobulin (B2M Hs.PT.58v.18759587, Tema Ricerca Srl) was used as the standard for the results. The amplification protocol consisted of three phases: preincubation at 95°C for 3 min, followed by 40 cycles of 15 s at 95°C, and an annealing phase for 1 min at 60°C. Each gene's expression levels were estimated using the $2^{-\Delta\Delta CT}$ method.

5.2.8 Statistical analysis

GraphPad Prism 5 software (GraphPad, San Diego, RRID: SCR_002798) was used to conduct one-way ANOVA and *post hoc* Tukey's multiple comparisons analysis. Statistical significance was calculated at $p < 0.05$. The comparative $2^{-\Delta\Delta CT}$ technique was used to analyze the gene expression data.

5.3 Results

5.3.1 Morphological features of the titanium disk surface

SEM observations showed the surface morphology of the two different considered surfaces, machined and TiN-coated titanium surfaces (Figure 6). SEM results showed that the titanium disk surface coated with TiN possesses a rougher surface than the machined disk (Figure 6A1). At a magnification of 2,000 times of SEM, changes in the titanium surface were observed (Figure 6B1).

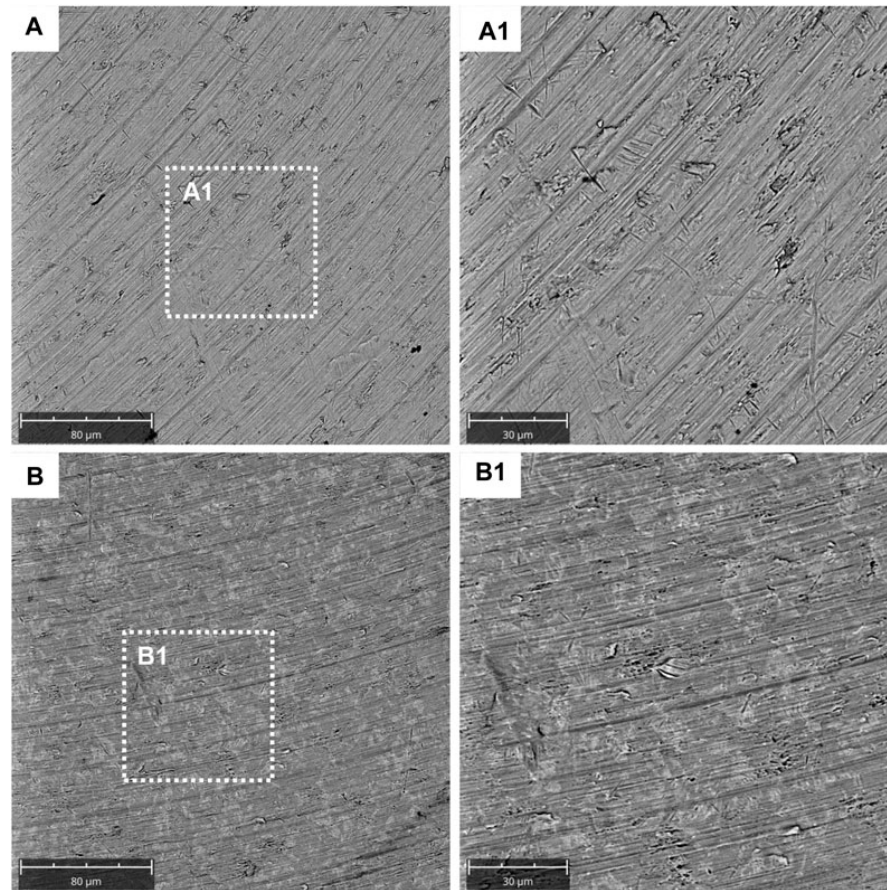


Figure 6. Characterization data for the machined and titanium nitride (TiN)-coated titanium surface. SEM analysis of the titanium machined surface: **(A)** Machined surface disk acquired at 1,000x; **(A1)** machined surface disk acquired at 2,000x; **(B)** TiN surface disk acquired at 1,000x; and **(B1)** TiN surface disk acquired at 2,000x.

5.3.2 Machined and TiN-coated disk implants influence the inflammatory pathway and the extracellular matrix deposition.

To study the inflammation response to the LPS-G stimulus, the TLR4/MyD88/NF- κ B p65/NLRP3 pathway in the hPDLSC culture was considered. TLR4 is implicated in the immune response; this receptor is activated in the presence of the cell wall component of Gram-negative bacteria, LPS. TLR4 can activate the MyD88 pathway responsible for the expression of the inflammatory cytokines. NF- κ B regulates many aspects of innate and adaptive immune functions and acts as a mediator in inflammatory responses. NF- κ B induces the expression of various proinflammatory genes, including those encoding cytokines and chemokines, and also participates in the regulation of the NLRP3 inflammasome. The NLRP3 inflammasome is one of the best characterized inflammasomes leading to the production and maturation of IL-1 β (Liu et al., 2022). The immunofluorescence observations reported the protein expression of TLR4/MyD88/NF- κ B p65/NLRP3 in hPDLSCs cultured with a machined disk implant,

hPDLSCs cultured with a machined disk implant stimulated with LPS-G, hPDLSCs cultured with a TiN-coated disk implant, and hPDLSCs cultured with a TiN-coated disk implant stimulated with LPS-G. The obtained representative images showed that the TLR4/MyD88/NF- κ B p65/NLRP3 pathway was notably upregulated in hPDLSCs cultured with machined disk implants stimulated with LPS-G for 24 h compared to the hPDLSCs cultured with machined and machined TiN-coated disk implants treated with LPS-G (Figure 7; Figure 8; Figure 9; Figure 10). Furthermore, the protein expression of fibronectin, vimentin, and vinculin was also evaluated by confocal microscopy. The results evidenced that fibronectin, vimentin, and vinculin were upregulated in hPDLSCs cultured with the machined TiN-coated disk implant untreated and stimulated with LPS-G compared with control samples, cells cultured with the machined disk (Figure 11; Figure 12; Figure 13), as also demonstrated by quantitative analysis represented as the arbitrary unit of fluorescence per cell surface unit ($F/\mu\text{m}^2$). Western blot analyses provided the protein quantitative expression in order to validate the immunofluorescence acquisition results (Figure 14; Figure 15).

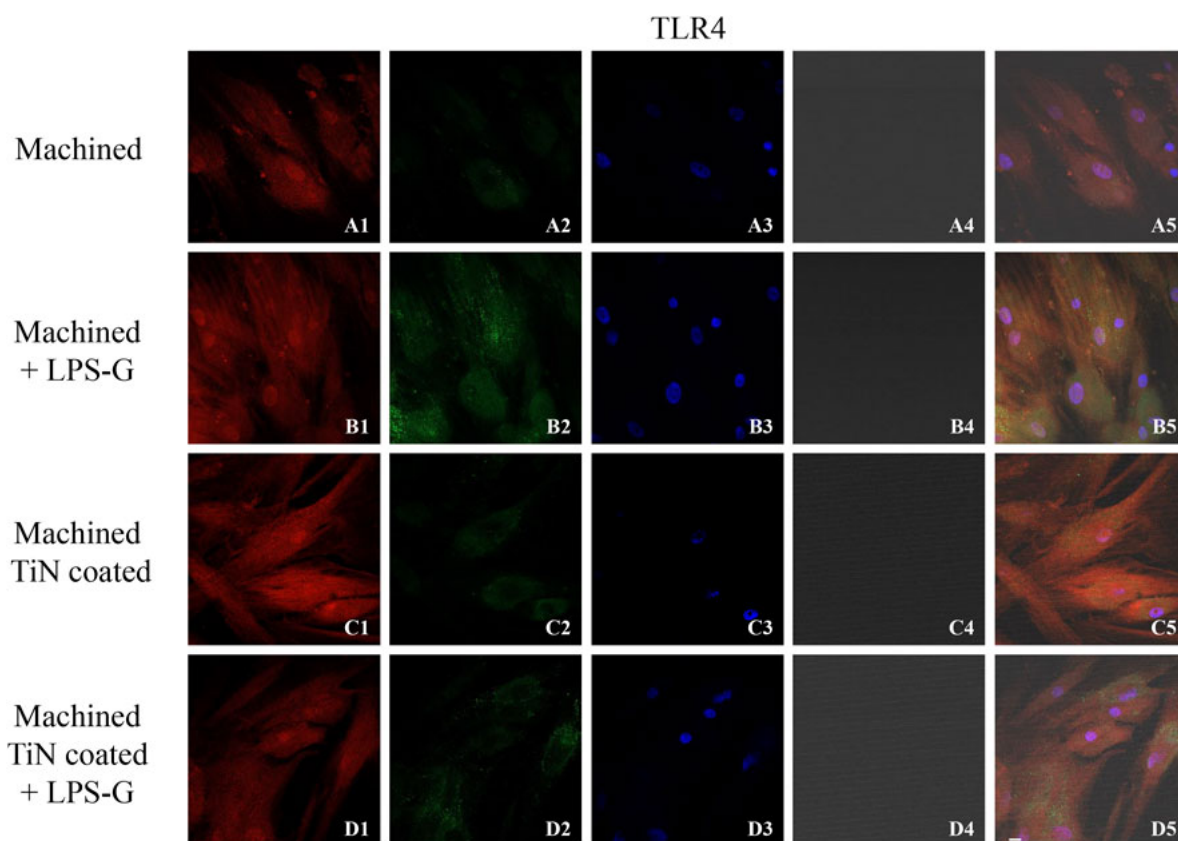


Figure 7. Level expression of TLR4 evaluated by confocal microscopy in hPDLSCs (A1–D5). TLR4 expression in hPDLSCs cultured with the machined disk, in hPDLSCs cultured with the machined disk and induced with LPS-G, in hPDLSCs seeded with the machined TiN-coated disk, and in hPDLSCs cultured with the machined TiN-coated disk and treated with LPS-G. Red fluorescence: TLR4 (A1–D1); green fluorescence: cytoskeleton actin (A2–D2); blue fluorescence: cell nuclei (A3–D3); titanium disk (A4–D4); and merge (A5–D5). Scale bar: 20 μm .

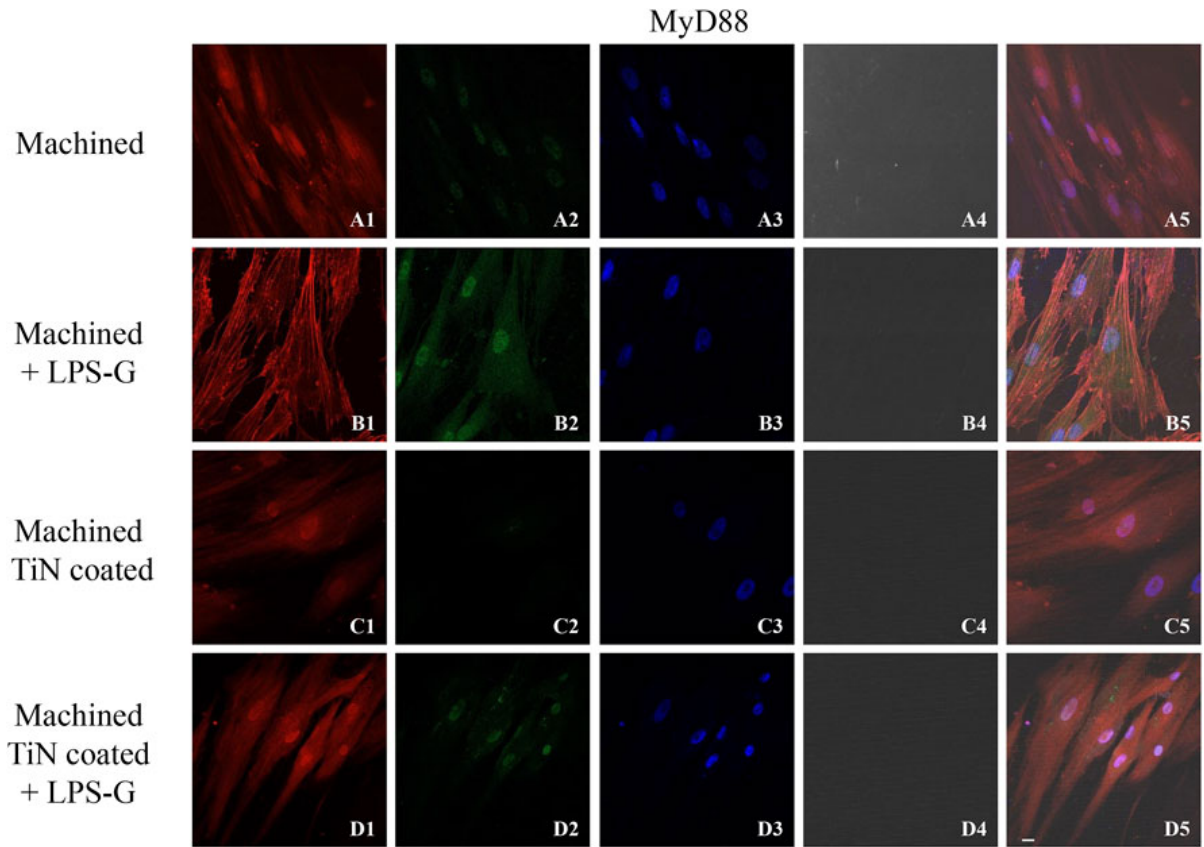


Figure 8. Expression of MyD88 investigated by confocal microscopy in hPDLSCs (A1–D5). MyD88 expression in hPDLSCs cultured with a machined disk, in hPDLSCs cultured with a machined disk and treated with LPS-G, in hPDLSCs seeded with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and stimulated with LPS-G: Red fluorescence: MyD88 (A1–D1); green fluorescence: cytoskeleton actin (A2–D2); blue fluorescence: cell nuclei (A3–D3); titanium disk (A4–D4); and merge (A5–D5). Scale bar: 20 μ m.

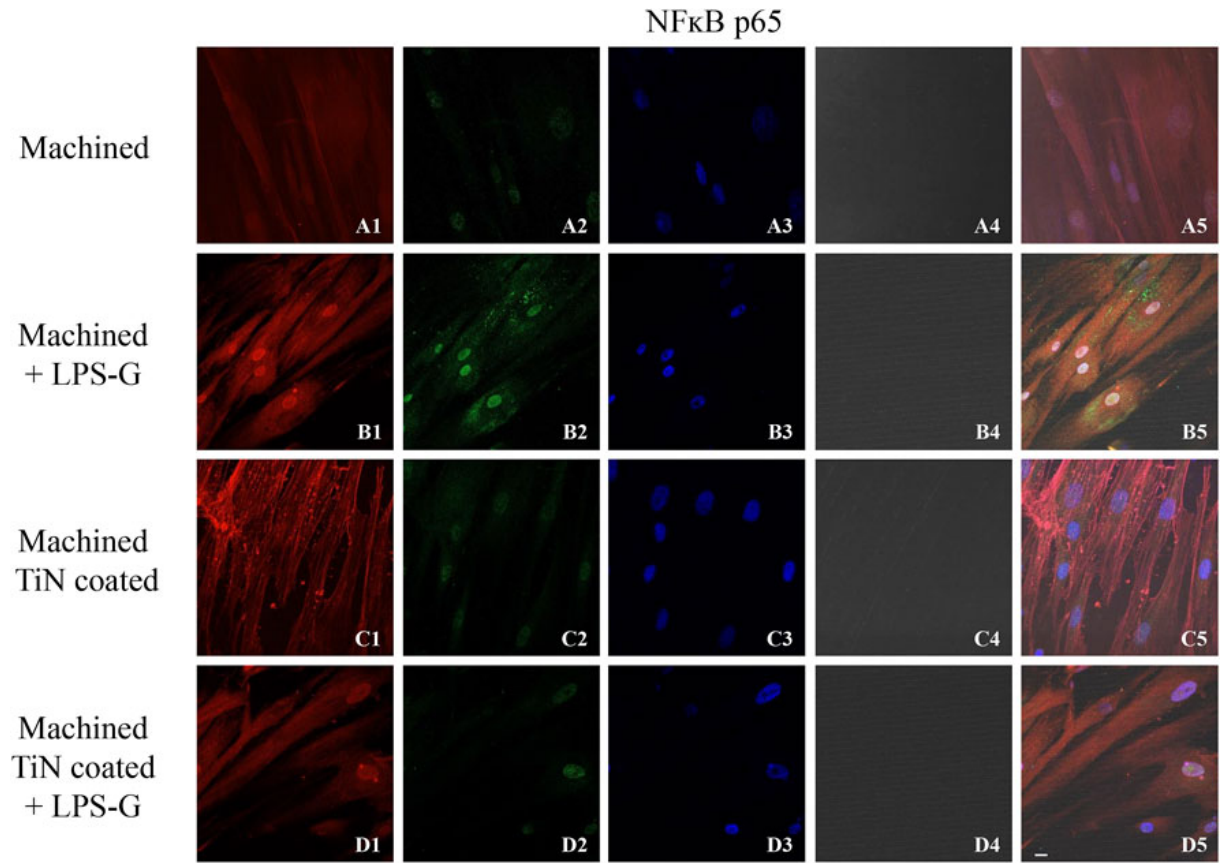


Figure 9. Expression of NF-κB evaluated by confocal microscopy in hPDLSCs (A1–D4). NF-κB expression in hPDLSCs cultured with a machined disk, in hPDLSCs cultured with a machined disk and treated with LPS-G, in hPDLSCs seeded with a machined TiN-coated disk, and in hPDLSCs seeded with a machined TiN-coated disk and stimulated with LPS-G: Red fluorescence: NF-κB p65 (A1–D1); green fluorescence: cytoskeleton actin (A2–D2); blue fluorescence: cell nuclei (A3–D3); titanium disk (A4–D4); and merge (A5–D5). Scale bar: 20 μm.

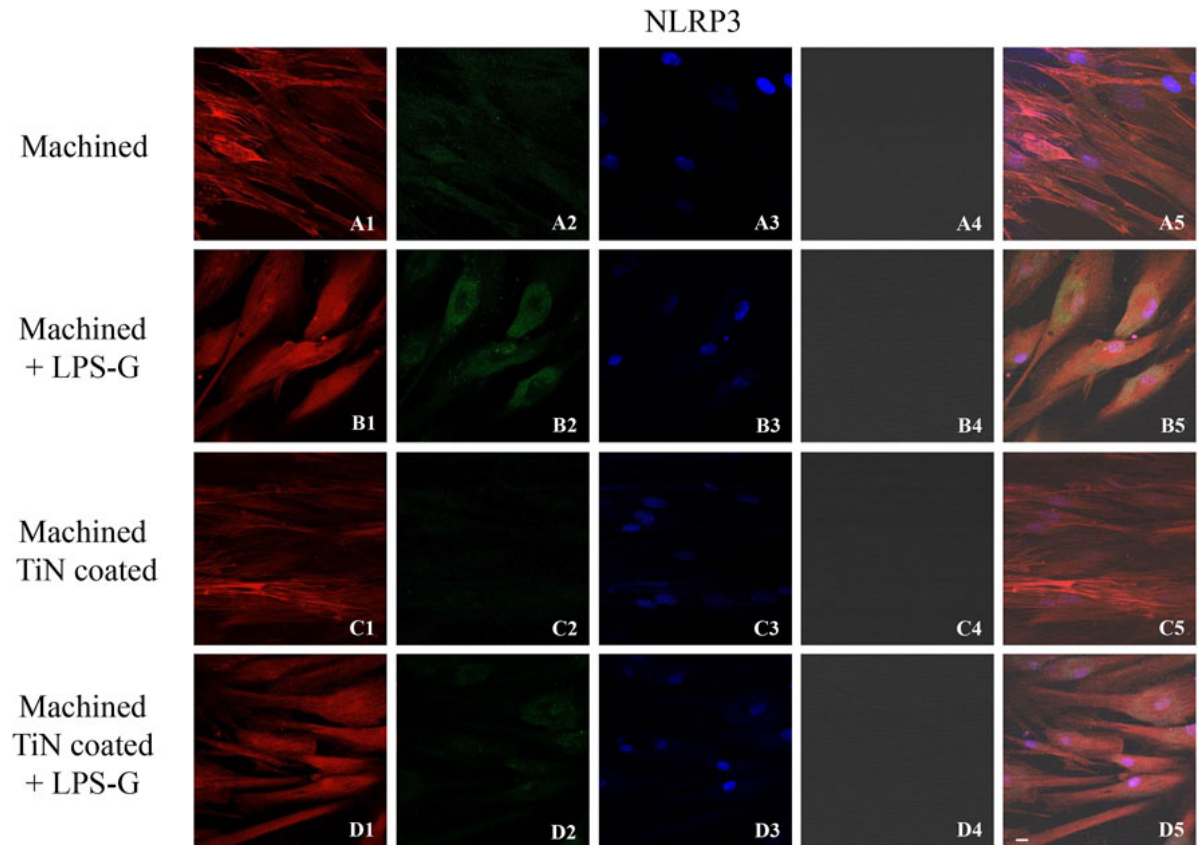


Figure 10. Expression of NLRP3 investigated by confocal microscopy in hPDLSCs (A1–D4). NLRP3 expression in hPDLSCs cultured with a machined disk, in hPDLSCs cultured with a machined disk and treated with LPS-G, in hPDLSCs cultured with a machined TiN-coated disk, and in hPDLSCs seeded with a machined TiN-coated disk and induced with LPS-G: Red fluorescence: NLRP3 (A1–D1); green fluorescence: cytoskeleton actin (A2–D2); blue fluorescence: cell nuclei (A3–D3); titanium disk (A4–D4); and merge (A5–D5). Scale bar: 20 μ m.

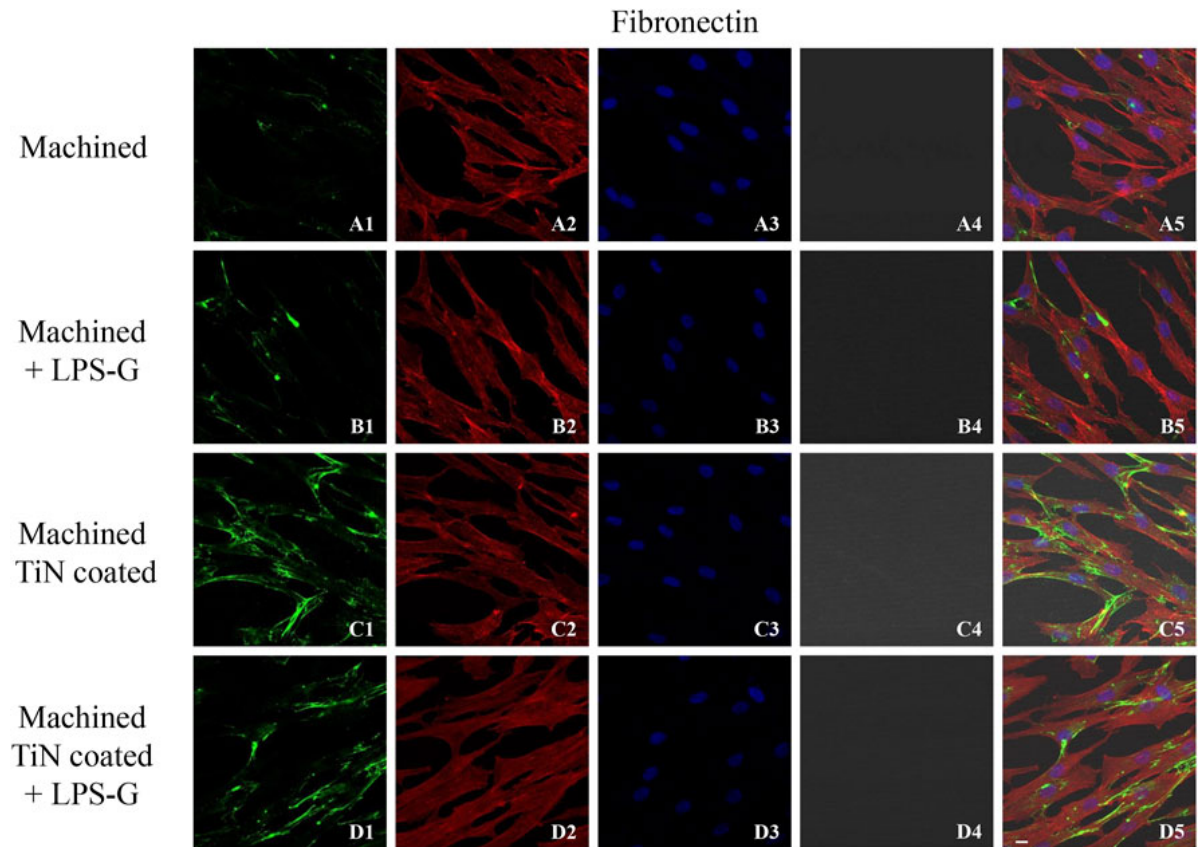


Figure 11. Expression of fibronectin studied by confocal microscopy in hPDLSCs (A1–D4). Fibronectin expression in hPDLSCs cultured with a machined disk, in hPDLSCs seeded with a machined disk and treated with LPS-G, in hPDLSCs cultured with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and induced with LPS-G: Red fluorescence: fibronectin (A1–D1); green fluorescence: cytoskeleton actin (A2–D2); blue fluorescence: cell nuclei (A3–D3); titanium disk (A4–D4); and merge (A5–D5). Scale bar: 20 μ m.

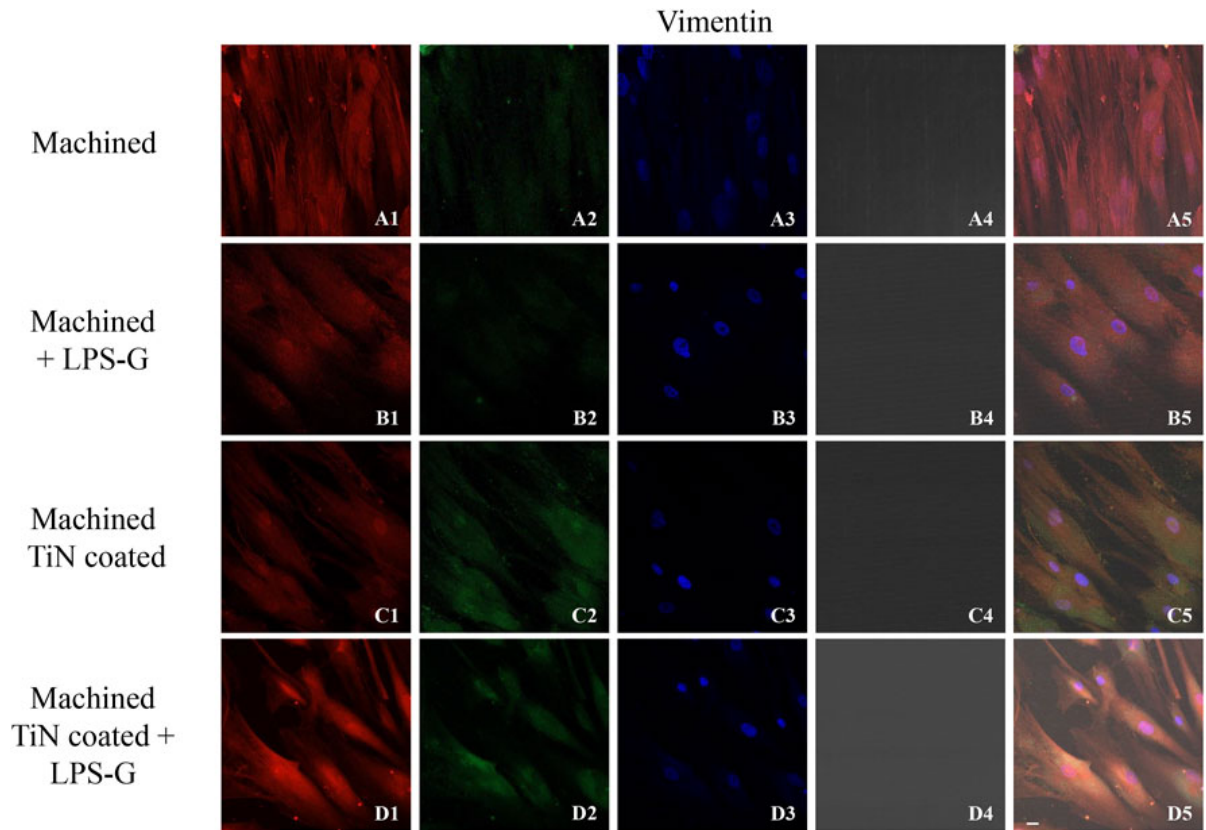


Figure 12. Expression of vimentin evaluated by confocal microscopy in hPDLSCs (A1–D4). Vimentin expression in hPDLSCs cultured with a machined disk, in hPDLSCs cultured with a machined disk and stimulated with LPS-G, in hPDLSCs cultured with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and treated with LPS-G: Red fluorescence: vimentin (A1–D1); green fluorescence: cytoskeleton actin (A2–D2); blue fluorescence: cell nuclei (A3–D3); titanium disk (A4–D4); and merge (A5–D5). Scale bar: 20 μ m.

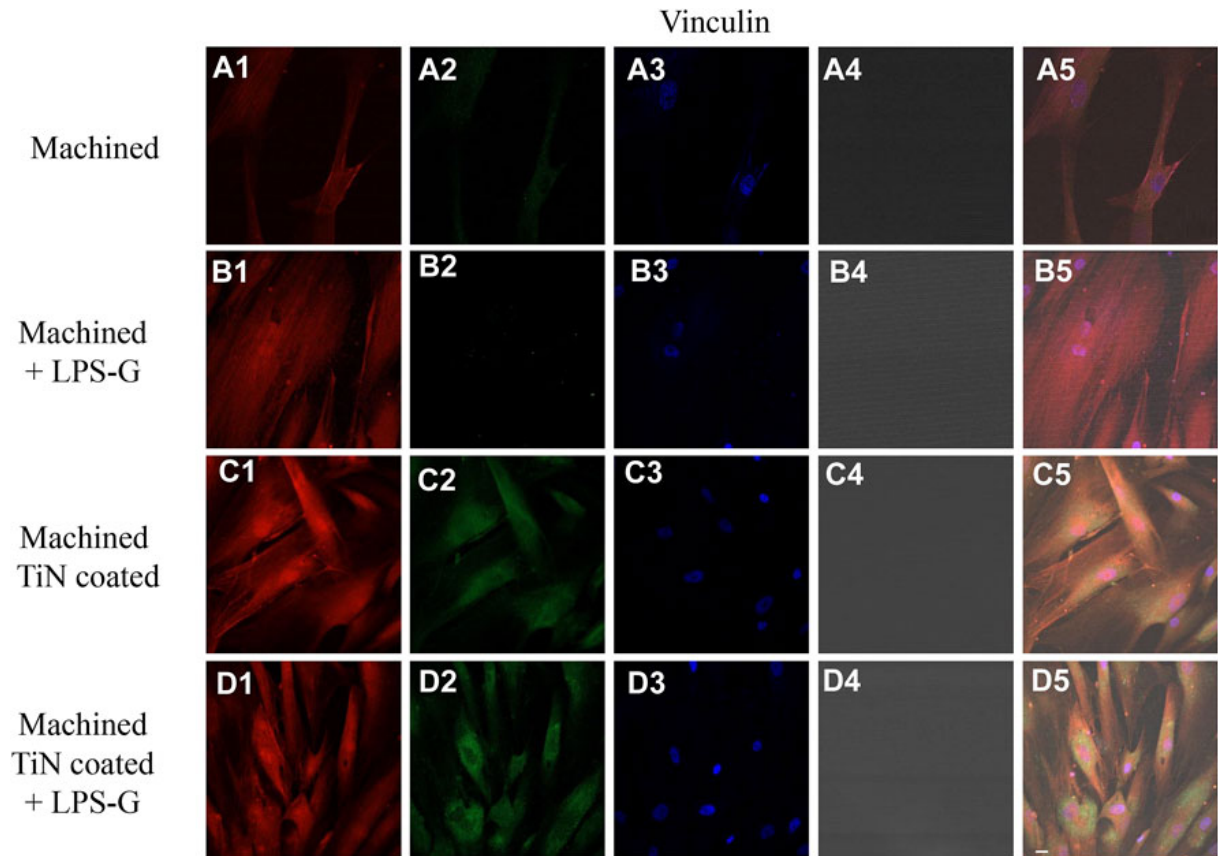


Figure 13. Expression of vinculin examined by confocal microscopy in hPDLSCs (**A1–D4**). Vinculin expression in hPDLSCs seeded with a machined disk, in hPDLSCs cultured with a machined disk and treated with LPS-G, in hPDLSCs cultured with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and induced with LPS-G: Red fluorescence: vinculin (**A1–D1**); green fluorescence: cytoskeleton actin (**A2–D2**); blue fluorescence: cell nuclei (**A3–D3**); titanium disk (**A4–D4**); and merge (**A5–D5**). Scale bar: 20 μ m.

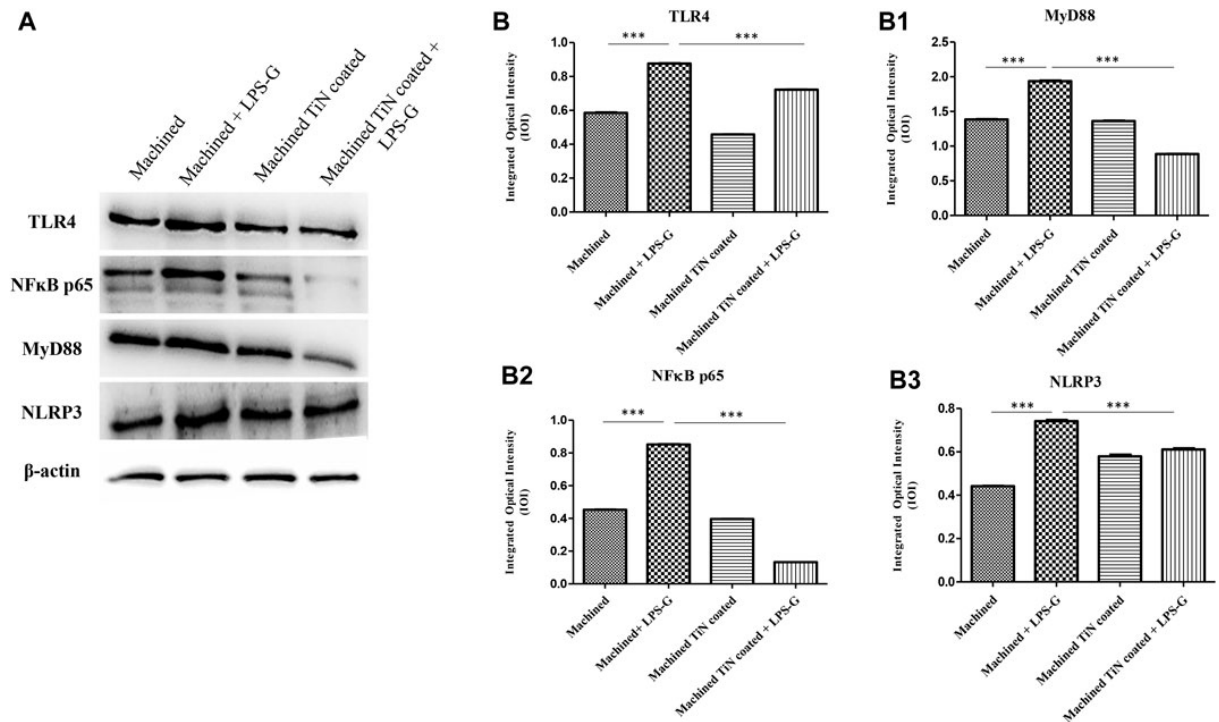


Figure 14. Western blotting analysis. TLR4, MyD88, NF-κB p65, and NLRP3 protein expression in hPDLSCs seeded with a machined disk, in hPDLSCs cultured with a machined disk and treated with LPS-G, in hPDLSCs seeded with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and stimulated with LPS-G: **(A)** Each membrane was probed with a β-actin antibody to validate the loading reliability. **(b–b3)** Histograms denote densitometric measurements of protein bands expressed as the mean of the integrated optical intensity (IOI) of three separate experiments. The error bars evidence the standard deviation ($\pm$ SD). * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

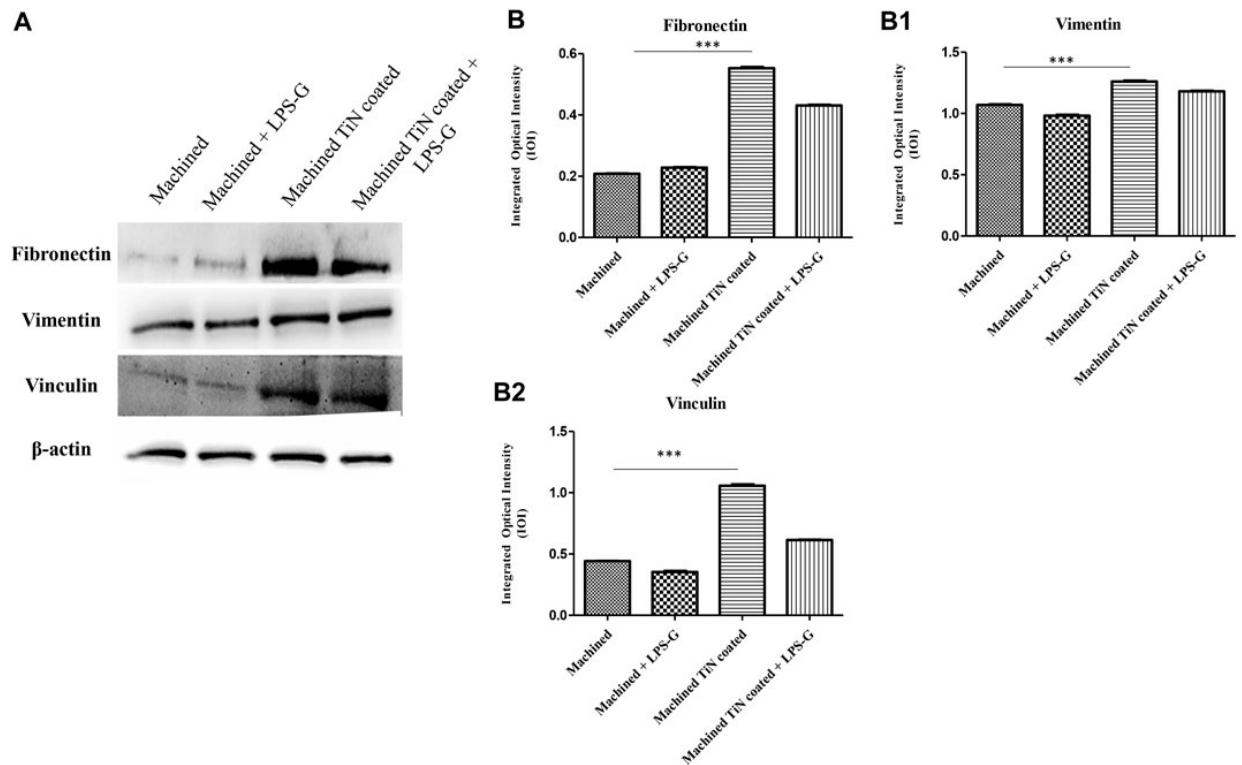


Figure 15. Western blotting analysis. Fibronectin, vimentin, and vinculin protein expression in the hPDLSCs seeded with a machined disk, in hPDLSCs seeded with a machined disk and treated with LPS-G, in hPDLSCs cultured with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and induced with LPS-G: **(A)** Each membrane was probed with a β -actin antibody to validate the loading reliability. **(b–b2)** Histograms denote densitometric measurements of proteins bands expressed as the mean of the integrated optical intensity (IOI) of three separate experiments. The error bars evidence the standard deviation ($\pm$ SD). * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

5.3.3 Gene expression

The bar graph exhibited the gene expression of TLR4/MYD88/RELA/NLRP3 and FN1/VIM/VCL by real-time PCR. The hPDLSCs cultured with the machined disk and stimulated with LPS-G reported a significantly higher gene expression of TLR4/MYD88/RELA and NLRP3 in comparison with the control samples, cells cultured with machined and machined TiN-coated disks and stimulated with LPS-G. Furthermore, FN1/VIM/VCL gene expression is higher in hPDLSCs cultured with the machined TiN-coated disk than in hPDLSCs cultured with the machined disk. The results obtained by RT-PCR validated the protein expression results (Figure 16).

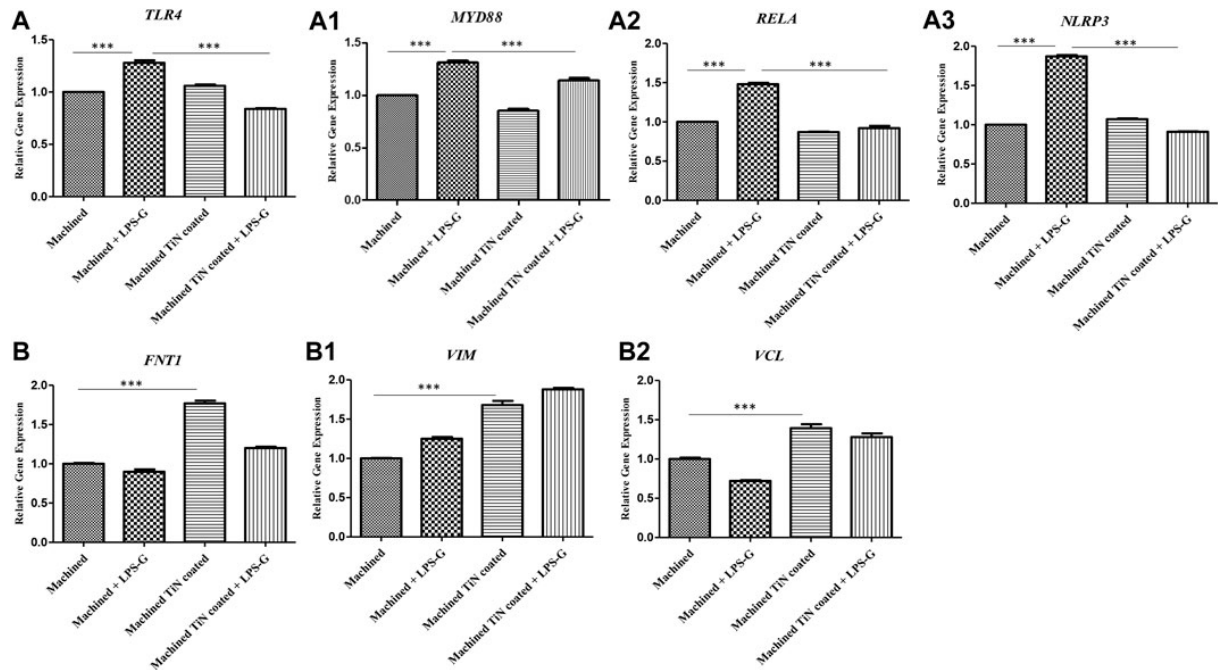


Figure 16. (a–a3) Histograms of RT-PCR showing the mRNA levels of TLR4, MYD88, RELA, and NLRP3. **(b–b2)** FNT1, VIM, and VCL in the hPDLSCs cultured with a machined disk, in hPDLSCs cultured with a machined disk and stimulated with LPS-G, in hPDLSCs cultured with a machined TiN-coated disk, and in hPDLSCs cultured with a machined TiN-coated disk and stimulated with LPS-G. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

5.4 Discussion (STUDY III)

Osseointegration is the crucial first step in the success of dental implants. It can be defined as biological processes that lead to a prompt attachment of bone tissue to the titanium, and the cellular adherence on the implant surfaces is the first step to ensure long-term clinical success.¹⁶⁷ Cell adhesion influences cell development, proliferation, and differentiation. Biological compounds, including the ECM, cell membrane, and cytoskeleton proteins, may also be present during the adhesion phase. These molecules cooperate to provide signal transduction, which facilitates the activity of transcription factors and subsequently controls gene expression.¹⁶⁸ Topographical alteration to improve cellular responses at the bone–implant interface has been explored in previous works on implant surfaces.¹⁶⁹ Coating with a suitable implant material is very important for avoiding its failure and to improve its durability and longevity. Innovative methods, such as physical and chemical approaches, are appropriate coating methods to stimulate the implant surfaces relatively to mechanical, biological, and other multifunctional properties. Coating the titanium alloy implants with titanium nitride by PVD creates a stable layer on the implant surface that alters its chemistry.

PVD is the most commonly used technique of adding TiN to orthopedic implants, and TiN is obtained by reacting pure titanium with nitrogen gas during a vapor phase prior to accumulation.¹⁰² A 48 million cycle wear test was conducted. Thin coatings of TiN and TiO₂ are applied using PVD techniques, allowing the surface chemical makeup and crystalline structure to alter while retaining the microroughness of the implant surface. PVD methods are engaged to produce films with thicknesses that range from a few to thousands of nanometers. Earlier studies demonstrated that TiN coating improves fibroblast cell adhesion, differentiation, and proliferation, in addition to decreasing bacterial adherence and biofilm production. The improvement of wear resistance and abrasive stiffness are fundamental characteristics of TiN film coatings to ameliorate the surface possessions of pure titanium.^{114,151}

The use of TiN hard thin-film coating in dentistry is strictly limited and recent, although it has been applied in the medical field to coat orthopedic implants (hip and knee replacement prostheses) more frequently.¹⁷⁰ Preliminary reports demonstrated that TiN coating enhances fibroblast cell proliferation, attachment, and differentiation, as well as reduces bacterial adherence and biofilm formation. Scarano et al. demonstrated that the implant surface covered by bacteria on TiN-coated implants was significantly lower than that of control implants ($p = .0001$). Grossner-Schreiber et al. examined the influence of titanium nitride coating on bacterial adhesion compared with the pure titanium surface, concluding that “a significant reduction in the number of adherent bacteria was observed on inherently stable titanium hard materials, such as TiN, compared to polished titanium,” with a statistically significant difference ($p = 0.0036$) showed that the percentage of dead bacteria was higher in the biofilms grown on the TiN-coated disks than those grown on the pure titanium disks.¹⁶³ Moreover, S. Datta et al. reported that TiN-coated implants have shown significant cytocompatibility with mouse fibroblast cells. It was also demonstrated that TiN-coated implants increased osteoblast cell proliferation compared with their sample controls, and the antibacterial activity of TiN-coated implants against different strains of *Streptococci* was also proven, which was bactericidal *in vitro*.¹⁷¹

Based on this purpose, in the current study, the biological outcomes of two diverse disk implant surfaces, machined and TiN coated, in an *in vitro* model of hPDLSCs were exploited in the inflammatory event throughout the modulation of the TLR4/MyD88/NF- κ B p65/NLRP3 pathway.

The first TLR identified in mammals was TLR4, which is implicated in the immune response. This receptor is activated in the presence of the cell wall component of Gram-negative bacteria, LPS.¹⁷² The activation of this pathway represents a physiological response of the organism to

the presence of infection. TLR4 can activate the MyD88 pathway capable of mediating the induction of type I interferons (IFNs) and interferon-inducible genes or a dependent MyD88 pathway responsible for the expression of inflammatory cytokines.¹⁷³ In the MyD88-dependent pathway, the activation of TLR4 drives MyD88, composed of a Toll/interleukin-1 receptor (TIR) domain and a death domain (DD), to recruit other molecules.

NF- κ B is involved in the transcription of genes implicated in the immune response and inflammation. The activation of NF- κ B can occur through a canonical or non-canonical signaling pathway. The canonical signaling pathway is related to the immune response as well as the activation of the TLR4 pathway induced by LPS. The transcription factor NF- κ B regulates many aspects of innate and adaptive immune functions and acts as a mediator in inflammatory responses. NF- κ B induces the expression of various proinflammatory genes, including those encoding cytokines and chemokines, and also participates in the regulation of the NLRP3 inflammasome. The NLRP3 inflammasome is one of the best characterized inflammasomes leading to the production and maturation of IL-1 β .¹⁷⁴

NF- κ B is a protein complex that controls DNA transcription, cytokine production, and cell survival. It is found in almost all cell types and is involved in cellular responses to stimuli, such as stress, cytokines, free radicals, heavy metals, ultraviolet radiation, and bacterial or viral antigens.¹⁷⁵ In cells not activated by inflammatory stimuli, NF- κ B is sequestered in the cytoplasm by inhibitory proteins called nuclear factor kappa-light-polypeptide-inhibitor the in B-cell inhibitor (I κ B). Upon stimulation, I κ B is phosphorylated by the I κ B kinase (IKK) complex, leading to its ubiquitination and degradation by the proteasome.¹⁷⁶ This releases NF- κ B, allowing it to translocate to the nucleus, where it can bind to specific DNA sequences and activate transcription of target genes. The NF- κ B pathway is associated with many important diseases, such as cancer, autoimmune diseases, chronic inflammation, metabolic disorders, and neurodegenerative diseases. NF- κ B plays a critical role in NLRP3 inflammasome activation. Activation of the NLRP3 inflammasome involves a two-signal process: priming and activation. The first signal, or priming signal, increases the transcription of pro-IL-1 β and NLRP3 through the activation of NF- κ B.¹⁷⁷

Our *in vitro* outcomes insinuate that hPDLSCs placed with the machined disk evidence an augmentation of the level expression of the inflammatory mediators TLR4/Myd88/NF- κ B p65/NLRP3 when stimulated with LPS-G or without stimulation. Instead, hPDLSC cells cultured with TiN-coated disks in the presence of LPS-G demonstrated a notable decrease in the TLR4/Myd88/NF- κ B p65/NLRP3 level expression compared to the cells cultured with the machined disk alone or in the presence of LPS-G. The decrease in the inflammatory mediators

detected in hPDLSCs seeded on the TiN-coated disk surface can be liable for reverting the inflammatory event.

In addition, to further sustain the results achieved, the gene expression of the fundamental markers implicated in cell adhesion, such as fibronectin, vimentin, and vinculin, was also evaluated.

Cellular attachment is crucial in cell communication and regulation, and it also plays a pivotal part in the establishment and preservation of tissue. The mechanical connections between a cell and its ECM can effect and regulate cell conduct and regenerative events.¹⁷⁸

Paschoal et al.¹⁷⁹ found that TiN coatings exhibited no cytotoxicity, irritation, or acute systemic toxicity responses. The biocompatibility of biomaterials is closely related to the behavior of the contacting cells, in particular, cell adhesion. Cell adhesion is fundamental in tissue reaction and impacts cell growth, proliferation, and differentiation and in the primary stability of the implant osseointegration process.¹⁸⁰ The cellular affinity to the surface relies on the ECM molecules and denotes a vital aspect for the biomaterial progress.¹³⁷ The titanium nitride-coated surface maintained the parameters of the titanium surface, and the nitrogen present on the TiN surface may interfere with the adsorbed proteins, as reported by a previous study.¹⁸¹ Manso et al. found that sputtered TiN coatings deposited on implant surfaces induced cell adhesion and proliferation.¹⁸²

In the present work, an augmentation in the gene expression of the major molecules implicated in cell adhesion events was found in hPDLSCs cultured with TiN-coated disks alone or with LPS-G. Specifically, a remarkable augmentation of FNT1/VIM/VCL transcribing correspondingly for fibronectin, vimentin, and vinculin underlines that the TiN-coated titanium disk encourages cell-to-cell interaction and cell connections with the neighboring context.

Fibronectin is a multidomain glycoprotein existing in body fluids and on cell surfaces with the primary role of connecting the cell to the ECM. Fibronectin has a ligand-binding site for ECM proteins, such as collagen, fibrin, and heparan sulfate proteoglycans. Fibronectin plays a fundamental part in cell attachment, development, migration, and differentiation, in addition to embryonic development and wound healing.¹⁸³

The contact of fibronectin with integrins, the main receptor proteins composed of α and β subunits, leads to a receptor conformational alteration and its activation. Integrins are bound to fibronectin adsorbed onto the substrate or incorporated into the insoluble matrix and linked to the actin through linker proteins such as vinculin, a membrane-cytoskeletal protein in focal adhesion plaques that is implicated in the link of integrin adhesion molecules to the actin cytoskeleton.¹⁸⁴

Vimentin, also known as the fibroblast intermediate filament protein, plays a vital role in stabilizing the intracellular structure. Vimentin interacts with $\beta 1$ and $\beta 3$ integrin subunits and is implicated in cell adhesion in a phosphorylation-dependent way. In addition, vimentin also works as a scaffold for other proteins and contributes to the control of cell migration, adhesion, and division across the direct connection with two other main filamentous factors, actin filaments and microtubules.¹⁸⁵

Even though the current *in vitro* study has limitations, promising results were achieved. These outcomes highlighted the anti-inflammatory properties and the ability of TiN-coated disks to ameliorate cell attachment capabilities, suggesting a better performance in terms of cell/surface interaction.

The future perspective of dental implantology aims at designing an implant surface with definite topography. This study evaluates in an *in vitro* model of hPDLSCs, from one side, the inflammatory event through the investigation of the TLR4/MyD88/NF- κ B p65/NLRP3 pathway and, from the other side, a comprehension of the interplay among proteins, cells, and the implant surface.

In this short-term *in vitro* study, cell adhesion factors increased significantly with nitride-coated titanium disks compared to the control uncoated titanium surfaces. The TiN-coated titanium surfaces can control the ECM factor issues and revert the inflammatory processes activated by LPS-G, which may represent an improvement in the biological performance of the dental implants.

6. Study IV. *A prospective, blinded, randomized controlled study on antibacterial activity of dental implants with coronal portion treated with anodic spark deposition.*

The *in vivo* study described in this chapter is currently ongoing and does not yet have final results. The main objective of the prospective, blinded, randomized controlled study is to assess the ability of anodic spark deposition-treated dental implants, which form anatase on their coronal surface, to reduce bacterial adhesion and plaque accumulation, as well as enhance osteogenic capacity. These implants will be compared to standard implants with a rough, acid-etched, and sandblasted coronal surface.

6.1 Introduction

The clinical success of titanium dental implants is closely associated with the osseointegration process and the bone-to-implant contact (BIC) percentage, which is influenced by the implant surface characteristics.³

In 2012, Zarb and Koka defined osseointegration as "a time-dependent healing process in which clinically asymptomatic rigid fixation of alloplastic materials is achieved and maintained within bone under functional loading."⁵

Implant surface topography plays a crucial role in the osseointegration of titanium implants and includes macroscopic, microscopic, and nanometric features of the implant surface.⁵⁰

In a recent literature review, Moraschini et al. reported a mean survival rate of 94.5% for dental implants, with a follow-up period of 10 years or more. The studies with the longest follow-up, lasting 20 years, showed a mean survival rate of 91.2%.²²

Criteria for long-term implant success have been proposed by several authors:

- The implant should exhibit mobility of less than 1 mm.
- There should be no continuous radiolucency around the implant.
- Vertical bone loss must be less than 0.2 mm annually after the first year.
- There should be an absence of signs and symptoms of infection, pain, neuropathies, or paresthesia.
- No pocket probing depth should exceed 5 mm.
- No bleeding on probing.^{23–25,186}

The failure of dental implant stability can be attributed to both biological and mechanical causes. Biological factors account for 20% of failures, often due to peri-implantitis caused by microbial plaque or bacterial infections.

Peri-implant diseases result from bacterial colonization on implant surfaces. Derks et al. demonstrated that the prevalence of peri-implant mucositis ranged from 19% to 65%, while peri-implantitis prevalence varied between 1% and 47%.³³

Peri-implant diseases can affect only the peri-implant mucosa (peri-implant mucositis) or may also involve the supporting bone (peri-implantitis).

The main clinical sign of inflammation is bleeding upon gentle probing (0.2 N), although other symptoms may include redness, swelling, and pus discharge. In cases of peri-implant mucositis, probing depths around the implant may be deeper than the baseline measurements taken after the prosthesis was placed. When these clinical signs are present, a radiograph showing no marginal bone loss can confirm the diagnosis of peri-implant mucositis. To diagnose peri-implantitis, an intra-oral radiograph is needed to identify progressive marginal bone loss.³⁴

The modification of dental implant surfaces has emerged as a key strategy for improving osseointegration and minimizing the occurrence of early implant failures.

Recently, a range of surface modification methods, including texturization, hydrophilization, coatings, and functionalization with molecules or nanoparticles, have been developed to promote osseointegration and, at the same time, enhance resistance to bacterial contamination.¹⁸⁷

In recent decades, anodic oxidation has gained popularity as a surface modification technique for titanium in biomedical applications, thanks to its notable advantages, straightforward processing method, and cost-effectiveness. It improves coating adhesion, interfacial bonding, and corrosion resistance, while offering easily tunable properties. Additionally, it enhances the mechanical compatibility of the coating by creating a porous structure, which has excellent potential for promoting cell colonization. Anodic spark deposition is a plasma-chemical-electrochemical technique used for surface treatment of implants, which enables the formation of oxides on anodic metals. Depending on the voltage and duration of the anodization process, pure titanium implants can have oxide layers of varying thicknesses. This method primarily results in the formation of anatase. During anodization, elements like calcium or phosphorus can be incorporated into the surface. The resulting oxide layer can positively affect cell growth by promoting cell adhesion and osseointegration. Moreover, the anodized surface provides mechanical benefits, such as enhanced corrosion resistance. Recent studies have also shown that the formation of surface oxides, particularly anatase, demonstrates strong antibacterial

properties.^{83,188} Ueda et al. observed excellent antibacterial activity (killing approximately 99% of bacteria) when the TiO₂ layer was rich in anatase and contained carbon, further improving its antibacterial effectiveness.⁸⁵ The prospective, double-blind, randomized controlled study aims to assess the ability of anodic spark deposition-treated dental implants, which form anatase on their coronal surface, to reduce bacterial adhesion and plaque accumulation, as well as enhance osteogenic capacity. These implants will be compared to standard implants with a rough, acid-etched, and sandblasted coronal surface.

6.2 Materials and methods

6.2.1 Surface Preparation by Manufacturer

In this study, 50 machined (control) and 50 titanium implants treated with anodic sparks deposition in their coronal portion (Half Treatment) (test) from Resista (Omegna, VB, Italy) were used. The implants were made of grade IV commercially pure titanium. The implants were automatically cleaned in a cavitation washer using detergents designed to eliminate processing grease and finish purification washes. After the decontamination process in a cold argon plasma reactor in a clean room, the implants were packaged in thermally sealed autoclaving trade bags. Gammatom S.r.l. (Italy) externally sterilized the object using 35 KGy of gamma radiation. Anodic spark deposition method was used to treat 50 implants in coronal portions. The coating did not affect the inherent texture of the Ti surface. The surface morphologies of the disks were characterized using a scanning electron microscope (SEM) (Jeol, JSM 5410, Tokyo, Japan), at a voltage of 10 kV.

6.2.2 Ethics statement

The protocol for this study was designed in accordance with the Helsinki Declaration, with the Good Clinical Practice Guidelines for clinical trials on medicinal products (GCP E6 R2:2017) and approved by the Ethical Committee for Biomedical Research of Chieti and Pescara. The adult patients have been recruited at the Department of Innovative Technologies in Medicine and Dentistry of the University “G.D’Annunzio” of Chieti-Pescara and signed the informed consent.

6.2.3 Study design

Eighteen partially edentulous patients afferent to department of “Oral Surgery” at University of studies “G.D’Annunzio” Chieti-Pescara, Abruzzo, Italy, were recruited for this prospective, blinded, randomized-controlled study. Patients needed at least two single implant supported restorations were incorporated in the present study.

6.2.4 Inclusion criteria

Patients between 21 and 75 years old of both sexes; partially edentulous who needed at least 2 single implant rehabilitations on both the upper and lower jaws.

6.2.5 Exclusion criteria

General contraindications to implant surgery; smoking more than 10 cigarettes per day; patients irradiated to the head or neck less than 2 years; patients undergoing chemotherapy for less than 1 year; patients with uncontrolled diabetes; pregnant and lactating women; post-extraction sites with acute or purulent infections; post-extraction sites with non-intact walls; post-extraction sites with implant-bone gap > 2 mm; serious disorders of clotting, patients with uncontrolled systemic or metabolic disease; drug and alcohol abuse; patients with periodontal disease (evaluated and recorded by plaque score (PS) and bleeding score (BOP) on four surfaces on each tooth > 25%) patients with poor oral hygiene and motivation; patients treated with bone augmentation surgical techniques < 6 months; patients participating in other clinical trials, in case they interfere with the application of the present protocol.

6.2.6 Randomization, Allocation Concealment and Blinding

Patients were randomised according to a split-mouth design to receive one HT implant and one full-treatment implant.

6.2.7 Clinical assessment

All implants were inserted (T0) by two operators (A.S. and S.O.), following a two-stage protocol and placed according to the manufacturer’s instructions. All the patients were subject to professional oral hygiene 1 week before, and a mouth rinse of chlorhexidine digluconate solution 0.2% for 2 min immediately before surgery to reduce the bacterial load. Local anesthesia was given with Articaine (Ubistesin 4%—Espe Dental AG Seefeld, D-82229

Seefeld, Germany) associated with epinephrine (1:100,000). The implants were positioned in the alveolus using a handpiece combined with a controlled torque handpiece and then, if necessary, inserted manually with the ratchet as indicated by the manufacturer. The upper approximal margin of the implant neck must be positioned at the level of the lowest bone peak. The closure of the flap above the implant was primarily intended with Vicryl 4.0 (FS-2, Ethicon, Somerville, MA, USA). Amoxicillin with clavulanic acid was administered, with a dose of 2 g/day for 6 days (Augmentin; Glaxo-Smithkline Beecham, Brentford, UK). The postoperative pain was controlled with NSAIDs, a cold/soft diet was suggested for 2 weeks, together with appropriate oral hygiene. The sutures were removed 10 days after implant insertion. The surgical protocol was performed as already described. Three months after insertion, the implants were uncovered and a standard healing transmucosal collar was applied. After 21 ± 7 days, the impression was taken with the specific transfers, and then, sent to the dental laboratory, which prepared the final customized collars and parallelized titanium abutments including the temporary element. The definitive gold ceramic crown was applied within the fifth month from implant placement.

6.2.8 Data Handling and Radiographic Analysis of Marginal Bone Level Changes

The implant's success was assessed according to the radiographic and clinical criteria of Papaspyridakos et al. 2012.¹⁸⁹ (1) absence of implant mobility; (2) absence of pain; (3) absence of recurrent peri-implant infection; and (4) absence of a continuous radiolucency around the implant. Data were collected in the specific patient's case report forms. In order to evaluate radiographic change of the peri-implant bone, intraoral radiograph, applying the parallel ray technique, was realized after implant surgery. To evaluate the MBL, intraoral analogic Rx was performed during each stage and processed on a digital software, since the method was considered of high precision for evaluations (E De Smet 1, 2002) with a precision less than 0.1 mm. The mean value between mesial and distal region was used as the primary outcome measure for this study. The commercially available Rinn film holders, used for intraoral radiographs applying the parallel X-ray technique, were customized using a silicone key for the exact reposition in every subject, in order to obtain a highly reproducible and faithful radiograph. Furthermore, during the first radiography, kilovolts, milliamperes, and seconds were registered and used in all the stages to obtain the same images. Radiographs were repeated at implant placement (T0), and at 6, 12, 24, 36 months follow-up.

6.2.9 Soft tissues evaluation

The analysis of peri-implant clinical parameters is essential for assessing the health of the soft tissues around implants. The plaque index (PI) was recorded at all timepoints to evaluate the presence or absence of dental plaque along the mucosal margin. The recording has been performed by two blinded calibrated operators and the mean value was considered for further statistical evaluations. Moreover, the bleeding on probing (BOP) was recorded to assess the presence/absence of bleeding after probing of the gingiva. Moderate force (<25 g) was used during the probing. Gingival tissue health using Gingival Index (Loe & Silness 1963) has been recorded as follows: 0 = Normal gingiva; 1 = Mild inflammation and slight edema and color change in absence of bleeding on probing; 2 = Moderate inflammation, reddened tissues, edematosis and bleeding on probing; 3 = Severe inflammation marked redness, edema, ulceration and tendency to bleeding.

6.2.10 Statistical analysis

The descriptive analysis of the data was carried out: the quantitative variables are presented with mean and standard deviation. The statistical evaluations were carried out by means of two sample *t* test to compare for each group of patients analyzed, the values detected at time T0 and the values measured at time T1. The level of statistical significance was set at 5% ($p < 0.05$). The normal distribution was evaluated by the Kilmogorov Smirnov test ($p\text{-value} > 0.05$).

6.3 Preliminary results

At the time of writing this thesis, the data from the *in vivo* study are not yet sufficiently complete for a definitive statistical evaluation. The sample size (6 patients) is currently too small to conduct robust statistical analyses, and the follow-up period (9 months) is too short to observe significant clinical effects. However, the data collected so far will be used to provide a preliminary overview of the results and possible trends. The mean values for bone resorption and clinical indices (bleeding on probing, plaque index, and gingival index), were calculated for both test and control implants. For bone resorption, the average for the test implants was 1.45 mm, while for the control implants, it was 1.47 mm. The clinical indices for the test implants were as follows: bleeding on probing 0.2, gingival index 0.6, and plaque index 0.6. For the control implants, the values were 0.8 for bleeding on probing, 0.8 for the plaque index, and 0.8 for the gingival index.

6.4 Discussion

The ongoing in vivo study aims to assess the clinical performance of dental implants with different surface treatments. However, the results presented in this phase are preliminary, derived from a small sample size (5 out of 18 patients expected in the protocol) and a relatively short follow-up period (9 months). Therefore, it is not possible to draw definitive conclusions from the observations made at this stage, as a more comprehensive analysis will be possible once the full sample and longer follow-up period are completed.

The limited number of patients included in the study at present does not allow for meaningful statistical analysis, hindering the ability to appropriately assess the differences between the implant groups. It is important to note that the final sample will consist of 18 patients, with a minimum follow-up of 3 years, which will ensure a long-term analysis of the clinical and biological performance of the implants. The collection of additional data and the extension of the follow-up period are crucial to provide the necessary statistical power to detect any significant differences between the surface treatments tested.

Regarding bone resorption and clinical indices (bleeding on probing, plaque index, and gingival index), the preliminary results show minimal difference between the test implants and control implants, with an average bone resorption of 1.45 mm for the test implants and 1.47 mm for the control implants. However, given the limited data available and the short follow-up period, these observations cannot lead to definitive conclusions. The clinical indices show a more favorable trend for the test implants, but their statistical relevance can only be assessed once the full sample is reached and the follow-up period extended, allowing for a proper evaluation of the impact of the surface treatments on the soft tissues around the implants.

7. Conclusions

This thesis analyzed the surface topography of dental implants, with a particular focus on the cellular and molecular responses, osteointegration stimulation, and bacterial adhesion associated with different implant surface treatments. The systematic review evaluated the effectiveness of TiN-coated implants and abutments in reducing initial biofilm formation, a critical factor in the development of mucositis. The findings suggest that TiN coatings can significantly reduce bacterial adhesion, thereby improving the long-term stability of dental implants. The reduction in biofilm formation is linked to the bio-corrosion properties of the TiN coating, which plays a key role in enhancing the implant surface's resistance to bacterial colonization and promoting better outcomes for patients. Despite the promising results, the review's major limitation was that only one human study was included in the analysis. Consequently, further clinical investigations with larger sample sizes and diverse patient populations are necessary to confirm these findings and fully understand the potential of TiN-coated implants in clinical settings. The first *in vitro* study focused on the evaluation of the interaction between human periodontal ligament stem cells (hPDLSCs) and two different titanium implant surface topographies (sandblasted (CTRL) and dual acid etched (TEST)). The primary aim was to assess the cytotoxicity, cellular adhesion capacity, and enhancement in the release of extracellular matrix (ECM) components in response to different surface treatments. The results demonstrated that both implant surfaces exhibited low cytotoxicity, with no significant adverse effects on cell viability. Microscopic analysis revealed that the TEST surface promoted better cellular adhesion compared to the CTRL surface, indicating a more favorable environment for cell attachment and proliferation. Immunofluorescence and Western blot analysis confirmed the upregulation of osteoblastic-related genes and the enhanced release of ECM components on the TEST surface. Furthermore, RT-PCR investigations indicated increased expression of genes associated with bone formation and osseointegration. These findings suggest that the surface topography of titanium implants plays a crucial role in modulating cell behavior, particularly in promoting ECM release and supporting the initial steps of osseointegration. The enhanced cellular responses observed on the TEST surface highlight the potential for improving implant surfaces to foster better bone integration and long-term implant stability. The second *in vitro* study aimed to evaluate the biological effects of two different dental implant surfaces—machined titanium and titanium nitride (TiN)-coated titanium—on inflammation levels using an *in vitro* model of human periodontal ligament stem cells (hPDLSCs). The focus was on the modulation of the TLR4/MyD88/NF- κ B p65/NLRP3

signaling pathway, which is triggered by *Porphyromonas gingivalis* lipopolysaccharide (LPS), a key factor in inflammation associated with implant failure. The results indicated that the TiN-coated titanium surfaces effectively modulated the inflammatory response. Specifically, TiN-coated implants suppressed the activation of the TLR4/MyD88/NF- κ B p65/NLRP3 pathway, leading to a reduction in inflammatory signaling at both the gene and protein expression levels. This suppression was contrasted with the machined titanium surfaces, which exhibited a more pronounced inflammatory response. Moreover, the study evaluated the effects of these surfaces on cell adhesion and extracellular matrix (ECM) deposition by assessing the expression of key proteins involved in these processes, including vimentin, vinculin, and fibronectin. TiN-coated surfaces promoted enhanced adhesion and facilitated ECM deposition, indicating a favorable environment for cell attachment and the early stages of osteointegration. Overall, the findings suggest that TiN-coated titanium implants may offer significant benefits in modulating inflammation and accelerating ECM apposition, thereby potentially improving the early stages of osteointegration and long-term implant success. Based on the literature review and in vitro studies, the in vivo study aimed to evaluate the clinical performance of titanium implants with different surface treatments, specifically implants treated with anodic spark deposition (TEST) and standard (CTRL) implants, within the same patient. While preliminary data are currently available, the study is ongoing with a larger sample size of 18 patients, and the follow-up period will extend over at least three years to allow for a more comprehensive analysis. The primary goal of this study is to assess whether TEST implants offer advantages over CTRL implants in terms of reducing peri-implant bone resorption and improving soft tissue indices.

In conclusion, the results of this research provide valuable insights into the improvement of dental implant surfaces, suggesting that surface modifications, such as TiN coatings, may enhance osteointegration and reduce inflammatory complications. However, further clinical studies are necessary to validate these findings and optimize the application of these technologies in long-term clinical settings.

8. References

1. Accioni F, Vázquez J, Merinero M, Begines B, Alcudia A. Latest Trends in Surface Modification for Dental Implantology: Innovative Developments and Analytical Applications. *Pharmaceutics*. 2022;14(2):455. doi:10.3390/pharmaceutics14020455
2. Buser D, Janner SFM, Wittneben JG, Brägger U, Ramseier CA, Salvi GE. 10-year survival and success rates of 511 titanium implants with a sandblasted and acid-etched surface: a retrospective study in 303 partially edentulous patients. *Clin Implant Dent Relat Res*. 2012;14(6):839-851. doi:10.1111/j.1708-8208.2012.00456.x
3. Smeets R, Stadlinger B, Schwarz F, et al. Impact of Dental Implant Surface Modifications on Osseointegration. *BioMed Research International*. 2016;2016(1):6285620. doi:10.1155/2016/6285620
4. Albrektsson T, Jacobsson M. Bone-metal interface in osseointegration. *J Prosthet Dent*. 1987;57(5):597-607. doi:10.1016/0022-3913(87)90344-1
5. Koka S, Zarb G. On osseointegration: the healing adaptation principle in the context of osseosufficiency, osseoseparation, and dental implant failure. *Int J Prosthodont*. 2012;25(1):48-52.
6. Cordioli G, Majzoub Z, Piattelli A, Scarano A. Removal torque and histomorphometric investigation of 4 different titanium surfaces: an experimental study in the rabbit tibia. *Int J Oral Maxillofac Implants*. 2000;15(5):668-674.
7. Albrektsson T, Johansson C. Osteoinduction, osteoconduction and osseointegration. *Eur Spine J*. 2001;10 Suppl 2(Suppl 2):S96-101. doi:10.1007/s005860100282
8. Frost HM. The biology of fracture healing. An overview for clinicians. Part II. *Clin Orthop Relat Res*. 1989;(248):294-309.
9. Hench LL, Wilson J. Surface-active biomaterials. *Science*. 1984;226(4675):630-636. doi:10.1126/science.6093253
10. Guglielmotti MB, Olmedo DG, Cabrini RL. Research on implants and osseointegration. *Periodontol 2000*. 2019;79(1):178-189. doi:10.1111/prd.12254

11. Liu Z, Liu X, Ramakrishna S. Surface engineering of biomaterials in orthopedic and dental implants: Strategies to improve osteointegration, bacteriostatic and bactericidal activities. *Biotechnol J*. 2021;16(7):e2000116. doi:10.1002/biot.202000116
12. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science*. 1999;284(5418):1318-1322. doi:10.1126/science.284.5418.1318
13. Giglio MJ, Giannunzio G, Olmedo D, Guglielmotti MB. Histomorphometric study of bone healing around laminar implants in experimental diabetes. *Implant Dent*. 2000;9(2):143-149. doi:10.1097/00008505-200009020-00006
14. Renou SJ, Guglielmotti MB, de la Torre A, Cabrini RL. Effect of total body irradiation on peri-implant tissue reaction: an experimental study. *Clin Oral Implants Res*. 2001;12(5):468-472. doi:10.1034/j.1600-0501.2001.120506.x
15. Marconi GD, Fonticoli L, Della Rocca Y, et al. Human Periodontal Ligament Stem Cells Response to Titanium Implant Surface: Extracellular Matrix Deposition. *Biology (Basel)*. 2021;10(9):931. doi:10.3390/biology10090931
16. Theocharis AD, Skandalis SS, Gialeli C, Karamanos NK. Extracellular matrix structure. *Adv Drug Deliv Rev*. 2016;97:4-27. doi:10.1016/j.addr.2015.11.001
17. Feng Y, Jiang Z, Zhang Y, et al. Stem-cell-derived ECM sheet–implant complexes for enhancing osseointegration. *Biomater Sci*. 2020;8(23):6647-6656. doi:10.1039/D0BM00980F
18. Niessen CM, Leckband D, Yap AS. Tissue Organization by Cadherin Adhesion Molecules: Dynamic Molecular and Cellular Mechanisms of Morphogenetic Regulation. *Physiological Reviews*. 2011;91(2):691-731. doi:10.1152/physrev.00004.2010
19. Feller L, Jadwat Y, Khammissa RAG, Meyerov R, Schechter I, Lemmer J. Cellular responses evoked by different surface characteristics of intraosseous titanium implants. *Biomed Res Int*. 2015;2015:171945. doi:10.1155/2015/171945
20. Sverzut AT, de Albuquerque GC, Crippa GE, et al. Bone tissue, cellular, and molecular responses to titanium implants treated by anodic spark deposition. *J Biomed Mater Res A*. 2012;100(11):3092-3098. doi:10.1002/jbm.a.34249

21. Aamodt JM, Grainger DW. Extracellular matrix-based biomaterial scaffolds and the host response. *Biomaterials*. 2016;86:68-82. doi:10.1016/j.biomaterials.2016.02.003
22. Moraschini V, Poubel LA da C, Ferreira VF, Barboza E dos SP. Evaluation of survival and success rates of dental implants reported in longitudinal studies with a follow-up period of at least 10 years: a systematic review. *International Journal of Oral and Maxillofacial Surgery*. 2015;44(3):377-388. doi:10.1016/j.ijom.2014.10.023
23. Albrektsson T, Zarb G, Worthington P, Eriksson AR. The long-term efficacy of currently used dental implants: a review and proposed criteria of success. *Int J Oral Maxillofac Implants*. 1986;1(1):11-25.
24. Karoussis IK, Salvi GE, Heitz-Mayfield LJA, Brägger U, Hämmerle CHF, Lang NP. Long-term implant prognosis in patients with and without a history of chronic periodontitis: a 10-year prospective cohort study of the ITI Dental Implant System. *Clin Oral Implants Res*. 2003;14(3):329-339. doi:10.1034/j.1600-0501.000.00934.x
25. Buser D, Mericske-Stern R, Bernard JP, et al. Long-term evaluation of non-submerged ITI implants. Part 1: 8-year life table analysis of a prospective multi-center study with 2359 implants. *Clin Oral Implants Res*. 1997;8(3):161-172. doi:10.1034/j.1600-0501.1997.080302.x
26. Misch CE, Perel ML, Wang HL, et al. Implant success, survival, and failure: the International Congress of Oral Implantologists (ICOI) Pisa Consensus Conference. *Implant Dent*. 2008;17(1):5-15. doi:10.1097/ID.0b013e3181676059
27. Mombelli A, Nyman S, Brägger U, Wennström J, Lang NP. Clinical and microbiological changes associated with an altered subgingival environment induced by periodontal pocket reduction. *J Clin Periodontol*. 1995;22(10):780-787. doi:10.1111/j.1600-051x.1995.tb00261.x
28. Brägger U, Bürgin WB, Hämmerle CH, Lang NP. Associations between clinical parameters assessed around implants and teeth. *Clin Oral Implants Res*. 1997;8(5):412-421. doi:10.1034/j.1600-0501.1997.080508.x
29. Mombelli A, Lang NP. Antimicrobial treatment of peri-implant infections. *Clin Oral Implants Res*. 1992;3(4):162-168. doi:10.1034/j.1600-0501.1992.030402.x

30. Scarano A, Khater AGA, Gehrke SA, et al. Current Status of Peri-Implant Diseases: A Clinical Review for Evidence-Based Decision Making. *Journal of Functional Biomaterials*. 2023;14(4):210. doi:10.3390/jfb14040210
31. Persson GR, Renvert S. Cluster of bacteria associated with peri-implantitis. *Clin Implant Dent Relat Res*. 2014;16(6):783-793. doi:10.1111/cid.12052
32. Kochar SP, Reche A, Paul P. The Etiology and Management of Dental Implant Failure: A Review. *Cureus*. 2022;14(10):e30455. doi:10.7759/cureus.30455
33. Derks J, Tomasi C. Peri-implant health and disease. A systematic review of current epidemiology. *J Clin Periodontol*. 2015;42 Suppl 16:S158-171. doi:10.1111/jcpe.12334
34. Heitz-Mayfield LJA. Peri-implant mucositis and peri-implantitis: key features and differences. *Br Dent J*. 2024;236(10):791-794. doi:10.1038/s41415-024-7402-z
35. Trubiani O, Toniato E, Di Iorio D, et al. Morphological analysis and interleukin release in human gingival fibroblasts seeded on different denture base acrylic resins. *Int J Immunopathol Pharmacol*. 2012;25(3):637-643. doi:10.1177/039463201202500310
36. Sanz AR, Carrión FS, Chaparro AP. Mesenchymal stem cells from the oral cavity and their potential value in tissue engineering. *Periodontol 2000*. 2015;67(1):251-267. doi:10.1111/prd.12070
37. Tatullo M, Marrelli M, Paduano F. The regenerative medicine in oral and maxillofacial surgery: the most important innovations in the clinical application of mesenchymal stem cells. *Int J Med Sci*. 2015;12(1):72-77. doi:10.7150/ijms.10706
38. Chen ST, Wilson TG, Hämmerle CHF. Immediate or early placement of implants following tooth extraction: review of biologic basis, clinical procedures, and outcomes. *Int J Oral Maxillofac Implants*. 2004;19 Suppl:12-25.
39. Zhou LL, Liu W, Wu YM, Sun WL, Dörfer CE, Fawzy El-Sayed KM. Oral Mesenchymal Stem/Progenitor Cells: The Immunomodulatory Masters. *Stem Cells Int*. 2020;2020:1327405. doi:10.1155/2020/1327405
40. Marconi GD, Fonticoli L, Guarnieri S, et al. Ascorbic Acid: A New Player of Epigenetic Regulation in LPS-gingivalis Treated Human Periodontal Ligament Stem Cells. *Oxid Med Cell Longev*. 2021;2021:6679708. doi:10.1155/2021/6679708

41. Zhang H, Sun SC. NF- κ B in inflammation and renal diseases. *Cell Biosci.* 2015;5:63. doi:10.1186/s13578-015-0056-4
42. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4⁺ T cells. *Science.* 2010;327(5969):1098-1102. doi:10.1126/science.1178334
43. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Front Immunol.* 2014;5:614. doi:10.3389/fimmu.2014.00614
44. Eliaz N. Corrosion of Metallic Biomaterials: A Review. *Materials (Basel).* 2019;12(3):407. doi:10.3390/ma12030407
45. Mishra SK, Chowdhary R. Evolution of dental implants through the work of per-ingvar branemark: A systematic review. *Indian J Dent Res.* 2020;31(6):930-956. doi:10.4103/ijdr.IJDR_587_18
46. Titanium Alloys for Dental Implants: A Review. Accessed February 2, 2025. <https://www.mdpi.com/2673-1592/2/2/11>
47. Chen X, Mao SS. Titanium dioxide nanomaterials: synthesis, properties, modifications, and applications. *Chem Rev.* 2007;107(7):2891-2959. doi:10.1021/cr0500535
48. Dambournet D, Belharouak I, Amine K. Tailored preparation methods of TiO₂ anatase, rutile, brookite: Mechanism of formation and electrochemical properties. *Chemistry of Materials.* 2010;22(3):1173-1179. doi:10.1021/cm902613h
49. Jacobs JJ, Gilbert JL, Urban RM. Corrosion of metal orthopaedic implants. *J Bone Joint Surg Am.* 1998;80(2):268-282. doi:10.2106/00004623-199802000-00015
50. Le Guéhennec L, Soueidan A, Layrolle P, Amouriq Y. Surface treatments of titanium dental implants for rapid osseointegration. *Dent Mater.* 2007;23(7):844-854. doi:10.1016/j.dental.2006.06.025
51. Yamaguchi Y, Shiota M, Fujii M, Shimogishi M, Munakata M. Effects of implant thread design on primary stability-a comparison between single- and double-threaded implants in an artificial bone model. *Int J Implant Dent.* 2020;6(1):42. doi:10.1186/s40729-020-00239-1

52. Zhu L, Luo D, Liu Y. Effect of the nano/microscale structure of biomaterial scaffolds on bone regeneration. *Int J Oral Sci.* 2020;12(1):6. doi:10.1038/s41368-020-0073-y
53. Pachauri P, Bathala LR, Sangur R. Techniques for dental implant nanosurface modifications. *J Adv Prosthodont.* 2014;6(6):498-504. doi:10.4047/jap.2014.6.6.498
54. Stanford CM. Surface modifications of dental implants. *Aust Dent J.* 2008;53 Suppl 1:S26-33. doi:10.1111/j.1834-7819.2008.00038.x
55. Barfeie A, Wilson J, Rees J. Implant surface characteristics and their effect on osseointegration. *Br Dent J.* 2015;218(5):E9. doi:10.1038/sj.bdj.2015.171
56. Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H. Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. *J Biomed Mater Res.* 1991;25(7):889-902. doi:10.1002/jbm.820250708
57. Gotfredsen K, Wennerberg A, Johansson C, Skovgaard LT, Hjørtting-Hansen E. Anchorage of TiO₂-blasted, HA-coated, and machined implants: an experimental study with rabbits. *J Biomed Mater Res.* 1995;29(10):1223-1231. doi:10.1002/jbm.820291009
58. Wennerberg A, Albrektsson T, Andersson B, Król J. A histomorphometric and removal torque study of screw-shaped titanium implants with three different surface topographies. *Clinical oral implants research.* Published online 1995. Accessed January 9, 2025. <https://www.semanticscholar.org/paper/A-histomorphometric-and-removal-torque-study-of-Wennerberg-Albrektsson/f90383eab50793c7f15ebcacfe37eec1592b4f3f>
59. Wennerberg A, Hallgren C, Johansson C, Danelli S. A histomorphometric evaluation of screw-shaped implants each prepared with two surface roughnesses. *Clin Oral Implants Res.* 1998;9(1):11-19. doi:10.1034/j.1600-0501.1998.090102.x
60. Brett PM, Harle J, Salih V, et al. Roughness response genes in osteoblasts. *Bone.* 2004;35(1):124-133. doi:10.1016/j.bone.2004.03.009
61. Busscher HJ, Rinastiti M, Siswomihardjo W, van der Mei HC. Biofilm formation on dental restorative and implant materials. *J Dent Res.* 2010;89(7):657-665. doi:10.1177/0022034510368644

62. Kolenbrander PE, Palmer RJ, Rickard AH, Jakubovics NS, Chalmers NI, Diaz PI. Bacterial interactions and successions during plaque development. *Periodontol 2000*. 2006;42:47-79. doi:10.1111/j.1600-0757.2006.00187.x
63. Paquette DW, Brodala N, Williams RC. Risk factors for endosseous dental implant failure. *Dent Clin North Am*. 2006;50(3):361-374, vi. doi:10.1016/j.cden.2006.05.002
64. Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ. Communication among oral bacteria. *Microbiol Mol Biol Rev*. 2002;66(3):486-505, table of contents. doi:10.1128/MMBR.66.3.486-505.2002
65. Kalykakis GK, Mojon P, Nisengard R, Spiekermann H, Zafiropoulos GG. Clinical and microbial findings on osseo-integrated implants; comparisons between partially dentate and edentulous subjects. *Eur J Prosthodont Restor Dent*. 1998;6(4):155-159.
66. Fürst MM, Salvi GE, Lang NP, Persson GR. Bacterial colonization immediately after installation on oral titanium implants. *Clin Oral Implants Res*. 2007;18(4):501-508. doi:10.1111/j.1600-0501.2007.01381.x
67. Steinberg D, Klinger A, Kohavi D, Sela MN. Adsorption of human salivary proteins to titanium powder. I. Adsorption of human salivary albumin. *Biomaterials*. 1995;16(17):1339-1343. doi:10.1016/0142-9612(95)91050-9
68. Berglundh T, Gotfredsen K, Zitzmann NU, Lang NP, Lindhe J. Spontaneous progression of ligature induced peri-implantitis at implants with different surface roughness: an experimental study in dogs. *Clin Oral Implants Res*. 2007;18(5):655-661. doi:10.1111/j.1600-0501.2007.01397.x
69. George A, Sanjay MR, Srisuk R, Parameswaranpillai J, Siengchin S. A comprehensive review on chemical properties and applications of biopolymers and their composites. *Int J Biol Macromol*. 2020;154:329-338. doi:10.1016/j.ijbiomac.2020.03.120
70. Zhang Y, Gulati K, Li Z, Di P, Liu Y. Dental Implant Nano-Engineering: Advances, Limitations and Future Directions. *Nanomaterials*. 2021;11(10):2489. doi:10.3390/nano11102489

71. Rupp F, Liang L, Geis-Gerstorfer J, Scheideler L, Hüttig F. Surface characteristics of dental implants: A review. *Dent Mater.* 2018;34(1):40-57. doi:10.1016/j.dental.2017.09.007
72. Gil J, Pérez R, Herrero-Climent M, Rizo-Gorrita M, Torres-Lagares D, Gutierrez JL. Benefits of Residual Aluminum Oxide for Sand Blasting Titanium Dental Implants: Osseointegration and Bactericidal Effects. *Materials (Basel).* 2021;15(1):178. doi:10.3390/ma15010178
73. Chen CJ, Ding SJ, Chen CC. Effects of Surface Conditions of Titanium Dental Implants on Bacterial Adhesion. *Photomed Laser Surg.* 2016;34(9):379-388. doi:10.1089/pho.2016.4103
74. Velasco-Ortega E, Alfonso-Rodríguez CA, Monsalve-Guil L, et al. Relevant aspects in the surface properties in titanium dental implants for the cellular viability. *Mater Sci Eng C Mater Biol Appl.* 2016;64:1-10. doi:10.1016/j.msec.2016.03.049
75. Ban S, Iwaya Y, Kono H, Sato H. Surface modification of titanium by etching in concentrated sulfuric acid. *Dent Mater.* 2006;22(12):1115-1120. doi:10.1016/j.dental.2005.09.007
76. Yeo ISL. Modifications of Dental Implant Surfaces at the Micro- and Nano-Level for Enhanced Osseointegration. *Materials (Basel).* 2019;13(1):89. doi:10.3390/ma13010089
77. Hallgren C, Reimers H, Chakarov D, Gold J, Wennerberg A. An in vivo study of bone response to implants topographically modified by laser micromachining. *Biomaterials.* 2003;24(5):701-710. doi:10.1016/S0142-9612(02)00266-1
78. Bellows CG, Heersche JNM, Aubin JE. The effects of fluoride on osteoblast progenitors In Vitro. *Journal of Bone and Mineral Research.* 1990;5(S1):S101-S105. doi:10.1002/jbmr.5650051361
79. Delgado-Ruiz R, Romanos G. Potential Causes of Titanium Particle and Ion Release in Implant Dentistry: A Systematic Review. *International Journal of Molecular Sciences.* 2018;19(11):3585. doi:10.3390/ijms19113585
80. Talib RJ, Toff MRM. Plasma-sprayed coating of hydroxyapatite on metal implants--a review. *Med J Malaysia.* 2004;59 Suppl B:153-154.

81. Kulkarni Aranya A, Pushalkar S, Zhao M, LeGeros RZ, Zhang Y, Saxena D. Antibacterial and bioactive coatings on titanium implant surfaces. *Journal of Biomedical Materials Research Part A*. 2017;105(8):2218-2227. doi:10.1002/jbm.a.36081
82. Hall J, Lausmaa J. Properties of a new porous oxide surface on titanium implants. *Appl Osseointegration Res*. 2000;1:5-8.
83. Kurze P, Drysmann W, Knöfler W. [Anodic oxidation using spark discharge--a new surface treatment method for medical technology]. *Stomatol DDR*. 1986;36(10):549-554.
84. Yerokhin AL, Nie X, Leyland A, Matthews A, Dowey SJ. Plasma electrolysis for surface engineering. *Surface and Coatings Technology*. 1999;122(2):73-93. doi:10.1016/S0257-8972(99)00441-7
85. Ueda T, Sato N, Koizumi R, et al. Formation of carbon-added anatase-rich TiO₂ layers on titanium and their antibacterial properties in visible light. *Dental Materials*. 2021;37(2):e37-e46. doi:10.1016/j.dental.2020.10.009
86. Jemat A, Ghazali MJ, Razali M, Otsuka Y. Surface Modifications and Their Effects on Titanium Dental Implants. *Biomed Res Int*. 2015;2015:791725. doi:10.1155/2015/791725
87. Simmons CA, Valiquette N, Pilliar RM. Osseointegration of sintered porous-surfaced and plasma spray-coated implants: An animal model study of early postimplantation healing response and mechanical stability. *J Biomed Mater Res*. 1999;47(2):127-138. doi:10.1002/(sici)1097-4636(199911)47:2<127::aid-jbm3>3.0.co;2-c
88. Camargo SEA, Roy T, Xia X, et al. Novel Coatings to Minimize Corrosion of Titanium in Oral Biofilm. *Materials (Basel)*. 2021;14(2):342. doi:10.3390/ma14020342
89. Ji MK, Park SW, Lee K, et al. Evaluation of antibacterial activity and osteoblast-like cell viability of TiN, ZrN and (Ti_{1-x}Zr_x)N coating on titanium. *J Adv Prosthodont*. 2015;7(2):166-171. doi:10.4047/jap.2015.7.2.166
90. Adell R, Lekholm U, Rockler B, Brånemark PI. A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. *Int J Oral Surg*. 1981;10(6):387-416. doi:10.1016/s0300-9785(81)80077-4

91. Malchiodi L, Scarano A, Quaranta M, Piattelli A. Rigid fixation by means of titanium mesh in edentulous ridge expansion for horizontal ridge augmentation in the maxilla. *Int J Oral Maxillofac Implants*. 1998;13(5):701-705.
92. Abrahamsson I, Zitzmann NU, Berglundh T, Linder E, Wennerberg A, Lindhe J. The mucosal attachment to titanium implants with different surface characteristics: an experimental study in dogs. *J Clin Periodontol*. 2002;29(5):448-455. doi:10.1034/j.1600-051x.2002.290510.x
93. Abrahamsson I, Berglundh T, Wennström J, Lindhe J. The peri-implant hard and soft tissues at different implant systems. A comparative study in the dog. *Clin Oral Implants Res*. 1996;7(3):212-219. doi:10.1034/j.1600-0501.1996.070303.x
94. Guo T, Gulati K, Arora H, Han P, Fournier B, Ivanovski S. Race to invade: Understanding soft tissue integration at the transmucosal region of titanium dental implants. *Dent Mater*. 2021;37(5):816-831. doi:10.1016/j.dental.2021.02.005
95. Lindhe J, Meyle J, Group D of European Workshop on Periodontology. Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. *J Clin Periodontol*. 2008;35(8 Suppl):282-285. doi:10.1111/j.1600-051X.2008.01283.x
96. Berglundh T, Persson L, Klinge B. A systematic review of the incidence of biological and technical complications in implant dentistry reported in prospective longitudinal studies of at least 5 years. *J Clin Periodontol*. 2002;29 Suppl 3:197-212; discussion 232-233. doi:10.1034/j.1600-051x.29.s3.12.x
97. Ong ES, Newman HN, Wilson M, Bulman JS. The occurrence of periodontitis-related microorganisms in relation to titanium implants. *J Periodontol*. 1992;63(3):200-205. doi:10.1902/jop.1992.63.3.200
98. Scarano A, de Oliveira PS, Leo L, Festa F, Carinci F, Lorusso F. Evaluation of a new antibacterial coating of the internal chamber of an implant via real time measurement of Volatile Organic Compounds (VOCs). *Front Biosci (Elite Ed)*. 2021;13(2):216-225. doi:10.52586/E879
99. Scarano A, Piattelli A, Polimeni A, Di Iorio D, Carinci F. Bacterial adhesion on commercially pure titanium and anatase-coated titanium healing screws: an in vivo human study. *J Periodontol*. 2010;81(10):1466-1471. doi:10.1902/jop.2010.100061

100. Lindhe J, Berglundh T, Ericsson I, Liljenberg B, Marinello C. Experimental breakdown of peri-implant and periodontal tissues. A study in the beagle dog. *Clin Oral Implants Res.* 1992;3(1):9-16. doi:10.1034/j.1600-0501.1992.030102.x
101. Mezger PR, Creugers NH. Titanium nitride coatings in clinical dentistry. *J Dent.* 1992;20(6):342-344. doi:10.1016/0300-5712(92)90021-4
102. Pappas MJ, Makris G, Buechel FF. Titanium nitride ceramic film against polyethylene. A 48 million cycle wear test. *Clin Orthop Relat Res.* 1995;(317):64-70.
103. Yenyol S, Bölükbaşı N, Bilir A, Çakır AF, Yenyol M, Ozdemir T. Relative Contributions of Surface Roughness and Crystalline Structure to the Biocompatibility of Titanium Nitride and Titanium Oxide Coatings Deposited by PVD and TPS Coatings. *ISRN Biomaterials.* 2013;2013:e783873. doi:10.5402/2013/783873
104. Wu S, Xu J, Zou L, et al. Long-lasting renewable antibacterial porous polymeric coatings enable titanium biomaterials to prevent and treat peri-implant infection. *Nat Commun.* 2021;12(1):3303. doi:10.1038/s41467-021-23069-0
105. Renvert S, Persson GR, Pirih FQ, Camargo PM. Peri-implant health, peri-implant mucositis, and peri-implantitis: Case definitions and diagnostic considerations. *J Clin Periodontol.* 2018;45 Suppl 20:S278-S285. doi:10.1111/jcpe.12956
106. Sitek R, Kamiński J, Adamczyk-Cieślak B, et al. Effect of Plasma Nitriding on Structure and Properties of Titanium Grade 2 Produced by Direct Metal Laser Sintering. *Metallogr Microstruct Anal.* 2022;11(6):852-863. doi:10.1007/s13632-022-00903-5
107. Shamseer L, Moher D, Clarke M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation. *BMJ.* 2015;350:g7647. doi:10.1136/bmj.g7647
108. Sterne JA, Hernán MA, Reeves BC, et al. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. *BMJ.* 2016;355. doi:10.1136/bmj.i4919
109. Sheth VH, Shah NP, Jain R, Bhanushali N, Bhatnagar V. Development and validation of a risk-of-bias tool for assessing in vitro studies conducted in dentistry: The QUIN. *The Journal of Prosthetic Dentistry.* Published online June 23, 2022. doi:10.1016/j.prosdent.2022.05.019

110. Tran L, Tam DNH, Elshafay A, Dang T, Hirayama K, Huy NT. Quality assessment tools used in systematic reviews of in vitro studies: A systematic review. *BMC Medical Research Methodology*. 2021;21(1):101. doi:10.1186/s12874-021-01295-w
111. Grössner-Schreiber B, Griepentrog M, Haustein I, et al. Plaque formation on surface modified dental implants. An in vitro study. *Clin Oral Implants Res*. 2001;12(6):543-551. doi:10.1034/j.1600-0501.2001.120601.x
112. Del Castillo R, Chochlidakis K, Galindo-Moreno P, Ercoli C. Titanium Nitride Coated Implant Abutments: From Technical Aspects And Soft tissue Biocompatibility to Clinical Applications. A Literature Review. *J Prosthodont*. Published online November 3, 2021. doi:10.1111/jopr.13446
113. Prachar P, Bartakova S, Brezina V, Cvrcek L, Vanek J. Cytocompatibility of implants coated with titanium nitride and zirconium nitride. *Bratisl Lek Listy*. 2015;116(3):154-156. doi:10.4149/bll_2015_031
114. Durual S, Pernet F, Rieder P, Mekki M, Cattani-Lorente M, Wiskott HWA. Titanium nitride oxide coating on rough titanium stimulates the proliferation of human primary osteoblasts. *Clinical Oral Implants Research*. 2011;22(5):552-559. doi:10.1111/j.1600-0501.2010.02033.x
115. Zhurakivska K, Ciacci N, Troiano G, et al. Nitride-Coated and Anodic-Oxidized Titanium Promote a Higher Fibroblast and Reduced Streptococcus gordonii Proliferation Compared to the Uncoated Titanium. *Prosthesis*. 2020;2(4):333-339. doi:10.3390/prosthesis2040031
116. Alsabeeha N, Swain M, Payne A. Clinical performance and material properties of single-implant overdenture attachment systems. *The International journal of prosthodontics*. 2011;24:247-254.
117. Scarano A, Piattelli M, Vrespa G, Caputi S, Piattelli A. Bacterial adhesion on titanium nitride-coated and uncoated implants: an in vivo human study. *J Oral Implantol*. 2003;29(2):80-85. doi:10.1563/1548-1336(2003)029<0080:BAOTNA>2.3.CO;2
118. Zhang Y, Zheng Y, Li Y, et al. Tantalum Nitride-Decorated Titanium with Enhanced Resistance to Microbiologically Induced Corrosion and Mechanical Property for Dental Application. *PLoS One*. 2015;10(6):e0130774. doi:10.1371/journal.pone.0130774

119. Han Y, Li X, Zhang Y, Han Y, Chang F, Ding J. Mesenchymal Stem Cells for Regenerative Medicine. *Cells*. 2019;8(8):886. doi:10.3390/cells8080886
120. Ruh AC, Frigo L, Cavalcanti MFXB, et al. Laser photobiomodulation in pressure ulcer healing of human diabetic patients: gene expression analysis of inflammatory biochemical markers. *Lasers Med Sci*. 2018;33(1):165-171. doi:10.1007/s10103-017-2384-6
121. Diomedede F, Merciaro I, Martinotti S, et al. miR-2861 is involved in osteogenic commitment of human periodontal ligament stem cells grown onto 3D scaffold. *J Biol Regul Homeost Agents*. 2016;30(4):1009-1018.
122. Portron S, Soueidan A, Marsden AC, et al. Periodontal regenerative medicine using mesenchymal stem cells and biomaterials: A systematic review of pre-clinical studies. *Dent Mater J*. 2019;38(6):867-883. doi:10.4012/dmj.2018-315
123. Sinjari B, Pizzicannella J, D'Aurora M, et al. Curcumin/Liposome Nanotechnology as Delivery Platform for Anti-inflammatory Activities via NFκB/ERK/pERK Pathway in Human Dental Pulp Treated With 2-HydroxyEthyl MethAcrylate (HEMA). *Front Physiol*. 2019;10:633. doi:10.3389/fphys.2019.00633
124. Tomokiyo A, Wada N, Maeda H. Periodontal Ligament Stem Cells: Regenerative Potency in Periodontium. *Stem Cells Dev*. 2019;28(15):974-985. doi:10.1089/scd.2019.0031
125. Diomedede F, Marconi GD, Guarnieri S, et al. A Novel Role of Ascorbic Acid in Anti-Inflammatory Pathway and ROS Generation in HEMA Treated Dental Pulp Stem Cells. *Materials (Basel)*. 2019;13(1):130. doi:10.3390/ma13010130
126. Pizzicannella J, Fonticoli L, Guarnieri S, et al. Antioxidant Ascorbic Acid Modulates NLRP3 Inflammasome in LPS-G Treated Oral Stem Cells through NFκB/Caspase-1/IL-1β Pathway. *Antioxidants (Basel)*. 2021;10(5):797. doi:10.3390/antiox10050797
127. Laino L, La Noce M, Fiorillo L, et al. Dental Pulp Stem Cells on Implant Surface: An In Vitro Study. *Biomed Res Int*. 2021;2021:3582342. doi:10.1155/2021/3582342
128. Yu YJ, Zhu WQ, Xu LN, Ming PP, Shao SY, Qiu J. Osseointegration of titanium dental implant under fluoride exposure in rabbits: Micro-CT and histomorphometry study. *Clin Oral Implants Res*. 2019;30(10):1038-1048. doi:10.1111/clr.13517

129. Matos GRM. Surface Roughness of Dental Implant and Osseointegration. *J Maxillofac Oral Surg.* 2021;20(1):1-4. doi:10.1007/s12663-020-01437-5
130. Dohan Ehrenfest DM, Coelho PG, Kang BS, Sul YT, Albrektsson T. Classification of osseointegrated implant surfaces: materials, chemistry and topography. *Trends Biotechnol.* 2010;28(4):198-206. doi:10.1016/j.tibtech.2009.12.003
131. Fischer K, Stenberg T. Prospective 10-year cohort study based on a randomized, controlled trial (RCT) on implant-supported full-arch maxillary prostheses. part II: prosthetic outcomes and maintenance. *Clin Implant Dent Relat Res.* 2013;15(4):498-508. doi:10.1111/j.1708-8208.2011.00383.x
132. Shibata Y, Tanimoto Y. A review of improved fixation methods for dental implants. Part I: Surface optimization for rapid osseointegration. *J Prosthodont Res.* 2015;59(1):20-33. doi:10.1016/j.jpor.2014.11.007
133. Marconi GD, Diomedede F, Pizzicannella J, et al. Enhanced VEGF/VEGF-R and RUNX2 Expression in Human Periodontal Ligament Stem Cells Cultured on Sandblasted/Etched Titanium Disk. *Front Cell Dev Biol.* 2020;8:315. doi:10.3389/fcell.2020.00315
134. Rupp F, Scheideler L, Rehbein D, Axmann D, Geis-Gerstorfer J. Roughness induced dynamic changes of wettability of acid etched titanium implant modifications. *Biomaterials.* 2004;25(7-8):1429-1438. doi:10.1016/j.biomaterials.2003.08.015
135. Albrektsson T, Wennerberg A. Oral implant surfaces: Part 1--review focusing on topographic and chemical properties of different surfaces and in vivo responses to them. *Int J Prosthodont.* 2004;17(5):536-543.
136. Ramaglia L, Postiglione L, Di Spigna G, Capece G, Salzano S, Rossi G. Sandblasted-acid-etched titanium surface influences in vitro the biological behavior of SaOS-2 human osteoblast-like cells. *Dent Mater J.* 2011;30(2):183-192. doi:10.4012/dmj.2010-107
137. Ahmad Khalili A, Ahmad MR. A Review of Cell Adhesion Studies for Biomedical and Biological Applications. *Int J Mol Sci.* 2015;16(8):18149-18184. doi:10.3390/ijms160818149

138. Gallant ND, Michael KE, García AJ. Cell Adhesion Strengthening: Contributions of Adhesive Area, Integrin Binding, and Focal Adhesion Assembly. *Mol Biol Cell*. 2005;16(9):4329-4340. doi:10.1091/mbc.E05-02-0170
139. García AJ, Boettiger D. Integrin-fibronectin interactions at the cell-material interface: initial integrin binding and signaling. *Biomaterials*. 1999;20(23-24):2427-2433. doi:10.1016/s0142-9612(99)00170-2
140. Bhatavadekar NB, Gharpure AS, Balasubramaniam N, Scheyer ET. In Vitro Surface Testing Methods for Dental Implants-Interpretation and Clinical Relevance: A Review. *Compend Contin Educ Dent*. 2020;41(3):e1-e9.
141. Pizzicannella J, Diomedea F, Gugliandolo A, et al. 3D Printing PLA/Gingival Stem Cells/ EVs Upregulate miR-2861 and -210 during Osteoangiogenesis Commitment. *Int J Mol Sci*. 2019;20(13):3256. doi:10.3390/ijms20133256
142. Diomedea F, Marconi GD, Cavalcanti MFXB, et al. VEGF/VEGF-R/RUNX2 Upregulation in Human Periodontal Ligament Stem Cells Seeded on Dual Acid Etched Titanium Disk. *Materials (Basel)*. 2020;13(3):706. doi:10.3390/ma13030706
143. Diomedea F, Zini N, Pizzicannella J, et al. 5-Aza Exposure Improves Reprogramming Process Through Embryoid Body Formation in Human Gingival Stem Cells. *Front Genet*. 2018;9:419. doi:10.3389/fgene.2018.00419
144. Pizzicannella J, Cavalcanti M, Trubiani O, Diomedea F. MicroRNA 210 Mediates VEGF Upregulation in Human Periodontal Ligament Stem Cells Cultured on 3DHydroxyapatite Ceramic Scaffold. *Int J Mol Sci*. 2018;19(12):3916. doi:10.3390/ijms19123916
145. Mazzatenta A, Marconi GD, Zara S, Cataldi A, Porzionato A, Di Giulio C. In the carotid body, galanin is a signal for neurogenesis in young, and for neurodegeneration in the old and in drug-addicted subjects. *Front Physiol*. 2014;5:427. doi:10.3389/fphys.2014.00427
146. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262

147. Junker R, Dimakis A, Thoneick M, Jansen JA. Effects of implant surface coatings and composition on bone integration: a systematic review. *Clin Oral Implants Res.* 2009;20 Suppl 4:185-206. doi:10.1111/j.1600-0501.2009.01777.x
148. Coelho PG, Jimbo R, Tovar N, Bonfante EA. Osseointegration: hierarchical designing encompassing the micrometer, micrometer, and nanometer length scales. *Dent Mater.* 2015;31(1):37-52. doi:10.1016/j.dental.2014.10.007
149. Li D, Ferguson SJ, Beutler T, et al. Biomechanical comparison of the sandblasted and acid-etched and the machined and acid-etched titanium surface for dental implants. *J Biomed Mater Res.* 2002;60(2):325-332. doi:10.1002/jbm.10063
150. Cervino G, Fiorillo L, Iannello G, Santonocito D, Risitano G, Cicciù M. Sandblasted and Acid Etched Titanium Dental Implant Surfaces Systematic Review and Confocal Microscopy Evaluation. *Materials (Basel).* 2019;12(11):1763. doi:10.3390/ma12111763
151. Zizzari VL, Marconi GD, De Colli M, et al. In Vitro Behavior of Primary Human Osteoblasts Onto Microrough Titanium Surface. *Implant Dent.* 2015;24(4):377-383. doi:10.1097/ID.0000000000000268
152. Manescu A, Giuliani A, Mohammadi S, et al. Osteogenic potential of dualblocks cultured with human periodontal ligament stem cells: in vitro and synchrotron microtomography study. *J Periodontal Res.* 2016;51(1):112-124. doi:10.1111/jre.12289
153. Gugliandolo A, Fonticoli L, Trubiani O, et al. Oral Bone Tissue Regeneration: Mesenchymal Stem Cells, Secretome, and Biomaterials. *Int J Mol Sci.* 2021;22(10):5236. doi:10.3390/ijms22105236
154. Shekaran A, García AJ. Extracellular matrix-mimetic adhesive biomaterials for bone repair. *J Biomed Mater Res A.* 2011;96(1):261-272. doi:10.1002/jbm.a.32979
155. Jung S, Böhner L, Hanisch M, Kleinheinz J, Sielker S. Influence of Implant Material and Surface on Mode and Strength of Cell/Matrix Attachment of Human Adipose Derived Stromal Cell. *Int J Mol Sci.* 2020;21(11):4110. doi:10.3390/ijms21114110
156. Rosset EM, Bradshaw AD. SPARC/osteonectin in mineralized tissue. *Matrix Biol.* 2016;52-54:78-87. doi:10.1016/j.matbio.2016.02.001

157. Kramer PR, Janik Keith A, Cai Z, Ma S, Watanabe I. Integrin mediated attachment of periodontal ligament to titanium surfaces. *Dent Mater.* 2009;25(7):877-883. doi:10.1016/j.dental.2009.01.095
158. Hamidouche Z, Fromigué O, Ringe J, et al. Priming integrin alpha5 promotes human mesenchymal stromal cell osteoblast differentiation and osteogenesis. *Proc Natl Acad Sci U S A.* 2009;106(44):18587-18591. doi:10.1073/pnas.0812334106
159. Pellegrini G, Francetti L, Barbaro B, Del Fabbro M. Novel surfaces and osseointegration in implant dentistry. *J Investig Clin Dent.* 2018;9(4):e12349. doi:10.1111/jicd.12349
160. Hendry JA, Pilliar RM. The fretting corrosion resistance of PVD surface-modified orthopedic implant alloys. *J Biomed Mater Res.* 2001;58(2):156-166. doi:10.1002/1097-4636(2001)58:2<156::aid-jbm1002>3.0.co;2-h
161. Ballo AM, Bjöörn D, Astrand M, Palmquist A, Lausmaa J, Thomsen P. Bone response to physical-vapour-deposited titanium dioxide coatings on titanium implants. *Clin Oral Implants Res.* 2013;24(9):1009-1017. doi:10.1111/j.1600-0501.2012.02509.x
162. Berglundh T, Lindhe J, Marinello C, Ericsson I, Liljenberg B. Soft tissue reaction to de novo plaque formation on implants and teeth. An experimental study in the dog. *Clin Oral Implants Res.* 1992;3(1):1-8. doi:10.1034/j.1600-0501.1992.030101.x
163. Brunello G, Brun P, Gardin C, et al. Biocompatibility and antibacterial properties of zirconium nitride coating on titanium abutments: An in vitro study. *PLoS One.* 2018;13(6):e0199591. doi:10.1371/journal.pone.0199591
164. Liu T, Zhang L, Joo D, Sun SC. NF- κ B signaling in inflammation. *Signal Transduct Target Ther.* 2017;2:17023-. doi:10.1038/sigtrans.2017.23
165. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci.* 2021;78(4):1233-1261. doi:10.1007/s00018-020-03656-y
166. Marconi GD, Gallorini M, Carradori S, Guglielmi P, Cataldi A, Zara S. The Up-Regulation of Oxidative Stress as a Potential Mechanism of Novel MAO-B Inhibitors for Glioblastoma Treatment. *Molecules.* 2019;24(10):2005. doi:10.3390/molecules24102005

167. Anselme K, Bigerelle M. Topography effects of pure titanium substrates on human osteoblast long-term adhesion. *Acta Biomater.* 2005;1(2):211-222. doi:10.1016/j.actbio.2004.11.009
168. Anselme K. Osteoblast adhesion on biomaterials. *Biomaterials.* 2000;21(7):667-681. doi:10.1016/s0142-9612(99)00242-2
169. Lange R, Lüthen F, Beck U, Rychly J, Baumann A, Nebe B. Cell-extracellular matrix interaction and physico-chemical characteristics of titanium surfaces depend on the roughness of the material. *Biomol Eng.* 2002;19(2-6):255-261. doi:10.1016/s1389-0344(02)00047-3
170. Harman MK, Banks SA, Hodge WA. Wear analysis of a retrieved hip implant with titanium nitride coating. *The Journal of Arthroplasty.* 1997;12(8):938-945. doi:10.1016/S0883-5403(97)90164-9
171. Chan CW, Quinn J, Hussain I, Carson L, Smith GC, Lee S. A promising laser nitriding method for the design of next generation orthopaedic implants: Cytotoxicity and antibacterial performance of titanium nitride (TiN) wear nano-particles, and enhanced wear properties of laser-nitrided Ti6Al4V surfaces. *Surface and Coatings Technology.* 2021;405(126714). doi:10.1016/j.surfcoat.2020.126714
172. Hallman M, Rämert M, Ezekowitz RA. Toll-like receptors as sensors of pathogens. *Pediatr Res.* 2001;50(3):315-321. doi:10.1203/00006450-200109000-00004
173. Duan T, Du Y, Xing C, Wang HY, Wang RF. Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity. *Front Immunol.* 2022;13:812774. doi:10.3389/fimmu.2022.812774
174. Liu T, Zhang L, Joo D, Sun SC. NF- κ B signaling in inflammation. *Signal Transduct Target Ther.* 2017;2:17023-. doi:10.1038/sigtrans.2017.23
175. Mulero MC, Huxford T, Ghosh G. NF- κ B, I κ B, and IKK: Integral Components of Immune System Signaling. *Adv Exp Med Biol.* 2019;1172:207-226. doi:10.1007/978-981-13-9367-9_10

176. Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel DV. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science*. 1997;278(5339):866-869. doi:10.1126/science.278.5339.866
177. Harder J, Franchi L, Muñoz-Planillo R, Park JH, Reimer T, Núñez G. Activation of the Nlrp3 inflammasome by *Streptococcus pyogenes* requires streptolysin O and NF-kappa B activation but proceeds independently of TLR signaling and P2X7 receptor. *J Immunol*. 2009;183(9):5823-5829. doi:10.4049/jimmunol.0900444
178. Barker TH. The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. *Biomaterials*. 2011;32(18):4211-4214. doi:10.1016/j.biomaterials.2011.02.027
179. Paschoal AL, Vanâncio EC, Canale L de CF, da Silva OL, Huerta-Vilca D, Motheo A de J. Metallic biomaterials TiN-coated: corrosion analysis and biocompatibility. *Artif Organs*. 2003;27(5):461-464. doi:10.1046/j.1525-1594.2003.07241.x
180. Chien CC, Liu KT, Duh JG, Chang KW, Chung KH. Effect of nitride film coatings on cell compatibility. *Dent Mater*. 2008;24(7):986-993. doi:10.1016/j.dental.2007.11.020
181. Cavalcanti IMG, Ricomini Filho AP, Lucena-Ferreira SC, et al. Salivary pellicle composition and multispecies biofilm developed on titanium nitrided by cold plasma. *Arch Oral Biol*. 2014;59(7):695-703. doi:10.1016/j.archoralbio.2014.04.001
182. Manso M, Ogueta S, Pérez-Rigueiro J, García JP, Martínez-Duart JM. Testing biomaterials by the in-situ evaluation of cell response. *Biomol Eng*. 2002;19(2-6):239-242. doi:10.1016/s1389-0344(02)00030-8
183. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. *Annu Rev Cell Dev Biol*. 2010;26:397-419. doi:10.1146/annurev-cellbio-100109-104020
184. Peng X, Nelson ES, Maiers JL, DeMali KA. New insights into vinculin function and regulation. *Int Rev Cell Mol Biol*. 2011;287:191-231. doi:10.1016/B978-0-12-386043-9.00005-0
185. Paulin D, Lilienbaum A, Kardjian S, Agbulut O, Li Z. Vimentin: Regulation and pathogenesis. *Biochimie*. 2022;197:96-112. doi:10.1016/j.biochi.2022.02.003

186. Albrektsson T. On long-term maintenance of the osseointegrated response. *Aust Prosthodont J*. 1993;7 Suppl:15-24.
187. Kunrath MF, Garaicoa-Pazmino C, Giraldo-Osorno PM, et al. Implant surface modifications and their impact on osseointegration and peri-implant diseases through epigenetic changes: A scoping review. *Journal of Periodontal Research*. 2024;59(6):1095-1114. doi:10.1111/jre.13273
188. Kaluđerović MR, Schreckenbach JP, Graf HL. Titanium dental implant surfaces obtained by anodic spark deposition - From the past to the future. *Mater Sci Eng C Mater Biol Appl*. 2016;69:1429-1441. doi:10.1016/j.msec.2016.07.068
189. Papaspyridakos P, Chen CJ, Singh M, Weber HP, Gallucci GO. Success criteria in implant dentistry: a systematic review. *J Dent Res*. 2012;91(3):242-248. doi:10.1177/0022034511431252