

***'Histological and Scanning Electron Microscopy Analyses of  
Bone/Implant Interface Using the Novel Bonelike® Synthetic Bone Graft'***

Journal of Ortopaedic Research Vol 24(2005): 953-958

M. Gutierrez, N. Sooraj Hussain, A. Afonso, L. Almeida, T. Cabral, M.A. Lopes, J.D. Santos

# Histological and Scanning Electron Microscopy Analyses of Bone/Implant Interface Using the Novel Bonelike<sup>®</sup> Synthetic Bone Graft

M. Gutierrez,<sup>1</sup> N. Sooraj Hussain,<sup>2,3</sup> M.A. Lopes,<sup>2,3</sup> A. Afonso,<sup>4</sup> A.T. Cabral,<sup>1</sup> L. Almeida,<sup>1</sup> J.D. Santos<sup>2,3</sup>

<sup>1</sup>FMUP—Faculdade de Medicina da Universidade do Porto, Hospital de São João, Largo Hernâni Monteiro, 4200 Porto, Portugal

<sup>2</sup>DEMM, Faculty of Engg., University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

<sup>3</sup>INEB—Instituto de Engenharia Biomédica, Laboratório de Biomaterias, Rua Campo Alegre, 823, 4150-180, Porto, Portugal

<sup>4</sup>FMDUP, Faculdade de Medicina Dentária da Universidade do Porto, Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal

Received 8 August 2005; accepted 31 October 2005

Published online 11 April 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20117

**ABSTRACT:** Synthetic bone grafts provide an alternative to autografts and allografts. Bonelike<sup>®</sup> is a patented synthetic bone graft that mimics the mineral composition of bone. The aim of the present work was to assess the biological behavior of Bonelike<sup>®</sup> graft in humans, before using the material in orthopedic applications of bone regeneration, for example, in opening wedge high tibial osteotomies for medial knee osteoarthritis. Bonelike<sup>®</sup> granules were implanted in cortical bone of 11 patients undergoing osteotomies, and new bone formation, osteoconductive properties, and resorption characteristics of the granules were analyzed. The granules ranged from 500 to 1000 µm and were implanted in the lateral aspect of the tibia. The patients' mean age was 59 years (range 48 to 70 years); there were eight women and three men, all suffering from medial compartment osteoarthritis of the knee. At surgery, a 1 × 1 × 1-cm cortical defect was created 3 cm distal to the entry point of the screws, in line with the long axis of tibia. The implanted Bonelike<sup>®</sup> graft sample was extracted for analysis during removal of the metallic prosthesis after implantation times of 6, 9, and 12 months. Radiological follow-up, scanning electron microscopy, histological analysis, and histomorphometric measurements were conducted on the retrieved samples to assess bone regeneration in the defect area. Osteoconductive capacity was demonstrated by extensive mature bone formation around the implanted granules and high levels of percentage bone-to-graft contact (from 67–84%). Bonelike<sup>®</sup> acted as an excellent bioactive scaffold, allowing the migration, proliferation, and differentiation of bone cells on its surface, and therefore regeneration of the defects was achieved in a rapid, controlled manner. Our results suggest that Bonelike<sup>®</sup> graft is an excellent candidate for orthopedic applications where rapid new bone formation is a fundamental requirement. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 24:953–958, 2006

**Keywords:** synthetic bone graft; Bonelike<sup>®</sup>; osteoconduction; clinical applications; histological analysis; orthopedics

## INTRODUCTION

Bone is a connective tissue impregnated with calcium and phosphate salts. The inorganic calcium salts, mainly calcium phosphate, provide the tissue with its ability to resist the compressive forces of weight-bearing and impact forces.<sup>1</sup> For decades, calcium phosphate ceramics have received a great deal of attention in clinical

applications as orthopedic and dental materials because of their ability to bond with the living tissues.<sup>2</sup> To improve the properties of calcium phosphate materials for clinical use, hydroxyapatite (HA), a major inorganic component of bone, has been used extensively as bone graft filler and as a coating for dental and orthopedic implants because of its osteoconductive properties.<sup>3–9</sup>

Synthetic bone grafts provide an alternative to autografts and allografts, but both have a number of well-documented disadvantages.<sup>10</sup> Autograft requires a second surgical procedure that can lead to infection and chronic pain in the harvest site and requires more hospital time than allograft or bone

Correspondence to: J.D. Santos (Telephone: (351) 22 50812419; Fax: (351) 22 50812419; E-mail: jdsantos@fe.up.pt)

© 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

graft substitutes. Allograft is attractive when a large bone segment must be replaced, but can also be used to fill small defects. The risk of post-operative infection is slightly higher than autograft. Allografts avoid the need for a harvesting procedure, sparing the patient unnecessary pain and expense, and are especially useful for "at-risk" patients, for whom a harvesting procedure is undesirable.

Synthetic bone grafts are available as blocks, granules, cements, gels, and strips. Block and granule products account for the majority of the market. Granules are frequently "poured" into the defect area, and are commonly used for posterior/lateral spinal fusion, filling cystic voids (from tumors), and in hip and knee revision arthroplasties.<sup>11</sup> Source appears to be the most important factor when choosing a graft for reconstruction; membranous and endochondral factors such as resorption must be considered for long-term outcome, and donor site and technical aspects of the actual harvest are important issues to consider. The ideal recipient site should provide good blood supply, adequate bony contact, and secure fixation.

Over the past decade, we have developed and characterized *in vitro* a glass-reinforced hydroxyapatite biomaterial (GR-HA) using human bone cells. This novel graft has been recently registered as Bonelike<sup>®</sup>.<sup>12-19</sup> From *in vivo* animal studies, Bonelike<sup>®</sup> stimulates more rapid new bone formation on granules and rod implants when compared to commercially available HA, particularly during early periods of implantation.<sup>20</sup>

The aim of the present work was to assess the biological behavior of Bonelike<sup>®</sup> graft in humans. While performing a high tibial osteotomy in 11 patients being treated for medial compartment osteoarthritis, Bonelike<sup>®</sup> graft in granular form was implanted in the cortical bone of the knee for implantation periods of 6, 9, and 12 months. Osteotomy is a treatment for osteoarthritis (OA), the most common joint disease, affecting more than 80% of people in the world. OA is characterized by progressive cartilage destruction in weight-bearing areas of the joints and osteophyte formation in nonweight-bearing regions. Symptomatic osteoarthritis of the knee is more common in women than men, and its prevalence increases with age from 7% of patients aged 65-70 years to 11.2% of those aged 80 years or above.<sup>21-24</sup> By implanting Bonelike<sup>®</sup> granules in cortical bone of patients undergoing osteotomies, the material's capacity to generate new bone formation, its osteoconductive properties, and its resorption characteristics could be analyzed.

## MATERIALS AND METHODS

Bonelike<sup>®</sup> granules ranging in size from 500-1000  $\mu\text{m}$  were produced as follows. First, a  $\text{P}_2\text{O}_5$ -based glass ( $65\text{P}_2\text{O}_5-15\text{CaO}-10\text{CaF}_2-10\text{Na}_2\text{O}$  in mol %) was prepared from reagent-grade chemicals by using a platinum crucible at 1400°C for 2 h. The glass was crushed in an agate mortar and sieved to a granules size less than 75  $\mu\text{m}$ . The Bonelike<sup>®</sup> composite was obtained by mixing 4% of glass with lab-prepared hydroxyapatite (HA) in isopropanol. The mixed powders were dried for 24 h at 60°C, sieved to less than 75  $\mu\text{m}$ , and then isostatically pressed at 200 MPa. Finally, the obtained Bonelike<sup>®</sup> was sintered at 1300°C for 1 h. A description of this preparation process was reported previously.<sup>12,25</sup>

Phase identification and quantification were performed from X-ray diffraction (XRD) and Rietveld analysis. XRD was performed on Bonelike<sup>®</sup> powder samples using a Siemens D5000 diffractometer with  $\text{Cu-K}\alpha$  radiation ( $\lambda = 1.5418 \text{ \AA}$ ). The scans were made in the range of 25-40° (2 $\theta$ ) with a step size of 0.02° and a count time of 2 s/step.

The clinical study took place between March 2002 and June 2003, and included eight women and three men, with a mean age of 59 years (range 47 to 70 years), who consented to participate under a protocol approved by the Ethic Commission of Hospital de São João (Porto, Portugal). The inclusion criteria were active patients with medial compartment OA of the knee, under the age of 70, with varus deformity less than 15° and no associated inflammatory disease. Mild-to-moderate patellofemoral OA was not a contraindication. In accordance with patient consent, the blade plates used in the osteotomies were removed at 6, 9, or 12 months post-operatively.

Preoperatively, all patients were clinically evaluated using the International Knee Score (IKS). The radiological protocol included: a standing anteroposterior view of both knees; a lateral view with the knee in 30° flexion; skyline views; and standing hip-knee-ankle radiographs to determine the global alignment of the limb and perform preoperative planning to obtain a correction to 2 or 3° valgus.

Under general or regional anesthesia, all patients submitted to a prior arthroscopy of the affected knee. Although this procedure has not been demonstrated to influence long-term clinical outcome, associated meniscal lesions could be treated and loose bodies removed. A 12-cm curved incision was made beginning midway between the patella and the fibular head and extended distally to the tibial crest. The fascia was opened, and a subperiosteal dissection made exposing the lateral tibia and tibiofibular ligaments. Division of superior tibiofibular ligaments was performed in six patients. In two, a proximal fibular osteotomy was performed. Larger corrections were required in five patients, so prior to the tibial approach, a fibular shaft osteotomy was done in the region between its middle and distal thirds.

A Coventry<sup>26</sup> type closing wedge high tibial osteotomy was done. The use of special instrumentation improved the precision of the bone cuts and application of the Giebel blade plate that was used to internally fix the osteotomy. Special care was given to keep the medial cortex intact. Correction control was achieved with intraoperative fluoroscopy.

To evaluate the biological behavior of Bonelike<sup>®</sup>, a 1 × 1 × 1-cm cortical defect was created 3 cm distal to the entry point of the screws, in line with the long axis of tibia. Approximately 2 g of Bonelike<sup>®</sup> granules were used to fill the cavity (Fig. 1) and covered with the original cortical bone. A suction drain was placed (and remained for 48 h), and the fascia, subcutaneous tissue, and skin were closed. At a second operation at a predetermined date, the Bonelike<sup>®</sup> samples were collected using the same surgical approach as before.

For histology, retrieved Bonelike<sup>®</sup>/bone samples were immediately placed in a neutral formaldehyde fixative solution (6%) for 7 days, and then dehydrated in an increased percentage of alcohol solutions (70, 80, 90, and 100%) and embedded in a methylmethacrylate resin. After polymerization, specimens were sectioned with a diamond saw and polished to the thickness of 40 ± 10 µm with a diamond disc. These sections were stained with hematoxylin/eosin and Solo-Chrome R and then examined using an Olympus BH-2 transmitted light microscope. For histomorphometry, the interface between Bonelike<sup>®</sup> and new bone formation was examined by scanning electron microscopy (JEOL JSM 630IF). Histomorphometric measurements were performed on unstained and stained sections observed by scanning electron and transmitted light microscopy, respectively. A series of micrographs were taken of each section to include all the implanted granules. Total average percentages of bone/Bonelike<sup>®</sup> granule contact<sup>27</sup> were



**Figure 1.** The radiograph revealed no radiolucent line between the granules and the host bone, showing that the material is integrated with surrounding bone.

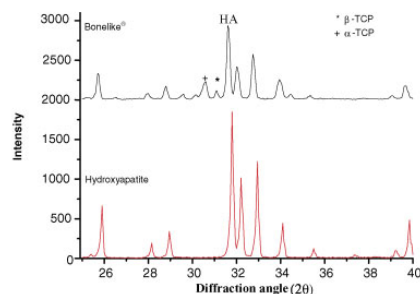
determined using a map measurement device. For statistical analysis, Student's *t*-test were performed using a 5% significance level.

## RESULTS

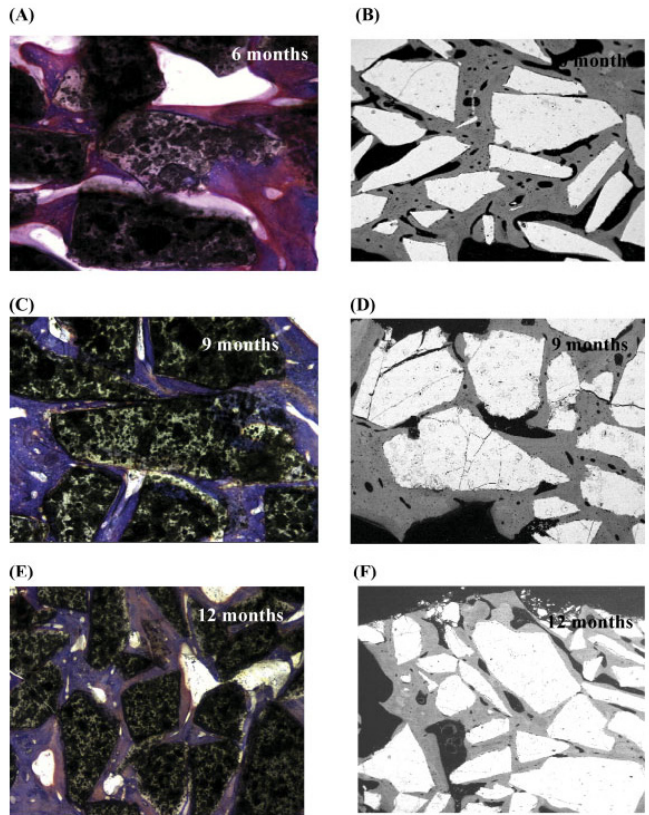
XRD analysis revealed that due to the reaction between the HA matrix and P<sub>2</sub>O<sub>5</sub>-based glass during the sintering process, the Bonelike<sup>®</sup> microstructure had a main crystalline phase of HA with β- and α-tricalcium phosphate (TCP) as secondary phases (Fig. 2). Rietveld analysis showed that the weight percentage of each phase was: HA = 69.2%, β-TCP = 8.8%, and α-TCP = 22.0%.

Radiographs revealed no radiolucent lines between the granules and host bone suggesting osteointegration (Fig. 1). Histology and scanning electron microscopy images showed increasing bone regeneration and maturation in the defect area with implantation time (Fig. 3). Neither inflammation nor fibrous tissue was seen surrounding the Bonelike<sup>®</sup> granules. Direct bone apposition as well as bridging between granules could be observed. Microscopic features after staining of bone sections revealed a mature bone even after 6 months of implantation. Some signs of granule surface degradation with simultaneous new bone ingrowth were also detected (Fig. 4).

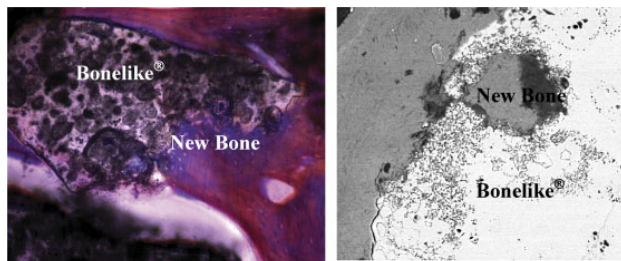
Table 1 summarizes the clinical data of the patients who underwent biopsy and shows the total average percentage of new bone-Bonelike<sup>®</sup> granule contact as determined from histology and scanning electron microscopy. Based on quantitative histomorphometry from approximately 43 assessed slices for the three different implant periods, the bone implant contact average ranged from 67 to 84% through the healing period. The



**Figure 2.** X-ray diffraction patterns of HA and Bonelike<sup>®</sup>. Bonelike<sup>®</sup> is composed of HA and β- and α-TCP phases.



**Figure 3.** Histological and scanning electron microscopy images at different implantation times: (A–B) 6 months, (C–D) 9 months, and (E–F) 12 months.



**Figure 4.** Degradation of Bonelike® granules observed by histology and scanning electron microscopy after 6 months of implantation.



**Table 1.** Clinical Details of Patients and Histomorphometric Results, Expressed as Bonelike<sup>®</sup>-Bone Contact (% ± Standard Deviation), Obtained by SEM and Histological Data (No Less Than 50 Granules Were Quantified)

Sample	Patients Details		Implantation Period (Months)	Bone Implant Contact (%)
	Age	Sex		
1	63	F	6	67 ± 10
2	63	M	6	
3	60	F	6	
4	53	M	9	75 ± 6
5	55	F	9	
6	70	F	9	
7	68	F	9	
8	54	F	12	84 ± 5
9	67	F	12	
10	51	M	12	
11	48	F	12	

values attained at 6 and 9 months were significantly lower than the value achieved at 12 months. As expected, an increase in percentage was observed with the implantation time, in accordance with the histology and scanning electron microscopy images (Fig. 3).

## DISCUSSION

Using a chemical route, single phase HA could be prepared (Fig. 2). The addition of P<sub>2</sub>O<sub>5</sub>-CaO glass cannot only reinforce the HA matrix, but also to lead to the formation of β- and α-Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> phases, depending upon the sintering cycle and the composition of the glass addition.<sup>15-19</sup> Furthermore, because CaO-P<sub>2</sub>O<sub>5</sub> can have a large range of compositions, Bonelike<sup>®</sup> graft can incorporate in its structure ions commonly found in human bone, such as fluorine and magnesium besides Ca and P.

Previous *in vitro* studies using both osteoblast-like cell lines and human osteoblasts showed that Bonelike<sup>®</sup> substrates support cell growth, differentiation, and mineralization by the formation of cell-mediated Ca-P crystals.<sup>13,28,29</sup> Furthermore, Bonelike<sup>®</sup> enhanced the mineralization process compared to unmodified HA, which was a clear indication of its osteoconductive behavior.

Histology is the most powerful method to examine the healing of bone defects.<sup>30</sup> Another commonly used method for quantitative assessment is based on the measurement of contact percentage between implant material and host

tissue. Our results (Table 1 and Fig. 3) revealed that after 6 months of implantation 67 ± 10% of the bone defect area was already regenerated and that after 12 months almost complete regeneration had been achieved, confirming previous results in animal experiments<sup>31</sup> and clinical applications.<sup>14</sup> New bone was formed from the contact with the bone defect border, which intimately contacted the granules, to the middle of the bone defect by the osteoconductive properties of Bonelike<sup>®</sup> granules. The amount of newly formed bone tissue correlated with the distance of the biopsy from the preexisting bone surface.

Biopsies contained mineralized matrix in the grafted area, and mature lamellar bone could be detected close to or in contact with Bonelike<sup>®</sup> granules even for the shortest implantation period. New bone formation appeared to be healthy with embedded osteocytes and active osteoblasts adjacent to osteoid seams. The presence of Haversian canals in close proximity to the bony surface implies bone remodeling around the graft material. The extensive osseointegration was achieved by bone deposition within the granules or directly on their surface, demonstrating a high quality implant/tissue interface.

The basic pattern of integration of Bonelike<sup>®</sup> seems to be the same in animals<sup>31</sup> and humans: no fibrous interface occurs, but instead, an intimate contact between mature new bone and graft material. A transcortical bone defect implanted with Bonelike<sup>®</sup> becomes vascularized and is invaded by newly formed bone, whereas the graft material is resorbed at a rate commensurate with bone formation (Fig. 4).

Many of the clinical human data reported in the literature regarding bone grafts is related to the repair of alveolar defects, ridge maintenance after tooth extraction, or for sinus lift procedures. Previous clinical applications of Bonelike<sup>®</sup> in maxillofacial surgery proved its osteoconductive properties.<sup>32</sup> In these studies, resorption of Bonelike<sup>®</sup> granules occurred simultaneously new bone formation, which correlates to the histological results from our current work.

In some clinical applications, bioresorbable phases like are particularly beneficial; β- and α-TCP<sup>33</sup> are more soluble than HA. Therefore, granule degradation in this study probably resulted from the phase chemical composition of Bonelike<sup>®</sup>. This phenomenon may allow the full regeneration of a bone defect at longer implantation times with complete bioresorption of Bonelike<sup>®</sup>.

Clinical results obtained thus far with Bonelike<sup>®</sup> have been promising, and studies are now being

conducted in other surgical applications, namely as macroporous scaffolds for knee osteotomies.

In conclusion, Bonelike<sup>®</sup> synthetic bone graft proved to be osteoconductive when implanted in the tibiae of humans, and rapid regeneration of bone defects was observed. The osteoconductive properties of Bonelike<sup>®</sup> result from its chemical composition, which mimics that of human bone through an innovative route of liquid phase sintering.

## ACKNOWLEDGMENTS

The authors express their grateful thanks to the FCT (Fundação para a Ciência e a Tecnologia) for their support in this project through Grant #BPD/6010/2001 and Mrs. Ana Mota for her technical assistance in the histological studies.

## REFERENCES

- Peter P, Heinrich R, Meinrad P. 2003. In: YH An, editor. Orthopaedic issues in osteoporosis. Boca Raton, FL: CRC Press.
- Hench LL, Wilson J. 1999. In: An introduction to bioceramics. Singapore: World Scientific Publishing Co. Pvt. Ltd.
- Corpe RS, Steffik DE, Whitehead RY, et al. 2000. Correlative experimental animal and human clinical retrieval evaluations. *Crit Rev Biomed Eng* 28:395–398.
- Kitsugi T, Yamamuro T, Nakamura T, et al. 1993. Four calcium phosphate ceramics as bone substitutes for non-weight bearing. *Biomaterials* 14:216–224.
- Koshino T, Murase T, Takagi T, et al. 2001. New bone formation around porous hydroxyapatite wedge implanted in opening wedge high tibial osteotomy in patients with osteoarthritis. *Biomaterials* 22:1579–1582.
- LeGeros RZ. 1991. In: Meyers HM, editor. Calcium phosphates in oral biology and medicine. New York: Karger; pp. 4–45.
- Macdonald DE, Betts F, Stranick M, et al. 2001. Physicochemical study of plasma-sprayed hydroxyapatite-coated implants in humans. *J Biomed Mater Res* 54:480–490.
- Oonishi H, Yamamoto M, Ishimaru H, et al. 1989. The effect of hydroxyapatite coating on bone growth into porous titanium alloy implants. *J Bone Surg Br* 71:213–216.
- Thomas KA. 1994. Hydroxyapatite coatings. *Orthopedics* 17:267–278.
- Doron I, Amy LL. 2003. Bone graft substitutes. *Oper Tech Plastic Reconstruct Surg* 9:151–160.
- Write S. 1999. The bone-graft market in Europe. Commentary extracted from a report entitled "Emerging technologies in orthopedics I: Bone graft substitutes, bone growth stimulators and bone growth factors." London: Datamonitor plc; pp. 591–595.
- Botelho CM, Brooks RA, Best SM, et al. 2004. Biological and physical-chemical characterization of phase pure HA and si-substituted hydroxyapatite by different microscopy techniques. *Key Eng Mater* 254:845.
- Costa MA, Gutierrez M, Almeida L, et al. 2004. In vitro mineralisation of human bone marrow cells cultured on bonelike<sup>®</sup>. *Key Eng Mater* 254–256:821–824.
- Gutierrez M, Hussain NS, Afonso A, et al. 2005. Biological behaviour of bonelike<sup>®</sup> graft implanted in the tibia of humans. *Key Eng Mater* 284–286:1041–1044.
- Lopes MA, Santos JD, Monteiro FJ, et al. 1998. Glass reinforced hydroxyapatite: a comprehensive study of the effect of glass composition on the crystallography of the composite. *J Biomed Mater Res* 39:244–251.
- Lopes MA, Monteiro FJ, Santos JD. 1999. Glass-reinforced hydroxyapatite composites: fracture toughness and hardness dependence on microstructural characteristics. *Biomaterials* 20:2085–2090.
- Lopes MA, Silva RF, Monteiro FJ, et al. 2000. Microstructural dependence of Young's and shear moduli of P<sub>2</sub>O<sub>5</sub> glass reinforced hydroxyapatite for biomedical applications. *Biomaterials* 21:749–754.
- Lopes MA, Knowles JC, Santos JD. 2000. Structural insights of glass-reinforced hydroxyapatite composites by Rietveld refinement. *Biomaterials* 21:1905–1910.
- Santos JD, Knowles JC, Reis RL, et al. 1994. Microstructural characterization of glass-reinforced hydroxyapatite composites. *Biomaterials* 15:5–10.
- Lopes MA, Santos JD, Monteiro FJ, et al. 2001. Push-out testing and histological evaluation of glass reinforced hydroxyapatite composites implanted in the tibia of rabbits. *J Biomed Mater Res* 54:463–469.
- Felson DT, Naimark A, Anderson J, et al. 1987. The prevalence of knee osteoarthritis in the elderly: the Framingham Study. *Arthritis Rheum* 30:914–918.
- Guincamp C, Pap T, Schedel J, et al. 2000. Gene therapy in osteoarthritis. *Joint Bone Spine* 67:570–571.
- Marc F, Serge P, Mario MLC, et al. 2003. Assessment of the test-retest reliability and construct validity of a modified lequesne index in knee osteoarthritis. *Joint Bone Spine* 70:520–525.
- Pendleton A, Arden N, Dougados M, et al. 2000. EULAR recommendations for the management of knee osteoarthritis: report of a task force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCSIT). *Ann Rheum Dis* 59:936–944.
- Santos JD, Hastings GW, Knowles JC. 1999. Sintered hydroxyapatite compositions and method for the preparation thereof. *Eur. Pat. WO 0068164*.
- Coventry MB. 1965. Osteotomy of the upper portion of the tibia for degenerative arthritis of the knee: a preliminary report. *J Bone Joint Surg Am* 47:984.
- Hing KA. 1996. Assessment of porous hydroxyapatite for bone replacement (Ph D Thesis). London: University of London.
- Lopes MA, Knowles JC, Santos JD, et al. 2000. Direct and indirect effects of P<sub>2</sub>O<sub>5</sub>-glass reinforced-hydroxyapatite composites and growth and function of osteoblast-like cells. *Biomaterials* 21:1165–1172.
- Lopes MA, Knowles JC, Santos JD, et al. 1998. Flow cytometry for assessing biocompatibility. *J Biomed Mater Res* 41:649–656.
- Ross MH, Romrell LJ, Kaye GI. 1995. *Histology—a text and atlas*. 3rd ed. Baltimore, MD: Williams & Wilkins.
- Lobato JV, Hussain NS, Botelho CM, et al. 2005. Assessment of the potential of Bonelike<sup>®</sup> graft for bone regeneration by using an animal model. *Key Eng Mater* 284–286:877–880.
- Duarte F, Lopes MA, Santos JD. 2004. Bonelike<sup>®</sup> composites for the regeneration of bone lesions. *Mater Sci Forum* 455–456:370–373.
- Laurencin CT. 2003. In: Bone graft substitutes. West Conshohocken, PA: ASTM International.