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Study of biofunctionalized titanium implants on an osteoporotic animal model

David Alfaiate



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Study of biofunctionalized titanium implants on an osteoporotic animal model

Doctoral thesis dissertation presented by David Alfaiate to apply for the degree of doctor at the University of Barcelona

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LIST OF ARTICLES IN THE THESIS

Thesis in classic format with 1 article annexed.

- The thesis consists of one general objective that include three specific objectives, and one articles.

Articles

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- Alfaiate D, Mas-Moruno C, Manuela C, Maria Ustrell J, Camara JA, Ferrer M, Manero JM, Manzanares-Céspedes MC. Surgical Bone Implantation Technique for Rat Tibia Models of Diabetes and Osteoporosis. J Vis Exp. 2024 Jul 5;(209). doi: 10.3791/66591. PMID: 39037246
- Q2, Impact Factor 1,2.

THESIS SUMMARY

TÍTULO

Estudio en modelo animal osteoporótico de implantes de titanio biofuncionalizados

INTRODUCCIÓN

Los dispositivos médicos basados en titanio para la implantación ósea son considerados actualmente el tratamiento estándar cuando se observan condiciones de salud favorables, de reparación y cicatrización. Se consideran parte de este grupo de dispositivos los implantes dentales, las prótesis articulares, los sistemas de fijación de fracturas y los de fijación espinal. Por lo tanto, su uso es esencial para asegurar el bienestar de la población. Sin embargo, el aumento en la esperanza de vida en Europa y Estados Unidos, verificada anualmente, comporta un aumento de enfermedades asociadas al envejecimiento de la población. En este contexto, la correcta integración de los implantes con el tejido óseo puede estar comprometida, principalmente por la existencia de ciertas patologías como el cáncer, la osteoporosis, o la diabetes, entre otras, que afectan la capacidad natural del hueso de regenerar, limitando el proceso de osteointegración de los implantes, necesario para la eficacia de dispositivos médicos de implantación ósea.

En particular, la osteoporosis se presenta como una enfermedad que reduce la densidad ósea mineral, fragilizando los huesos, y por lo tanto está asociada al riesgo de fracturas óseas, como son los casos de fractura de pelvis, cadera, tobillo y alrededor de implantes existentes. Estas fracturas además requieren la máxima estabilidad para su reparación. Es importante, como se mencionó anteriormente, que la técnica de reparación de fracturas permita una recuperación efectiva, que muchas veces resulta sumamente difícil, ya sea por la morfología característica del hueso osteoporótico, que presenta una delgada capa cortical y una parte medular con mayor porosidad, o por el hecho que el hueso tiene un tiempo de regeneración más lento y por tanto más susceptible a la fractura.

Por este motivo, es importante desarrollar implantes con superficies activas que permitan una estimulación específica de la adhesión y diferenciación celular para

promover una reparación ósea más rápida y de mejor calidad en escenarios en los que estos procesos están comprometidos.

HIPÓTESIS

- Nuevos revestimientos de implantes biofuncionalizados estimulan una respuesta favorable en el proceso de osteointegración, comparados con los no biofuncionalizados.

OBJETIVOS

- Desarrollar y perfeccionar una técnica quirúrgica para colocar un implante en la tibia de la rata, capaz de ser reproducible fácilmente y con máxima efectividad.
- Testar nuevas superficies de implantes de titanio funcionalizadas con moléculas con propiedades osteoinductivas en ratas con osteoporosis, donde la capacidad de osteointegración se encuentra afectada.

METODOS

En una muestra de 40 ratas, se formaron 2 grupos de ratas de $n=20$ donde, ensayando en cada grupo una superficie de implante biofuncionalizada diferente, identificadas como condición A (con peptidomiméticos que se unen a la integrina $\alpha v \beta 3$) y condición B (con peptidomiméticos que se unen a la integrina $\alpha 5 \beta 1$), y por último las condiciones C y D que se identifican como grupo control (sin modificación en la superficie del implante) de cada una de las condiciones anteriores.

En cada grupo de $n=20$, será aplicada la eutanasia a las 4 semanas a 10 ratas (tiempo 1) y a las 12 semanas (tiempo 2) a otras 10 ratas. Los implantes se han colocado entre la cresta tibial proximal y el límite posterior de la cara medial del hueso de la tibia mediante un método quirúrgico descrito en detalle y optimizado por nosotros. Cada rata tendrá un implante biofuncionalizado (condición A o B) en una tibia y un implante control (condición C o D) en la otra tibia.

La técnica quirúrgica utilizada fue cuidadosamente desarrollada y optimizada para evitar el traumatismo óseo a todos los niveles, y mejorar tanto el postoperatorio de los animales como el resultado final de la osteointegración.

Para el ensayo se empleó un método de doble ciego, asegurando que ni los cirujanos que implantaron los dispositivos dentales, ni el equipo responsable de procesar y analizar las muestras conocieran el tipo de implantes que se estaban utilizando. Esto se logró mediante un sistema de codificación único para cada implante.

Después de las eutanasias y del procesamiento de las muestras, la interacción de los implantes con el tejido óseo se analizó mediante microscopia electrónica de barrido y microscopía óptica utilizando el procedimiento de tinción Masson-Goldner.

RESULTADOS PRINCIPALES

En cuanto a la técnica quirúrgica, todos los implantes fueron osteointegrados correctamente, sin reacciones inflamatorias ni infecciosas.

Se pudo observar que sólo los implantes modificados exhibieron ganancia de volumen cortical y reversión osteoporótica debido a la bio-activación en todo el hueso.

Para las 3 condiciones, A, B y Control (C y D), los valores de bone-implant contact (BIC), se ven positivamente influenciados principalmente por la técnica quirúrgica según el contacto interno entre el implante y el hueso cortical, sin que esa variable afecte la estabilidad de los implantes y éxito en el proceso de osteointegración.

CONCLUSIONES

Este estudio nos permitió concluir lo siguiente:

La técnica quirúrgica desarrollada y optimizada en este estudio permite de manera efectiva colocar implantes en la tibia de un modelo animal de rata con osteoporosis. Además, se ha proporcionado un protocolo detallado para su adecuada reproducibilidad por parte de otros investigadores.

Los implantes biofuncionalizados estimulan una respuesta biológica favorable en el proceso de osteointegración, revirtiendo el patrón óseo de la osteoporosis, no sólo a través de la densidad, sino también a través del aumento del espesor óseo.

INTRODUCTION

The pursuit of overall well-being for the population is a constant path that has always been sought through techniques and technological means. Its goal is the maintenance or recovery of general health beyond the ideal well-being indexes to be achieved. To reach these happiness and health parameters, different medical devices have been developed with the goal of restoring certain functions that are lost due to illness, accident, or even aging. Some of these functions are restored and recovered through bone-implantable medical devices. This group of devices includes dental implants, joint prostheses, fracture fixation systems, and spinal fixation systems, among others. Currently, most are made of titanium, which can have different chemical compositions and alloys.

Currently, titanium medical devices for bone implantation are considered relatively safe under favorable health conditions for repair and healing, but they present greater limitations when the ideal biological conditions are not present. In the United States, in 2004, failures in joint replacement procedures accounted for 86,000 surgical revisions, costing a total of 32 billion dollars (1). Currently, more than 2.2 million bone graft procedures are performed in dentistry and orthopedics, making it the second most common type of transplant after blood transplants (2). Recent studies estimate that the number of hip and knee surgeries due to implant failures could exceed 3.6 million by the year 2030 (1).

The aging of the population and the increase in life expectancy, approaching closer to 90 years, bring inevitable consequences in the increase of diseases associated with the aging population. In this context, some biological reactions may be compromised, mainly because of the existence of certain pathologies such as cancer, osteoporosis, diabetes, among others, which affect the natural ability of the bone to regenerate, limiting the process of osseointegration, necessary for the effectiveness of bone implantation medical devices. To overcome this obstacle, the development of different implant surfaces that stimulate biological reactions by the bone tissue may be the solution, thus ensuring the osseointegration process.

1. Osteointegration

The first studies on the phenomenon of osteointegration were developed by Per-Ingvar Brånemark in the 60s, 70s and 80s (3). However, it was the early studies related to microcirculation in rabbit bones in the 1950s that demonstrated that titanium chambers were permanently fixed in the bone. He observed that the bone was so attached to the titanium oxide layer that to separate it, it was necessary to fracture it (4). Later it was observed that the alveolar bone was capable of forming a direct connection with alloplastic screw-like material such as a titanium implant, when placed in a surgically created wound (5). Today it is known that the osteointegration of titanium implants is a complex biological process that includes interactions between immunoinflammatory responses, angiogenesis and osteogenesis, all greatly influenced by the physical and chemical characteristics of the implant surface (6). It is a process that requires an initial mechanical fixation followed by a functional connection over a long period of time between the implant and the wall in its surroundings (7).

It is currently defined as osteointegration, when there is no progressive relative movement between the implant and the bone with which it is in direct contact (8).

For the biological process to occur, stability is required between the medical device, in this case an implant or osteosynthesis screw, and the bone. Primary implant stability is crucial for the successful healing process around the implant site (9). A stable implant with minimal micromotion between the bone and implant fosters optimal tissue growth, including critical processes like angiogenesis and osteogenesis (10). This stability concept also occurs in a healing of fracture, where instability between bone ends can lead to the formation of cartilage instead of new bone (11, 12). In extreme cases, this scenario can result in pseudoarthrosis, a debilitating condition. Furthermore, it has already been emphasized that movement at a fracture site may extend the expression of key genes, such as the Indian hedgehog gene, which plays a vital role in regulating the maturation of chondrocytes (13). On the other hand, excessive motion around an implant tends to promote the growth of fibrous connective tissue rather than bone tissue (14). A micromotion exceeding 150 μm can result in fibrous tissue attaching to the implant surface instead of bone integration (15). Consequently, it is advised to restrict early weight bearing on cementless orthopedic implants. It is worth noting that a controlled

degree of micromovement, likely less than 25-50 μm , is necessary to facilitate bone formation in both the healing of fractures and the integration of implants (16).

Osseointegration results from a cascade of cellular and extracellular biological events at the implant-bone interface, until the implant surface is surrounded by new bone (17). This cascade of events (Figure 1) (18) is regulated by growth factors and cell differentiation factors, released at the bone-implant interface (19).

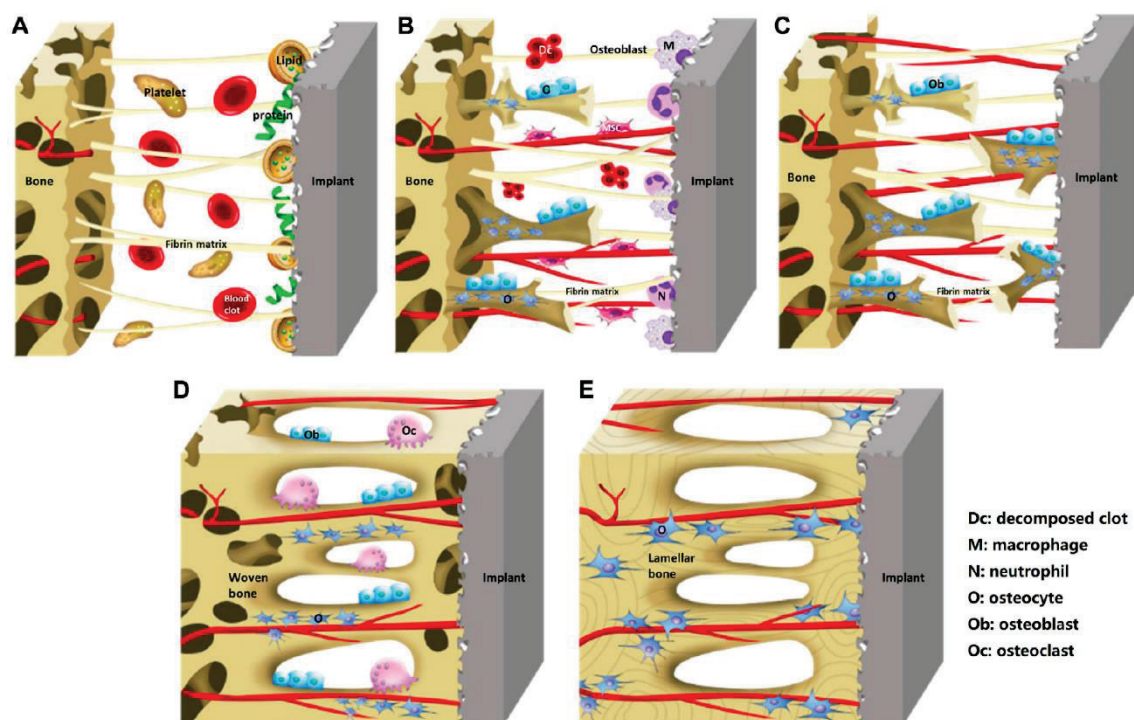


Figure 1(18): Schematic of the process of implants OI. The process of Osseointegration includes:

A- The formation of the blood clot and fibrin matrix. **B-** Angiogenesis and woven bone formation. **C-** Distance osteogenesis and contact osteogenesis. **D-** Newborn woven bones fill up the gap and bone remodeling. **E-** Woven bones transform into lamellar bones.

The initial biological element to make contact with an endosseous implant is blood. This includes blood cells like erythrocytes, platelets, and inflammatory cells, such as polymorphonuclear granulocytes and monocytes, which leave the post-capillary spaces and move into the tissue that envelops the implant. Once caught at the interface of the implant, these blood cells become activated, leading to the release of cytokines along with various soluble factors that promote growth and differentiation (19).

Neutrophils are the forefront responders arriving at the implant site, reaching peak levels between 24 to 48 hours post-implantation. Shortly thereafter, monocytes undergo rapid transformation into macrophages, becoming the predominant leukocytes in the area after 48 hours (20). This influx of leukocytes into the capillaries around the implant is primarily activated by cytokines released from platelets, such as β -thromboglobulin and platelet-derived growth factor (PDGF) (21). A significant consequence of this leukocyte activation is the secretion of inflammatory mediators, notably including cytokines like interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor alpha (TNF- α), and macrophage colony-stimulating factor. These mediators play essential roles in orchestrating the initial inflammatory response critical for the healing process following implantation (20).

Within 24 hours of injury, much like inflammatory mediators, members of the tissue growth factor β (TGF- β) superfamily, including bone morphogenic proteins (BMPs) and growth and differentiation factors (GDFs) superfamily are also expressed (22). These factors play a significant role in stimulating bone formation at sites of fracture. Clinically, BMP-2 and BMP-7 are employed in North America to boost the healing process of non-union fractures (23). Additionally, osteoinductive factors, responsible for steering the differentiation of osteogenic cells from mesenchymal cells, have demonstrated positive outcomes in promoting bone regeneration during peri-implant healing in live animal studies (24).

The initial interaction of blood cells with an implant is crucial for clot formation and subsequent healing processes. Upon contact with the implant surface, platelets undergo significant morphological and biochemical transformations, including adhesion, spreading, and aggregation. These changes are accompanied by key intracellular modifications, such as the induction of phosphotyrosine, an increase in intracellular calcium, and the hydrolysis of phospholipids. The resultant fibrin matrix serves not only as a physical scaffold facilitating osteoconduction but also promotes the migration and osteoinduction of osteogenic cells within the healing site. These osteogenic cells are instrumental in forming osteoid tissue and new trabecular bone, which undergoes remodeling into lamellar bone, achieving direct osseointegration with the implant surface (19, 25, 26).

From the first day following implantation, osteoblasts and mesenchymal stem cells (MSCs) initiate migration and attachment to the implant surface. Their activity leads to the deposition of bone-specific proteins, contributing to the formation of a non-collagenous matrix layer. This layer plays a pivotal role in regulating cellular adhesion and the binding of minerals to the implant surface. An early product of this interaction is a calcified, afibrillar layer, comparable in composition to bone cement lines and laminae limitans. It forms a continuous layer approximately 0.5 mm thick, enriched in essential elements such as calcium and phosphorus, and proteins including osteopontin and bone sialoprotein. This layer represents a crucial step in the integration of the implant with the surrounding bone tissue (26, 27).

Peri-implant osteogenesis occurs in two distinct modes: via distance osteogenesis and contact osteogenesis, relative to the host bone. Distance osteogenesis involves the formation of new bone trabeculae that extend from the host bone towards the implant, while contact osteogenesis describes bone formation that initiates at the implant surface and extends towards the healing bone. This network of trabeculae ensures the implant's biological fixation and includes marrow spaces rich in mesenchymal cells and expansive blood vessels. A thin layer of calcified and osteoid tissue laid down by osteoblasts covers the implant surface. Furthermore, blood vessels and mesenchymal cells occupy the gaps not filled with calcified tissue, contributing to the overall process of osteointegration (10, 19, 28).

The initial phase of peri-implant bone healing is characterized by the rapid deposition of a new calcified matrix directly on the implant surface. Following this, the organized formation of woven bone and bone trabeculae occurs. This phase is crucial, featuring a highly active area with a broad surface that merges seamlessly with marrow spaces laden with vascular and mesenchymal cells. The richly vascularized marrow tissue plays a pivotal role, supporting the mononuclear precursors to osteoclasts. This environment facilitates the accelerated remodeling of bone trabeculae, a process that occurs faster than in cortical bone (10).

Early formation of peri-implant trabecular bone plays a vital role in anchoring tissue, ensuring the biological fixation of the implant, typically initiating within 10 to 14 days post-surgery. Biological fixation, distinct from primary mechanical stability achieved

during implant insertion, relies on biophysical factors such as mechanical fixation, bio-mimetic surface characteristics, and optimal spacing between the implant and host bone. This type of fixation is notably facilitated by rough implant surfaces. Over time, woven bone undergoes gradual remodeling, gradually transitioning to lamellar bone with enhanced mineralization. By the three-month mark following implantation, a combination of woven and lamellar bone textures can be observed, indicative of the ongoing bone healing processes around titanium implants (29).

The contact and distance osteogenesis contribute significantly to its secondary stabilization within the host bone. This shift occurs from the initial primary stabilization, established through the secure fit of the implant within the host bone during implantation, to the subsequent secondary stabilization resulting from the development of woven bone around the implant (30). As time progresses, the effectiveness of primary stabilization diminishes due to the natural turnover process where the host bone in direct contact with the implant perishes and is reabsorbed by osteoclasts. The secondary stability of the implant hinges on potential bone bonding, particularly if the implant's surface topography supports contact osteogenesis. In instances where the implant's surface texture is intricate in three dimensions, featuring pores and undercuts, there is a possibility for the cement line to intertwine with the implant surface, facilitating bone bonding. If the implant lacks the necessary topographical complexity, bone growth takes place through distance osteogenesis, potentially resulting in a lack of bone bonding (31).

When bone is in contact with an implant surface, it changes its structure to adapt to the stress and mechanical forces acting on it. The turnover of mature bone around implants that have successfully integrated into the bone is evidenced by the presence of specialized bone cells, such as osteoclasts, osteoblasts, mesenchymal cells, and blood vessels near the implant. As the bone around the implant undergoes remodeling, new bone formations known as osteons form circularly around the implant, aligning their long axes parallel to the implant's surface and perpendicular to the implant's long axis. The production of osteoid tissue by osteoblasts indicates active bone formation or osteogenesis occurring in the vicinity. This remodeled bone tissue extends outward from the implant surface up to approximately 1 mm, showcasing the significant structural

changes that occur as the bone adapts to the presence and integration of the implant (10, 29).

Several factors inhibit the successful integration of implants with bone. These include excessive implant mobility and micromotion (15, 32), inadequate porosity of the implant surface (33), as well as external factors such as receiving radiation treatment (34, 35) or being on certain medications like cyclosporin A, methotrexate, and cis-platinum (36-38). Additionally, drugs like warfarin and low molecular weight heparins (39), along with non-steroidal anti-inflammatory drugs, specifically selective COX-2 inhibitors (40, 41), selective serotonin reuptake inhibitors (SSRIs) (42, 43), or Proton Pump Inhibitors (PPIs) (44), can impact osseointegration. Furthermore, individual patient factors such as diabetes (45, 46), osteoporosis, rheumatoid arthritis, older age, poor nutrition, smoking habits, and renal problems can also act as barriers to the successful fusion of implants with bone tissue (47-49).

Although there are several factors that can negatively influence osteointegration, these changes are not always observed in a clear and significant way (50).

2. Epidemiology of Osteoporosis

Osteoporosis is defined as a “skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture (51)”.

This disorder represents a significant non-communicable disease, and it is the most prevalent bone condition, impacting one in three women and one in five men over the age of 50 globally (52-54). The primary clinical outcome of osteoporosis is the occurrence of fragility fractures (55). Research indicates that experiencing an initial fracture significantly heightens the risk of subsequent fractures (56). With the swift aging of the global population and shifts in lifestyle habits, the incidence of osteoporosis and associated fractures has risen sharply and is projected to continue to increase substantially in the future. Given its widespread prevalence, osteoporosis is regarded as a critical public health issue (55, 57).

It is estimated that 75 million individuals across Europe, the USA, and Japan are affected by osteoporosis (58). In Asia, osteoporosis is significantly underdiagnosed and undertreated, even among high-risk patients who have previously sustained fractures. This issue is particularly prevalent in rural regions. In populous countries like China and India, a large portion of the population resides in rural areas (60% in China), where hip fractures are often managed conservatively at home rather than through surgical intervention in hospitals (59).

Globally, osteoporosis is believed to impact 200 million women—approximately one-tenth of women aged 60, one-fifth of those aged 70, two-fifths of women aged 80, and two-thirds of women aged 90 (58). A survey conducted by the International Osteoporosis Foundation (IOF) across 11 countries highlighted that postmenopausal women often deny their personal risk, have limited discussions about osteoporosis with their healthcare providers, and encounter barriers to diagnosis and treatment before experiencing their first fracture, leading to significant underdiagnosis and undertreatment of the condition (60). Moreover, the aging populations worldwide will contribute to a considerable rise in the incidence of osteoporosis among postmenopausal women (61).

In the United States, an average of 2 million fractures is observed in patients with osteoporosis, representing an approximate cost of 19 billion dollars (62). The low bone density in these patients presents a decrease in mechanical resistance to implant removal, observing a loosening in the stability of the implants in the spine by around 18 to 27% (63-65), as well as a high risk of fixed fracture in osteoporotic patients in approximately 5 to 23% of cases (66-68).

A report named "SCOPE 2021: a new scorecard for osteoporosis in Europe" examines the state of osteoporosis in the European Union as of 2019. According to this report, approximately 25.5 million women and 6.5 million men were estimated to be living with osteoporosis across the EU, including Switzerland and the United Kingdom. In that year, approximately 4.3 million new cases of fragility fractures were reported, which included 827,000 hip fractures, 663,000 vertebral fractures, 637,000 forearm fractures, and 2.15 million fractures classified as "other," affecting areas such as the pelvis, ribs, humerus, tibia, fibula, clavicle, scapula, sternum, and various femoral fractures. The financial toll of

new and existing fragility fractures in 2019 was estimated to be €57 billion. In the EU27+2 (all 27 EU countries + Switzerland and UK), the study found that there were 248,487 fracture-related deaths that year. The number of these deaths is comparable to or even surpasses some leading causes of mortality, including lung cancer, diabetes, and chronic lower respiratory diseases. The population aged 50 and older is anticipated to grow by 11.4% from 2019 to 2034, and it is expected that the annual occurrence of osteoporotic fractures in the EU27+2 will rise by 25%. Alarming, a significant number of individuals who have suffered an osteoporosis-related fracture or who are at a high risk of fractures are not receiving treatment, and the rate of high-risk patients who are being treated is decreasing (69).

Regarding fractures as a consequence of osteoporosis, it is important to understand their significance, both in terms of the changes these fractures require in the patients adaptability to life referred as DALYs (Disability Adjusted Life Years), Figure 2 (70), and in recognizing that these fractures pose a life risk for the patients (Figure 3). This risk, as previously mentioned, in fact, represents a higher mortality rate than many other diseases (69).

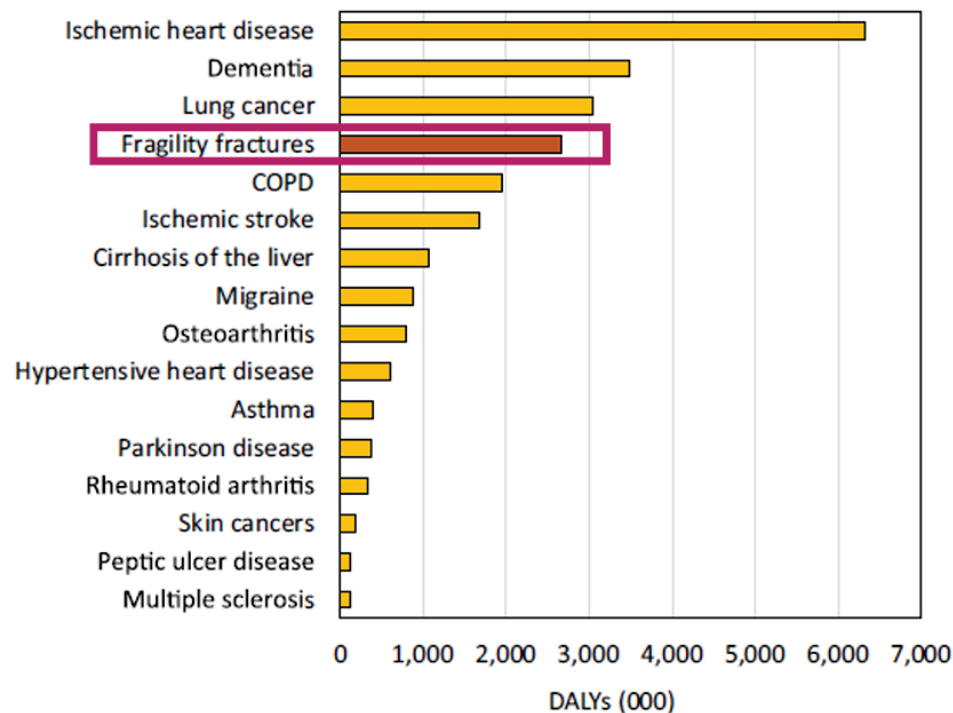


Figure 2 (70): DALYs by disease in 6 European countries in 17 selected non-communicable diseases- IOF 2018. Disability burden expressed in DALYs is greater than many other common diseases.

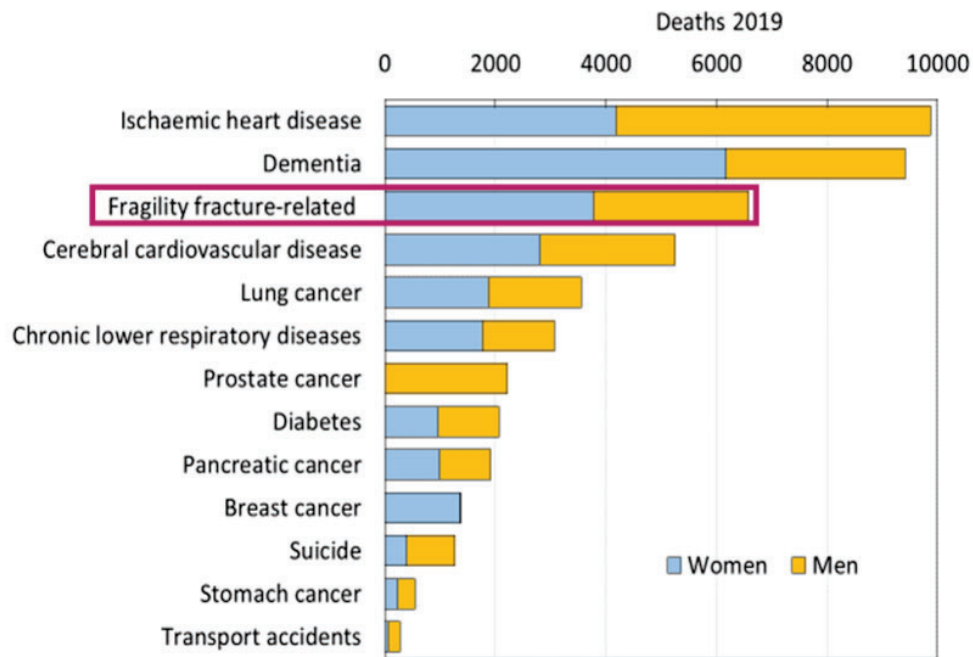


Figure 3 (69): Comparison between number of deaths and relevant causes in 2019 (Sweden)

If we consider only hip fractures, the data is worrying even today. Morbidity rates in the first year following a hip fracture indicate that 40% of patients are unable to walk independently (70). Additionally, 80% experience limitations in other activities, such as driving, significantly impacting their quality of life (70). Alarmingly, there is a mortality rate of up to 20% within that first year in some countries (71). It is estimated that there were 248,487 osteoporotic bone fractures related to death in the EU27+2 in 2019 (69).

Furthermore, these statistics illuminate the broader impact of osteoporosis on healthcare systems, highlighting the urgent need for effective prevention and management strategies. Effective interventions can not only reduce the incidence of fractures but also improve patients' overall health outcomes and quality of life.

Looking to the future, the results are even less promising. A significant increase in fragility fractures due to osteoporosis is projected over the coming years. Estimates suggest a 24.8% increase between 2019 and 2034 (Figure 4), translating to a rise from 4.28 million fractures in 2019 to 5.34 million by 2034 (69). This upward trend underscores the

necessity for increased awareness, early screening, and innovative treatment approaches to address this growing public health concern.

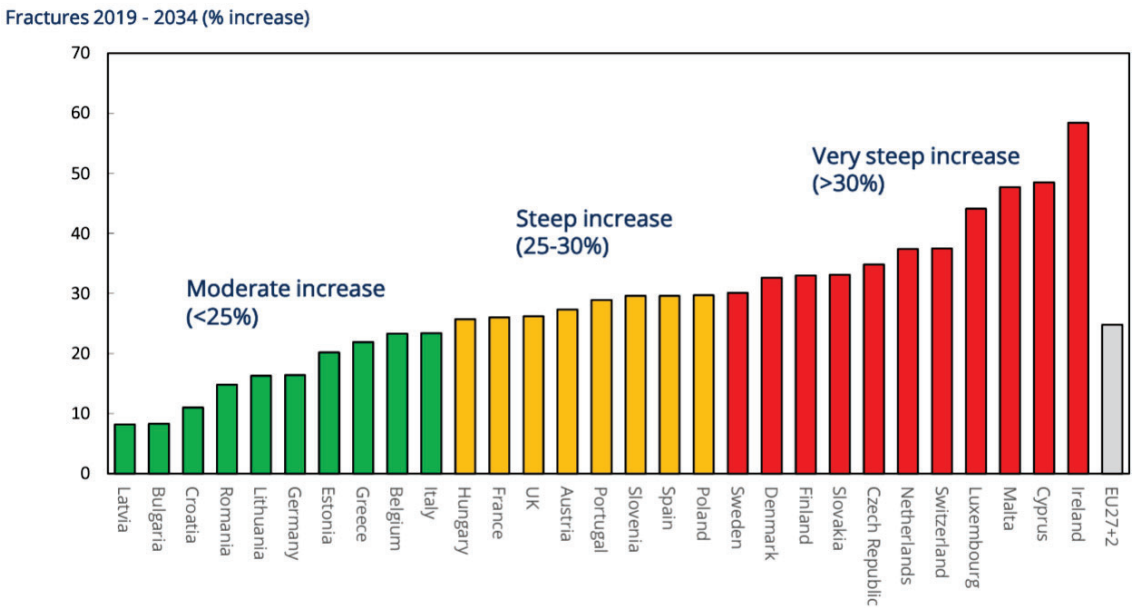


Figure 4 (69): The percentage increase in the number of fragility fractures between 2019 and 2034 in the EU27+2.

Furthermore, osteoporosis clinical management is not recognized as a specialty in most countries, with osteoporotic patients being mostly managed by other specialties (Figure 5). However, the recognition of the impact of this disease on society has led to osteoporosis being acknowledged as a component of specialty training. This wide variation may reflect inconsistencies in patient care and the training of primary care physicians (72).

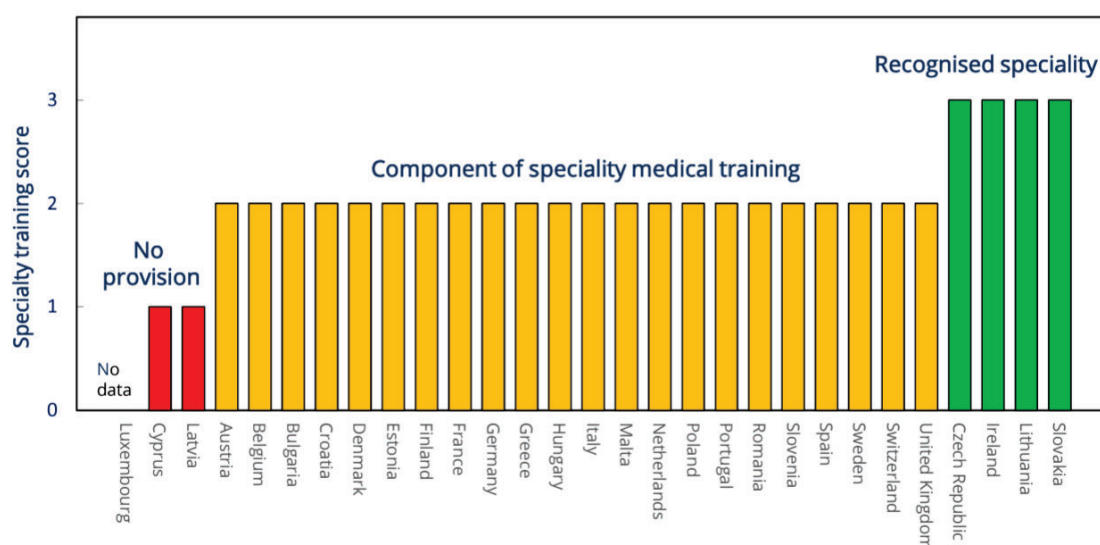


Figure 5 (69): Manage of osteoporosis as a recognized specialty or as a medical training.

The global prevalence of osteoporosis among women is reported to be 23.1% (95% CI 19.8–26.9), while the prevalence among men is reported at 11.7% (95% CI 9.6–14.1). Subgroup analysis results indicate that the highest prevalence of osteoporosis in men occurs in Asia, whereas among women, the highest prevalence is found in Africa (73). According to a systematic review and meta-analysis conducted in 2021, it is important to note that no studies have been conducted on men in Africa (73).

Iran was also identified as having the one of the highest prevalence at 77.3% in a study in 2008 with post-menopausal women who had done BMD test (Bone Mineral Density) (74). However, a systematic review regarding the prevalence of osteoporosis in postmenopausal Iranian women, published in 2022, presents even more worrying data, indicating that only 18.7% of women exhibited no changes. Despite these numbers, the review subdivides the bone changes into osteoporosis and osteopenia, with percentages of 33.7% and 47.6% respectively, resulting in a total of 81.4% of women with bone fragility (75).

3. Osteoporosis and Osteointegration

Key features of osteoporosis can be observed through skeletal histomorphometry, showing fewer trabeculae, reduced trabecular bone volume, and notable differences in microstructure compared to healthy bone. Additionally, variations in bone remodeling, or turnover, can be seen in individuals with osteoporosis, with some experiencing low, normal, or increased rates of bone remodeling (76).

Fractures in patients with osteoporosis often present a slower healing process (77, 78). Healing a fractured bone typically involves immobilization through conservative methods like casts or orthoses, or through surgical intervention with osteosynthesis implants such as screws, plates, or nails. However, treating fractures in osteoporotic bones poses specific challenges. The reduced density and increased brittleness of osteoporotic bone can cause it to fracture into smaller, more fragmented pieces, leading to more intricate and complex fractures compared to fractures in healthy bone (79).

Surgeons need high levels of skill to precisely reduce fractures and use effective implants to maintain stability. Elderly people with osteoporotic fractures face issues like reduced balance, coordination, and vision, leading to uncoordinated limb loading and a higher risk of falls (80, 81).

Inability to follow weight-bearing restrictions after surgery can overload the implanted structures, requiring maximum stability for immediate full weight-bearing. Furthermore, the presence of other health conditions alongside osteoporosis in the elderly increases the risk of complications post-fracture, necessitating quick, minimally invasive surgeries and prompt mobilization for better recovery outcomes (82).

Osteoporotic bone's weakened mechanical properties, like porous cancellous and thin cortical bone, make it less resistant to rigid implants, often resulting in complications such as implant loosening or fractures near the implants (83). Overall, managing fractures in elderly individuals with osteoporosis involves establishing stable fracture fixation that can support full weight-bearing with no restrictions, ensuring a successful healing process. To address this, osteosynthesis implants must be engineered to resist loosening or be supported further to prevent failure. Achieving durable and stable fracture fixation capable of supporting unrestricted weight is particularly vital in the treatment of fractures

in older patients suffering from osteoporosis. This focus includes fractures prevalent in elderly patients with osteoporosis, such as those in the pelvis, hip, ankle, and surrounding existing implants, all requiring the capacity to bear full weight (84).

Aging and osteoporosis significantly alter various cellular processes, including cell proliferation, synthetic activity, responsiveness of cells to local factors, and the quantity of MSCs (49). These biological changes, combined with variations in the biomechanical and microarchitectural characteristics of bone, contribute to the higher risk of failure in the osteointegration of biomaterials implanted into aged and osteoporotic bones. Specifically, factors such as TGF- β 1, IL-1, and IL-6 play crucial roles in bone regeneration and the osteointegration of biomaterials. TGF- β 1 is a key promoter of osteointegration, while the interleukins are notably present at the interface of bone-biomaterial in cases of aseptic implant failures (85, 86).

Local factors significantly contribute to bone loss observed in senile and post-menopausal conditions through their mediation of bone remodeling. For example, the level of TGF- β 1, an anabolic cytokine, is negatively associated with age and post-menopausal osteoporosis, indicating a decrease in anabolic activities essential for bone maintenance (87). Conversely, levels of IL-6, a catabolic cytokine involved in bone resorption, rise in aged and post-menopausal osteoporotic patients. Furthermore, the post-menopausal decrease in estrogen levels aids IL-6 induced bone resorption and the differentiation of osteoclast precursors into mature osteoclasts (88).

In considering both foundational and practical research, the principal reasons for the elevated failure risk of biomaterials implanted into osteoporotic bone can be summarized as follows: alterations in bone's structural and mechanical properties; a decline in MSCs numbers; an increase in osteoclast numbers and activity; an imbalance between anabolic and catabolic factors influencing bone remodeling; and abnormal bone cell responses to hormonal, growth factor and cytokine stimuli (hormones, growth factors and cytokines), as well as to mechanical stress (17).

There are two main types of osteoporosis: primary and secondary. Primary osteoporosis predominantly affects postmenopausal women and older men without any specific underlying health condition, primarily due to the natural aging process. On the other

hand, secondary osteoporosis is linked to other diseases or medications that contribute to the weakening of bones. Remarkably, up to 30% of postmenopausal women and between 50 to 80% of men have factors that predispose them to osteoporosis, underscoring the prevalence and complexity of managing this condition (89).

Despite being a well-explored topic, there is still a need to enhance the integration between metallic implants and bone, particularly in cases of osteoporosis due to the low bone density and strength (77, 78).

Moreover, due to the slower bone repair observed in patients with osteoporosis, as well as its characteristic low density, surface modifications, e.g. improving the roughness of the implants, may be required to ensure an adequate bone to implant contact. In this regard, osteoinductive coatings on the surface of implants capable of stimulating osteoblastic activation (90), as well as the osteogenic differentiation of MSCs at the implant site, appear to be promising. For instance, it has been observed that implants functionalized with selective coatings for $\alpha 5\beta 1$ integrins displayed improved osteointegration rates in both healthy and osteoporotic rats (91).

In dentistry, implant devices offer a solution that significantly enhances the quality of life for partially and fully edentulous patients. Despite the effect of osteoporosis on the process of osseointegration, the outcome in dentistry appears to be favorable when compared to orthopedics. Implant survival rate of 98% in osteoporotic patient was observed, which is comparable to patients without osteoporosis (50). Other systematic review revealed a similar failure rate between osteoporotic patients and healthy individuals, suggesting no significant discrepancy in implant failure rates between the two groups (92).

Despite these findings, the varying bony composition between the maxilla and mandible has been suggested to potentially lead to diverse outcomes in the osseointegration of dental implants among osteoporotic patients (93), suggesting further in-depth analysis of this variable in future studies.

Regardless of antiresorptive drug therapy, it becomes imperative to educate patients on the contraindications of antiresorptive drug therapy, particularly the potential development of medication-related osteonecrosis of the jaw (MRONJ), which has severe

effects on their oral health and well-being (94, 95). However, the uncertainty remains about this risk in patients under antiresorptive drugs who undergo oral surgeries like dental implant placements, as highlighted by the American Association of Oral and Maxillofacial Surgeons (96).

Several reviews have not found statistically significant differences in the osseointegration process between patients with antiresorptive drug treatment and those without (50, 97-99). Nonetheless, in other studies, it was observed that Immediate loading with antiresorptive drug treatment was considered as a risk factor for implant loss (100, 101).

4. Integrins

Integrins are pivotal in cell adhesion, acting as bidirectional, heterodimeric cell surface receptors that play a key role in how cells interact with the extracellular matrix (ECM) proteins (102). Through their engagement with ECM ligands, integrins initiate signal transduction pathways inside the cell, facilitating cell migration and adhesion. Since their identification in the late 1980s, integrins have been deeply studied for their structure, mechanisms of ligand recognition, and biological roles, making them the most extensively researched adhesion receptors to date (103).

There are at least 24 known integrin subtypes, which are formed through the noncovalent pairing of 18 alpha (α) and 8 beta (β) subunits. Both α and β subunits are type-I membrane proteins featuring a large ectodomain, a typically short noncatalytic cytoplasmic domain, and a single transmembrane domain (104). The ability and affinity of integrins to bind their ligands is dynamically regulated by cellular signals, a process known as "inside-out" signaling (105).

On the other hand, the binding of ECM ligands to integrins leads to structural changes, separation of transmembrane helices, and clustering into oligomers, triggering "outside-in" signal transduction (105, 106). It's noteworthy that while ligands can bind to integrins in their resting state, effective signal transduction necessitates the disassociation of the integrin's transmembrane helices followed by oligomerization (Figure 6) (107).

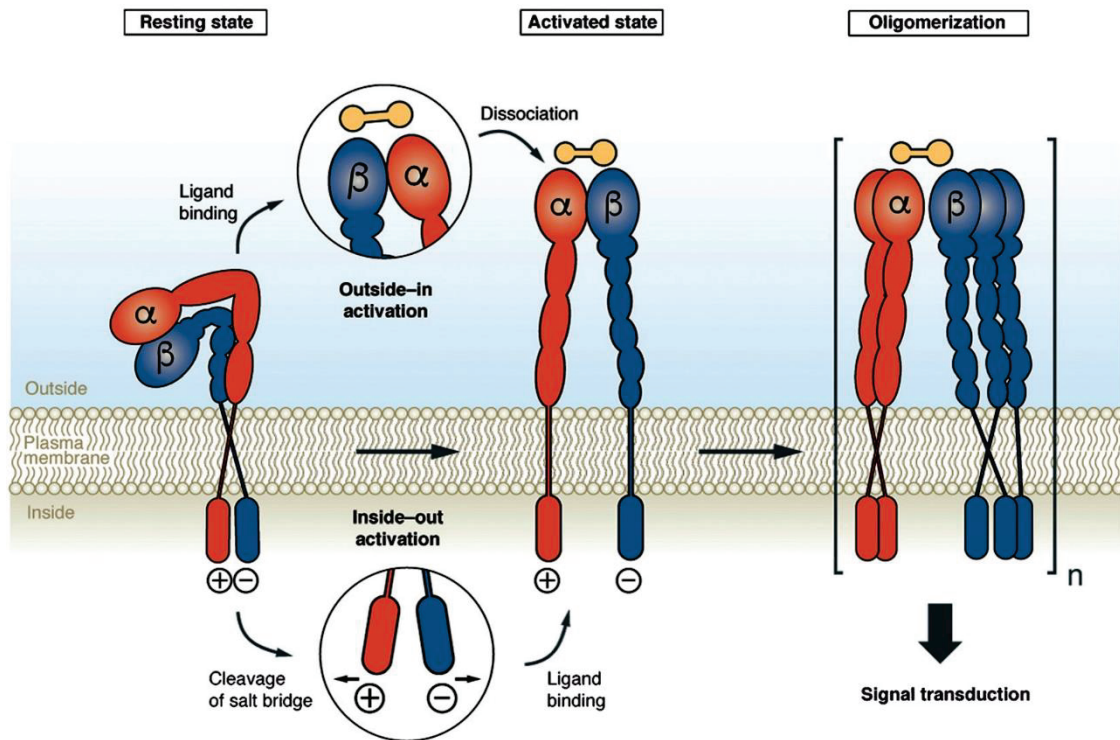


Figure 6 (107): Representation of integrin activation mechanisms: Illustrating the transition from 'Inside-Out' to 'Outside-In' Signaling. Highlights include the initial low-affinity, bent conformation of integrins, the intracellular events leading to the breaking of salt bridges and subsequent transmembrane helix rearrangement, culminating in a high-affinity, extended state ready for ECM binding. Further showcased is the role of ECM ligand engagement in stabilizing this high-affinity state at focal adhesions, emphasizing the crucial process of integrin oligomerization for effective signal transduction into the cell.

Therefore, integrins serve as crucial bidirectional signaling entities that regulate cell polarity, adhesion, and survival, illustrating their intricate and multifaceted roles in cell biology (108).

During the process of cell adhesion, integrins are essential in mediating force transmission in focal adhesions to proteins in the ECM, a mechanism known as mechanotransduction (109). This activity of integrins is crucial for the processes of embryogenesis, the development of tissues, the formation of new blood vessels (angiogenesis), and the functioning of the immune system (110). Furthermore, integrins play significant roles in various diseases including thrombosis, osteoporosis, the development and progression of tumors, the spread of cancer cells (metastasis), and inflammation (111). Given these biological roles in both health and disease, it is understandable why integrins have become prime targets for developing medications aimed at treating a wide array of medical conditions (112, 113).

Grasping the biological importance of integrins is critical for the creation of new medications that are both highly effective and have fewer side effects. However, the development in this area has been slow due to a lack of integrin-specific ligands. This issue is exemplified by the ubiquitous integrin-binding peptide, RGD (114). While numerous integrins recognize and bind to the ECM via an RGD-specific mechanism, they can still distinguish among various natural ligands that contain the same RGD sequence (115). Factors such as additional binding domains, the characteristics of neighboring amino acids, and the shape and manner in which the RGD sequence is presented to integrins, all contribute to this specificity.

Therefore, significant research over the past thirty years has focused on identifying the structural attributes that dictate specific interactions with integrins. Particularly, integrins $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$, which are crucial for cell adhesion and differentiation, the formation of new blood vessels (angiogenesis), and cancer growth, have been highlighted as promising targets for therapeutic intervention (116, 117).

Achieving distinction between the similar integrins $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$ has generally been challenging for cyclic peptides (118). Most of the selectivity between these receptors has been attained through synthetic RGD-based peptidomimetics. The foundation for developing these specific ligands was laid by comprehensive studies on the structure-activity relationship along with the crystallography of $\alpha\text{v}\beta\text{3}$ (119) and homology models for $\alpha\text{5}\beta\text{1}$ (120). These molecular compounds have shown promise in potentially serving as integrin antagonists for purposes such as cancer therapy, tumor imaging, and detailed biophysical research to uncover the precise functions of these crucial integrins. Alongside these developments, ECM proteins and short synthetic peptides have been utilized to enhance the bioactivity of various materials by promoting cell adhesion processes on their surfaces. A significant amount of research has demonstrated that activating integrin and signaling on the surface of bioinert materials effectively encourages cell attachment, growth, and differentiation. This approach has led to the creation of a new wave of biomaterials for use in tissue engineering and regenerative medicine. However, surprisingly, the adaptation of peptidomimetics for surface coating has been limited, and most strategies in this area have leaned towards using RGD-containing peptides and proteins, which lack specificity for integrin receptors (121).

Different information can be found in the literature regarding the roles of integrins $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$, which do not always seem to be in agreement. These variations can be attributed to different factors.

Often, studies examine ECM ligands' affinity primarily for a single integrin subtype, like $\alpha\text{v}\beta\text{3}$ or $\alpha\text{5}\beta\text{1}$, leaving their interaction with other subtypes less explored. Consequently, the influence of an integrin on biological processes might be overstated while overlooking the roles of others. Additionally, integrin expression varies significantly across different cell types and can be affected by culture conditions and substrates, complicating comparisons across studies. It is also important to recognize that there is functional redundancy among integrins, meaning the inhibition of one may be compensated by another (107).

The impact of $\alpha\text{v}\beta\text{3}$ and $\alpha\text{5}\beta\text{1}$ integrins on MSCs differentiation is receiving considerable attention due to its relevance in stem cell therapy and the design of biomaterials that guide cell behavior. There is a growing interest in controlling stem cell states, whether to maintain undifferentiation or to trigger specific differentiation pathways. Beyond traditional differentiation signals like growth factors, the microenvironment is emerging as a valuable tool for directing stem cell fate, although the complete role of integrins is yet to be fully understood. Positive effects of the $\alpha\text{5}\beta\text{1}$ subtype on osteogenesis have been observed, including the upregulation of osteogenic markers and activities beneficial for bone integration inducing osseointegration of implants (122, 123) and ectopic bone formation (124, 125).

Further reinforcing this trend, a decrease in $\alpha\text{5}\beta\text{1}$ levels was associated with bone loss in an animal model of skeletal unloading (126).

Conversely, contrasting reports exist regarding $\alpha\text{v}\beta\text{3}$, with some studies indicating it suppresses bone differentiation (122, 127), whereas others suggest it enhances bone matrix mineralization (128).

Interestingly, research has shown that a cyclic RGD peptide targeting $\alpha\text{v}\beta\text{3}$ can encourage MSCs towards bone formation, indicated by increased osteogenic markers and cell spreading on coated surfaces (129). In contrast, a linear RGD peptide, less affine to $\alpha\text{v}\beta\text{3}$, led to myogenic differentiation. However, the focus on $\alpha\text{v}\beta\text{3}$ bone-forming potential has

been limited due to its known association with bone resorption, highlighted by its high expression in osteoclasts and its involvement in inhibiting bone resorption when interfered (130).

This effect is attributed to the $\alpha v\beta 3$ -dependent migration of osteoclasts (131). Moreover, $\alpha v\beta 3$ role extends to cell migration across various cell types and is notably a key player in both physiological and pathological angiogenesis, critical for tumor metastasis and progression (132). Initial tumor stages may activate the "angiogenic switch" through hypoxia, leading to growth factor secretion and integrin upregulation. $\alpha v\beta 3$ interaction with ECM ligands facilitates new blood vessel formation, aiding tumor sustenance (133). While $\alpha v\beta 3$ has been linked with angiogenic promotion in tumors, evidence from integrin-deficient mice suggests its role, though significant, is not indispensable for angiogenesis (134).

The function of $\alpha 5\beta 1$ in angiogenesis remains to be fully clarified. Its association with the epidermal growth factor receptor (EGFR) (135) and increased expression in angiogenic processes and tumors imply a tumor-promoting capacity (136). Yet, its effect appears to be context-dependent, varying between tumor promotion and inhibition. Recent findings indicate that αv and $\alpha 5$ integrins might work together or interchangeably in vascular remodeling, further complicating our understanding of these integrins' roles in angiogenesis and tumor dynamics (137).

Functionalizing surfaces with peptidomimetics that selectively target specific integrins, such as $\alpha v\beta 3$ and $\alpha 5\beta 1$, provides a powerful approach to advancing our understanding of integrin functions in cellular processes. Being able to directly interact with a particular integrin subtype presents new opportunities to investigate how integrin-mediated signals influence various biological activities, including stem cell differentiation, where the distinct contributions of each integrin subtype are not fully established (107).

The ability to trigger specific integrin activation allows for the customization of cellular responses and the manipulation of how cells interact with biomaterials, particularly beneficial in regenerative medicine. For instance, peptidomimetics hold promise for enhancing the integration of implants with bone. Exploring their effectiveness in regenerating other tissues and organs represents an exciting frontier (107).

Therefore, further research into these molecules is essential. Considering the critical roles that $\alpha\text{v}\beta3$ and $\alpha5\beta1$ integrins play in guiding MSCs toward becoming bone cells, surfaces selective for these integrins could be important in directing MSC differentiation into the osteoblastic lineage. This potential of peptidomimetic-coated biomaterials to foster bone development was already showed in *in vivo* studies in rat calvarial defects (138). However, its potential using other, more complex models, remains to be studied.

Few studies have focused on the osteogenic potential of $\alpha\text{v}\beta3$, as this receptor has traditionally been investigated for its role in bone resorption. In fact, osteoclasts are the cell type with the highest expression of $\alpha\text{v}\beta3$ integrin *in vivo* (107). Table 1 summarizes some of the properties of $\alpha\text{v}\beta3$ and $\alpha5\beta1$ integrins.

$\alpha\text{v}\beta3$	- Expressed in osteoblasts. - Support for the union, extension and formation of focal contacts. - Increased mineralization of the matrix. - Role in bone resorption. - Osteoclasts have the highest expression <i>in vivo</i>.
$\alpha5\beta1$	- Expressed in osteoblasts. - Support for the union, extension, and formation of focal contacts. - Role in the induction of osteogenesis. - Regulates the expression of osteogenic markers. - Regulates alkaline phosphatase (ALP) activity <i>in vitro</i>. - Induces osseointegration of implants and ectopic bone formation <i>in vivo</i>.

Table 1: Properties of $\alpha\text{v}\beta3$ and $\alpha5\beta1$ integrins.

5. Implant Device

An implant is a medical device defined as implantable if it is partially or entirely introduced, surgically or medically, into the human body and is intended to remain there after the procedure. Thus, it is a medical device created to replace, assist, or enhance some missing biological structure (139).

Medical implants are devices or tissues placed inside or on the surface of the body. Many implants are prostheses intended to replace missing body parts. Others deliver medication, monitor body functions, or provide support to organs and tissues. Some implants are made from skin, bone, or other body tissues, while others are made from metal, plastic, ceramics, or other materials. They can be placed permanently or removed once they have served their function and are no longer needed (140).

The risks associated with medical implants include surgical risks during placement or removal, infection, and implant failure. Some individuals may also have reactions to the materials used in implants. Over time, an implant can move, break, or stop functioning properly, necessitating additional surgery to repair or replace the implant (139-141).

The use of metals for replacing structural components in the human body is increasingly common. Among all materials, titanium and its alloys are being preferred over cobalt-chromium alloys, expanding their applications. It is noteworthy that only 10% of the total production is allocated to obtaining titanium in metallic form, with 50% of this used for manufacturing the Ti6Al4V alloy, primarily employed in orthopedic implants. The other 90% is directed towards producing titanium dioxide, utilized as a protective anti-corrosion paint (142).

Being a transition metal, titanium's allure lies in its exceptional corrosion resistance in biological environments, coupled with an outstanding level of biocompatibility shared by very few materials (143).

The high reactivity of titanium complicates the obtaining of pure metal, which is why, for commercial applications, commercially pure titanium (cp) is utilized, with varying levels of impurities in its composition. The 'American Society for Testing and Materials' (ASTM) standardizes cp titanium into four grades with the Technical Standard Number ASTM F67,

defined by the concentration of other elements. Similarly, the mechanical properties of titanium are strongly influenced by its purity grade (144).

However, the Ti6Al4V alloy with the Technical Standard Number ASTM F136, despite not being considered cp, particularly due to the presence of aluminum and vanadium, exhibits significantly superior mechanical properties. Therefore, it is still a commonly used alloy in dental implants, but especially in medical applications involving high levels of stress, where cp titanium cannot be applied, such as orthopedic prostheses, which ensures load transmission to bone tissues over a long time, which is necessary when damaged hard tissues are replaced by prostheses (144). Nonetheless, the Ti6Al4V alloy presents the possibility of releasing vanadium and aluminum, which could lead to a toxic effect (145).

The mechanical properties of the different grades of titanium, corresponding to Standard Number ASTM F67 and F136, can be observed in Table 2 (146).

Ti grade	ASTM	O	Fe	H	C	N	Ti	TS (MPa) ^a	YS (MPa)	E (GPa)	HB
Ti grade 1	F67	0.18	0.20	0.015	0.08	0.03	Balance	240	170	100	120
Ti grade 2	F67	0.25	0.30	0.015	0.08	0.03	Balance	345	275	100	160
Ti grade 3	F67	0.35	0.30	0.015	0.08	0.05	Balance	450	380	100	200
Ti grade 4	F67	0.40	0.50	0.015	0.08	0.05	Balance	550	483	102	250
Ti grade 5 ^b	F136	0.13	0.25	0.012	0.08	0.05	Balance	860	795	115	
Cortical bone								186		5–30 ^c	

^a Mechanical properties requirements for annealed wire with diameter higher than 3.18 mm.
^b The compositional requirement Ti G5 shall meet the following: Al: 5.5–6.5 and V: 3.5–4.5.
^c The bone modulus depending on the type of the bone and the direction of measurement.

Table 2 (146): Standard ASTM number, chemical requirements (max mass%), tensile strength (TS), yield strength (YS), modulus of elasticity (E) and Brinell hardness (HB) of cp Ti, Ti alloy G5 and bone properties. Based on Technical Standards ASTM F67 and ASTM F13.

Cp titanium and Ti6Al4V alloy, primarily used in dental implants and orthopedic prostheses, despite being a biocompatible material with good mechanical properties, lack osteoinductive capacity. This means that, while they support bone growth on their surface, they do not actively promote bone formation. Therefore, strategies are being developed to modify the material's surface and enhance its characteristics with the objective of obtaining a bioactive surface capable of interacting with the bone surrounding the implant. The most commonly used strategies to achieve this bioactive surface involve roughness control (physical modification), the application of a chemical

treatment on the surface (inorganic chemical modification), or the use of bioactive and biomimetic coatings (organic chemical modification) (147, 148).

Recently, the scientific community has been paying particular attention to a strategy that involves the functionalization of biomaterials or biofunctionalization as a tool to enhance and control cell-biomaterial interactions with the objective to allowing to influence the behavior of cells that come into contact with the biomaterial in order to improve the biological response of the organism to the material from which the implant is made (107, 149).

These changes are of particular interest for application in all types of intraosseous medical devices, such as those used in orthopedics, like orthopedic implants, and in dentistry, like dental implants.

Orthopedic implants can be broadly classified into two categories: permanent joint replacements and temporary fracture fixation devices.

Permanent orthopedic implants include hip, knee, ankle, shoulder, elbow, wrist, and finger joints, which are designed to last throughout the patients' lifetime within the human body. On the other hand, temporary orthopedic implants, which consist of plates, screws, nails, wires, and intramedullary pins, are employed to repair broken or fractured bones and are used for a relatively short duration, to allow the bones to heal (150).

Dental implants, in contrast, are inert devices made from non-biological, alloplastic materials that are placed within the jaw to address tooth loss and assist in the replacement of missing orofacial structures which may be due to trauma, tumors, or birth defects. The endosseous implant stands out as the predominant type of dental implant. It is typically shaped like a screw or cylinder and is implanted into a pre-drilled area within the dentoalveolar or basic bone (151).

6. Animal Model

The common choice of rats as experimental subjects is due to the fact that they are easy to breed and relatively inexpensive compared to larger animal models. The emergence of new procedures, such as the reliable reproduction of a disorder, e.g., osteoporosis or diabetes, makes this model especially useful to analyze the potential use of treatments and/or the influence of the disease in the biological response to drugs and surgical devices or procedures (152, 153).

The rat's bone mass gain occurs mostly during the first 6 months of life, although some authors believe that the long bone grows constantly for at least a year with a progressive increase in length (152). With aging, there is a transition from modelling to remodeling which does not occur in all cases equally throughout the bones (153). Female Sprague Dawley rats grow more slowly than male rats and achieve a lower peak in weight than male rats (152).

Continuous bone elongation and varied bone remodeling dynamics in rats are factors that have to be taken into account when addressing human health issues; however it has not yet been possible to find any experimental research that shows either lifelong rat bone development or the species inability to remodel bone (152). If the experimentation starts around 10 months of age, a margin of at least 1 mm from the growth plate of the tibia should be left intact due to this longitudinal bone growth, an issue to be considered in dental implant studies (153). Hormones are also a key parameter in bone research, since at 8 months of age, male rats were found to have 22% greater bone width and 33% greater breaking strength than females in the tibia (154).

The reliable reproduction of a disorder is thus very important in orthopedics and implant dentistry, since osseointegration of an orthopedic screw or a dental implant is a complex process that depends on numerous factors influencing the systemic response to the device implantation into the bone. Systemic disorders like osteoporosis and diabetes are known to affect the success rate in orthopedics and implant dentistry, so the reliable reproduction of those disorders in rat models can be applied to explore ways to overcome these limitations.

The rat tibia, due to the ease of access, relatively good volume, and the flat shape on the medial plate, makes it suitable for surgical bone implantation experiments (155, 156) and it has been used in numerous research studies exploring the effects of implant surface on osseointegration (155-157). A growing number of studies assess the effects on osseointegration of coatings and substances added to the implant surface in both healthy animals (158) and in compromised animals affected by diabetes or osteoporosis (159-165).

The number of implant devices placed in one rat tibia is limited and can differ depending on the type of study. Depending on the number of implants or study conditions, the dimension of the devices must be adapted to minimize surgical trauma. In studies with one implant a nearly human-size implant can be placed (2.0 mm in diameter and 4 to 5 mm in length) and bi-cortical anchorage can be achieved (157, 158, 166, 167). The dimension of the implants in multi-implant protocols should adopt an appropriate implant size (1.5 mm in diameter and 2.5 mm in length) (155, 168).

HYPOTHESIS

H1: The development of a precise surgical technique for implant placement in the rat tibia will result in optimal primary stability and maximum success in the osteointegration process, and will allow for the replication of the surgical technique by other researchers, ensuring consistent and reliable outcomes.

H2: The presence of a peptidomimetic selective for integrin $\alpha 5\beta 1$ improves cellular activity, favoring the process of osteointegration of titanium implants.

H3: The presence of a peptidomimetic selective for integrin $\alpha v\beta 3$ improves cellular activity, favoring the process of osteointegration of titanium implants.

Null Hypotheses

HO1: The developed surgical technique for implant placement in the rat tibia does not have a significant effect on the success of the osteointegration process; therefore, it does not ensure consistent and reliable outcomes, which ultimately inhibits replication by other researchers.

HO2: The presence of a peptidomimetic selective for integrin $\alpha 5\beta 1$ does not improve cellular activity, not favoring the process of osteointegration of titanium implants.

HO3: The presence of a peptidomimetic selective for integrin $\alpha v\beta 3$ does not improve cellular activity, not favoring the process of osteointegration of titanium implants.

OBJECTIVES

General Objectives:

- Evaluate the osteointegration process of newly developed biofunctionalized intraosseous implant surfaces in an animal model, under osteoporosis conditions. (Correspond to the published article)

Specific Objectives:

1. Develop and improve a surgical technique on the rat tibia, so that it can be easily reproduced and used with a high success rate. (Correspond to the published article)
2. Evaluate the process of osteointegration of titanium implants functionalized with a peptidomimetic selective for $\alpha v \beta 3$ integrin in an animal model of osteoporosis, studying the effect on the surrounding host tissues. (present in the article but not detailed).
3. Evaluate the process of osteointegration of titanium implants functionalized with a peptidomimetic selective for $\alpha 5 \beta 1$ integrin in an animal model of osteoporosis, studying the effect on the surrounding host tissues. (present in the article but not detailed)

Note: A second article is in process, specifically to the specific objectives 2 and 3.

MATERIAL AND METHODS.

1. Ethics Committee

All experimental procedures have been carried out in strict accordance with European legislation (Directive 2010/63/EU), Spanish legislation (Royal Decree 53/2013) and the Generalitat de Catalunya (Decree 214/97) on the protection of animals used. for experimental and other scientific purposes.

All the animal procedures and handlings followed the Ethics Committee for Animal Experimentation of the Vall D'Hebron Institut de Recerca, with the following registration number: 72/18 CEEA.

2. Sample and Group Organization

40 Sprague Dawley rats with osteoporosis were used; divided into 2 groups to test 2 different biofunctionalized surfaces. In all rats, 1 biofunctionalized implant (Condition A or B) and 1 control implant (Condition C or D), non-biofunctionalized, will be placed in each rat in different tibias (1 implant per tibia).

- a. Group of 20 Sprague Dawley rats with osteoporosis and 1 implant with Condition A in one tibia and 1 implant with Condition C or D in the other tibia:
 - i. 10 rats: euthanized 4 weeks (Time 1) after implant placement.
 - ii. 10 rats: euthanized 12 weeks (Time 2) after implant placement.
- b. Group of 20 Sprague Dawley rats with osteoporosis and 1 implant with Condition B in one tibia and 1 implant with Condition C or D in the other tibia:
 - i. 10 rats: euthanized 4 weeks (Time 1) after implant placement.
 - ii. 10 rats: euthanized 12 weeks (Time 2) after implant placement.

Two types of biofunctionalized surfaces will be used:

- A. Peptidomimetic with affinity and selectivity for $\alpha v \beta 3$ integrin.
- B. Peptidomimetic with affinity and selectivity for $\alpha 5 \beta 1$ integrin.

A double-blind method was employed for the study, ensuring that neither the surgeons implanting the dental devices nor the team responsible for processing and analyzing the samples were aware of the type of implants being used. This was achieved through a unique coding system for the implants, designated as XYZW, where "X" stood for the individual animal (numbered 1 to 40), "Y" indicated the side of the tibia where the implant was placed ("D" for right and "E" for left), "Z" reflected the type of implant (categorized as A, B, C, or D), and "W" represented the time post-implantation when the evaluation was to occur, that is, the moment of sacrifice (1 for 4 weeks and 2 for 12 weeks).

3. Biological Materials and Medical Devices

3.1. Biological materials

40 ovariectomized Sprague Dawley females and 5 sham animals were purchased from an animal supplier laboratory. Based on vendor recommendations, three months after neutering, the experiment started. This waiting period ensured the disappearing of sexual hormones. Mean weight 484 g and mean age 12 weeks. Osteoporosis was confirmed at the time of surgery based on micro-computed tomography (micro-CT) bone analysis, which reflected an average of 20% bone loss compared to sham group.

3.2. Implants

Grade 3 titanium implants (2 mm length, 1.8 mm diameter) were provided by Soadco S. L. (Andorra). All implants were ultrasonically cleaned in cyclohexane (3 x 2 min), acetone (1 x 1 min), Milli-Q water (3 x 2 min), ethanol (3 x 2 min), acetone (3 x 2 min), and dried with nitrogen gas. Prior to implantation, the implants were first soaked in Milli-Q water and then sterilized by immersion in 70% ethanol (v/v) for 10 min. After this time, the implants were transferred to sterile Eppendorf tubes and kept under sterile conditions until the surgery.

4. Surgical Procedure

4.1. Anesthesia/pharmacology and preparation of animals

Presurgical analgesia was conducted using buprenorphine 0.05 mg/kg (Buprecare 0.3 mg/mL, Animalcare, York, UK) and meloxicam 2 mg/kg (Metacam 5 mg/mL, Boehringer Ingelheim, Ingelheim am Rhein, Germany). Both administrations were done through subcutaneous injections 10-15 minutes before the surgical procedure starts.

Intrasurgical anesthesia was performed with inhaled isoflurane (Isoflo, Le Vet Pharma, Oudewater, Netherlands), at a ratio of 5% in fresh air during the induction phase and 3% for maintenance. The induction of anesthesia was conducted in a specific chamber for rats, while the isoflurane supply during the surgical procedure was guaranteed using a conical adaptation for the rat nose.

4.2. Preparation for Surgery

Once anesthetized, the body temperature of the animal was measured using a rectal probe. An electronically controlled heating pad gave heat support during the surgical procedure.

The skin of the knee should be completely clean in order to work in a sterile way. For this purpose, the hair was trimmed with an electric shaver and the remanent fur removed with hair remover cream.

An aseptic surgical field was obtained cleaning the skin of the knee with a rotation of iodine (Betadine, Mylan, WV, United States) and ethanol at 70% (v/v), using sterile swaps. The cleaning movements are always from inside to outside of the theoretical incision line, and never going back again. A minimum of three consecutive rotations are required (iodine-ethanol-iodine).

For the Isolation of the operating field, a sterile surgical drape was fenestrated over the animal, to leave the leg exposed through the central fenestration (Figure 7).



Figure 7: Surgical field.

4.3. Surgery

4.3.1. Bone exposure

A full thickness incision (Figure 8) with an average length of 1 cm vertical along the proximal border of the antero-medial face, in the metaphysis region, was done to expose the bone (Figure 9).



Figure 8: Leg stabilization; Surgical incision.



Figure 9: Bone exposure after the surgical incision.

To perform this incision, is necessary to pull the skin against the underlying bone and hold the leg firmly while making the incision. This will keep the skin incision in the desired location and help create a clean incision. The cleaning of the surgical window should be made with a compress soaked with saline solution, as light bleeding is expected.

The rat skin is thin and sagging or loose. Stabilization of the skin is crucial and required.

Total detachment of the region was performed with small periosteal elevators.

Exposure of the bone until identification of the insertion of the *tibialis cranialis muscle*, the *gracilis* and the gastrocnemius lateral head muscle (Figure 10) in the posterior border of the medial aspect of the tibia.

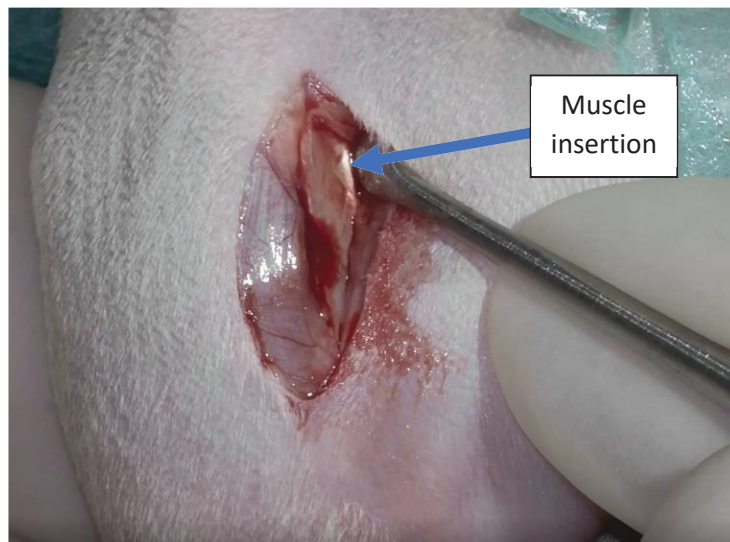


Figure 10: Identification of the muscle insertion of the *tibialis cranialis muscle*, the *gracilis* and the gastrocnemius lateral head muscle, avoiding any muscle injury.

4.3.2. Drilling process

The correct region to initiate the perforation is located between the proximal tibial crest and the posterior limit of the medial face of the tibia bone, contiguous to the insertion of the *tibialis cranialis muscle*, the *gracilis* and the gastrocnemius lateral head muscle, avoiding any muscle injury. Also, the correct location should be at $5 \text{ mm} \pm 2 \text{ mm}$ from the tibial plateau.

All drilling processes were made with a maximum of 150 rpm (rotations per minute) under saline solution irrigation at a temperature close to 20°C with a surgical electric motor with a 20:1 reduction contra-angle. Only two drills were required.

The drilling process started with a lance pilot drill (Figure 11) at a depth of 2.4 mm under saline solution irrigation.

A second drill was used (Figure 12) with a twist design, reaching a depth of 2.4 mm with a diameter of 1.6 mm under saline solution.



Figure 11: Lance pilot drill.



Figure 12: Twist drill.

4.3.3. Implant placement

The implant was inserted in an intermediary piece (Figure 13) connected to the 20:1 reduction contra-angle.

The cleaning of the residual chemical sterilant was made by rotating the implant in the contra-angle connected to the surgical motor, with saline solution irrigations simultaneously for 10 seconds (Figure 14).



Figure 13: Implant inserted in the intermediary piece.



Figure 14: Cleaning of the residual chemical sterilant.

Placement of titanium implants (2 mm lengthwise and 1.8 mm in diameter) was done with the intermediate piece at 20 rpm with measurement of torque in real time (Figures 15 and 16).

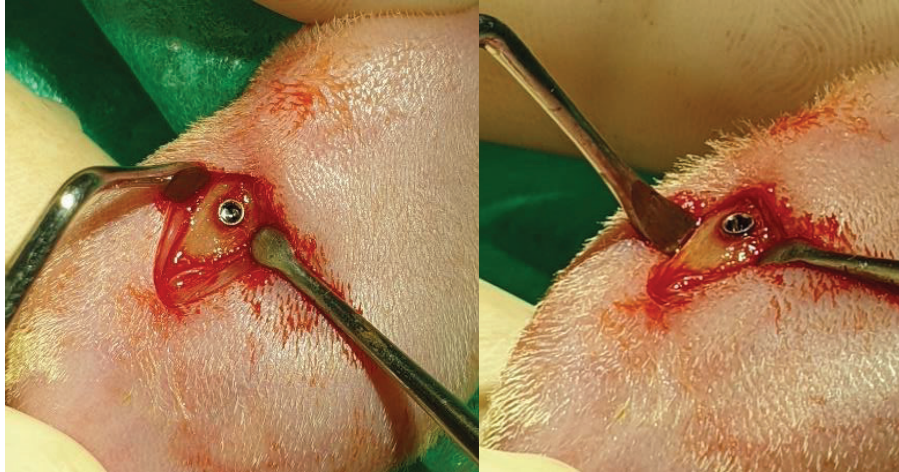


Figure 15 and 16: Titanium implant placed.

4.3.4. Wound closure

Simple internal suture to approximation of muscle tissue was made with 4/0 monofilament synthetic resorbable suture (Glyconate) (Figure 17 and Figure 18).



Figure 17: Internal (muscle) suture procedure.



Figure 18: Internal (muscle) suture completed.

Intradermal suture for skin approximation was made with 4/0 monofilament synthetic resorbable suture (Glyconate) (Figure 19 and Figure 20).

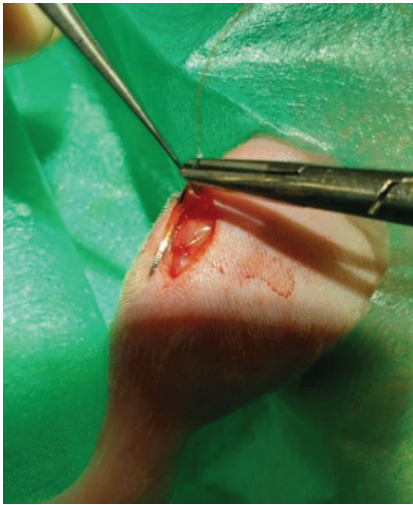


Figure 19: Intradermal suture of the skin procedure.



Figure 20: Intradermal suture of the skin completed.

4.4. Postsurgical Micro-CT scan

Once the surgery was finished, a micro-CT scan was performed to confirm the correct location of the implant. The distance between the proximal aspect of the implant and the surface of the tibial plateau was measured. This value will be helpful for the standardization of the technique.

The micro-CT study was performed with a Quantum Fx micro-CT (Perkin Elmer, MA, US). The acquisition parameters used were Field of View 5 mm, spatial resolution 0.0001 mm³, 50 kV, 200 μ A and acquisition time of 3 minutes (Figure 21).

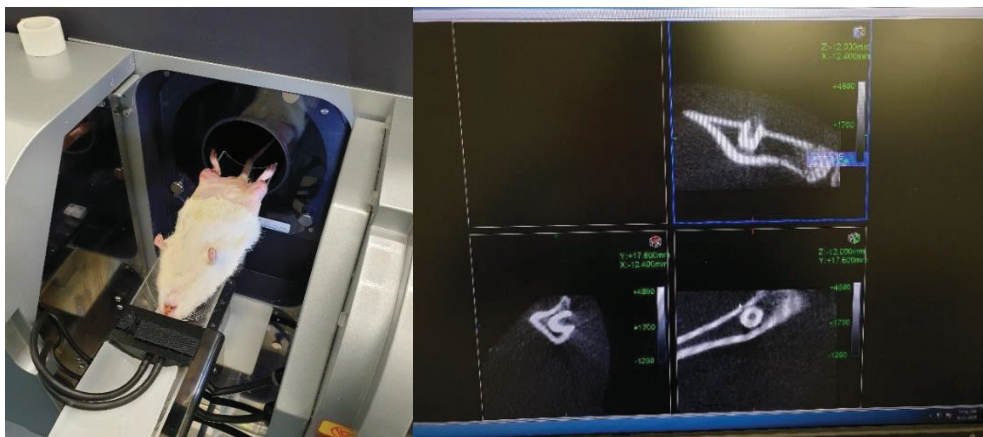


Figure 21: Positioning and accommodation of the rat to perform the Micro-CT scan.

5. Post-Surgical Animal Handling Phase

5.1. Postoperative care

After the imaging acquisition, the rat was moved back to the cage and controlled until its complete recovery. This takes 5 to 10 minutes approximately.

8 hours after the end of the surgery, a new dose of analgesia was administrated to the animal, using the same dose and routes as previously described. This dosing pattern of analgesia was repeated for 48 hours.

A daily examination of the surgical wound was done, checking for wound infections, suture release or dehiscence. The sutures were removed 15 days after surgery.

5.2 Euthanasia

After the implantation time (either 4 or 12 weeks), the euthanasia of the animals was carried out in a CO₂ chamber.

5.3 Micro-CT scan analysis

A postsurgical imaging study was performed at the Vall D'Hebron Institut de Recerca to assess the correct placement of the implants after euthanasia. Representative images are shown in Figure 22. Moreover, the analysis of micro-CT data allowed for the calculation of bone-implant contact (BIC).

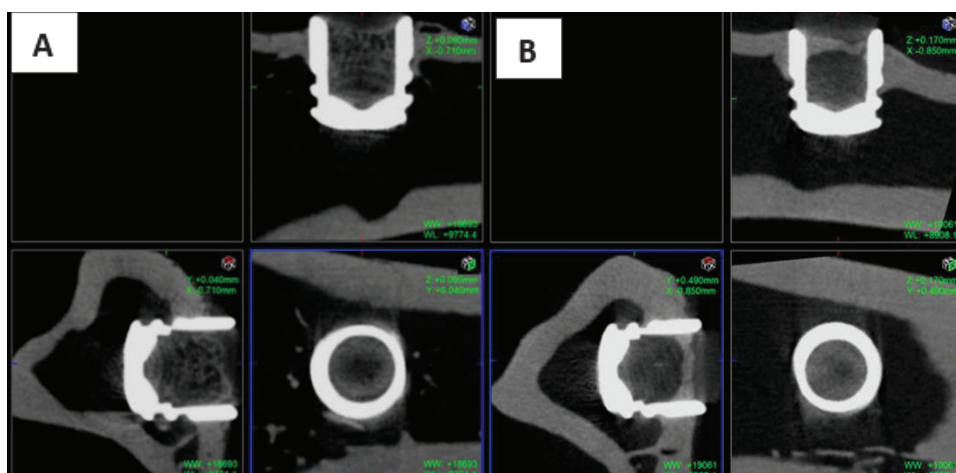


Figure 22: Representative postsurgical micro-CT images of titanium implants placed in the tibiae of osteoporotic rats after A - 4 weeks and B - 12 weeks of implantation. Each image is divided in three sections, corresponding to the x-, y- and z-views, respectively.

The images acquisition was conducted using the parameters previously described, and the analysis of the BIC was done dividing the bone area (mm²) by the implant area (mm²) calculated at the cortical region, adapting a protocol already described in the literature (169).

6. Histological and Histomorphometrical Analysis

6.1. Sample preparation

This section details the procedures undertaken for preparing the samples for both quantitative and qualitative analyses. It encompasses the entire workflow from sample collection, through processing and preparation stages, to the final setup for analysis. The methodologies applied aim to maintain the integrity of the samples while optimizing them for the most accurate and reliable analytical outcomes. Histology and histomorphometric analysis were carried out at the BBT group (Biomaterials, Biomechanics and Tissue Engineering) of the Universitat Politècnica de Catalunya.

6.1.1 Dehydration process

Sample preparation begins with the dehydration process. To this end, the samples were immersed in solutions with an increasing concentration of ethanol (30, 50, 70, 96 and 100 % v/v) until reaching 100%, with the aim of dehydrating the bone tissue and being able to place it later in resin. The samples were covered and placed in a box on the orbital shaker to achieve maximum possible contact between the sample and the ethanol for 48 hours at 50-100 rpm.

Once all the samples have been dehydrated, a cut is made with the band saw of the cutter (Figure 23) in each of the bones approximately 2 millimeters from the implant. The ethanol in which the samples are found is preserved.

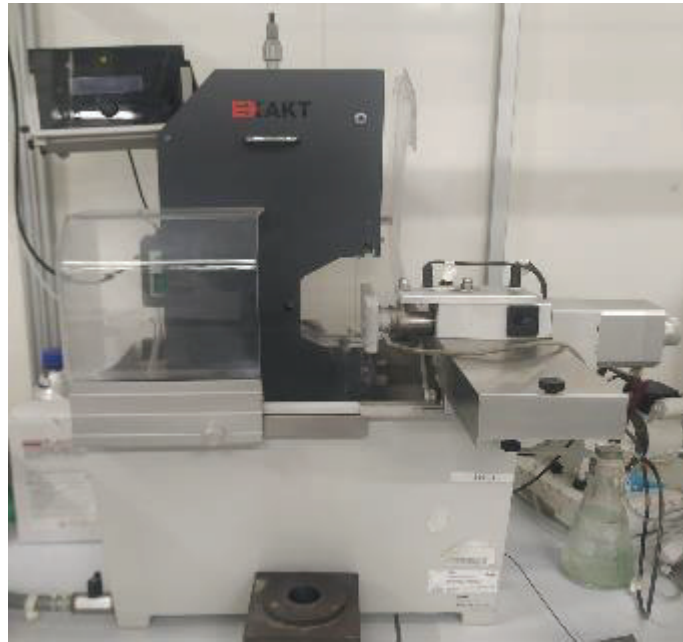


Figure 23: Cutter with belt saw.

The bone part that does not contain the implant was discarded. The part containing the implant was instead returned to its corresponding container in ethanol. The samples were kept in an orbital shaker until the next step of the sample preparation process was carried out.

6.1.2. Resin infiltration process.

The next step in the process is resin infiltration, which was performed in ethanol solutions with increasing resin concentration (30, 50, 70, 100%). The resin must have between 7-10 grams of catalyst for the resin to polymerize. For this study, the catalyst used was benzoyl peroxide (*Luperox*). Approximately 7 grams of catalyst were weighed, placed in the resin container, and carefully shaken so that everything was properly integrated.

It should be noted that the handling of the resin must be done under the laboratory's fume hood, and that each time the resin concentration is increased, the samples must be kept in the orbital shaker for 48 hours. The samples in the shaker were put in a cardboard box and covered with aluminum foil to prevent light from reaching the samples and the resin from polymerizing during the infiltration.

Ethanol was removed from the sample containers, being careful not to spill the samples or the paper with the code that accompanies it. Next, the containers were filled with 10 mL of the solution that was prepared for each of the resin concentrations, starting with the 30% resin concentration. The jars were covered and left in the orbital shaker as explained before.

After the first 48 hours, the change to 50% resin and subsequent concentrations was made following the same procedure as before.

The amount (mL) of resin and ethanol used to obtain the 800 mL of resin-ethanol mixture for the 80 samples (10 mL per sample) according to increasing concentrations is shown below (Table 3).

Resin concentration	Resin (mL)	Ethanol (mL)
30%	240	560
50%	400	400
70%	560	240
100%	800	-

Table 3: Amount (mL) of resin and ethanol used for the different resin concentrations.

Once the samples were at a 100% resin concentration, instead of placing them in the shaker, they were placed in a vacuum desiccator for a minimum of 24 hours.

To place the samples in the vacuum desiccator, the samples were first grouped into groups of 4, since this will be useful for the next step of the process due to the 4-sample capacity of the polymerizer. The lids of the containers were removed, wrapped in aluminum foil, and small holes were made in the upper part to ensure that a vacuum was created inside the container (Figure 24-A). Finally, the samples were placed in the desiccator (Figure 24-B).

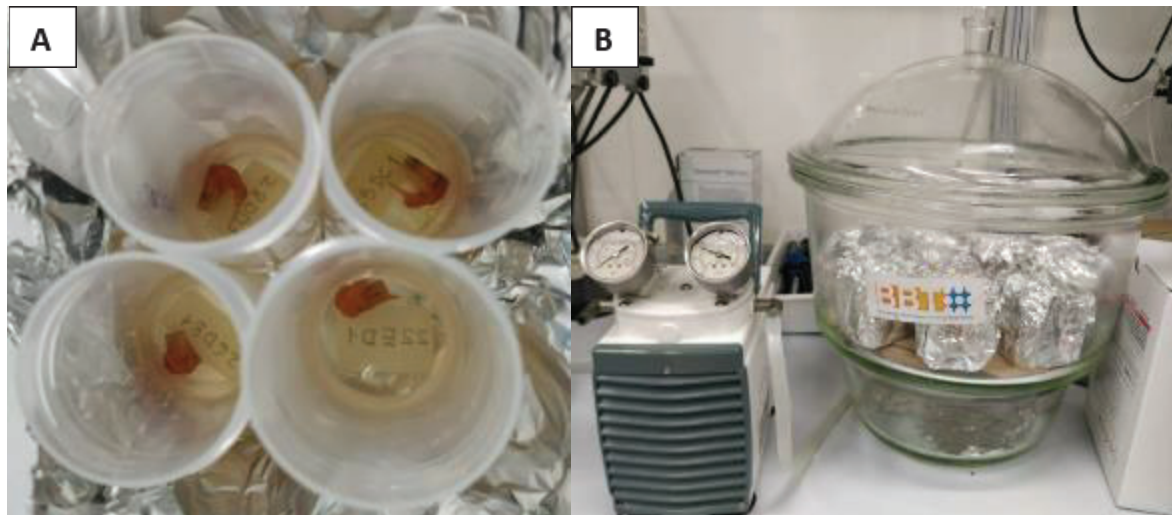


Figure 24: A - Samples in groups of 4 in aluminum foil to put in the vacuum desiccator. B - Vacuum desiccator with samples inside.

6.1.3 Polymerization process

After leaving the samples 24h in the vacuum desiccator, which seeks to maximize the infiltration of the resin into the samples, the polymerization of the samples can be done. To do this, the first step is to remove a group of 4 samples from the desiccator and carefully unwrap them.

Two molds of two samples can be inserted into the polymerizer at a time, allowing 4 samples to be polymerized. (Figure 25-B). Each sample was placed in the polymerizer mold with the region of interest (part where the implant is) at the bottom. In addition, the resin liquid must be poured out and the corresponding code paper must be placed to identify the sample. The paper was placed on the side of the mold to avoid polishing or cutting it later. In this case, a piece of Pasteur pipette was used as a support to keep the sample upright inside the mold (Figure 25-A).

The samples were left in the polymerizer for 1 cycle of 24 hours (12 hours of UV light + 12 hours of white light). The procedure is repeated until all 80 samples were polymerized.

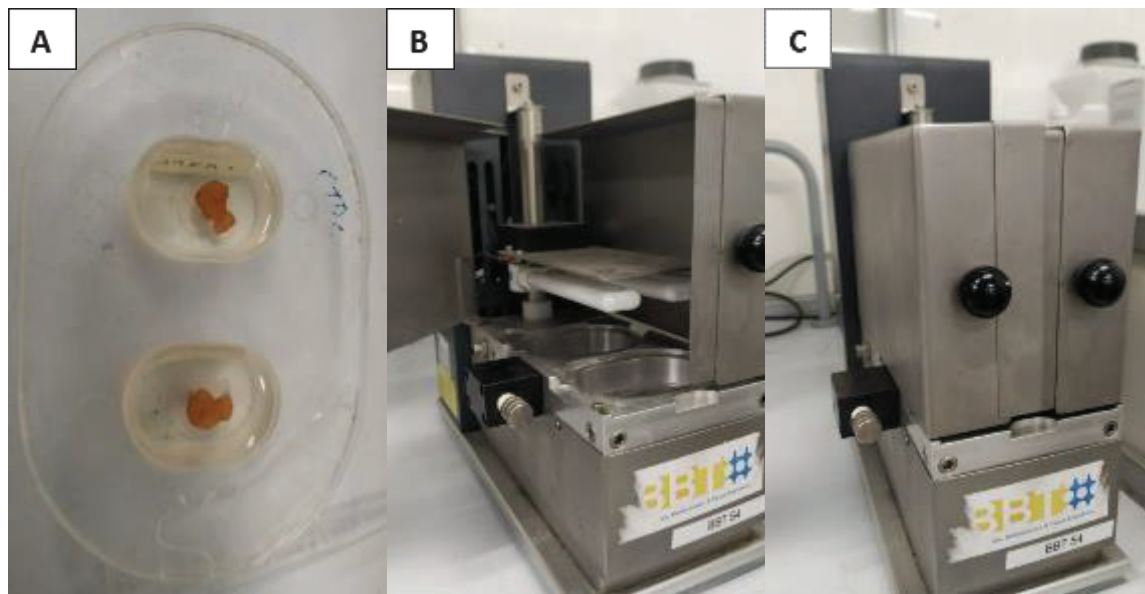


Figure 25: A - Mold with the samples to introduce into the polymerizer. B - Interior of the polymerizer. C - Exterior of the polymerizer.

Once the cycle of polymerization was finished, a solid block of resin was obtained with the implant inside. With the samples unmolded, the next step can begin, which consists of polishing the samples.

6.1.4. Polishing process

First, each of the polymerized and demolded samples was taken and hooked with double-sided tape to a slide object holder on the face of the implant, to polish the burr derived from the polymerization process. The sample code was marked on the back of each slide with a permanent marker.

The samples were polished for approximately 1 minute with a silicon carbide (SiC) P800 disc.

Once the burr was polished, the arrangement of the samples on the slide was changed so that it can be cut with the cutter. Now the polished part of the burr was hooked against the slide, thus leaving the implant on top. It was marked laterally with a permanent marker where the implant was located in the resin block to cut with the cutter in that area and be able to obtain two halves when the cut was made.

Both halves were marked with the sample code and the half was selected where there was better visibility of the implant, and in which the implant had better characteristics. The other half was saved in case it was needed in the future.

Then, half of the sample chosen was attached to its corresponding slide. Each sample must be polished on the histology polisher (Figure 26) with P1200 and P4000 SiC discs for the necessary time until the desired result is achieved.



Figure 26: Polishing machine with SiC disc.

6.1.5. Cathodic sputtering or sputtering process

The *sputtering* process is a standard method for preparing non-conductive samples prior to observation by SEM. Since the samples in this study are initially non-conductive, they must go through a prior sputtering process.

First, a circular carbon adhesive was hooked onto a pin and the polished resin block containing the sample was placed on one side (with the implant facing up). Next, an

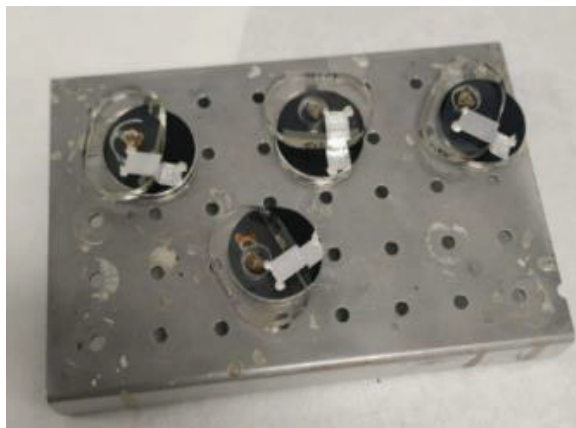


Figure 27: Samples prepared with carbon adhesive disc, belt and painted with silver paint.

aluminum tape was hooked between the sample and the carbon adhesive so that there was conductivity between both parts. Finally, the 4 corners of the tape were painted with silver to further secure and improve conductivity (Figure 27).

Before introducing the samples into the sputtering machine, the graphite tip was manually prepared and placed in the corresponding location on the machine (Figure 28-A). The settings were configured (*multi-pulse, no. evap. pulses = 7*), the samples introduced, and the action executed. Due to the arrangement of the samples on the pins, there was a capacity of 4 or 5 samples per sputtering process. Sputtering was carried out correctly if sparks were visible.

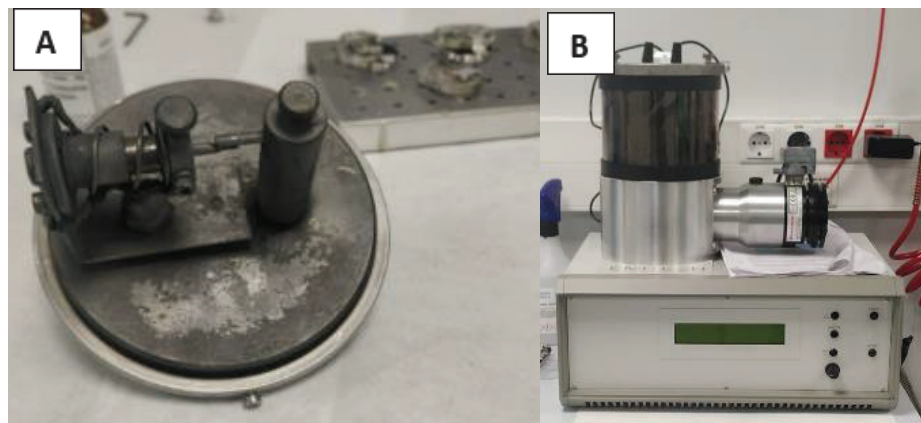


Figure 28: A - Graphite tip. B - Cathodic spraying machine (sputtering).

6.2. SEM imaging process

6.2.1. Scanning electron microscope (SEM)

The scanning electron microscope (SEM) is a type of electron microscope that uses electrons instead of visible light to form an image, currently giving it the highest possible image resolution.

The resolution level of a microscope is limited by the wavelength of the medium used to form the images. Visible light has a fixed wavelength between 200-500 nm. Electrons have the same type of wave structure as visible light, but their wavelength can vary with speed. By increasing the acceleration voltages, the wavelength is smaller and, consequently, a higher level of resolution is obtained (170). The SEM is made up of an

electron gun that acts as an emitter; a lens system, responsible for focusing the electron beam produced by the emitter and converting it into a beam with a very small cross section when it reaches the surface of the sample; a scanning system, to scan the surface of the sample; one or several detection systems, which allow capturing the result of the interaction of the electron beam with the sample in the form of an electrical signal, and various systems that allow observing the result of the insertion in the scanning process (Figure 29) (170).

In addition, for the assembly to function properly, certain vacuum requirements must be met to avoid interactions between electrons and gas molecules.

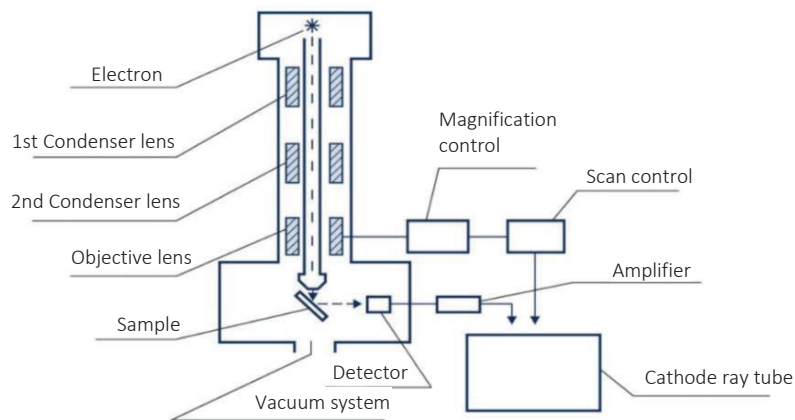


Figure 29 (170): Operating diagram of a bar electron microscope (SEM).

To generate the image, the SEM produces a focused and highly accelerated beam of electrons at the top of the microscope that follows a vertical path. The beam travels into a vacuum through electromagnetic fields and lenses, which focus the beam downward toward the sample (Figure 30) (171). Electrons accelerated in SEM carry significant amounts of kinetic energy, and this energy is dissipated as a variety of signals produced by electron-sample interactions when the incident electrons decelerate in the solid sample. Secondary electrons and backscattered electrons (BSEs) are commonly used to image samples: secondary electrons are more valuable for showing morphology and topography in samples and BSEs are more valuable for illustrating contrasts in composition in multiphase samples (170).

Detectors collect these x-rays, BSEs, and secondary electrons and convert them into a signal that is sent to a display. The resulting image will have varying contrasts due to differences in sample thickness, atomic number, crystal orientation, and wave interference.

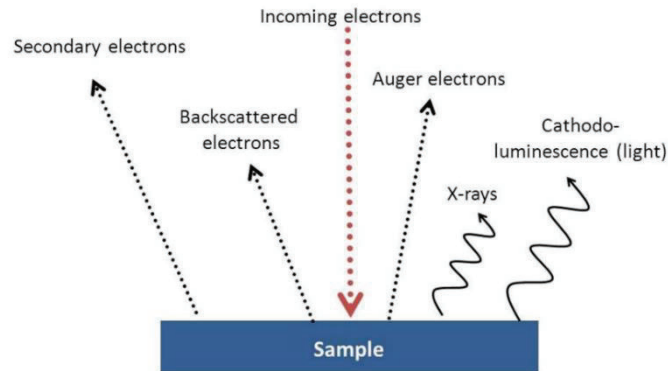


Figure 30 (171): Basic diagram of the different signals generated after interaction between beam of electrons and sample.

6.2.2. Imaging

Through the sputtering process, the samples are conductive and, therefore, ready to be processed by SEM (Figure 31). First, the SEM tray must be removed to arrange the pins that contain the samples. The implant was always oriented in the same way (in this case upwards, in a U shape) and the tray plate was adjusted 2 mm below the sample that protrudes the most.



Figure 31: Scanning electron microscope (SEM).

All images were obtained using the high voltage = 20 V, spot size = 3.0 and the magnifications were set to 500x. SEM observation of the samples was carried out by a sequential scan of the surface, acquiring high-magnification images that were then

merged (i.e., *stitching*). All images obtained (Figure 32) must be encoded with their corresponding code and adjusted in brightness and contrast to obtain optimal results.

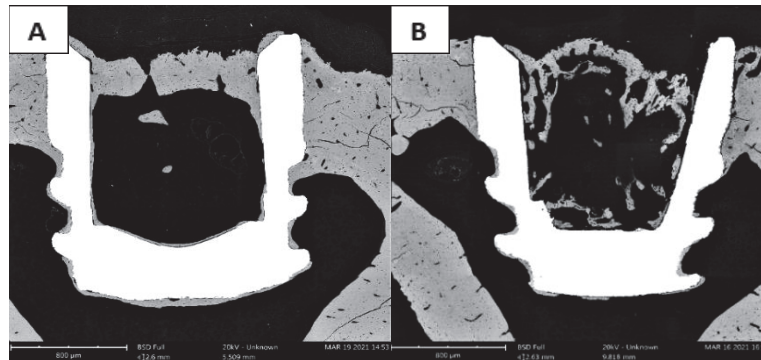


Figure 32: Representative images obtained using SEM. A - Sample 1DA2. B - Sample 28DD1.

6.2.3 Histological staining process

Once all the samples were visualized by SEM, the next step was to perform the histological staining. Through sample selection based on quality, and to ensure that the data analyzed presented the highest level of integrity, quality, resolution, and representative of all samples, from the 80 available samples, 39 were selected with the help of Dr. M^a Cristina Manzanares, member of the Human Anatomy and Embryology Unit of the University of Barcelona. This sample size is appropriate for the conditions being analyzed and is consistent with existing literature. Below is the code of the samples that were stained (Table 4).

Time 1	Time2	
21EC1	1DA2	11DC2
24DD1	1EC2	12DD2
25DA1	2DB2	12EB2
26DB1	2ED2	13DA2
26ED1	3DC2	15DC2
27DC1	3EA2	16EB2
28DD1	5DA2	17DA2
30DB1	5EC2	19DC2
32EB1	6DB2	20EB2
33DA1	6ED2	
34ED1	7DC2	
35EA1	7EA2	
37DA1	8DD2	
38ED1	10DB2	
39DC1	10ED2	

Table 4: Samples selected for histological staining.

The first step for the staining was to remove the pin and the aluminum strip from the selected samples. They were then hooked onto a slide previously marked with the code of each sample and polished with P4000 SiC discs to remove the rest of the silver paint and the carbon coating.

The samples were then polymerized for 15 minutes with a monomer (*Technovit*) in a polymerizing lamp (Figure 33-A, B). To do this, a drop of monomer was deposited in the area of the sample where the implant was located, the slide was introduced into the polymerizing lamp and another slide was placed on top, to ensure that when applying pressure during the polymerization the sample is kept between both slides (Figure 33-C, D).

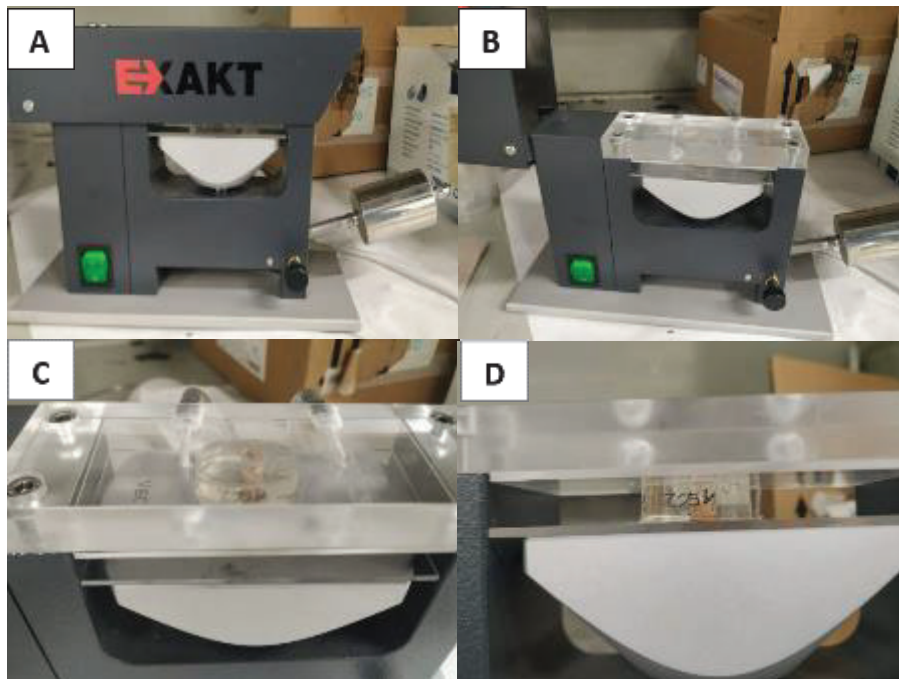


Figure 33: A - Closed polymerizer with the sample placed. B - Open polymerizer with the sample placed. C and D - Sample 1EC2 with the two slides in the polymerizer.

After 15 minutes, the sample was removed from the polymerizer, and the lower slide was removed so that the implant was hooked against the upper slide. Next, a cut was made with the cutter at a distance of about 200 μm from the slide. The cut part of the sample was saved, and everything was labeled. The part of the sample that remained attached

to the slide was polished with P1200 and P4000 SiC disks until reaching a thickness below 100 μm .

To be able to stain several samples at the same time, a hole was made at one end of the slide. The samples were placed on a bar, separating each one with a washer so that there was a small space, and the stain can soak in well (Figure 34).



Figure 34: Samples polished to less than 100 μm and placed on the bars to facilitate the staining process.

The procedure followed to stain the samples using the Masson-Goldner staining kit (*Sigma-Aldrich*) is explained step by step below:

1. First, the yellowish SF light green was placed in an oven at 60 °C to warm it up, since it will be used in the last steps of the staining.
2. It is important to clean the surface of the samples with a 50% ethanol-acetone solution (v/v) to make the resin more porous before starting the staining process.
3. Once the samples were clean, they were stained for 15 minutes in Weigert's ferric hematoxylin.
4. Then, the dye samples were removed and cleaned for 5 minutes with water.
5. The samples were then stained for 7 minutes with Masson's solution (Goldner).
6. After the corresponding time, the samples were cleaned with 2% acetic acid.
7. They were then stained with phosphomolybdic acid orange G for 5 more minutes.
8. The samples were cleaned again with 2% acetic acid.

9. They were stained next for 15 minutes in yellowish SF light green at 60 °C (previously placed in the oven).
10. After 15 minutes, the samples were briefly bathed in distilled water to remove all the acetic acid.
11. Once the entire staining process was finished, all the samples were dried and removed from the bar to be held individually again (Figure 35-A).
12. Once all the samples were dry, a drop of DPX (Distyrene, a plasticizer, and Xylene) was deposited on top of each sample with a coverslip (Figure 35-B). Samples were left to dry until the next day to be able to observe the samples by optical microscopy.

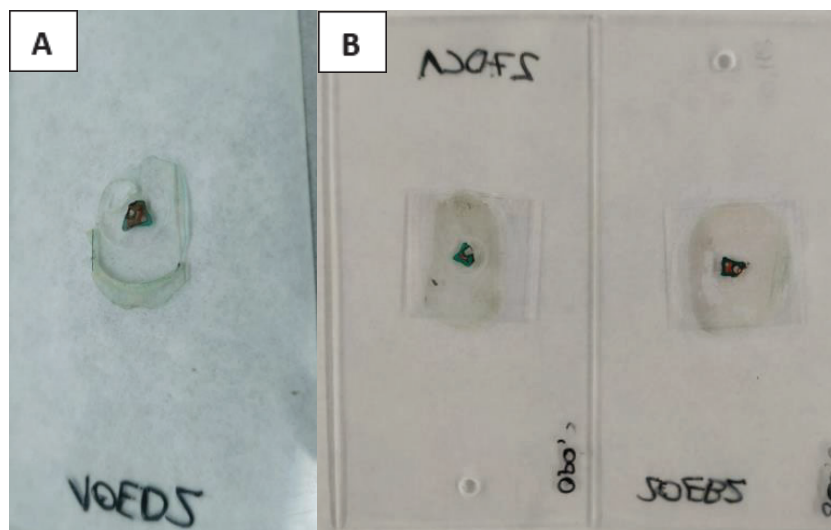


Figure 35: A - Stained sample 10ED2. B -Sample 27dc1 and 20EB2 with DPX drop and coverslip.

Once the explanation of the sample preparation process has been completed, the procedure followed to perform the quantitative analysis (Section 6.3), the statistical analysis (Section 6.4) and the qualitative analysis of the samples (Section 6.5) is explained.

6.3. Quantitative analysis – BIC calculation

The bone-implant contact (BIC) for each histological section is calculated by quantifying the length of the implant surface in contact with the bone tissue, compared to the total length of the implant surface, expressed as a percentage. To do this, SEM images are analyzed with "*ImageJ*", an open-source image processing software.

Firstly, the outer perimeter of the implant was considered as the maximum possible BIC (100%). To do this, with a previously binarized image (Figure 36), the internal part of the implant (green color) is subtracted from the total perimeter (yellow).

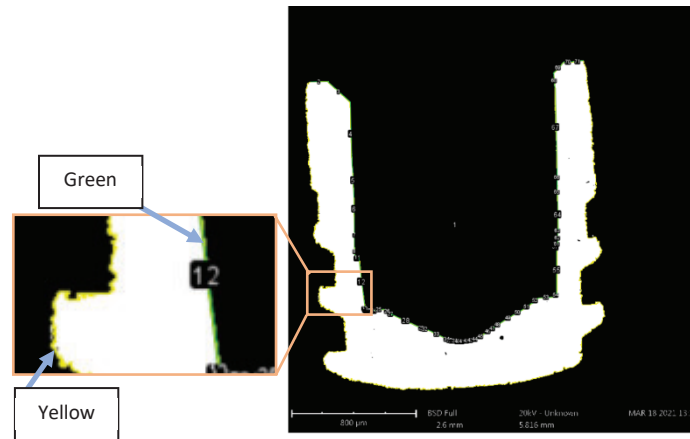


Figure 36: Determination of Bone-Implant Contact (BIC) based on SEM of the sample 2DB2. External perimeter calculation of the implant with binarized image. Exterior perimeter = total perimeter (yellow) – interior perimeter (green).

Secondly, the distance where there is contact between the bone and the implant was obtained. To achieve this, the original image (not binarized) was used and the external implant area where there is no contact with the bone calculated (red color). In this way, the area without contact with bone is subtracted from the previously calculated outer perimeter to obtain the region where contact does exist (Figure 37).

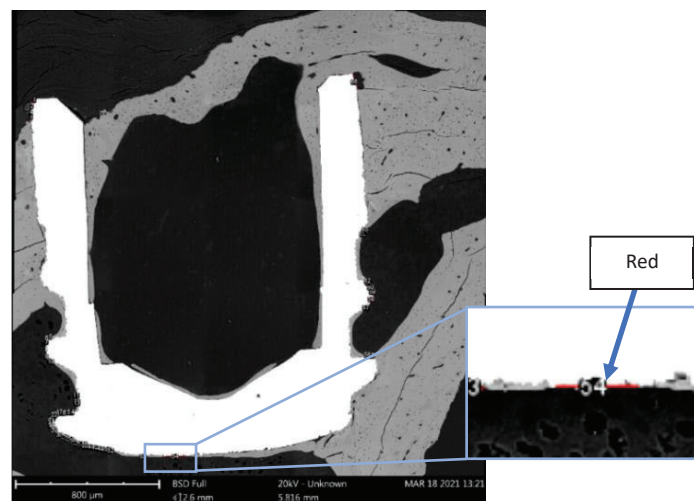


Figure 37: Determination of Bone-Implant Contact (BIC) based on (SEM) of sample 2DB2. Calculation of the implant region without contact with bone.

The BIC (%) is finally calculated as the ratio between the section where there is contact with the bone and the total external perimeter of the implant.

Below is the BIC calculation of the 2DB2 sample as an example.

Table 5 shows the calculation of the BIC (%) of the 2DB2 sample. On one hand, the value of the exterior is obtained by subtracting the sum of the interior perimeter values (4,848 mm) from the total perimeter of the implant (13,763 mm), resulting in a value of 8,915 mm corresponding to the value of the outer perimeter of the implant. On the other hand, the value of “total bone” (7.438 mm) is obtained by subtracting the “total No Bone” value (1.477 mm) from the previously obtained external perimeter value (8.915 mm).

Knowing this, the BIC is calculated as the quotient between “total bone” and “exterior” and multiplying the result obtained by 100 to obtain the result in percentage, thus obtaining a BIC of 83.43% for the 2DB2 sample (Table 5).

Sample code	2DB2
Total (yellow)	13,763
Internal (green)	4,848
External	8,915
Total No Bone	1,477
Total Bone	7,438
BIC (%)	83,4324173

Table 5: Example BIC calculation of 2DB2 sample.

During the SEM analysis and BIC calculation, it was observed that some implants were in contact with two cortical regions (such as the sample 2DB2, explained above). This observation was defined a “bicorticality” and is shown in Figure 38. As this could likely affect the calculation of BIC, it was decided to recalculate the BIC for those samples that presented bicorticality, using only half of the implant to perform the calculations (the part of the implant not presenting bicorticality).

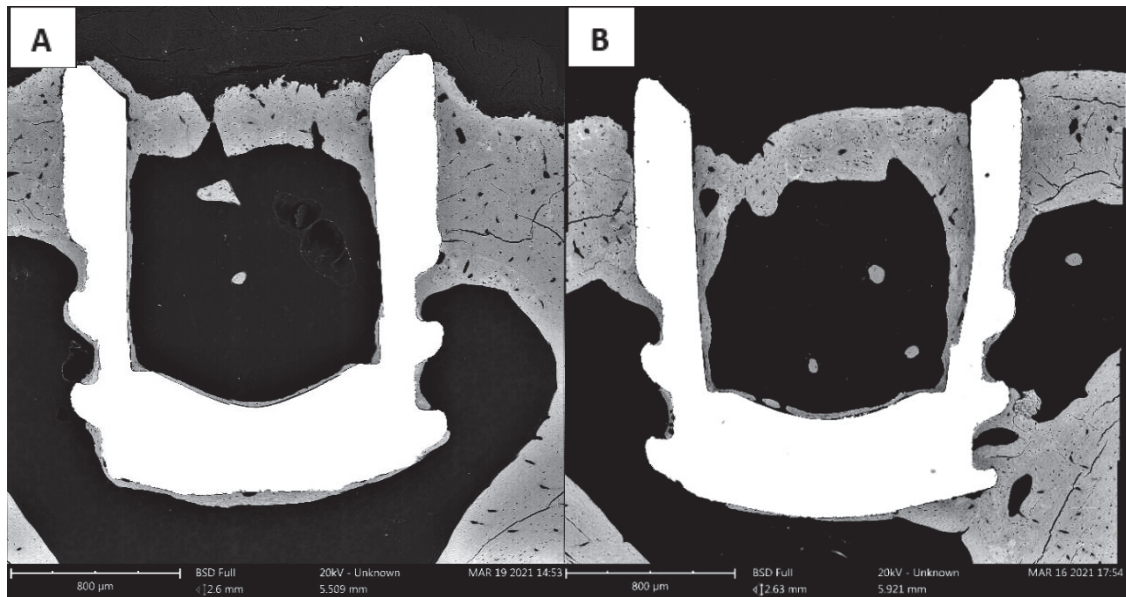


Figure 38: Example of a sample that presents bicorticality on one side of the implant and one that does not. A - Sample 1DA1 without bicorticality. B - Sample 1EC2 that presents bicorticality on one side of the implant.

In this way, the same procedure as described above was followed to calculate the BIC, adapting the calculations to fit the half of the implant that does not present bicorticality, as shown below.

The BIC calculation of sample 2DB2 (Figure 39) is shown again as an example of a sample that presents bicorticality on one side of the implant. Since the sample presents bicorticality on the right side, the BIC (%) is calculated from the left side of the sample.

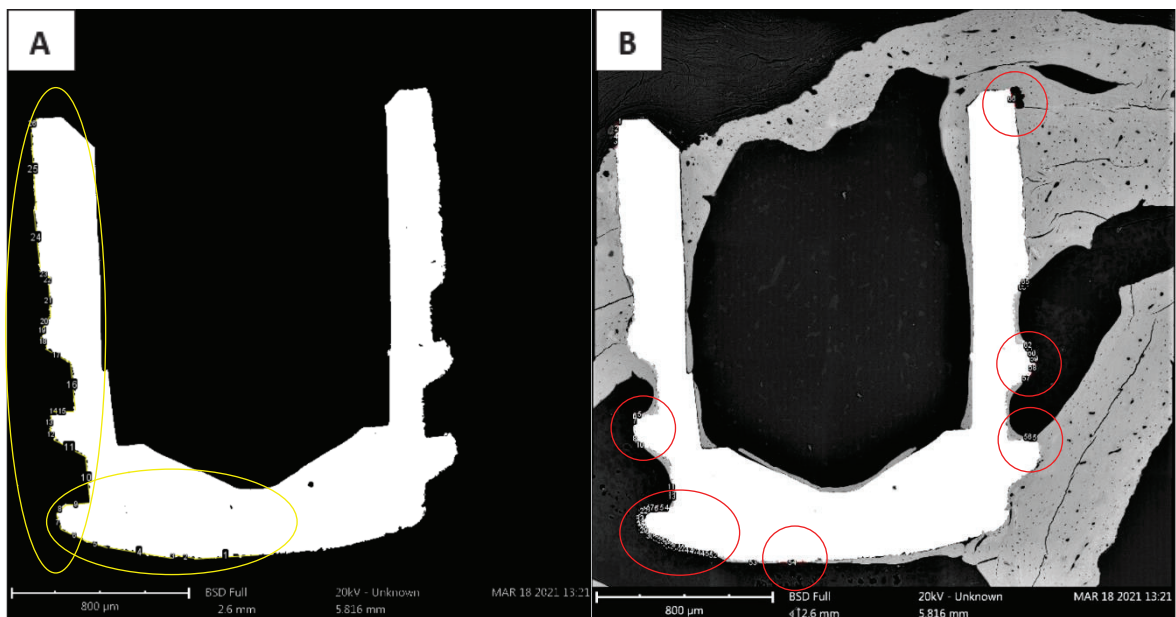


Figure 39: Determination of bone-implant contact (BIC) based on SEM of sample 2DB2. A - Calculation of half the outer perimeter of the implant with binarized image (yellow). B - Calculation of the implant region without contact with bone (red).

Table 6 shows the calculation of the BIC (%) of the 2DB2 sample considering its condition of bicorticality on one side of the implant. On the one hand, the value of the outer half is obtained by adding the values in the “outer half” column, thus obtaining a value of 3.149 mm. On the other hand, the value of “total bone” (2.076 mm) is obtained by subtracting the value of half of the external perimeter previously obtained (3.149 mm) with the sum of the areas of the external implant where there is no contact with bone called “total No bone” (1,073 mm).

Sample code	2DB2
Outer Half	3,149
Total No Bone	1,073
Total Bone	2,076
BIC (%)	65,9256907

Table 6: Example BIC calculation for a sample that presents bicorticality.

Knowing this, the BIC is calculated as the ratio between “total bone” and “exterior”. Multiplying the result obtained by 100 to obtain the result in percentage, a BIC of 65.93% is obtained for sample 2DB2 (considering bicorticality) compared to the value of 83.43% (without considering bicorticality) previously obtained for the same sample.

The procedure explained to calculate the BIC of the entire implant (without considering bicorticality) is applied for the 80 samples. For samples that present bicorticality on one side of the implant, the BIC of half of the implant is also calculated.

6.4. Statistical analysis

As previously mentioned, the study was carried out with 40 rats, each having two implants (one per tibia), thus resulting in a total of 80 samples.

To analyze the BIC values (%), three groups were made: the first considering the BIC values of the samples in their entirety, without taking into account bicorticality (Total);

The second grouping considering only the values of those samples with bicortical support on one side of the implant (Bicortical) and, finally, the third grouping considering all the samples but using the corresponding BIC value according to whether they presented bicorticality on one side of the implant or no (Total-bicortical).

In this way, for the Total and Total-bicortical groups, the sample size (N) for conditions A and B for both times is equal to 10. Conditions C and D are combined into a single condition called CONTROL, with which increases the N up to 20 (Tables 7 and 8).

CONDITION A	CONDITION B	CONDITION CONTROL (C-D)	
21DA1	22DB1	21EC1	22ED1
23EA1	24EB1	23DC1	24DD1
25DA1	26DB1	25EC1	26ED1
27EA1	28EB1	27DC1	28DD1
29DA1	30DB1	29EC1	30ED1
31EA1	32EB1	31DC1	32DD1
33DA1	34DB1	33EC1	34ED1
35EA1	36EB1	35DC1	36DD1
37DA1	38DB1	37EC1	38ED1
39EA1	40EB1	39DC1	40DD1

Table 7: Samples belonging to each of the conditions for the Total and Total-Bicortical groupings for time 1. N=10 for conditions A and B. N=20 for the CONTROL condition.

CONDITION A	CONDITION B	CONDITION CONTROL (C-D)	
1DA2	2DB2	1EC2	2ED2
3EA2	4EB2	3DC2	4DD2
5DA2	6DB2	5EC2	6ED2
7EA2	8EB2	7DC2	8DD2
9DA2	10DB2	9EC2	10ED2
11EA2	12EB2	11DC2	12DD2
13DA2	14DB2	13EC2	14ED2
15EA2	16EB2	15DC2	16DD2
17DA2	18DB2	17EC2	18ED2
19EA2	20EB2	19DC2	20DD2

Table 8: Samples belonging to each of the conditions for the Total and Total-bicortical groupings for time 2. N=10 for conditions A and B. N=20 for the CONTROL condition.

However, if only the samples that present bicorticality on one side of the implant are considered, that is, if we work with the Bicortical grouping, the N varies depending on the condition and time (Tables 9 and 10).

CONDITION A	CONDITION B	CONDITION CONTROL (C-D)	
21DA1	24EB1	21EC1	39DC1
23EA1	26DB1	25EC1	22ED1
25DA1	28EB1	27DC1	24DD1
31EA1	32EB1	29EC1	26ED1
33DA1	34DB1	31DC1	30ED1
35EA1	36EB1	33EC1	32DD1
39EA1	40EB1	35DC1	34ED1
		37EC1	

Table 9: Samples belonging to each of the conditions for the Bicortical grouping for time 1. N=7 for conditions A and B. N=15 for the CONTROL condition.

CONDITION A	CONDITION B	CONDITION CONTROL (C-D)	
3EA2	2DB2	1EC2	2ED2
5DA2	6DB2	5EC2	6ED2
13DA2	8EB2	7DC2	12DD2
15EA2	12EB2	13EC2	14ED2
17DA2	14DB2	15DC2	18ED2
	18DB2	17EC2	20DD2
	20EB2		

Table 10: Samples belonging to each of the conditions for the Bicortical grouping for time 1. N=5 for condition A, N=7 for condition B and N=12 for the CONTROL condition.

The statistical analysis of the calculated BIC values was done using Minitab. Specifically, two types of statistical tests were carried out.

First, a Grubbs test was performed to detect outliers. Outliers may be indicative of data that belongs to a different population than the rest of the established samples. For this

study, the hypothesis used was “The *smallest or largest data value is an outlier*”, a two-sided test that can detect outliers from the smallest or largest data value. The level of significance (α) used to compare it with the probability value (p) to decide whether or not the hypothesis can be rejected was 0.05. A significance level of 0.05 indicates a 5% risk of concluding that a difference exists when there is no actual difference.

Second, an analysis of variance (ANOVA) is performed to test the hypothesis that the means of two or more populations are equal. For this project, the populations correspond to each of the conditions. ANOVA evaluates the importance of one or more factors by comparing the means of the response variable at different levels of the factors. In this case, the null hypothesis states that all population means are equal, while the alternative hypothesis states that at least one is different. Additionally, to obtain more detailed information about the differences between specific means, the Tukey multiple comparisons method is used.

6.5. Qualitative analysis – Histological analysis by optical microscopy

Qualitative analysis of the images obtained by optical microscopy can be performed once 24 hours have passed after staining, when the DPX drop has dried. The optical microscope (Figure 40) used to view the samples allowed us to observe different morphological characteristics of the cells and tissues.

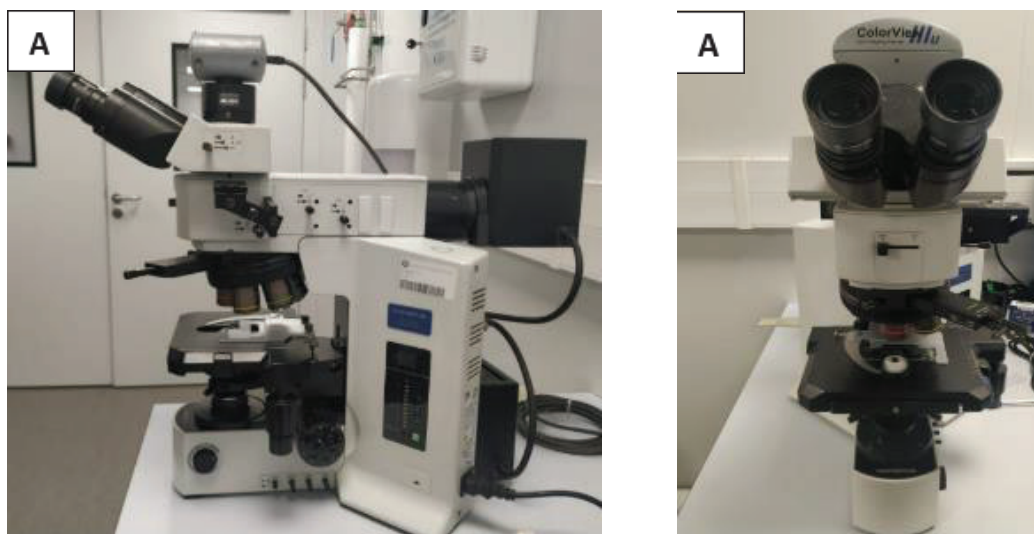


Figure 40: A - Optical microscope, lateral view. B - Optical microscope, front view.

Images were obtained using Image Analysis Software that turns the microscope into a high-performance analysis station. In this way, the samples were analyzed one by one in order to carry out the qualitative analysis.

In Figure 41, the images obtained of sample 33DA1 are shown as an example.

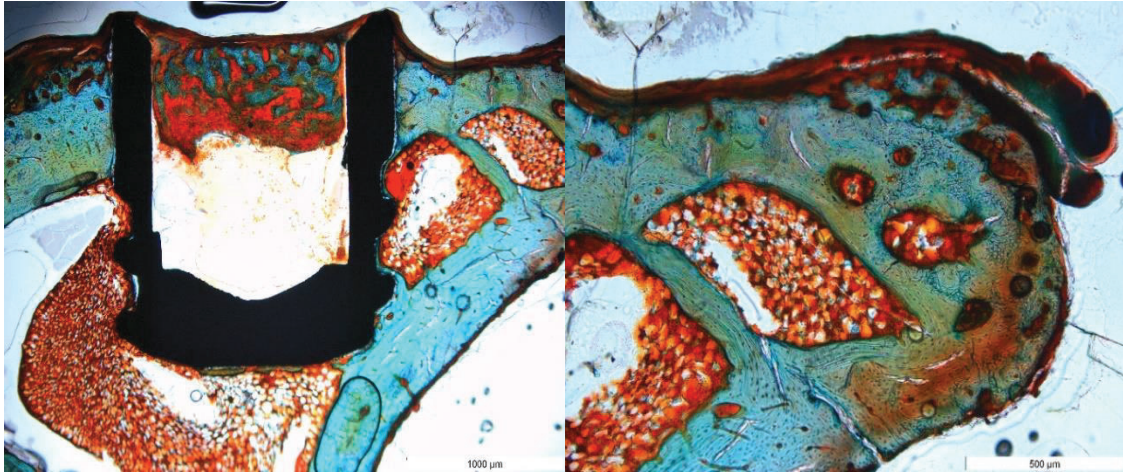


Figure 41: Example of images obtained by optical microscope of sample 33DA1.

RESULTS

1. Surgical Procedure

It is important to mention that the animal model used in this study presents certain constraints due to the condition of osteoporosis. These constraints are reflected during the surgical procedure, in particular regarding the manipulation of hard and soft tissues.

It is possible to observe greater bleeding during the drilling procedure. In addition, the implant placement process becomes difficult. Some complications may occur, such as small cracks in the bone, altering the progression of the implant during its entry, more unstable due to the lack of bone resistance on the lateral walls of the implant. This limitation requires manual pressure and stabilization during placement, making it more difficult to control until achieving the correct positioning of the implant.

Despite the array of anticipated challenges, the postoperative periods proceeded remarkably well, devoid of any complications typically associated with surgical interventions. Notably, the decision to make the incision away from the precise region of the implant, coupled with the meticulous separation of tissues while preserving their integrity (172), and ensuring that muscle insertions remained intact, facilitated a double-layer suture completed under ideal conditions. This strategic approach underscores the importance of surgical precision and care in maintaining the structural and functional integrity of the tissues involved.

The placement site of the implant, chosen with reference to muscle insertions to achieve uniform bone density at the selected location, appeared to be an exemplary choice. This area demonstrated excellent anatomical characteristics in terms of both volume and bone density, free from significant complications. Such conscientious planning and execution highlight the critical role of anatomical knowledge and surgical foresight in optimizing outcomes.

Moreover, the implementation of a slow drilling system with continuous irrigation using saline solution emerged as an atraumatic technique (173, 174). This method significantly contributed to the preservation and recovery of both hard and soft tissues, thereby

fostering a conducive environment for healing and osseointegration. The atraumatic nature of this technique illustrates its viability in enhancing post-surgical recovery.

All implants exhibited impressive stability during their insertion (table 11), culminating in the successful osseointegration of all implants. This result manifests as a 100% success rate in terms of osseointegration, as confirmed by the obtained BIC values. This success rate is a testament to the meticulous surgical technique and the body's intrinsic healing capabilities.

Nevertheless, upon further analysis, as detailed in point 3 of this chapter, it becomes apparent that the interaction related to the contact between the implant and the cortical bones in the interior aspect of the bone tibia varies throughout the sample. This variability underscores the inherent challenge in precisely controlling this aspect of the procedure. Consequently, it necessitates a segmented analysis based on the relationship between the implant and the intimately contacting cortical bones. Such an analysis is crucial in understanding the nuances of the bone-implant interface and in refining surgical strategies to achieve even more consistent outcomes.

Model	<i>Right leg</i>	<i>Left leg</i>
Osteoporosis	6.11 ± 0.96	6.49 ± 1.34

Table 11: Average of maximum implant insertion torque (N-cm).

2. Qualitative Results

The samples were qualitatively analyzed using SEM and optical microscopy after staining.

Shown below are selected images for each condition and time alongside the observed outcomes. When conducting the qualitative analysis, the groupings of samples (Total, Bicortical, and Total-bicortical) were not taken into account. These groupings are primarily useful for statistical and quantitative analyses.

Condition A (implants functionalized with $\alpha\beta 3$ -selective peptidomimetic) – Time 1 (4 weeks)

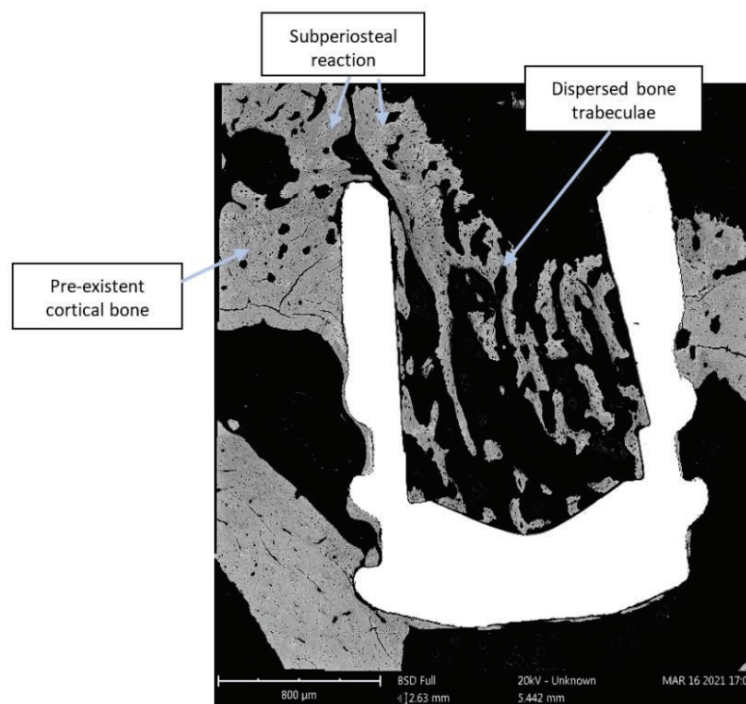


Figure 42: Sample 21DA1 acquired using SEM.

In sample 21DA1 (Figure 42), a subperiosteal reaction is observed that forms a type of posterior callus, and also invades the open intra-implant space with loose chondroid tissue trabeculae. Under SEM vacuum, the pre-existing cortex fractures. Additionally, a laminar reconstruction is visible on the right side, showing trabeculae reaching the inside of the implant to support its stability.

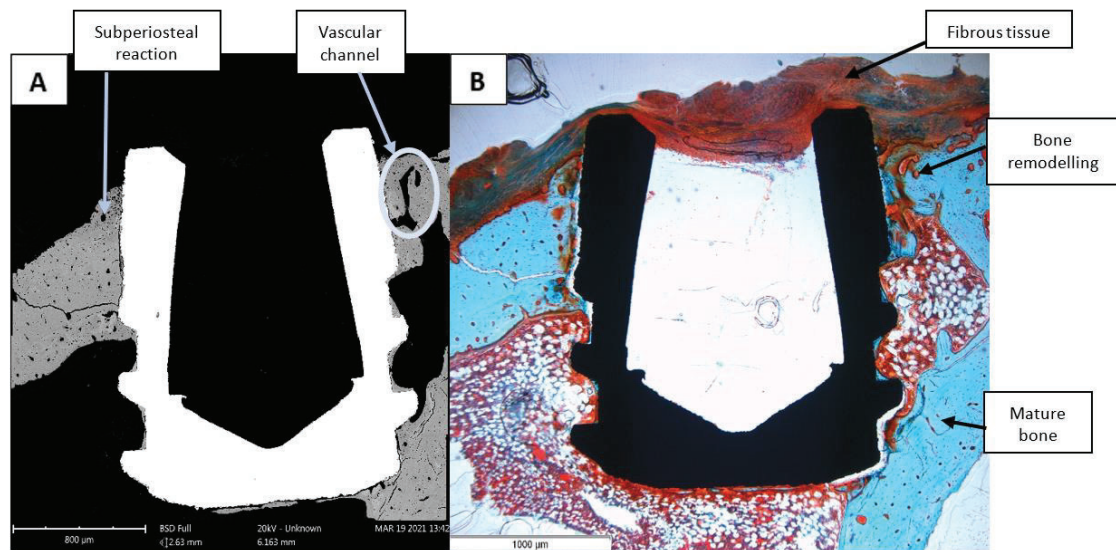


Figure 43: Sample 25DA1. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 25DA1 (Figure 43) shows bicortical support on one side of the implant. In the optical microscopy image, mature bone appears blue, and fibrous tissue appears red (Figure 43-B). The early osteoid formation is identifiable as calcifying ECM. In the SEM image (Figure 43-A), a subperiosteal reaction and a distinct vascular channel are visible, also well identified in sample 35EA1 (Figure 44) distinguishable by their structure, form, and histological features.

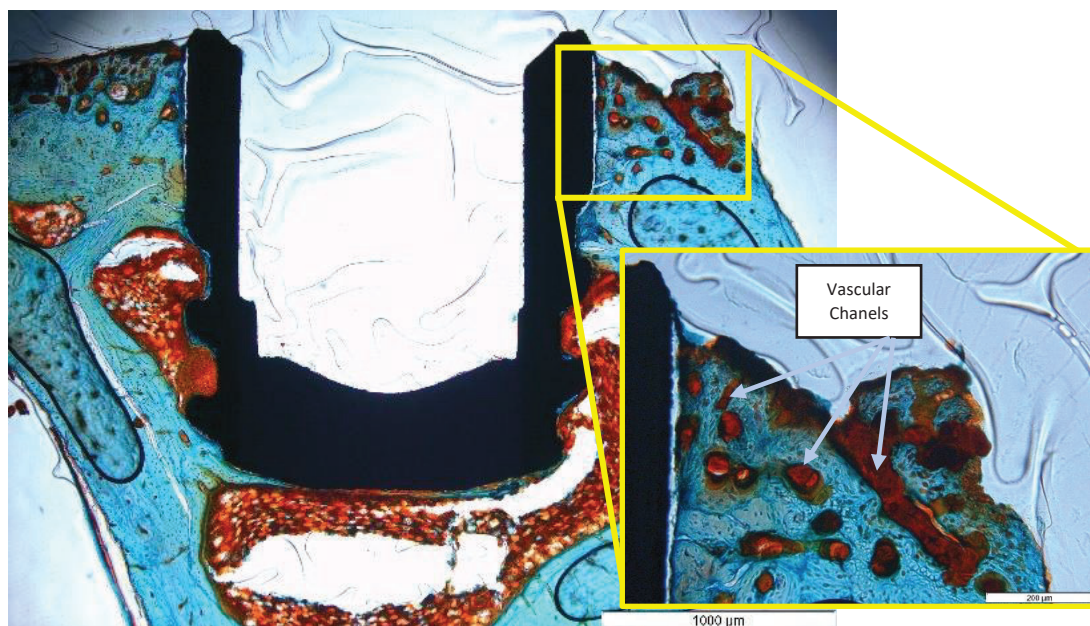


Figure 44: Sample 35EA1. Image obtained by optical microscopy.

This vascular response is observed to be accompanied by a periosteal reaction related to membranous ossification, as can be identified in the sample 33DA1 (Figure 45).

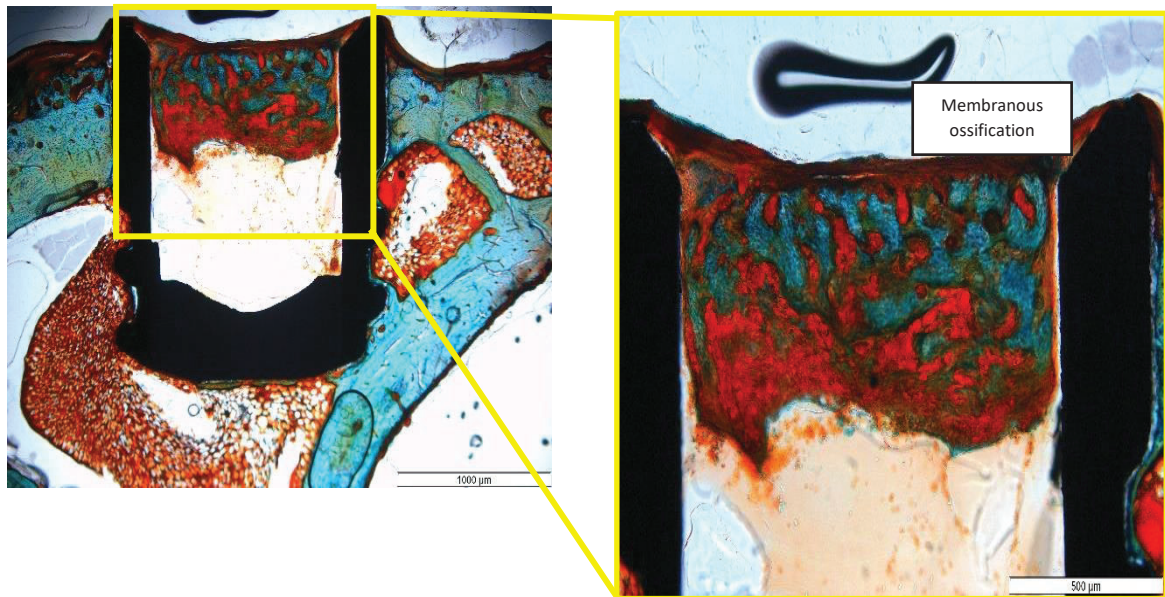


Figure 45: Sample 33DA1. Image obtained by optical microscopy.

The observations for condition A at time 1 can be summarized across several key points:

- **Cortical Support:** Around 70% of the samples show bicortical presence on one side of the implant.
- **Membranous Ossification:** All samples demonstrate signs of membranous ossification, a process where bone formation occurs directly from connective tissue, highlighting the body's natural response to the implant.
- **Periosteal Reactions:** Classic reactions of the periosteum, the outer layer of bone tissue, are evident in these samples. This suggests an active biological response, leading to the reconstruction of the cortex layer of the bone.
- **Vascular Response:** The initial reaction to the surgery implant placement, is vascular, indicating that blood supply and circulation changes are among the first steps in the tissue's response to the implant surgery, fostering a healing environment.
- These topics outline the complex interplay of bone growth, healing processes, and biological responses to the implants observed in an early phase.

Condition A (implants functionalized with $\alpha\beta 3$ -selective peptidomimetic) – Time 2 (12 weeks)

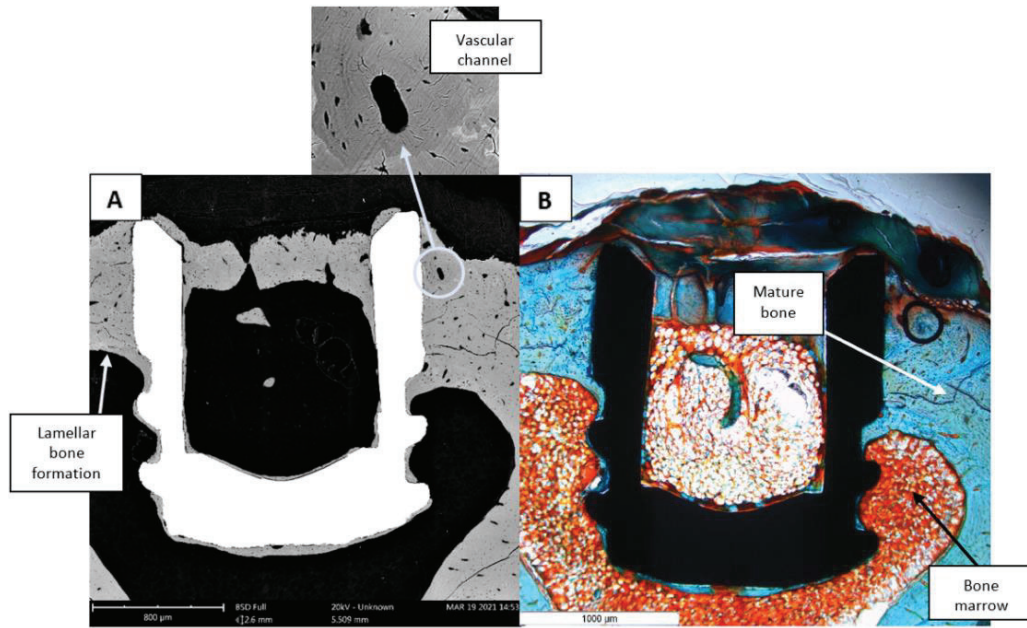


Figure 46: Sample 1DA2. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 1DA2 (Figure 46) shows no bicortical support around the implant, yet the bone remains stable. A vascular channel (Figure 46-A) is encased in laminar deposits, indicating it could be a precursor to an osteon. The development has predominantly been in the form of lamellar bone, signifying that the mature bone is consistent in both dense and cancellous forms. Additionally, bone marrow is present within the implant (Figure 46-B), a highly beneficial outcome demonstrating growth lines internally. The marrow displays a reddish hue, characteristic of hematopoiesis, due to the abundance of red blood cells.

Sample 7EA2 (Figure 47), demonstrates not only a clear generalized bone maturation around the implant, but also an increase in bone volume. This generalized augmentation, endosteal and periosteal, results in thickness, support, and BIC augmentation.

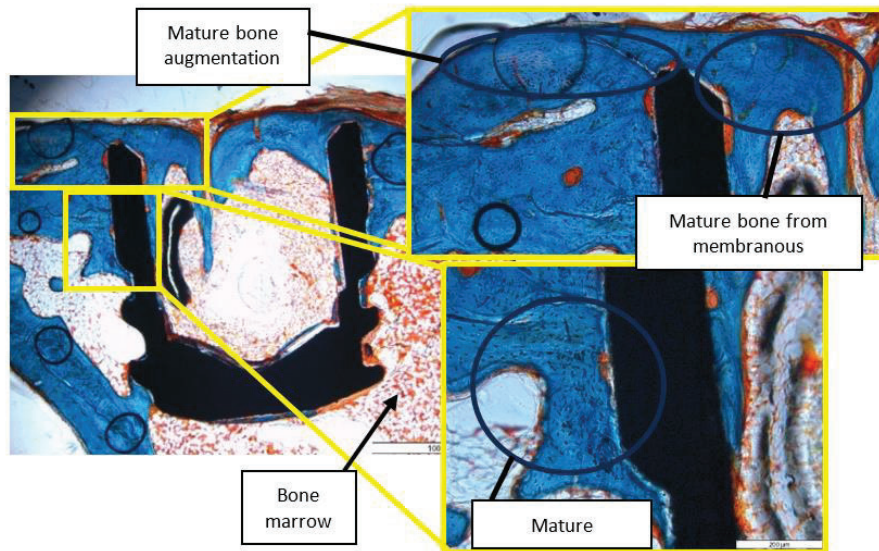


Figure 47: Sample 7EA2. Image obtained by optical microscopy.

Sample 3EA2 (Figure 48) demonstrates tissue regeneration, indicative of a perfect restoration ("restitutio ad integrum") of tissue following injury, a contrast to repair processes that result in scar formation with properties distinct from the original tissue. Furthermore, there's evidence of an ongoing periosteal reaction that contributes to filling the inside of the implant. Meanwhile, some faint areas, suggestive of remnants from the original cortex, are barely visible (Figure 48-A). This time, there's a notable presence of marrow cells (Figure 48-B), which play a role in mitigating osteoporotic conditions, alongside visible bone growth encircling the marrow.

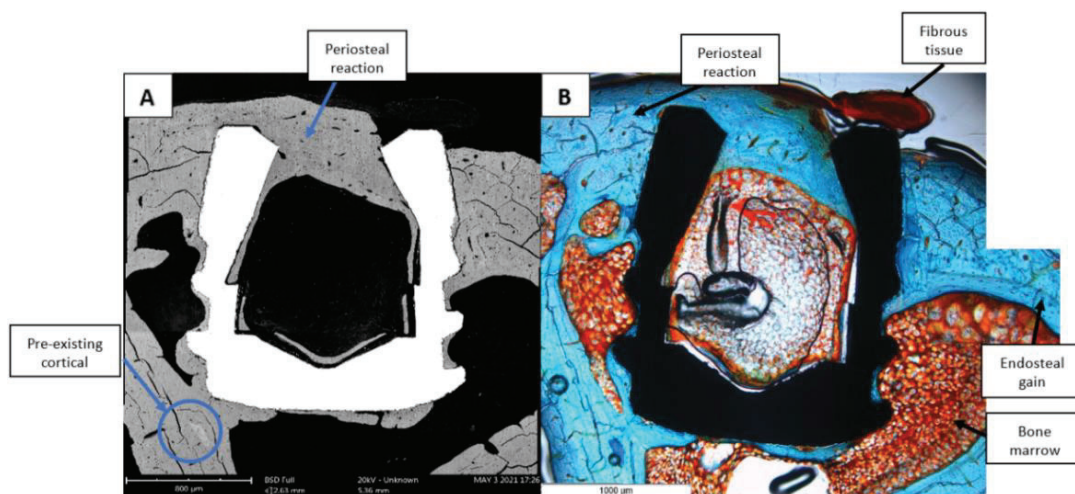


Figure 48: Sample 3EA2. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Regarding condition A, time 2, the analysis reveals significant findings across several key topics:

- **Cortical Support:** 50% of the examined samples display bicortical support on one side of the implant.
- **Tissue Maturity:** There's a noticeable advancement in tissue maturity when comparing these observations to the initial time 1 condition. This suggests a positive progression in the healing and integration process.
- **Structural Continuity and Bone Growth:** The samples exhibit structural continuity and are characterized by vigorous bone growth activity around the bone marrow. This indicates a healthy, ongoing process of bone regeneration and adaptation in response to the implant.
- **Increase in Tibial Cortical Thickness:** This active bone growth leads to a measurable increase in the thickness of the tibial cortex. Notably, this thickening affects both the periosteum (the outer layer of the bone) and the endosteum (the inner layer), signifying a comprehensive enhancement in bone density and strength around the implant area.

Condition B (implants functionalized with $\alpha\beta 3$ -selective peptidomimetic) – Time 1 (4 weeks)

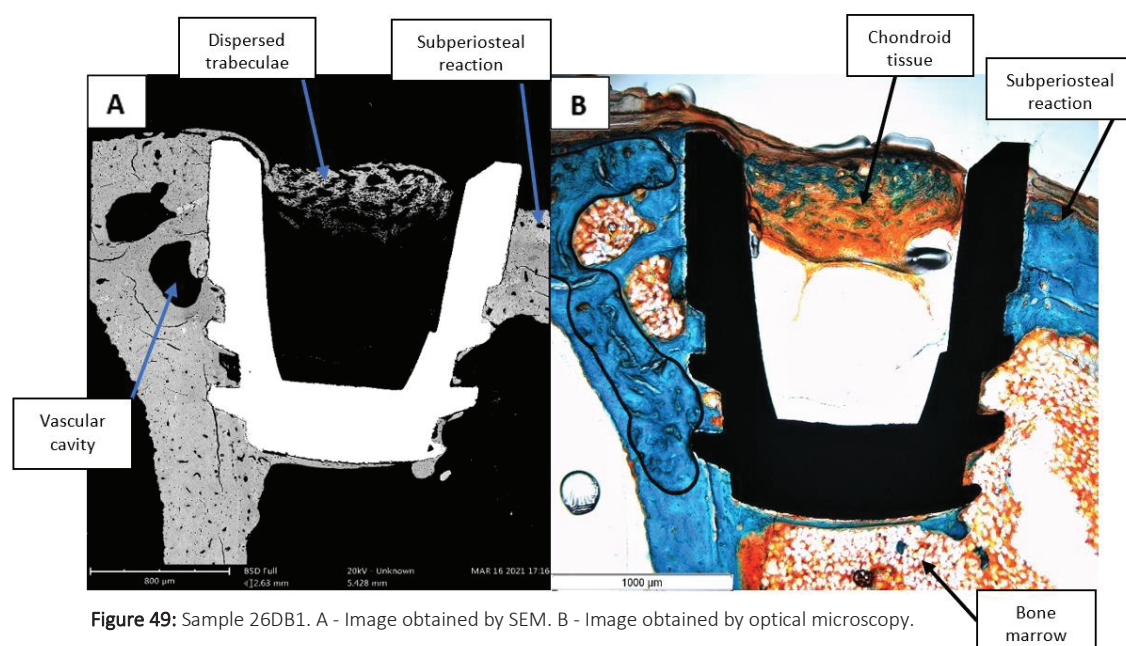


Figure 49: Sample 26DB1. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 26DB1 (Figure 49) shows bicortical support on one side of the implant. This sample reveals active remodeling of the cortical bone alongside the initial formation of membranous ossification, indicated by dispersed trabeculae. Similarly, the presence of chondroid tissue (observed in Figure 49-B) represents an early stage before membranous ossification begins. Additionally, the transparent areas within the bone marrow are identified as adipocytes.

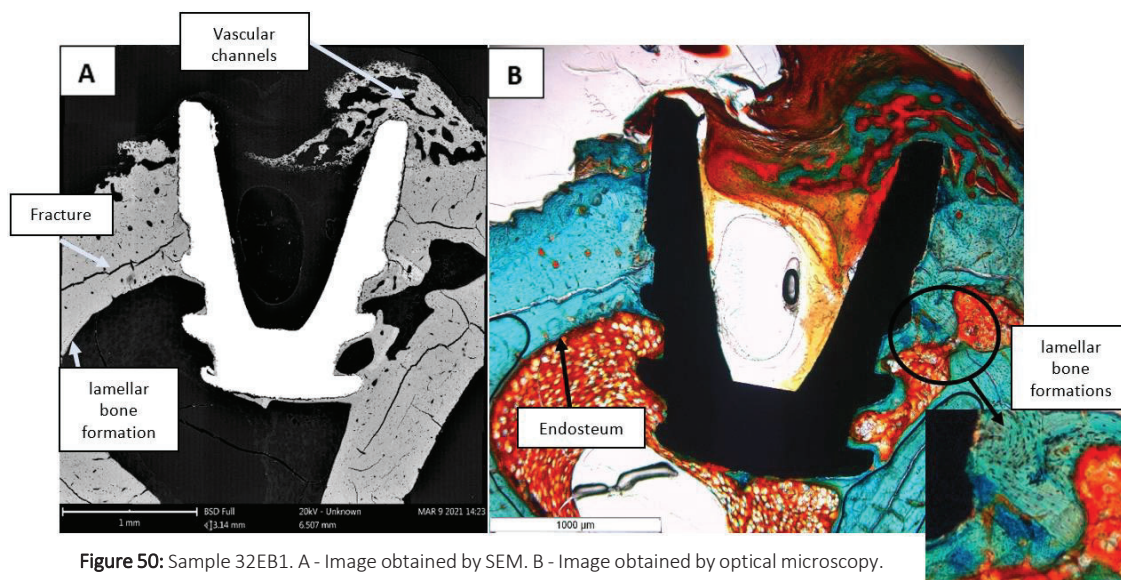


Figure 50: Sample 32EB1. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 32EB1 (Figure 50) presents bicortical support on one side of the implant. In this case, vascular channels are observed that begin to invade the interior of the implant in the image obtained by SEM (Figure 50-A) and in that obtained by optical microscopy (Figure 50-B). The fractures observed correspond to the pre-existing cortex which, when subjected to the vacuum of the SEM, fractures. Lamellar formations and a large number of osteocytes can be seen (Figure 50-B), as well as the activation of the endosteum and ossification by the marrow (endomembranous ossification).

Sample 30DB1 (Figure 51) presents no bicortical support, showing the same tissue reactions where we can identify: angiogenesis, surrounded by non-calcified osseous extracellular matrix apposition lining the numerous vascular channels and chondroid tissue formation.

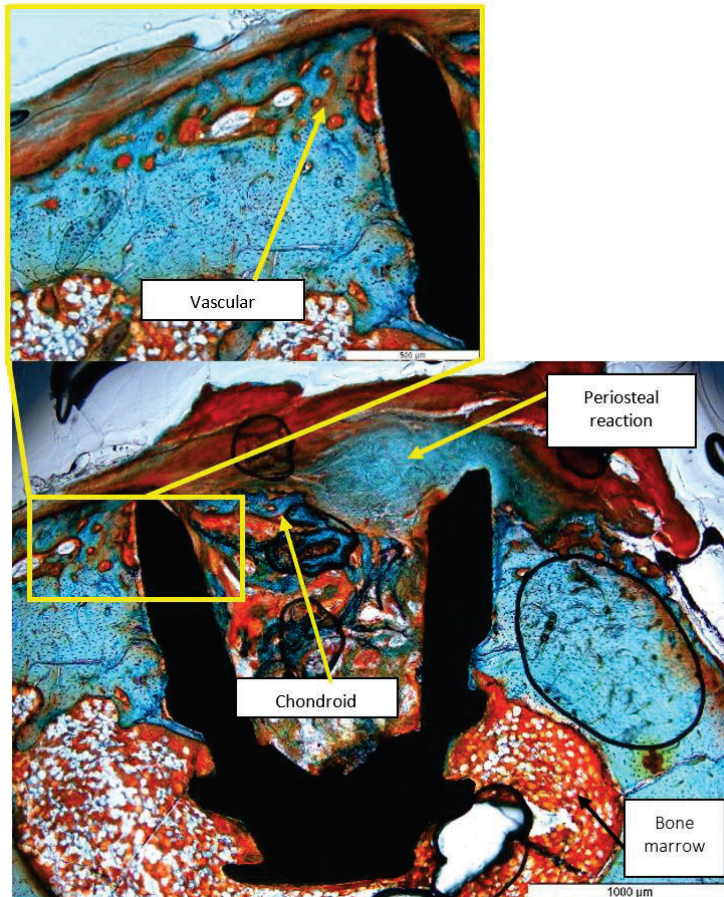


Figure 51: Sample 30DB1. Image obtained by optical microscopy.

The findings for condition B at time 1 are summarized under key topics as follows:

- **Cortical Support:** Similar to condition A, time 1, 70% of the samples display bicortical support on one side of the implant.
- In comparison to condition A, more subtle responses are noted for condition B at this time point. Despite the subtle nature of the responses, the outcomes are positive and indicate progress in the healing process.

Condition B (implants functionalized with $\alpha\beta 3$ -selective peptidomimetic) – Time 2 (12 weeks)

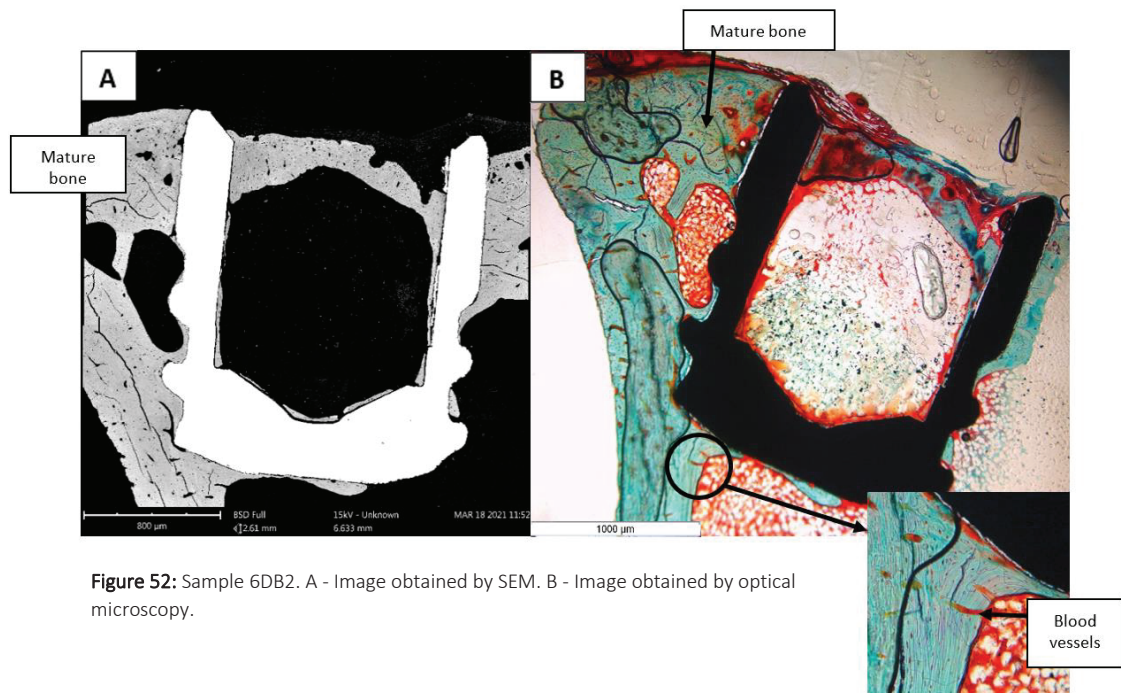


Figure 52: Sample 6DB2. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 6DB2 (Figure 52) exhibits bicortical support on one side of the implant. A distinct neovascularization, or the formation of new blood vessels, is evident. The periosteum, the outermost layer of bone, is clearly visible, playing a crucial role in fracture healing due to its rich blood supply. Overall, the sample shows highly mature tissue with a solid, uniformly laminar bone structure (clearly displaying laminar formations with oriented collagen fibers in Figure 52-B).

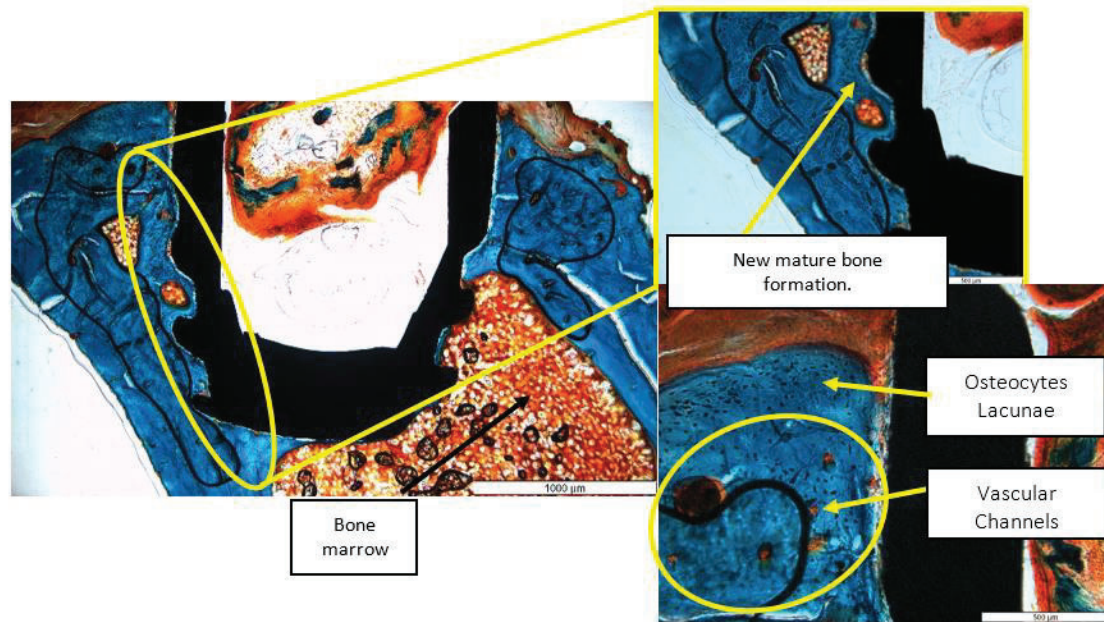


Figure 53: Sample 20EB2. Image obtained by optical microscopy.

Sample 20EB2 (Figure 53) shows bicortical support on one side of the implant. This sample also shows blood vessels as a result of the angiogenesis process. Good tissue reaction and integrations can be observed due to the new mature bone observed as well as the abundance of osteocyte *lacunae*.

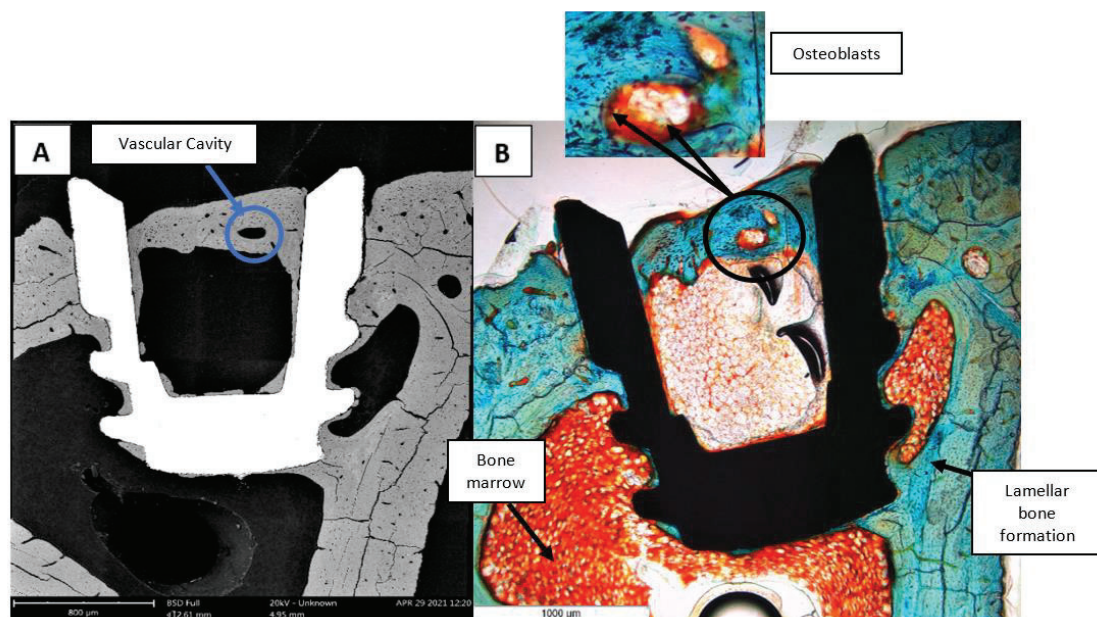


Figure 54: Sample 12EB2. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 12EB2 (Figure 54) displays bicortical support on one side of the implant. The observation includes an uninterrupted cortex and the presence of both vascular and medullary cavities (Figure 54-A). Near the vascular cavity (Figure 54-B), osteoblasts transitioning into osteocytes (bone remodeling) can be seen. Lamellar structures are apparent, along with bone marrow showing a reddish hue, and lighter color regions within the implant attributed to adipocytes.

The findings for condition B at the second observation point are summarized under key topics as follows:

- **Cortical Support:** At time 2, 80% of the samples exhibit bicortical support on one aspect of the implant, indicating strong bone integration.
- **Tissue Maturity and Stability:** Overall, the samples show mature tissues and appear stable, signifying successful healing and integration processes.
- **Staining Differences:** It's interesting to observe color variations observed during staining in the bone. The observed variations are indicative of distinguishing between old and new tissues, providing insights into bone remodeling and regeneration.

Condition Control (non-functionalized titanium) – Time 1 (4 weeks)

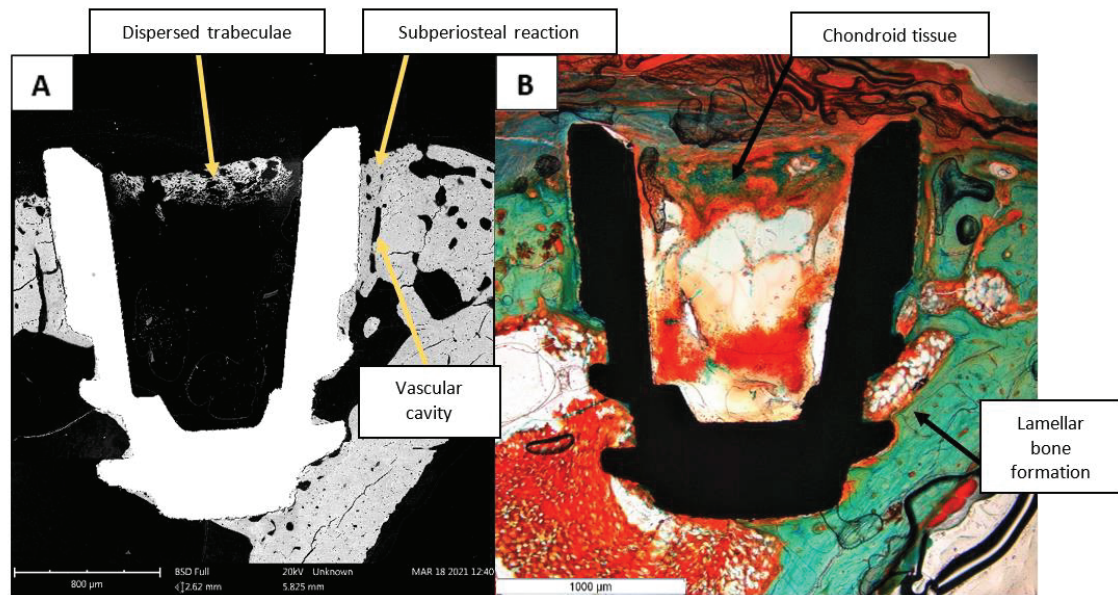


Figure 55: Sample 21EC1. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 21EC1 (Figure 55) demonstrates bicortical support on one side of the implant. Many vascular cavities often connect continuously with the medullary cavities (Figure 55-A). Once again, there are scattered trabeculae present, but in this instance, they do not extend all the way to the interior of the implant. Abundant chondroid tissue is visible (Figure 55-B), acting as a precursor to membranous ossification. The sample shows active neovascularization, triggering an active response.

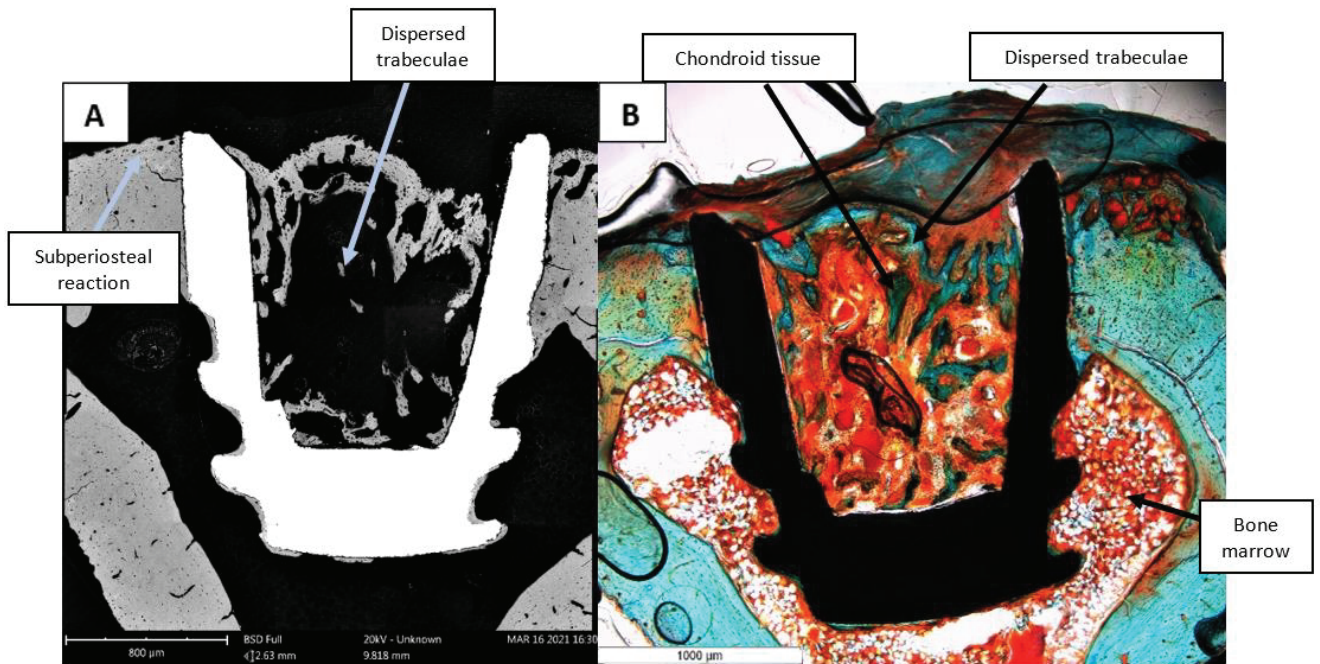


Figure 56: Sample 28DD1. A - Image obtained by SEM. B - Image obtained by optical microscopy.

Sample 28DD1 (Figure 56) does not display bicortical support on either side of the implant. Once more, a classic periosteal reaction is visible that extends into the interior of the implant. Trabeculae reappear within the implant along with chondroid tissue, contributing to its osteoconductive interior feature. Neovascularization can be observed originated from the periosteum.

The observations for the CONTROL condition at time 1 are summarized under key topics as follows:

- **Cortical Support:** For this condition, 80% of the samples exhibit bicortical support on one side of the implant, indicating a significant level of bone integration.
- **Overall Response:** Across the samples, a positive response is noted however in a more localized reaction compared to the previous conditions at the same time.

Condition Control (non-functionalized) – Time 2 (12 weeks)

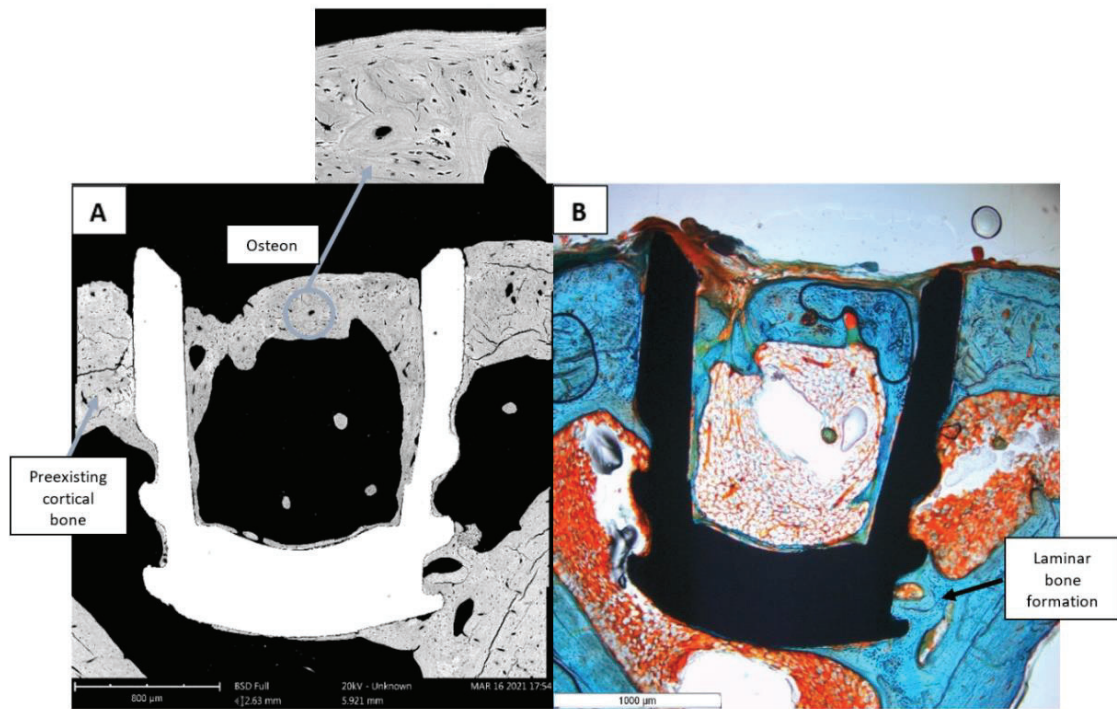


Figure 57: Sample 1EC2. A - Image obtained by SEM. B - Image obtained by optical microscopy.

In sample 1EC2 (Figure 57), external and internal laminar tissue progressing appropriately are observed. Blood vessels are growing, showing a significant bone growth continuity throughout the implant, extending into its interior. Additionally, an osteoclast (Howship's) *lacuna* surrounded by material is visible. This sample represents classic Haversian ossification by osteon formation. Osteonal system remodeling is noticed in tissue with remnants of immature tissue, indicating a positive trend but with lesser performance compared to previous conditions.

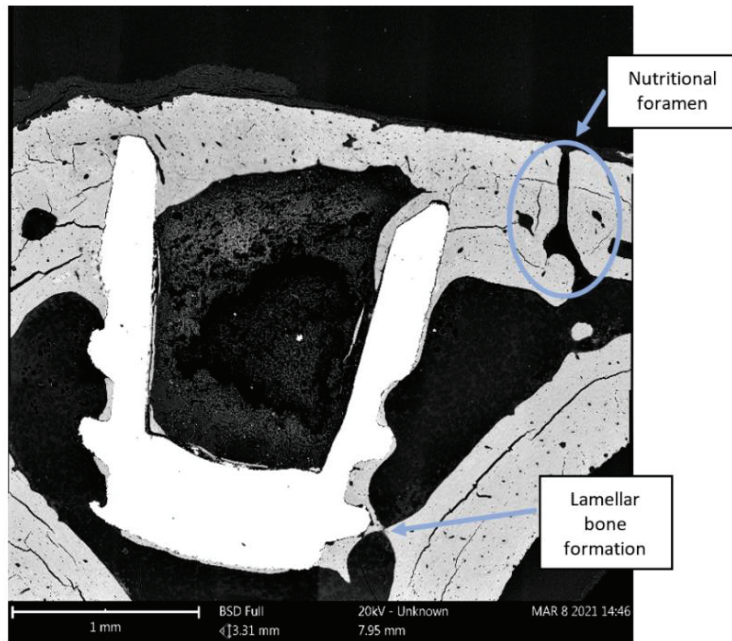


Figure 58: Sample 6ED2. Image obtained by SEM.

In sample 6ED2 (Figure 58), mature and stable tissue is observed. The implant appears well integrated into the bone. Evidence points to the presence of a nutrient foramen, which are openings in long bones that allow for the entry and exit of blood vessels. Bone formation has extended into the internal area of the implant.

The findings for the CONTROL condition at time 2 are as follows, detailed by key points:

- **Cortical Support:** 70% of the samples exhibit bicortical support on one side of the implant, indicating robust bone integration.
- **Tissue Maturity:** Mature tissues are evident, with continuity, observed in the majority of samples.
- **Bone Characteristics:** Although mature lamellar bone is present, there is no indication of osteoporosis reversal or significant changes in bone structure noted in the samples.

3 Quantitative/Statistical Analysis Results

The quantitative and statistical analysis of the samples has been carried out based on the calculated BIC (%) values.

As mentioned above, different BIC values have been calculated depending on the group of samples made (Table 12): the first group (Total) corresponds to the 80 samples, without taking into account bicorticality; the second group (Bicortical) is made up only of the samples that present bicorticality on one side of the implant and, finally, the third group (Total-bicortical) is made up again of the 80 samples, but this time considering the corresponding BIC in function whether they present bicorticality or not.

3.1. Group 1 – Total

Total	The total BIC of the 80 samples is considered.
Bicortical	Only the BIC of samples that present bicorticality on one side of the implant is considered.
Total-Bicortical	The BIC that best suits each of the samples is considered, depending on whether they present bicorticality on one side of the implant or not.

Table 12: Groups of samples with which the project works.

Initially, the BIC (%) of all the samples was calculated, without taking into account whether or not they present bicortical support on any side of the implant (“Total” group). The results obtained are the following.

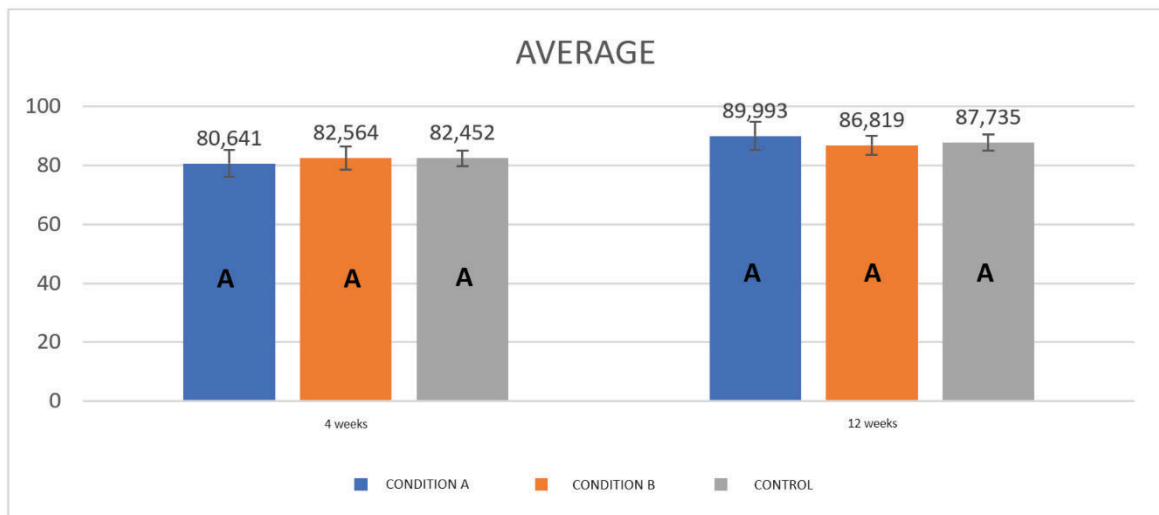


Figure 59: Graph with error bars for Total grouping. Shows the average of the conditions for time 1 (4 weeks) and time 2 (12 weeks). Condition A – $\alpha\beta3$ integrin. Condition B – $\alpha5\beta1$ integrin. Condition C and D – CONTROL – Ti without modifications. There are no statistically significant differences between the conditions for time 1 and 2.

On the one hand, it can be seen that 4 weeks after the experimental animals had been sacrificed, conditions B and CONTROL presented a similar BIC (%), slightly higher than that of condition A (Figure 59). However, through an analysis of variance (ANOVA) it was determined that the 3 conditions (A, B, CONTROL) share the same letter (A), and thus there are no statistically significant differences between the 3 conditions for time 1.

On the other hand, when the experimental animals were sacrificed 12 weeks after the intervention, the average BIC of all samples has increased, implying a higher contact of the implants with bone, in agreement with the qualitative data (please see previous section). Now condition A becomes the one with the highest BIC (89.99%), while condition B becomes the one with the lowest average (86.82%). However, the statistical analysis reveals that there are not statistically significant differences either between groups.

The standard deviation of each of the conditions is calculated during time 1 (4 weeks) and time 2 (12 weeks). Each of the graphs (Figure 60) shows the individual BIC (%) calculated for each of the samples of the condition (blue color), the average of the condition (orange color), the maximum limit of the standard deviation (color gray) and the minimum limit of the standard deviation (yellow).

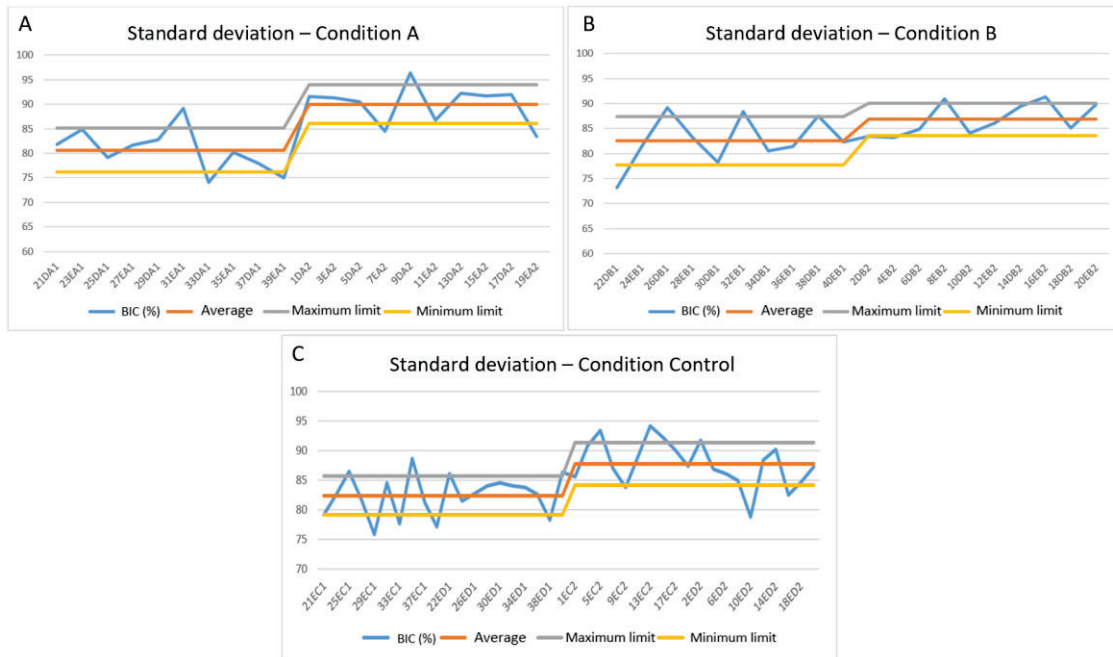


Figure 60: Standard deviations of each of the conditions for time 1 (4 weeks) and time 2 (12 weeks) for group 1-Total. A - Condition A - αvβ3 integrin. B - Condition B - α5β1 integrin. C - Condition C and D – CONTROL – Ti without modifications. There are no outliers in any of the conditions for time 1 and 2.

In conditions A (Figure 60-A) and B (Figure 60-B) the majority of the samples are within the range of the maximum and minimum limits of the standard deviation, so the values are assumed as acceptable results. Data that is outside the range could be considered not very relevant to the experiment. However, since we are working with a distribution of results with very dispersed values, the values that stand out cannot be considered irrelevant. The CONTROL condition (Figure 60-C) shows an even greater dispersion of results than the other two conditions.

All this is corroborated with the outliers test carried out where it is observed that for both time 1 and time 2, the value of p for each of the conditions is greater than the value of the significance level ($\alpha = 0,05$). It is concluded that there are no outliers in any of the 3 conditions for any of the times.

3.2. Group 2 – Bicortical

With the BIC values obtained from the 80 samples, it was observed that a large number of the samples presented bicortical support on one side of the implant. Approximately 75% of the samples for time 1, and 67.5% of the samples for time 2 presented bicortical support in one half of the implant. We wanted to know how this bicorticality affects the results.

Taking this into account, it was decided to recalculate the BIC only for those samples that presented bicorticality in a part of the implant (“Bicortical” grouping) and obtain the average and standard deviation to be able to compare it with the results obtained previously.

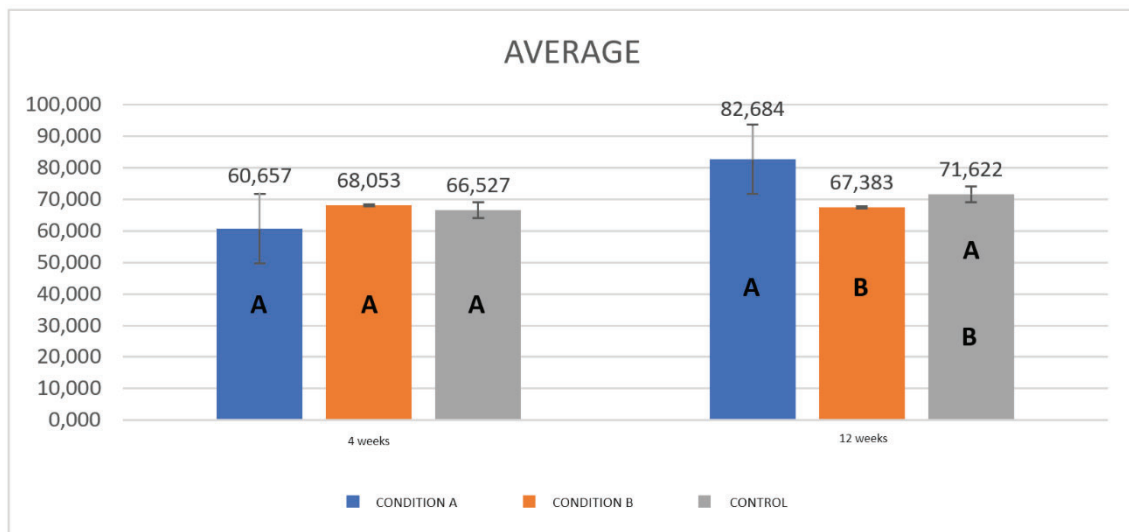


Figure 61: Graph with error bars for the Bicortical grouping. Shows the average of the conditions for time 1 (4 weeks) and time 2 (12 weeks). Condition A – $\alpha\beta3$ integrin. Condition B – $\alpha5\beta1$ integrin. Condition C and D – CONTROL – Ti without modifications. There are no statistically significant differences between the conditions for time 1. There are statistically significant differences between condition A and B for time 2.

The first thing that can be observed is a general decrease in the average BIC obtained for the 3 conditions (Figure 61). Condition A for time 1 again presents the lowest of the averages (60.66%), while condition B presents the highest of the 3 conditions (68.05%). As was the case for the “Total” grouping at time 1, the ANOVA analysis determines that there are no statistically significant differences between the groups.

At 12 weeks, a great change is observed in the BIC (%) for condition A (82.68%), making it the one with the highest average. The CONTROL condition has also increased the average, reaching a BIC of 71.62%. On the contrary, the average for condition B decreases, falling to 67.38%. On this occasion, the CONTROL condition presents both letters (A and B), which suggests that it shares a letter with each of the other two conditions and, therefore, the differences are not statistically significant. On the other hand, it is observed that condition A and B do not share a letter, which determines that there are statistically significant differences between these two groups.

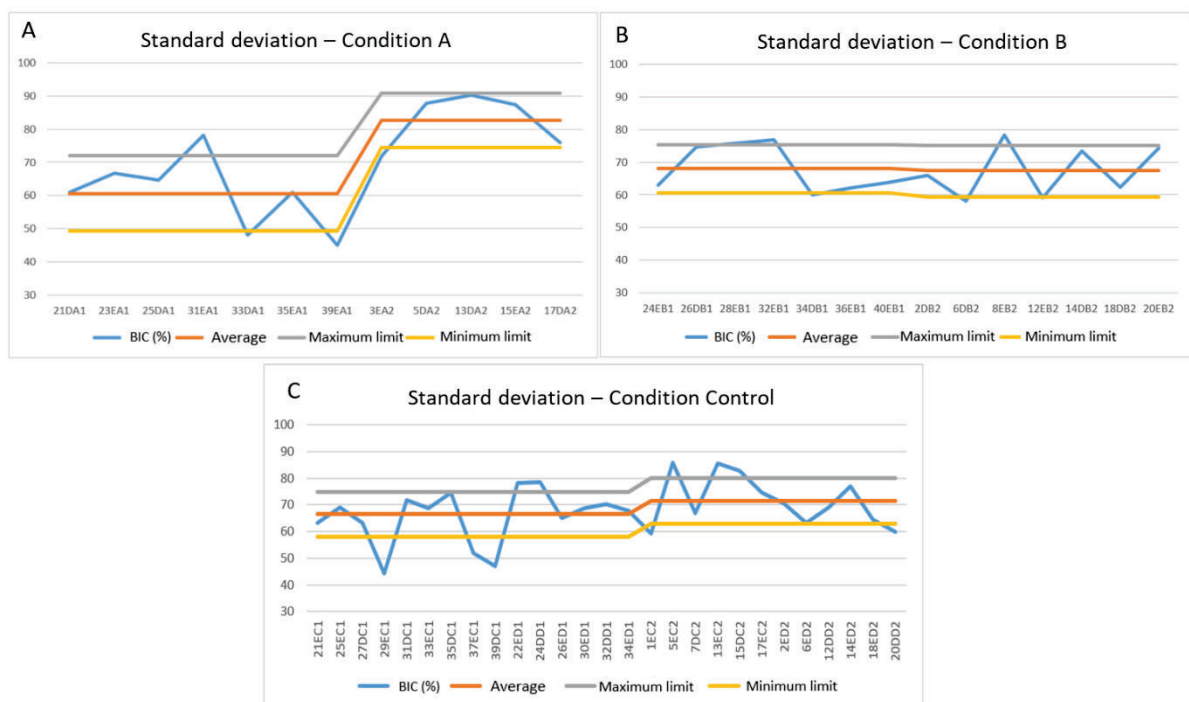


Figure 62: Standard deviations only for samples that present bicorticality on one side of the implant for time 1 (4 weeks) and time 2 (12 weeks) for the 2-Bicortical group. A - Condition A - $\alpha\beta3$ integrin. B - Condition B - $\alpha5\beta1$ integrin. C - Condition C and D – CONTROL – Ti without modifications. There are no outliers in any of the conditions for time 1 and 2.

The standard deviation of each of the conditions is calculated during time 1 (4 weeks) and time 2 (12 weeks). On this occasion, the greatest change in the average is observed in condition A (Figure 62-A) when the samples are observed 12 weeks after having performed the surgery. Condition B (Figure 62-B) in comparison presents a non-remarkable change in the average from time 1 (4 weeks) to time 2 (12 weeks). In conditions A and B, it is observed that most of the data obtained are within the band formed between the maximum and minimum limits of the standard deviation.

The outlier test carried out reveals that for both time 1 and time 2, the value of p for each of the conditions is greater than the value of the significance level ($\alpha = 0,05$). Due to this, it is concluded that there are no values atypical in any of the 3 conditions for any of the times.

3.3. Group 3 – Total-Bicortical

Once the results were obtained for the samples considering (group 2 – Bicortical) and without considering (group 1 – Total) the bicortical support on one side of the implant, it was decided that it was more effective to use the most appropriate BIC value for each sample (“Total-bicortical” grouping).

It was decided to calculate the average and standard deviation again to compare with the results obtained previously.

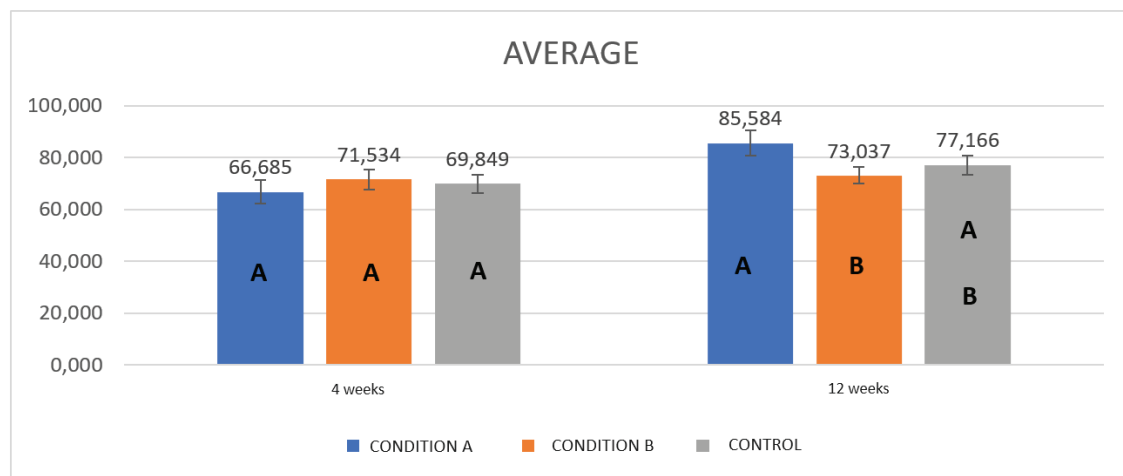
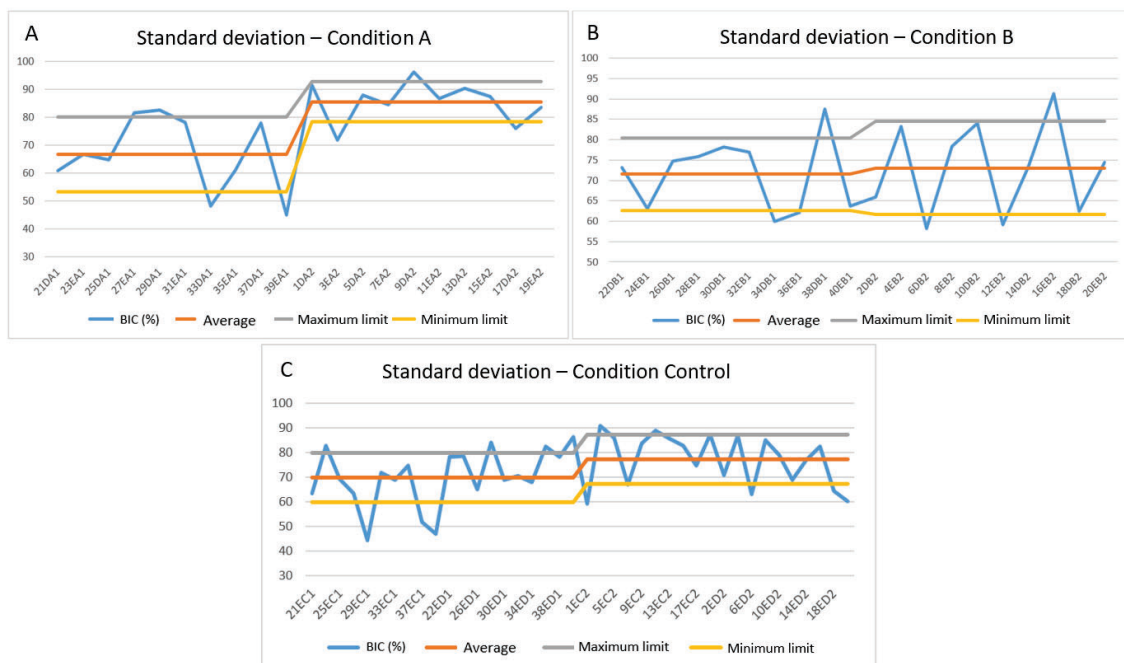


Figure 63: Graph with error bars for the Total-Bicortical grouping. Shows the average of the conditions for time 1 (4 weeks) and time 2 (12 weeks). Condition A – $\alpha v \beta 3$ integrin. Condition B – $\alpha 5 \beta 1$ integrin. Condition C and D – CONTROL – Ti without modifications. There are no statistically significant differences between the conditions for time 1. There are statistically significant differences between condition A and B for time 2.

Similarly to what was observed for Group 2, the average BIC values for this group are decreased in comparison to Group 1 (Figure 63). In particular, the maximum BIC value for time 1 is the one presented by condition B (71.53%), while the minimum average is the one presented by condition A (66.68%). Such differences, however, are not statistically different.

As expected, the BIC values for time 2 are again lower than those that can be observed for Group 1. Condition A maintains the maximum average (85.58%) as happened previously especially in group 2, while condition B is the one that now presents the lowest average (73.04%). The average for the CONTROL condition is greater for time 2 than for time 1. On this occasion, as happens for the Bicortical grouping at time 2, the CONTROL condition presents both letters (A and B), which establishes that the differences are not statistically significant (Figure 63). On the other hand, condition A and B do not share a letter, so it is determined that there are statistically significant differences between both conditions.



Once again, the statistical analysis determines that p is greater than the significance level value and, therefore, the null hypothesis is approved, and it is determined that no outliers have been found in any of the conditions for both times.

In order to make a comparison of all conditions, the results obtained are shown according to time and the grouping carried out (Tables 13 and 14).

TIME 1	AVERAGE		STATISTICAL SIGNIFICANCE	ATYPICAL VALUES
Grouping/condition*	Maximum	Minimum		
Total	B	A	NO	NO
Bicortical	B	A	NO	NO
Total-bicortical	B	A	NO	NO

Table 13: Comparison of the results obtained for time 1 according to the groupings made for each of the conditions.

*Condition A - integrin $\alpha\beta3$. Condition B - integrin $\alpha5\beta1$. Condition C and D – CONTROL – Ti without modifications.

TIME 2	AVERAGE		STATISTICAL SIGNIFICANCE	ATYPICAL VALUES
Grouping/condition*	Maximum	Minimum		
Total	A	B	NO	NO
Bicortical	A	B	YES (A-B)	NO
Total-bicortical	A	B	YES (A-B)	NO

Table 14: Comparison of the results obtained for time 2 according to the groupings made for each of the conditions.

*Condition A - integrin $\alpha\beta3$. Condition B - integrin $\alpha5\beta1$. Condition C and D – CONTROL – Ti without modifications.

On the one hand, it can be seen that none of the condition's present outlier values for any of the two times (Tables 13 and 14). This means that unusually large or small observations cannot be seen in the results in the condition and, therefore, there are no disproportionate effects in the statistical results.

On the other hand, it is observed that, in all the groupings carried out, for time 1, condition B presents the maximum of the averages while condition A presents the minimum (Table 13). On the other hand, for time 2, condition A becomes the one with the highest average and condition B the lowest (Table 14). Precisely at time 2 there are two groupings that present statistically significant differences between conditions A and B (Table 14), and in both cases condition A presents the highest average.

As has been seen, condition A presents the maximum of the averages 12 weeks after having performed the surgery. This indicates that the level of osseointegration in implants coated with $\alpha\text{v}\beta\text{3}$ integrin-binding peptides is greater than for $\alpha\text{5}\beta\text{1}$ -integrin-binding-coated implants or greater than unmodified implants.

DISCUSSION

1. Surgical Procedure

Although the rat is a widely used model for the study of osseointegration, it is important to define and describe a reproducible surgical technique for an adequate placement of the implants, which may serve as a guide for the scientific community. Moreover, the fact that certain diseases, such as osteoporosis and diabetes, alter bone metabolism, implies stronger demands on the correct design of surgical procedures.

Despite the considerable amount of scientific literature in the field, there are only a few scientific articles describing detailed surgical techniques (175, 176). And yet, the vast majority omit crucial surgical information, thus preventing the possibility of replicating the techniques described.

In our surgical protocol, we can define several critical steps to take into consideration throughout the surgical procedure, such as the location of the incision, the precise location for drilling and implant placement, the drilling technique, the exact dimension of the implant and the final drill used to the insertion of the implant.

1.1. Incision

In the literature the exact location of the incision region is generally vague, with no scientific basis for this purpose (177). The incision should be made in a different region from that of the implant placement. It should be avoided that the incision and consequent suture are located exactly above the placed implant, in order to allow the total integrity of the osteogenic properties of the detached periosteum (172) above the implant. This should allow optimizing peri-implant bone formation and even within the implant itself, free of any trauma, poor healing, fibrosis or even the risk of bacterial colonization in the suture area. In our method, the incision is detailed, being made in the proximal tibial crest, preventing it from being exactly in the same position where the implant is placed.

1.2. Location for implant placement

Regarding the region for implant placement, it is important that the bone density is as uniform as possible throughout the sample. The measurement is usually performed with reference to the tibial plateau. Since rats are not all the same size longitudinally, an anatomical reference is important to allow placing the implants in the same region.

In our method, we also used as an anatomical reference the region contiguous to the insertion of the tibialis cranialis muscle, the gracilis and the gastrocnemius lateral head muscle. Being a region of muscular insertions, a homogeneous bone density is expected throughout the sample. However, there are scarce references in the recent literature to such criteria for implant placement, which explains the heterogeneity of the morphological analysis reported (178, 179).

1.3. Drilling method

As for the drilling method, it is important to accurately state the type/dimensions of the drill(s), the drilling speed, usually measured in rpm (revolutions per minute) as well as the temperature of the saline solution. According to several studies focused on the necrotic reaction caused by drilling, these 2 factors are precisely referred to as essential to avoid an extensive necrotic area and consequent formation of fibrous tissue (175, 180). However, there are several articles that use drilling methods that exceed these biological limits (181) and others that simply omit this information (155, 182-185).

In our methodology, the information is clear, and we chose to be very careful during the drilling procedures to avoid surgical trauma and consequent tissue necrosis. We opted for a safety drilling procedure without exceeding 150 rpm, identified as safe in relation to the temperature produced in the bone, even without saline solution, and at the same time is considered as an effective drilling in terms of its speed (174). It also allows good control of the drill during the procedure (173). Even so, we chose to maintain irrigation and cooling with saline solution at a temperature of approximately 20 °C.

1.4. Implant Size

Another relevant information frequently omitted is the size of the implant used (181), which makes it impossible to interpret the relationship between the size of the last drill and the size of the implant. This discrepancy is important in assessing stability of the

implant, with a deficit may lead to the lack of stability and in excess may lead to excess pressure and an ischemic-necrotic reaction.

However, it is important to understand that for primary stability to occur, there should be a difference between the diameter of the final drill and the implant to be placed, the implant being slightly larger in diameter. This important surgical parameter for obtaining primary stability is not always consistent with the literature, and it is possible to see methodologies where the last drill has a larger diameter than the implant placed (155).

In our study, the technique is explained with utmost detail, allowing its accurate reproduction by other researchers.

In summary, our method describes all surgical procedures in detail and allows observing the different surgical difficulties in both diseases. Moreover, our protocol stands out as a valid method given that all samples with direct contact with the bone were obtained with stable $V_{(BIC)}$ values. However, it differs from the majority of experimental studies existing in the literature, because it presents a detailed and justified technique, thus allowing its exact reproduction, and can therefore be used to study the effect of different implant surfaces in different diseases, even those that affect bone density.

2. Effect of functionalized implants on osteointegration process.

A recent scoping review on the peptides applied in dentistry (186) reveals that the application of these active biomolecules can be used to modify the surfaces of tooth implants or restorative biomaterials, as well as to increase their osseointegration and guide tissue regeneration. Previous histological and histomorphometrical results in biofunctionalization applied both to synthetic bone grafts (187) and dental implants (188) shown that biofunctionalization improves both the quantitative and the qualitative parameters of osteointegration. The qualitative parameters, obtained by histological observation and ultramicroscopic analysis, showed a constant presence of vascular structures in the alveolar spaces, that maintains a stable membranous formation throughout the different experiment's timelines (187, 188). After adequate preparation, the samples were qualitatively analyzed using SEM and optical microscopy following staining with similar techniques as the ones described by Cirera et al (187, 188).

Implants functionalized with $\alpha\text{v}\beta 3$ -selective peptidomimetic (Condition A).

Time 1 (4 weeks)

Our findings reveal a combination of membranous ossification and periosteal reactions, showcasing vital processes in bone development and healing. Membranous ossification (Figure 45 in the Results chapter), where bone forms directly from connective tissue, highlights the body's natural response to the implant. Classic periosteal reactions, reflecting responses of the outer layer of bone tissue, suggest an active biological response contributing to the reconstruction of the bone cortex. Furthermore, the initial reaction to surgical implant placement manifests as a vascular response, indicating early changes in blood supply and circulation that foster a healing environment. These elements collectively outline the complex interactions of bone growth, healing processes, and biological responses observed in the early phases following implant placement previously described in the literature (187-189).

In our study, we observed a significant vascular response post-implant surgery (Figure 44 in the Results chapter), highlighting the pivotal role of altered blood circulation and supply as the initial step in fostering a healing environment within the tissue.

This observation resonates with the extensive research conducted over the past two decades on cyclic peptides containing the RGD sequence, elucidating the critical involvement of $\alpha\beta3$ integrin in driving angiogenesis (189). The successful utilization of PET radiopharmaceuticals, including ^{18}F -Galacto-RGD, has underscored the importance of $\alpha\beta3$ integrin in mediating cell adhesion, migration, and angiogenic processes (190-194).

Notably, the upregulation of $\alpha\beta3$ expression has been observed not only in cancer cells but also in cell types involved in ECM remodeling (194, 195), indicating its broader implications in tissue response mechanisms and healing dynamics. Studies utilizing RGD-PET agents have revealed the diverse roles of $\alpha\beta3$ integrin across various pathophysiological conditions, with a particular focus on cardiovascular diseases (195) like atherosclerosis.

The presence of $\alpha\beta3$ integrin in endothelial cells and macrophages within atherosclerotic plaques suggests its involvement in promoting angiogenesis and potentially contributing to plaque instability (196-198).

Moreover, the essential role of $\alpha\beta3$ integrin in orchestrating myocardial repair post-myocardial infarction further underscores its significance in facilitating angiogenesis within the infarcted myocardium. Recent research utilizing RGD-PET agents in cardiovascular settings has reinforced the central role of $\alpha\beta3$ integrin in angiogenic processes, highlighting its multifaceted functions and potential therapeutic implications in promoting tissue healing and vascular responses (199, 200).

During angiogenesis, different integrins on activated endothelial cells likely play a crucial role in controlling essential adhesive connections with various proteins in the ECM, including fibronectin, vitronectin, laminin, collagen types I and IV, von Willebrand factor, fibrinogen, and denatured collagen. Each of these interactions may govern specific biological processes like cell movement, growth, and specialization. Additionally, the process of angiogenesis in different tissues may rely on particular interactions between

endothelial cells and ECMs that differ significantly in their composition of adhesive proteins(201).

Among the diverse integrin subunit combinations expressed on cell surfaces, integrin $\alpha\beta3$ stands out for its intriguing expression pattern in vascular cells during angiogenesis and vascular remodeling. This integrin acts as a receptor for various ECM ligands containing the RGD sequence, including vitronectin, fibronectin, fibrinogen, and others. In adults, integrin $\alpha\beta3$ is generally limited in tissue distribution, not commonly found on epithelial cells, and only minimally expressed in specific cell types like intestinal, vascular, and uterine smooth muscle cells. It is also present in a small fraction of activated leukocytes, macrophages, and osteoclasts, contributing to immune function and bone resorption. Additionally, some invasive tumors such as metastatic melanoma and late-stage glioblastoma exhibit $\alpha\beta3$ expression, enhancing the malignant characteristics of the tumor (202).

Endothelial cells experiencing growth factor exposure or involved in angiogenesis within tumor, wound, or inflammatory environments exhibit elevated levels of $\alpha\beta3$ (190, 203-205). Recent research indicates that $\alpha\beta3$ could potentially serve as a valuable diagnostic or prognostic marker for tumors. Notably, studies show that the anti- $\alpha\beta3$ mAb LM609 conjugated to a gadolinium-containing liposome effectively targets blood vessels associated with tumors in rabbits, as visualized through magnetic resonance imaging (206).

The pivotal role of integrin $\alpha\beta3$ in key processes of angiogenic blood vessel formation supports the notion that this integrin promotes the viability of stimulated endothelial cells. Evidence from studies involving systemic administration of $\alpha\beta3$ antagonists in animals undergoing angiogenesis reveals blood vessels with elevated levels of apoptotic endothelial cells. These findings imply that the activation of $\alpha\beta3$ on vascular cells may trigger a vital signaling cascade necessary for the survival and differentiation of vascular cells during angiogenesis in vivo. Specifically, the ligation of $\alpha\beta3$ plays a crucial role in maintaining the sustained activation of MAP kinase induced by angiogenic growth factors (207).

Even though $\alpha\beta3$ is known to play a role in angiogenesis, not many studies have explored how it is expressed in response to osteogenic mechanical loading. The first study investigating this found that after applying WBF loading, the $\beta3$ integrin subunit showed minimal expression on blood vessels on days 1 and 3 but was abundantly expressed on vessels around newly formed woven bone on days 7 and 14 (208). These results support the idea that angiogenesis starts around day 3 during the healing of stress fractures, and they align with reports on wound healing showing the presence of the $\alpha\beta3$ receptor in new blood vessels but not in mature ones (209). The strong expression of the $\beta3$ integrin in blood vessels observed even on day 14 (208) suggests that vascular remodeling continues as the density of the woven bone increases from day 7 to 14 (210).

Implants functionalized with $\alpha\beta3$ -selective peptidomimetic (Condition A).

Time 2 (12 weeks)

The findings from our study, related with the mature bone augmentation in all cortical regions, as can be seen in Figure 47 in the Results chapter, highlight notable advancements in tissue maturity, indicating a positive progression in the healing and integration process when compared to the initial observations. There is also a demonstration of structural continuity and vigorous bone growth activity around the bone marrow, pointing to a healthy, ongoing process of bone regeneration and adaptation in response to the implant. Further, this active bone growth culminates in a measurable increase in the thickness of the tibial cortex. This thickening, affecting both the periosteum (the outer layer of the bone) and the endosteum (the inner layer), signifies a comprehensive enhancement in bone density and strength around the implant area.

The noted advancements in tissue maturity and the overall healing process observed in our study may be intricately linked to the interaction of the $\alpha\beta3$ integrin with its surrounding cellular and ECM environments. The $\alpha\beta3$ integrin, known for its critical role in bone biology, including osteoclast-mediated bone resorption and osteoblast-mediated bone formation, could provide valuable insights into how these processes contribute to the healing and integration observed.

The correlation between the overexpression of $\alpha\text{v}\beta 3$ and enhanced osteoblast growth provides a compelling insight into the critical role this integrin plays in bone biology. Osteoblasts, the cells responsible for new bone formation, exhibit a higher proliferation rate when $\alpha\text{v}\beta 3$ is overexpressed (211). This suggests that $\alpha\text{v}\beta 3$ integrin is not just a participant in the adhesion and migration of osteoblasts but is also a potent stimulator of their proliferation (212). This contrasts other reports indicating it suppresses bone differentiation (122, 127).

The structural continuity and the vigorous bone growth activity around the bone marrow suggest a dynamic interplay where $\alpha\text{v}\beta 3$ may facilitate the attachment of osteoblasts to the ECM as has already been demonstrated in the case of SaOS-2 cells (213). This is crucial for the regeneration phase, where the formation of new bone tissue is paramount. As already discussed, $\alpha\text{v}\beta 3$ integrin's role in angiogenesis (190-194) could also support this vigorous bone growth by enhancing the vascularization in the implant area, ensuring a sufficient nutrient and oxygen supply for bone tissue regeneration.

The measurable increase in the thickness of the tibial cortex, affecting both the periosteum and the endosteum, may further emphasize the contribution of $\alpha\text{v}\beta 3$ to bone strength and density. By mediating the signaling pathways that control the deposition of new bone matrix proteins (214, 215) and its subsequent mineralization, $\alpha\text{v}\beta 3$ could be enhancing the mechanical properties of the bone.

However, contrasting findings elucidated by other studies indicate a nuanced aspect of $\alpha\text{v}\beta 3$ role. An overexpression of $\alpha\text{v}\beta 3$ may lead to a hindrance in matrix mineralization, a process pivotal to bone strength and health, as this overexpression typically encourages cell differentiation, a critical phase that could paradoxically slow down mineralization (216).

According to some studies, this inhibition mechanism of matrix mineralization is partly attributed to decreased activity of alkaline phosphatase, a crucial enzyme in the mineralization process, alongside a reduced expression of type I collagen and bone sialoprotein (BSP), with an accompanying increase in osteopontin expression. Under normal conditions, matrix mineralization occurs within an environment rich in alkaline phosphatase, either in matrix vesicles or along collagen fibrils (217). Given that BSP acts

as a nucleation center for hydroxyapatite, essential for mineralization (218, 219), a reduction in these key components due to $\alpha\beta3$ overexpression inherently diminishes the matrix's ability to mineralize. Compounding this effect, overexpressed $\alpha\beta3$ potentially captures BSP on the cell surface, thereby preventing it from contributing to matrix assembly and, consequently, mineral deposition. Additionally, because of osteopontin role in inhibiting the formation of hydroxyapatite(220), an increase in osteopontin levels within $\alpha\beta3$ -overexpressing cells might further delay the mineralization process.

According to these findings, If the mineralization process is delayed in favor of osteoblastic cell differentiation through the action of overexpressed $\alpha\beta3$, this process could potentially justify ongoing bone growth before the mineralization phase, offering significant advantages through bone apposition, such as increased mechanical properties of the bone. This phenomenon underscores the intricate balance between cell differentiation and mineralization in bone development, highlighting the potential for enhanced bone formation and strength through optimized cellular processes.

Understanding the complex role of $\alpha\beta3$ in these processes opens up new avenues for therapeutic strategies aimed at enhancing bone repair and regeneration, particularly in the context of improving implant integration. Targeting the $\alpha\beta3$ integrin pathway could lead to the development of novel treatments that specifically enhance bone healing and integration, leading to faster recovery times and improved outcomes for patients requiring implants.

Implants functionalized with $\alpha5\beta1$ -selective peptidomimetic (Condition B)

Time 1 (4 weeks)

Compared to condition A at time 1, in condition B for the same time it is possible to find positive reactions where processes related to angiogenesis, as well as chondroid tissue and osteoid layer, can be observed. Despite this relatively positive response related to the healing process, it appears more subtle compared to condition A in all biological processes (Figure 51 in the Results chapter).

The role of $\alpha5\beta1$ in angiogenesis is still not fully understood. Its connection with the epidermal growth factor receptor (EGFR) (135) and heightened expression during

angiogenic processes and in tumors suggests a potential tumor-promoting function (136). The impact of $\alpha 5\beta 1$ seems to be influenced by contextual factors, leading to variations between tumor promotion and inhibition. Recent studies suggest that αv and $\alpha 5$ integrins may collaborate or function interchangeably in vascular remodeling, adding complexity to our comprehension of these integrins' involvement in angiogenesis and tumor behavior (137).

However, distinct growth factors can trigger specific pathways of angiogenesis that involve different integrins (221). For instance, integrin $\alpha v\beta 3$ is active in the bFGF (basic fibroblast growth factor) and TNF- α angiogenesis pathways, while $\alpha v\beta 5$ is involved in the VEGF and TGF- α pathways (221). The impact of integrin $\alpha 5\beta 1$ antagonists on angiogenesis induced by various growth factors has also been examined. When TNF- α or IL-8 stimulated angiogenesis, antibodies targeting integrin $\alpha 5\beta 1$ hindered the process, although they were ineffective in blocking VEGF-induced angiogenesis. Interestingly, while antibodies against integrin $\alpha v\beta 5$ effectively halted VEGF-induced angiogenesis, both peptide and nonpeptide antagonists of integrin $\alpha 5\beta 1$ failed to do so (222).

When antibodies against integrin $\alpha 5\beta 1$ and $\alpha v\beta 3$ were separately or combinedly used on bFGF-stimulated chorioallantoic membranes from chicken (CAMs), no additional inhibitory effects were observed (222).

All these findings suggest that integrin $\alpha 5\beta 1$ is involved in the same angiogenic pathway as integrin $\alpha v\beta 3$ (222).

The angiogenic function, which involves the formation of new blood vessels, is crucial for bone healing and bone formation. Additionally, supporting cell adhesion and proliferation is another fundamental process that plays a vital role in various biological functions, including bone healing. This process has been observed to be essential for creating a conducive environment for cell growth and tissue regeneration during the bone healing process (223).

While some authors have not observed any effect of this receptor on cell growth, both in vivo and in vitro (124), other studies indicate that blocking $\alpha 5\beta 1$ significantly reduces the expression of the transcription factor c-Fos, which is linked to cell proliferation (224). Furthermore, several studies have identified a positive role of the $\alpha 5\beta 1$ subtype in

stimulating osteogenesis. This receptor has been demonstrated to increase the expression of osteogenic markers and alkaline phosphatase activity in laboratory settings and to promote the integration of implants (122, 124, 125). Additionally, it has been shown to induce ectopic bone formation in vivo (225).

Thus, our results are consistent with the positive biological responses observed by other authors in terms of improved healing and osseointegration due to $\alpha 5\beta 1$ stimulation; these favorable outcomes are not observed when compared with Conditions C and D, i.e., implants without biofunctionalization.

Implants functionalized with $\alpha 5\beta 1$ -selective peptidomimetic (Condition B)

Time 2 (12 weeks)

In our samples, the formation of new blood vessels is evident (Figure 53 in the Results chapter), highlighting active angiogenesis. The periosteum, the outermost layer of bone, is distinctly visible and plays a crucial role in fracture healing thanks to its rich blood supply. Overall, the samples display highly mature tissue characterized by a solid, uniformly laminar bone structure, suggesting successful healing and integration processes, also confirmed by the abundance of osteocyte *lacunae*. Additionally, color variations observed during the staining of the bone provide further insights. These variations help distinguish between old and new bone tissues, offering valuable information on the processes of bone remodeling and regeneration.

An increase of mature lamellar bone around the implant is observed in contact with the cortical bone, which results in a significant thickening of the bone. This phenomenon demonstrates the importance of osteoblastic activity, indicating an active process of bone formation. The presence of this mature bone tissue and its positive interaction with the cortical bone suggest an effective consolidation process and a healthy response of the body to the presence of the implant. This increase in bone thickness around the implant is a positive indicator of proper integration of the implant with the surrounding bone tissue, promoting a strong anchorage and potential longevity of the implant in the site.

This osteoblastic response aligns with the existing literature. As previously described, $\alpha 5 \beta 1$ integrins are particularly pivotal in managing osteoblast adhesion (226), and also critical for the survival of osteoblasts and for the mineralization of bone. Suppression of $\alpha 5 \beta 1$ expression has been shown to result in reduced survival rates of osteoblasts (227) and significant bone loss (227, 228). Indeed, its prominent role in MSC osteogenic differentiation and bone formation and repair has been reported by several authors (124, 225, 229). It has been documented that the differentiation of MSCs into osteoblasts through the $\alpha 5 \beta 1$ integrin entails the activation of FAK/ERK1/ERK2, PI3K, and IGF2/IGFBP2 signaling pathways (230), indicating the potential involvement of multiple pathways in mediating the integrin's impact. In the context of bone biology, the Wnt signaling pathway is recognized as a critical regulator of osteoblast formation. However, the specific molecular mechanisms through which $\alpha 5 \beta 1$ integrin enhances osteogenic differentiation remain to be fully elucidated.

Non-functionalized implants (Condition C and D)

Time 1 (4 weeks)

In examining the details of the observed bone healing and regeneration processes within non-functionalized implants (i.e. control conditions) at time 1, several distinct but interrelated phenomena are evident. The following analysis synthesizes observations from vascular activity, trabecular formation, and the presence of chondroid tissue to develop a broader understanding of the osteogenic dynamics occurring within these samples.

Firstly, the continuity between the vascular and medullary cavities, as shown in Figure 55-A in the Results chapter, suggests a robust integration between the host bone and the implant environment. This connectivity is essential for maintaining a sustainable nutrient supply, which is vital for ongoing bone health and regeneration. Despite the presence of trabeculae, their incomplete extension into the interior of the implant indicates a transition phase in the structural integration process. This incomplete integration further underscores the dynamic and gradual nature of bone remodeling within implanted regions.

Abundant chondroid tissue, as highlighted in Figure 55-B in the Results chapter, serves as a critical precursor to membranous ossification, an essential part of bone formation where osteoblasts directly deposit bone matrix. The presence of this tissue points to an active osteogenic front, characterized by preparatory groundwork for new bone formation.

Moreover, the observation of active neovascularization across these samples is significant. This new blood vessel formation from the periosteum not only indicates metabolic activity but also suggests an enhanced biological response to the implant. This neovascularization facilitates the transport of essential minerals and osteoprogenitor cells, crucial for bone formation and healing.

A classic periosteal reaction extending into the interior of the implant is also observed, indicative of a reparative mechanism wherein the periosteum thickens and aids in stabilizing and integrating the implant with native bone structures. As trabeculae reappear within the implant alongside chondroid tissue, it marks a significant step toward achieving an osteoconductive interior, which supports the ingrowth of new bone.

The overall response across the samples indicates a positive, albeit localized, biological reaction similar to previous conditions at the same time. This localization might suggest a concentrated area of bioactivity which could indicate zones of intense remodeling or areas where further intervention may be required to encourage widespread osteointegration.

In summary, the observed behaviors, including vascular integration, chondroid tissue presence, and periosteal reactions, are intertwined elements that depict an active process of bone repair and implant integration and adaptation. Such a comprehensive view offers vital insights into the mechanisms at play in bone regeneration within implants.

Non-functionalized implants (Condition C and D)

Time 2 (12 weeks)

After 8 weeks of healing following implant placement in our osteoporotic rat tibia model, the healing process exhibits notable characteristics indicative of bone regeneration.

Rigorous observations reveal external and internal laminar tissue progress, alongside significant blood vessel growth throughout the implant, extending into its interior. The presence of an osteoclast surrounded by material indicates active bone remodeling consistent with classic Haversian ossification. Although signs of osteonal system remodeling and remnants of immature tissue suggest a positive trend, the healing shows a slightly reduced performance compared to previous conditions, highlighting the impact of functionalized implants on healing intensity.

In addition to mature and stable tissue within the samples, evidence of a nutrient foramen, crucial for blood vessel ingress and egress in long bones, is noted. This insertion point indicates a robust vascular network supporting bone growth. Further, bone formation extends deeply into the implant, signifying successful integration and healing in the internal area.

While mature lamellar bone is evident in the samples, no clear reversal of osteoporosis or significant structural alterations are noted. This steady bone structure, although mature, suggests a lack of profound changes in bone density or quality over the healing period, failing to deliver substantial improvements in the osteoporotic condition.

The detailed observations of bone healing in the osteoporotic rat tibia model elucidate the intricate processes of bone regeneration and the impact of functionalization on healing outcomes. The nuanced healing trends seen in the presence of functionalized implants versus non-functionalized ones underscore the importance of targeted interventions for addressing specific bone conditions, leading to more robust and effective bone regeneration strategies.

However, when comparing non-functionalized implants to conditions with functionalized implants containing $\alpha 5\beta 1$ and $\alpha v\beta 3$ -selective peptidomimetics, a distinction in healing intensity is observed. The healing with the non-functionalized implant appears to be

normal but less robust or subtle in comparison to the enhanced healing seen with the functionalized implants.

Globally, we can conclude that condition A, which involves implants with the RGD peptidomimetic with specific affinity to integrin $\alpha\beta3$, exhibits the best characteristics. This receptor is of particular interest due to its involvement in various biological functions such as wound healing, angiogenesis, tumor growth and metastasis (132, 213), as well as osteoclast-mediated bone resorption(130). Additionally, some studies have shown that osteoclasts are the cell type with the highest in vivo expression of integrin $\alpha\beta3$ (130). However, $\alpha\beta3$ stimulation has also been correlated with increased osteogenic differentiation and bone formation (129), as it is clearly shown in our study.

Condition B, which involves implants with the RGD peptidomimetic with specific affinity to integrin $\alpha5\beta1$, has characteristics very similar to those of condition A, but slightly more moderate. The observed results show significant activity and growth around the medulla. Studies have indicated that integrin $\alpha5\beta1$ supports cell adhesion and proliferation (226), as well as bone formation (124, 225, 229).

Regarding conditions C and D, which refer to implants that have not been functionalized, adequate host responses were also observed, which are indicative of an adequate surgical protocol; however, bone growth and healing processes seem to be more discrete and locally reactive compared to those observed in conditions A and B.

The qualitative results obtained by observation of the images obtained by SEM and optical microscopy for each condition can be summarized in table 15.

	Discussion Highlights Corollary
CONDITION A	Great activity and growth around the bone marrow. The peptide activates bone marrow cells that manage to reverse the characteristics of osteoporosis. There is a gain in the tibial cortical thickness, both periosteum and the endosteum.
CONDITION B	Great activity and growth around the marrow, but this time a little more moderate. There is gain in cortical thickness and reversal of osteoporosis.
CONTROL CONDITION	Positive but more local reaction. The pre-existing cortex is remodeled, but there are no changes in the bone structure, there is no gain in volume. There is no evidence of osteoporotic reversal.

Table 15: Condition A – $\alpha\beta3$ integrin. Condition B – $\alpha5\beta1$ integrin. Condition C and D – CONTROL – Ti without modifications.

3. Critical Analysis: Strengths and Limitations of the Study.

Strengths of the Study

The positive aspects appear evident. The surgical technique demonstrates predictability and safety for this animal model. The detailed description of the procedure, both in the thesis and the accepted publication article, facilitates its reproducibility in numerous other studies with this model.

Tissue response to the peptidomimetics was positive compared to the control group. This response aims to address one of the main problems affecting the well-being of osteoporotic patients, particularly in cases of difficult recovery after traumatic bone fractures or in implant-supported oral rehabilitation.

The whole procedure enabled clear and interpretable imaging results.

Limitations of the Study

The animal numbers have been a constant concern, making it difficult to calculate the minimum number of animals required for significant results. Ethical considerations and study efficiency are also important factors. Additionally, this model exhibits significant metabolic differences, making it challenging to reproduce results in larger animals or humans.

In terms of the surgical technique, despite positive results in most aspects, there is a limitation in reproducing the exact positioning for contact between the implant and the bone cortices on the inner side of the tibia. This limitation requires sample grouping in segments, potentially necessitating a significant increase in the number of animals.

Regarding the use of peptidomimetics, although the response was positive, the full extent of secondary effects remains unclear. The complex primary and secondary actions of peptidomimetics, sometimes contradictory depending on the stage of healing and other metabolic actions occurring concurrently, complicate understanding their effects.

Future Perspectives

For the surgical procedure, testing in different conditions will enhance its reproducibility. This can include using the same model with different pathologies or implants with varying surface properties.

Regarding the implant surface, further identical and parallel studies focusing on other tissue behaviors should be conducted to optimize understanding of the peptidomimetics reaction. Additionally, ongoing research on other peptidomimetics and methods to enhance the quality of life for individuals suffering from osteoporosis-related fractures is of crucial importance.

As a future perspective, adapting this study to larger animals will offer valuable insights into their reactions in specimens that are metabolically closer to humans.

CONCLUSIONS

In summary, the analysis conducted on the samples has led to several key findings:

1. Our surgical protocol stands out as a valid method given that all samples showed osseointegration without complications, with direct contact with bone-implant contact values.
2. This surgical protocol allows its exact reproduction and can therefore be used to study the effect of different implant surfaces in osteoporosis.
3. The preparation of the samples enabled qualitative and quantitative analysis through SEM and optical microscopy. The high percentage of bone-implant contact (BIC) observed is attributed more to the surgical procedure than surface modifications. The presence of osteoporotic bone tissue did not significantly affect implant survival rates.
4. Qualitative analysis highlighted that implant with peptidomimetics inducing $\alpha\beta3$ integrin activation showed robust bone growth around the marrow, leading to increased tibial cortical thickness. This activation helped reverse osteoporotic characteristics. The implants functionalized with $\alpha\beta3$ -selective peptidomimetics exhibited more mature bone formation in the represented by the cortical aspect compared to those functionalized with $\alpha5\beta1$ -selective peptidomimetics.
5. Comparing modified and unmodified implants, while both showed good osseointegration, only modified implants exhibited cortical volume gain and osteoporotic reversal due to bioactivation throughout the bone.
6. Quantitative analysis demonstrated that implants with $\alpha\beta3$ integrin peptidomimetics had higher BIC compared to those with $\alpha5\beta1$ integrins. There were no significant outliers in the results, indicating consistent statistical outcomes.
7. The presence of bicorticality in samples did not impact implant stability and was independent of the conditions. However, significant differences in BIC were observed between conditions A ($\alpha\beta3$ integrin) and B ($\alpha5\beta1$ integrin) when analyzing bicortical and total-bicortical groupings separately at time 2, with condition A showing higher averages in both cases.

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ANNEXES

Article acceptance letter for publication from the Editor of the Journal JOVE and the published article.

31/05/24, 02:53

View Letter

Date:

Apr 26, 2024

To:

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Surgical Bone Implantation Technique for Rat Tibia Models of Diabetes and Osteoporosis

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Abstract

The rat has long served as a valuable animal model in implant dentistry and orthopedics, particularly in studying the interactions between biomaterials and bone tissue. The rat's tibia is frequently chosen due to its easy surgical access through thin tissue layers (skin and muscle) and the flattened shape of its medial face, facilitating the surgical insertion of intraosseous devices. Additionally, this model enables the induction of specific diseases, mimicking various clinical conditions to assess biological responses to different implant conditions like geometry, surface texture, or biological cues. However, despite its robust cortical structure, certain intraosseous devices may require adaptations in design and size for successful implantation. Therefore, establishing standardized surgical methods for manipulating both soft and hard tissues in the implantation region is essential for ensuring proper implant or screw device placement, particularly in fields like implant dentistry and orthopedics. This study included eighty Sprague Dawley rats divided into two groups based on their respective diseases: Group 1 with osteoporosis and Group 2 with Type 2 Diabetes. Implantations were performed at 4 weeks and 12 weeks, with the same surgeon following a consistent surgical technique. A positive biological response was observed, indicating complete osseointegration of all implants placed. These results validate the success of the surgical protocol, which can be replicated for other studies and serve as a benchmark for the biomaterials community. Notably, osseointegration values remained stable at both 4 weeks and 12 weeks for both disease models,

demonstrating a durable integration of the implant over time and emphasizing the establishment of an intimate bone connection as early as 4 weeks.

Introduction

The common choice of rats as experimental subjects is due to the fact that they are easy to breed and relatively inexpensive compared to larger animal models. The emergence of new procedures, such as the reliable reproduction of a disorder, e.g., osteoporosis or diabetes, makes this model especially useful for analyzing the potential use of treatments and/or the influence of the disease in the biological response to drugs and surgical devices or procedures^{1,2}.

The rat's bone mass gain occurs mostly during the first 6 months of life, although some researchers believe that the long bone grows constantly for at least a year with a progressive increase in length¹. With aging, there is a transition from modeling to remodeling, which does not occur in all cases equally throughout the bones². Female Sprague Dawley rats grow more slowly than male rats and achieve a lower peak in weight than male rats¹. Continuous bone elongation and varied bone remodeling dynamics in rats are factors that have to be taken into account when addressing human health issues; however, it has not yet been possible to find any experimental research that shows either lifelong rat bone development or the species' inability to remodel bone¹. If the experimentation starts around 10 months of age, a margin of at least 1 mm from the growth plate of the tibia should be left intact due to this longitudinal bone growth, an issue to be considered in dental implant studies². Hormones are also a key parameter in bone research since at 8 months of age, male rats were found to have 22% greater bone width and 33% greater breaking strength than females in the tibia³.

The reliable reproduction of a disorder is thus very important in orthopedics and implant dentistry since osseointegration of an orthopedic screw or a dental implant is a complex process that depends on numerous factors influencing the systemic response to the device implantation into the bone. Systemic disorders like osteoporosis and diabetes are known to affect the success rate in orthopedics and implant dentistry, so the reliable reproduction of those disorders in rat models can be applied to explore ways to overcome these limitations.

The rat tibia, due to the easy surgical access, moderate bone volume, and the flat shape on the medial plate, makes it suitable for surgical bone implantation experiments^{4,5}, and it has been used in numerous research studies exploring the effects of implant surface on osseointegration^{4,5,6}. A growing number of studies assess the effects on osseointegration of coatings and substances added to the implant surface in both healthy animals⁷ and in compromised animals affected by diabetes or osteoporosis^{8,9,10,11,12,13,14}.

The number of implant devices placed in one rat's tibia is limited and can differ depending on the type of study. Depending on the number of implants or study conditions, the dimensions of the devices must be adapted to minimize surgical trauma. In studies with one implant, a nearly human-size implant can be placed (2.0 mm in diameter and 4 to 5 mm in length), and bi-cortical anchorage can be achieved^{6,7,15,16}. The dimensions of the implants in multi-

implant protocols should adopt an appropriate implant size (1.5 mm in diameter and 2.5 mm in length)^{4,17}.

The present study aims to describe a standardized surgical protocol for titanium implant placement on the tibia of two rat models: the osteoporosis and the diabetes rat model. Moreover, this study permits testing the surgical protocol to assess different types of implant surface biofunctionalization and its effect on osseointegration.

A sample of 80 rats was divided into two groups. In group 1, 40 ovariectomized Sprague Dawley females and 5 sham animals were selected, with a mean weight of 484 g and a mean age of 12 weeks. Based on vendor recommendations (see **Table of Materials**), three months after neutering, the experiment started. This waiting period ensured the disappearance of sex hormones. Osteoporosis was confirmed at the time of surgery based on micro-computed tomography (micro-CT) bone analysis, which reflected an average of 20% bone loss compared to the sham group. Group 2 consisted of 40 BBDR (Bio Breeding Diabetes Resistant) genetically modified Sprague Dawley rats with type II diabetes. The mean weight was 730 g, and the average age was 12 weeks. Prior to surgery, the diabetic status was confirmed with three consecutive days of glucose measurements with results higher than 200 mg/dL. Glucose was measured with a glucometer in 6 h of fasting, and a blood drop was collected by tail puncture.

Grade 3 titanium implants measuring 2 mm in length and 1.8 mm in diameter were used. All implants were ultrasonically cleaned in cyclohexane (3 times for 2 min), acetone (once for 1 min), deionized water (3 times for 2 min), ethanol (3 times for 2 min), and acetone (3 times for 2 min) using an ultrasound bath (230 VAC, 50/60 Hz, 360 W). Then, the samples were dried with nitrogen gas, and a nitrogen beam at 0.5 bar

was applied directly onto the samples. Prior to implantation, the implants were first soaked in deionized water and then sterilized by immersion in 70% ethanol (v/v) for 10 min. After this, the implants were transferred to sterile microcentrifuge tubes, and kept under sterile conditions until the surgery.

Protocol

All experimental procedures were conducted in accordance with the European Community Guidelines for the protection of animals used for scientific purposes (Directive 2010/63/EU) as implemented in Spanish law (Royal Decree 53/2013) and Generalitat de Catalunya regulations (Decree 214/97). Ethics approval for all animal procedures and handling was obtained from the Ethics Committee for Animal Experimentation of the Vall D'Hebron Institut de Recerca (registration number 72/18 CEEA). For the osteoporotic model, female Sprague Dawley rats with an average weight of 484 g and an average age of 12 weeks were utilized. As for the diabetic model, genetically modified female BBDR (Bio Breeding Diabetes Resistant) rats with an average weight of 730 g and an average age of 12 weeks were employed. All animals were sourced from a commercial supplier. The specific details of the animals, reagents, and equipment utilized in the study are listed in the **Table of Materials**.

1. Anaesthesia/pharmacology and preparation of animals

1. Administer presurgical analgesia using buprenorphine at 0.05 mg/kg and meloxicam at 2 mg/kg through subcutaneous injections 10-15 min before starting the surgical procedure.
2. Perform intra-surgical anesthesia with inhaled isoflurane: initiate at 5% in fresh air during induction and maintain at 3%. Induce anesthesia in a rat chamber and maintain the

isoflurane supply with a conical nose adaptation during surgery.

2. Preparation for the surgery

1. Measure the anesthetized animal's body temperature with a rectal probe and use an electronically controlled heating pad for thermal support throughout the surgical procedure. Eye ointment must be applied prior to surgery beginning to avoid corneal dryness.

NOTE: If necessary, the ointment must be reapplied after checking eye dryness.

2. Clean the skin of the knee thoroughly, ensuring sterility. Trim hair with an electric shaver and apply hair removal cream to eliminate any remaining fur.
3. Obtain an aseptic surgical field by cleaning the knee skin in a pattern of iodine and 70% (v/v) ethanol using sterile swaps, starting from inside and moving outward the incision line without retracing. Perform a minimum of three consecutive cleaning rotations (iodine-ethanol-iodine).
4. Isolate the operating Field by positioning a sterile surgical drape fenestrated over the animal, exposing the leg through the central opening (**Figure 1**).

3. Surgery

1. Surgical exposure
 1. Make a full-thickness skin incision of approximately 1 cm in length vertically along the proximal border of the anteromedial face of the tibia, in the metaphysis region to expose the bone (**Figure 2**).
 2. Stabilize the leg and pull the skin taut against the underlying bone while making the incision, ensuring a clean incision stays in the correct location. Manage

by cleaning the expected light bleeding with a compress soaked in saline solution (**Figure 2**).

NOTE: The rat skin is thin and sagging or loose. Stabilization of the skin is crucial and required.

3. Fully detach the area using small periosteal elevators (**Figure 3**).
4. Expose the bone until identification of the insertion of the *tibialis cranialis muscle*, the *gracilis*, and the *gastrocnemius lateral head muscle* in the posterior border of the medial aspect of the tibia, as a fibrous white tissue firmly adhered to the bone (**Figure 3**).

NOTE: It is important to identify this group of muscular insertions to enable the implant to be placed in a bone region with similar characteristics and stimuli across the entire sample, regardless of the rat's size.

2. Drilling process

1. Begin the drilling process at the correct region between the proximal tibial crest and the posterior limit of the medial face of the tibia bone, contiguous to the insertion of the *tibialis cranialis muscle*, the *gracilis*, and the *gastrocnemius lateral head muscle*, avoiding any muscle injury.¹

NOTE: The correct location should be 5 mm ± 2 mm from the tibial plateau.

2. Drill with a maximum of 150 rpm (rotations/min) under saline solution irrigation at a temperature close to 20 °C with a surgical electric motor with a 20:1 reduction contra-angle.

NOTE: Only two drills were required.

3. Start with a lance pilot drill (**Figure 4**) at a depth of 2.4 mm under saline solution irrigation.

NOTE: Each drill had a maximum of 10 uses.

4. As a second drill, use (**Figure 4**) a twist design drill at a depth of 2.4 mm with a diameter of 1.6 mm under saline solution.

NOTE: Each drill had a maximum of 10 uses.

3. Implant placement

1. Insert the implant with an intermediary piece (**Figure 5**) attached to the 20:1 reduction contra-angle.
2. Before placing the implant, clean the implant from any residual chemical sterilant by rotating it in the contra-angle with simultaneous saline irrigation for 10 s (**Figure 5**).

3. Place the titanium implant (2 mm lengthwise and 1.8 mm in diameter) using the intermediate piece at 20 rpm while monitoring real-time torque value, registering the maximum insertion torque.

NOTE: An initial difficulty in inserting the implant is expected due to the difference between the final drill and the implant, as well as the cylindrical profile of the implant; however, this initial difficulty is quickly normalized as soon as the implant penetrates the initial cortical bone.

4. Finish implant insertion before it completely passes the cortical bone where it is inserted, that is, the flat medial face of the tibia.

NOTE: At the final moment of implant insertion, it is important to leave the implant slightly outside the cortical bone or level with the cortical bone where it is inserted, that is, the flat medial face of the tibia, to ensure primary stability (**Figure 6**).

4. Wound closure

1. Suture the muscle tissue borders with simple internal sutures using a 4/0 monofilament synthetic resorbable suture (Glyconate) (**Figure 7**).
2. Perform skin closure with an intradermal suture using a 4/0 monofilament synthetic resorbable suture (Glyconate) (**Figure 7**).

4. Micro-CT scan

1. After completing the surgery and still under general anesthesia, conduct a micro-CT scan to confirm proper implant placement.
2. Remove the rat from the surgery bed and place it on the scanning bed. Locate the operated leg using the micro-CT live scan mode and center the Field of View on the implant.

NOTE: Recommended acquisition parameters are Field of view of 5 mm, spatial resolution of 0.0001 mm^3 , 50 kV, 200 μA , and acquisition time of 3 min.

3. Once the scan is acquired, confirm the correct distance between the proximal aspect of the implant and the surface of the tibial plateau, according to step 5.2.1.

NOTE: This value will be helpful for the standardization of the technique (**Figure 8**).

5. Postoperative care

1. Following imaging acquisition, return the rat to its cage and monitor until full recovery.

NOTE: This takes approximately 5-10 min, depending on the animal model. Diabetic rats are expected to remain anesthetized longer and have a longer recovery time due to the metabolic changes associated with diabetes.

2. Administer postoperative analgesia 8 h after surgery using the same dose and technique as previously

described. Repeat this dosing pattern of analgesia at 48 h.

3. Suture removal time

1. Examine the surgical wound daily for infection, suture integrity, or other issues, and remove sutures 15 days post-surgery.

6. Euthanasia

1. Euthanize the animals using a CO₂ chamber, according to the National Institutes of Health (NIH) guidelines, with a CO₂ fill rate of 30%-70% of the chamber volume/min after the implantation time (4 weeks or 12 weeks).

7. Postsurgical analysis

1. For both models (osteoporosis and diabetes), remove the tibia by disarticulation after euthanasia for further analysis.

NOTE: Regarding the micro-CT analysis, data allowed for calculating bone-implant contact (BIC). The CT acquisition was conducted using the previously described parameters, and the BIC analysis was performed by dividing the bone area (mm²) by the implant area (mm²) calculated at the cortical region, adapting a protocol already described in the literature¹⁸. Representative images are shown for each model, osteoporosis (**Figure 9**) and diabetes (**Figure 10**).

Representative Results

Surgical phase

It is important to mention that both animal models used in this study present certain constraints due to the induced diseases.

These constraints regarding the manipulation of hard and soft tissues are reflected during the surgical procedure.

In the diabetic model, the rat is larger, making it difficult to stabilize the legs during surgical procedures. This increases the surgical time and, consequently, the anesthesia time, which requires a longer recovery time and, therefore, requires greater vigilance in the postoperative period.

As for the osteoporotic model, the requirements were very different, being more concentrated at the time of bone manipulation. In this case, it is possible to observe greater bleeding during the drilling procedure. In addition, the implant placement process becomes more difficult. Some complications may occur, such as small cracks in the bone, altering the progression of the implant during its entry and making it more unstable due to the lack of bone resistance on the lateral walls of the implant. This limitation requires more manual pressure and stabilization during placement, making it more difficult to control until the correct positioning of the implant is achieved.

Regarding the maximum implant insertion torque, relatively different values were observed for the two diseases (**Table 1**). In general, the maximum average insertion torque observed for the osteoporotic model was higher than for the diabetic model. With these results, it is interesting to understand the effect that diabetes has on bone density and bone resistance to implant insertion, as this condition appears to have a more severe effect on the mechanical properties of bone compared to osteoporosis.

Postsurgical analysis

The adequate placement of the titanium implants after 4 weeks and 12 weeks of implantation was analyzed by micro-CT imaging. Representative images are provided for both

osteoporotic (**Figure 9**) and diabetic (**Figure 10**) rats. Overall, the implants were correctly implanted through the cortical and trabecular tibiae, showing intimate and continuous contact with the cortical part, and without any signs of inflammation or adverse reactions, thus proving the success of the surgical protocol for both models of pathology.

Collectively, the analysis of the images also allowed for calculating the ratio of bone area (mm^2) versus implant area (mm^2), referred to as $V_{\text{(BIC)}}$, at the cortical region, and

comparing the performance of the surgical procedure over time and within models. As shown in **Figure 11**, $V_{\text{(BIC)}}$ values remained constant for the osteoporotic model at 4 weeks and 12 weeks, indicating a stable integration of the implant over time and highlighting that an intimate connection with bone is ensured already at 4 weeks. Of note, very similar values were obtained for the diabetic model, thus showing the adequacy of the study protocol for both models of pathology.

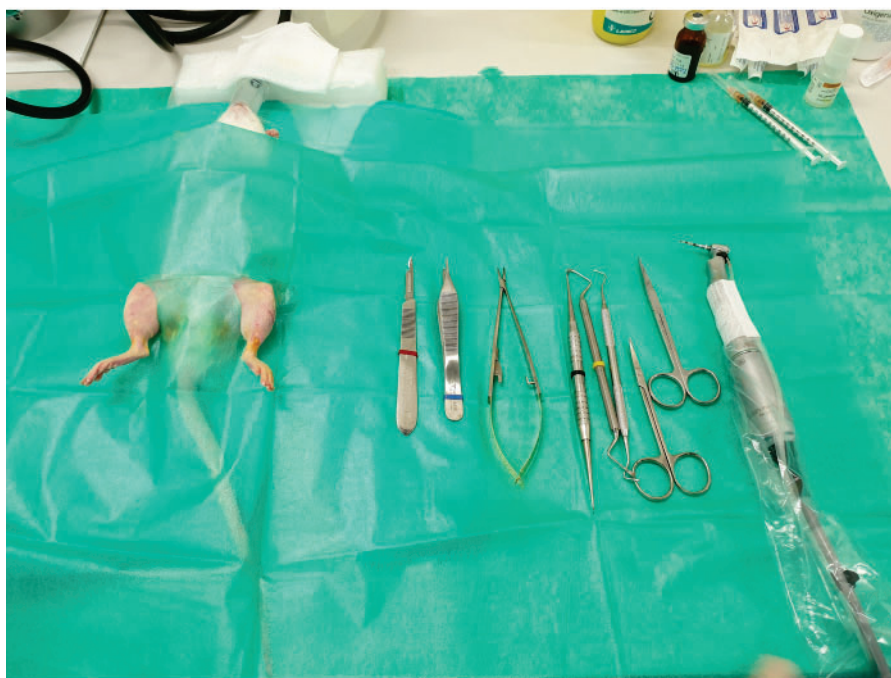


Figure 1: Surgical field. The surgical field showing the correct position of the rat and exposure of the area to be intervened, adaptation of the conical adaptor to the airways for maintenance of the anesthesia during the procedure, as well as the instruments necessary for the intervention. [Please click here to view a larger version of this figure.](#)



Figure 2: Surgical incision. Correct stabilization of the rat's paw and, at the same time, fixing the thin mobile skin to obtain a correct incision. [Please click here to view a larger version of this figure.](#)



Figure 3: Bone exposure and muscle insertion Identification. Left side: total detachment of the tissues with the consequent bone exposure. To preserve and avoid damage to the insertion of the *tibialis cranialis muscle*, the *gracilis*, and the *gastrocnemius lateral head* muscles, it is important to correctly identify the fibrous white tissue firmly adhered to the bone, visible in the figure on the right side. [Please click here to view a larger version of this figure.](#)

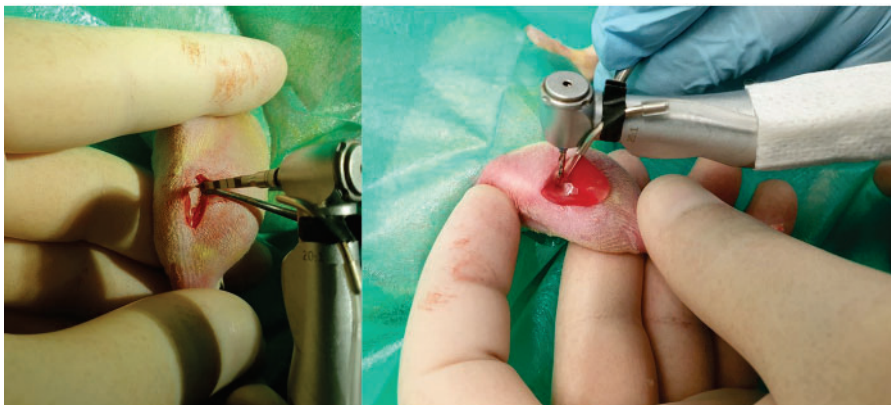


Figure 4: Drilling sequence. The drilling process sequence begins with the lance pilot drill on the left, followed by the twist drill on the right. To ensure a stable and safe drilling procedure, it is important to maintain both leg and soft tissue stabilization during drilling. [Please click here to view a larger version of this figure.](#)

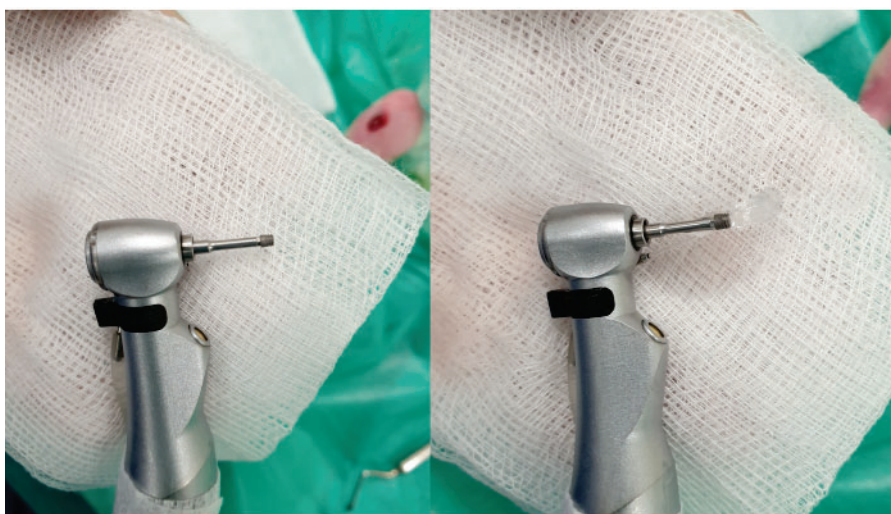


Figure 5: Preparation for implant insertion. The implant is inserted on an intermediate piece to be submitted to the cleaning process with a saline solution while the implant rotates at 20 rpm. This intermediate piece allows the insertion of the implant into the bone with an electronic device. This device ensures control of the implant insertion speed and records the real-time insertion torque. [Please click here to view a larger version of this figure.](#)

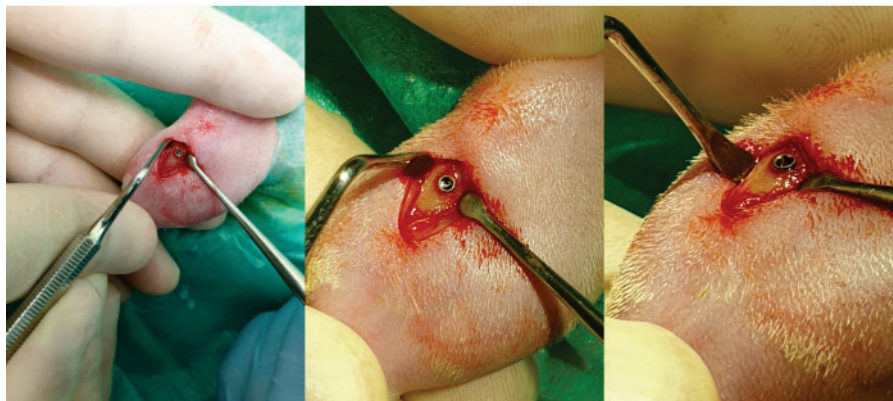


Figure 6: Titanium implant placement. Placing the implant within the bone requires its placement on the flat face of the medial bone, away from the proximal border of the anterior-medial face of the bone. [Please click here to view a larger version of this figure.](#)



Figure 7: Wound closure. The steps for closing the wound are observed in 2 different planes. Left: the first, deeper plane is made with simple stitches to approximate the edges of the muscle tissue. Right: the second, more superficial plane is carried out with an intradermal suture to approximate the skin while preventing the knot from being in an externalized position. [Please click here to view a larger version of this figure.](#)

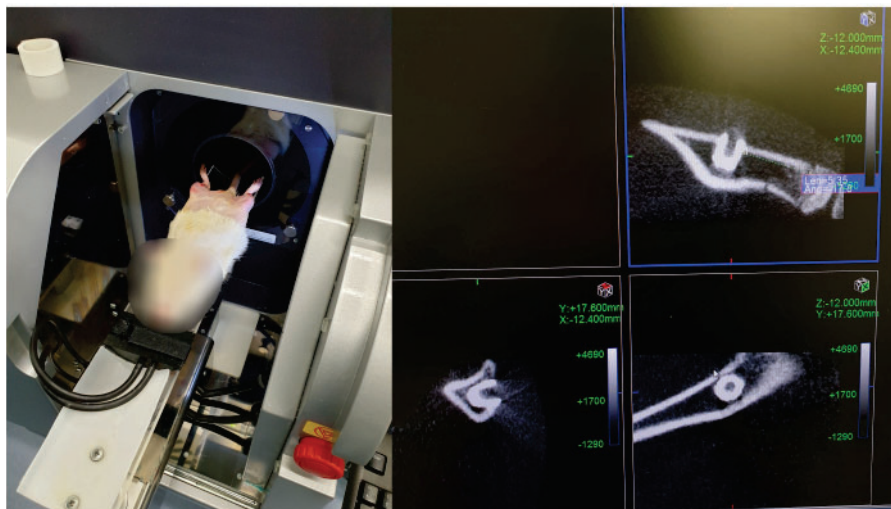


Figure 8: Micro-CT scan of implant placement after surgical procedure. The figure illustrates the positioning of the rat in the Micro-CT scanner, and the images of the implant position are divided into three sections, corresponding to the x-, y-, and z-views, respectively. The implant is pointed with a red arrow in the different views. [Please click here to view a larger version of this figure.](#)

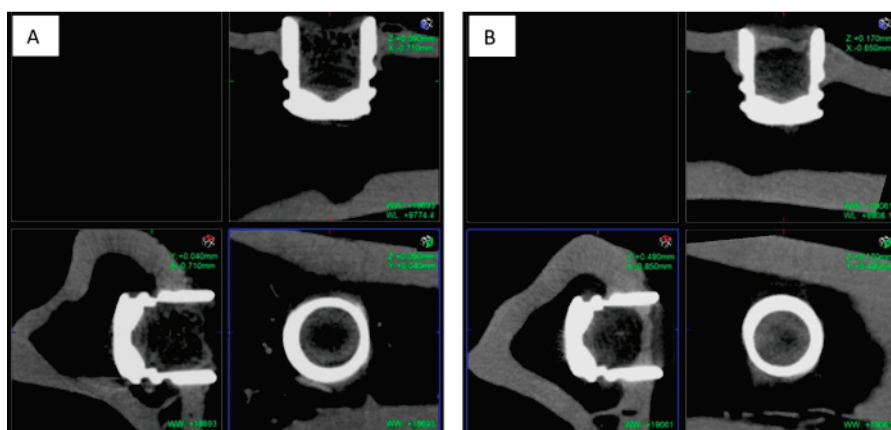


Figure 9: Micro-CT scan of an implant placed in the tibia of an osteoporotic rat. Representative postsurgical micro-CT images of titanium implants placed in the tibiae of osteoporotic rats after (A) 4 weeks and (B) 12 weeks of implantation. Each image is divided into three sections, corresponding to the x-, y- and z-views, respectively. [Please click here to view a larger version of this figure.](#)

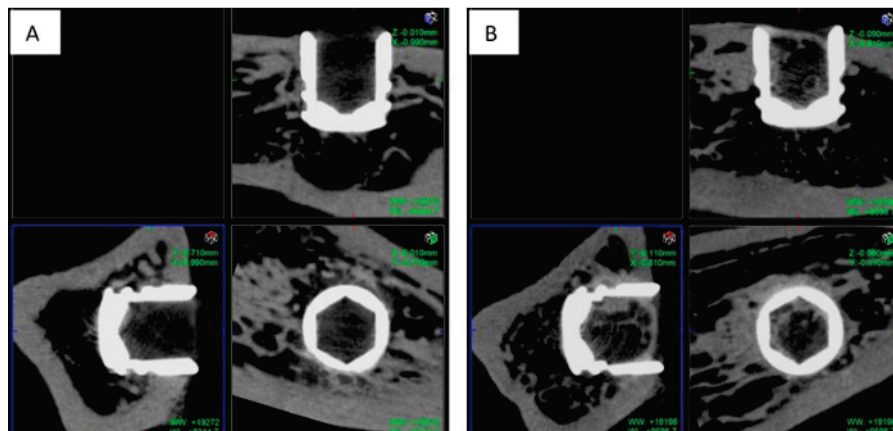


Figure 10: Micro-CT Scan of an implant placed in the tibia of a diabetic rat. Representative postsurgical micro-CT images of titanium implants placed in the tibiae of osteoporotic rats after (A) 4 weeks and (B) 12 weeks of implantation. Each image is divided into three sections, corresponding to the x-, y- and z-views, respectively. [Please click here to view a larger version of this figure.](#)

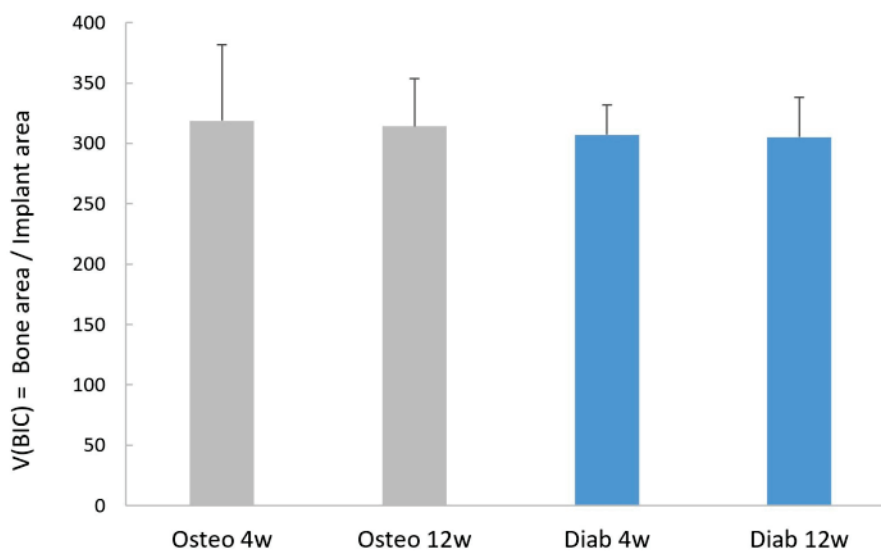


Figure 11: Bone-implant contact (BIC) analysis. $V_{(BIC)}$ values calculated as bone area (mm^2) *100/ implant area (mm^2) from micro-CT images for the osteoporotic (Osteo) and diabetic (Diab) models after 4 weeks (4 w) and 12 weeks (12 w) post-implantation. [Please click here to view a larger version of this figure.](#)

	Maximum implant insertion torque [N·cm]	
Model	Right leg	Left leg
Osteoporosis	6.11 ± 0.96	6.49 ± 1.34
Diabetes	4.79 ± 1.70	4.90 ± 1.76

Table 1: Maximum insertion torque. Differences in maximum torque values obtained during implant insertion between diabetic rats and osteoporotic rats. Diabetic rats present lower maximum torque values than osteoporotic rats.

Discussion

Although the rat is a widely used model for studying osseointegration, it is important to define and describe a reproducible surgical technique for adequately placing implants. Such a technique could serve as a guide for the scientific community. Moreover, the fact that certain diseases, such as osteoporosis and diabetes, alter bone metabolism implies stronger demands for correctly designing surgical procedures. The rat compares favorably with other animal models since it presents the main features of both osteoporosis (spontaneous and low-impact fractures) and diabetes and diet-induced obesity without requiring costly specialized facilities and long, expensive experiments, and postoperative control and maintenance¹⁹. The rat's small size, high fecundity, and short life facilitate large-scale, time-efficient experiments while meeting the three R requirements of animal experiments (replacement, reduction, and refinement)²⁰.

Despite the considerable amount of scientific literature in the field, there are only a few scientific articles describing detailed surgical techniques. Yet, the vast majority omits crucial surgical information, thus preventing the possibility of replicating the techniques described.

In this surgical protocol, several critical steps need to be considered throughout the surgical procedure, such as the

location of the incision, the precise location for drilling and implant placement, the drilling technique, the exact dimensions of the implant, and the final drill used for implant insertion.

In the literature, the exact location of the incision is often imprecise, lacking a scientific basis for its placement. The incision should be made in a different region from that of the implant placement. It is important to avoid placing the incision and consequent suture directly above the implanted area to maintain the integrity of the periosteum detached above the implant. An incision and initial detachment of the full-thickness flap can disrupt the blood supply and damage the periosteum, potentially leading to a disturbance in periosteal cell activity during new bone formation, as observed in other studies²¹. This approach aims to optimize peri-implant bone formation and minimize trauma, poor healing, fibrosis, or the risk of bacterial colonization in the suture area. In this method, the incision is detailed and made in the proximal tibial crest, ensuring it is not directly above the implant placement site.

When determining the region for implant placement, it's crucial to ensure uniform bone density across the sample. Typically, measurements are referenced to the tibial plateau. Given the longitudinal size variation among rats, an anatomical reference is necessary to consistently place implants in the same region. In this method, the area

adjacent to the insertion of the *tibialis cranialis*, *gracilis*, and *gastrocnemius lateral head* muscles serves as a reliable anatomical reference. This region, characterized by muscular insertions, is expected to exhibit homogeneous bone density throughout the sample.

When discussing the drilling method, it's crucial to provide accurate details about the type/dimensions of the drill(s), the drilling speed (usually measured in rpm), and the temperature of the saline solution. These factors are essential in preventing an extensive necrotic area and subsequent formation of fibrous tissue^{22,23}, as highlighted in several studies focused on drilling-induced necrotic reactions. However, some articles exceed these biological limits²⁴ in their drilling methods, while others omit this crucial information entirely^{4,25,26,27,28}. This study emphasizes the meticulous precautions taken during drilling procedures to reduce surgical trauma and tissue necrosis. We opted for a safety drilling procedure with a speed capped at 150 rpm, a level considered safe in terms of bone temperature even without saline solution. This approach is effective in terms of drilling speed and allows for good drill control during the procedure^{29,30}. Additionally, we maintained irrigation and cooling using a saline solution at approximately 20 °C to further mitigate any potential thermal effects.

Another relevant piece of information that is frequently omitted is the size of the implant used. This omission makes it impossible to interpret the relationship between the size of the final drill and the size of the implant. This discrepancy is crucial in assessing the stability of the implant, which in a deficit may lead to instability, while in excess, it may cause excess pressure and an ischemic-necrotic reaction.

It's crucial to note that for primary stability to be achieved, there should be a slight difference in diameter between the

final drill and the implant, with the implant being slightly larger in diameter. This critical surgical parameter for attaining primary stability is not always consistent in the literature, as some methodologies feature a final drill with a larger diameter than the implant placed⁴. Given that this region is characterized by muscular insertions, a homogeneous and robust bone density is expected throughout the sample. This characteristic helps avoid the primary limitation reported for rat tibial interventions, namely the longitudinal growth of the tibia over time³¹. Additionally, this method allows for temperature control, addressing one of the causes of complications and subsequent morbidity²³. However, similar to other studies, this study also faces a limitation concerning the need to adapt the device due to variations in tibia size^{4,17}.

Another concern related to the number of animals used is the importance of selecting an appropriate sample size. This is crucial for maximizing the impact of research outcomes while minimizing animal usage. Such a strategy not only improves the efficiency and reliability of studies but also emphasizes the ethical considerations in scientific research. Continued efforts should be made to optimize this efficiency.

In summary, the present method provides a detailed description of all surgical procedures and allows for the observation of various surgical challenges in both diseases. Furthermore, this protocol stands out as a valid method because all samples with direct contact with the bone achieved stable $V_{(BIC)}$ values. However, it differs from the majority of experimental studies in the literature by presenting a detailed and justified technique, thereby allowing for exact reproduction. Consequently, it can be used to study the effect of different implant surfaces in various diseases, including those affecting bone density.

Disclosures

We hereby declare that there is no conflict of interest regarding this scientific article.

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