UNIVERSIDADE CATÓLICA PORTUGUESA | INSTITUTO DE CIÊNCIAS DA SAÚDE

ADVANCES USING HYDROXYAPATITE AS A BIOMATERIAL IN BONE REGENERATION

Dissertação apresentada à Universidade Católica Portuguesa

Para obtenção do grau de Mestre em Medicina Dentária

Por:

Margarida Fernandes Alves Pereira

Viseu, 2014

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Orientador: Professora Doutora Ana Leite Oliveira

Co-orientador: Doutor Filipe Araújo

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“Learning is the only thing the mind never exhausts, never fears, and never regrets.”

Leonardo Da Vinci

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Resumo

Objectivo: A presente monografia tem como objectivo a análise e discussão de novas estratégias de preenchimento ósseo, utilizando como matriz-base a hidroxiapatite, que promovam um comportamento bioactivo e consequentemente a regeneração. Também englobará a comparação destas novas abordagens com abordagens mais convencionais. Pretende-se dar resposta a várias questões, tais como: quais os mediadores que promovem/participam na regeneração óssea, de que forma estes mediadores permitem a regeneração óssea, bem como as possíveis vantagens do uso de modelos tridimensionais com a forma exacta do defeito que se pretende reconstruir.

Metodologia: Foi efectuada uma pesquisa bibliográfica do estado da arte, analisando estudos publicados nos últimos 5 anos de investigação de produtos em fase clínica de desenvolvimento e de novas metodologias ainda em fase pré-clinica (estudos in-vitro e in-vivo). A componente prática deste trabalho irá consistir na análise de materiais de preenchimento ósseo comerciais (ex: Cerabone®, Bio-Oss® and NuOss®), por Microscopia Electrónica de Varrimento, Difracção de Raios X, Espectroscopia de Energia Dispersiva e Área superficial especifica.

Resultados: A caracterização dos biomateriais compostos por matriz óssea bovina inorgânica revelou que estas se trataram de matrizes ósseas com excesso de cálcio. As diferenças da razão molar Ca/P não foram significativas. As amostras revelaram diferenças quanto ao aspecto cristalino da superfície, uma vez que o Cerabone® apresentava aspecto cristalino relativamente aos restantes substitutos ósseos. Foi estabelecida uma relação entre o grau de cristalinidade com a temperatura de processamento.

Relativamente a incorporação de biomoléculas em scaffolds de HA os resultados globais mostram que levam a melhor bioactividade e diferenciação osteogénica, melhorando a proliferação e diferenciação celular, estimulam a formação óssea mais rápida, com baixa taxa de reabsorção. Este último fato tem uma importância crucial, pois a reabsorção lenta mantém o volume levando a uma boa dimensão do novo osso.

Conclusão: A incorporação de biomoléculas em matrizes ósseas osteocondutoras melhora o processo de osteogénese.

Palavras-chave: Hidroxiapatite, osso, regeneração óssea, fosfato de cálcio, Bio-Oss®.

Abstract

Objective: This thesis aims at analyzing and discussing new strategies for bone filling, using hydroxyapatite as a template, which is able to promote a bioactive behavior and consequently regeneration. The new approaches using hydroxyapatite will be compared with the more conventional strategies. This thesis intends also to answer several questions such as: what are the mediators that promote/participate in bone regeneration, how these mediators allow bone regeneration, as well as the possible advantages of using three-dimensional models with the exact shape of the defect that needs to be filled.

Methodology: A bibliographic search of the state of the art was performed, examining studies published in the last 5 years of research, products in clinical phase of development and new methodologies still in pre-clinical phase (*in vitro* and *in vivo* studies). The practical component of this thesis will consist in the analysis of commercial bone filling hydroxyapatite material (eg, Bio-Oss ®) by Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), X-Ray Diffraction (XRD) and Specific surface area.

Results: The characterization of biomaterials composed of bovine bone inorganic matrix revealed that those are bony matrix with excess of calcium. The differences in the molar ratio Ca / P were not significant. The samples showed differences in the crystalline surface appearance, since Cerabone® presented a crystalline appearance comparatively to the remaining bone substitutes. A relationship between the degree of crystallinity and the processing temperature has been established. The overall results showed that the incorporation of biomolecules in HA scaffolds lead to better bioactivity and osteogenic differentiation by improving cell proliferation and differentiation which stimulates faster bone formation with low rate resorption. This last fact has a crucial importance because the slow resorption keeps the volume leading to a good dimension of the new bone.

Conclusion: The incorporation of biomolecules in bone osteoconductive matrix improves the process of osteogenesis.

Keywords: hydroxyapatite, bone, bone regeneration, calcium phosphate, Bio-Oss®.

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A	
APDC	Apical pulp derived cells
aAC	Atelocollagen
AF	Amniotic fluid
AB	Autogenous bone grafts
B	
BET	Brunauer, Emmett and Tellerum
BM	Bone marrow
BCP	Biphasic calcium phosphate
BFP	Buccal fat pad
BMAC	Bone marrow aspiration concentrate
BMP7	Bone morphogenetic protein -7
BMP-2	Bone morphogenetic protein 2
BHC	Bovine hydroxyapatite/ collagen
β-TCP	Beta-tricalcium phosphate
C	
Col-I	Type I Collagen fibers
CAD/CAM	Computer-aided design/computer-aided manufacturing
D	
DO	Distraction osteogenesis
DA	Dopamine
E	
EDS	Energy Dispersive X-ray Analysis
F	
FA	Fluorapatite
G	
GTR	Guided tissue regeneration
H	
HA	Hydroxyapatite
hAFSCs	Human amniotic fluid stem cells
hMSCs	Human mesenchymal stem cells

HA/PLLA	Hydroxyapatite/ Poly-L-lactide
HA-TCP	Hydroxyapatite-Tricalcium phosphate
HA-pDA	Polydopamine coated nano-Hydroxyapatite
Hap	Porous spherical hydroxyapatite
HLA	Human leukocyte antigen
L	
LB	Lamelar bone
M	
MPa	Megapascal
MAP	Mitogen-activated protein
MgHA	Magnesium-enriched hydroxyapatite
N	
n-HA/PA	Polyamide nanohydroxyapatite
nano-HA	Nanoscale hydroxyapatite
NACl	Sodium chloride
O	
OAC	Oroantral communication
OAF	Oroantral fistula
oAFMC	Ovine amniotic fluid mesenchymal cells
P	
PRGF	Platelet- rich growth factor
PCBM	Particulate cancelous bone and marrow
PDGF	Platelet derived growth factor
pDA	Polydopamine
PLGA	poly(D,L-lactide-co-glycolic acid)
R	
rhBMP-2	Recombinant human bone morphogenetic protein-2
RUNX-2	Runt-related transcription factor 2
S	
SEM	Scanning Electron Microscopy
SDF1	Stromal-delivered factor-1
SIM/PLGA/Hap	Simvastatin /poly(lactic-co-glycolic acid)/hydroxyapatite
Skelite	Hydroxyapatite tricalcium phosphate scaffold

T	
TMJ	Temporomandibular joint
TGFB3	Transforming growth factor b3
TGF-β	Transforming growth factor-β
U	
USSCs	Unrestricted somatic stem cells
UCB	Human umbilical cord blood
W	
W/WB	Woven bone
X	
XRD	X-Ray Diffraction

Advances Using Hydroxyapatite as a Biomaterial in Bone Regeneration

Introduction

1 Introduction

Since the term *bone regeneration* started to be used, several strategies have been established and then put aside, for the implementation of new and more promising ones. Currently, different options for bone regeneration are already available. However, new advances have been made and there are several studies that suggest alternatives with the potential to overcome the current options.

Bone cells, growth factors, mesenchymal cells and others or cues that stimulate host cell recruitment can be added to hydroxyapatite [1]. At the same time there have been new developments in biomodelation such as the production of implants with the shape of the bone defect for three-dimensional reconstruction techniques [2].

In the current thesis we will discuss the existing techniques to bone regeneration and compare them with the new ones using hydroxyapatite scaffolds. This study will also describe the principal mediators which are involved in this remodeling process.

In order to understand this, it is essential to realize the components and processes involved in bone formation.

1.1 Bone

Bone is a specialized connective tissue that is responsible not only for the support and protection of the organs, but also for the locomotion. Moreover, it is a dynamic tissue so, when exposed to injury, changes of stress, vascular, endocrine, genetic and nutritional influences, it presents a structural alteration. The bone can undergo regeneration rather than repair with formation of scar tissue, a capacity which few organs are capable of [3].

It contains organic and inorganic components. The organic constituents are type I collagen fibers and other organic molecules that make up 70% of the total bone composition. The majority of the inorganic constituents are nano-sized crystals of hydroxyapatite and they relate to the remaining 30 %. The inorganic components are responsible for hardness and the organic ones are responsible for elasticity.

There are two types of bones: long bones which include the tibia and femur and flat bones to which craniomaxillofacial (CMF) bones belong (i.e. cranial vault, maxilla, mandible, and frontal region of the facial skeleton). The bones of the CMF are the neurocranium and facial bones and like other bones in our skeleton, they also provide protection for organs and attachments for oral-facial soft tissues, so they have an important role in functionality and aesthetics [4].

These two types of bone have many differences. Regarding their embryonic formation, the CMF bones are made of cranial neural crest cells, but the axial skeleton is formed from paraxial mesoderm showing distinct embryonic lineages [4]. Also the ossification process happens by two different methods, i.e. intramembraneous ossification and endochondral ossification. Flat bones formation occurs by intramembranous ossification.

During this process, mesenchymal cells differentiate into progenitor cells and then into osteoblasts. The last ones line up around blood vessels and produce and deposit new bone matrix. At the end of this process the cancellous bone exists within the cortical bone achieving the typical arrangement of the bone. The mechanical strength of the bone is accomplished through cortical bone (100-230 MPa), specifically through the organization of its osteons.

Figure 1. The osteonal concentric ring structure from 50 to 500 μm in diameter [5].

The osteon is formed by lamellar rings of cells which surround a central microchannel. These rings are made by the secretion of Type –I Collagen (Col-I) and for osteoblasts.

The structural organization of the bone, specifically the distribution of Hydroxyapatite crystals along the fibers of collagen, confers high compressive strength and toughness to the cortical bone [5].

Cancellous bone has a porous appearance because of the network of the trabeculae. It is found below the cortical bone and has support function. The organization of this type of bone consists of incomplete osteon, contrasting to cortical bone [6].

From a clinical therapeutic point of view, the CMF skeleton is associated with the oral cavity, which increases the infection risk. It has also bigger risk of tumor metastases to CMF bone from near tissues because in this area numerous lymph nodes, nerves and vasculature structures can be found [4].

Bone maintenance is accomplished through bone cells. These cells are osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Bone is a dynamic tissue. Osteoprogenitor cells have the potential to differentiate into osteoblasts that are responsible for the synthesis of inorganic components of bone matrix (collagen, proteoglycans and glycoproteins). Bone has cells which promote bone resorption, the osteoclasts and osteocytes which are mature bone cells [7].

The following table takes an example a tooth extraction to describe the structural changes in bone tissue (table 1) [8].

Table 1. Description of structural changes in bone tissue after a tooth extraction [8].

Time after extraction	**Structural changes**
At day 1	The socket is occupied by a coagulum which contained erythrocytes and platelets that were surrounded in a fibrous matrix. The bundle bone and principal fibers from periodontal ligament (Sharpey's fibers) were in contact with the coagulum.
At day 3	A granulation tissue richly vascularizes and takes the place of the coagulum.
At day 7	In the primary matrix newly formed blood vessels were found, as well as various types of leukocytes and collagen fibers, which take the place of the residual periodontal ligament and granulation tissue.
At day 14	The majority of bundle bone had disappeared, and the woven bone takes its place, adjacent to the newly formed blood vessels, spreading from the old bone of the socket walls toward the center of the socket.
At day 30	The resorption of woven bone suggests that the remodeling process had begun.
At day 60	The marginal mucosa is separated from the socket by hard tissue bridges, and bone marrow replaced woven bone at the center of the socket.
At day 90	The woven bone begins to be replaced by lamellar bone.
At day 120 and 180	The majority of the woven bone had been replaced by lamellar bone.

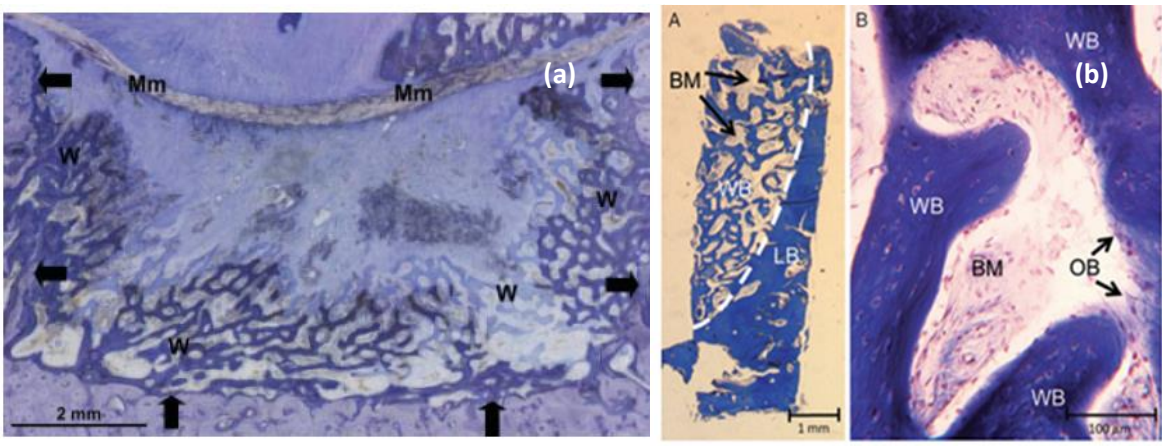


Figure 2. (a) Four weeks after defect preparation and without bone substitutes application. Woven bone (W). Section stained with toluidine blue [9]. (b) Twenty three weeks after tooth extraction and without bone substitutes application. Woven bone (WB) and bone marrow (BM) are in coronal part. Lamellar bone (LB) is in the apical part [10].

1.2. Causes of bone loss in the oral cavity

There are several causes for bone loss, such as the loss of teeth, bone fracture and trauma [5, 11, 12] , the insertion of dental implants, cyst enucleation and other benign lesions [13], tumor resection [14], oroantral communication (OAC) and periodontal disease [15, 16]. Although they are not causes of bone loss, the cleft palate [17] and distraction osteogenesis [18] could use biomaterials of bone regeneration for management or faster bone consolidation.

The loss of maxillary molars leads to rapid horizontal and vertical resorption of alveolar bone. This loss occurs because there is an absence of intraosseous stimulation by periodontal ligaments [3, 8, 19] and by improved activity of osteoclasts in Schneider's membrane, which causes pneumatisation of sinuses in a few months [3, 20].

The extraction of maxillary teeth can sometimes cause clinical complications, like oroantral communications, that could develop into oroantral fistula [21].

In some cases, patients with insufficient bone height in the posterior maxilla need a maxillary sinus grafting. This is a common procedure for subsequent implant placement for prosthodontic rehabilitation [22-27].

On places of teeth extraction, it is possible to find bone remodeling of the alveolar crest. The buccal bone wall, in the first three months after extraction, suffers a loss of 50%, reducing the global crestal width to 3.87 and 3.79mm [28].

In conclusion, compared with conventional socket healing, the post-extraction alveolar bone remodeling can be significantly reduced, however, it is important to mention that socket shrinkage occurs always [28].

Hossein Behnia et all [17] suggests that secondary alveolar bone grafting is essential for the management of cleft palate patients.

Finally, distraction osteogenesis is a procedure that requires a long term bone consolidation. The use of biomaterials for bone regeneration can accelerate bone formation and compression [18].

1.3. Hydroxyapatite

There are three groups of materials able to function as a scaffold or a carrier system: biological materials, ceramic or glass materials and polymeric materials. The ceramic or glass materials group has been researched over the last three decades, and hydroxyapatite was one of the first alloplastic materials to be used as bone regeneration scaffold [29].

Hydroxyapatite (HA) can be found in its natural form. It is the major inorganic component of human bone. But it can also be found in its synthetic form, namely calcium phosphate, whose composition is $Ca_{10}(PO_4)_6(OH)_2$. The main difference between natural HA and synthetic HA is the substitution of phosphate groups by carbonate groups. It produces a structure favorable for angiogenesis, is non-toxic, has high chemical stability and it causes less inflammation and antigenic reaction; nevertheless, it also has some limitations, such as slow resorption, which may be overcome when the hydroxyapatite is used together with other substances [12].

Regarding its stability and solubility, hydroxyapatite has different behavior depending on how it is processed. Sintered HA is treated at high temperatures which leads to absence of carbonate and therefore exhibits high crystallinity. This feature provides greater stability but results in a much lower degradation rate /solubility. On the contrary precipitated HA has lower crystallinity and consequent increased solubility, due to its carbonate content. In consequence its bioactivity is higher due to the release of Ca and P ions during the biodegradation process.

Hydroxyapatite has been widely used as a biomaterial for bone regeneration because it has several interesting properties, such as: rapid bone adaptation, lack of fibrous tissue formation, intimate adherence implant/tissue and lower healing time. In general hydroxyapatite, as a biomaterial for bone regeneration, has optimum properties in terms of osteoconductivity and biocompatibility [30]. It has a biological and mechanical behavior that is close to the bone, but it is still deficient in the field of osteoinduction [31].

To overcome this shortcoming, bone cells, growth factors, mesenchymal cells and others can be added to stimulate host cell recruitment, to create superior biofuncionality comparatively to his individual constituents. Biomodelation, the production of implants with the three-dimensional shape of the bone defect, can be achieved using in reconstruction techniques.

1.4 Hydroxyapatite from natural origin

In the last decade, many synthetic bone substitutes have been produced. They involve high level of technology, are time consuming and expensive. For these reasons, natural bone substitutes are presently being explored.

In addition, there is an ever-growing concern to develop clean, non-toxic and environmentally friendly procedures for hydroxyapatite synthesis. It is necessary to reuse waste not only because waste materials are accumulating but also because natural raw materials are being exhausted [32].

Hydroxyapatite has been extracted of several natural sources, such as fish bones, bovine bones, teeth and bones of pig, fish scales, oyster shells, eggshells, cuttlefish shells [33-42].

Also natural hydroxyapatite is preferred to synthetic hydroxyapatite, because the last one has better metabolic activity and preserves chemical composition and structure of the precursor material [33].

At the moment, several bone substitutes of animal origin are already available, more specifically from bovine and porcine origin. Some examples of biomaterials of

bovine origin are Bio-Oss®, Cerabone® and NuOss®. There are also biomaterials of porcine origin such as Apatos® that are already being commercialized [29].

Hydroxyapatite synthesized from eggshells has been highly studied, due to its abundance and for this reason it has been suggested as a candidate material for Maxillofacial and cranio surgery. Every day tons of eggshells are thrown away which contributes to pollution and health problems. Similarly to human bone, chicken eggshells contain trace elements, such as Na, Mg, and Sr [32, 35].

This hydroxyapatite can also be used as a scaffold for adhesion of proteins, peptides, lipids and for drug delivery [34].

To extract HA some procedures are required. First the eggshells are dehydrated in a heat chamber at 808C. This procedure is important because it minimizes pollution and eliminates most organic components. Then the eggshells are crushed to produce a kind of flour formed by fine particles. There are methods that are able to preserve the structure of precursors, which give them a higher superficial area and, consequently, a bigger osteoconductivity behavior [43].

Another example of an hydroxyapatite source in bio-waste is fish. This source is potentially lucrative because it is widely available and biologically safe after treatments.

For example, Portugal consumes about 60.000 tonnes per year of Cod fish. After consumption, enormous quantities of bones are displaced which could be used to obtain compounds with high added value [36].

They require simple and inexpensive methods that reduce the costs of expensive and high purity reagents [42].

At the moment there is a growing global demand for hydroxyapatite as implant or coating material. HA extraction from bio-waste has many advantages such as high availability and cheap treatments and it has a low impact on the environment.

1.5 Smart materials

Smart biomaterials are those able to establish associations with the surrounding cells and tissues. They could provide chemical and physical cues that induce tissue repair and regeneration, achieving the three physicochemical characteristics which are biocompatibility, osteoconductivity and osteoinductivity (osteogenesis) properties.

The recognition of these facts led to many efforts to achieve modulation of the surface and bulk properties or to provide biomolecules.

The goal of internal modification is to mimic the native tissue and for to that happen chemical or physical modification can be introduced to the biomaterial. These modifications offer cues to the surrounding cells allowing the cells recognize the biomaterial and improve target function like adhesion, migration, proliferation and tissue differentiation.

Many biomolecules can be incorporated such as peptides, adhesive proteins. The external modulation is related to the delivery of biofactors such as chemical drugs, proteins and nucleic acids. Delivery systems refer to bone grafts substitutes that can be loaded with biomolecules that enhance bone formation and have methods for controlled delivery [44]. After incorporation they can be delivered in a controlled fashion. The main targeted tissues where smart biomaterials are used include bone and teeth [1].

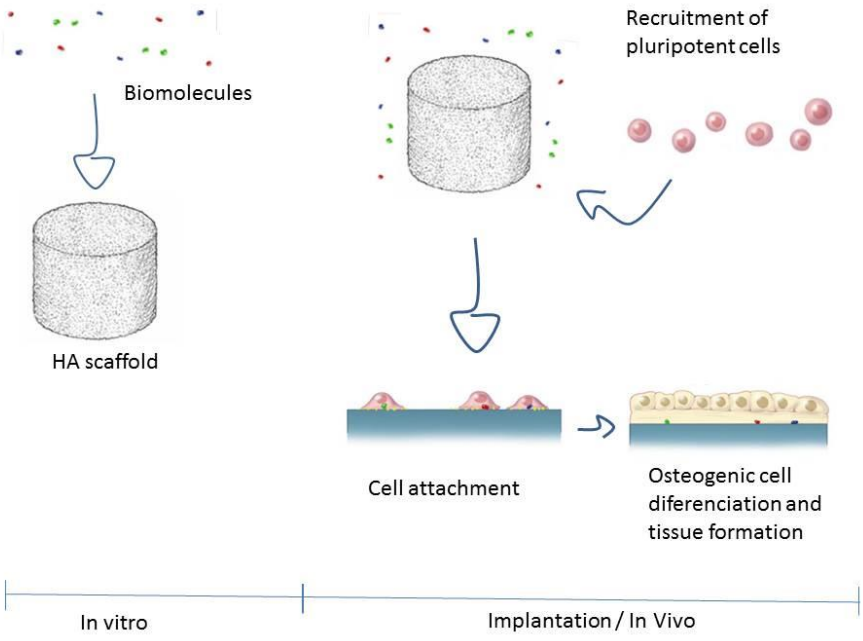


Figure 3 Physical and chemical modifications induce after biomolecules were implanted into scaffold [4].

1.6 Mediators involved in regeneration

Wei Ji *et al* [4] divided biomolecules for CMF bone regeneration in two groups according to their molecular weight (5KDa) namely Small and Large biomolecules. It will be presented a classification scheme/table according to information ceded by these authors.

Table 2. Biomolecules for CMF bone regeneration

Biomolecules for CMF bone regeneration				Ref.
Large Molecules	Inflammatory cytokines and chemokines	Pro-infammatory cytokines (IL-1, TNF-a) Pro-woundhealing cytokines IL-4, IL-3 Chemokines SDF-1a	Cytokines are secreted by macrophages and inflammatory cells, and they can be divided in two groups: pro-inflammatory cytokines and pro-wound healing cytokines. The first ones are responsible for initiating the inflammatory cascate and the second ones cease the production of pro-inflammatory cytokines and then led to bone healing. Chemokines promote the recruitment of cells to a desire site, they also enhance BMPs efficiency by adding more osteoprogenitor cells.	4
	Morphogenetic Factors	TGF-b superfamily (BMP), fibrobast growth factors (FGF), sonic hedgehog (SHH) and Wingless and intrelated proteins (Wnts), insulin-like growth (IGFs), platelet-derived growth factor (PDGF), growth hormone, parathyroid hormone and vitamin D.	These factors contribute in a cascade of events that lead to bone formation induction. They participate in important roles such as: migration, mesenchymal condensation, proliferation. They also mediate osteoblast activity.	3,14,17 ,18,22, 23,29,3 0,37,44 ,45,46, 47,48,5 0,52,53 ,54,58, 59,60
	Angiogenic factors	VEGF, Ang	Bone needs a blood supply to receive oxygen and nutrients. It has been demonstrated that vascular-endothelial growth factor (VEGF) dependent pathways and angiopoietin (Ang)-dependent pathways are involved in bone formation.	4

Small molecules	Short Peptides		Instead of applying entire proteins, synthetic peptides can be administrated into a scaffold. These have similar biological functions to complex structures. They can promote differentiation of osteoblast precursor cells and activate osteoblast to support bone regeneration.	4
	Anti-infection molecules	Tetracyclines, penicillins (amoxicillin), metronidazole, cefalosporins and antimicrobial peptides (AMPs)	To avoid alveolar bone loss by infection, several anti-microbial infections treatments are available. For that reason, these molecules are necessary to achieve bone regeneration.	4
	Anti-tumor molecules	Anti-tumor molecules can be added for reconstruction of the skeletal malignancies and in surgical removal of metastases in other to decrease tumor growth.	Anti-tumor molecules can be added for reconstruction of the skeletal malignancies and in surgical removal of metastases in other to decrease tumor growth.	4
	Anti-osteoporotic molecules	Anti-resorptive drugs (bisphosphonates, calcitonin, estrogen), statins, parathyroid hormone (PTH).	These factors inhibit osteoclast activity to reduce bone loss by promoting a balance of bone remodeling.	4

Several mediators are commonly associated to the bone matrix to induce and enhance bone regeneration [3,14,17,18,22,23,29,30,37,44-48,50,52-54,58-60]. These can include cells, biomolecules, extracellular matrix (ECM) analogues or combinations of these.

1.6.1. Mesenchymal Stem cells

Mesenchymal stem cells (MSCs) are a type of stem cells able to differentiate in multiple phenotypes that is why their use has been exponential in the field of therapeutic approaches for bone substitution. They contribute to the restoration of structure and functionality of a variety of injured tissues or organs [23] through its previous implantation into a scaffold and subsequently in the patient [31].

We can categorize MSCs in two major groups, such as pluripotent (embryonic) cells and multipotent (adult) cells. Contrarily to what happens in pluripotent cells, multipotent cells are limited in the number of cell types they can differentiate into.

Some sources of pluripotent cells are amniotic membrane, amniotic fluid and umbilical cord blood. These embryonic cells are easily available and accessible, have the ability to differentiate into multiple types of tissues from all three embryonic germ layers and have immune-modulatory and anti-inflammatory properties [23].

However, despite all its advantages, this source of cells raises some concerns about the possibility of infection, immunogenicity, and tumourgenicity, which has limited their application [45].

According to Esmaeil Biazar *et al* [46], the use of Human umbilical cord blood (UCB) is a rich source of hematopoietic stem cells and it stands less chances of rejection, because of lower expression levels of human leukocyte antigen (HLA) (tissue incompatibility complex).

The most common source of adult MSCs has been the bone marrow (BM) and blood stream, but this type of cells can be found in adipose tissues (AF) [45, 47]. Adipose derived stem cells (ADSCs) are isolated from adipose tissue and are considered to be an appropriate source of MSCs [45, 48], due to their abundance, their easy accessibility and their ability to differentiate towards osteogenic, adipogenic, myogenic, and chondrogenic lineages *in vitro*, when treated with the appropriate inducing factors.

Bone marrow can be aspirated and seeded in different carriers. This, combined with MSCs, can improve bone formation, but the addition of growth factors, like of xenogenic growth factors (such as fetal calf serum) may increase human applicability of

these cells [17]. However, BM-MSCs harvesting and processing show some limitations such as donor morbility.

Also, new studies have reported that cells derived from apical pulp of human developing tooth with immature apex, APDCs (apical pulp-derived cells) show high proliferation activity and multilineage differentiation potential, which can been used in vivo in hard tissue engineering [49].

It is important to emphasize that several studies have tested that MSCs have proliferative and osteogenic differentiation capabilities. Although MSC delivery into synthetic or natural scaffolds results in a lower bone formation in comparison with autogenous bone graft, further studies have demonstrated the improvement of bone formation with various growth and differentiating factors and cell carriers [30].

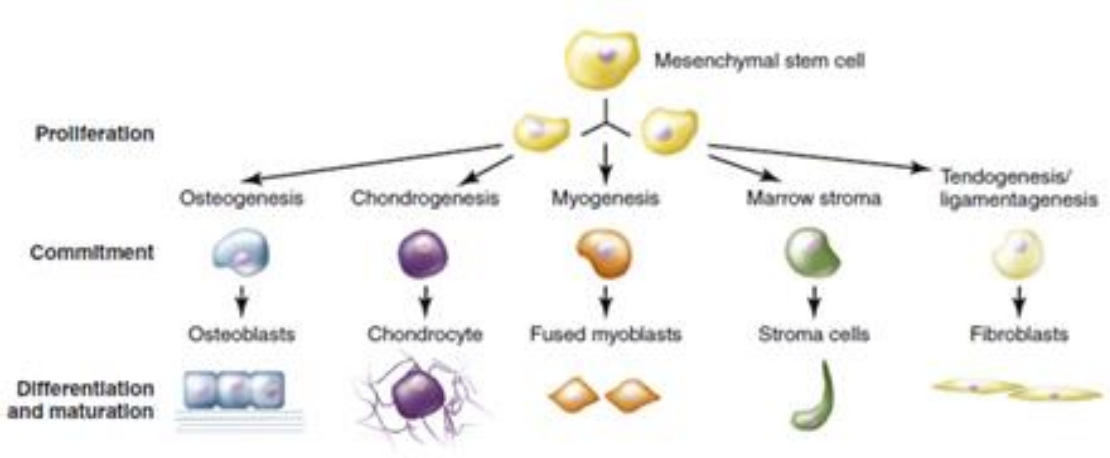


Figure 4 Mesenchymal cells and their different differentiation pathways [29].

1.6.2 BMPs

BMPs belong to the TGF-β (transforming growth factor-β) group which play an essential role in inducing mesenchymal stem cells to differentiate into osteoblasts by regulating important downstream targets as well as cross signaling pathways to produce bone tissue [3, 50].

Also these proteins are resisting to noggin inhibition, and this characteristic is responsible to BMPs' potent osteogenic activity [50, 51].

Several recombinant forms of BMP, such as BMP- 2,BMP-7 and BMP-9, hold promise to promote bone regeneration. These BMPs stimulates bony regeneration and reduces healing time, accelerate bone formation and maturation [14, 52, 53]. However BMPs also have some disadvantages such the high cost and rapid loss of their bioactivity in physiological conditions [11, 51].

Although BMPs can improve bone regeneration by enhance scaffold osteoconductivity, in some studies were addressed that nano-HA, nano scale particules made of hydroxyapatite, also enhance BMP expression. Mizuki Suto *et al* [54] demonstrated that nano-HA is able to induce phosphorylation of p38 mitogen –activated protein (MAP) kinase pathway and this phosphorylation increases BMP-2 expressionat gene and protein levels which regulate bone formation.

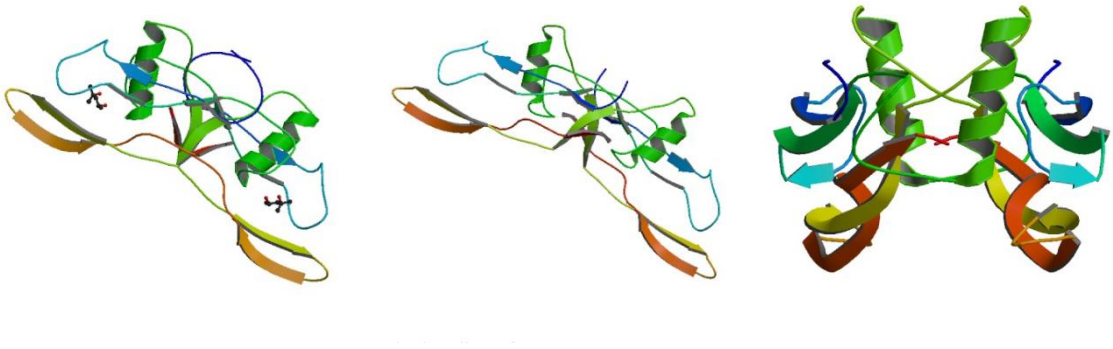


Figure 5. The main bone morphogenic proteins used in CMF bone regeneration, respectively BMP-2, BMP-7 and BMP-9 (http://www.rcsb.org/pdb/home/home.do.)

1.6.3 Growth factors/ Platelet rich plasma

Platelet rich plasma (PRP) contains multiple growth factors, nominally platelet-derived growth factor(PDGF), transforming growth factor-1(TGF), transforming growth factor-2, insulin-like growth factor(IGF), vascular endothelial growth factor(VEGF), epithelial growth factor(EGF), platelet factor 4, interleukin (IL)-1, platelet-derived angiogenesis factor, platelet derived endothelial growth factor, epithelial cell growth factor, but also have osteocalcin, osteonectin, fibrinogen, vitronectin, fibronectin, and thrombospondin. [22, 55] The platelets' release these growth factors (GFs) from granules when they are activate by via the addition of thrombin.

The GFs have many important activities like macrophage activation, chemotaxis, and mitogenesis of osteoblast precursors, inhibiting osteoclast formation or stimulating vascular ingrowth also have anti and pro-inflammatory cytokines responsible for pro-inflammatory response in wound healing.

When seeded in a carrier PRPs generate an angiogenic and osteogenic microenvironment, allowing for the creation of endogenous mechanisms which can lead to an increase in the rate of bone formation through cell proliferation, migration and differentiation. In conclusion PRP have the capacity for irreversible damage [22, 55].

This thesis describes some GFs, like nerve growth factor (NGF), stromal-derived factor-1 (SDF1). These GFs can be seeded in the scaffold to release bioactive cues that are responsible for the recruitment of endogenous cells and for inducing angiogenesis, This events will consequently lead to the improvement of bone formation [18, 56]

1.6.4. Extracellular matrix (ECM)

To improve the bioactivity of carrier materials extracellular matrix (ECM) proteins, including collagen, fibronectin and peptides have been investigated.

There are two methods to immobilize ECM proteins or peptides onto the implant material's surface which are physical adsorption and chemical covalent conjugation.

Physical adsorption is a simple process, but has the inconvenient that simply adsorbed molecules are frequently released with a burst in the initial stage.

For prolonged periods of time chemical conjugation is more effective, allowing successive regulation of cell behaviors. This technique requires multistep, complicated procedures such as surface activation or functionalization steps requiring plasma or chemical treatments.

Examples in this category are dopamine and simvastatin. Dopamine (DA) can experience a self-polymerization and adhere onto almost any solid surface in alkaline solution without surface pretreatments. It enhances bioactivity and osteogenic differentiation and improves osteointegartion [51].

Simvastatin is prescribed in patients who need to reduce blood cholesterol levels. Statins stimulate BMP-2 gene expression in osteoblasts, increases expression of osteogenic marker genes such Runt-related transcription factor 2 (RUNX-2), osteocalcin, osteopontin, and alkaline phosphatase. These alterations improve bone formation.

This thesis approaches the bone regeneration using different materials for bone replacement. It is important to discuss the normal bone healing in order to understand the processes involved.

In the tables 3, 4 and 5 were illustrated some modifications using hydroxyapatite as a template. All of these strategies aim to promote a bioactive behavior and consequently regeneration. This thesis analyses the recent studies published in the last 5 years of research products in clinical phase of development and new methodologies still in preclinical phase (studies *in vitro* and *in vivo*).All selected papers refer to approaches using Ha scaffolds in association with biomolecules for craniomaxillofacial bone regeneration. Because the used terms were so specific, the number of papers that met the inclusion criteria is reduced. Nevertheless the results obtained are a representative of the newest approaches for bone regeneration.

Although many advances have been made in tissue engineering, more concretely in bone regeneration, there are several limitations that need to be overcome.

Objectives

2. Objectives

The following objectives are defined in the present thesis:

1. To understand and develop knowledge about bone tissue, particularly its constituents and structure as well as the normal process of healing.

2. To identify the main causes of bone loss in particular for the maxillofacial area.

3. To analyze the potential of hydroxyapatite based biomaterials for bone regeneration.

4. To study hydroxyapatite composition and chemistry according to its origin and processing route.

5. To evaluate new strategies for promoting bone filling a bioactive behavior and consequently regeneration.

6. To identify key mediators involved in the new strategies for bone filling.

7. To analyze by Scanning Electron Microscopy, Energy Dispersive Spectroscopy, X-ray Diffraction, Specific surface area a commercially bone filling materials (eg BioOss ®, Cerabone ® and NuOss®).

The proposed study aims to collect data on new strategies and HA-based materials that promote bone filling and a bioactive behavior and consequently regeneration. This study is important since it allows for identifying new trends and strategies for bone regeneration including the use of mediators that participate and promote the process of bone regeneration.

Materials and Methods

3. Materials and Methods

3.1. Bibliographic search

A bibliographic search was performed for the state of the art that was limited to the past 5 years. Research studies of new biomaterials and strategies using hydroxyapatite either in clinical trials as in *in vivo* and *in vitro* studies were analyzed.

The search engines used were PubMed and Science Direct. Several combinations were made like: hydroxyapatite AND bone regeneration, cell recruitment AND hydroxyapatite, maxillary defects AND hydroxyapatite, CAD CAM AND hydroxyapatite, maxillary sinus AND hydroxyapatite and periodontal defects AND hydroxyapatite.

The studies focus on bone regeneration using hydroxyapatite scaffolds with the addition of mediators that release biological cues and potentiate bone regeneration.

After the bibliographic search process finalized there were found 27 articles meeting the inclusion criteria and 33 articles about conventional approaches for comparison with the first addressed, which totalized 61 articles that were used for developing this thesis.

The exclusion criteria were: book chapters, conference proceedings, *in-vivo* studies with a small amount of samples (less than 8) and studies that do not use hydroxyapatite as a template to promote bone regeneration.

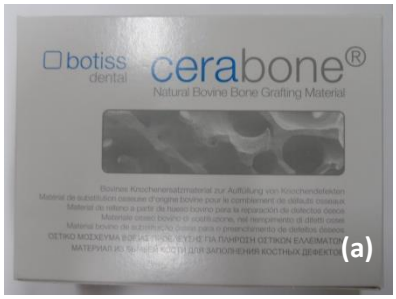
3.2. Practical component

In this work there commercial bone filler materials (eg Bio-Oss ®, Cerabone ® and NuOss ®) typically used for filling bone defects in the oral cavity have been purchased and the microstructure of its particles analyzed by Scanning Electron Microscopy, Energy Dispersive Spectroscopy and X- Ray Diffraction. The Average particle size and the Specific surface area were calculated using Coulter Fluid Module (LS) Particle Size Analyser 230 and using an adsorption isotherm Brunauer, Emmett and Tellerum (BET).

3.2.1. Materials

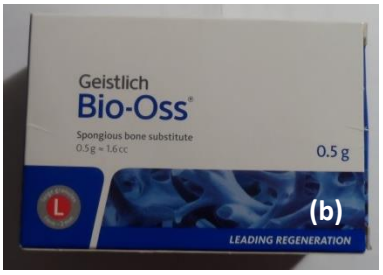
Cerabone ®

CeraBone® is a mineral matrix made of bovine bone produced by Botiss. This bone substitute has granule size between 0.25mm and 1mm and it was supplied by Klockner.



Bio-Oss ®

Bio-Oss® is a bone substitute from bovine origin produced by Geistlich Pharma AG (Switzerland). Bio-Oss used in this work has granule size between 0.25 mm and 1mm and it was supplied by Inibsa.



NuOss ®

NuOss® is a mineral matrix made of bovine bone produced by AceSurgical. This bone substitute has a granule size between 0.25mm and 1mm and it was Supplied by Henry Schein®.

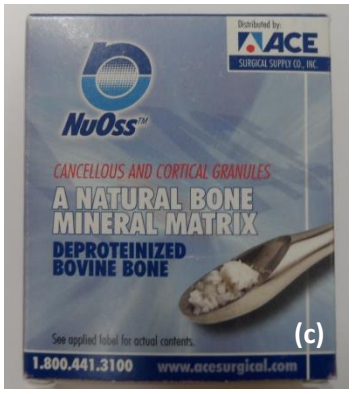


Figure 6. Bone substitutes: Cerabone® (a), Bio-Oss® (b) and NuOss® (c).

3.2.2. Biomaterials characterization

Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Analysis (EDS)

The scanning electron microscope used for analyzing the morphology of these three samples was Hitachi SU-70, Hitachi High-Technologies Europe, GmbH, Germany, with an acceleration voltage of 15 kV and a beam current of 43 μA.

The samples were placed in an aluminum holder and fixed with adhesive tape carbon. After fixing the particles on the support, it was placed in a carbon evaporator (Emitech K950 X) where there was carbon deposition on the particles. This procedure was essential because without it, it would not possible to make the observation and characterization of the samples.

The composition analysis of these three biomaterials was also made by secondary electron diffraction (EDS, Hitachi SU-70, Hitachi High-Technologies Europe, GmbH, Germany).

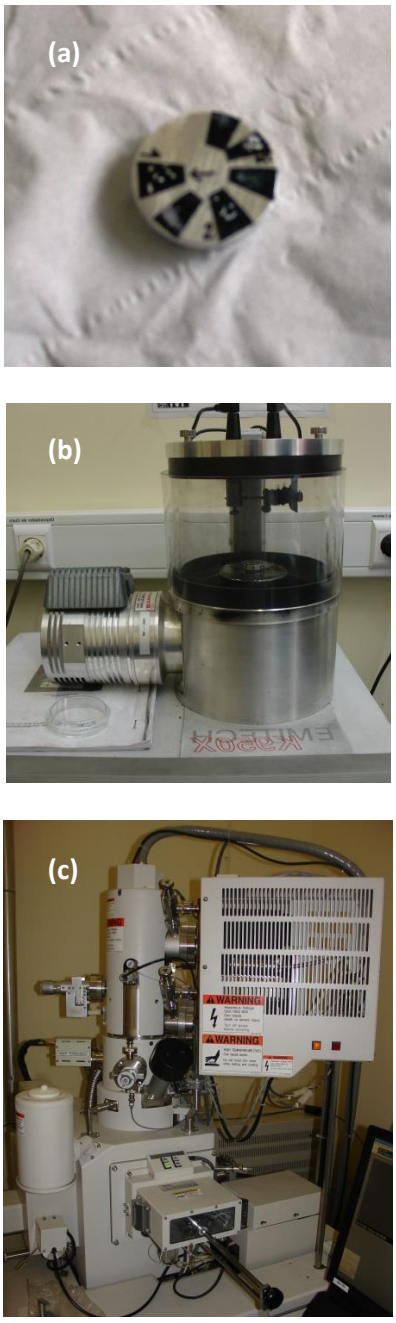


Figure 7. Aluminum holder (a), Carbon sputter (b) and scanning electron microscope (c)

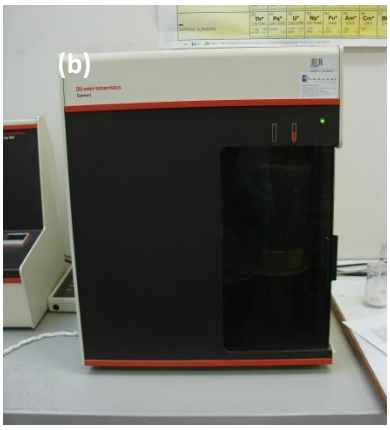
X-Ray Diffraction (XRD)

The crystal phases of the three samples were detected in a X-Ray Diffractometer (X' PERT –PRO), C Series with radiation copper Kα, λ = 1.540596 Å.

The following parameters were stipulated to collect data: 2θ of 10-80 °; step width 2θ of 0.026 °; time per step 46s.

The crystalline phases present in the samples were identified using a database.

The degree of crystallinity can be calculated by two methods. The first method calculates the degree of crystallinity through dividing the intensity of the valley between the planes (112) and (300) over the intensity of the plane (300) minus one, whereas the second method used the width at half height of the peak corresponding to the reflection (002).



Specific Surface Area

The specific surface area was calculated by nitrogen adsorption with the assistance of Micromeritis a Gemini 2680 (Norcross, USA) using the adsorption isotherm Brunauer, Emmett and Tellerum (BET) after degassing the powder in the Micromeritis Flow prep 060 (Norcross, USA).

The particles were prepared using nitrogen and afterwards heated at 35ºC.

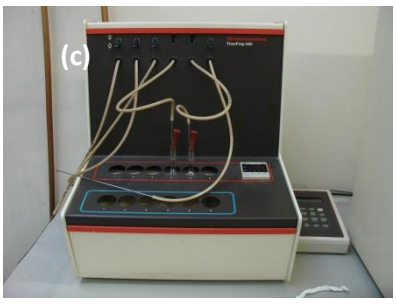


Figure 8. (a**)** X-ray diffractometer, (b) Micromeritis and (c) Device for samples' preparation

Results

4. Results and discussion

From the bibliographical research we found 27 articles meeting the inclusion criteria. Figure 1 represents the percent of the studies meeting the criteria, published *per year* between 2009 and 2013.

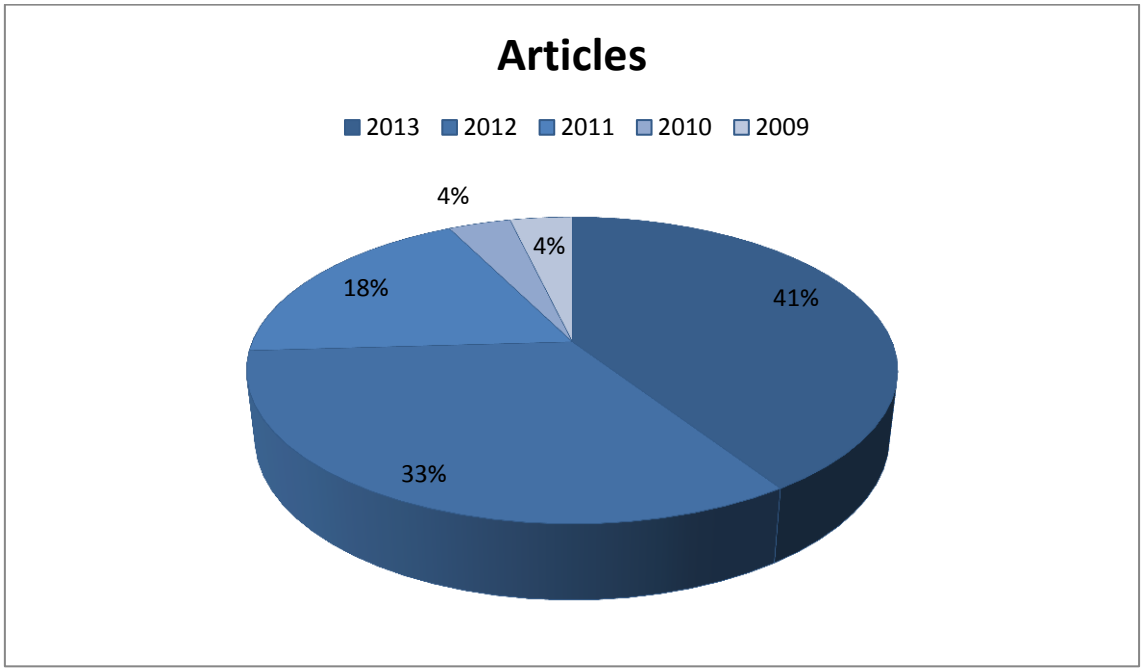


Figure 9. Percentage of relevant papers published per year between 2009 and 2013.

A gradual increase of papers concerning the aim of this study was observed from 2009 to 2013. The majority of the papers concerning the aim of this study were published in 2012 and 2013, which means that the interest for the use of HA has substancially increased.

In Figure 2 are presented the studies that meet the inclusion criteria. These studies can be divided in: *in vitro*, *in vivo* and clinical trials.

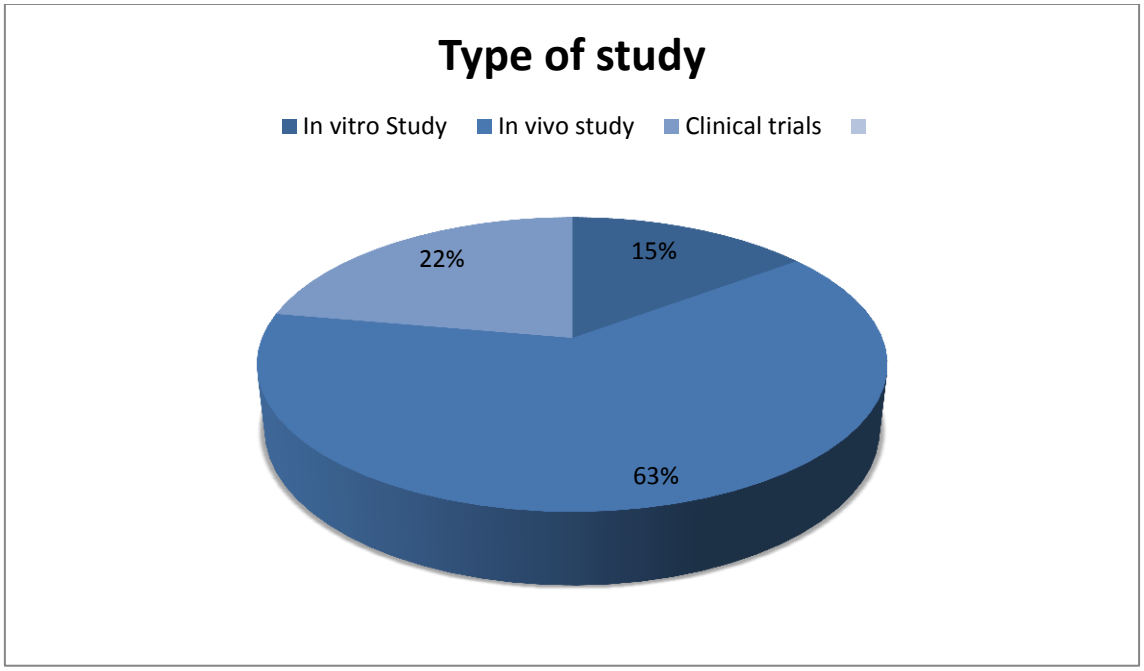


Figure 10. Percentage of type of study that includes, in vitro studies, in vivo studies and clinical trials published between 2009 and 2013.

From the 27 papers analysed, 63 % represented in vivo studies, 22 % represented clinical studies and 15% represented in vitro studies. It is noted that most of the papers found were in vivo studies, followed by clinical trials and with a low percentage, in vitro studies.

Most of the studies address by this subject are still in pre-clinical phase (*in vivo* studies) but there is a limited number of clinical trials which means a period (longer than 5 years) in which the *in vitro* studies were performed. Thanks to the encouraging results of these studies, developing the same trials in humans is being considered.

4.1. Clinical trials.

In Table 3, [2, 3, 17, 22, 57, 58], the clinical trials are presented including the underlying innovation, aim of the study, local of the defect/time of follow-up, methodology and results.

Table 3. Description of the clinical trials found in the bibliographic search

References	Innovation	Aim of the study	Local of the defect/ time of follow-up	Methodology	Results
Jihua Li *et al* [2]	CAD/CAM	Report a case of computer-aided design/computer-aided manufacturing (CAD/CAM) and rapid prototyped nanoscale hydroxyapatite/polyamide (n-HA/PA) composite construction for a mandibular condyle defect created during mandibular angle osteotomy.	Mandibular condyle defect 24 moths	CAD-CAM was used to reconstruct the missing segment by mirroring the healthy mandible. The individualized mandibular condylar implant was designed and fabricated from n-HA/PA. The implant then was fixed with L-type plates but before the previously resected bone margin was redefined.	The case report suggests that CAD/CAM and rapid prototyped n-HA/PA implants can be a viable alternative to autologous bone offering precise implants for mandibular condyle substitution. The lateral pterygoid is not attached to the new condyle and some movements had no assistance.
Giuseppe Corinaldesi *et al* [3]	rhBMP-7	Evaluate the quantity and quality of bone regeneration with recombinant human bone morphogenetic protein-7 (rhBMP-7) to augmentation of the floor of maxillary sinus	Floor of the maxillary sinus Four moths	Nine patients with mean age of 50 years who required bilateral augmentation, were recruited to participate in this study. Two groups was made: Test group: Injection of Osigraft (eptotermin 3,5mg in collagen 1g) reconstituted with saline solution 2,5ml and sodium chloride 9ml/ml and inorganic bovine hydroxyapatite 0,5g was grafted. Test group: graft of inorganic bovine hydroxyapatite 2g reconstituted with saline solution 2,5ml Both sinus grafts were covered with resorbable collagen membrane and closerd with non-resorbable sutures.	Radiologic results: More gain in height on the test side after sinus lift. Less resorption volume after 4 months in the test group. Histologic results: rhOP-1 gave poor results, with less new bone formed than with inorganic bovine HA. Histomorphometric results shows more newly formed bone in control sites.
Marcus Jager *et al* [57]	Bone Marrow	Investigate the potency of bone marrow aspiration concentrate (BMAC) to augmentation bone grafting and support bone healing.	Local bone defect 6 moths	BMAC was obtained from the posterior iliac crest. Fifty percent of the local defect was treated with autologous bone graft and the remaining by BMAC mixed with porous hydroxyapatite or applied onto a porcine collagen sponge.	New bone formation was detected in both BMAC- HA and BMCA- Col, but bone formation appeared significantly early in the HA group.
Paul W. Poeschl *et al* [22]	PRP	Evaluate the effect of platelet-rich plasma (PRP) on new bone formation and remodeling after grafting of the maxillary sinus with an algae-derived HA AlgOss/C Graft/Algipore	Maxillary sinus Seven moths	Study group-mixture of collected bone, algae-derived hydroxyapatite AlgOss/C Graft/Algipore and a combined addition of PRP Control group- mixture of collected bone, algae-derived hydroxyapatite AlgOss/C Graft/Algipore	Significantly better resorption of algae-derived hydroxyapatite AlgOss/C Graft/Algipore and increased new bone formation in apical region when PRP was used.
Hossein Behnia *et al* [17]	Mesenchymal stem cells PDGF	Evaluate the enhancing effect of recombinant platelet derived growth factor(PDGF) on human mesenchymal stem cells(hMSCs) in secondary alveoloplasty.	Alveolar defects 3 moths	hMSCs were collected from iliac bone aspirate and combined with PDGF which was extracted from blood. Then were mounted on a scaffold and transferred to the defect.	There was successful healing suggesting that the use or recombinant platelet derived growth factor with hMSCs may enhance the regeneration capacity of the cells.

Yu Zhang *et al* [58]	PRF	Evaluate the effect of PRF on bone regeneration in sinus augmentation with Bio-Oss.	Maxillary sinus 6 moths	Test group- Was grafted Bio-Oss in combination with PRF Control group- in this group was grafted Bio-Oss alone After 6 months bone biopsies was performed	There were no significant differences between groups. In this study both groups had proximally amount of newly formed bone and residual bone substitute (Bio-oss).

In this table were included some modifications such as, incorporation of biomolecules like BMP [3], BM/MSCs [17, 57] and PRP [22, 58] behaviour were also analysed. Also it can be found a study where a matrix is produced with the same shape of the defect [2].

4.2. Pré-clinical studies

4.2.1. In vivo studies

The *in vivo* studies are present in Table 4, including their innovation, aim of the study, local of the defect/time of follow up, methodology and results.

Table 4. Description of the *in vivo* studies found in the bibliographic search

References	Innovation	Aim of the study	Local of the defect/ time of follow-up	Methodology	Results
Rania M. El Backly *et al* [55]	PRP Skelite Cell recruitment	Evaluate the influence of lyophilized platelet rich plasma (PRP) on bone regeneration in combination with silicon stabilized hydroxyapatite tricalcium phosphate scaffold (Skelite)	Rabbit calvarial defect Four, eight and sixteen weeks	Group1-PRP with skelite Group2-PRP gel Group3-Skelite particles	The PRP addition delivered key cryptic factors for bone regeneration. This factors increase progenitor cell recruitment, collagen and bone matrix deposition, and created a bridging interface between the scaffold and bone.
L.Ciocca *et al* [31]	CAD-CAM MSCs	Evaluate the efficacy of mesenchymal stem cells (MSCs) and CAD-CAM customized in pure and porous hydroxyapatite (HA) scaffolds for condyle substitution.	Temporomandibular joint – condyle (sheep) Four moths	HA scaffolds with 70% total porosity volume were prototype using CAD-CAM MSCs were obtained from the aspirated iliac crest bone marrow, and platelets from the venous blood. Histological preparation and histomorphometric analyses were made. In six animals were implanted two condyles, one with and one without MSCs.	The condyle with MSCs showed good organization and maturation of osteoblastic and osteoclastic cells. Also condyle with MSCs showed higher bone formation.
N. Pourebrahim *et al* [45]	Stem cells	are bone regeneration of tissue-ɛered bone from adiposed- derived stem ıd autogenous bone graft	Maxillary alveolar cleft defect Fifteen and sixty days after	Bilateral defects was created and repair with tissue engineered bone graft from adipose-derived stem cells on one side and corticocancellous tibial auto graft on the other side.	Bone formation on the autograft sides was higher than on the stem cell sides, but tissue engineered bone can provide an acceptable alternative.
Hossein Behnia *et al* [30]	MSCs PRGF	Evaluate Nanobone as a carrier construct for mesenchymal stem cells (MSCs) and platelet- rich growth factor (PRGF)	Calvary defects Six and twelve weeks	Four 8 mm defects were created in 8 New Zealand rabbits. Each defect was treated with one of the folling treatments; Group 1- nHA alone Group 2-nHA with Group3-nHA with autogenous MSCs Group 4-nHA with autogenous PRGF and MSCs	More newly bone was found in the group receiving the nHA/PRGF/MSC combination was significantly more than that in other 3 groups. Also in the MSC-PRGF group was present more trabecular bone.
Esmaeil Bizar *et al* [46]	Stem cells Injectable nanoparticular scaffold USSCs	Evaluate if calcium phosphate such as hydroxyapatite (HA)/fluorapatite(FA), with chitosan gel filled with unrestricted somatic stem cells (USSCs) can healing calvarial bone .	Calvarial defects 28 days	Group 1- scaffold filled with USSCs Group2- scaffold without cells Group3- control group no scaffold and no cells were added.	Greater amounts of regenerated new bone was found in the scaffolds with USSCs
Teodelinda Mirabella *et al* [47]	hAFSCs	Evaluate the recruitment of host´s progenitor cells to sites of human amniotic fluid stem cells (hAFSCs) implantation seeded onto HA scaffolds	Subcutaneously on the back of mice 1,2,4and 8 weeks	For each time point was implanted on three mice four scaffolds (2 scaffolds with hAFSC and 2 control scaffolds were empty)	After 8 weeks, the empty implanted control scaffold was poorly populated by cells and no organized tissues were evidence, while in hAFSCs –seeded scaffolds they are present and there is evidences of immature bone formation.
Paolo Berardinelli *et al* [23]	oAFMC	Evaluate if magnesium-enriched hydroxyapatite (MgHA)/ collagen –based scaffold engineered with ovine amniotic fluid mesenchymal cells (oAFMC) could increase bone regeneration process.	Sinus augmentation Forty five and ninety days	Group1- oAFMC implanted on a scaffold Group 2- scaffold alone	MgHA)/ collagen –based scaffold showed high bone deposition and stimulated a more rapid angiogenic reaction than scaffold alone.

Jae-Kook Cha *et al* [53]	BMP-2	Evaluate the efficacy of bone morphogenetic protein 2 (BMP-2) in a bovine hydroxyapatite/ collagen (BHC) carrier to augment bone formation in a canine nasal sinus model.	Sinus 20 weeks	Group1- BHC alone Group2-BHC loaded with BMP-2 at 0.1mg/mL Group3- BHC loaded with BMP-2 at 0.5 and 1,5mg/mL	Histometric analysis showed significantly enhanced bone formation for the BMP-2 groups compared with control. Even the low concentration induces osteogenic activity.
Jian Cao *et al* [18]	Col/nHA/Alg hydrogel hNGFβ	Assess the effects of the injectable NGF-carrying collagen/nanohydroxyapatite/alginate hydrogel on bone formation	Bone formation on mandibular distraction osteogenesis Fourteen days	Thirty-five New Zeland white rabbits underwent bilateralmadibular distraction osteogenesis (DO) (at a rate of 0.75 mm per 12 hours for 6 days) These animals were divided into 4 groups. Group 1 – receive injections of 0,2ml Col/nHA/Alg hydrogel containing 20 µg hNGFβ Group 2- receive injections of 20 ug hNGFB in 0.2 mL isotonic saline Group 3- receive injections of 0,2ml Col/nHA/Alg hydrogel alone Group 4-receive injections of 0.2 mL isotonic saline alone. Plain radiographs and scans (dual- energy X-ray was taken; mechanical tests and bone histology and histomorphometry analyses were made.	No difference in regenerated bone dimensions was found among the 4 groups. Bone histology and histomorphometric analysis Group 1- well organized woven bone and lamellar bone, with signs of callus remodeling and no fibrous or cartilaginous tissues Bone consolidation and remodeling were most advanced in group 1. Bone volume is higher in group 1 Radiographic examination Bone mineral density and the maximum load was higher in group 1
Stephan T. Becker *et al* [14]	CAD-CAM rhBMP-2	Evaluate if synthetic hydroxyapatite and tricalcium phosphate (TCP) blocks made by computer assited designed (CAD) could serve as precise scaffolds for intramuscular bone induction (endocultivation).	Intramuscular endocultivation 8 weeks	Group1- 3-D printed HA and TCP Group 2- bovine HA (control) Was added a central channel and rhBMP-2 to each group.	All materials tested showed new bone formation, but higher rates of bone density in HA groups.
Akira Matsuo *et al* [59]	PCBM	Evaluate if custom-made bioresorbable composites of a raw particulate hydroxyapatite/poly-L-lactide (HA/PLLA) with particulate cancelous bone and marrow (PCBM) can improve bone quality for mandibular reconstruction.	Bone defects on mandible 12 moths	Group 1- PRP and PCBM were harvest and mixed into custom-made HA/PLLA. Group2- titanium tray was fixed by screws	No significant difference in buccal side between the HA/PLLA type and the titanium type. In lingual side, the HA/PLLA type, shows increased bone volume, sufficient bone quality and it was easily and more adapted to the mandible.
Ji Woong Jang *et al* [52]	rhBMP-2	Evaluate if HA /beta-tricalcium phosphate (β-TCP) ratio of 20/80 imbued with recombinant human bone morphogenetic protein (rhBMP-2) enhances new bone formation and to determinate the dose-dependent response of rhBMP-2.	Calvarial defects 2 and 8 weeks	Calvarial defects were made in rats, and filled with biphasic calcium phosphate (BCP) with different rhBMP-2 doses. There was also one control group without rhBMP-2	rhBMP-2 induces new bone formation at all doses, showing greater bone formation in rhBMP-2 groups than in control groups.
Wei Shui *et al* [50]	BMP9	Analyze the in vivo osteoconductive activities and bone regeneration of three scaffolds, type I collagen, hydroxyapatite-tricalcium phosphate (HA-TCP) and demineralized bone matrix (DBM), using BMP9-expressing C2C12 osteoblastic progenitor cells.	Subcutaneous implantation 1,2and 4 weeks	Three scaffolds materials, type I collagen, HA-TCP and DBN, were implanted with BMP9-expressing C2C12. There was a fourth group without carrier.	Confirmation of the osteogenic activity of BMP9 in C2C12 cells. Robust ectopic bone formation was detected in collagen sponge and HA-TCP.
Masaaki TAKECHI *et al* [60]	PLGA aAC	Analyse a 3-dimensional scaffold consisting of biodegradable poly(D,L-lactide-co-glycolic acid)(PLGA) (75/25) with hydroxyapatite particles containing atelocollagen (aAC) in regard to its properties and biocompatibility.	Subcutaneous implantation 3 days and 3 weeks	Group1- PLGA with HA Group2-PLGA +HA mixed with aAC power (1:1) Group3- PLGA +HA mixed with aAC power (1:2)	PLGA-aAC was superior in terms of cell attachment and proliferation for bone regeneration compared to PLGA alone.

X. Juan Sun *et al* [61]	BMP-2	Evaluate if OsteoBome scaffold in combination with bMSCs modified by BMP-2 gene can increase vertical bone height.	Maxillary sinus 2 and 4 weeks	Bone marrow was obtained from fibula and then modified with AdBMP-2. Group A- AdBMP-2-bMSCs on OsteoBone GroupB- AdRGFP-bMSCs on OsteoBone.	Although there was no differences in augmentation height between groups, there was more new bone formation on group A.
S.T.Becker *et al* [62]	BMP-2	Compare BMP application on hydroxyapatite blocks at different times for heterotopic bone induction.	Latissimus dorsi muscle 1, 2, 4, 8 weeks	On the right side was implanted one block that served as a control group and where BMP-2 was applied simultaneous at the application of the block of Hap. On the left: Group 1- BMP-2 delayeted 1 week Group2- BMP-2 delayeted 2 weeks Group3- BMP-2 delayeted 4 weeks	Bone formation was less effective on delayed application of BMP than simultaneous application, but render greater neovascularization.
Wei Xiao *et al* [44]	Ha microspheres BMP-2	Evaluate if hollow hydroxyapatite microspheres can serve as a carrier for controlled released of BMP-2 in bone regeneration.	Calvarial defect 3 and 6 weeks	Group1- hollow HA microspheres Group2- hollow HA microspheres loaded with BMP-2(1u per defect) Group3- hollow HA microspheres loaded with BMP-2 (1u per defect) and coated with PLGA (5omg ml^{-1}) Group4- hollow HA microspheres loaded with BMP-2 (1u per defect) and coated with PLGA(200mg ml^{-1})	Was observed markedly greater bone formation on the defects implanted with BMP-2 –loaded microspheres and limited bone formation on control group (group 1).

In this table were included some modifications such as, incorporation of biomolecules like BMP [14, 44, 50, 52, 53, 61, 62], GF [18], BM/MSCs [23, 30, 31, 45-47] and PRP [55, 59] behaviour were also analysed. Also can be found a study where cell are recruited, named cell homing [55] and two where are produced matrix with the same shape of the defect [14, 31].

4.2.2. In vitro studies

The in vitro studies are presented in Table 5, including their innovation, the aim of the study, time of follow-up, methodology and results.

Table 5. Description of the *in vitro* found in the bibliographic search

References	Innovation	Aim of the study	Time of follow-up	Methodology	Results
Yuhua Sun *et al* [51]	Peptide decoration	Evaluate the bioactivity and the osteogenic differentiation of the bone using peptide decorated nano-hydroxyapatite	1,3and 5 days	The nano-HA was coated by one-step pH-induced polymerization of dopamine, an then the peptide was grafted onto polydopamine (pDA) coated nano-HA(HA-pDA) through catechol chemistry	Peptide decorated nano-HA demonstrated better bioactivity and osteogenic differention
Min-Ho Hong *et al* [63]	Spherical hydroxyapatite	Presentation of the preparation process of the porous spherical hydroxyapatite (Hap) granules with a novel method for fabrication of bone void filler.	14 days	Pore and channel structures of spherical granules were obtained by adjusting the ratio of water to Hap power and the amount of sodium chloride (NACl). Dexamathasone (Dex) added to Hap the drug release behavior.	Hap granules showed a potential as bone void filler with ability of controlled drug released.
Mizuki Suto *et al* [54]	nanohydroxyapatite	Demonstrate if nano-HA increases BMP-2 expression at gene and protein levels	15days	To the nano-HA suspension was added a monolayer of human periodontal ligament (PDL) cells and 3ml of culture medium with 5% foetal bovine serum (FBS) and 50ug/ml ascorbic acid.	Nano-HA increased the gene expression level of BMP-2 through activation of the p38 mitogen-activated protein (MAP) kinase pathway.
J.R.Overman *et al* [48]	hASCs	Test if differentiation progress of human adipose stem cells (hASCs), produce more gene products associated with process such as angiogenesis and bone remodeling.	0,4,14 and 21 days	hASCs were treated with BMP-2 and seeded on plastic or on BCP	The BMP-2 has an effect on osteogenic differentiation of hASCs on BCP (60% HA and 40% b-tricalcium phosphate) helping in bone repair.

In this table were included some modifications such as, incorporation of biomolecules like BMP [48, 54] and drugs [63], behavior were also analyzed.

4.3. Biomaterials characterization

4.3.1. Scanning Electron Microscopy (SEM)

Figure 9 presents the SEM micrographs of the three studied graft materials, Cerabone®, Bio-Oss® and NuOss®.

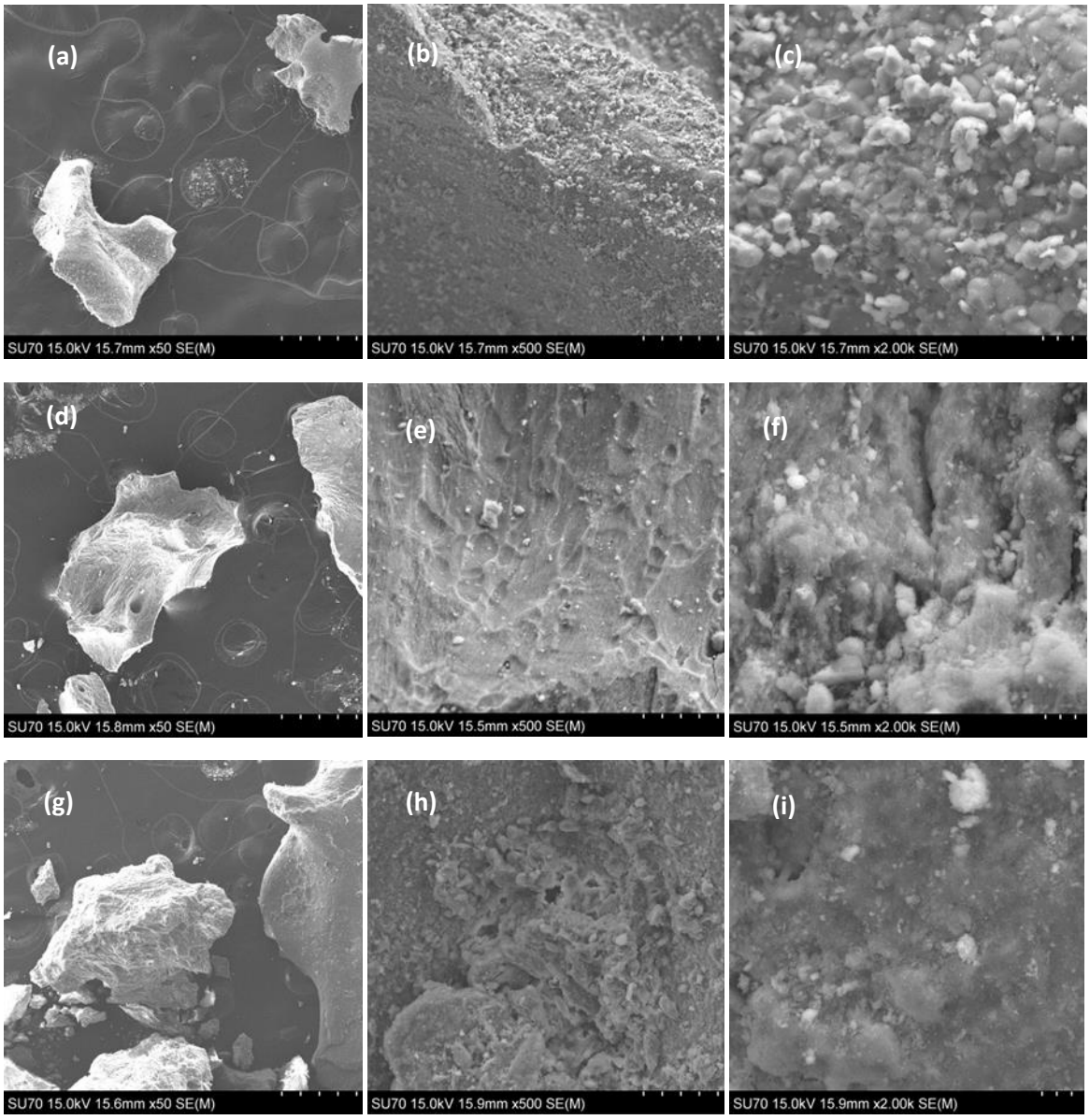


Figure 11. SEM images showing the surface morphology of Cerabone®, Bio-Oss® and Nu Oss® at 50xmagnification (**a,d,g**), at 500x magnification (**b,e,h**) and at 2000x magnification (**c,f,i**), respectively.

All the materials presented strong similarities at low magnification (20x) with respect to the structure and morphology of the particles (Figure 9.a), d), and g)). However when increasing the magnification (500x, 2000x)(Figure 9. b), c), e), f), h), i)) it is possible to detect different surface topographies. Only on Cerabone® (Figure 9. B), c)) a surface coating of fine blister/bubble-like crystal conglomerations with smooth surface and clear contour was visible. A common characteristic shared by these three samples was low porosity. The average particle size was 0.86 mm for Cerabone®, 0.82 mm for Bio-Oss® and 1.4 mm for NuOss®, whereas the average pore diameter was 24 µm (Cerabone®) 55µm (Bio-Oss®) and 90µm for NuOss® . In Figure 9c. the presence of pores with a medium diameter of 1.8 µm for Cerabone® sample can be observed. There is a discrepancy between the values obtained by SEM analysis and data presented by the manufacturer (100-1500 µm).

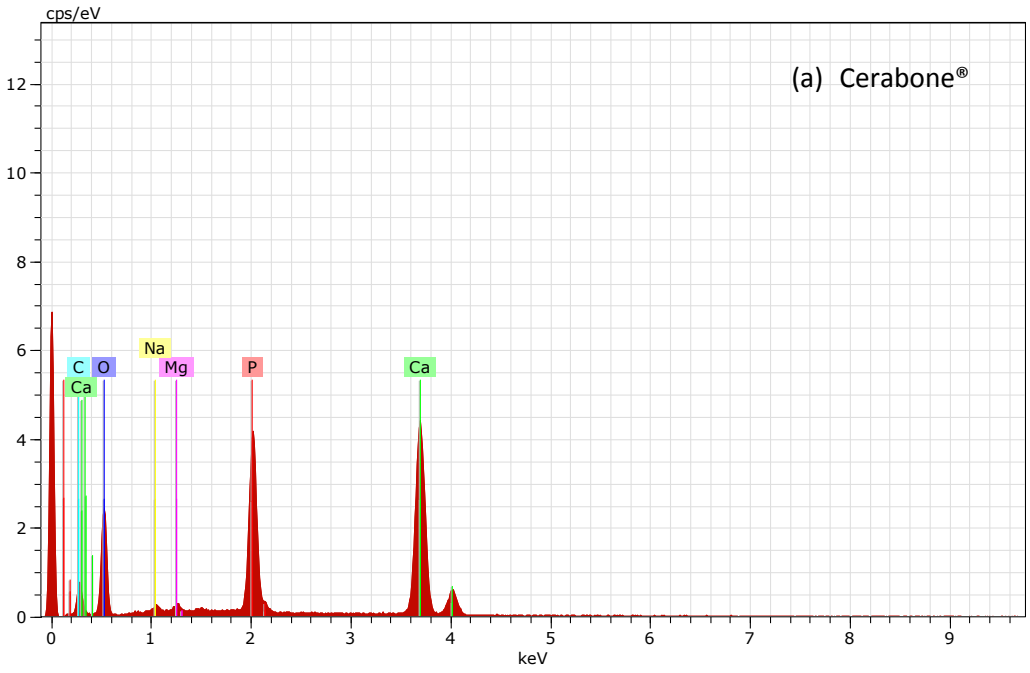
4.3.2. Energy Dispersive X-Ray Analysis (EDX)

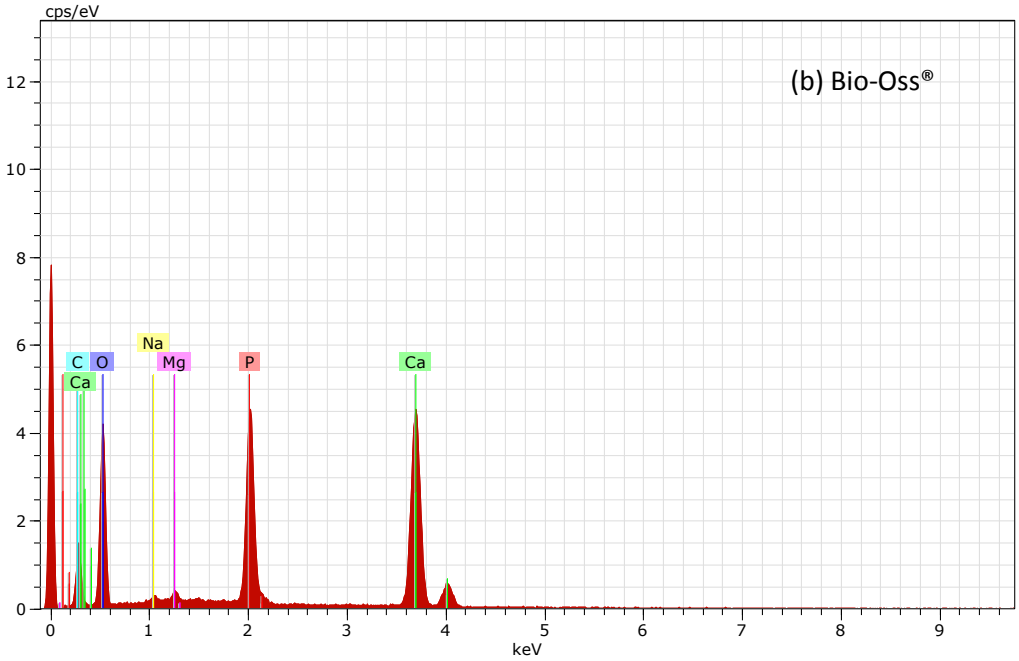
The atomic composition of Cerabone®, Bio-Oss® and NuOss® obtained by EDX analysis and the respective Ca/P ratios are presented in Table 6. The typical spectrum obtaing for these type ceramic materials is illustrated in Figure 12.

Table 6. Atomic composition of the three materials obtained by EDX analysis.

Atomic %			
	CeraBone®	**Bio-Oss®**	**NuOss®**
Phosphorus	8,92	6,83	10,30
Calcium	17,52	13,32	20,73
Oxygen	55,32	57,85	46,13
Carbon	17,52	21,34	21,75
Magnesium	0,30	0,33	0,42
Sodium	0.43	0,20	0,67
Ca/P	1,96%	1,95%	2,01%

In addition to calcium, phosphorus and oxygen, which are present in large quantities in these samples, also traces of sodium and magnesium were detected. The presence of these last two elements is explained by the simple fact that we are analyzing bone substitutes of natural origin (bovine). The results obtained, allowed to calculate the proportion of Ca / P, which was 1,96% for Cerabone®, 1,95% for Bio-Oss® and 2.01% for NuOss®. There was no statistical difference in the Ca/P ratios between the three bone substitutes. The Ca/P ratios were higher comparatively to human bone (1,67%) for all biomaterials [64].





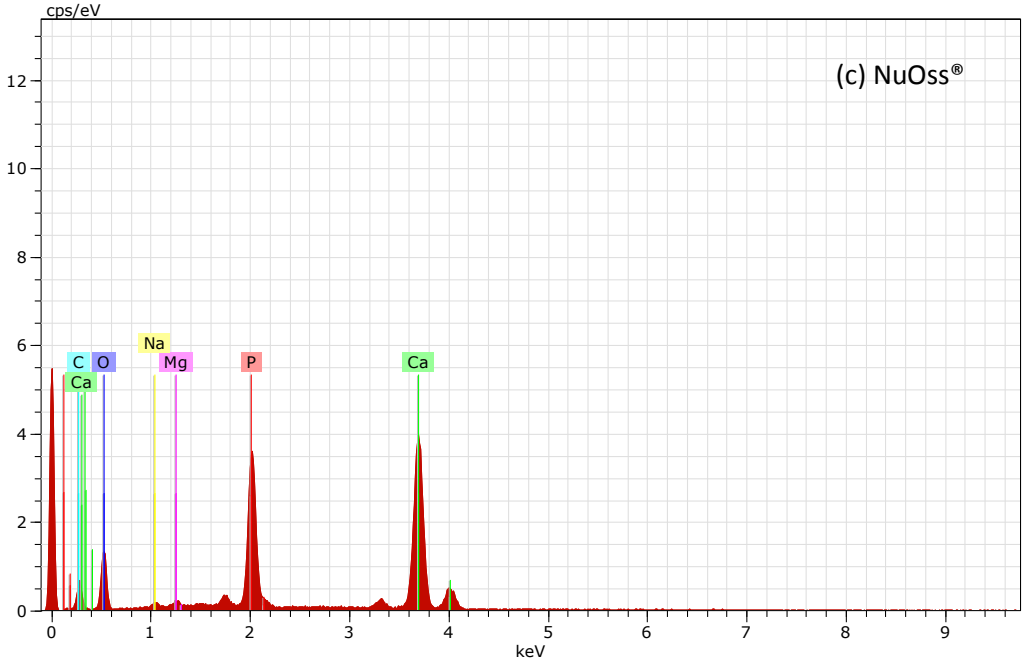
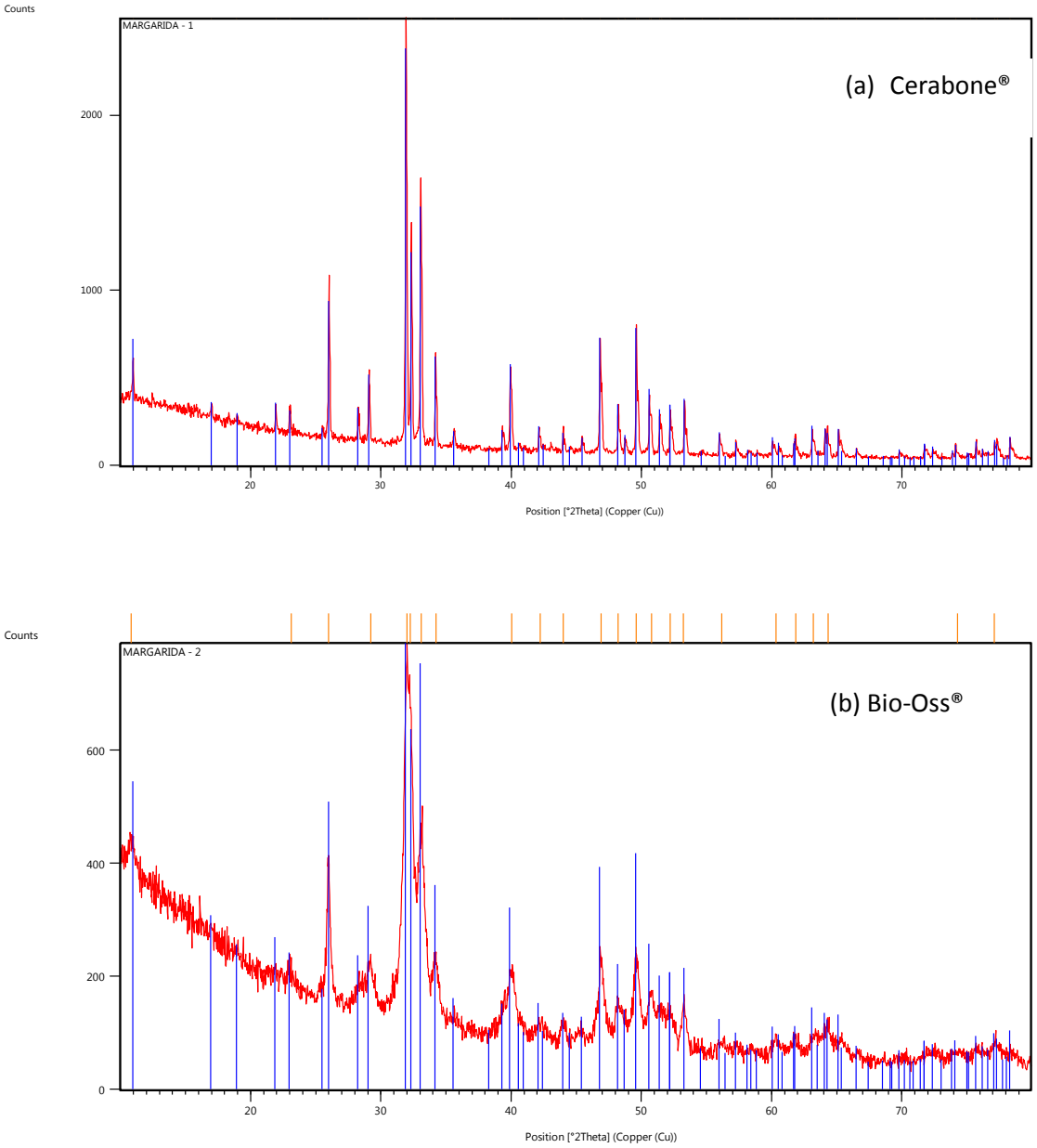


Figure 12. Typical EDS spectrum obtained for the studied ceramic materials, (a) Cerabone®, (b) Bio-Oss® and (c) NuOss ®

4.3.3. X-ray Diffraction (XRD)

Figure 13 presents the XRD spectra of (a) Cerabone®, (b) Bio-Oss® and (c) NuOss® where it is possible to detect the main characteristic peaks assigned to HA.



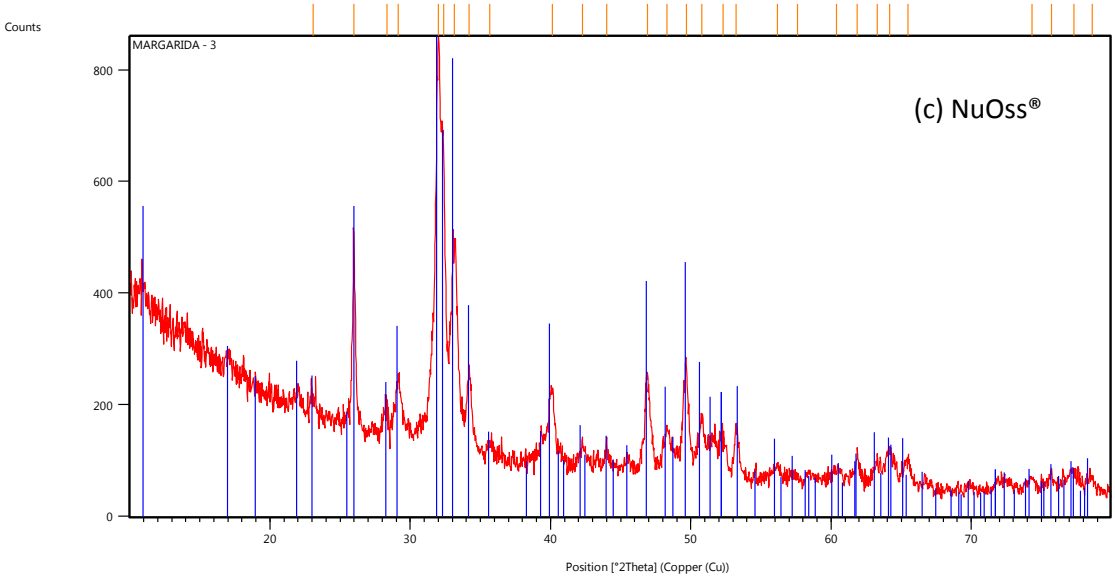


Figure 13. XRD spectra of (a) Cerabone®, (b) Bio-Oss® and (c) NuOss®.

All bone substitutes spectra are assigned to the reference hydroxyapatite spectra [04-010-6315].

The peaks with big intensity could be observed in the diffraction angles 2θ 26º,32º,33º and 40º associated to the crystal plans (002), (211), (112) and (300) respectively.

Cerabone® showed higher crystallinity (0,81) as compared to Bio-Oss® (0,43) and NuOss® (0,49). The Bio-Oss® and NuOss® spectra presented larger peaks than Cerabone® spectrum which support the values above enunciated.

4.3.4 Specific Surface Area

The values of the specific surface area of hydroxyapatite degassed for five hours at 35 ° C were 79, 69 m^2/g for Bio-Oss®, 54, 67 m2/g for NuOss® and not possible of being calculated for Cerabone®.

Table 7. Specific Surface Area m^2/g calculated for the studied materials.

SPECIFIC SURFACE AREA m^2/g		
Bio-Oss®	NuOss®	Cerabone®
79.6894 ± 0.6587 m²/g	54.6706 ± 0.3874 m²/g	A positive BET surface area was not calculated.

Discussion

5. Discussion

In the bibliographical search, it was possible to gather the most recent works that display the state of the art regarding HA scaffolds in association with biomolecules for craniomaxillofacial bone regeneration. All of these strategies aim to promote a bioactive behavior and, consequently, regeneration.

5.1 Clinical studies

Jihua Li *et al.* [2] reported a reconstruction of mandibular condyle which was lost during a mandibular angle reduction (plastic surgery). The condyle was fabricated from nanoscale hydroxyapatite/polyamide (n-HA/PA) with the assistance of computed tomography (CT) data, and computer-aided design/computer-aided manufacturing (CAD/CAM) for rapid prototyped printing. These results allowed the restoration of facial contour, symmetry and normal occlusion which was achieved by mirroring the healthy mandibular condyle. However, the patient maintains a slight deviation during mouth opening and some movements had no assistance because the lateral pterygoid was not attached to the new condyle. Also further studies with more cases are needed to validate the approach.

Giuseppe Corinaldesi *et al.* [3] evaluated the quantity and quality of bone regeneration with recombinant human bone morphogenetic protein-7 (rhBMP-7) and deproteinised bone substitute to augmentation of the floor of maxillary sinus. The overall results showed that more gain in height on the test side after sinus lift and less resorption volume after 4 months although new bone formation was also found in control sites. In spite animal studies using BMP-2 obtained more newly bone formation, in this paper the same did not happened.

Marcus Jager *et al.* [57] evaluated the potency of bone marrow aspiration concentrate (BMAC) to augmentation bone grafting and support bone healing. The overall results showed new bone formation was detected in both BMAC- HA and BMCA- Collagen groups , but bone formation appeared significantly early in the HA group.

Paul W. Poeschl *et al.*[22] investigated the effect of platelet-rich plasma (PRP) on new bone formation and remodeling after grafting of the maxillary sinus with an

algae-derived HA AlgOss/C Graft/Algipore. The results suggested significantly better resorption of algae-derived hydroxyapatite AlgOss/C Graft/Algipore and increased new bone formation in apical region when PRP was used and no significant differences in coronal portion in both group. This study proves that PRP can enhance bone regeneration but remember the importance of the addition of collected bone which had bony cells.

Hossein Behnia *et al.* [17] analyzed the enhancing effect of recombinant platelet derived growth factor (PDGF) on human mesenchymal stem cells (hMSCs) in secondary alveoloplasty. The results of this study suggest that the combination of platelet derived growth factor with hMSCs give to cells the regenerative capacity, but the results are far being satisfactory. It would be interested if the authors of this study had a control group to compare the results.

Yu Zhang *et al.* [58] evaluated the effect of PRF on bone regeneration in sinus augmentation with Bio-Oss. The results suggest no significant differences between groups. In this study both groups had proximally amount of newly formed bone and residual bone substitute (Bio-Oss). Although in vitro studies suggest greater bone formation when added PRF to Bio-Oss in this study this fact not happened. The authors hypothesized that the absence of precursor cells could be the reason of less bone formation.

Although clinical studies are still at a very early stage, these have revealed promising results. The results of previous studies revealed that the incorporation of biomolecules (such as rhBMP-7, PRP, hMSCs and PRF) and cells increase bone formation and decrease resorption rate. This last fact has a crucial importance because the slow resorption keeps the volume leading to a good dimension of the new bone. Also these studies showed that new bone appeared significantly early in the HA matrix in comparison to others matrix. In oral cavity theses new approaches for bone regeneration were applied preferentially on the floor of maxillary sinus, on the mandibular condyle and on the alveoli.

It is important to note that only the addiction of biomolecules is not enough to enhance bone formation. The presence of precursor cells or bony cells is necessary since

only in combination with biomolecules they can receive regenerative capacity. Despite of these encouraging results there are still few studies and low number of subjects. Further studies need to be performed in order to generate a representative amount of results that can be extrapolated to achieve a predictable method of bone engineering.

5.2 Pré-clinical studies

5.2.1In vivo studies

Several in vivo studies were performed in last years, showing encouraging results. These studies have a big significance because they predict tissues reactions to the scaffold implantion.

Rania M. El Backly *et al.*[55] evaluated the influence of lyophilized platelet rich plasma (PRP) on bone regeneration in combination with silicon stabilized hydroxyapatite tricalcium phosphate scaffold (Skelite). The results of this study suggest that the addition of PRP increased bone formation contrarily to what happen in control group and Skelite group. The PRP when associated with osteoconductive scaffold increase progenitor cell recruitment, collagen and bone matrix deposition, and created an interface between bone and the scaffold.

L. Ciocca *et al.* [31] evaluated the efficacy of mesenchymal stem cells (MSCs) in CAD-CAM customized in pure and porous hydroxyapatite (HA) scaffolds for condyle substitution. The MSCs presence accelerated bone formation and showed more volume of new bone also exhibited good organization and maturation of osteoblastic and osteoclastic cells.

N. Pourebrahim *et al.* [45] compare bone regeneration of tissue- engineered bone from adiposed- derived stem cell and autogenous bone graft. The authors of this study demonstrate that were more bone formation on the autograft sides than on the stem cell sides, but tissue engineered bone can provide an acceptable alternative avoiding the disadvantages of the autograft.

Hossein Behnia *et al.* [30] evaluated Nano-HA as a carrier construct for mesenchymal stem cells (MSCs) and platelet- rich growth factor (PRGF) . This study showed that more newly bone was found in the group receiving the nHA/PRGF/MSC

combination and it was significantly more than that in others 3 groups. The authors of this study suggested that nano-crystaline HA has high level of porosity that may be responsible for higher absorbance of biofactors and consequently of higher rate of bone formation.

Esmaeil Biazar *et al.* [46] evaluated if calcium phosphate such as hydroxyapatite (HA)/fluorapatite(FA), with chitosan gel filled with unrestricted somatic stem cells (USSCs) can healing calvarial bone defects. The overall results indicated that greater amounts of regenerated new bone were found in the scaffolds with USSCs. The results of this study suggested that small size HA has a good interaction with the surrounding cells and also has an increased protein absorption and cell adhesion.

Teodelinda Mirabella *et al.* [47] evaluated the recruitment of host´s progenitor cells to sites of human amniotic fluid stem cells (hAFSCs) implantation seeded onto HA scaffolds. The results showed that the empty implanted control scaffold was poorly populated by cells and no organized tissues were evidence, while in hAFSCs –seeded scaffolds they are present and there is evidences of immature bone formation. The authors have proven that the presence of hAFSCs led to recruitment of cells and the cascade of events which induced bone formation.

Paolo Berardinelli *et al.* [23] evaluated if magnesium-enriched hydroxyapatite (MgHA)/ collagen –based scaffold engineered with ovine amniotic fluid mesenchymal cells (oAFMC) could increase bone regeneration process. The overall results suggested that MgHA)/ collagen –based scaffold showed high bone deposition and stimulate a more rapid angiogenic reaction. oAFMC provides an easy and cheap source of MSCs that could differentiate into osteoblast cells.

Jae-Kook Cha *et al.* [53] evaluated the efficacy of bone morphogenetic protein 2 (BMP-2) in a bovine hydroxyapatite/ collagen (BHC) carrier to augment bone formation in a maxillary sinus. Histometric analysis showed significantly enhanced bone formation for the BMP-2 groups even the low concentration induces osteogenic activity.

Jian Cao *et al.* [18] assessed the effects of the injectable NGF-carrying collagen/nanohydroxyapatite/alginate hydrogel on bone formation. The results showed that no difference in regenerated bone dimensions was found among 4 groups, but the new bone was well organized, has more density and volume in group 1 (receive

injections of 0,2ml Col/nHA/Alg hydrogel containing 20 μg hNGFβ) . Bone dimensions were similar which could be explained because of the enzymatic degradation. hNGF is rapidly eliminated from the organism so different delivery systems should be investigated. Another alternative could pass for delayed administration of this growth factor. Nerveless this approach should not be put aside because NGF can stimulate differentiation and also inhibit apoptosis if osteoblast cells.

Stephan T. Becker *et al.* [14] evaluate if synthetic hydroxyapatite and tricalcium phosphate (TCP) blocks made by computer assisted designed (CAD) could serve as precise scaffolds for intramuscular bone induction (endocultivation).The results of this study suggest that HA groups presented higher rates of bone density. The authors prove that BMPs play an important role in bone regeneration once they are able to recruit pluripotent cells and after differentiate them into osteogenic cells. Also this paper showed that HA scaffolds yielded more bone formation than TCP scaffolds.

Akira Matsuo *et al.* [59] evaluated if custom-made bioresorbable composites of a raw particulate hydroxyapatite/poly-L-lactide (HA/PLLA) with particulate cancelous bone and marrow (PCBM) can improve bone quality for mandibular reconstruction. The overall results suggest no significant difference in buccal side between the HA/PLLA type and the titanium type but in lingual side, the HA/PLLA type, shows increased bone volume, sufficient bone quality and it was easily and more adapted to the mandible. Also it was proved that mandibular reconstruction using HA/PLLA reduce operating times because the tray is reabsorb after one year. In this paper HA/PLLA trays had delayed bone formation, fact that was not expect because it contains HA.

Ji Woong Jang *et al.* [52] evaluated if HA /beta-tricalcium phosphate (β-TCP) with recombinant human bone morphogenetic protein (rhBMP-2) enhances new bone formation and determinate the dose-dependent response of rhBMP-2. The authors proved that even low doses of rhBMP-2 induce new bone formation, showing greater bone formation in rhBMP-2 groups than in control groups. The absence of dose-dependent response to rhBMP-2 cannot be extrapolated because others factors must be considered like type of carrier, animal species and defect site.

Wei Shui *et al.* [50] analyzed the in vivo osteoconductive activities and bone regeneration of three scaffolds, type I collagen, hydroxyapatite-tricalcium phosphate

(HA-TCP) and demineralized bone matrix (DBM), using BMP9-expressing C2C12 osteoblastic progenitor cells. The results showed robust and mature cancellous bone formation in collagen sponge and HA-TCP. This fact can be justified due the low cellularity of DBM and because this carrier has glycerol as a bonding agent. Glycerol is toxic and can cause cell death.

Masaaki TAKECHI *et al.* [60] analyzed a 3-dimensional scaffold consisting of biodegradable poly(D,L-lactide-co-glycolic acid)(PLGA) (75/25) with hydroxyapatite particles containing atelocollagen (aAC) in regard to its properties and biocompatibility. PLGA-aAC was superior in terms of cell attachment and proliferation for bone regeneration compared to PLGA alone. The authors of this study attempted to create a better scaffold combining different properties of various materials.

X. Juan Sun *et al.* [61] evaluated if OsteoBome scaffold in combination with bMSCs modified by BMP-2 gene can increase vertical bone height. The results showed that there was more new bone formation on group modified by BMP-2 suggesting bMSCS infected by AdBMP-2 increased differentiation into osteoblastic cells and bone formation.

S. T. Becker *et al.* [62] compared BMP application on hydroxyapatite blocks at different times for heterotopic bone induction. The overall results suggested that delayed application of BMP led to a smaller amount of bone. This finding may be explained because the HA matrix is resorbed during the inflammatory phase and before the application of BMP. After this initial phase the soft tissues invade where HA matrix is placed and prevent the spread of BMP within the matrix.

Wei Xao *et al.* [44] investigated if hollow hydroxyapatite microspheres can serve as a carrier for controlled released of BMP-2 in bone regeneration. Was observed markedly greater bone formation on the defects implanted with BMP-2 loaded microspheres and limited bone formation on control group. HA microspheres showed the ability to release BMP-2 at desirable doses avoiding the adverse effects associated with the use of high dosages of this protein.

Different animal models were used, such as rats, rabbits, dogs, sheep and mice. In all of these animals defects were created and then these received HA scaffolds in conjugation with biomolecules. Some examples of biomolecules used on these new

methods were PRP, MSCs, PRGF, USSCs, hAFSCs, oAFMC, BMP-2, hNGFB, BMP-9, PCBM, PLGA and aAC.

The incorporation rend more and faster bone formation, increase progenitor cell recruitment, bone matrix deposition, created an interface between bone and the scaffold, also exhibited good organization and maturation of osteoblastic and osteoclastic cells.

The size of HA particles were tested and showed to have high level of porosity that may be responsible for increased protein absorption and cell adhesion and consequently for more bone formation.

Autograft may still the gold standard, but tissue engineered for bone regeneration can provide an acceptable alternative avoiding the disadvantages of the first cited.

5.2.2 In vitro studies

In vitro studies represent an essential phase. They are the starting point for the development of new advances, in this specific case, on the development of methods to added a bioactive behavior and enhance bone regeneration.

Yuhua Sun *et al.* [51] evaluated the bioactivity and the osteogenic differentiation of the bone using peptide decorated nano-hydroxyapatite. The overall results of this study suggest that peptide decorated nano-HA demonstrated better bioactivity and osteogenic differention by improving cell proliferation and differentiation.

In previous studies [48, 54] BMP-2 has a higher expression in nano-HA scaffolds and improve osteogenic differentiation of hASCs facilitating bone regeneration.

Min-Ho Hong *et al.* [63] presented a preparation process of the porous spherical hydroxyapatite (Hap) granules for bone void filler. The results suggest that porous spherical HA granules cause less inflammatory reaction and led to fast bone formation.

The overall results showed that the incorporation of biomolecules in HA scaffolds lead to better bioactivity and osteogenic differention by improving cell proliferation and differentiation stimulate faster bone formation.

5.3. Biomaterials characterization

5.3.1. Scanning Electron Microscopy (SEM)

The particle size, its topography and porosity are important factors for osteogenesis during bone regeneration [65].

The bone substitutes studied in this thesis are constituted by bovine bone inorganic matrix. Yet, despite possessing the same origin they have different morphology.

This can be explained by the processing temperature. The heat treatment above 1000° C used for the removal of organic content, resulted in an increase of the crystallites and crystallinity [66].

At low magnifications the three samples showed similarities in the particle size, the morphology and the structure of particles, but at high magnifications, the differences can be seen more clearly. It can also observe the reduced pore size (average pore diameter that is low 24 μm) in Cerabone® sample.

5.3.2. Energy Dispersive X-Ray Analysis (EDS)

The results obtained, allowed to calculate the proportion of Ca/P, which was 1,96% for Cerabone®, 1,95% for Bio-Oss® and 2.01% for NuOss®. The Ca/P ratios were higher comparatively to human bone (1,67%) for all biomaterials [64].

Calcium ions in excess were observed (Ca / P ratio > 1.67). The process of formation of these bone substitutes intervenes directly in the molar ratio of calcium and phosphorus. Consequently we conclude that higher processing temperatures lead to the formation of more crystalline bone substitute [67]. The crystallinity will dictate the behavior of bone matrix in vivo, or the behavior of a biodegradable material into biological fluids. It can be stated that samples with a greater molar ratio Ca / P will be more stable / crystalline and therefore less soluble [67].

The human bone is not composed solely of hydroxyapatite also has in its constitution carbonate. The carbonate ion can be seen in samples of bone substitutes lower crystallinity and is responsible for checking the samples greater affinity to

extracellular matrix proteins [66]. In bone matrices processed at higher temperatures than 1000 ° C the carbonate ion decreases [66]. The samples of Bio-Oss® (21, 34%) and NuOss® (21, 75%) have presented more carbon ions comparatively to Cerabone® (17, 52%).

Our results do not meet the images obtained by XRD, because the bone substitute with bigger ratio Ca / P is the one with less crystallinity. The composition of trace elements (Magnesium and Sodium) revealed very similar results.

Nevertheless, since the EDX was conducted with a particle of each sample and we have to take into account the natural composition variability of the biological bone. In future more replicates should be used in order to achieve statistically significant results.

5.3.3. X-ray Diffraction (XRD)

The x-ray diffraction allows for a qualitative study of the crystalline phases, but also allows calculating the degree of crystallinity and crystal size.

In the diffractograms of low crystalline samples, the peaks are larger, complicating the identification of the correct value of the valley between the peaks (112) and (300), and for this reason the second method is used for the calculation of crystallinity in amorphous samples.

The Cerabone® sample was the one with the highest degree of crystallinity (0,81).

For the crystal size, we can say that it is indirectly proportional to the width of peaks obtained by powder diffractogram, the enlargement of the peaks corresponding to smaller crystals. Also smaller crystals lead to high specific surface area [68]. A correspondence between the crystal size and the processing temperature was also established, being the biggest crystals formed at higher temperatures.

Relatively to cell behavior, smaller crystals show a more favorable topography for cell attachment / osteoblast adhesion [69].

5.3.4. Specify surface area

The values of specific surface area ranging from 0 79.69 m2/g to 0 m^2/g. The Bio-Oss® sample showed a higher specific surface area of the three samples [70].

A high specific surface area might indicate increased porosity. The porosity is essential as it is responsible for improving the mechanical integration between the bone substitute and bone, thereby providing a better mechanical stability at the interface, but also increasing the level of resorption [70].

It was not possible to calculate the specific surface area of Cerabone® sample, however it is conceivable that this sample will have less surface area because it was processed at higher temperatures. There is an indirect proportionality between the specific surface area and temperature [71].

Investigations involving the incorporation of biomolecules in bone substitutes have gained great interest. The possibility for attributing a bioactive behavior or functionalize the bone matrix is now possible thanks to the incorporation biomolecules (such as proteins) and cells that enhance bone regeneration.

Different bone matrices are available. Studies show that in case of hydroxyapatite matrices, new bone formation occurs significantly sooner as compared to other matrices. Beta tricalcium phosphate (BTP) is rapidly absorbed which does not happen with hydroxyapatite scaffolds [72], mostly if this is stabilized at high temperatures. This rapid resorption is detrimental to bone regeneration, since volumetric maintenance is needed in order to achieve a good volume of new bone [72].

In this thesis there was the opportunity of performing a brief characterization of three commercial bone substitutes: Bio-Oss®, Cerabone® and NuOss®. They were analyzed and compared by SEM, EDS, XRD and specific surface area.

Important differences were found regarding their crystallinity. The processing temperatures which these filling bone materials are subject play a critical role on the expected behavior vivo. We can say that high processing temperatures lead to the synthesis of samples with a higher degree of crystallinity [66].

In the literature numerous studies were found which address the relationship between the degree of crystallinity and the *in vivo* behavior of hydroxyapatite samples synthesized at different temperatures [69]. These studies demonstrate that a surface topography of bone matrices with lower degree of crystallinity is more favorable for cell attachment [69]. The degree of crystallinity can still influence the chemical composition. For processing temperatures above 1000 °C (e.g., Cerabone®> 1200 °C) it is not possible to find carbonate ions in their composition. Carbonate ions are present in natural bone, since it is processed at low temperature (37ºC). They are responsible for the affinity of extracellular matrix proteins [66]. It is possible to find these ions in Bio-Oss® and NuOss® materials due to their processing conditions. This means that these two products are more biomimetic, as they are closer to natural bone.

Based on the literature, it was also possible to observe, that regarding its morphology, amorphous crystallites have a higher surface area and smaller crystallite size (refs). Smaller crystal size improves the osteoblasts' adhesion and subsequent bone formation [69, 73]. Cerabone® reveled particles and crystals with larger size in comparison with Bio-Oss [72]. In this way, Cerabone® has lower adsorption capacity and therefore is better indicated for maintaining the space/volume stability [72]. However, although Bio-Oss® presents a lower crystallinity in comparison with Cerabone®, the degree of resorption is also low.

The incorporation of biomolecules on the HA matrix (or other CaP) and its recognized ability to enhance bone regeneration was studied in this work. It can be noted that within the studied biomolecules BMPs were the most studied group [53].

However, the incorporation of these biomolecules may not be sufficient to promote bone formation. The presence of precursor cells/bone cells are sometimes required, for the attaining the required regenerative capacity [58]. This highlights the importance of assessing the status of the patient bone in terms of its quality in order to ensure the best outcome possible.

In the bibliographical search performed in the first part of this work, for the last five years and using Sciencedirect and Pubmed, only Bio-Oss® has appeared in our results, which indicates that this commercial material had a higher impact among the scientific community to justify its continuous study and improvement. The reason for this is related with their physic-chemical properties, such as, the smallest particle size

observed in SEM trials, the medium crystallinity (0.43) obtained by X-ray diffraction and higher specific surface area (79.69 m2 / g), properties which favor the ability to interact with extracellular matrix proteins [66].

As far as NuOss® is concerned, it has very similar characteristics to Bio-Oss®. Howeve,r the NuOss® showed lower surface area and larger particle size, which will affect the behavior in vivo referred above. Nevertheless, there are articles that advocate a similar performance between the two biomaterials, assuming that this biomaterial does not stand out as much, is should be just a matter of marketing. In these studies Bio-Oss® is tested in conjunction with biomolecules in terms of quality and amount of new bone formed [74].

Conclusions and future perspectives

6. Conclusions and future perspectives

Up to recently, the possibility of inducing bone regeneration in a defect site seemed unimaginable. However the latest developments the fields of tissue engineeringand regenerative medicine have made this goal achievable.

There are several causes of bone loss, such as loss of teeth, bone fracture and trauma, the insertion of dental implants, cyst enucleation and other benign lesions, tumor resection, oroantral communication and periodontal disease.

Many biomaterials have been proposed to play the role of bone substitutes. The autogenous bone graft is considered the gold standard for repair and regeneration of bony defects, but it have some disadvantages such the limited amount of bone and donor morbidity. For these reasons other bone substitutes have been investigated, being bone-like ceramic materials particularly interesting. The ideal bone substitute should have the three main physicochemical characteristics, which are biocompatibility, osteoconductivity and osteoinductivity (osteogenesis) properties.

Hydroxyapatite is one of the most well-researched bone substitutes. It has rapid bone adaptation, no fibrous tissue formation, intimate adherence implant/tissue and low healing time, but also has some limitations, such as slow resorption and deficiency of osteoinduction. This is explained because when HA are treated with high temperatures to lower the risk of viral transmission, the soft tissues and cells are removed.

To overcome this shortcoming different adjunctive elements are presently being considered to be incorporated in HA like growth factors, cells and others to stimulate host cell recruitment, to create superior biofuncionality as compared to his individual constituents. These biomolecules generate modifications and consequently these modifications offer cues to the surrounding cells allowing them to recognize and attach to the biomaterial and improve target function like proliferation, migration and tissue differentiation. The final goal is to mimic the native tissue.

However, the majority of the studies that address this subject are still in pre-clinical phase and there is a limited number of clinical trials. For this reason more studies on regeneration of bone defects are needed.

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